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DISSERTATION

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„Phylogenetic analysis of the Drusinae subfamily (Insecta, Trichoptera)“

verfasst von

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The first caddisflies were described by Linnaeus in the genus *Phryganea*, then comprising 17 species (Linnaeus 1758). Establishing this genus, he used it to summarize several primarily aquatic "Insecta Neuroptera", lumping together Plecoptera, Trichoptera, Diptera and Megaloptera (Holzenthal et al. 2007a). Of the 25 (including five non-Trichoptera) species he described, 17 are still valid (Fischer 1962, 1966a, 1968; Holzenthal et al. 2007a). However, Linnaeus must have noted the characteristic cases of some Trichoptera larvae: knowing their remarkable constructs, the derivation of φρύγανον ['bundle of sticks'] is easily explained. It is a quite fitting, albeit somewhat simplifying description of a typical caddisfly case constructed of plant material. Similarly, common names for this group can be deduced from the cases constructed by some members of the group (e.g., 'kofferjuffers' [Dutch common name for Trichoptera larvae], 'Köcherfliegen' [German common name for any member of the group]).

The Order Trichoptera was proposed by Kirby (1815) in a somewhat lengthy (but self-reflective) and pious footnote, which, acknowledging observations of Réaumur and De Geer and discussing similarities of wing morphology and metamorphosis of certain groups (including Lepidoptera and Trichoptera), reaches a systematic apogee in denominating the order of Trichoptera (Kirby 1815, Holzenthal et al. 2007a, Wichard & Wagner 2015). The name derives the autapomorphic wing cover of adults, typically composed of hair [but also wing-scales are present in some taxa, e.g., in *Monocentra lepidoptera* Rambur, or *Pseudoleptocerus chirinensis* Kimmins (Schmid 1956, Huxley & Barnard 1988)] – and loosely translates to 'the hairy wings' -'Trichoptera' (θρίξ: 'hair' + πτερόν: 'wing'). Trichoptera are holometabolous insects with eggs, larval and pupal stages typically living in freshwater habitats, and terrestrial adult stages. Amongst the European fauna the genus *Enoicyla* represents a rare exception in exhibiting terrestrial immature stages, and has attracted much attention of naturalists and scientists (e.g., MacLachlan 1868, Ritsema 1873, Whitehead 2007).

Currently, the order comprises over 13,000 described species (summarized in Holzenthal et al. 2007a), of which about 1,000 occur in Europe (Malicky 2005). Taxonomic efforts recently demonstrated that species richness of Trichoptera might be underestimated, particularly in hot-spots of biodiversity (cf. Holzenthal et al. 2007a), but also in Europe (e.g., Kučinić et al. 2011, Oláh 2010, Oláh 2011, Oláh et al. 2012, Oláh et al. 2013, Oláh and Kovács 2013, Oláh et al. 2014, Previšić et al. 2014a, Graf et al. 2015). Total species number of Trichoptera was estimated to be about 50,000, with a maximum of species richness in the Oriental region (Schmid 1984, de Moor & Ivanov 2008).

Systematically, (Trichoptera + Lepidoptera) are treated as Amphiesmenoptera, a group most likely originating in the late Triassic (Misof et al. 2014). Amphiesmenoptera is considered a monophylum (cf. Beutel & Pohl 2006, Beutel et al. 2010, Misof et al. 2014), defined by several autapomorphies. Homogametic males, presence of a spermathecal gland and bursa copulatrix, and, most strikingly, a spinneret formed of the hypopharynx at the opening of the larval labial silk glands are present in both Lepidoptera and Trichoptera (Krenn 2007, Beutel et al. 2010).

Trichoptera are widely accepted as monophyletic (Kjer et al. 2001, Kjer et al. 2002, Beutel & Pohl 2006, Kjer et al. 2006), and exhibit a number of synapomorphies (summarized by Morse 1997, Holzenthal et al. 2007a, Malm et al. 2013), e.g., the

modified mouthparts of adults. Phylogenetic relationships within Trichoptera have been intensively studied, scrutinizing morphological, ecological, molecular and behavioural characteristics to estimate taxonomic relationships. Within Trichoptera, three suborders are traditionally distinguished, primarily based on maxillar palp morphology, silk usage in larvae, and characteristics of the pupal cocoon: monophyletic Annulipalpia exhibit terminal segments of maxillar palps seemingly consisting of single annuli and construct silken nets as larvae and permeable pupal cocoons prior to pupation; monophyletic Integripalpia exhibit terminal segments of maxillar palps with rounded tips and unmarked cuticle and larvae that construct portable cases of silk and various plant, detrital and/or mineral particles, which are modified to serve as permeable pupal cocoons; paraphyletic 'Spicipalpia' exhibit maxillar palps bearing a terminal tip and larvae that construct either nets or cases in the last larval stage and construct a semipermeable pupal cocoon (cf. Martynov 1924 [suggesting Annulipalpia and Integripalpia], Ross 1967 [suggesting the three suborders Rhyacophiloidea, Hydropsychoidea, Limnephiloidea], Wiggins & Wichard 1989, Wiggins 2004, Holzenthal et al.

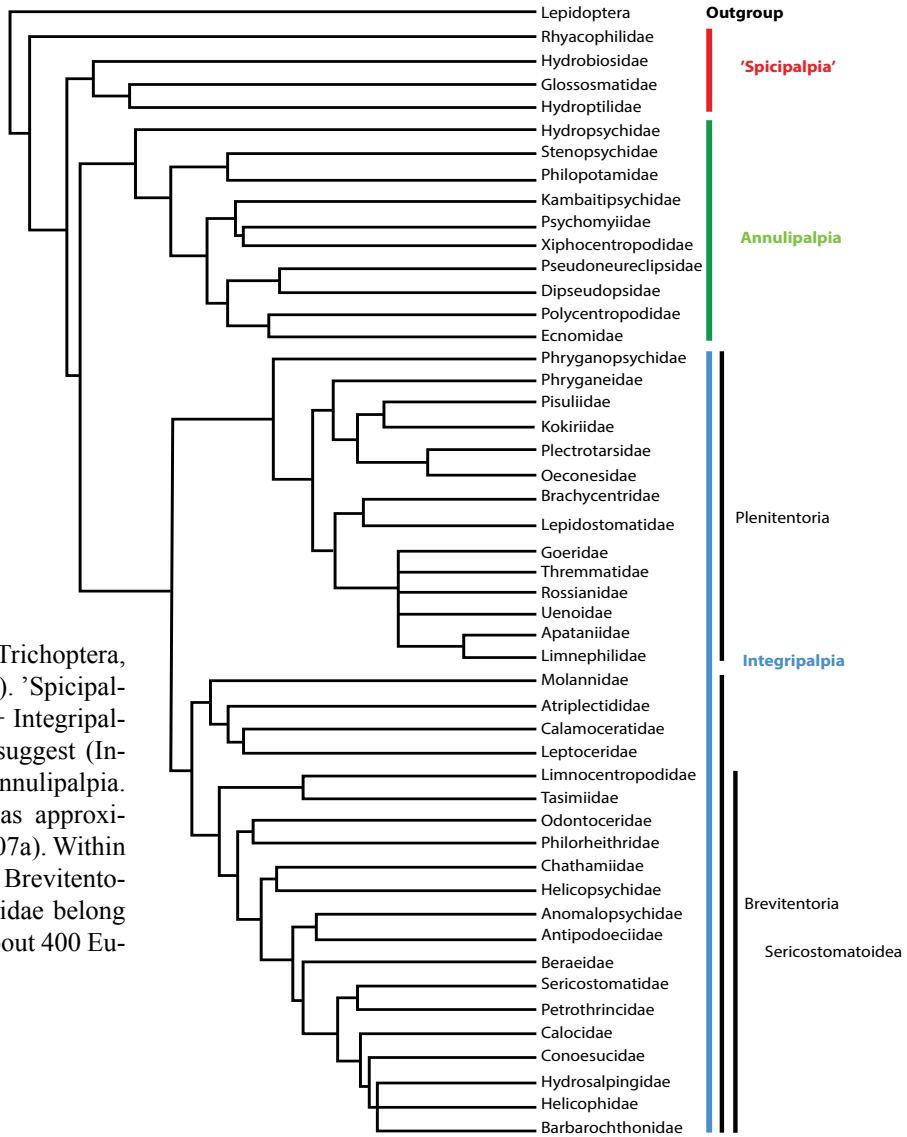

Figure 1. Phylogeny of extant Trichoptera, modified after Malm et al. (2013). 'Spicipalpia' are sister to (Annulipalpia + Integripalpia). Holzenthal et al. (2007a) suggest (Integripalpia + 'Spicipalpia') + Annulipalpia. The position of Rossianidae was approximated after Holzenthal et al. (2007a). Within Integripalpia, Plenitentoria and Brevitentoria are distinguished. Limnephilidae belong to Plenitentoria, and comprise about 400 European species.

2007a, Malm et al. 2013). Recently, relationships of Trichoptera suborders were re-examined using molecular methods (Holzenthal et al. 2007a, Malm et al. 2013). Annulipalpia and Integripalpia were found to be monophyletic, and well supported. 'Spicipalpia' however, were not recovered as monophyletic (Holzenthal et al. 2007a, Malm et al. 2013). Rather, paraphyletic 'Spicipalpia' were found to be basal to Integripalpia + Annulipalpia (Malm et al. 2013). Alternatively, Holzenthal et al. (2007a) suggest Annulipalpia as basal sister to ('Spicipalpia' + Integripalpia).

Within Integripalpia, two groups are distinguished, so-called Brevitentoria and Plenitentoria, each comprising a number of families (Holzenthal et al. 2007a, Malm et al. 2013). The Limnephilidae are the largest family of Plenitentora, comprising approximately 900 described species (Holzenthal et al. 2010) worldwide, to which the European fauna contributes 394 species in 52 genera (Malicky 2005, Graf et al. 2008). European limnephilidae are characterized by exploiting a high diversity of habitats (Graf et al. 2008). Within Limnephilidae, one of the most species rich groups is the subfamily Drusinae (Malicky 2005, Graf et al. 2008). The last exhaustive treatment of this group was conducted by Fernand Schmid (1956) who identified seven genera of Drusinae and six species groups within the largest genus, *Drusus*. Venation characteristics of male wings (in particular the presence of specialized A2 and A3 veins in the male hindwings forming a pouch that harbours long vein bristles of unknown function) served as primary characters to define both the subfamily and genera (Schmid 1956). Schmid (1956) examined 37 out of a total of 41 known species, of which 35 are still valid. Since then, one new genus and many new species have been described, resulting in roughly 100 known extant species in eight genera: *Anomalopterygella* Fischer, 1966b; *Cryptothrix* McLachlan, 1867; *Drusus* Stephens, 1837; *Ecclisopteryx* Kolenati, 1848; *Hadimina* Sipahiler, 2002; *Leptodrusus* Schmid, 1955; *Metanoea* McLachlan, 1880; *Monocentra* Rambur, 1842 (Sipahiler 2002, Malicky 2004 Malicky 2005, Graf et al. 2008, Kučinić et al. 2011, Oláh 2010, Oláh 2011, Oláh and Kovács 2013, Previšić et al. 2014a, Vitecek et al. 2015, Vitecek et al. submitted).

The species groups in the genus *Drusus* proposed by Schmid (1956) under the impression of a majorly homogenous group were based primarily on the formation of the intermediate appendages (paraproct *sensu* Snodgrass 1935). Although not entirely satisfied ['*Un seul caractère ne pourrait, semble-t-il, conduire qu'á un arrangement artificiel...'* (Schmid 1956, p.17, lines 34-35)], he considered the groups he proposed as sufficiently natural (Schmid 1956).

A first molecular phylogenetic study on the subfamily Drusinae revealed the prevalence of three distinct evolutionary lineages, reflecting feeding ecology of larvae (Figure 2) (Pauls et al. 2008; Graf et al. 2009): (1) carnivorous filterers (developing toothed shredder-like mandible edges, modifications of head capsules, and additional filtering spines on legs and the first abdominal sternum), (2) omnivorous shredders (exhibiting toothed shredder mandibles, but lacking additional spines and filtering bristles), (3) epilithic grazers (developing spoon-shaped mandibles without teeth and lacking additional spines and filtering bristles). Further, Pauls et al. (2008) rejected most of the generic concepts and species groupings that Schmid (1956) had proposed based primarily on the morphology of adult male terminalia. In particular, the monotypic genus *Cryptothrix* was found to be close-

ly related to the filtering carnivorous Drusinae, whereas the genera *Drusus* and *Ecclisopteryx* were identified as paraphyletic with *Anomalopterygella*, *Metanoea* and *Cryptothrix* nested within (Pauls et al. 2008). The genus *Drusus* was found to comprise members of all three larval feeding types (Pauls et al. 2008, Graf et al. 2009).

Ecology of Drusinae

The majority of extant Drusinae are cold-stenotopic, restricted to Eurasian mountain ranges (Figure 3). Drusinae features many endemic species (Malicky 2005, Graf et al. 2008, Oláh 2010, Oláh 2011, Kučinić et al. 2011), but also a number of widely distributed taxa (e.g., *A. chauvinina*, *D. annulatus*, and *D. discolor* (Graf et al. 2008)). Unfortunately, no species-specific traits could yet be identified in Drusinae that could serve to estimate a species dispersal capacity and flight ability. Potentially, wing anatomy and size could be a

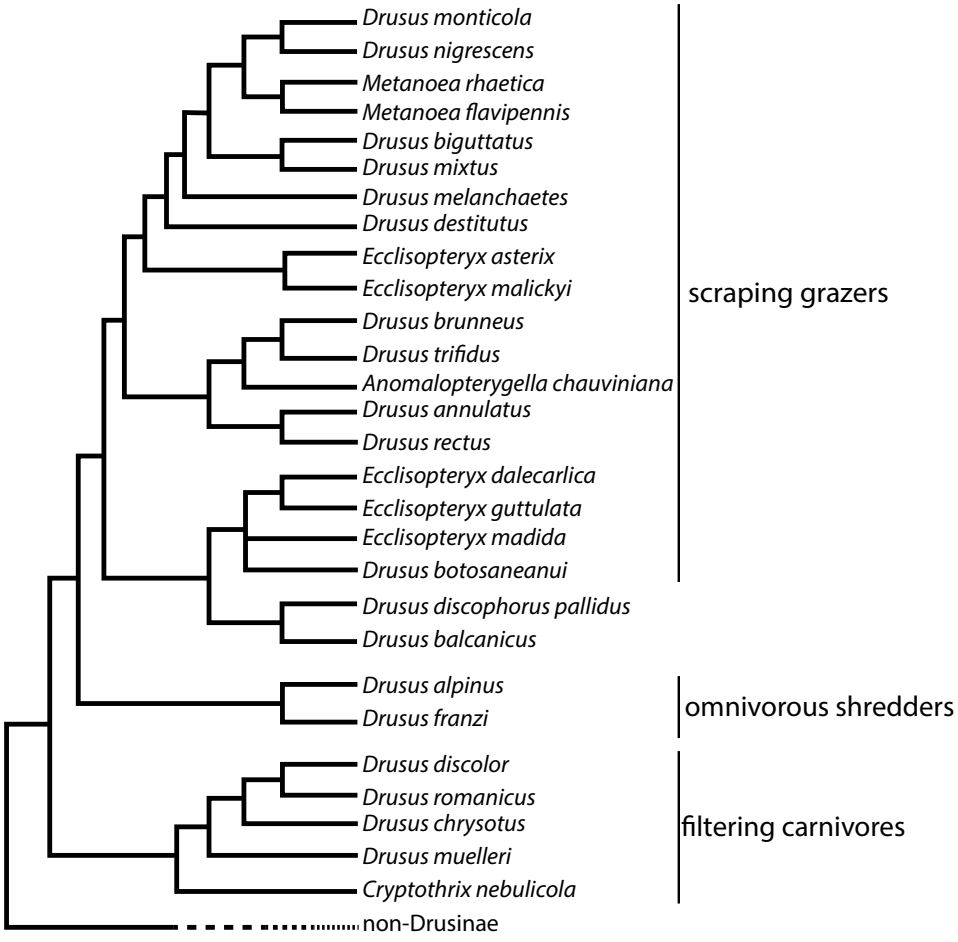

Figure 2. Phylogeny of extant Drusinae as estimated using Bayesian inference on a 1468 bp, 53 terminal taxa dataset (Pauls et al. 2008). Feeding types were recovered as monophlyetic [with the exception of filtering carnivores, which are recovered as unsupported (*C. nebulicola* + (*D. muelleri* + *D. chrysotus* + (*D. discolor* + *D. romanicus*))), in which the inner clade is highly supported], but relationships between and within groups were not resolved. Larvae of *D. balcanicus* and *D. discophorus pallidus* were not known at that time, the recent description of the larva of *D. balcanicus* confirms the position of this species among scraping grazers (Waringer et al. 2015). Modified after Pauls et al. (2008).

proxy for the dispersal capacity in Drusinae. (cf. Müller-Peddinghaus 2011).

Speciation of Drusinae putatively peaked during glaciation periods, as indicated by distribution patterns of haplotypes within species (Pauls et al. 2006). Some species exhibit highly disjunct distribution patterns, indicating perseverance of evolutionary lineages during several glaciation periods (Previšić et al. 2009, 2014b). Additionally, the majority of Drusinae seems limited in their dispersal capacity, another trait that enhances speciation in geographically isolated lineages. These factors might have contributed to the high number of endemic Drusinae species (Graf et al. 2008).

Further, Drusinae exhibit high habitat specificity and almost exclusively inhabit crenal sections of cold streams (Graf et al. 2008). Thus, susceptibility of Drusinae to habitat modification and global change is high (cf. Hering et al. 2009, Bálint et al. 2011). Anthropogenic habitat modification linked to extraction of water for tourism or hydroelectricity is particularly threatening to crenal biota, such as Drusinae (cf. Foster 1991, Barquín & Scarsbrook 2008, Cantonati et al. 2012). Fluctuations of climatic conditions are expected to increase in the next decades (IPCC 2014), potentially altering temperature conditions in Drusinae habitats and thus affecting a wide variety of crenobiont taxa.

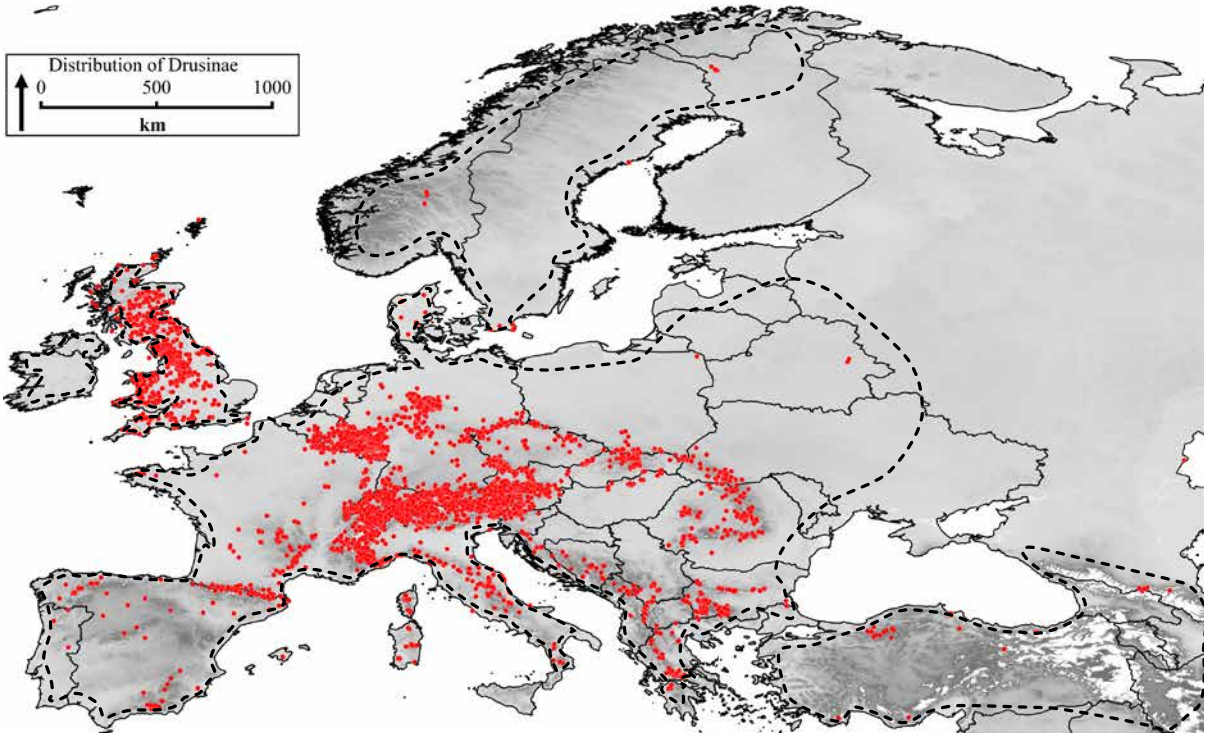

Figure 3. Distribution of Drusinae. Potential area of Drusinae is marked as dotted line, point distribution data of Drusinae (as collated in DAET) are depicted in red. Drusinae were predominately recorded in European mountain regions. Although a number of Drusinae species have been described from Anatolia, and abundance of Drusinae in the Caucasus is high (Graf, pers. comm.), these areas are underrepresented in the present data. Reports of *D. simplex* from Iran (Schmid 1956, Graf pers. comm.) are not depicted.

Data on reproductive cycles of Drusinae are scarce. From what little is known, uni- to semivoltine reproductive cycles have been observed (Graf et al. 2008). Flight and emergence period range from spring to autumn, with the majority of species on the wing during summer and autumn (Graf et al. 2008).

Feeding ecology of Drusinae larvae is remarkably diverse, and comprises omnivorous shredders, filtering carnivores and scraping grazers (Pauls et al. 2008, Graf et al. 2009). Filtering carnivores exhibit peculiar head morphology in larval stages, and morphological adaptations to filter-feeding. Morphological adaptations in passive filter-feeders are known from most filter-feeding Diptera and all filter-feeding Ephemeroptera. These groups typically develop specialized structures (body appendages, etc.), whereas the majority of filter-feeding Trichoptera employ silken nets for filter feeding (Merrit & Wallace 1984, McCafferty & Bae 1992). Particularly the Annulipalpia *sensu* Holzenthal et al. (2007) & Malm et al. (2013) construct specialized nets adapted to the predominating hydrological constraints of their ecological niche (Holzenthal et al. 2007, Malm et al. 2013). Diversity of net bauplans is well documented in some groups (e.g., Merrit & Wallace 1984), as are net-tending behaviour and filtering capacity (Runde & Hellenthal 2000, Wallace & Malas 1976, Malas & Wallace 1977). Also, specificity of hydrological niches for different net types was demonstrated (Georgian & Wallace 1981). Constant effects of noxious substances on net-building behaviour of Hydropsychidae have been identified, and can be used to assess stream ecosystem pollution (e.g. Wendt-Rasch et al. 1998; Tessier et al. 2000a, b, c). Despite the heterogeneity of behavioural adaptations to filter-feeding in net-spinning European Trichoptera, only few genera of Annulipalpia develop specialized mouthparts to exploit fine particulate organic matter (FPOM), whereas the majority develops shredder-like mouthparts (W. Graf, J. Waringer, unpubl. data).

Integripalpia filter-feeders, however, exhibit morphological adaptations and use their whole body as filtering structure, forfeiting their potential to produce and use silk to construct filtering devices. Usually, filtering bristles are accommodated on legs, heads, or sterna, and employed in a stereotypical manner, as can be observed in the European genera *Allogamus*, *Brachycentrus*, and *Drusus* (Graf et al. 2008). Additionally, modifications of legs (e.g. elongation of femora in *Brachycentrus*), or heads (as in Drusinae) are common. Interestingly, the South-East Asian genus *Limnocentropus* exploits a similar niche, and also develops modified legs armed with numerous filtering bristles (Wiggins 1969, Wallace & Merrit 1980, Graf pers. comm.).

Significance of Trichoptera larvae in water quality monitoring

Prevalence and abundance of macrozoobenthos taxa is widely used to monitor water quality, particularly in Europe, North America and Australia (Buffagni et al. 2006, Wright et al. 2000, Smith et al. 1999, Barbour & Yoder 2000, European Union Water Framework Directive (EU WFD): European Commission 2000, Hering et al. 2006). Larval stages of Ephemeroptera, Plecoptera and Trichoptera are primary indicator taxa in contemporary assessment schemes. Differential sensitivity of Trichoptera larvae to environmental parameters renders them ideal quality elements to estimate effects of (anthropogenic) pollution and habitat modification (Moog et al. 2002, Graf et al. 2002,

Dohet 2002, Waringer et al. 2013c).

Species-level identification of larvae provides improved resolution for water quality monitoring, and species-specific information is crucial to fully exploit the potential of caddisflies as indicator organisms (e.g., Goethals 2002, Schmidt-Kloiber & Nijboer 2004). Extant Drusinae are currently covered disjunctly in taxonomic tools (Waringer et al. 2013a, Waringer et al. 2013b, Previšić et al. 2014a): only about 50% of all Drusinae larvae are known. As all identifiable members of Drusinae are used as bio-indicators and sensitive species in biological monitoring (Moog et al. 2002, Graf et al. 2002), larval keys to this highly diversified group are crucial. Thus, taxonomic tools and information on Drusinae species autecology are necessary to improve and adapt existing assessment schemes.

Due to their habitat requirements Drusinae may further serve as indicators of high-quality habitats in alpine and montane regions, imparting crucial information for conservation measures.

Aims of the study

Drusinae are a group of aquatic insects that features high numbers of endemics, complex evolutionary history and diverse feeding ecology. Thus they represent an ideal model to study evolutionary ecology of Trichoptera. Main objective of the project was to revise Drusinae based on molecular and morphological data obtained from all available lifestages. Moreover, taxonomic richness of Drusinae was assessed by means of a vast collection campaign, classic taxonomic tools and molecuar phylogenetics.

Adequacy of methods employed were tested in an exemplary study aiming to identify the position of the monotypic genus *Cryptothrix*, and to assess evolutionary patterns in the complexity of head capsule modifications in a subgroup of Drusinae, the filtering carnivores.

I spent the past three years together with my colleagues in a joint effort to generate data and background information necessary to achieve this feat. In the course of this endeavour, several manuscripts were published, some submitted, and some are still on their way. The chapters presented are a selection of manuscripts that I contributed to the project.

Chapter I deals with taxonomy of *Ecclisopteryx* species in the Western Balkans, and identifies two new species that were hitherto overlooked.

Chapter II presents a new species of *Drusus* from the Western Balkans, and presents a key to a subgroup of Western Balkan Drusinae.

Chapter III presents two new species of filtering carnivore *Drusus* from the Western Balkans, and summarizes morphological features of filtering carnivorous Drusinae *sensu* Pauls et al. (2008).

Chapter IV re-evaluates the phylogeny of Drusinae larval feeding groups. In this chapter, results of species tree analysis, and larval and adult autapomorphies of feeding groups are presented. Further, potential evolutionary trends in modifications of heads of filtering carnivorous Drusinae larvae, and the validity of the genus *Cryptothrix* are discussed. Additionally, a new species of filtering carnivorous Drusinae is presented, and its status and potential implication of its position in the filtering carnivore clade are discussed. This chapter lays the base for the revision of the whole group in identifying morphological characteristics of Drusinae feeding groups.

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Chapter I

Manuscript

Cryptic diversity of caddisflies in the Balkans: the curious case of Ecclisopteryx species (Trichoptera: Limnephilidae)

This manuscript deals with cryptic diversity of Drusinae in the Balkans and highlights the importance of alphataxonomic studies in a supposedly well-studied area.

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I supplied illustrations, generated and analysed the genetic dataset partly, designed the keys and wrote the manuscript as equally contributing first author together with Ana Previšić and Wolfram Graf.

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Cryptic diversity of caddisflies in the Balkans: the curious case of *Ecclisopteryx* species (Trichoptera: Limnephilidae)

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Abstract

Adults and larvae of two new cryptic, endemic caddisflies, *Ecclisopteryx keroveci* sp.n. and *Ecclisopteryx ivkae* sp.n., are described and illustrated from the Western Balkans. Phylogenetic analysis (Bayesian MCMCMC) and association of different life history stages in both cryptic species were achieved through comparison of morphological characters and mitochondrial (mtCOI and mtLSU) and nuclear (nuWG) gene sequence data. The new species form a sister clade to the widely distributed *E. dalecarlica* and *E. guttulata*, with which they were formerly misidentified. Adults differ from each other and other species in the genus by the uniquely shaped inferior appendages in males and segment X in females. The larvae differ from each other and their congeners in the shape of the pronotum, and presence and constitution of additional spines on the parietalia. Larvae of both species are grazers and prefer stony substrate. *Ecclisopteryx keroveci* sp.n. has a wide distribution in the Western Balkans, while *E. ivkae* sp.n. is endemic to Dalmatia. Our findings demonstrate the significance of the Western Balkans as a freshwater biodiversity hotspot, and accentuate the importance of research focused on freshwater biodiversity and biogeography in southern Europe.

Key words

Biodiversity, Drusinae, larval morphology, adult morphology, phylogeny.

1. Introduction

Cryptic species are typically defined as two or more distinct species that are classified as a single nominal species, mostly due to seemingly identical morphology (Bickford et al. 2007). A broader definition describes cryptic species as "those that cannot be identified by conventional means" (Ross 1974). "Conventional means" have changed historically, and numerous methods examining ecology, behaviour and genetics have been used to discover cryptic species (e.g. recognition of distinctive mating signals in various animals; review in Howard & Berlocher 1998, variability in allozyme markers; review in Avise 2004, etc.). However, the dis-

covery of cryptic species has increased in recent years in different taxa, habitats, and regions, facilitated by the use of DNA barcoding among other methods (e.g. Pfenninger & Schwenk 2007; Zakšek et al. 2009; Pauls et al. 2010; Jackson et al. 2014; Weiss et al. 2014). Expectably, high cryptic diversity occurs in poorly surveyed and geographically isolated regions, such as the Balkan Peninsula (e.g. Francuski et al. 2011; Klobučar et al. 2013; Tsuomani et al. 2013). In fact, many cryptic species have been discovered recently in different freshwater taxa in the Balkans, including fishes (Tsuomani et al. 2013), crayfishes (Klobučar et al. 2013), amphipods (Weiss et al. 2014), and caddisflies (Oláh et al. 2012).

Within Europe, highest species diversity and endemism of Trichoptera (caddisflies) fauna are recorded in ecoregions (sensu Illies 1978) covering diverse mountain areas (e.g. Iberic-Macaronesian Region [ER1], Italy, Corsica and Malta [ER3], Hellenic Western Balkan [ER6], The Carpathians [ER10], Alps [ER4] etc.; Graf et al. 2008; Graf & Schmidt-Kloiber 2011). In particular, high numbers of endemic species are common in cold adapted montane groups, such as Drusinae (Limnephilidae) (Malicky 2005; Graf et al. 2008; Oláh 2010, 2011; Kučinić et al. 2011). Considering the remarkable distribution patterns of Drusinae (Graf et al. 2008) and their complex evolutionary history (Pauls et al. 2006; Previšić et al. 2009, 2014), more endemics and cryptic species are likely to be discovered, particularly in poorly explored areas, such as the Balkans.

The subfamily Drusinae comprises eight genera, including *Ecclisopteryx* Kolenati, 1848, with five species (Schmid 1956; Pauls et al. 2008). The genus is restricted to Europe. Three species have relatively wide and partially overlapping ranges: *Ecclisopteryx dalecarlica* Kolenati, 1848, *Ecclisopteryx guttulata* (Pictet, 1834) and *Ecclisopteryx madida* (McLachlan, 1867) (Graf et al. 2008; Graf & Schmidt-Kloiber 2011; Fig. 1A). *Ecclisopteryx dalecarlica* was previously reported from throughout the Western Balkans (e.g., Slovenia: Urbanič 2004; Croatia: Previšić & Popijač 2010; Vučković 2011; Vučković et al. 2011; Bosnia and Herzegovina: Stanić-Koštroman 2009; Kosovo: Ibrahimi 2011). In older literature, however, *E. guttulata* was listed from only part of the Western Balkan region (e.g., Bosnia: Radovanović 1935; Marinković-Gospodnetić 1970).

The remaining two *Ecclisopteryx* species are endemic to the Karawanken and Julian Alps (*Ecclisopteryx asterix* Malicky, 1979) and the Lessinian Alps (*Ecclisopteryx malickyi* Moretti, 1991) (Graf et al. 2008, 2011; Graf & Schmidt-Kloiber 2011; Fig. 1B). Monophyly of the genus *Ecclisopteryx* within Drusinae was rejected by a multigene phylogenetic study on the subfamily (Pauls et al. 2008). *Ecclisopteryx* species formed two distant lineages consisting of closely related species (*E. madida* + *E. guttulata* + *E. dalecarlica* and *E. malickyi* + *E. asterix*) that were corroborated by larval morphology and geographic distribution (Pauls et al. 2008; Graf et al. 2011).

Ecclisopteryx specimens similar to *E. dalecarlica* were collected in eastern Bosnia and Herzegovina and Dalmatia (southern Croatia). Comparison of morphological characters and molecular genetic sequence data [mitochondrial cytochrome oxidase *c* subunit I (mtCOI) and ribosomal large subunit (mtLSU = 16S) and nuclear wingless (nuWG)] with *Ecclisopteryx* specimens from other parts of the Western Balkan region and central Europe enabled us to distinguish two new *Ecclisopteryx* species that were previously overlooked and/or misidentified. Thus, in the current paper we describe the morphological features of males and females as well as the larvae of two new species. We also summarise the most important morphological characteristics enabling their identification and successful separation from each other and the other Drusinae. Moreover, we define distribution ranges of these cryptic species in the Western Balkans and discuss distribution patterns of the more widespread *E. dalecarlica* and *E. guttulata*.

2. Material and methods

2.1. Material

Specimens of *Ecclisopteryx keroveci* sp.n. were collected at the mouth of the Jabučica River where it joins the Sutjeska River (Table 1). Adults were collected using a UV light trap and larvae were collected by handpicking. Adults of *Ecclisopteryx ivkae* sp.n. were collected in the Glavaš spring and 2 sites in the upper reach of the Cetina River using a sweeping net (Table 1). Larvae were collected in the Glavaš spring by handpicking.

Collected specimens were stored in 70 and 96% EtOH for morphological and molecular analysis, respectively. All collected specimens are deposited in the Faculty of Science, University of Zagreb (Croatia), the Institute of Hydrobiology and Aquatic Ecosystem Management, University of Natural Resources and Applied Life Sciences, Vienna (Austria), and the Biology Centre, Oberösterreichisches Landesmuseum, Linz (Austria). Terminology for larval morphological features follows Wiggins (1998) and Waringer & Graf (2011). Nomenclature of primary setae and setal areas follows Wiggins (1998). Nomenclature of male terminalia follows Nielsen (1957).

To delineate the two new species from remaining *Ecclisopteryx* species and maximise geographic coverage, we compared *Ecclisopteryx* specimens from the entire Western Balkan region in the current study (Table 1). For both morphological characters and mtCOI, mtLSU and nuWG sequence data, we also compared *E. dalecarlica* specimens from Northern, Central and Eastern Europe and *E. guttulata* from Central Europe (Table 1). Sequence data were taken from Pauls et al. (2008) and Previšić et al. (2014); additional specimens used for comparative morphology were provided by the many colleagues listed in Table 1 and the Acknowledgements.

Adult male and female of *E. dalecarlica* were redrawn based on material collected in Norway (Table 1).

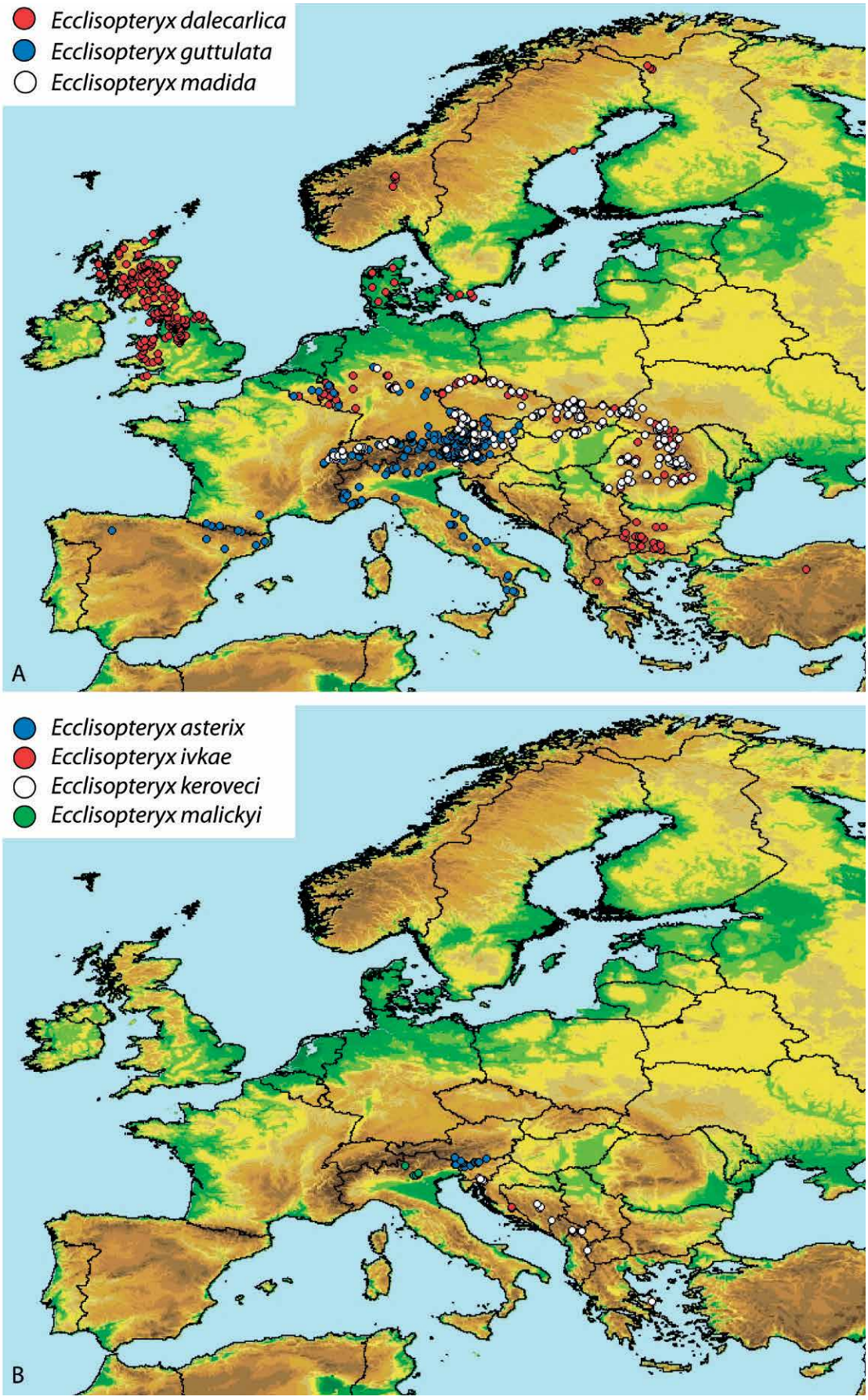

Fig. 1. Distribution of *Ecclisopteryx* species; **A**: widespread and **B**: endemic species (based on data from the Distribution Atlas of European Trichoptera [DAET; the BioFresh EU project–Biodiversity of Freshwater Ecosystems: Status, Trends, Pressures and Conservation Priorities]).

Table 1. Information on specimens used in this study. *Historical collection, geographic reference is provided for the closest town, Pazarić, as the exact location is not known; **Outgroup taxa in phylogenetic analysis. LT = locus typicus, IM = imagines, adults, M = male, F = female, Lv = larvae.

Locality (country, name)	Longitude dec.	Latitude dec.	Altitude (m)	No of specimens and stage/sex		Specimen Code	GenBank Accession Nos			Collector	Publication
				Morphology	Molecular genetic analyses		mtCOI	nuWG	mtLSU		
***Ecclisopteryx dalecarlica* Kolenati, 1848**											
AT, Bruck/Lafnitz	N 47.4396	E 15.9138	560	1 IM/M						Graf	this study
AT, Ritterkamp, Kamp Aqem	N 48.52887	E 15.10722	581	4 Lv						Graf	this study
SK, Vysoké Tatry (high Tatra Mts.), Podbanské, Béla river	N 49.14017	E 19.90247	934	5 IM (3M + 2F)						Graf	this study
D, Spessart, Jossa below Sahlensee	N 50.218548	E 9.484726	290		Lv	ED001	EU215112	EU215165	EU215218	Lohse	Pauls et al. 2008
D, Spessart, Jossa below Sahlensee	N 50.218548	E 9.484726	290		Lv	ED002	EU215113	EU215166	EU215219	Lohse	Pauls et al. 2008
N, Hedmark, Folldal, Streitlie	N 62.09520	E 9.96412	804	2 IM (1M + 1F)	IM/M	fEda0801M	KM001830	KM001819	KM001825	Andersen	this study
N, Hedmark, Folldal, Streitlie	N 62.09520	E 9.96412	804		IM/F	fEda0802F	KM001829	KM001820	KM001826	Andersen	this study
RO, Țarcu Mts., Poiana Mărului	N 45.403056	E 22.540556	638		IM/F	Dsp023	EU215106	EU215159	EU215212	Balint	Pauls et al. 2008
RO, Țarcu Mts., Poiana Mărului	N 45.403056	E 22.540556	638		IM/F	Dsp031	EU215107	EU215160	EU215213	Balint	Pauls et al. 2008
RO, Carlibaba, Tibau Valley	N 47.464228	E 24.842512	980	IM/F	IM/F	fEda0701F	KM001831	KM001818	KM001824	Neu	this study
BG, Rhodope Mts., stream close to Teshel, Devin	N 41.666389	E 24.365556	870	IM/F	IM/F	fEda0901F	KM001828	KM001818	KM001827	Neu	this study
RO, Făgăra Mts., Bâlea Valley	N 45.665299	E 24.554063	800	IM/F		fEda0601F				Balint, Theissinger & Taubmann	this study
RO, Făgăra Mts., Bâlea Valley	N 45.665299	E 24.554063	800	IM/F		fEda0602F					this study
RO, Sibiului Mts (Cindrel), Râu Sadu	N 45.624	E 24.033	770	IM/M		fEda0401M					this study
RO, Sibiului Mts (Cindrel), Râu Sadu	N 45.624	E 24.033	770	IM/F		fEda0402F					this study
RO, Muntii Ciucas, Sacele, stream close to Babarunca	N 45.513889	E 25.848333	960	IM/M	IM/M	fEda0501M	KM001833		KM001822	Neu	this study
RO, Muntii Ciucas, Sacele, stream close to Babarunca	N 45.513889	E 25.848333	960	IM/F	IM/F	fEda0502F	KM001832		KM001823	Neu	this study
***Ecclisopteryx ivkae* Previšić, Graf & Vitecek, sp.n.**											
HR, Cetina River, Crveni most	N 43.960347	E 16.429489	370	12 IM (5M + 7F)	IM/M	fEda0101M	KM001813	KM001799	KM001806	Previšić	this study
HR, Cetina River, Crveni most	N 43.960347	E 16.429489	370		IM/M	fEda0102M	KM001815	KM001801	KM001808	Previšić	this study
HR, Cetina River, Crveni most	N 43.960347	E 16.429489	370		IM/F	fEda0101F	KM001812	KM001798	KM001805	Previšić	this study
HR, Cetina River, Glavaš spring (LT)	N 43.976697	E 16.430150	386	12 IM (6M + 6F)	IM/F	fEda0102F	KM001814	KM001800	KM001807	Previšić	this study
HR, Cetina River, Glavaš spring (LT)	N 43.976697	E 16.430150	386	8 Lv	Lv	fDsp3301L	KM001811	KM001797	KM001804	Kučinić & Previšić	this study
HR, Cetina River, Glavaš spring (LT)	N 43.976697	E 16.430150	386		Lv	fEda1201L	KM001816	KM001802	KM001809		this study
HR, Cetina River, Glavaš spring (LT)	N 43.976697	E 16.430150	386		Lv	fEda1202L	KM001817	KM001803	KM001810		this study
HR, Cetina River, Vinalić	N 43.936253	E 16.443441	375	1 IM/M						Previšić	this study

Table 1. continued.

Locality (country, name)	Longitude dec.	Latitude dec.	Altitude (m)	No of specimens and stage/sex		Specimen Code	GenBank Accession Nos			Collector	Publication
				Morphology	Molecular genetic analyses		mtCOI	nuWG	mtLSU		
***Ecclisopteryx keroveci* Previšić, Graf & Vitecek, sp.n.**											
*BIH, Pazarić, SW of Sarajevo, valley of Krupa River	N 43.786	E 18.166	630	1 IM/M						Winneguth	this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765	40 IM (20M + 20F)	IM/M	fDs30101M	KM001785	KM001761	KM001773	Ivković, Mihaljević, Miliša & Previšić	this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		IM/M	fDs30102M	KM001786	KM001762	KM001774		this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		IM/M	fDs30103M	KM001787	KM001763	KM001775		this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		IM/M	fDs30105M	KM001788	KM001764	KM001776		this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		IM/F	fDs30106F	KM001789	KM001765	KM001777		this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		IM/F	fDs30109F	KM001790	KM001766	KM001778		this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		IM/M	fDs30113M	KM001851				this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		IM/M	fDs30114M	KM001852				this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		IM/F	fDs30115F	KM001853				this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		IM/F	fDs30116F	KM001854				this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765	10 Lv	Lv	E1JAL1	KM001847	KM001836	KM001842	Graf & Previšić	this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		Lv	E1JAL2	KM001848	KM001837	KM001843		this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		Lv	E2JAL1	KM001849	KM001838	KM001844		this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		Lv	E2JAL2	KM001850	KM001839	KM001845		this study
BIH, Željeznica River, upper reach	N 43.898666	E 17.952901	650	2 IM/M						Stanić-Koštroman	Stanić-Koštroman 2009
HR, Čabranka, spring reach	N 45.60126	E 14.64043	589	4 IM (3M + 1F); 2 Lv	IM/M	EdIM1	FJ002686		FJ002818	Bokan, Kučinić, Popijač & Previšić	Previšić et al. 2009; this study

Table 1. continued.

Locality (country, name)	Longitude dec.	Latitude dec.	Altitude (m)	No of specimens and stage/sex		Specimen Code	GenBank Accession Nos			Collector	Publication
				Morphology	Molecular genetic analyses		mtCOI	nuWG	mtLSU		
***Ecclisopteryx keroveci* Previšić, Graf & Vitecek, sp.n.**											
HR, Kupa, bridge before Čabranka mouth	N 45.524417	E 14.700383	292	2 IM (1M + 1F)						Popijač	Previšić & Popijač 2010
HR, Velika Belica, bridge at Kuželj	N 45.475514	E 14.805144	242	1 IM/M		fEda1001M	KM001794	KM001770	KM001782	Popijač	Previšić & Popijač 2010
KS, Pejë, Lumbardhi and Pejës rivers	N 42.66128	E 20.25958	587	2 IM (1M + 1F)						Ibrahimi	Ibrahimi 2011
MK, Radika river, Monastery St. Jovan Bigorski NE of Debar	N 41.623611	E 20.606111	694	2 IM/M						Chvojka	this study
MN, Brodavac, right tributary of Peručica	N 42.68587	E 19.73636	960	2 IM (1M + 1F)	IM/M	fEda0201M	KM001791	KM001767	KM001779	Miliša	this study
MN, Brodavac, right tributary of Peručica	N 42.68587	E 19.73636	960		IM/F	fEda0202F	KM001792	KM001768	KM001780	Miliša	this study
MN, Grncar, Gusinje	N 42.565944	E 19.833389	922	2 IM (1M + 1F)	IM/F	fEda1101F	KM001795	KM001771	KM001783	Graf	this study
MN, Grncar, Gusinje	N 42.565944	E 19.833389	922		IM/M	fEns0101M	KM001796	KM001772	KM001784	Graf	this study
MN, Peručica	N 42.69472	E 19.75661	884	5 IM (4M + 1F)	IM/M	fEda0301M	KM001793	KM001769	KM001781	Previšić	this study
***Ecclisopteryx asterix* Malicky, 1979**											
AT, Soboth, Krumbach tributary	N 46.716667	E 15.066667	1130		Lv	EastDDest002	EU215111	EU215164	EU215217	Graf & Pauls	Pauls et al. 2008
AT, Karawanken, Babniakgraben	N 46.5201	E 14.2345	683		Lv	East003	EU215110	EU215163	EU215216	Graf	Pauls et al. 2008
SLO, Julian Alps, Radovna stream	N 46.4303	E 13.963	717	4 IM (3M + 1F)	IM/M	fEas0101M	KM001760	KM001757	KM001754	Olah	this study
SLO, Julian Alps, Radovna stream	N 46.4303	E 13.963	717		IM/M	fEas0102M		KM001758	KM001755	Olah	this study
SLO, Julian Alps, Radovna stream	N 46.4303	E 13.963	717		IM/M	fEas0103M		KM001759	KM001756	Olah	this study
***Ecclisopteryx guttulata* (Pictet, 1834)**											
AT, Ybbs at Lunz	N 47.856	E 15.023	600	12 Lv						Graf	this study
AT, Jogland, Lafnitz tributary	N 47.43	E 15.48	1170		IM/M	Egut009	EU215114	EU215167	EU215220	Graf & Pauls	Pauls et al. 2008
ES, Pyrenees, Val d'Aran, Salardu	N 42.706361	E 0.896944	1220	4 IM (3M + 1F)	IM/M	fEgu0101M	KM001750	KM001742	KM001746	Graf	this study
ES, Pyrenees, Val d'Aran, Salardu	N 42.706361	E 0.896944	1220		IM/F	fEgu0102F	KM001751	KM001743	KM001747	Graf	this study
ES, Pyrenees, Val d'Aran, Salardu	N 42.706361	E 0.896944	1220		IM/M	fEgu0103M	KM001752	KM001744	KM001748	Graf	this study
ES, Pyrenees, Val d'Aran, Salardu	N 42.706361	E 0.896944	1220		IM/M	fEgu0104M	KM001753	KM001745	KM001749	Graf	this study
***Ecclisopteryx madida* (McLachlan, 1867)**											
RO, Bucegi Mts., Valea Dobresti, Cariera Lespezi, Pietrele Albe	N 45.287451	E 25.405600	889		IM/M	EM001	EU215115	EU215168	EU215221	Pauls & Ujvarosi	Pauls et al. 2008
SVK, Hronec	N 48.8	E 19.6	550		Lv	EM002		KM001727	KM001733	Graf	this study
SVK, Hronec	N 48.8	E 19.6	550		Lv	EM003		KM001728	KM001734	Graf	this study
AT, Nockberge, St. Oswald stream	N 46.864432	E 13.787671	1570		IM/M	EM004	EU215116	KM001729	EU215222	Graf	Pauls et al. 2008
AT, Nockberge, St. Oswald stream	N 46.864432	E 13.787671	1570		IM/M	EM005		KM001730	KM001735	Graf	this study
AT, Nockberge, St. Oswald stream	N 46.864432	E 13.787671	1570		IM/M	EM006	KM001739	EU215169	KM001736	Graf	Pauls et al. 2009

Table 1. continued.

Locality (country, name)	Longitude dec.	Latitude dec.	Altitude (m)	No of specimens and stage/sex		Specimen Code	GenBank Accession Nos			Collector	Publication
				Morphology	Molecular genetic analyses		mtCOI	nuWG	mtLSU		
***Ecclisopteryx madida* (McLachlan, 1867)**											
AT, Bruck/Lafnitz	N 47.4396	E 15.9138	560		IM/M	fEma0101M	KM001740	KM001731	KM001737	Graf	this study
AT, Bruck/Lafnitz	N 47.4396	E 15.9138	560		IM/M	fEma0102M	KM001741	KM001732	KM001738	Graf	this study
***Ecclisopteryx malickyi* Moretti, 1991**											
IT, springbrook near Camposilvano SE of Rovereto, Monti Lessini, Trentino	N 45.748231	E 11.151095	1171		IM/F	Emal001	EU215223	EU215170	EU2015117	Graf	Pauls et al. 2008
IT, springbrook near Camposilvano SE of Rovereto, Monti Lessini, Trentino	N 45.748231	E 11.151095	1171		Lv	Emal002	KM001726		KM001725	Graf	this study
** ***Drusus discolor* (Rambur, 1842)**											
HR, Čabranka, spring reach	N 45.60126	E 14.64043	589		IM/M	DdCAIM1	KC881331	KM001835	KM001841	Sivec	Previšić et al. 2014, this study
** ***Allogamus uncatus* (Brauer, 1857)**											
AT, Gampadelsbach, Vorarlberg	N 47.03638	E 9.88972	1555		IM/M	AUn003	KM001846	KM001834	KM001840	Graf	this study

Illustrations were prepared as described by Thomson & Holzenthal (2010). Briefly, pencil drawings were produced using a camera lucida mounted on a compound microscope, and digitally edited and inked.

2.2. DNA extraction and PCR amplification

DNA extraction and amplification were performed as outlined by Pauls et al. (2008) and Previšić et al. (2009) for the 541-bp-long fragment of the mitochondrial cytochrome oxidase *c* subunit I (mtCOI) using primers S20 and Jerry (Simon et al. 1994; Pauls et al. 2006), a 346-bp-long fragment of the nuclear wingless gene (nuWG) using primers WGbDrrev (5'-ACCCTCTCC-CGCARCACATTGAG) and WgbDrfwd 5'-CTTGCTG-GATGCGTCTGCC), and a 362-bp-long fragment of the mitochondrial large ribosomal subunit gene (mtLSU) using primers LeptoF and LeptoR (Malm & Johanson 2008). Sequences were edited manually using the program Geneious R7 (Biomatters Ltd., New Zealand) and aligned using MAFFT v.7 (Katoh & Standley 2013). Sequences were deposited in GenBank under accession nos: KM001724–KM001854. In addition, published sequences of all *Ecclisopteryx* species (Pauls et al. 2008; Table 1) were included in the alignment and intra- and interspecific uncorrected *p*-distances were calculated in Mega 4.0.1 (Tamura et al. 2007) based on the 541-bp-long fragment of the mtCOI. For *p*-distances a colour heat map was drawn using the package 'pheatmap' in R (version 3.0.2, R Core Team 2013).

2.3. Phylogenetic reconstruction

To examine *Ecclisopteryx* species delineation and association of specimens from the Western Balkans, we infered a phylogeny using all available mtCOI, mtLSU and nuWG sequences of *Ecclisopteryx* species (Table 1). As outgroups taxa we used *Drusus discolor* (Limnephilidae: Drusinae) and *Allogamus uncatus* (Limenphilidae: Stenophylacini) (Table 1). According to the Akaike Information Criterion (AIC) test implemented in MrModeltest 2.2 (Nylander 2004) the following models of DNA substitution were identified as best-fit for particular data sets: mtCOI: Hasegawa–Kishino–Yano+Invariant+Gamma (HKY+I+G), mtLSU: General time reversible+Gamma (GTR+G), and nuWG; Hasegawa–Kishino–Yano+Gamma (HKY+G). The phylogeny was estimated using a Bayesian Metropolis-coupled Monte Carlo Markov Chain (MCMCMC) method with the program MrBayes 3.2. (Ronquist & Huelsenbeck 2003) using concatenated sequences of the three genes. The matrix contained 6.5% missing data with 9 individuals missing one and 4 individuals missing two gene regions, respectively (Table 1). Two parallel runs were performed with four chains each (10 million generations, sampling every 1000th generation). The likelihood scores were plotted against generation time using Tracer 1.4 (Drummond &

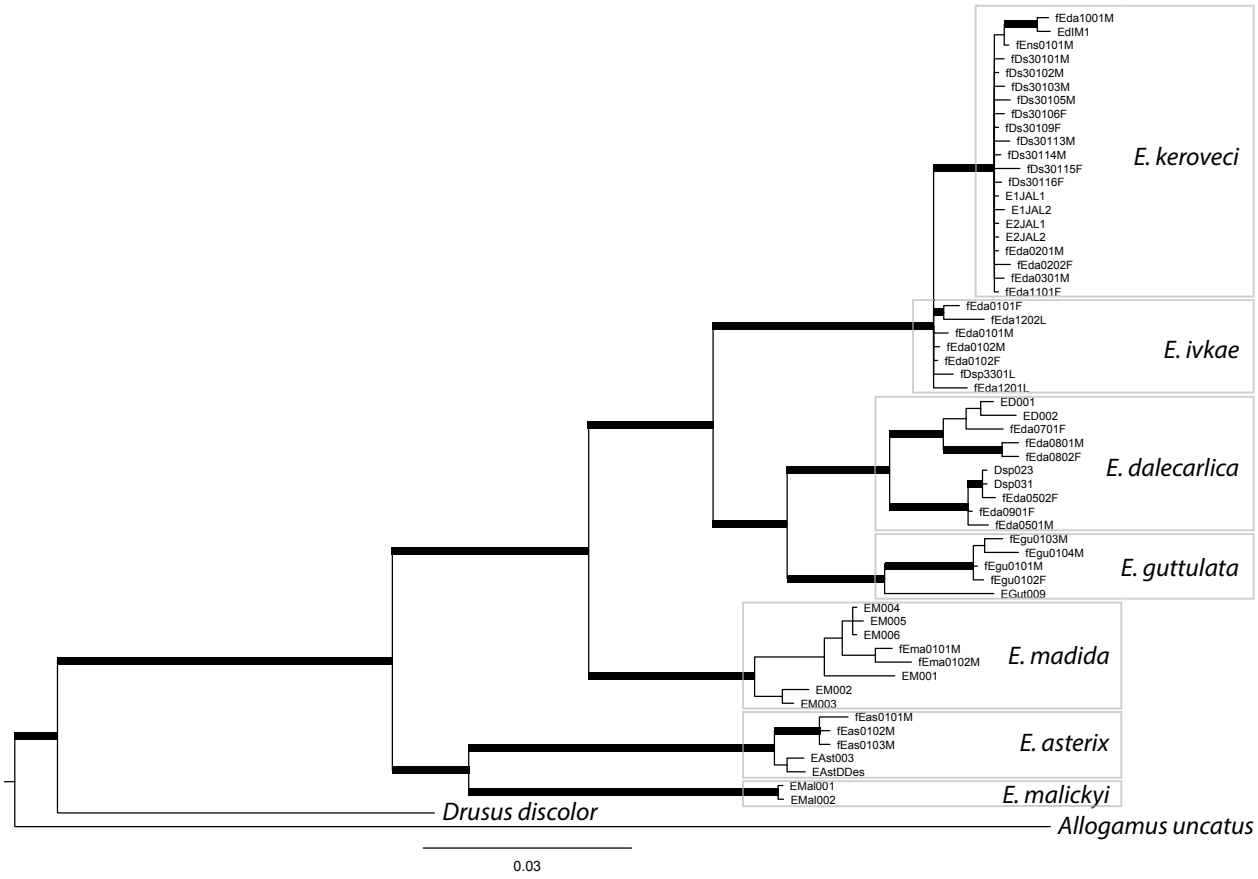

Fig. 2. Rooted Bayesian phylogenetic tree of seven *Ecclisopteryx* species based on the partial mitochondrial (mtCOI, mtLSU) and nuclear (nuWG) gene sequences. *Drusus discolor* and *Allogamus uncatus* were used as outgroup taxa. Bold lines bear nodes with posterior probabilities ≥ 0.95.

Rambaut 2007) to determine the number of generations needed to reach the stationary phase. Consequently, the initial 3000 trees were discarded as burn-in and the remaining trees used to create a 50% majority rule consensus tree.

3. Results

3.1. *Ecclisopteryx* species delimitation

In a B/MCMCMC phylogeny based on concatenated partial sequences of mtCOI, mtLSU and nuWG, with the exception of *E. ivkae* sp.n., monophyly of each putative *Ecclisopteryx* species was highly supported (pp≥0.95; Fig. 2). *Ecclisopteryx dalecarlica* specimens from different parts of its range (i.e., Norway, Germany, Bulgaria and Romania, Table 1) formed a highly supported clade, as did *E. guttulata* specimens (pp≥0.98; Fig. 2). All specimens of *E. keroveci* sp.n. including the *Ecclisopteryx* larvae collected from the Jabučica River also formed a highly supported monophyletic clade (pp=0.99; Fig. 2). *Ecclisopteryx keroveci* sp.n. and *E. ivkae* sp.n. formed a highly supported monophyletic clade (pp=1; Fig. 2); however, *E. ivkae* sp.n. haplotypes occured in a basal polytomy, thus this species was not recovered as monophyletic (Fig. 2). *Ecclisopteryx dalecarlica* + *E. guttulata* were the sister clade to *E. keroveci* sp.n. + *E. ivkae* sp.n., and this relationship was also highly supported (p=0.99; Fig. 2).

Mitochondrial COI haplotypes (fragment length 440 bp) of *E. keroveci* sp.n. adults and larvae sampled at the mouth of the Jabučica River were either identical or differed at a maximum of 5 nucleotide positions (= 1.1%). Overall, mtCOI haplotypes of *E. keroveci* sp.n. differed across the whole region by at most 8 nucleotide positions (21 specimens); hence, intraspecific uncorrected *p*-distances ranged from 0–1.8% (Fig. 3). A similar case was observed in *E. ivkae* sp.n., as mtCOI haplotypes from the two sequenced sampling sites (both at the Cetina River) were found to differ by maximally 4 nucleotide positions (7 specimens), with intraspecific uncorrected *p*-distance ranging from 0–0.9% (Fig. 3). Overall, interspecific uncorrected *p*-distances of mtCOI haplotypes ranged from 1.6–2.7% between *E. keroveci* sp.n. and *E. ivkae* sp.n., 8.2–10.5% between *E. dalecarlica* and *E. keroveci*, and 7.7–9.5% between *E. dalecarlica* and *E. ivkae* sp.n. (Fig. 3). Uncorrected *p*-distances of the same mtCOI fragment between the other *Ecclisopteryx* species ranged from 6.1–13.2% (Fig. 3).

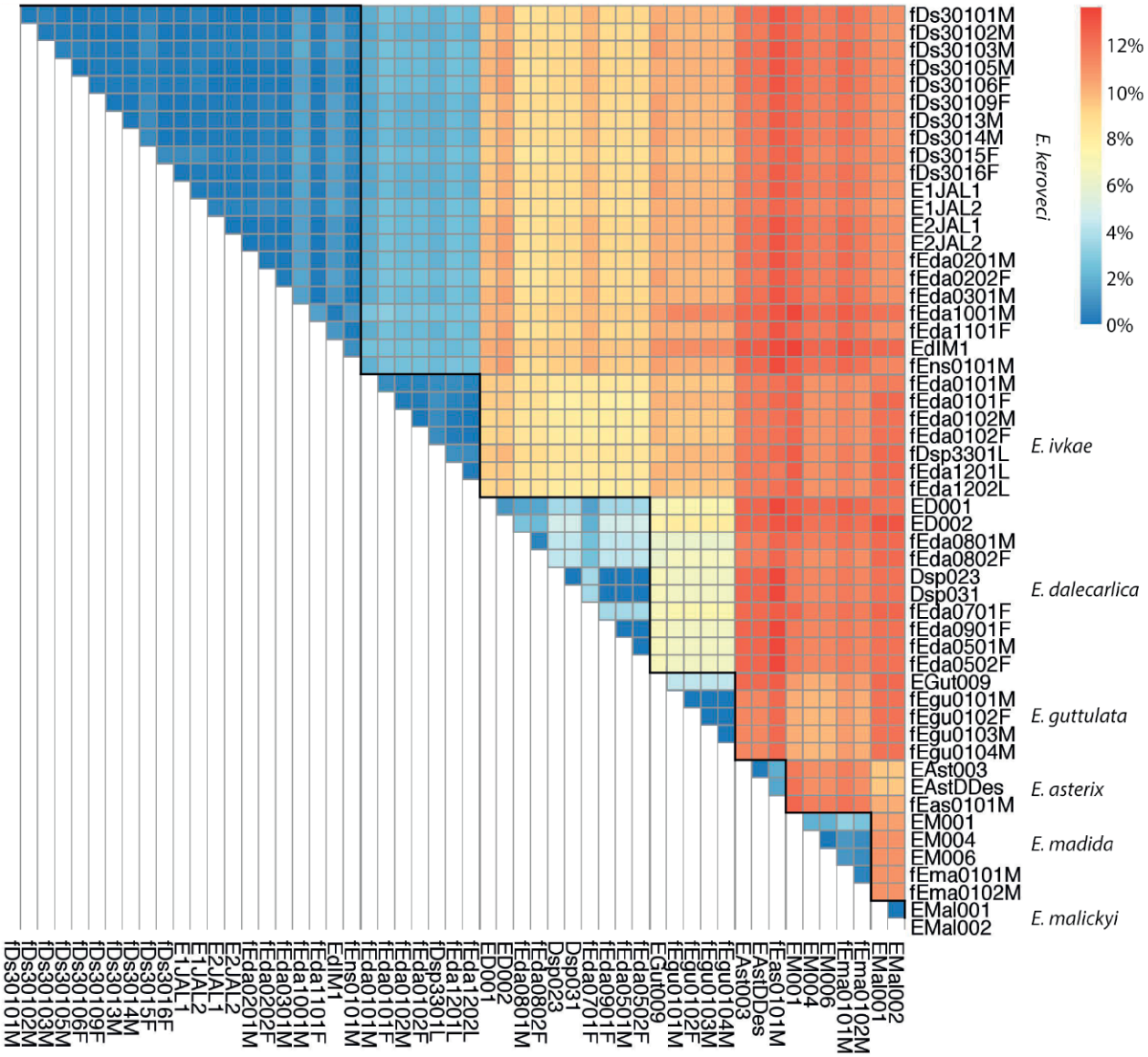

Fig. 3. Colour heat map showing inter- and intraspecific uncorrected *p*-distances of the partial mitochondrial COI gene sequence (440 bp) between seven *Ecclisopteryx* species. Intraspecific *p*-distances are outlined by the black line. For detailed information on haplotypes see Table 1.

3.2. Description of *Ecclisopteryx keroveci* Previšić, Graf & Vitecek sp.n.

Adults. General appearance: light brown; sternites and tergites brown; cephalic, thoracic and abdominal setal areas pale, yellowish; body setation light brown; legs light brown; haustellum and intersegmental teguments pale, whitish. Male maxillary palps 3-segmented. Spur formula (male and female): 1-2-3. Forewing length: male 9.8–12.1 mm (N=20), female 10–12.5 mm (N=20).

Male terminalia (Fig. 4A–E): Tergite VIII brown with lighter areas around alveoli and somewhat darker stripe medially lacking setae; setation concentrated posteriorly, around spinate area, anterior part of tergite VIII with few setae. Spinate area mushroom-shaped in dorsal view and flanked by membraneous areas.

Segment IX rhombus-shaped in lateral view, transversely dilated: in ventral view distance from lateral most point of segment IX to straight anteroposterior line originating from lateral most point of inferior appendages is approximately 10% of total width of segment IX on each side.

Superior appendages (cerci sensu Snodgrass 1935) in lateral view round, simple, without further modifications. Intermediate appendages (paraprocts sensu Snodgrass 1935) reduced as typical for genus, membraneous dorsal and ventral protuberances rounded. Inferior appendages (gonopods sensu Snodgrass 1935) in lateral view bipartite with well sclerotized dorsal part and less sclerotized, seemingly membranous, ventral part; dorsal part prolonged caudally with distinct dorsally turned tip: with broad and shallow lateral concavity, somewhat bifurcated with 2 rounded tips of unequal length (dorsal

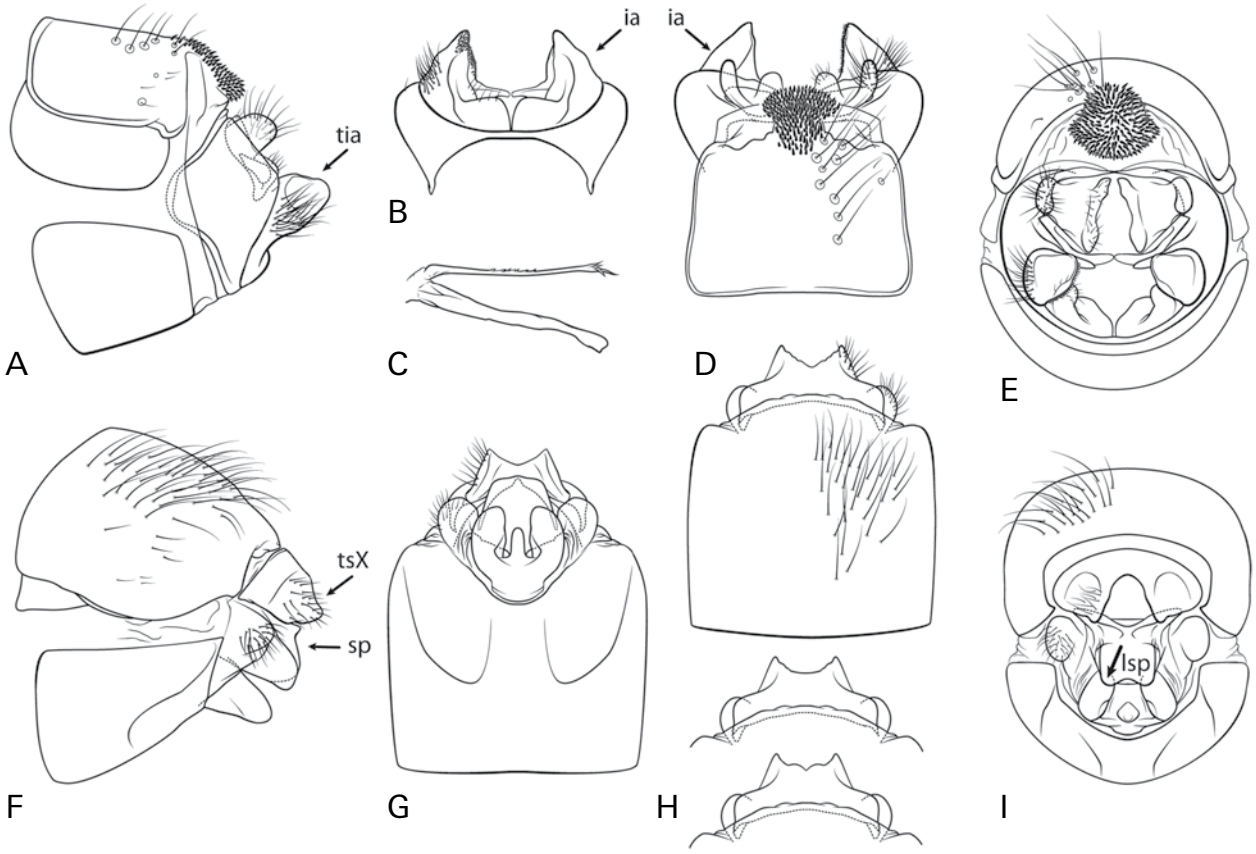

Fig. 4. *Ecclisopteryx keroveci* sp.n.; male genitalia, **A**: lateral view, **B**: ventral view, **C**: aedeagus and parameres, **D**: dorsal view and **E**: caudal view; female genitalia, **F**: lateral view, **G**: ventral view, **H**: dorsal view and variability in female genitalia, dorsal view and **I**: caudal view. — Abbreviations: tia = tip of inferior appendages, ia = inferior appendages, tsX = tip of segment X, sp = supragenital plate, lsp = lobes of supragenital plate.

one shorter than ventral one), in dorsal view triangular and slender with tips separated by small indentation, caudal parts and median margins strongly sclerotized and covered with spines; setation of appendices inferiores concentrated laterally on dorsal part, forming setal brush.

Aedeagus slender with distinct terminal protuberance (in lateral view only) and parameres of equal length. Parameres fused at their bases, with 2 major concentrations of thorn-like spines on dorsal surface; several well developed distal thorn-like spines and medial group of smaller thorn-like spines, the latter with bulbous bases.

Female terminalia (Fig. 4F–I): Lateral lobe of segment IX membraneous, triangular in lateral view with dorsal sclerotized setose part, the latter evenly rounded in dorsal and ventral view. Segment X wider proximally than distally, in dorsal view with 2 lateral lobes and median triangular excision of varying shape (Fig. 4H); lateral lobes laterally slightly concave, tips sharp and distinct in dorsal and ventral views, curved somewhat dorsally in lateral view; approximately as long as supragenital plate; ventrally unsclerotized. Supragenital plate in lateral view quadrangular with small dorsal process; in ventral view quadrangular; in caudal view quadrangular with 2 indistinct ventral lobes. Vulvar scale with 3 lobes in ventral view: 2 lateral lobes, roundly oval with converging tips; 1 median, well developed, about half as long as lateral lobes and of greater length than width.

Fifth instar larvae. Body length of larva 10–13.1 mm, head width 1.3–1.5 mm (N = 10). Case slightly curved, consisting of mineral particles (Fig. 7A), 10.2–13.7 mm long, slightly attenuating posteriorly (width at anterior opening 2.8–4.5 mm and at posterior opening 1.4–2.9 mm).

Head: Light to chestnut brown with dark muscle attachment spots, with yellowish-white rings around the eyes (Fig. 7B). 18 pairs of primary setae (# 1, 4, 10, 11 white; 13, 16 light brown, rest dark brown) and additional spines on parietalia present (i.e. between eyes and anterior head margin; N = 12–20, light brown, 100–300 µm length) (Figs. 7B, 8A). Frontoclypeus bell-shaped; carinae bearing antennae; ventral apotome bell-shaped, yellowish-brown. Mandibles typical for grazers, lacking teeth.

Thorax: Pronotum light to chestnut brown, with dark muscle attachment spots, posterior margin thickened and darkly striped (Fig. 7B,C). Dorsal profile in lateral view lacking distinct ridge, in dorsal and lateral view medially with a delicate step (Figs. 7B, 8B). Two setal rows along anterior border of pronotum: (1) dense fringe of short, curved, fine, yellow setae; (2) widely-spaced, continuous row of long, straight, dark setae meeting at anterior

pronotal midline; in total, 110–130 dark setae of varying lengths (100–300 µm) distributed over each pronotal half. Small, white recumbent setae present on pronotal surface. Prosternal horn present.

Mesonotal sclerites light brown, with dark muscle attachment spots and lateral and posterior margins darkly sclerotised (Fig. 7C). Anterior mesonotal setal group (*sa*1) consisting of 7–13 setae; posterior group, *sa*2 (26–41 setae) and lateral group, *sa*3 (30–40 setae) connected, not clearly separated.

Metanotum divided into 3 pairs of light brown sclerites. Anteromedian sclerites (*sa*1) ellipsoid, distance between them smaller than their length (Fig. 7C); 19–30 setae per sclerite. Posteromedian sclerites small (*sa*2), with 16–21 setae; lateral sclerites (*sa*3) with 30–40 setae. A row of setae present between posteromedian sclerites (*sa*2); a small setal group of 20–30 setae present between each lateral (*sa*3) and posteromedian sclerite.

Legs light brown to yellowish with numerous setae on coxae, trochanters and femora; tibiae and tarsi bearing less setae. Foreleg coxa, femur and tibia each wider than those of mid- and hind legs. Whole dorsal and ventral margins of all coxae and femora covered with setae. Forefemora each with 3–6 yellow and 2–5 dark setae on ventral-edge, midfemora each with 6–13 dark and hind femora each with 6–10 dark setae on ventral edge. Additional setae present at both anterior and posterior faces of all femora; ventral trochanteral brush present at distal section of foretrochanters. Setae present at ventral margin (proximal and distal sections) of all trochanters. Dorsal setae only at distal third of mid- and hind tibiae.

Abdomen: First abdominal segment with 1 dorsal and 2 lateral fleshy protuberances. Setal areas *sa*1, *sa*2 and *sa*3 fused, resulting in continuous transverse row of setae anterior to the dorsal protuberance, reaching to the dorsal section of each lateral protuberance. Setal group posterior to dorsal protuberance lacking (Fig. 7C). Lateral protuberances lacking posterior sclerites; in front of each lateral protuberance lies continuous band of anterolateral setae, linking with each dorsal and ventral *sa*3 setal group. First abdominal sternum with fused setal areas *sa*1, *sa*2 and *sa*3, resulting in a continuous field of setae, with small sclerites at the base of individual setae.

Single filamentous gills present on segments II–VII. Dorsal pre- and post-segmental gills present on segments II–VII; ventral pre- and post-segmental gills present on segments II–VII. Lateral pre-segmental gills present on segments II and V and post-segmental gills on segments II–IV. Lateral fringe extends from anterior border of segment III to anterior border of segment VIII. Number of posterodorsal setae on segment VIII 2–6.

Etymology. The name of *keroveci* was given in honour of our colleague, Prof. Dr. Mladen Kerovec, who has enthusiastically supported our studies of Drusinae in the Balkans for many years.

Type material. *Ecclisopteryx keroveci* sp.n.: Holotype ♂: Bosnia and Hercegovina, mouth of Jabučica River, N 43.29022 E 18.61733, 765 m asl, 04.vii.2012, leg. Previšić A., Ivković M., Mihaljević Z., Miliša M.; deposited in the Biology Centre, Oberösterreichisches Landesmuseum, Linz, Austria. Paratypes: 30 ♂ and 49 ♀, same data; deposited in the first author's collection at the Faculty of Science in Zagreb. 10 5th instar larvae, same location, 14.v.2008 and 02.vi.2009, leg. Previšić A., Graf W.

3.3. Description of *Ecclisopteryx ivkae* Previšić, Graf & Vitecek sp.n.

All morphological characters of adults and 5th-instar larvae identical to *Ecclisopteryx keroveci* sp.n. except:

Adults. General appearance: brown; cephalic, thoracic and abdominal setal areas pale, yellowish to light brown; body setation light brown to brown. Forewing length: male 10.4–12.5 mm (N=7), female 12.4–14.0 mm (N=9).

Male terminalia (Fig. 5A–E): Tergite VIII brown, lacking a darker median stripe; setation evenly distributed over the whole surface of tergite VIII with larger setae posteriorly. Superior appendages (cerci sensu Snodgrass 1935) in lateral view somewhat elongated. Dorsal part of inferior appendages (gonopods sensu Snodgrass 1935) in lateral view not turned dorsally, with a deep and broad lateral concavity, faintly bifurcated with 2 tips of more or less equal length, in dorsal view broadly triangular with the tips separated by a distinct indentation. Parameres fused at their bases, with 3 major concentrations of thorn-like spines on dorsal surface: several well developed distal thorn-like spines and medial group of smaller thorn-like spines divided into 2 groups of thorn-like spines.

Female terminalia (Fig. 5F–I): Segment X in dorsal view with 2 lateral lobes and a deep and round median excision leaving 2 distinct median protrusions in lateral, ventral and dorsal view; tips of lateral lobes rounded, not curved dorsally in lateral view, somewhat longer than the supragenital plate. Supragenital plate quadrangular in lateral and ventral view; in caudal view hourglass-shaped. Vulvar scale with 3 lobes in ventral view: 2 lateral lobes, quadrangular with converging tips; 1 median lobe, about half as long as the lateral lobes and triangular in shape.

Fifth instar larva. Body length of larva 8.0–9.5 mm, head width 1.5–1.7 mm (N=8). Case slightly curved, consisting of mineral particles (Fig. 7D), 7.5–10.8 mm long, slightly attenuating (width at anterior opening 2.8–4.8 mm and at posterior opening 1.7–1.8 mm).

Head: Brown to brownish black with granular surface sculpturing. Primary setae # 1, 4, 10, 11 white; the remaining setae dark brown; 1–7 dark brown spines on parietalia (200–375 µm length) present (Figs. 7F, 8C,D).

Thorax: Pronotum brown to brownish black with granular surface sculpturing (Fig. 7E). Dorsal profile in lateral view with small but distinct ridge, in dorsal view medially with a delicate step-like structure (Figs. 7F, 8D). In total, 110–120 dark setae of varying lengths (100–300 µm) distributed over each pronotal half. Mesonotum sclerites chestnut brown, with dark muscle attachment

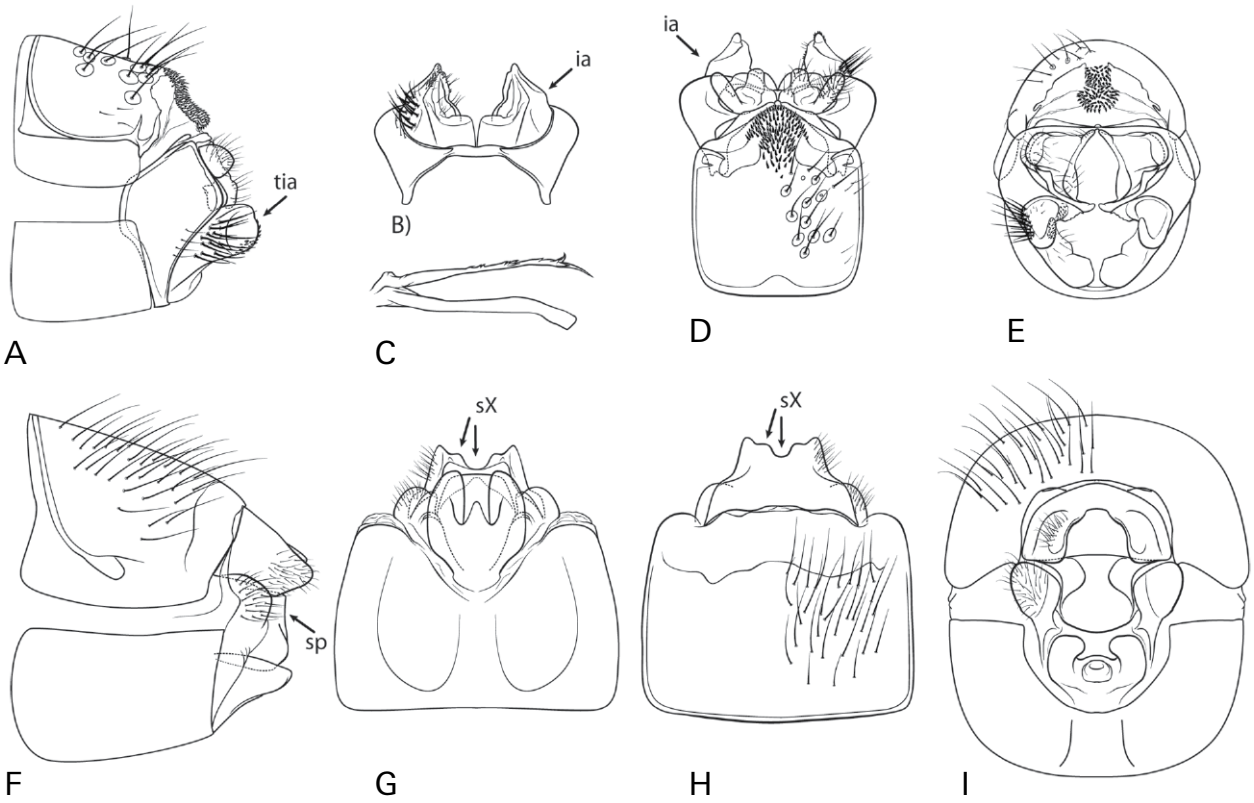

Fig. 5. *Ecclisopteryx ivkae* sp.n.; male genitalia, **A**: lateral view, **B**: ventral view, **C**: aedeagus and parameres, **D**: dorsal view and **E**: caudal view; female genitalia, **F**: lateral view, **G**: ventral view, **H**: dorsal view and **I**: caudal view. — Abbreviations: tia = tip of inferior appendages, ia = inferior appendages, sp = supragenital plate, sX = segment X.

spots and lateral and posterior margins darkly sclerotised (Fig. 7E,F). Anterior mesonotal setal group (*sa*1) consisting of 3–7 setae; posterior group, *sa*2 (20–32 setae) and lateral group, *sa*3 (28–40 setae) connected, not clearly separated. Anteromedian metanotum sclerites (*sa*1) quadrangular, distance between them smaller than their length (Fig. 7E); 16–28 setae per sclerite. Posteromedian metanotum sclerites small (*sa*2), with 15–19 setae; lateral sclerites (*sa*3) with 20–32 setae. A row of setae present between posteromedian sclerites (*sa*2); a small setal group of 16–22 setae present between each lateral (*sa*3) and posteromedian sclerite.

Legs chestnut brown with numerous setae on coxae, trochanters and femora; tibiae and tarsi with only small number of setae. Forefemora each with 3–4 yellow and 2–5 dark ventral-edge setae, midfemora each with 6–9 dark and hind femora each with 3–7 dark ventral edge setae. Number of posterodorsal setae on segment VIII 4–9.

Etymology. The name of *ivkae* was given in honour of Ivka Previšić, the first author's grandmother.

Type material. *Ecclisopteryx ivkae* sp.n.: Holotype ♂: Cetina River, Glavaš spring N 43.976697 E 16.430150, 386 m asl, 02.vi.2011, leg. Previšić A.; deposited in the Biology Centre, Oberösterreichisches Landesmuseum, Linz, Austria. Paratypes: 4 ♂ and 2 ♀, same data; 1 ♂ and 1 ♀ 31.v.2005, leg. Previšić A.; 1 ♀ 07.vi.2007, leg. Graf W; 2 ♀ 02.vi.2012, leg. Previšić A.; deposited in the first author's collection at the Faculty of Science in Zagreb. 8 5th instar larvae: same location, 04.x.2013 (N = 4, leg. Kučinić M.) and 07.xi.2013 (N = 4, leg. Previšić A.).

3.4. Differential diagnosis of *Ecclisopteryx keroveci*, *E. ivkae*, *E. dalecarlica* and other Drusinae species

Adult males. *Ecclisopteryx keroveci* and *E. ivkae* males are morphologically most similar to *E. dalecarlica* males, but differ distinctly in several features. They can be separated using the following key:

1 Tips of inferior appendages distinctly indented in lateral view (Fig. 6A; arrow tia), inferior appendages elongate and slender in ventral view (Fig. 6B; arrow ia): ..... ***E. dalecarlica***

1' Tips of inferior appendages lacking a distinct indentation in lateral view, inferior appendages more or less triangular and broad in ventral view (Figs. 4A,B & 5A, B; arrows tia & ia): ..... **2, *E. ivkae* & *E. keroveci***

2 Posterior edge of tip of inferior appendages ventrally elongated and arched dorsally (Fig. 4A; arrow tia), tips in dorsal view lacking a distinct shoulder (Fig. 4D; arrow ia): ..... ***E. keroveci***

2' Posterior edge of tip of inferior appendages more or less straight in lateral view, lacking a clear ventral elongation (Fig. 5A; arrow tia), tips in dorsal view with a distinct shoulder (Fig. 5D; arrow ia): ..... ***E. ivkae***

Adult females. *Ecclisopteryx keroveci* and *E. ivkae* females are morphologically most similar to *E. dalecarlica* females, but differ in several features. Species can be distinguished using the following key:

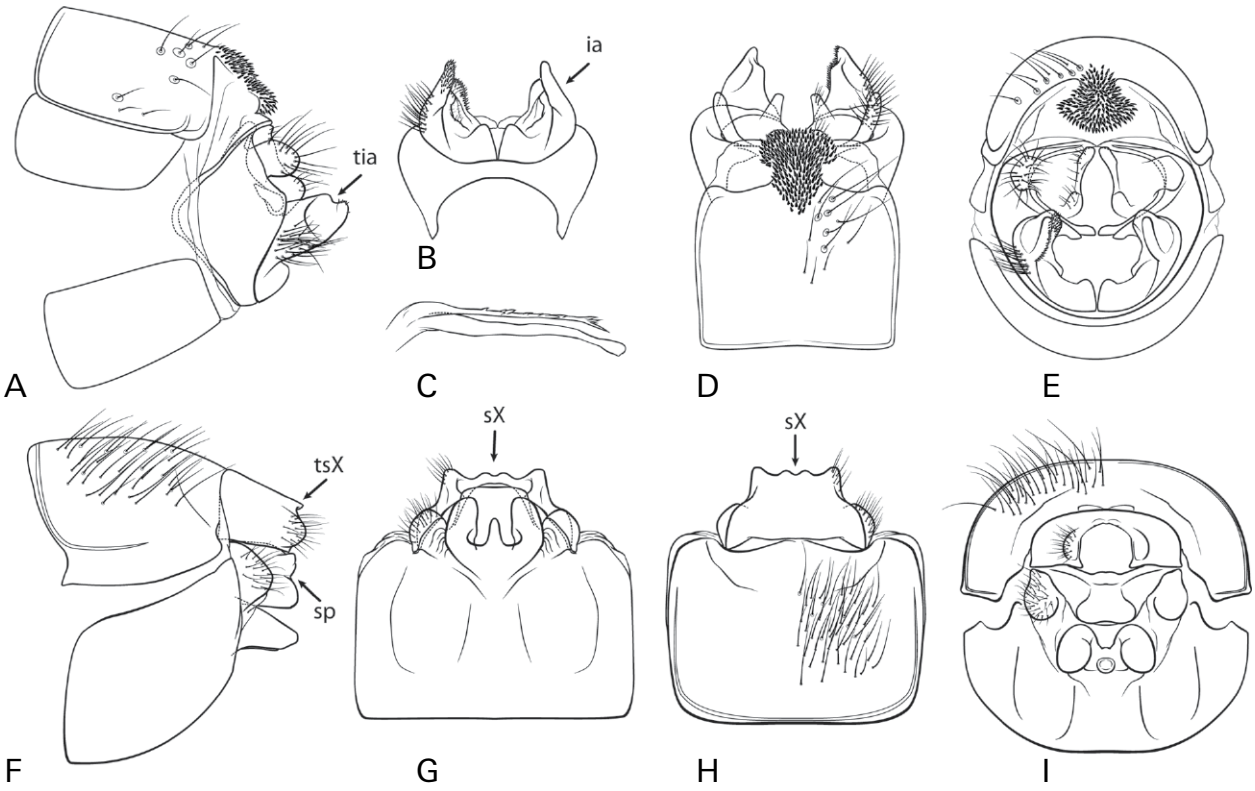

Fig. 6. *Ecclisopteryx dalecarlica* Kolenati, 1848; male genitalia, **A**: lateral view, **B**: ventral view, **C**: aedeagus and parameres, **D**: dorsal view and **E**: caudal view; female genitalia, **F**: lateral view, **G**: ventral view, **H**: dorsal view and **I**: caudal view. — Abbreviations: tia = tip of inferior appendages, ia = inferior appendages, sp = supragenital plate, tsX = tip of segment X, sX = segment X.

1 In lateral view, segment X with a distinct protuberance (Fig. 6F; arrow tsX) and supragenital plate with a sharp median incision (Fig. 6F; arrow sp); and in dorsal and ventral view segment X with 2 distinct median lobes, somewhat shorter than outer lobes (Fig. 6G,H; arrows sX): ..... ***E. dalecarlica***

1' In lateral view, tips of segment X somewhat curved dorsadly (Fig. 4F; arrow tsX) and supragenital plate longer ventrally than dorsally (Fig. 4F; arrow sp), in caudal view with 2 distinct ventral lobes (Fig. 4I; arrow lsp): ..... ***E. keroveci***

1" In lateral view, supragenital plate lacking an indentation (Fig. 5F; arrow sp); in dorsal and ventral view, segment X with distinct, round median incision, leaving 2 mediolateral lobes (Fig. 5G,H; arrows sX): ... ***E. ivkae***

Larvae. A summary of morphological features for the identification of Limnephilidae and Drusinae larvae was given by Waringer (1985). Within the framework of the limnephilid key by Waringer & Graf (2011), *E. keroveci* is keyed together with *E. dalecarlica* and *Drusus trifidus*, whereas *E. ivkae* is keyed together with *E. guttulata* and *E.madida*.

(A) The fifth instar larva of *E. keroveci* can be separated from the larva of *D. trifidus* and *E. dalecarlica* using the following key:

1 Colouration of head capsule and body sclerites blackish brown; additional spines lacking on parietalia; additional spines on pronotum short and yellow; lateral fringe extending from anterior margin of abdominal segment III to first 1/3 of segment VIII: ..... ***D. trifidus***

1' Colouration of head capsule and body sclerites yellow or brown (Figs. 7B,C, 8A,E); additional spines present on parietalia (Figs. 7B, 8A,E,F); additional spines on pronotum long and brown (Fig. 7B) or short and blackish brown (Fig. 8F); lateral fringe extending from anterior margin of abdominal segment III to end of segment VIII: ..... **2, *E. dalecarlica & E. keroveci***

2 Colouration of head capsule and body sclerites yellow (Fig. 8E,F); additional spines on parietalia and pronotum blackish, stout and of roughly the same length (80–100 µm; Fig. 8E,F): ..... ***E. dalecarlica***

2' Colouration of head capsule and body sclerites light brown to chestnut brown (Figs. 7B, 8A); the additional spines on parietalia and pronotum light brown, pointed, varying in length (100–300 µm; Figs. 7B, 8A): ..... ***E. keroveci***

(B) The fifth instar larva of *E. ivkae* can be separated from the larvae of *E. guttulata* and *E. madida* using the following key:

1 Colouration of head capsule and body sclerites brownish-red (Fig. 8G,H); pronotum with a pronounced median notch in anterior view (Fig. 8G): ..... ***E. guttulata***

Table 2. Synoptic key for the diagnosis of the larvae of the genus *Ecclisopteryx* and *Drusus trifidus* (Limnephilidae: Drusinae). Distribution of *Ecclisopteryx* species following Graf et al. 2008, 2011; Graf & Schmidt-Kloiber 2011, unpublished data; endemicity indicated by a cross (†); [1] also present on British Islands.

Species	Parietalia: presence and number of additional spines per parietale	Lateral fringe extending on abdominal segments:		Mesonotal sclerites, colour:	Pronotum with		Distribution
		beginning at	ending at		sharp ridge (lateral view)	pronounced median notch (anterior view)	
E. keroveci†	yes (12–20)	anterior margin of III	end of VIII	light brown to chestnut brown	no (Fig. 8B)	no (Fig. 8A)	Western Balkan region
E. ivkae†	yes (1–7)	anterior margin of III	end of VIII	brownish-black	yes (Fig. 8D)	no (Fig. 8C)	Dalmatia
E. dalecarlica[1]	yes (stout, 14–20)	anterior margin of III	end of VIII	yellow	no (Fig. 8F)	no (Fig. 8E)	central & northern Europe
E. guttulata	yes (≥ 16)	anterior margin of III	first third of VIII	brownish-red	yes (Fig. 8H)	yes (Fig. 8G)	central & southern Europe
E. madida	yes (> 20)	anterior margin of III	first third of VIII	black	yes	no (Fig. 8I)	central–eastern Europe
E. asterix†	no	last third of III	first third of VIII	brownish-black	no	no	Karawanken/ Soboth
E. malickyi†	no	last third of III	first third of VIII	brownish-black	no	no	Lessinian Alps
D. trifidus	no	anterior margin of III	first third of VIII	anterior: brown to brownish-black, posterior: beige	no	no	central & western Europe

1' Colouration of head capsule and body sclerites blackish brown (Figs. 7D–F, 8C,D); pronotum without a pronounced median notch in anterior view (Fig. 8C,I): ..... **2, *E. ivkae* & *E. madida***

2 Number of additional spines on each parietale is 1–7 (Figs. 7F, 8C,D); lateral fringe ending at the end of abdominal segment VIII: ..... ***E. ivkae***

2' Number of additional spines on each parietale is > 20 (Fig. 8I); lateral fringe ending at the first 1/3 of abdominal segment VIII: ..... ***E. madida***

The most important morphological features enabling separation of all *Ecclisopteryx* species (and *D. trifidus*) are summarised in Table 2 and Fig. 8. For reliable identification, distribution ranges of these species should also be kept in mind: *D. trifidus* does not occur in the Dinaric Western Balkan ecoregion (ER5; Graf et al. 2008; Graf & Schmidt-Kloiber 2011) (Table 2); *Ecclisopteryx asterix* and *E. malickyi* are very restricted in their distribution ranges and are not known from ecoregion ER5 (Graf et al. 2008, 2011; Graf & Schmidt-Kloiber 2011). Also, larvae of the two latter species differ from all other *Ecclisopteryx* species by lacking additional spines on the parietalia, and are easily differentiated from larvae of *E. keroveci* and *E. ivkae* by various features summarized in Table 2.

3.5. Ecology, habitat and phenology of *Ecclisopteryx keroveci*

Larval habitat characteristics at the type locality of *E. keroveci* (mouth of the Jabučica River) indicate a preference for rhithral sections of streams. The collection site is approximately 9 km downstream from the spring, where the stream is about 4 m wide and has a mean current velocity of 0.1 ms^{-1} in July; water temperature in July 2012 was 12.7°C (10.00 am). However, strong discharge dynamics have been observed. Substrate was mainly composed of larger fractions, i.e., megalithal (30%), macrolithal (35%) and mesolithal (25%), and rocks were substantially covered with algae. Larvae of *E. keroveci* were collected on the surface of algae-covered stones, which, in agreement with larval mandibular morphology, indicates that *E. keroveci* is a grazer (Pauls et al. 2008; Waringer et al. 2010). Adults of *E. keroveci* were collected in early July 2012; however data on its flight period are still incomplete.

At the mouth of the Jabučica River, *E. keroveci* was sympatric with the trichopterans *Rhyacophila armeniaca* Guerin, 1834, *Rhyacophila balcanica* Radovanović, 1953, *Rhyacophila moscaryi* Klapálek, 1894, *Rhyacophila nubila* (Zetterstedt, 1840), *Rhyacophila obliterata* McLachlan, 1863, *Rhyacophila trescavicensis* Botosaneanu, 1960, *Rhyacophila tristis* Pictet, 1834, *Glossosoma conformis* Neboiss, 1963, *Glossosoma discophorum* Klapálek, 1902, *Agapetus ochripes* Curtis, 1834, *Synagapetus slavorum* Botosaneanu, 1960, *Diplectrona atra* McLachlan, 1878, *Hydropsyche dinarica* Marinković-Gospodnetić, 1979, *Hydropsyche instabilis* (Curtis, 1834), *Hydropsyche mostarensis* Klapálek, 1898, *Hydropsyche tabacarui* Botosaneanu, 1960, *Polycentropus excisus* Klapálek, 1894, *Polycentropus flavomaculatus* (Pictet, 1834), *Polycentropus ierapetra dirfis* Malicky, 1974, *Lype reducta* (Hagen, 1868), *Brachycentrus montanus* Klapálek, 1892, *Micrasema minimum* McLachlan, 1876, *Drusus biguttatus* (Pictet, 1834), *Limnephilus hirsutus* (Pictet, 1834), *Potamophylax luctuosus* (Piller & Mitterpa-

Fig. 7. *Ecclisopteryx keroveci* sp.n., larva; **A**: larva in its case, **B**: head and pronotum, left lateral view, **C**: thorax and first abdominal segment, dorsal view. *Ecclisopteryx ivkae* sp.n., larva; **D**: larva in its case, **E**: thorax, dorsal view and **F**: head and pronotum, left lateral view. Scale bar is 1 mm.

cher, 1783), *Lepidostoma basale* (Kolenati, 1848), *Odontocerum albicorne* (Scopoli, 1763), *Beraeamyia schmidi* Botosaneanu, 1960, *Oecismus monedula* (Hagen, 1859), *Sericostoma flavicorne* Schneider, 1845 and the plecopterans *Perla marginata* (Panzer, 1799), *Perla pallida* Guerin, 1838, *Dinocras megacephala* (Klapálek, 1907), *Isoperla tripartita* Illies, 1954 and *Chloroperla russevi* Braasch, 1969 (Marinković-Gospodnetić 1970, our data). Hence, the caddisfly community at the mouth of the Jabučica River is quite species rich (e.g. Previšić et al. 2007). The finding of *Polycentropus ierapetra dirfis* is particularly noteworthy, since it was previously recorded only from the Hellenic Western Balkan ecoregion (ER6; Graf et al. 2008; Graf & Schmidt-Kloiber 2011; Ma-

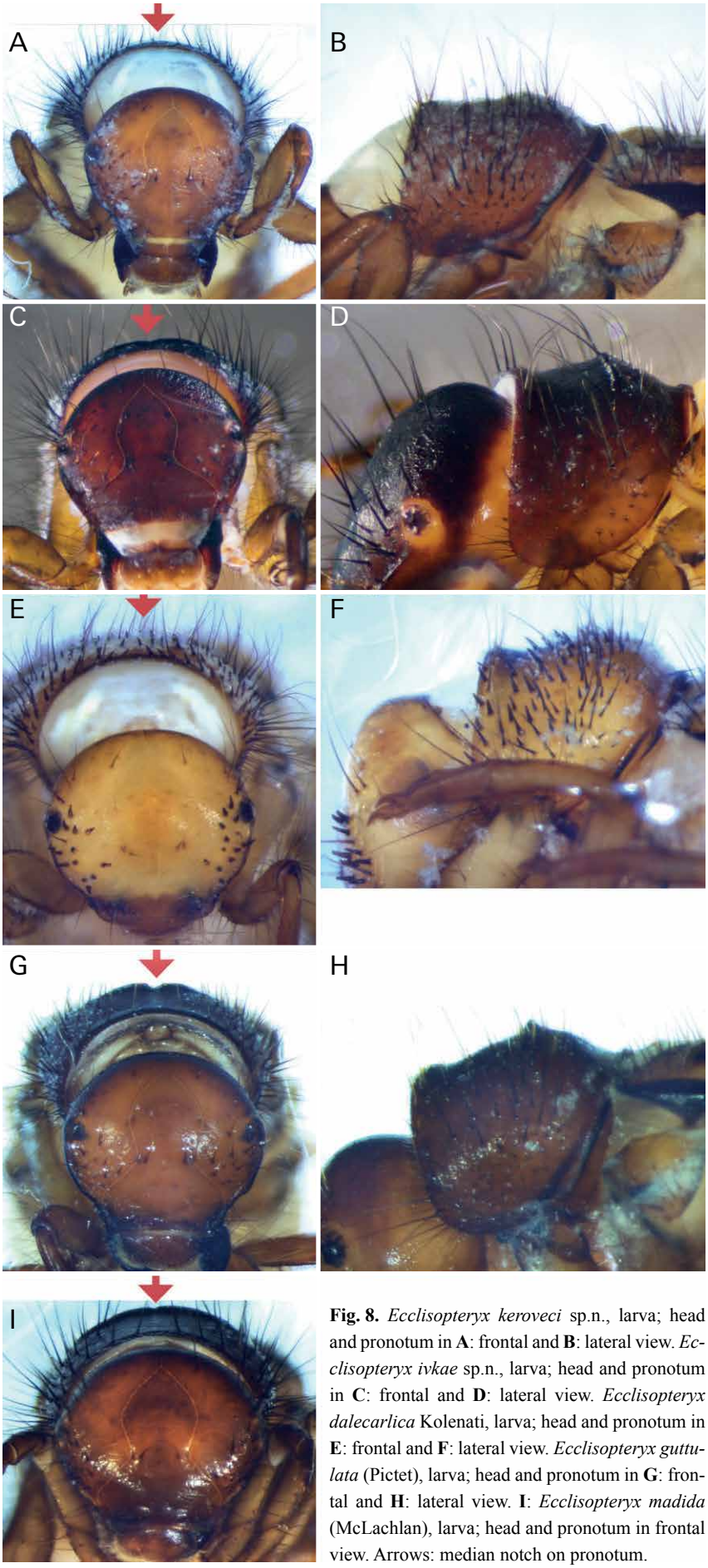

Fig. 8. *Ecclisopteryx keroveci* sp.n., larva; head and pronotum in **A**: frontal and **B**: lateral view. *Ecclisopteryx ivkae* sp.n., larva; head and pronotum in **C**: frontal and **D**: lateral view. *Ecclisopteryx dalecarlica* Kolenati, larva; head and pronotum in **E**: frontal and **F**: lateral view. *Ecclisopteryx guttulata* (Pictet), larva; head and pronotum in **G**: frontal and **H**: lateral view. **I**: *Ecclisopteryx madida* (McLachlan), larva; head and pronotum in frontal view. Arrows: median notch on pronotum.

LICKY 2004). This collection site represents the northwesternmost distribution of the species and considerably extends its range to the Dinaric Western Balkan ecoregion (ER5 sensu ILLIES 1978).

3.6. Ecology, habitat and phenology of *Ecclisopteryx ivkae*

Larval habitat characteristics at the type locality of *E. ivkae* (Cetina River, Glavaš spring) indicate a preference for crenal sections of streams. Larvae were collected approximately 50 m downstream of the spring. Here the stream was about 5 m wide, with a mean current velocity of 0.21 ms^{-1} and water temperatures ranging from 8.4 to 12.9°C throughout the year (POPIJAČ 2007). Substrate was mainly composed of smaller fractions (microlithal and mesolithal) with some larger stones and submersed vegetation. Larvae of *E. ivkae* were collected on the surface of algae-covered stones, which, in concordance with larval mandibular morphology, indicates that *E. ivkae* is also a grazer (PAULS et al. 2008; WARINGER et al. 2010). Additionally, adults were collected at the site 2.4 km downstream, indicating a possible preference for rhithral sections as well. Adults were generally collected in late May and early June (in 2005, 2007 and 2012).

At Glavaš spring, *E. ivkae* was sympatric with the trichopterans *Rhyacophila balcanica* Radovanović, 1953, *Rhyacophila fasciata* Hagen, 1859, *Glossosoma discophorum* Klapálek, 1902, *Hydropsyche* sp., *Tinodes dives* (Pictet, 1834), *Annitella apfelbecki* Klapálek, 1899, *Chaetopteryx fusca* Brauer, 1857, *Grammotaulius nigropunctatus* (Retzius, 1783), *Limnephilus flavicornis* (Fabricius, 1787), *Limnephilus lunatus* Curtis, 1834, *Limnephilus vittatus* (Fabricius, 1798), *Micropterna nycterobia* McLachlan 1875, *Micropterna testacea* (Gmelin, 1789), *Stenophylax permistus* McLachlan, 1895, *Allogamus uncatus* (Brauer, 1857), *Halesus digitatus* (Schrank, 1781), *Sericostoma flavicorne* Scheider, 1845 and *Odontocerum albicorne* (Scopoli, 1763) and the plecopterans *Leuctra mortoni* Kempny, 1899, *Nemoura cinerea* (Retzius, 1783), *Protonemura autumnalis* Rauser, 1956, *Protonemura hrabei* Rauser, 1956, *Isoperla illyrica* Tabacaru, 1971, *Isoperla inermis* Kacanski & Zwick, 1970, *Isoperla tripartita* Illies, 1954, and *Brachyptera tristis* (Klapálek, 1901) (POPIJAČ & SIVEC 2010; WARINGER et al. 2009; our data).

4. Discussion

4.1. Species delimitation and larval affiliation

Association of adults and larvae of the two new species collected at the mouth of the Jabučica River and the spring reach of the Cetina River, respectively, is supported by molecular genetic analyses at both intra- and interspecific levels. Additionally, larvae collected at both localities exhibit a unique combination of morphological characters and are clearly distinct from each other and the other *Ecclisopteryx* species (see 3.4. for details).

Despite the tremendous importance of species level identification of larvae in applied science (e.g., water quality monitoring, conservation biology) (e.g. HERING et al. 2004), ca. 60% of Drusinae larvae remain unknown (WARINGER et al. 2013). In particular, species level identification offers enhanced resolution of trait-environment relationships, particularly in ecologically sensitive taxa. Thus, species-level resolution has the potential to improve the quality of ecological assessments that use caddisflies or other sensitive aquatic insects as quality indicators (e.g. SCHMIDT-KLOIBER & NIJBOER 2004). The morphological characteristics of the larva of *E. keroveci* and *E. ivkae* now allow clear identification of these species and will enable better ecological characterisation of Drusinae in the region. When this information is integrated in national and international databases it will be of great value for further use in ecological investigations.

The B/MCMCMC phylogenetic analysis presented here to discriminate the species is based on the combination of three gene fragments (mtCOI, mtLSU, nuWG) previously demonstrated to successfully resolve phylogenetic relationships among species of Drusinae (PAULS et al. 2008). The phylogeny suggests a highly supported sister clade relationship of (*E. keroveci* + *E. ivkae*) + (*E. dalecarlica* + *E. guttulata*) and monophyly of all putative *Ecclisopteryx* species except *E. ivkae*, which is recovered in a basal polytomy (Fig. 2). Interspecific *p*-distances of mtCOI sequence data between the two new species, *E. keroveci* and *E. ivkae* (1.6–2.7%), are similar to the observed intraspecific variability of the same mtCOI fragment in populations of some Drusinae species (e.g. KUČINIĆ et al. 2008; PAULS et al. 2009; PREVIŠIĆ et al. 2009, 2014). However, in several closely related Drusinae species, interspecific *p*-distances of the same mtCOI fragment are similar or even lower than observed between *E. keroveci* and *E. ivkae* in the current study (e.g., WARINGER et al. 2007; KUČINIĆ et al. 2011). Further, intraspecific *p*-distances of the mtCOI gene fragment in *E. keroveci* show relatively high divergence of easternmost and westernmost populations (e.g., 1.6% between haplotypes from eastern Montenegro (Brodavac) and western Croatia (Velika Belica), 1.8% between haplotypes from eastern Bosnia (Sutjeska NP) and the latter, Fig. 3). Nevertheless, the morphological and molecular data at hand suggests that these specimens all belong to a single species.

In contrast to the highly variable mtCOI gene, differences between closely related species in nuWG and particularly in mtLSU are much lower (PAULS et al. 2008), and thus provide less information. Probably for this reason the B/MCMCMC phylogeny does not fully support the delimitation of the two species, as *E. ivkae* forms a basal polytomy. Including additional (or more variable) loci would improve the present analysis. However, in our case, the striking differences in larval morphology of northern European populations of *E. dalecarlica* initially

led us to think that two or more new species might occur, especially since larval characters in Drusinae are stable and vary little among species (e.g. Kučinić et al. 2011; Waringer & Graf 2011; Waringer et al. 2013). Furthermore, as indicated in our study, morphology of male and female genitalia of *E. keroveci* and *E. ivkae* differs distinctly between these species and *E. dalecarlica* (see 3.4 for details).

Thus, the integration of genetic and morphological differences, justifies the separation of *E. ivkae* and *E. keroveci* as two distinct species, as both taxa exhibit a unique combination of both genetic and morphological character states. Additional and equally comprehensive studies might uncover the existence of other localised allopatric lineages in *Ecclisopteryx* species in the Balkans, regardless of their current taxonomic status.

4.2. Distribution of *E. keroveci* and *E. ivkae*

With the addition of the 2 new *Ecclisopteryx* species, this genus currently comprises 7 species: 3 species relatively widespread in Europe, 1 regional endemic, and 3 micro-endemics (Table 2 & Fig. 1; Graf et al. 2008, 2011; Graf & Schmidt-Kloiber 2011). Unlike the 2 *Ecclisopteryx* range restricted endemics confined to different parts of the Alps (*E. malicky* is known exclusively from the south-central Alps, and *E. asterix* from the south-eastern Alps; Graf et al. 2008, 2011; Graf & Schmidt-Kloiber 2011), *E. keroveci* and *E. ivkae* show rather unique distribution patterns in the Western Balkans (Fig. 1B). Although both are allopatric and endemic, *E. keroveci* seems to have a wider, disjunct distribution in the region.

Previous records of both *E. dalecarlica* (Urbanič 2004; Stanić-Koštroman 2009; Previšić & Popijač 2010; Ibrahimi 2011; Vučković 2011; Vučković et al. 2011) and *E. guttulata* (Radovanović 1935; Marinković-Gospodnetić 1970) in the region are misidentifications of these 2 new, formerly unknown cryptic endemics. This conclusion is further supported since specimens from most the localities where *E. dalecarlica* and/or *E. guttulata* were presumed to have been collected were available for the current study; these all proved to be either of the 2 new species. The only historical record we could examine is a single male from central Bosnia, collected at the end of the 19th century, from Klapálek's collection (the National Museum, Prague, Czech Republic). It was originally listed as *E. guttulata*, but it proved to be *E. keroveci.* The findings of Radovanović (1935) and Marinković-Gospodnetić (1970) could not be checked because the material was not available for study.

4.3. The Western Balkans – a hotspot of freshwater biodiversity under threat

The description of *E. keroveci* and *E. ivkae* increases the number of endemic Western Balkan caddisflies: of 36 Drusinae species distributed in the region, 28 are endemics. Most of these are restricted to very small geographic areas (Graf et al. 2008; Oláh 2010, 2011; Graf & Schmidt-Kloiber 2011; Oláh & Kovács 2013). In the Dinaric Western Balkan ecoregion (ER5) 64% of Drusinae species are endemic (14 of 22 species); similarly, in the Hellenic Western Balkan ecoregion (ER6) 65% are endemic (13 of 20; Graf et al. 2008; Oláh 2010, 2011; Graf & Schmidt-Kloiber 2011; Oláh & Kovács 2013).

Hence, the Western Balkans is a diversity centre for highland caddisflies inhabiting isolated "island habitats" such as coldwater springs and streams (e.g. Drusinae, *Chaetopteryx* species; Kučinić et al. 2013). Such habitat preferences coupled with low dispersal abilities, specific life history traits, and historical processes causing further fragmentation and isolation of habitats (e.g., karstification; Previšić et al. 2009, 2014) most likely result in high diversification rates. A remarkable degree of cryptic diversity was recently observed in the Western Balkans not only in groundwater fauna (e.g. Zakšek et al. 2009), but also in widespread and commonly known surface aquatic species, such as the crayfish *Austropotamobius torrentium* (Klobučar et al. 2013) and the amphipod *Gammarus fossarum* (Weiss et al. 2014). Furthermore, a considerable fraction of the existing endemic diversity across various animal groups is considered cryptic in many parts of Europe, e.g., in isolated southern European mountain ranges (Essl et al. 2013). All this indicates that high degrees of cryptic diversity can be expected in many groups of aquatic organisms in the Balkans, and highlights the need for more comprehensive research of insufficiently investigated freshwater biodiversity and biogeography.

Near natural streams in all Western Balkan countries are increasingly threatened by human activities, especially the increase in construction of small power plants (Freyhof 2012; Schwarz 2012). According to Freyhof (2012), construction of dams and its inevitable side effects pose the most serious threat to freshwater diversity in the Balkans, the most important "hotspot" of European threatened biodiversity. Such adverse environmental impacts also threaten the existence of yet-to-be-discovered cryptic species.

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Chapter II

Manuscript

Description of a new species of *Wormaldia* from Sardinia and a new *Drusus* species from the Western Balkans (Trichoptera: Philopotamidae; Limnephilidae).

This manuscript describes two new species of Trichoptera for the Mediterranean region, and provides a key to the larval stages of the *Drusus bosnicus*-group.

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I supplied illustrations, generated and analysed the genetic dataset partly, designed the keys and wrote the manuscript as equally contributing first author together with W. Graf as senior author. Additionally, I performed the duties of the corresponding author.

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RESEARCH ARTICLE

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Description of a new species of *Wormaldia* from Sardinia and a new *Drusus* species from the Western Balkans (Trichoptera, Philopotamidae, Limnephilidae)

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Abstract

New species are described in the genera *Wormaldia* (Trichoptera, Philopotamidae) and *Drusus* (Trichoptera, Limnephilidae, Drusinae). Additionally, the larva of the new species *Drusus crenophylax* **sp. n.** is described, and a key provided to larval *Drusus* species of the *bosnicus*-group, in which the new species belongs. Observations on the threats to regional freshwater biodiversity and caddisfly endemism are discussed.

The new species *Wormaldia sarda* **sp. n.** is an endemic of the Tyrrhenian island of Sardinia and differs most conspicuously from its congeners in the shape of segment X, which is trilobate in lateral view. The new species *Drusus crenophylax* **sp. n.** is a micro-endemic of the Western Balkans, and increases the endemism rate of Balkan Drusinae to 79% of 39 species. Compared to other Western Balkan *Drusus*, males of the new species are morphologically most similar to *D. discophorus* Radovanovic and *D. vernonensis* Malicky, but differ in the shape of superior and intermediate appendages. The females of *D. crenophylax* **sp. n.** are most similar to those of *D. vernonensis*, but differ distinctly in the outline of segment X. Larvae of *D. crenophylax* sp. n. exhibit toothless mandibles, indicating a scraping-grazing feeding ecology.

Keywords

Caddisfly, Europe, larval key, taxonomy, conservation, Mediterranean, hydropower

Introduction

The Mediterranean area is a flora and fauna biodiversity hot-spot. The Tyrrhenian islands and the Balkans, in particular, are noteworthy for their high number of plant endemics (Médail and Quezél 1997, 1999; Nikolić et al. 2008; Fenu et al. 2010; Bacchetta et al. 2012), and mammal and invertebrate endemics (Holdhaus 1924, Vigne 1992, Muccedda et al. 2002, Griffiths et al. 2004, Grill et al. 2007). Freshwater biodiversity has recently become a focus of attention throughout Europe, including the Mediterranean region with the Western Balkans and Sardinia (e.g., di Sabatino 2003, Zakšek et al. 2009, Tierno de Figueroa et al. 2013, Klobučar et al. 2013, Weiss et al. 2014).

The genus *Wormaldia* currently comprises 204 species (Morse 2014) of which 36 species occur in Europe (Malicky 2005, Graf et al. 2008). Most species are widely distributed, but also several apparently highly endemic species have been described (Graf et al. 2008, Martínez-Menéndez and González 2011). Aquatic stages of the genus, with few exceptions, prefer crenal and rhithral sections of alpine to lowland streams, are caseless and behave as passive filter feeders using characteristic nets (Graf et al. 2008). Species in the genus exhibit characteristic male genitalia, but also comparatively high variability, particularly of the phallic structures (Malicky 2004, Martínez-Menéndez and González 2011, Neu pers. comm.), resulting in the description of several subspecies.

The genus *Drusus* is in the subfamily Drusinae Banks, and comprises 84 species (Malicky 2004, 2005; Kučinić et al. 2011a; Oláh 2010, 2011; Oláh and Kovács 2013). Larvae of the group prefer eucrenal to epirhithral sections of cold alpine or montane streams and brooks. Feeding ecology of *Drusus* larvae is complex, and three different feeding guilds can be distinguished based on the shape of larval mandibles and leg setation: filtering carnivores, omnivorous shredders, and scraping grazers (Pauls et al. 2008, Graf et al. 2009). Taxonomic richness of Drusinae is particularly high in the Western Balkans, including a high number of micro-endemics (Malicky 2004; Graf et al. 2008; Oláh 2010, 2011; Kučinić et al. 2011a, b; Oláh and Kovács 2013, Previšić et al. 2014a, b).

In this paper we describe a new species of *Wormaldia* and a new grazer *Drusus* species, including a key to the hitherto known larval stages of the *bosnicus*-group, in which *Drusus crenophylax* sp. n. belongs.

Materials and methods

Adults were collected using sweep nets and immature stages by handpicking. Collected specimens were stored in 70% and 96% EthOH, for morphological and molecular analyses, respectively.

50 Male and female genitalia were examined after being cleared in either KOH or lactic acid. Nomenclature of male genitalia of *Wormaldia* McLachlan follows Nielsen (1957, for *Wormaldia occipitalis* Pictet), nomenclature of male genitalia of *Drusus* follows Nielsen (1957, for *Limnephilus flavicornis* Fabricius) using the simplifying terms "superior appendages" for the lateral processes of segment X (cerci *sensu* Snodgrass

Table 1. Detailed list of *Drusus* specimens used for mtCOI analysis. Abbreviations: M adult male, F female; L larva; U unknown.

Species	Locality	Specimen ID/Stage	Accession #	Collectors
D. crenophylax	44°32.932'N, 17°23.562'E	fDsp4501M/M	KP793082	Dmitrović, Šukalo
D. crenophylax	44°33.003'N, 17°23.580'E	fDsp4502L/L	KP793083	Dmitrović, Šukalo
D. crenophylax	44°33.003'N, 17°23.580'E	fDsp4503L/L	KP793081	Dmitrović, Šukalo
D. crenophylax	44°33.003'N, 17°23.580'E	fDsp3401F/F	KP793084	Dmitrović, Šukalo
D. crenophylax	44°33.003'N, 17°23.580'E	fDsp3402F/F	KP793085	Dmitrović, Šukalo
D. vernonensis	41°0.887'N, 21°10.448'E	DdphPEIM1/M	KC881524	Kučinić, Graf
D. vernonensis	41°0.887'N, 21°10.448'E	DdphPEIM2/M	KP793087	Kučinić, Graf
D. vernonensis	41°0.887'N, 21°10.448'E	DdphPEIM3/M	KP793086	Kučinić, Graf
D. discophorus	Macedonia, Jablanica Mts.	fDds0110M/M	KP793089	Kučinić
D. discophorus	Macedonia, Jablanica Mts.	fDds0112F/F	KP793088	Kučinić

1935), and "intermediate appendages" for the sclerite and the anterior process of segment X (paraproct *sensu* Snodgrass 1935). Nomenclature of larval morphological features follows Wiggins (1998) and Waringer and Graf (2011), nomenclature of primary setae and setal areas follows Wiggins (1998). Illustrations were prepared according to Thomson and Holzenthal (2010) in which pencil drawings made with a camera lucida are digitized, edited and inked in Adobe Illustrator (v. 16.0.4, Adobe Systems Inc.).

Molecular genetic sequence data were used to support larval association and assess relationships to previously described *Drusus* species. DNA extraction and amplification of a 541-bp-long fragment of the mtCOI gene using standard primers (forward primer: Jerry, Simon et al. 1994, reverse primer: S20, Pauls et al. 2006) was performed as outlined by Pauls et al. (2008) and Previšić et al. (2009b). Sequences were edited manually using Geneious version R7 (http://www.geneious.com, Kearse et al. 2012) and aligned using MAFFT (Katoh and Standley 2013). Sequences were deposited in GenBank under Accession nos: KC881524, KP793081–KP793089 (Table 1). Inter- and intraspecific genetic distances (uncorrected *p*-distances) were calculated in Mega 4.0.1 (Tamura et al. 2007).

Taxonomy

***Wormaldia sarda* Graf & Malicky, sp. n.**
http://zoobank.org/F02C5CF5-9043-463F-809B-FCD5D2B8FBD2

Material examined. Holotype. 1 male pupa, holotype: Sardinia, Gola di Gorruppo; 40°11.122'N, 9°30.104'E; 350 m a.s.l.; 28.03.2001; leg. Monika Hess, Ulrich Heckes; currently in coll. W. Graf, will deposited in the Biologiezentrum des Oberösterreichischen Landesmuseums, Linz, Austria.

Type locality. Italy, Sardinia.

Diagnosis. Morphology of the male terminalia suggests placement of the new species in *Wormaldia*. The species is unique in the European Trichoptera fauna, and easily differentiated from all other *Wormaldia* species by the combination of the fol-

lowing characters: (1) presence of median subtriangular protrusion in the distal half of the harpago, (2) membraneous dorsoproximal portion and trilobate lateral portions of segment X, and (3) distinct sclerotized structures visible on the invaginated phallus.

Description. *Adults* (in pupa). Habitus dark, sclerites and tergites brown; cephalic and thoracic setal areas pale; cephalic, thoracic and abdominal setation dark brown; legs light brown, proximally darker; haustellum and intersegmental integument pale cream. Wings brown mottled with golden patches. Male maxillary palp 5-segmented. Spurformula 2–4–4 in males.

Male genitalia (Fig. 1A–D). Segment IX in lateral view subrectangular, bulging anteriad; dorsal quarter reduced to a narrow transverse bridge, ventral 3/4ers broad (Fig. 1A). Segment X in lateral view trilobate: unpaired dorsal lobe strongly convex with a bicuspid apex, dorsoproximally membraneous; 1 lateromedian lobe, subovate, pointed on either side; 1 ventral lobe, posteriad, pointed on either side (Fig. 1A, B). Superior appendages suboval, curved dorsad in lateral view, flat with a rounded apex in dorsal and ventral view (Fig. 1A, C, D). Invaginated phallus terminally with a dorsal pair of sclerotized, laterad divergent tines and a ventral sclerotized plate; internally with 4 distinct tines (Fig. 1A, D). Coxopodite subovate in lateral view, ventrally with a sharp mediolaterad ridge (Fig. 1A, C). Harpago subovate in lateral view, in ventral view distally with a median subtriangular serrated protrusion flattened dorsoventrally (Fig. 1A, D).

Mature pupa (Fig. 2D–F). Mandibles tubular, dilated at the apex (Fig. 2E,F). Abdominal dorsal sclerites as in Fig. 2D.

Female and larva unknown.

Etymology. The species epithet refers to the island of Sardinia, the type locality.

***Drusus crenophylax* Graf & Vitecek, sp. n.**
http://zoobank.org/4FBB2D55-59BD-46AB-8E39-B34F2D892C79

Material. Holotype. 1 male: Bosnia and Herzegovina, Cvrcka river; 44°32.932'N 17°23.562'E; 393 m a.s.l.; 01.10.2014; leg. Dejan Dmitrović, Goran Šukalo; specimen identifier: fDsp4501M. Paratypes: 2 females: Bosnia and Herzegovina, Spring of Cvrcka river, Vilenjska vrela; 44°33.003'N, 17°23.580'E; 456 m a.s.l.; 12.09.2012; leg. Dejan Dmitrović; specimen identifiers: fDsp3401F, fDsp3402F. 4 males, 3 females, 19 larvae: Bosnia and Herzegovina, Spring of Cvrcka river, Vilenjska vrela; 44°33.003'N, 17°23.580'E 456 m a.s.l.; 12.09.2012; leg. Dejan Dmitrović, Goran Šukalo; specimen identifiers for 3 larvae: fDsp4502L, fDsp4503L, fDsp4504L. Holotype and paratypes currently in coll. W. Graf, will deposited in the Biologiezentrum des Oberösterreichischen Landesmuseums, Linz, Austria.

52 **Type locality.** Bosnia and Herzegovina, Republika Srpska, Cvrcka River.

Diagnosis. Males of the new species are most similar to *Drusus discophorus* Radovanovic and *D. vernonensis* Malicky, but exhibit (1) subtriangular superior appendages in lateral view, (2) subtriangular, low tip of the intermediate appendage in lateral view, and (3) simple, rounded tips of intermediate appendages in caudal view. *Drusus disco-*

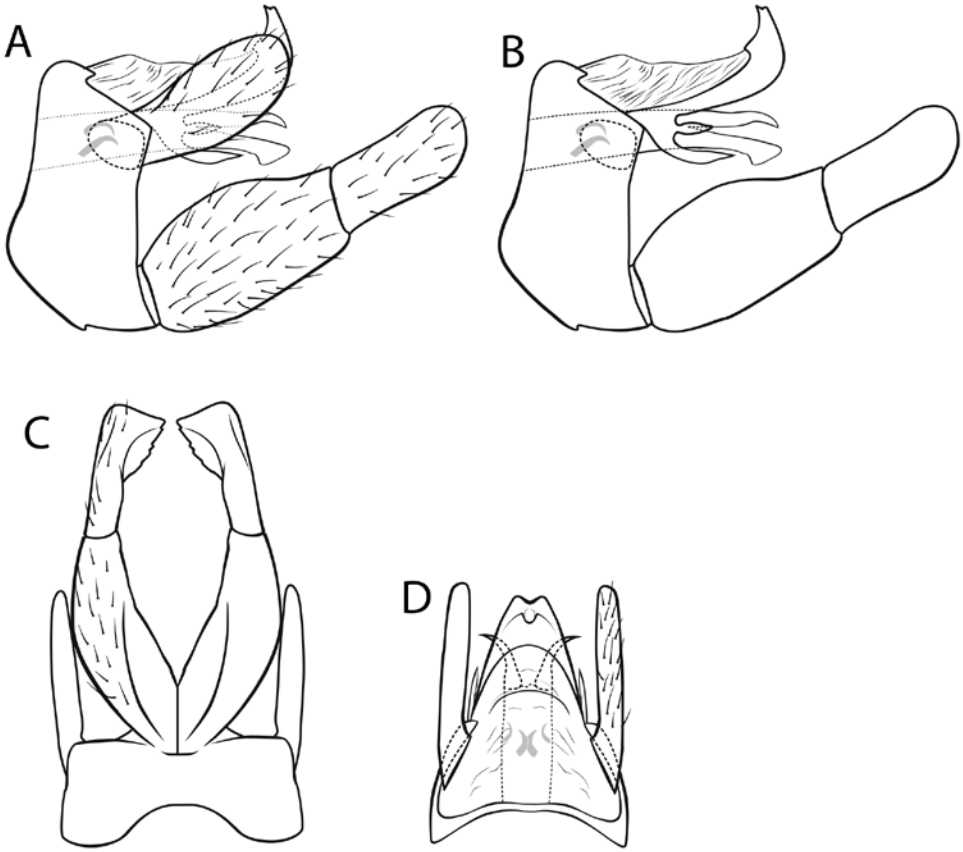

Figure 1. Male genitalia of *Wormaldia sarda* sp. n. **A** right lateral view, intact **B** right lateral view, superior appendage removed **C** ventral view **D** dorsal view.

phorus males have suboval superior appendages and a high round tip of the intermediate appendage in lateral view; *D. vernonensis* males have round superior appendages in lateral view and trilobate tips of intermediate appendages in caudal view.

Females of the new species show the reduced median lobe of the vaginal sclerite and high base of the lateral lobe of segment IX as typical for Balkan Drusinae, and are most similar to *Drusus vernonensis*, but exhibit (1) a sharp dorsal notch of segment X in lateral view, and (2) segment X with 2 round median lobes in dorsal view. *Drusus vernonensis* females have a rounded dorsal outline of segment X and lack the median lobes of segment X.

Larvae of the new species are most similar to *Drusus klapaleki* Marinković-Gospodnetić and *D. serbicus* Marinković-Gospodnetić, but exhibit (1) a semicircular area dorsomedially on the pronotum anterior the pronotal ridge void of white recumbent setae, (2) lateral gills, and (3) a subtriangular pronotal ridge in lateral view. Larvae of *D. klapaleki* have white recumbent setae covering the whole pronotum, and larvae of *D. serbicus* lack lateral gills and have an annular pronotal ridge.

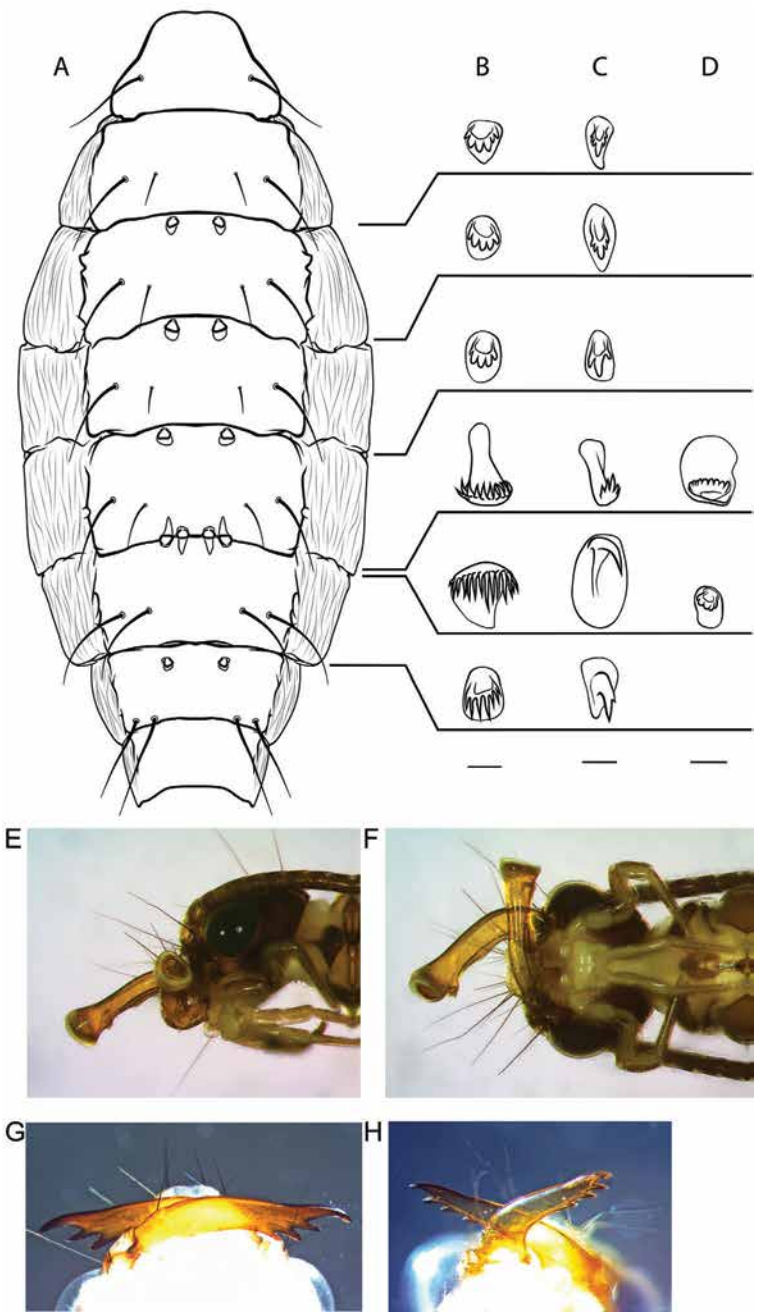

Figure 2. Pupal characteristics of *Wormaldia sarda* sp. n., *Philopotamus montanus*, and *Wormaldia* spp. **A** generalized pupal abdomen in dorsal view, depicting the position of the dorsal sclerites **B** dorsal sclerites of *Philopotamus montanus* **C** dorsal sclerites of *Wormaldia occipitalis* **D** dorsal sclerites of *W. sarda* sp. n. **E** head of *W. sarda* pupa in left lateral view **F** head of *W. sarda* pupa in ventral view **G** pupal mandibles of *Philopotamus montanus* in ventral view **H** pupal mandibles of *Wormaldia copiosa* in ventral view. Scale bars: 100 μm (**B**); 50 μm (**C, D**).

Description. *Adults*. Habitus dark; sclerites and tergites brown; cephalic and thoracic setal areas pale; cephalic, thoracic and abdominal setation blond; legs light brown to fawn, proximally darker; haustellum and intersegmental integument pale, whitish. Wings smoky, with dark setae. Male maxillary palp 3-segmented. Forewing length 11–13.2 mm, spur formula 1–3–3 in males; forewing length 13–14.5 mm, spur formula 1–3–3 in females.

Male genitalia (Fig. 3A–E). Tergite VIII dark brown, in dorsal view cranially distinctly incised, with lighter areas around fused alveoli; setation concentrated at laterocranial borders of spinate areas; spinate area as two ± triangular laterocaudal lobes medially connected by a band of spines, embracing a medial, indent less sclerotized area (translucent in cleared specimens) with scarce spines. Ninth abdominal segment (IX) ventrally wider than dorsally in caudal view; in lateral view medially with a sharp caudad protrusion and a ventral protrusion, embracing the base of the inferior appendices. Superior appendages in lateral view subtriangular, somewhat Y-shaped with a shorter dorsal and a longer ventral protrusion separated by a slight indentation. Intermediate appendages in lateral view blocky with 2 tips, the proximal sharp, the distal high, rounded, rough; in dorsal view the tips parallel, extending laterally: a bar-shaped, laterally rounded distal tip and a sharp proximal tip, separated by a rounded excision with round edges; in caudal view approximately triangular, tips rounded. Inferior appendages (gonopods *sensu* Snodgrass 1935) in lateral view proximally wide, medially slightly constricted with a slight dorsal triangular protrusion, curved dorsadly in the slender posterior third; in dorsal, ventral and caudal view proximal part laterad, distal part approximately straight in dorsoventral plane, curved dorsad; in caudal view tips distinctly slender; setal alveoli fused, creating a rugged, less sclerotized ventral area. Parameres simple, with a distinct medial thorn-like spine and 2 proximal spines in the proximal half.

Female genitalia (Fig. 3F–I). Segment IX setation abundant, concentrated in the caudal half; lateral lobe of segment IX membraneous, in lateral view oblique triangular, the ventral edge about twice as long as the dorsal edge, with a dorsal sclerotized setose part protruding caudally; in dorsal and ventral view slender, projecting lateradly; in caudal view dorsal sclerotized setose part somewhat triangular. Segment X in lateral view with a proximal and a distal part, defined by a sharp dorsal notch; in dorsal view trapezoidal, with rounded shoulders, 2 small dorsal median lobes, and distally with 2 triangular, sharp-tipped lateral lobes, each with a lateral rounded setose and a small median rounded protrusion; ventrally unsclerotized, open. Supragenital plate in lateral view sinuously-edged quadrangular with a small, rounded dorsal protrusion, caudal line slightly indent; in ventral view quadrangular, in caudal view quadrangular, dorsally slightly wider than ventrally. Vulvar scale in lateral view triangular, rather straight, longer than the supragenital plate; in ventral view slender with 3 lobes: 2 lateral lobes, digitiform, roundly oval, straight; 1 median, short (reduced), of greater width than length: length approximately 1/6th of that of lateral lobes.

Fifth instar larva (Fig. 4A–I). Head capsule hypognathous, finely granulated with a field of microspinules dorsal to each eye, dark brown dorsally, fading to yellow ven-

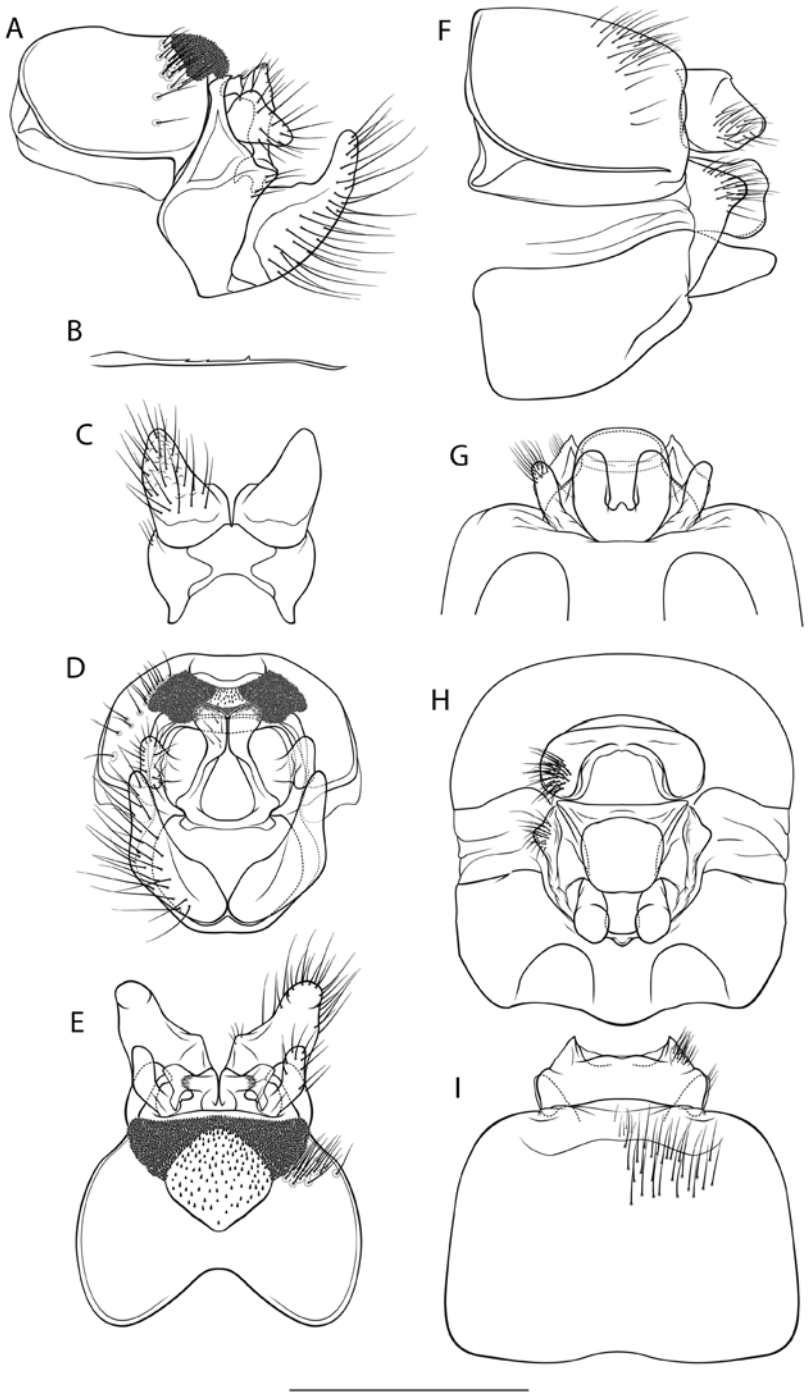

Figure 3. Genitalia of *Drusus crenophylax* sp. n. **A–E** male genitalia: **A** right lateral view **B** paramere in right lateral view **C** ventral view **D** caudal view **E** dorsal view **F–I** female genitalia: **F** right lateral view **G** ventral view **H** caudal view **I** dorsal view. Scale bar: 1 mm.

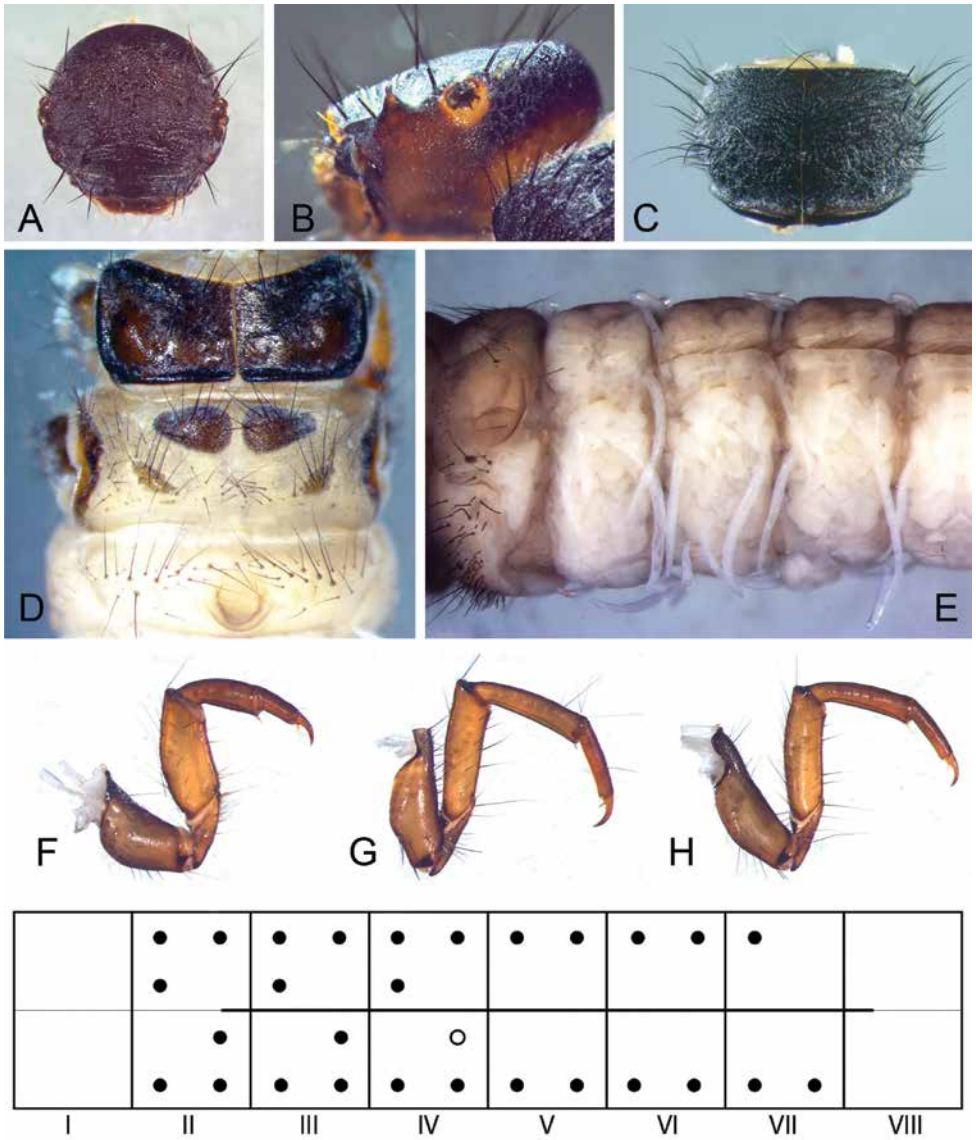

Figure 4. Larval characteristics of *Drusus crenophylax* sp. n. **A** head, frontal view **B** head, left lateral view **C** pronotum dorsal view **D** meso- and metathorax with abdominal segment I, dorsal view **E** abdominal segments I-V, left lateral view **F** left thoracic leg I, frontal view **G** left thoracic leg II; frontal view **H** left thoracic leg III, frontal view; bottom, gill and lateral line diagram, positions of gills are depicted as black circles, position of lateral line bold.

trally; 18 pairs of primary setae present: #1, 4, 6, 10, 12, 13 yellow and #6, 13 short, inconspicuous, the rest dark brown, long (Fig. 4A); antennae located on high carinae, each carina about as high as long, both strongly curved mediad (Fig. 4B); mandibles toothless. Pronotum dark brown, coarsely granulated; distinct medial ridge present,

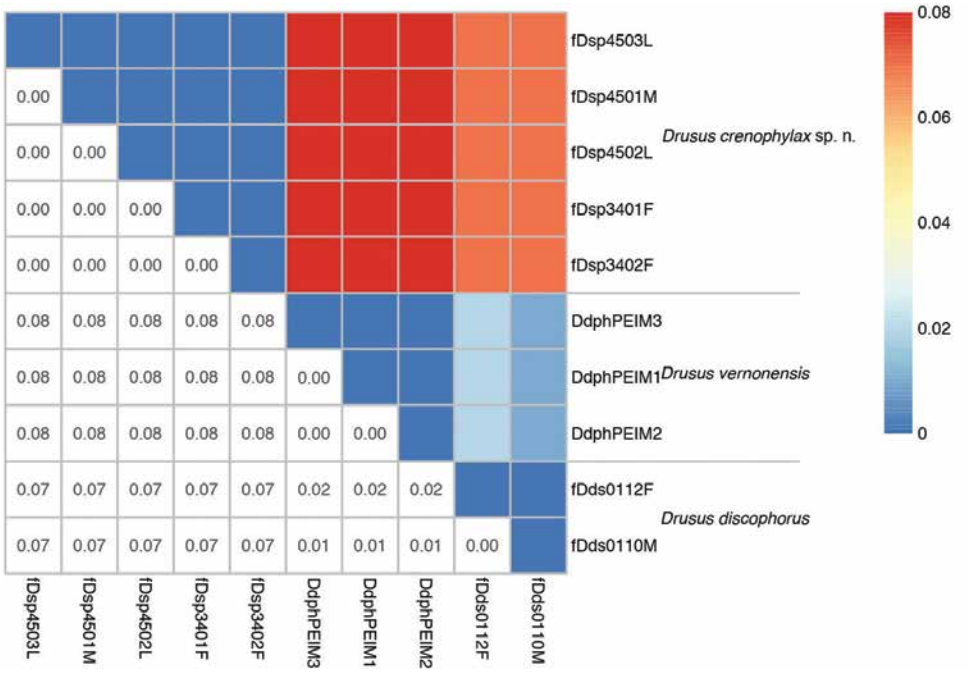

Figure 5. Distance matrix (lower left) and colour heat map (upper right) showing uncorrected inter- and intraspecific *p*-distances of the partial mtCOI sequence (541 bp) between *Drusus crenophylax* sp. n., *D. vernonensis* and *D. discophorus*. For detailed information on the haplotypes, see Table 1.

rounded, steeper anteriorly in lateral view; recumbent white setae present, but lacking in a semicircular area anterior the pronotal ridge (Fig. 4C); pronotal horn present. Mesonotum completely covered by 2 sclerites, dark brown, with darker apodemes; edges black; *sa*1 comprising 4–6 setae, *sa*2 and *sa*3 connected, comprising 28–34 setae in total on each sclerite (Fig. 4D). Metanotum with 3 pairs of sclerites: anteriomedian sclerites subtriangularly ovoid, dark brown with 11–19 setae; posteromedian sclerites rhomboid, pale brown, with 13–15 setae; lateral sclerites long, curved dorsally in lateral view, pale brown fading to yellow ventrally with a dark median spot and 21–25 setae (Fig. 4E). Legs yellow-light brown, dorsally and distally darker (Fig. 4F–H). Abdomen white (Fig. 4G), dorsal gills from II praesegmental position to VI praesegmental position, lateral gills from II praesegmental position to IV praesegmental position, ventral gills from II prasegmental position to VII postsegmental position; lateral line from last quarter of II to first quarter of VIII (Fig. 4I); abdomen I with 1 dorsal and 2 lateral protuberances, posterior sclerites absent on lateral protuberances, setal areas *sa*1–3 fused dorsally and ventrally (Fig. 4D, E), sternum bearing 2 setae with distinct basal plates; abdomen VIII with 2 long and 2–4 short posterodorsal setae on either side; abdomen IX with 1 posterodorsal seta on either side, dorsal sclerite IX semicircular, pale brown with 7 long and several shorter setae. Case simple, constructed of mineral particles.

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Molecular species delimitation and larval affiliation. Analysis of the genetic distance of mtCOI between *Drusus crenophylax* sp. n. and the in the adult stage morpho-

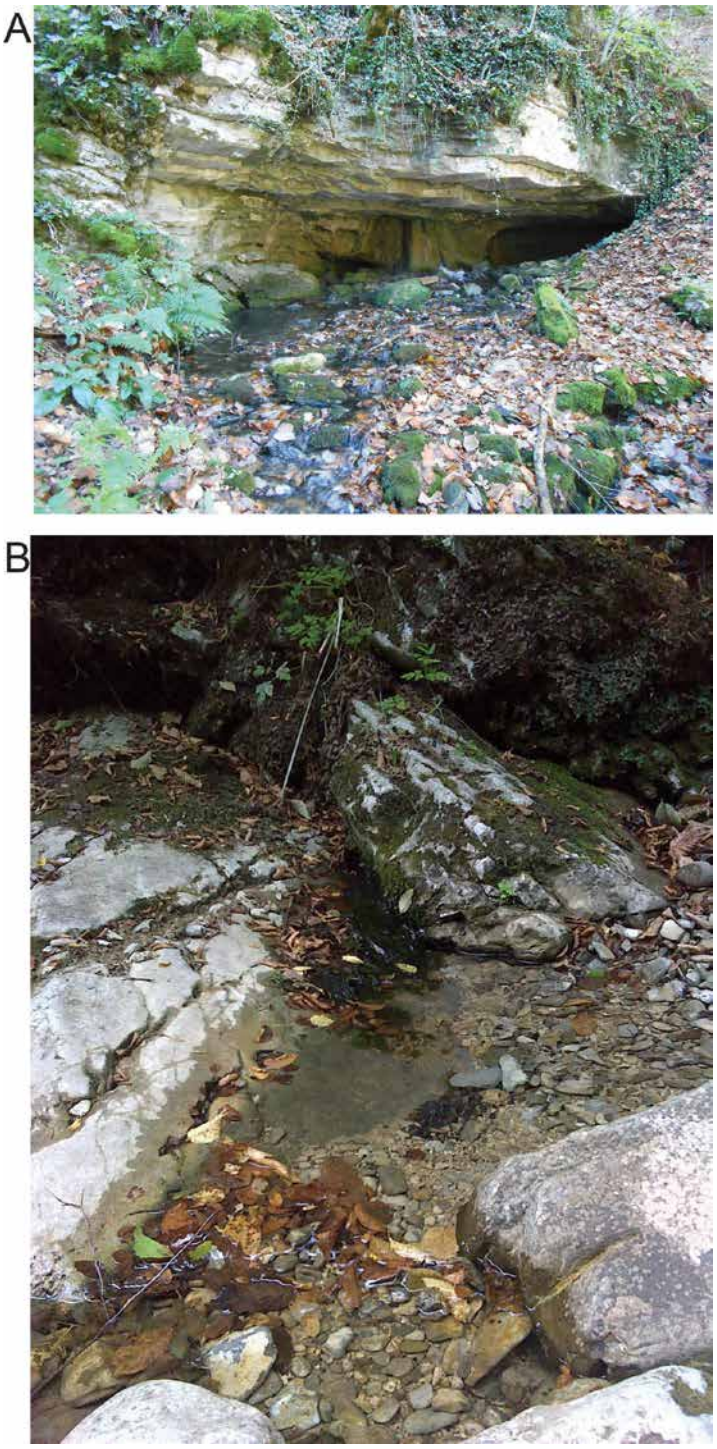

Figure 6. Habitat of *Drusus crenophylax* sp. n. at the type locality. **A** collection site of the larval paratypes **B** collection site of the male holotype.

logically most similar species, *D. discophorus* and *D. vernonensis*, clearly supports the recognition of the new species. Uncorrected *p*-distances recorded in a fragment of the mtCOI gene (ranging from 2–8%; Fig. 5), agree with the interspecific distances commonly recorded in Limnephilidae (e.g., Graf et al. 2005; Kučinić et al. 2011a; Previšić et al. 2014a, b) and other caddisfly families (e.g., Hydropsychidae; Pauls et al. 2010). Also, all haplotypes of *Drusus crenophylax* sp. n. adults were completely identical to another and those of undescribed *Drusus*-larvae collected at the locus typicus, enabling confident affiliation of larvae and adults of *D. crenophylax* sp. n.

Ecology and distribution. Drusinae species typically are members of crenal species communities, and mainly inhabit crenal sections of cold streams. Larval *D. crenophylax* were collected at eucrenal sections of the Cvrcka River (Fig. 6A, B) and behave as epilithic grazers, as indicated by mandible morphology (Pauls et al. 2008, Graf et al. 2009). Based on regional collection data, we assume that the species is a micro-endemic restricted to the watershed of the Cvrcka river.

Etymology. The species epithet is a compound name, combining κρηνον ('well, spring, fountain' in Ancient Greek) and φυλαξ ('guard, keeper, protector' in Ancient Greek), terms that reflect the high degree of niche specificity of *Drusus* species, the majority of which inhabit crenal sections of streams (Graf et al. 2008).

Key to Drusinae larvae of the *bosnicus*-group

Drusinae have evolved into three distinct subclades reflecting feeding ecology of larvae (Pauls et al. 2008, Graf et al. 2009). The grazer clade *sensu* Pauls et al. 2008 represents the largest clade, comprising over 70 species in several subclades (Malicky 2004, 2005; Kučinić et al. 2011a; Oláh 2010, 2011; Oláh and Kovács 2013). Larvae of scraping grazers species characteristically develop toothless mandibles (Pauls et al. 2008, Graf et al. 2009, Waringer and Graf 2011). In the Western Balkans, the grazing *bosnicus*-group represents a group of morphologically similar endemics and comprises according to Marinković-Gospodnetić (1976) *Drusus bosnicus* Klapálek, *D. klapaleki* Marinković-Gospodnetić, *D. medianus* Marinković-Gospodnetić, *D. plicatus* Radovanović, *D. radovanovici* (Marinković-Gospodnetić), *D. ramae* Marinković-Gospodnetić, *D. septentrionis* (Marinković-Gospodnetić) and *D. vespertinus* Marinković-Gospodnetić (Kučinić et al. 2011a).

Larvae of the *bosnicus*-group also develop, with the exception of *D. ramae* (Kučinić et al. 2010), a field of microspinules close to each eye (Kučinić et al. 2011a, b; Waringer et al. 2015). Further, carinae of *D. bosnicus*, *D. radovanovici*, *D. septentrionis* and *D. medianus* are high and curved mediad. Larvae of *D. crenophylax* sp. n. share those characters and can be integrated in the following dichotomous key (Waringer et al. 2015):

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1 Head with flat vertex ..... ***Drusus bosnicus*** (Kučinić et al. in press)
– Vertex evenly rounded **2**
2 Pronotum with thin long, yellow setation .....
..... ***Drusus radovanovici*** (fig. 17 in Kučinić et al. 2011a)

– Pronotum without thin long, yellow setation **3**
3 Pronotum with numerous short, white, recumbent setae **4**
– Pronotum without numerous short, white, recumbent setae ..... ***Drusus septentrionis*** (figs 4, 5 in Kučinić et al. 2008)
4 Dorsal pronotal hump smoothly rounded ..... ***Drusus medianus*** (fig. 43 in Kučinić et al. 2010, figs 20–22 in Kučinić et al. 2011b)
– Dorsal pronotal hump with distinct ridge **5**
5 Anterior metanotal sclerites narrowly subtriangular (width / length ratio ≥ 2.0) ..... ***Drusus vespertinus*** (Previšić et al. 2009a)
– Anterior metanotal sclerites broadly subtriangular (width / length ratio < 2.0) **6**
6 In lateral view, dorsal pronotal ridge annular, posterior section sharply descending ..... ***Drusus serbicus*** (Waringer et al. 2015)
– In lateral view, posterior section of dorsal pronotal ridge gently descending 7
7 White recumbent setae cover the entire pronotum ..... ***Drusus klapaleki*** (Kučinić et al. 2011b)
– White recumbent setae lacking in a semicircular area anterior to the pronotal ridge ..... ***Drusus crenophylax*** **sp. n.**

Discussion

Systematic significance of *Wormaldia sarda* sp. n.

The Tyrrhenian islands and Sardinia in particular have been renowned for their relictual fauna and flora for a long time (Holdhaus 1924) and represent one of the Mediterranean biodiversity hotspots (Grill et al. 2007). *Wormaldia sarda* sp. n. represents an addition to the distinct Sardinian biodiversity. As no species similar to *W. sarda* sp. n. are recorded from neither northern Africa nor mainland Europe, it is likely that this species is restricted to Sardinia, as are several other species such as *Crunoecia irrorata sarda* Curtis, *Stactobia ericae* Malicky or *Hydropsyche sattleri* Tobias (Graf et al. 2008). However, the geological history and geographic proximity of the Tyrrhenian islands – Sardinia and Corsica in particular (Vigliotti et al. 1990) – suggest that some species may occur on both islands. For instance, *Leptodrusus budtzi* Ulmer or *Micrasema togatum* Hagen occur also on Corsica, or other Mediterranean islands (Graf et al. 2008).

The distinct apomorphic characters, particularly the modified segment X and the very different pupal characters (mandibles, dorsal abdominal sclerites; Fig. 2D–F), might warrant establishing a new genus for this species. The pupal characteristics alone are strikingly different from those of either *Wormaldia* or *Philopotamus* (Lepneva 1964; Fig. 2). However, since pupae of only three species of *Wormaldia* are described (Nielsen 1942, Lepneva 1964) the range of genus-level pupal characters remains unknown. Further, modifications of segment X are common in southeast Asian species

of *Wormaldia* (Malicky 2010). Tooth-like structures on segment X similar to the ones observed in *W. sarda* sp. n. are present in *Wormaldia* species from Thailand (e.g., *W. acheloos* Malicky & Chantaramongkol, *W. congina* Malicky & Chantaramongkol, *W. lot* Malicky & Chantaramongkol), or Sulawesi (*W. otaros* Neboiss). Nevertheless, *Wormaldia* species with a phallus shaped as in *W. sarda* sp. n. have not yet been described. Since the whole genus is in need of revision (Malicky 2005, Malicky unpubl. data), we refrain, in the interest of taxonomic stability, from creating a new genus.

Aquatic diversity of the Western Balkans under threat

Endemic freshwater species are particularly vulnerable to global change and (anthropogenic) habitat degradation (Hering et al. 2009, Tierno de Figueroa et al. 2010, Bálint et al. 2011, Conti et al. 2014). The Balkans is rich in apparently endemic freshwater species (Griffiths et al. 2004). Recent taxonomic efforts in the Western Balkans increased the number of endemic Drusinae taxa to 31 of 39 described Drusinae species (Previšić et al. 2014b, Vitecek et al. unpubl. data). Further, several endemic species of *Chaetopteryx* were recently described from the Western Balkans (Oláh et al. 2012, Kučinić et al. 2013) indicating the need for further systematic investigations on an underestimated diversity of southeastern Europe.

The construction of hydropower dams in emerging economies is currently one of the greatest threats to freshwater biodiversity (Zarfl et al. 2014). Small hydropower plants fed by small cold-water mountain rivers such as the Cvrcka River are currently under construction throughout the Western Balkans (Freyhof 2012, Schwarz 2012), and gravely threaten the habitats that harbour endemic highland caddisflies such as Drusinae (Previšić et al. 2014a, Vitecek et al. unpubl. data, this study), or *Chaetopteryx* species (Kučinić et al. 2013). The description of *Drusus crenophylax* sp. n. highlights the importance of biodiversity research in southern Europe, and demonstrates that the currently prevailing energy policy will likely result in the loss of known and unknown biodiversity.

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S. Vitecek and W. Graf contributed equally to this manuscript. F. Hoppeler, Germany, assisted in the generation of genetic sequence data.

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Chapter III

Manuscript

Description of two new filtering carnivore *Drusus* species (Limnephilidae: Drusinae) from the Western Balkans

This manuscript describes two new species of filtering carnivorous Drusinae from the Western Balkans, and summarizes morphological features of males of filtering carnivorous Drusinae.

The manuscript was submitted to ZooKeys for publication.

I supplied illustrations, species descriptions and drafted the manuscript as first author. Additionally, I performed the duties of the corresponding author.

Description of two new filtering carnivore *Drusus* species (Limnephilidae: Drusinae) from the Western Balkans

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Abstract
Two new species of the genus *Drusus* (Trichoptera, Limnephilidae, Drusinae) are described. Additionally, observations on aquatic biodiversity and threats to regional and micro-endemic aquatic fauna are discussed.
The new species *Drusus krpachi* sp. n. is a micro-endemic of the Korab Mountains, Macedonia; the new species *D. puskasi* sp. n. is a micro-endemic of the Prokletije Mountains, Albania. Both new species are most similar to *D. macedonicus* but differ from the latter in the shape of segment IX, the shape of the tips of the intermediate appendages in lateral view, the shape of the inferior appendages, and the form and shape of the parameres.
These additions to the Western Balkan fauna demonstrate the significance of this region for European biodiversity and further highlight the importance of faunistic studies in Europe.

Keywords: Caddisfly, aquatic diversity, Mediterranean, taxonomy, conservation, Southern Europe

Introduction

The Western Balkans harbour high biodiversity including a high numbers of endemic species. This has been attributed to historic climate conditions and the highly diverse geology of the region (e.g., Neubauer 2002, Reed et al. 2004, Chiari et al 2011) that permitted perseverance of taxa in glacial refugia (e.g., Tzedakis 2004, 2009; Médail & Diadema 2009), and the diverse climatic conditions and rich geological history resulting in a high diversity of habitats. Thus, the Western Balkans are rich in (endemic) plant (e.g., Eastwood 2004, Petrova & Vladimirov 2010, Mereďa et al. 2011, Redžić 2011) and vertebrate and invertebrate species (e.g., Bianco 1998, Bănărescu 2004, Kryštufek 2004, Griffiths & Frogley 2004, Bohlen et al. 2006, Guéoruiev 2007, Deltshev 2010, Pešić & Glöer 2013). The Western Balkans have further been identified as a major hotspot of aquatic biodiversity, with high rates of endemism and cryptic diversity (Bănărescu 2004, Previšić et al. 2014a). Potentially, the diverse geology of the region and historic climate conditions also shaped the observed patterns of distribution and speciation of aquatic biodiversity.
Global change and its detrimental effects on biodiversity (e.g., Hering et al. 2009, Bálint et al. 2011) recently focused scientific attention on freshwater biodiversity throughout Europe, including the

Western Balkans (e.g., Zakšek et al. 2009, Klobučar et al. 2013, Weiss et al. 2014).
Faunal studies on Western Balkan aquatic biodiversity recovered intriguing biogeographic patterns, and several new species (e.g., Petkovski et al. 2009, Pešić & Glöer 2013, Vitecek et al. 2015). Investigations on the caddisfly fauna of the Western Balkans further identified several factors including karstification as drivers of speciation (Previšić et al. 2009, 2014b). Taxonomic richness of Drusinae (Trichoptera, Limnephilidae) is particularly high in the Western Balkans, including a high number of micro-endemics (Malicky 2004; Graf et al. 2008; Oláh 2010, 2011; Kučinić et al. 2011a,b; Oláh and Kovács 2013; Previšić et al. 2014a, b; Vitecek et al. 2015; Ibrahimi et al. submitted).
The subfamily Drusinae Banks currently comprises 110 species in 8 genera (Malicky 2004, 2005; Oláh 2010, 2011; Oláh and Kovács 2013; Previšić et al. 2014a; Vitecek et al. 2015; Ibrahimi et al. submitted), the majority of which are crenobiont (Graf et al. 2008), and features three different larval feeding groups: filtering carnivores, omnivorous shredders, and scraping grazers (Pauls et al. 2008, Graf et al. 2009). Adults of each feeding group are characterized by a set of synapomorphies (Vitecek et al. submitted). In contrast to males of the other feeding types, filtering carnivorous Drusinae males exhibit laterally positioned gland openings at the fifth abdominal sternite and parallel wing veins in the hind wing anal field (depicted in Vitecek et al. submitted). The largest, paraphyletic genus *Drusus* (Pauls et al. 2008) comprises 86 species of all feeding types (Malicky 2004, 2005; Kučinić et al. 2011a; Oláh 2010, 2011; Oláh and Kovács 2013; Vitecek et al. 2015; Ibrahimi et al. submitted), including several filtering carnivorous species (Graf et al. 2008, Vitecek et al. submitted). The monotypic genus *Cryptothrix* also behaves as filtering carnivore (Bohle 1987, Graf et al. 2008), and thus represents another filtering carnivorous Drusinae *sensu* Pauls et al. 2008 — the systematic position of this genus is discussed in Vitecek et al. (submitted).
We describe two new micro-endemic filtering carnivore *Drusus* species, and provide re-descriptions of filtering carnivorous Drusinae *sensu* Pauls et al 2008.

Materials and Methods
Adults were collected using sweep nets and by handpicking. Collected specimens were stored in 96% EthOH.
Male and female genitalia were examined after being cleared in either KOH or lactic acid. Nomenclature of male genitalia of *Drusus* follows Nielsen (1957, for *Limnephilus flavicornis* Fabricius) using the simplifying terms "superior appendages" for the lateral processes of segment X (cerci *sensu* Snodgrass 1935), and "intermediate appendages" for the sclerite and the anterior process of segment X (paraproct *sensu* Snodgrass 1935). Nomenclature of larval morphological features follows Wiggins (1998) and Waringer & Graf (2011), nomenclature of primary setae and setal areas follows Wiggins (1998). Illustrations were prepared according to Thomson & Holzenthal (2010) in which pencil drawings made with a camera lucida are digitized, edited and inked in Adobe Illustrator (v. 16.0.4, Adobe Systems Inc.).

Taxonomy
Descriptions of the new species

Drusus krpachi sp. n. Kučinić, Graf and Vitecek
Material examined. Holotype. 1 male: Macedonia, Mavrovo National Park, Korab Mountains, česma Elem; N41.857, E 20.625; leg. Kučinić, Krpač, Mihoci; 15.VIII.2011. Currently deposited in coll. W. Graf, will be deposited in the Croatian Natural History Museum, Zagreb, Croatia.
Type locality. Macedonia, Korab Mountains.
Diagnosis. Males of the new species are most similar to *D. macedonicus*, but exhibit (1) a distally straight ventral half of segment IX; (2) a dorsally straight tip of the intermediate appendage distinctly separated by a proximal indentation and with small proximal and distal rough protrusions; (3)

a conical inferior appendage with a proximal dorsal triangular protrusion; (4) parameres with three tines in the distal third in dorsal view. *Drusus macedonicus* males have a distally concave ventral half of segment IX, intermediate appendages with two rough rounded dorsad protrusions but lacking a distinct proximal indentation, distally tapering inferior appendages, and parameres with a single tine in the distal third in dorsal view.

Description. *Adults*. Habitus yellow; sternites and tergites fawn; cephalic and thoracic setal areas pale; cephalic and thoracic setation blond, abdominal setation scarce, blond legs fawn; haustellum and intersegmental integument pale, whitish; wings yellow with blond setae on veins and the membrane. Male maxillary palp 3-segmented. Forewing length 11 mm, spur formula 1–3–3 in males. *Male genitalia* (Fig. 1). Tergite VIII fawn, setae absent; spinate area in lateral view approximately flat with a slight dorsad protrusion in the anterior half, in dorsal view suboval, flanked by membraneous, less sclerotized areas. Ninth abdominal segment (IX) with a rounded lateral protrusion in the dorsal half; dorsally roughly as wide as ventrally in caudal view; in lateral view ventral half distally straight. Superior appendages in lateral view suboval, caudally elongated, length:height ~ 2.5:1; in dorsal view slightly concave medially. Intermediate appendages in lateral view with a subtriangular tip, rough areas concentrated on the more dorsal proximal and the more ventral distal part; in dorsal view tips separated, oval, distally converging; in caudal view approximately triangular. Inferior appendages in lateral view conical, proximally wide, distally slender, with a proximal triangular protrusion; in ventral and dorsal view with a small median tip and a slight notch. Parameres simple, in dorsal view with 3 thorns in the distal third: 2 mediolateral, 1 dorsal.

Female and pupa unknown, larva described and keyed out in Vitecek et al. (submitted).

Etymology. Named for V. Krpač, Macedonian entomologist and collector of the species.

Distribution. This species is a micro-endemic of the Korab Mountains, Hellenic Western Balkans (ecoregion 6, Illies 1978) (Fig. 11).

Comments. This species is also recovered as closely related to *D. macedonicus* (see Vitecek et al. submitted).

Drusus puskasi sp. n. Oláh and Vitecek

Material examined. Holotype. 1 male: Albania Shkoder County, Shkoder District, Prokletije Mts, beech forest with brook above Okol; N42.42258, E19.76127; leg. Puskas 05.IX.2013. Currently deposited in coll. W. Graf, will be deposited in János Oláh Private Collection under national protection of the Hungarian Natural History Museum, Budapest, Hungary.

Type locality. Albania, Prokletije Mountains.

Diagnosis. Males of the new species are most similar to *D. macedonicus*, but exhibit (1) a sharp mediocaudad protrusion of segment IX; (2) a dorsally straight and rough tip of the intermediate appendage distinctly separated by a proximal indentation (3) a distinctly slender and constricted distal half of the inferior appendage in lateral view. *Drusus macedonicus* males have a mediocaudad and a ventrocaudad protrusion of segment IX, intermediate appendages with two rough rounded dorsad protrusions but lacking a distinct proximal indentation, and to a lesser degree constricted inferior appendages.

Description. *Adults*. Habitus yellow; sternites and tergites fawn; cephalic and thoracic setal areas pale; cephalic and thoracic setation blond, abdominal setation scarce, blond legs fawn; haustellum and intersegmental integument pale, whitish; wings yellow with blond setae on veins and the membrane. Male maxillary palp 3-segmented. Forewing length 10.9 mm, spur formula 1–3–3 in males. *Male genitalia* (Fig. 2). Tergite VIII fawn, setae scarce; spinate area in lateral view approximately flat with a slight dorsad protrusion in the anterior half, in dorsal view suboval, flanked by membraneous, less sclerotized areas. Ninth abdominal segment (IX) with a rounded lateral protrusion in the dorsal half; dorsally roughly as wide as ventrally in caudal view; in lateral view with a sharp median caudad protrusion. Superior appendages in lateral view suboval, caudally elongated, length:height

~ 2.5:1, in dorsal view medially concave. Intermediate appendages in lateral view with a subtriangular, dorsally rough tip; in dorsal view tips separated, wedge-shaped, approximately parallel; in caudal view approximately triangular. Inferior appendages in lateral view subtriangular, proximally somewhat bulbous, distally slender and distinctly constricted; in ventral view with a longitudinal groove delimiting a median lobe; in ventral and dorsal view with a small median tip and a slight notch. Parameres simple, in lateral view with 1 thorn in the distal third.
Female, pupa and fifth instar larva unknown.
Etymology. Named for G. Puskas, Hungarian entomologist and collector of the species.
Distribution. This species is a micro-endemic of the Prokletije Mountains, Hellenic Western Balkans (ecoregion 6) (Fig. 11).
Comments. This species is also recovered as closely related to *D. macedonicus* (see Vitecek et al. submitted).

Re-descriptions of filtering carnivore Drusinae *sensu* Pauls et al. 2008
Cryptothrix nebulicola McLachlan
Material examined. 1 male: Italy, Torino, Traversella, Fondo, Burdeivier brook; leg. Vincon; 12.VII.2012. 12 males: Italy, San Marco Pass; leg. Graf; 14.VIII.2000.
Type locality. Switzerland, Canton of Valais, Maienwang (Grimselpass).
Description. *Adults.* Habitus dark; sternites and tergites brown; cephalic and thoracic setal areas pale; cephalic, thoracic and abdominal setation blond; legs light brown to fawn, proximally darker; haustellum and intersegmental integument pale, whitish; wings dark, with dark setae. Male maxillary palp trisegmented. Forewing length 8–10 mm, spur formula 1–2–2 in males.
Male genitalia (Fig. 3). Tergite VIII brown, with lighter areas around alveoli; setation abundant, ubiquitous; spinate area squarish in dorsal view, flanked by membraneous, less sclerotized areas. Ninth abdominal segment (IX) with a rounded wedge-shaped protrusion in the dorsal half, dorsally wider than ventrally in caudal view. Superior appendages in lateral view elongated caudally, suboval with dorsad tips slightly curved, in dorsal view medially concave. Intermediate appendages in lateral, dorsal and caudal view dorsally with 2 distinct tips, the proximal tip rounded, rough, the distal tip pointed, smooth; in caudal view approximately an isoceles trapezium. Inferior appendages (gonopods *sensu* Snodgrass 1935) in lateral view roughly triangular with a concave ventral outline; tips converging in dorsal and ventral view; in ventral view with a longitudinal groove delimiting a median lobe. Parameres simple, rodlike, medially and distally somewhat bulbous.
Female depicted in Schmid (1956), Malicky (2004); larva keyed by Waringer & Graf (2011), Vitecek et al. (submitted); pupa unknown.
Distribution. The species is a regional endemic of the Western Alps (ecoregion 4) (Fig. 11).

Drusus chrysotus Rambur
Material examined. 12 males: Austria, Krumbach, Soboth; N 46.723, E15.0555; leg. Graf; 20.V.2004.
Type locality. France, Rhône-Alpes, Haute-Savoie, Chamonix valley.
Description. *Adults.* Habitus: light brown to yellow; sternites and tergites light brown, abdominal tergite VII with a distinct saddle; cephalic and thoracic setal areas pale; cephalic and thoracic setation blond, abdominal setation scarce, short, dark; legs fawn, proximally darker; haustellum and intersegmental integument pale, whitish; wings light brown to yellow with dark setae on the veins and blond setae on the membrane. Male maxillary palp trisegmented. Forewing length 14–16 mm, spur formula 1–3–3 in males.
Male genitalia (Fig. 4). Tergite VIII light brown, with short, pale, translucent setae; spinate area in lateral view with a distinct hump, in dorsal and caudal view tripartite, flanked by membraneous, less sclerotized areas. Ninth abdominal segment with a distinct rounded protrusion in the dorsal half;

dorsally as wide as ventrally in caudal view. Superior appendages in lateral view caudally elongated, subtriangular, in dorsal view medially concave. Intermediate appendages in lateral view with a long, broad, rough tip, in dorsal view fused to an oval structure; in caudal view subtriangular. Inferior appendages in lateral view conical, short; in ventral and dorsal view blunt, with a blunt, short median tip; in ventral view with a longitudinal groove delimiting a median lobe. Parameres simple with several thorns on a common base in the distal third.
Female depicted in Schmid (1956), Malicky (2004); larva keyed by Waringer & Graf (2011), Vitecek et al. (submitted); pupa described in Bohle (1987).
Distribution. This species is widely distributed, occuring in and around the Alpine arc (ecoregion 4), the Western and Central Highlands (ecoregions 8 & 9) and was also found in the northern part of the Dinaric Alps (ecoregion 5) (Fig. 11).

Drusus discolor Rambur
Material examined. 3 males: France, Mt. Canigou; N42.4864, E2.4139; leg. Graf; 12.VII.2012. 2 males: France, St. Pierre de la Martin; N42.9597, E0.8290; leg. Graf; 22.VII.2012. 7 males: Austria, Gurkursprung; leg. Wieser; 13.VII.1997. 22 males: Switzerland, Val Munstair; N46.5852, E10.4544; leg. Graf; 20.VII.2006. 1 male: Montenegro, Brodavac, right tributary of Peručica; N42.6859, E19.7364; leg. A. Previšić; 10.VII.2013.
Type locality. France, Rhône-Alpes, Haute-Savoie, Chamonix valley.
Description. *Adults.* Habitus fawn to brown; sternites and tergites fawn to brown; cephalic and thoracic setal areas pale; cephalic, thoracic and abdominal setation blond; legs fawn, proximally darker; haustellum and intersegmental integument pale, whitish; wings blond-brown, with blond-brown setae on the veins and blond setae on the membrane. Male maxillary palp trisegmented. Forewing length 12-15 mm, spurformula 1–3–3.
Male genitalia (Fig. 5). Tergite VIII light brown, setation scarce; spinate area in lateral view with a distinct hump, in dorsal view suboval, flanked by membraneous, less sclerotized areas. Ninth abdominal segment with a distinct rounded protrusion in the dorsal half; dorsally roughly as wide as ventrally in caudal view. Superior appendages in lateral view caudally elongated, suboval, in dorsal view medially concave. Intermediate appendages in lateral view with a rounded, rough, dorsal tip; in dorsal view the tips separate, oval, posteriorly orientated mediadly; in caudal view subtriangular. Inferior appendages in lateral view conical; in ventral view with a median tip and a distinct notch; in ventral view with a longitudinal groove delimiting a median lobe. Parameres simple with a single bulbously based thorn in the distal third.
Female depicted in Schmid (1956), Malicky (2004); larva keyed by Waringer & Graf (2011), Vitecek et al. (submitted); pupa unknown.
Distribution. This species is one of the most wide-spread Drusinae species, covering all major European mountain ranges from the Carpathians to the Pyrenees (ecoregions 1-10) (Fig. 11).

Drusus macedonicus Schmid
Material examined. 1 male: Macedonia, Jablanica Mt., Labunište; N41.271841, E20.558136; leg. Kučinić and Krpač; 19.IX.2013. 1 male: Macedonia, Pelister Mt., springs of Caparska reka; N41.003889, E21.167944; leg. Graf and Previšić; 07.VII.2010.
Type locality. Macedonia, Pelister Mountains.
Description. *Adults.* Habitus yellow; sternites and tergites fawn; cephalic and thoracic setal areas pale; cephalic and thoracic setation blond, abdominal setation scarce, blond; legs fawn; haustellum and intersegmental integument pale, whitish; wings yellow with blond setae on veins and the membrane. Male maxillary palp trisegmented. Forewing length 10-12 mm, spur formula 1–3–3.
Male genitalia (Fig. 6). Tergite VIII fawn, setation lateral, scarce; spinate area in lateral view approximately flat, in dorsal view suboval, flanked by membraneous, less sclerotized areas. Ninth

abdominal segment with a somewhat sharp protrusion in the dorsal half; dorsally as wide as ventrally in caudal view. Superior appendages in lateral view caudally elongated; suboval in diameter, length:height ~ 2.5:1; in dorsal view proximally slightly concave medially. Intermediate appendages in lateral view with two rough tips: 1 curved dorsoposteriad, 1 central, rounded; in dorsal view tips adjacent, parallel; in caudal view subtriangular. Inferior appendages in lateral view approximately conical, proximally wide, distally slender; in ventral and dorsal view with a median tip and a notch, separated by a slight notch; in ventral view with a longitudinal groove delimiting a median lobe. Parameres simple, with a single dorsal thorn in the distal third.
Female depicted in Schmid (1956), Malicky (2004); larva keyed by Vitecek et al. (submitted); pupa unknown.
Distribution. This species is a micro-endemic of the Pelister and Jablanica Mountains, Hellenic Western Balkans (ecoregion 6) (Fig. 11).

Drusus meridionalis Kumanski
Material examined. 10 males: Bulgaria, Vihren, Pirin Mountains, Okotovo-Banserishka, marshy spring; N41.7389, E23.4462; leg. Keresztes, Török, Kolcsár; 23.VIII.2013.
Type locality. Bulgaria, Rila and Pirin Mountains.
Description. *Adults.* Habitus yellow to brown; sternites and tergites yellow to brown; cephalic and thoracic setal areas pale; cephlic and thoracic setation blond, abdominal setation scarce, short, dark; legs yellow to light brown, proximally darker; haustellum and intersegmental integument pale, whitish; wings yellow to fawn, with blond setae on the veins and the membrane. Male maxillary palp trisegmented. Forewing length 12-14 mm, spur formula 1–3–3 in males.
Male genitalia (Fig. 7). Tergite VIII yellow to brown, setae absent; spinate area in lateral view approximately flat, in dorsal view suboval, somewhat squarish, flanked by membraneous, less sclerotized areas bearing single setae. Ninth abdominal segment with a distinct, somewhat rounded protrusion in the dorsal half; in caudal view ventrally wider than dorsally. Superior appendages in lateral view caudally elongated; suboval in diameter, length:heigth~3:1; in dorsal view proximally slightly concave medially. Intermediate appendages in lateral view with a rounded, rough tip, in dorsal view 2 separate parallel tips; in caudal view subtriangular. Inferior appendages in lateral view conical; in ventral and dorsal view slender with a slight median tip and a shallow notch; in ventral view with a longitudinal groove delimiting a median lobe. Parameres simple, with a single, bulbously based thorn in the distal third.
Female depicted in Kumanski (1973), Malicky (2004); larva keyed by Vitecek et al. (submitted); pupa unknown.
Distribution. This species is a micro-endemic of the Eastern Balkans (ecoregion 7) (Fig. 11).
Comments. This taxon was formerly described as subspecies but was elevated to species rank, as it is morphologically distinct from *D. romanicus*, differentially distributed, and further recovered as well separated from the latter species in phylogenetic analyses (Pauls et al. 2009, Vitecek et al. submitted).

Drusus muelleri McLachlan

Material examined. 1 male: Switzerland, Furkapass; N46.5888, E8.4327; leg. Graf; 21.VII.2006.
Type locality. Switzerland, Canton of Uri, Hospental.
Description. *Adults.* Habitus dark; sternites and tergites brown; cephalic and thoracic setal areas pale, cephalic and thoracic setation blond, abdominal setation scarce, short, dark; coxa, trochanter, femur brown, tibia and tarsi fawn; haustellum and intersegmental integument pale, whitish; wings brown, smoky, with dark setae on the veins and blond setae on the membrane. Male maxillary palp trisegmented. Forewing length 11–13 mm (Malicky 2004), spur formula 1–3–3 in males.
Male genitalia (Fig. 8). Tergite VIII brown, setae absent; spinate area spinate area in lateral view

convex, in dorsal view approximately trilobate, flanked by membraneous, less sclerotized areas. Ninth abdominal segment with a sharp wedge-shaped protrusion in the dorsal half; dorsally wider than ventrally in caudal view. Superior appendages in lateral view caudally distinctly elongated; length:width~5:1, tip approximately round in diameter, in dorsal view proximally concave medially. Intermediate appendages in lateral view with a rough round tip dorsally, in dorsal view two separate, wedge-shaped tips; in caudal view subtriangular. Inferior appendages in lateral view conical; in ventral and dorsal view with a median tip and a distinct notch; in ventral view with a longitudinal groove delimiting a median lobe. Parameres simple, with a single dorsal thorn in the distal third.
Female depicted in Schmid (1956), Malicky (2004), larva keyed by Waringer & Graf (2011), Vitecek et al. (submitted); pupa unknown.
Distribution. This species is a regional endemic of the Western Alps (ecoregion 4) (Fig. 11).

Drusus romanicus Murgoci and Botosaneanu
Material examined. 1 male: Romania, Apuseni Mts., Garda de Sus, tributary of Ariesul Mare; N46.4508, E22.7982; leg. Oláh, Bajka, Balogh, Borics; 29.V.2013. 1 male: Romania, Apuseni Mts., Muntii Giaului, Stiunea Muntele Baisorii, Lupinus stream; leg. Oláh, Balogh, Fekete; 18.06.2013. 1 male: Romania, Retezat Mts, Bucara Stream, 150 m below Bucara lake; N45.3570, E22.8753; leg. Bajka, Balogh, Borics, Borics; 10.VIII.2013.
Type locality. Romania, Carpathian Mountains, spring areas of the Ialomita stream.
Description. *Adults.* Habitus brown to light brown; sternites and tergites brown to light brown; cephalic and thoracic setal areas pale; cephalic, thoracic and abdominal setation blond; legs light brown, proximally darker; haustellum and intersegmental integument pale, whitish; wings brown, proximally lighter, with blond setae on veins and membrane. Male maxillary palp trisegmented. Forewing length 12-14 mm, spur formula 1–3–3.
Male genitalia (Fig. 9). Tergite VIII brown, setae present; spinate area in lateral view approximately flat, in dorsal view suboval, flanked by membraneous, less sclerotized areas. Ninth abdominal segment with a distinct, somewhat rounded protrusion in the dorsal half; in caudal view ventrally wider than dorsally. Superior appendages in lateral view caudally elongated; approximately round in diameter, length:heigth~4.5:1; in dorsal view proximally distinctly concave medially. Intermediate appendages in lateral view with a rounded, rough tip, in dorsal view 2 separate laterad tips; in caudal view subtriangular. Inferior appendages in lateral view conical, long; in ventral and dorsal view slender with a slight median tip and a shallow notch. Parameres simple, with a median hook-shaped tip bearing several smaller thorns.
Female and pupa unknown; larva keyed by Vitecek et al. (submitted).
Distribution. This species is a regional endemic of the Western and Southern Carpathians (ecoregion 10) (Fig. 11).

Drusus siveci Malicky
Material examined. 5 males: Bosnia and Herzegovina, Sutjeska National Park, stream close to Čermerno; N43.2650, E18.5927; leg. Previšić, Miliša; 04.VII.2012.
Type locality. Montenegro, Andrijevica, Gnjili Potok.
Description. *Adults.* Habitus yellow to fawn; sternites and tergites fawn; cephalic and thoracic setal areas pale; cephalic, thoracic and abdominal setation blond; legs yellow to fawn; haustellum and intersegmental integument pale, whitish; wings fawn, with blond to brown setae on the veins and blond setae on the membrane. Male maxillary palp trisegmented. Forewing length 10–12 mm, spur-formula 1–3–3.
Male genitalia (Fig. 10). Tergite VIII fawn, setation abundant; spinate area in lateral view approximately flat, in dorsal view oval, flanked by membraneous, less sclerotized areas. Ninth abdominal segment with a distinct, rounded protrusion in the dorsal half; dorsally wider than ventrally in cau-

dal view. Superior appendages in lateral view caudally elongated, suboval, in dorsal view slightly concave medially. Intermediate appendages in lateral view with a pointed, hook-like tip; in dorsal view the tips fused, arching dorsad; in caudal view subtriangular. Inferior appendages in lateral view conical, blunt, ventral line somewhat concave; in ventral view with a median tip and a distinct notch; in ventral view tip clearly rounded. Parameres simple, with a single bulbously based thorn in the distal third.

Female and pupa unknown; larva keyed by Vitecek et al. (submitted).

Distribution. This species is a micro-endemic of the Dinaric Western Balkans (ecoregion 5) (Fig. 11).

Discussion

Drusinae micro-endemics of the Western Balkans

Morphology of the new species as well as molecular phylogenetics (Vitecek et al. submitted) suggest a nested position in filtering carnivorous Drusinae *sensu* Pauls et al. 2008 (comprising *Drusus discolor*, *D. muelleri*, *D. chrysotus*, *D. siveci*, *D. romanicus*, *D. meridionalis*, *Cryptothrix nebulicola*). The new *Drusus* species presented here are morphologically similar to *D. macedonicus* Schmid. However, they differ distinctly in the formation of the male genitalia (particularly the intermediate appendage), and are discretely distributed. Also, they are well delineated in phylogenetic analysis (Vitecek et al. submitted). To our current knowledge, the new species are micro-endemics of single mountain ranges.

Interestingly, the type localities of the new species are in close vicinity the known range of *D. macedonicus* (Fig. 11). Such small-scale distribution of distinct Drusinae species is well documented from the Western Balkans (Marinković-Gospodnetić 1976; Kučinić et al. 2011a; Oláh 2010, 2011; Oláh and Kovács 2013; Previšić et al. 2014a, b; Vitecek et al. 2015). Similarly, other taxa exhibit comparable distribution patterns, in which single mountain ranges represent the main range of a species, or evolutionary lineage (Ursenbacher et al. 2008, Zogaris et al. 2008, Stevanović et al. 2009, Karaman et al. 2011). The intriguing distribution patterns exhibited by some radiations potentially result from the geological history of the region and historic and present-day climate conditions. Small-scale speciation of Drusinae presumably is facilitated by intrinsic traits of the subfamily, such as their occurrence at higher elevations (Pauls et al. 2006, 2009), a putatively low dispersal potential (Müller-Peddinghaus 2011) [although dispersal potential of single species might be surprisingly high as inferred by Geismar et al. (2015)], and might be further enhanced by habitat fragmentation, e.g., via regional karstification. Occurrence of Drusinae could therefore serve as proxy for the occurrence of other aquatic invertebrate taxa, particularly to crenobiont taxa exhibiting the same or similar traits.

Western Balkan aquatic diversity

The Western Balkans represent a hot-spot of species richness and endemicity in Europe (cf. Kryštufek & Reed 2004, Guéorguiev 2007, Kenyeres et al 2009, Jaskuła 2011). Particularly inhabitants of isolated habitats like coldwater springs and streams, caves or the profundal of large lakes contribute to high species richness of the region (e.g., Petkovski et al. 2009, Wilke et al. 2010, Pešić & Glöer 2013). Such taxa probably are more susceptible to drivers of speciation, such as climatic and geological processes (e.g., karstification, see Previšić et al. 2009, 2014b), especially if their dispersal potential is low.

The description of the two new micro-endemic *Drusus* species augments the number of Western Balkan Drusinae species. Drusinae diversity in the Western Balkans currently comprises 40 species including 13 species (30 %) that were discovered since 2010, of which 32 are endemics occurring exclusively in Western Balkans (Graf et al. 2008, Oláh 2010, 2011; Schmidt-Kloiber & Hering 2012; Oláh & Kovács 2013; Previšić et al. 2014b; Vitecek et al. 2015; Ibrahimi et al. submitted,

pers. comm.; this study).
Global change and (anthropogenic) habitat modification are among the greatest threats to endemic and micro-endemic freshwater species (Hering et al. 2009, Tierno de Figueroa et al. 2010, Bálint et al. 2011, Conti et al. 2014). Water extraction for tourism, agriculture, and, most importantly, hydroelectricity are key drivers of anthropogenic habitat modification (Foster 1991, Polhemus 1993, Dudgeon 2006). Hydropower plants were identified as the greatest threat to the European freshwater biodiversity (Zarfl et al. 2014,Freyhof 2012, Schwarz 2012; riverwatch.eu).
Recent taxonomic efforts in the Western Balkans demonstrated the significance of the region for European biodiversity. The description of the two new species *D. krpachi* and *D. puskasi* highlight, in combination with those studies, the significance of the region. However, progressing socio-economic change and, thus, anthropogenic habitat modification threaten the freshwater biodiversity of the Western Balkans, and potentially will result in the loss of yet-to-be discovered species.

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Figure captions

Figure 1. Male genitalia of *Drusus krpachi* sp. n. A, left lateral view; B, paramere, dorsal view; C, caudal view; D, dorsal view; E, ventral view. Scale bar denotes 1 mm. Del. Vitecek.

Figure 2. Male genitalia of *Drusus puskasi* sp. n. A, left lateral view; B, paramere, dorsal view; C, caudal view; D, dorsal view; E, ventral view. Scale bar denotes 1 mm. Del. Vitecek.

Figure 3. Male genitalia of *Cryptothrix nebulicola*. A, left lateral view; B, paramere, dorsal view; C, caudal view; D, dorsal view; E, ventral view. Scale bar denotes 1 mm. Del. Vitecek.

Figure 4. Male genitalia of *Drusus chrysotus*. A, left lateral view; B, paramere, dorsal view; C, caudal view; D, dorsal view; E, ventral view. Scale bar denotes 1 mm. Del. Vitecek.

Figure 5. Male genitalia of *Drusus discolor*. A, left lateral view; B, paramere, dorsal view; C, caudal view; D, dorsal view; E, ventral view. Scale bar denotes 1 mm. Del. Vitecek.

Figure 6. Male genitalia of *Drusus macedonicus*. A, left lateral view; B, paramere, dorsal view; C, caudal view; D, dorsal view; E, ventral view. Scale bar denotes 1 mm. Del. Vitecek.

Figure 7. Male genitalia of *Drusus meridionalis*. A, left lateral view; B, paramere, dorsal view; C, caudal view; D, dorsal view; E, ventral view. Scale bar denotes 1 mm. Del. Vitecek.

Figure 8. Male genitalia of *Drusus muelleri*. A, left lateral view; B, paramere, dorsal view; C, caudal view; D, dorsal view; E, ventral view. Scale bar denotes 1 mm. Del. Vitecek.

Figure 9. Male genitalia of *Drusus romanicus*. A, left lateral view; B, paramere, dorsal view; C, caudal view; D, dorsal view; E, ventral view. Scale bar denotes 1 mm. Del. Vitecek.

Figure 10. Male genitalia of *Drusus siveci*. A, left lateral view; B, paramere, dorsal view; C, caudal view; D, dorsal view; E, ventral view. Scale bar denotes 1 mm. Del. Vitecek.

Figure 11. Distribution of filtering carnivore Drusinae. Single records of micro-endemic species are depicted as symbols, stroked or filled areas denote ranges of more widely distributed species with a higher number of occurrence records.

Figure 1

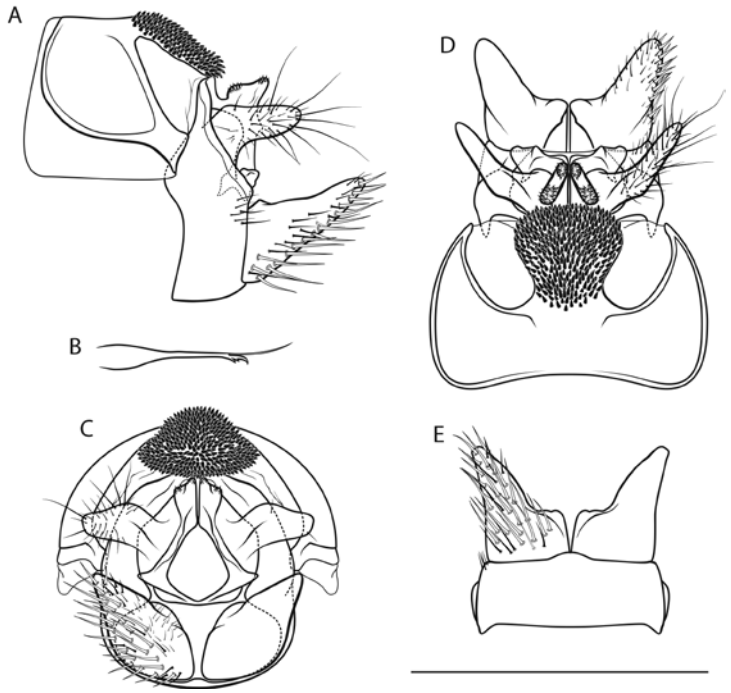
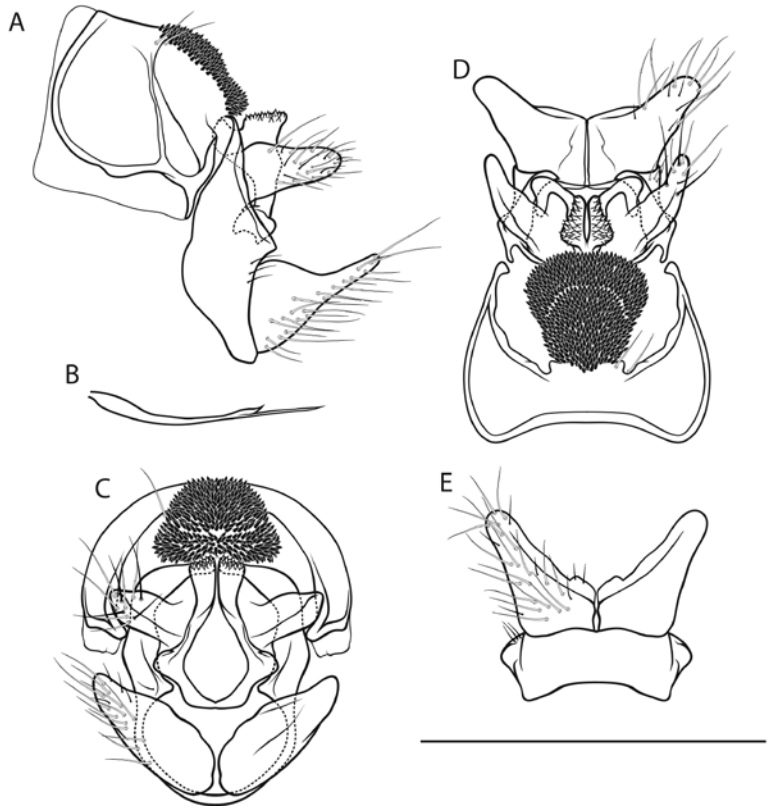
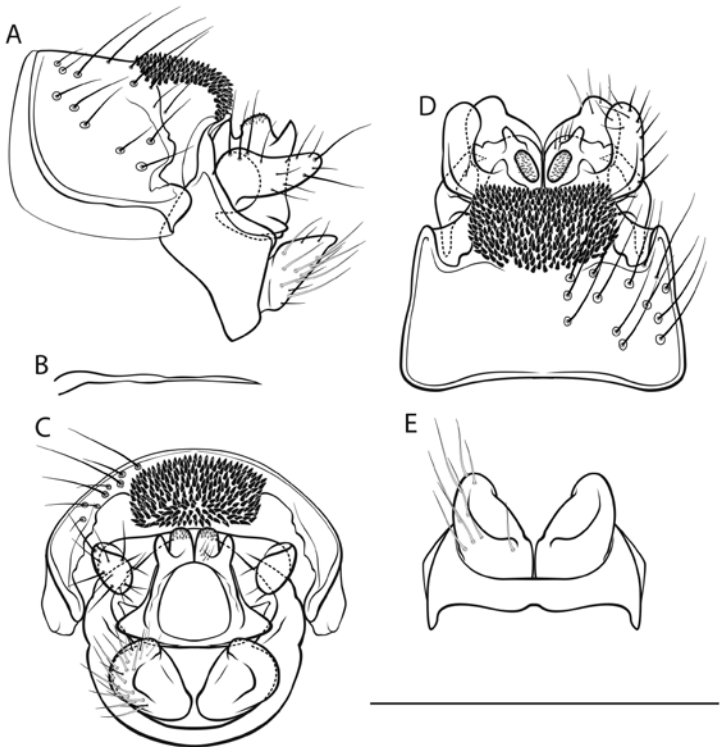


Figure 2





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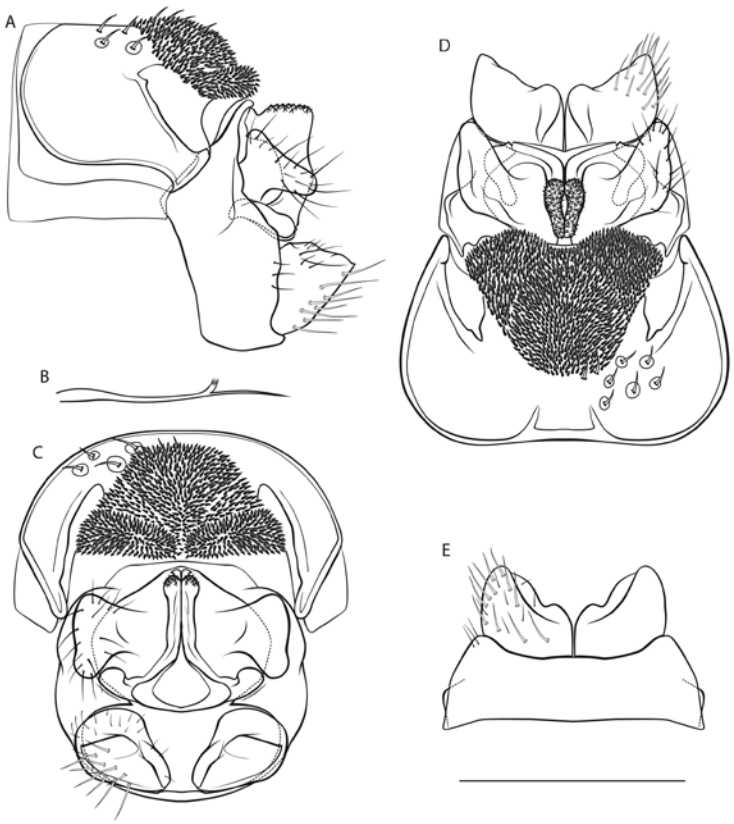

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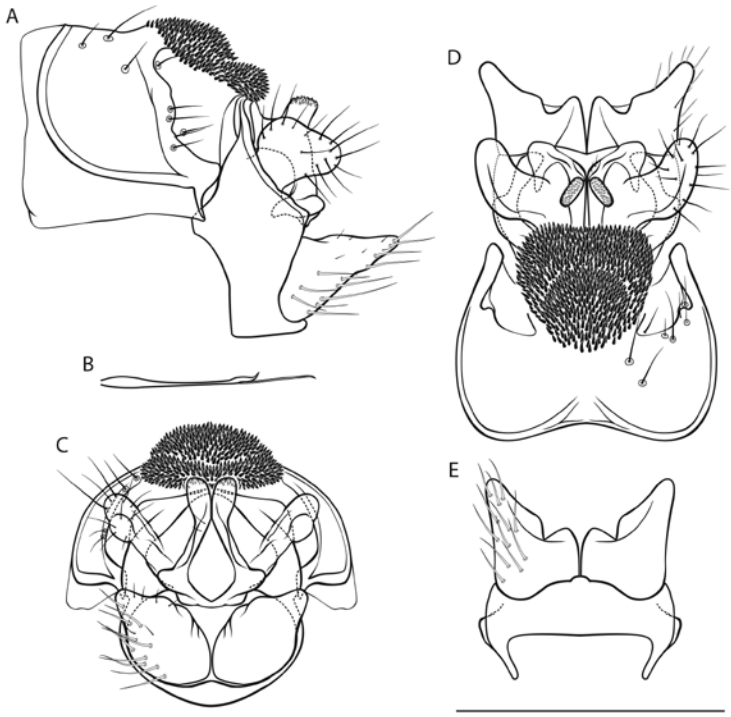


Figure 5

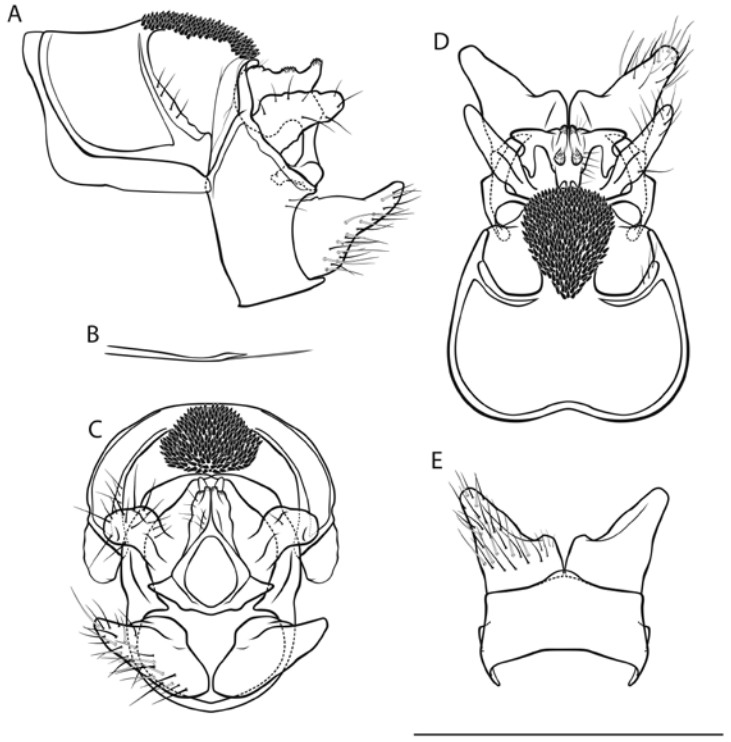


Figure 6

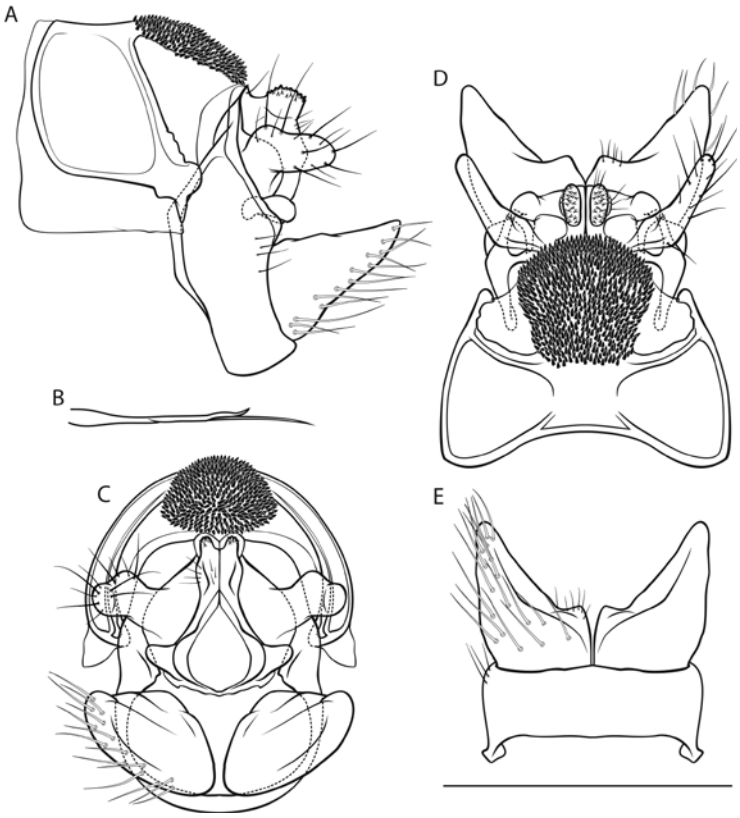
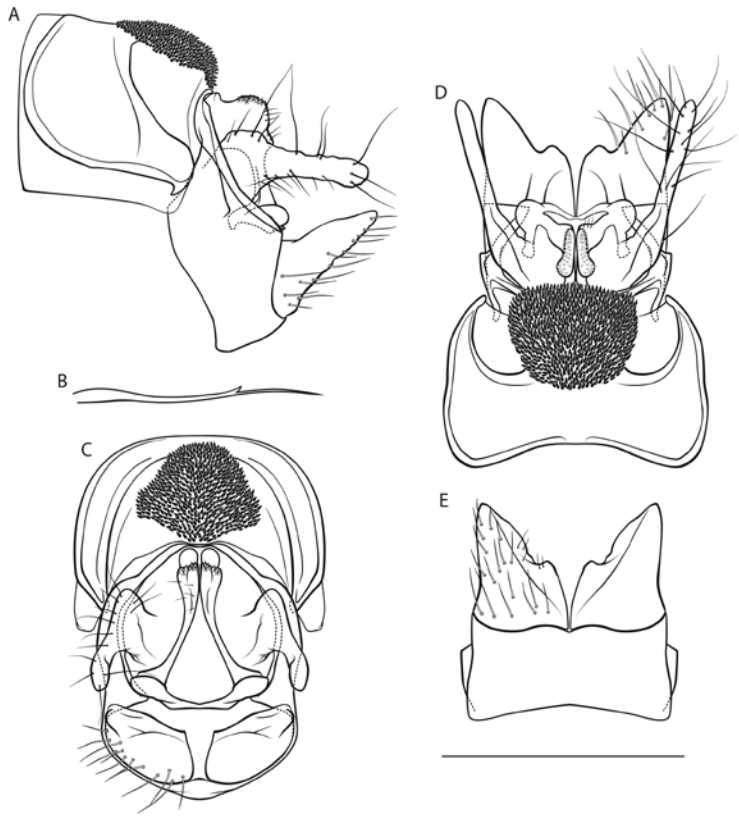


Figure 7



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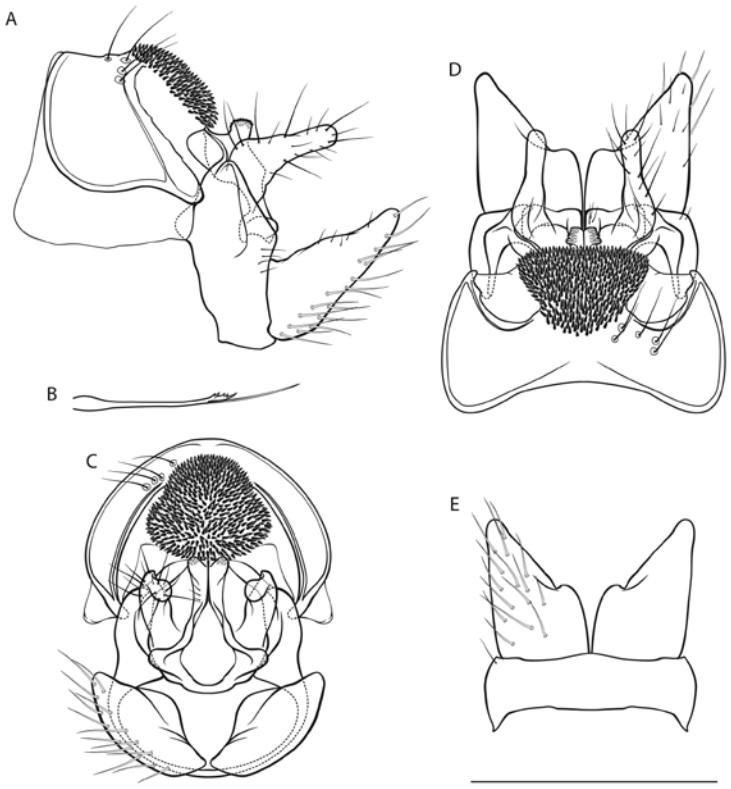

Figure 9

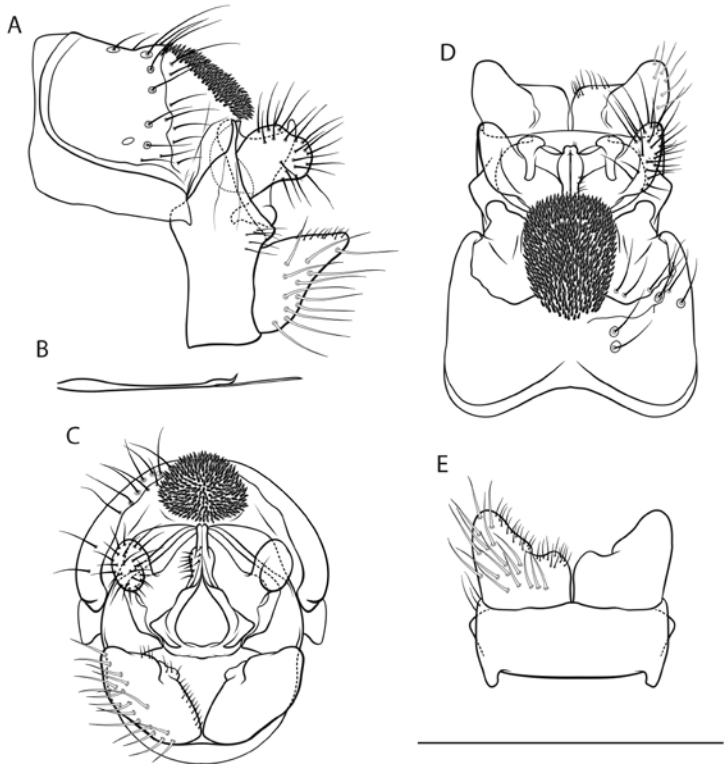

Figure 10

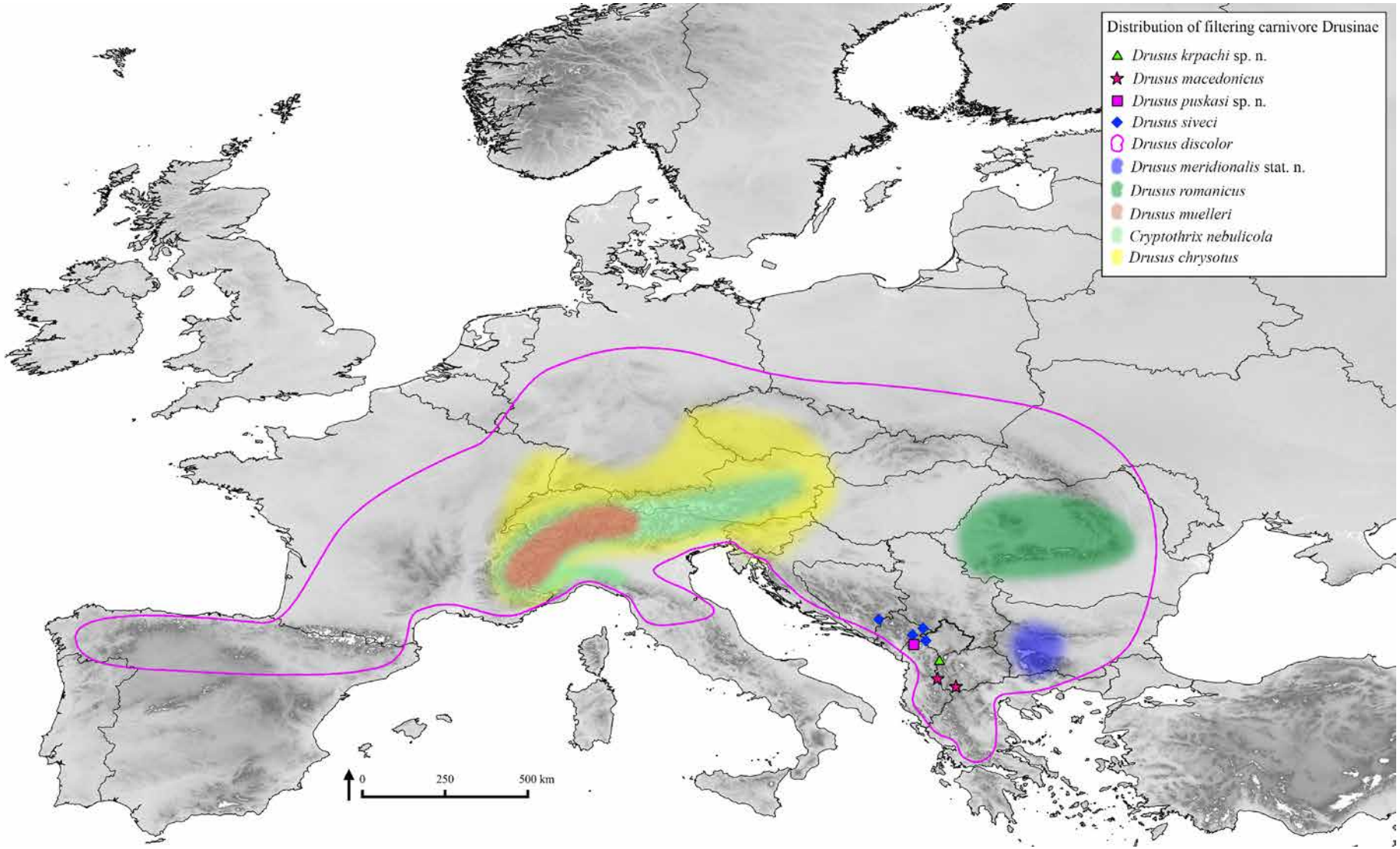
Distribution of filtering carnivore Drusinae
Drusus krpachi sp. n.
Drusus macedonicus
Drusus puskasi sp. n.
Drusus siveci
Drusus discolor
Drusus meridionalis stat. n.
Drusus romanicus
Drusus muelleri
Cryptothrix nebulicola
Drusus chrysotus
0
250
500 km

Figure 11

Chapter IV

Manuscript

A hairy case: The evolution of filtering carnivorous Drusinae (Limnephilidae, Trichoptera)

This manuscript focuses on evolutionary trends in head capsule modifications of filtering carnivorous Drusinae, discusses the status of the monotypic genus Cryptothrix, describes two new species of filtering carnivores, provides a key to larvae of filtering carnivorous Drusinae and postulates another 2 new species.

The manuscript was accepted for publication at Molecular Phlogenetics and Evolution.

I supplied illustrations, generated and analysed the genetic dataset, designed the keys and wrote 90% of the manuscript as equally contributing first author together with Wolfram Graf. Additionally, I executed the duties of the corresponding author.

A hairy case: The evolution of filtering carnivorous Drusinae (Limnephilidae, Trichoptera)

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Running title: Evolution of filtering carnivorous Drusinae

Abstract. The caddisfly subfamily Drusinae BANKS comprises roughly 100 species inhabiting mountain ranges in Europe, Asia Minor and the Caucasus. A 3–gene phylogeny of the subfamily previously identified three major clades that were corroborated by larval morphology and feeding ecologies: scraping grazers, omnivorous shredders and filtering carnivores. Larvae of filtering carnivores exhibit unique head capsule complexities, unknown from other caddisfly larvae. Here we assess the species-level relationships within filtering carnivores, hypothesizing that head capsule complexity is a derived state based on the simple shapes observed in the other feeding groups. We summarize the current systematics and taxonomy of the group, clarify the systematic position of *Cryptothrix nebulicola*, and present a larval key to filtering carnivorous Drusinae.
We infer relationships of all known filtering carnivorous Drusinae and 34 additional Drusinae species using Bayesian species tree analysis and concatenated Bayesian phylogenetic analysis of 3805 bp of sequence data from six gene regions (mtCOI5-P, mtCOI3-P, 16S mrDNA, CADH, WG, 28S nrDNA), morphological cladistics from 308 characters, and a total evidence analysis.

All analyses support monophyly of the three feeding ecology groups but fail to fully resolve internal relationships. Within filtering carnivores, variation in head setation and frontoclypeus structure may be associated with progressive niche adaptation, with less complex species recovered at a basal position. We propose that diversification of complex setation and frontoclypeus shape represents a recent evolutionary development, hypothetically enforcing speciation and niche specificity within filtering carnivorous Drusinae.

Keywords: phylogeny; new species; *BEAST; species tree; larval key; filter-feeding

1. Introduction

Aquatic invertebrates have evolved to a staggering diversity of different feeding types (e.g., grazers, shredders, scrapers, gatherers, filter feeders, predators, and piercers (Cummins and Klug, 1979; Mecom, 1972; Wallace and Merrit, 1980). In particular, feeding ecology of caddisfly larvae is highly diverse (Graf et al., 2008; Mackay and Wiggins, 1979), rendering them fundamental participants in nutrient and energy fluxes in aquatic and the adjacent terrestrial ecosystems (Covich et al., 1999, Wallace and Webster, 1996).

In benthic insects, passive filter-feeding has evolved in Ephemeroptera, Trichoptera and Diptera. Most filter-feeding Diptera and all filter-feeding Ephemeroptera develop specialized structures, whereas the majority of Trichoptera employ silken-nets for filter feeding (McCafferty and Bae, 1992; Merrit and Wallace, 1984). Within Trichoptera, most families of Annulipalpia *sensu* Malm et al. (2013) are net-spinning filter-feeders that construct specialized nets, befitting their ecological niche (Holzenthal et al., 2007; Malm et al., 2013). Despite the heterogeneity of behavioural adaptations in net-spinning European Trichoptera, only few genera of Annulipalpia develop specialized mouthparts to exploit fine particulate organic matter (FPOM), whereas the majority develops shredder-like mouthparts (W. Graf, J. Waringer, unpubl. data). Filter-feeding Integripalpia do not construct filtering nets, but rather employ their whole body as a filtering structure. Members of the European genera *Allogamus*, *Brachycentrus*, and *Drusus* (Graf et al., 2008) and the South-East Asian genus *Limnocentropus* (Wiggins, 1969; Wallace and Merrit, 1980; Graf unpubl. data) exhibit filtering bristles on legs and sometimes on sterna, as well as modifications of the legs (e.g. elongation of femora in *Brachycentrus*) or heads (as in Drusinae) as morphological adaptations to filter-feeding. Drifting macrozoobenthos, and large organic particles were found to primarily consitute food particles foraged for in *B. subnubilus* (Majecki et al., 1997), and some species of filtering carnivorous Drusinae. (Bohle, 1983, 1987; Graf unpubl. data). Particularly larvae of Chironomidae and Ephermeroptera are preyed upon by filtering carnivorous Drusinae (Bohle, 1983, 1987; Graf unpubl. data), whereas fine particulate organic matter seems to be of lesser importance.

Understanding evolution of feeding modes and associated traits can help us to peruse evolutionary

ecology of Trichoptera, particularly with respect to evolutionary constraints on niche exploitation. To this end, the subfamily Drusinae represents an ideal model, as it exhibits unusually high feeding diversity including unique and complex feeding strategies among caddisflies (Graf et al., 2009; Pauls et al., 2008).

The limnephilid subfamily Drusinae comprises 8 currently recognized genera: *Anomalopterygella* Fischer, 1966; *Cryptothrix* McLachlan, 1867; *Drusus* Stephens, 1837; *Ecclisopteryx* Kolenati, 1848; *Hadimina* Sipahiler, 2002; *Leptodrusus* Schmid, 1955; *Metanoea* McLachlan, 1880; *Monocentra* Rambur, 1842. Most Drusinae are cold-stenotopic species restricted to Eurasian mountain ranges. The highly disjunct distribution patterns of some Drusinae species reflect the complex evolutionary history of the group with long-time persistence of distinct lineages (e.g. through multiple glacial cycles) and high levels of small scale allopatric speciation (Graf et al., 2008; Pauls et al., 2006; Previšić et al., 2009, 2014b). Thus, the subfamily features many endemic species (Graf et al., 2008; Malicky, 2005; Kučinić et al., 2011; Oláh, 2010, 2011).

The last exhaustive treatment of this group was conducted by Schmid (1956), who identified 7 genera of Drusinae and 6 species groups within the largest genus *Drusus*. He defined the subfamily mainly by venation characteristics of the male wings, and the presence of specialized A2 and A3 veins in the male hindwings, which form a pouch that harbours long vein bristles of unknown function (Schmid, 1956). Schmid (1956) examined 37 out of a total of 41 known species, of which 35 are still valid. Since then, 1 new genus and many new species have been described, resulting in roughly 100 known extant species (Graf et al., 2008; Malicky, 2004, 2005; Kučinić et al., 2011; Oláh, 2010, 2011; Oláh and Kovács, 2013; Previšić et al., 2014a; Sipahiler, 2002).

A first molecular phylogenetic study on the subfamily Drusinae revealed the prevalence of three distinct evolutionary lineages, reflecting feeding ecology of larvae (Graf et al., 2009; Pauls et al., 2008): (1) carnivorous filterers (developing toothed shredder-like mandible edges, modifications of head capsules, and additional filtering spines on legs and the first abdominal sternum), (2) omnivorous shredders (exhibiting toothed shredder mandibles, but lacking additional spines and filtering bristles), (3) epilithic grazers (developing spoon-shaped mandibles without teeth and lacking additional spines and filtering bristles). Further, Pauls et al. (2008) rejected most of the generic concepts and species groupings that Schmid (1956) had proposed based primarily on the morphology of adult male terminalia. In particular, the monotypic genus *Cryptothrix* was found to be closely related to the filtering carnivorous Drusinae, whereas the genus *Drusus* was identified as paraphyletic with *Anomalopterygella*, *Metanoea,* polyphyletic *Ecclisopteryx*, and *Cryptothrix* nested within (Pauls et al., 2008). The genus *Drusus* was found to comprise members of all three larval feeding types (Graf et al., 2009; Pauls et al., 2008).

Taxonomic knowledge enabling larval identification were hitherto presented for 47 Drusinae species only (references in Previšić et al., 2014a; Vitecek et al. 2015; Waringer et al., 2013a, b). Larval stages of Ephemeroptera, Plecoptera and Trichoptera are crucial quality elements for contemporary biological water quality monitoring approaches (AQEM consortium, 2002; Barbour et al., 1999; Barbour

and Yoder, 2000; Graf et al., 2002). Particularly the differential sensitivity of caddisfly species to minute environmental changes renders them ideal to monitor pollution or anthropogenic disturbance (Graf et al., 2002), particularly at species level (Waringer et al., 2013c). As all identifiable members of Drusinae are used as bio-indicators and sensitive species in biological monitoring (Moog et al., 2002), larval keys to this highly diversified group are crucial. Currently available larval keys cover roughly 50% of all known species, and need to be extended in order to exploit the full bioindicative potential of Drusinae.

In this study, we examine whether adult apomorphies exist for the feeding groups *sensu* Pauls et al. (2008). Further, to resolve phylogenetic relationships within filtering carnivorous Drusinae we infer phylogenetic relationships in a Bayesian framework using sequence data from 6 loci (mtCOI5-P, mtCOI3-P, 16SmrDNA, CADH, WG, 28SnrDNA) and 308 morphological characters in separate species tree and concatenated Bayesian inference on the molecular data set, cladistic analysis on the morphological dataset, and total evidence analysis based on the combined molecular and morphological dataset. We aim to identify potential evolutionary trends in larval head setation and head capsule complexity, and clarify the systematic position of two recently described species (Vitecek et al. submitted), *Cryptothrix* and an unassociated new larva of filtering carnivorous Drusinae displaying remarkable, potentially transitional characters. Additionally, we provide re-descriptions of all known filtering carnivorous Drusinae larvae and present descriptions and larval keys to identify the hitherto unidentifiable larvae of the *Drusus discolor* group *sensu* Pauls et al. (2008).

2. Materials and Methods

2.1. Taxon sampling

Adult specimens were collected using sweep nets and light traps; larvae were collected by handpicking (Table A.1). Specimens were stored in 70% and 96% EtOH for morphological and molecular analysis, respectively.

2.2. Comparative morphological analysis and coding of adult and larval characters

Morphological characteristics of male terminalia were examined in cleared specimens. Specimens were cleared using either the Qiagen Blood and Tissue Kit for DNA-extraction according to the manufacturer's recommendation and subsequent KOH-treatment (Böhm et al., 2011), or KOH-treatment. Nomenclature of male terminalia follows Nielsen (1957, for *Limnephilus flavicornis* Fabricius) using the simplifying terms "superior appendages" for the lateral processes of segment X (cerci *sensu* Snodgrass 1935), and "intermediate appendages" for the sclerite and the anterior process of segment X (paraproct *sensu* Snodgrass 1935). Larval morphological features were examined following Wiggins (1998) and Waringer and Graf (2011). Nomenclature of primary setae and setal areas follows Wiggins (1998). Illustrations were prepared according to Holzenthal (2008) and Thomson and Holzenthal (2010) in which pencil drawings made with a camera lucida are digitized, edited and inked in Adobe Illustrator (v. 16.0.4, Adobe Systems Inc.). We used a Nikon SMZU-1000 stereomicroscope (larval

heads, male terminalia) or a Wild M20 compound microscope (details of male terminalia) for comparative morphological examination of specimens.
All morphological characters used in the present study have been newly defined. Each character was evaluated for scoring consistency, shared presence in 2 or more taxa, and unique presence in 1 taxon. Characters were included if they could be clearly identified and delimited. Non-additive binary presence/absence coding of characters was used, based on the following assumptions: 1) the subfamily Drusinae was demonstrated to represent a monophyletic entity (Pauls et al., 2008); 2) assuming 1) to be true, we hypothesize a primordial set of characters that was present in the Drusinae ancestor; 3) presence of a certain character in a taxon is a derived homologous state shared with all other taxa that develop this character, and, vice versa, shared absence of a certain character in several taxa represents a homologous plesiomorphic state, as this putatively was the character state in a hypothetical Drusinae ancestor. If a character could not be assessed, it was scored as 'missing'. However, states of binary coding do not imply assumptions regarding the plesiomorphic or derived nature of characters. Definitions of morphological characters and the morphological matrix used in phylogenetic analysis are provided in the supplementary material (Appendix A, B).

2.3. DNA extraction, amplification and sequencing
Whole genomic DNA was extracted from the abdomen or the thorax of adult or larval specimens using the DNEasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. Standard PCR procedures and primers were used (Table 1). PCR reactions were set up in 10µl reactions. Unpurified PCR products were sequenced on an ABI 3177XL capillary sequencer at BiK-F using the PCR primers and two additional internal primers for D2 (D2UP-4 and D2DN-B, Zhou et al., 2007).

2.4. Datasets, sequence alignment and phylogenetic analysis
Phylogeny of Drusinae was inferred using a 42 species (40 ingroup species, 107 terminal taxa), 6 loci (mtCOI5-P, mtCOI3-P, 16SmrDNA, CADH, WG, 28SnrDNA), 3805 bp molecular dataset (Table A1). Additionally, phylogenetic relationships of Drusinae were estimated using a 41 terminal taxa (39 ingroup species), 308 character morphological dataset (Appendix B) and a 41 terminal taxa (39 ingroup species), 4113 character combined morphological and molecular dataset.

Sequences were edited in Geneious R6 (Kearse et al., 2012) and aligned using MAFFT v7 (Katoh and Standley, 2013) as implemented in Geneious R6. Nucleotide substitution models for each partition were selected according to the Bayesian Information Criterion in the model test module of Mega v5.1 (Tamura et al., 2011) (Table 2). For phylogenetic analysis, the 16SmrDNA and 28SnrDNA fragments were not partitioned.
Phylogenetic relationships were inferred using Bayesian Inference (BI) through BEAST v1.8 (Drummond et al., 2012) and MrBayes 3.2 (Ronquist et al., 2012).

To examine heterogeneity of phylogenetic signal among data partitions, ≥0.95 posterior probability topologies of B/MCMCMC single gene and combined data analyses were examined. Single gene analyses were performed for each partition in MrBayes 3.2, implementing the respective substitution models. Four parallel runs with 6 chains each were carried out ($10x10^6$ generations, sampling every 1000^{th} generation). Stationary distribution of runs in the same optimal tree space was assumed if the average standard deviation of split frequencies reached values below 0.01. Additionally, MrBayes parameter files were examined in Tracer v1.6 (Rambaut et al., 2014) to assess if runs had reached a stationary phase and converged on model parameters. A maximum clade credibility tree was estimated based on trees sampled by MrBayes after discarding the first 2,500 trees of each run as burn-in using TreeAnnotator v1.8 (Drummond et al., 2012). Lack of incongruencies among individual data partitions was assumed to indicate homogeneity of phylogenetic signal from each respective partition. Data sets were concatenated for BI if there were no incongruencies.

Bayesian inference of the concatenated dataset (mtCOI5-P + mtCOI3-P + 16SmrDNA + CADH + 28S) was performed ($10x10^6$ generations, sampling every 1000^{th} generation) in 4 independent runs with 6 chains each to obtain gene trees. Performance of the B/MCMCMC analyses was scrutinized as stated above.

In addition to a concatenated analysis, a species tree analysis was performed to infer a species tree from separate gene trees using *BEAST (Heled and Drummond, 2010) as implemented in BEAST. Species identity (as determined by classical taxonomy) was used as species trait. Genealogical relationships between species were inferred assuming a Yule speciation tree prior, and running a species tree analysis of $60x10^6$ generations, sampling every 10000^{th} generation. The analysis was run 4x independently to assure topological convergence among runs. *BEAST log files were examined in Tracer v1.6 to assess when runs had reached a stationary phase. Support for tree topologies estimated by *BEAST analysis was assessed by constructing a maximum clade credibility tree running TreeAnnotator v1.8 after discarding the first 2,500 trees as burn-in.

Phylogenetic analysis of a purely morphological and a combined dataset was performed in MrBayes 3.2 following Wright and Hillis (2014) to assess adequacy of morphological character coding in species-level phylogenetic analysis of limnephilid Trichoptera. For the morphological data partition, rate of variation was assumed to follow a gamma-shaped distribution. Tree space was sampled every 1000^{th} generation for $5x10^6$ generations in 4 parallel runs with 6 chains each for both the morphological and the combined dataset.

Systematic suggestions concerning the subfamily Drusinae will be made based on the results of the species tree analysis only, as this method provides more accurate estimations of phylogenetic trees (Heled and Drummond, 2010).

3. Results

3.1. Phylogenetic inference

3.1.1. Molecular dataset

In all analyses monophyly of Drusinae, and monophyly of larval feeding groups within Drusinae were highly supported.

In the species tree analysis of our data set (Fig. 1) omnivorous shredders (Clade S), filtering carnivores (Clade F) and scraping grazers (Clade G) were well delineated and highly supported (≥95% posterior probability). The topology suggests a dichotomous diversification within Drusinae, with a split between highly supported scraping grazers (Clade G, Fig. 1) and (omnivorous shredders + filtering carnivores) (Clades C and S, Fig. 1). Within the highly supported filtering carnivores, *Cryptothrix nebulicola* is returned as basal sister to a highly supported clade (Clade 1, Fig 1A) comprising filtering carnivorous *Drusus* spp. Within filtering carnivorous *Drusus* spp., (*D. muelleri* + *D.* sp. Valchiusella I) form a highly supported sister species pair to all other members of the group. In the latter group the tree topology suggests *D. chrysotus* as basal to a highly supported clade (Clade 2, Fig. 1) comprising *D.* sp. nov. Valchiusella II + *D.* discolor + *D.* sp. Bucegi + *D. siveci* + D. *romanicus* + *D. meridionalis* stat. nov. + *D. macedonicus* + *D. krpachi* + *D. puskasi* Relationships between species are not resolved (i.e., lack strong support) in the latter clade except for (((*D. discolor* + *D.* sp. Bucegi) + *D. siveci*) + *D. romanicus*) and ((*D. macedonicus* + *D. krpachi*) + *D. puskasi*) (Clades 3 and 4, Fig. 1). A B/MCMCMC gene tree analysis (Fig. A. 1) based on concatenating the same dataset procured a similar topology with the following differences: 1) Gene tree analysis suggests filtering carnivores (Clade C, Fig. A. 1) opposed to (scraping grazers + omnivorous shredders) (Clades G and S, Fig. A. 1). This relationship is, however, not strongly supported; 2) Within the filtering carnivore clade, gene tree analysis suggests an unsupported basal position of *C. nebulicola* as sister to the other, highly supported, carnivorous Drusinae (Clade 1, Fig. A. 1); 3) the gene tree analysis suggests (*D.* sp. nov. Valchiusella II + *D. chrysotus*) (Clade 2, Fig. A. 1) as sister to the other filtering carnivorous *Drusus* spp. (Clade 3, Fig. A. 1); 4) relationships between species were not resolved, except for ((*D. discolor* + *D.* sp. Bucegi) + *D. siveci*) and ((*D. macedonicus* + *D. krpachi*) + *D. puskasi*) (Clades 4 and 5, Fig. A. 1); 5) gene tree analysis returns *D. meridionalis* stat. nov. unsupported sister to *D. romanicus* (Fig. A. 1).

Hypothesis testing. We performed hypothesis testing to assess if alternative topologies not displayed in the consensus or maximum credibility trees allow for alternative placements of *C. nebulicola* as well as *D*. sp. nov. Valchiusella II. Alternative composition of clades was assessed from the last 15000 trees from the species tree analysis using functions from the package 'ape' (Paradis et al. 2004) in a specialized R script (R Core Team 2013). Alternative topologies concerning the placement of *D.* sp. nov. Valchiusella II and their frequencies are given in Table 3. No alternative topologies concerning the placement of *C. nebulicola* were found in the Bayesian species tree sample.

3.1.2. Morphological and combined datasets

The analysis of a 41 taxa morphological dataset comprising 308 male, female, and larval characters suggested monophyletic Drusinae and monophyletic omnivorous shredders as sister to (filtering carnivores + scraping grazers) (Fig. A. 2A). However, this relationship is not supported. The phyloge-

netic analysis of the morphological dataset did not support any of the deeper nodes, and only terminal splits between morphologically highly similar sister species are supported. Filtering carnivores are not supported. Also, internal nodes within filtering carnivores are not supported. The topology suggests the following strongly supported sister species groups: (*D. muelleri* + *D.* sp. Valchiusella I), (*D. krpachi* + *D. puskasi*), and (*D. discolor* + *D. siveci*). Relationships between the other taxa, and the sister species groups are not resolved.

A B/MCMCMC analysis of a concatenated data set comprising 3805 bp molecular and 308 morphological characters suggests strongly supported monophyletic Drusinae, and monophyletic feeding groups within Drusinae (Fig. A. 2B). Scraping grazers are opposed to an unsupported clade comprising omnivorous shredders + filtering carnivores. Regarding the filtering carnivores, tree topology suggests *C. nebulicola* as basal sister to all other filtering carnivorous *Drusus* spp. Within these (*D. muelleri* + *D.* sp. Valchiusella I) is basal to a highly supported clade comprising ((*D. chrysotus* + *D.* sp. nov. Valchiusella II) + ((*D. discolor* + *D. siveci*) + ((*D. romanicus* + *D. meridionalis* stat. nov.) + ((*D. macedonicus* + *D. krpachi*) + *D. puskasi*))). Internal nodes of the latter clade are highly supported except for the relationship between (*D. romanicus* + *D. meridionalis* stat. nov.) and ((*D. macedonicus* + *D. krpachi*) + *D. puskasi*).

3.2. Taxonomic differentiation of Drusinae feeding groups

3.2.1. Morphological diagnosis of filtering carnivorous Drusinae

The three feeding groups are well defined by unique characters in larval and adult morphology. Filtering carnivores within Drusinae develop a unique combination of morphological synapomorphies that define this group: (1) straight hindwing A2 and A3 in males (Fig. 2a) [opposed to differentially modified hindwing venations of omnivorous shredders (Fig. 2b), or scraping grazers (Fig. 2d)]; (2) a posterolateral position of the duct opening of the male abdominal sternite V gland (Fig. 2c) [a character shared with omnivorous shredders, but distinct from the duct opening position in scraping grazers (Fig. 2e)]; (3) an incomplete, i.e., ventrally unfused, anal tube lacking modifications of the tips in females; (4) toothed larval mandibles; (5) absence of carinae, (6) presence of forked lamellae (*sensu* Wiggins 1998) dorsal the lateral line, (7) presence of filtering bristles on the first abdominal sternite, and (8) a modified head capsule of larvae (Fig. 3). This combination of adult and larval characters separates the filtering carnivores from representatives of epilithic grazers and omnivorous shredders within Drusinae.

3.2.2. Morphological diagnosis of larvae and adults of filtering carnivorous Drusinae

Larvae of carnivorous Drusinae are easily recognized and differentiated from all other Drusinae larvae by the following combination of characters: (1) larval mandibles with terminal teeth; (2) absence of carinae; (3) presence of filtering bristles on legs and the first abdominal sternite; (4) a modified head capsule that is characterized by a sharp bend of the frontoclypeus between the eye and the anteclypeus in lateral view, optionally with modified setae or a woolly layer of hair (Table 4).

Species-specific formations of the head capsule allow grouping of larvae of filtering carnivorous Drusinae. Based on the presence or absence of flocculent hair or setae, three distinct groups can be distinguished: 1) *C. nebulicola*, *D. muelleri*, *D.* sp. Valchiusella I, *D. chrysotus*; 2) *D.* sp. nov. Valchiusella II; 3) *D. discolor*, *D. siveci*, *D. meridionalis* stat. nov., *D. romanicus*, *D. macedonicus*, *D. krpachi*. Members of the first group exhibit a hair- and setaeless, moderately modifed head capsule, that is either flattened or concave in frontal view (Fig. 4, a-c). The only member of the second group exhibits a distinct setation, a unique morphological feature among Drusinae (Fig. 3i). Members of the third group exhibit flocculent hair that covers head and pronotum, and a protruding median bulge of the frontoclypeus with species-specific ramifications in frontal view (Fig. 4d-i). Within this group, ramification complexity is lowest in *D. macedonicus*, which exhibits only 2 ventrolateral protrusions of the median bulge (Fig. 4i). *Drusus krpachi* and *D. meridionalis* stat. nov. each exhibit 6 protrusions of the median bulge, all of which extend to the border of the frontoclypeus in *D. krpachi*, but only two (ventrolateral and dorsal) in *D. meridionalis* stat. nov. (Fig. 4f,h). The other species of group 3) exhibit 8 protrusions of the median bulge (Fig. c,e,g). Additionally, *D. romanicus* and *D. meridionalis* stat. nov. exhibit indentations of the parietalia in frontal view (Fig. 4f,g).

Larvae of *D. puskasi* and *D.* sp. Bucegi are not known, but putatively also exhibit flocculent hair on heads and pronota as well as species-specific formations of the frontoclypeus.

Morphologically, *D. krpachi* and *D. puskasi* males are most similar to *D. macedonicus*, but differ in the shapes of the superior, intermediate and inferior appendages (Vitecek et al., submitted). Further, all filtering carnivorous Drusinae with the exception of *D. discolor* exhibit discrete distribution patterns (Fig. 5).

Detailed (re-)descriptions of filtering carnivorous Drusinae larvae, including a key to the last larval stages, are provided in the supplementary material (Appendix C).

4. Discussion

4.1. Phylogenetic relationships and taxonomy of the filtering carnivores

Larval feeding groups were returned as monophyletic in the current analysis, confirming prior results from Pauls et al. (2008). We also found the same pattern of paraphyly concerning the genera *Drusus* and *Ecclisopteryx*: both genera were found in several clades in all phylogenetic trees. Interestingly, morphological similarities of larvae in the grazer clade were again found to be predictive value for phylogenetic grouping of clades (Waringer et al., 2015).

All analyses support a monophyletic filtering carnivore clade with *C. nebulicola* as a basal member of the filtering carnivores. While not significantly supported in all cases, all analyses suggest a close relationship of *C. nebulicola* to the other filtering carnivorous Drusinae, and exclusion of *C. nebulicola* from the other main clades (shredders and grazers). Larvae of *C. nebulicola* were found to behave as carnivores via gut content analysis (Bohle, 1987), and to develop a setation pattern on legs and the first abdominal sternite remarkably similar to all other filtering carnivores. Male *C. nebulicola* share hind wing and abdominal sternite V characters with all members of the filtering carnivores. Thus,

morphological, phylogenetic and ecological data suggests to group *C. nebulicola* with the other filtering carnivores within Drusinae. Therefore, the validity of the genus is questionable. The logical consequence would be to omit *Cryptothrix* and to include the species in *Drusus*, applying the principle of priority. However, the genus *Drusus* is paraphyletic (Pauls et al., 2008; this study). Thus, nomenclatural steps regarding generic status should only be undertaken upon revision of the entire subfamily.
Species tree analysis suggests *D. meridionalis* stat. nov. as sister to *D. macedonicus* + *D. krpachi* + *D. puskasi*, whereas gene tree analysis suggests this species as sister to *D. romanicus*. However, in both analyses *D. meridionalis* stat. nov. is well delineated from *D. romanicus*. The two taxa are also clearly distinguishable by morphological characteristics of the male terminalia and the larval frontoclypeus. Together our results clearly support raising *D. meridionalis* stat. nov. to species rank.
Concerning the status of the putative new species, phylogenetic inference strongly supported all potentially new species (*D.* sp. Valchiusella I, *D.* sp. nov. Valchiusella II, *D.* sp. Bucegi), as well as the most recently described species *D. krpachi* and *D. puskasi* (Vitecek et al., submitted). The other potential new species are difficult to address, as they either exhibit only minute differences in the male terminalia to already known species, or do not differ in larval morphology (Appendix C). We therefore refrain from describing them based on the current knowledge.
Of particular interest is *D.* sp. Bucegi, as this putative new species is represented by a single individual that is morphologically close to *D. romanicus*, which is also corroborated by nuclear markers, whereas mitochondrial markers indicate a close relationship to *D. discolor*. Potentially, this individual might be the result of a hybridization event, as both *D. discolor* and *D. romanicus* were present at the collection location and historic introgression was previously inferred among the species (Pauls et al., 2009). Hybridization in caddisflies was demonstrated to produce adult males with terminalia sharing characters of both parent species (Malicky and Pauls, 2012). Alternatively, infection with *Wolbachia* sp. (cf. Hurst and Jiggins, 2005; Kondo et al., 2002) or incomplete lineage sorting (cf. Degnan and Rosenberg, 2006; Pollard et al., 2006) might result in an individual exhibiting similar characteristics. To clarify the situation, we would need to analyse more specimens, assess infestation levels of *Wolbachia* sp., and putatively perform hybridization experiments in order to identify factors that could lead to a specimen exhibiting such characteristics. This, however, is beyond the scope of this study and we therefore refrain from a formal description of this form at this time.
Both *D.* sp. Valchiusella I and *D.* sp. nov. Valchiusella II are well delineated and highly supported by both gene tree and species tree analysis. However, differences of specimens between *D.* sp. Valchiusella I and *D. muelleri* are minute or absent (cf. Vitecek et al., submitted; Appendix C). Also, the type locality of the 'lost' species *D. chapmani* is in the vicinity of Locarno, Switzerland (Malicky, 2005; McLachlan, 1901) close to where we found *D.* sp. Valchiusella I. This further complicates taxonomic decisions. Currently, we refrain from describing this species without more comparative material from the whole range of *D. muelleri*.
The outstanding larval habitus of *D.* sp. nov. Valchiusella II would justify the description of this taxon. However, we consider it odd to describe a species based on the larva alone, and thus refrain from

the description of this species.

4.2. Performance of morphological data in phylogenetic reconstruction of Drusinae

Phylogenetic reconstruction of Drusinae based exclusively on morphological data allows species delimitation and identification of sister species but fails to resolve deeper nodes and the relationships in the group. This is most likely because we attempted to reconstruct phylogenetic relationships within Drusinae on species level. A reduced dataset encompassing exclusively characters that distinguish morphologically similar lineages will most likely produce supported deeper nodes, but fail to resolve final taxa. Further, selection and coding of species-level characters for the morphological dataset is delicate, as species (following the morphological species concept (Wheeler and Meier, 2000)) are defined by the stability of their unique combination of morphological characters. Larval characters in particular might be misleading, as the risk of assessing adaptations to certain ecological constraints rather than phylogenetic signal is prevalent. Adult genital characters might not be susceptible to ecological constraints, but are difficult to assess as they have been shown to vary over populations (Kučinić et al., 2013; Malicky, 2004; but see Oláh et al., 2012, 2013, 2014). Overall, this demonstrates the significance of molecular data and inaptitude of a purely morphological approach for species-level reconstruction of Drusinae phylogeny.

4.3. Significance of larval head modifications in filtering carnivorous Drusinae

Larval heads seem to follow a certain pattern of development within the filtering carnivore clade. Tree topology of species tree analysis suggests a basal position of *C. nebulicola,* (*D. muelleri* + *D.* sp. Valchiusella I) and *D. chrysotus* in the carnivore clade, species which share a hairless, and moderately modified (either flattened or concave) larval head capsule. These species are followed by a strongly supported clade comprising all other carnivorous Drusinae in which larvae develop a modified setation or flocculent hair on their strongly modified head capsule. Of particular interest is *D.* sp. nov. Valchiusella II, as this taxon seemingly is basal to all species in which larvae develop flocculent head hair in species tree analysis. Therefore, this taxon might represent a 'missing link' concerning hair development in carnivorous Drusinae, given the intriguing head setation and absence of flocculent hair. At present, however, although our testing of alternative topologies did not find evidence to convincingly reject the basal position (Table 3), the topologies are not sufficiently well resolved to be certain. Interestingly, within the clade where species that develop cephalic hirsuteness, a pattern of increasing diversification of frontoclypeus structures is evident. However, no assumption on the direction of diversification can be made, since structural complexity increases in a seemingly random manner within the clade. Nevertheless, species-specificity of frontoclypeus structures allows identification of larval stages of *D. discolor*, *D. siveci*, *D. romanicus*, *D. meridionalis* stat. nov., *D. macedonicus*, and *D. krpachi* that hitherto were indistinguishable from another, extending the number of identifiable Drusinae larvae from 48 to 53 (Botosaneanu, 1959; Previšić et al., 2014a; Vitecek et al., 2015; Waringer et al., 2010; Waringer and Graf, 2011). Considering the feeding habits of filtering carnivorous

Drusinae (Bohle, 1983, 1987; Graf et al., 2008), we assume an ecological significance of the distinct and diverse larval head morphology that developed in this group. The flocculent hair observed in this group might, e.g. mimic a certain type of substrate, thus attracting potential prey organisms (Bohle, 1983). Additionally or alternatively, the anatomy of the larval heads may aid in maintaining the stereotypical filtering posture first described in *D. discolor* (Bohle, 1983; Graf, unpubl. data). Interestingly, the most simple frontoclypeus observed in filtering carnivorous Drusinae is developed by *D. macedonicus* larvae which live in streamlets under rocks and within moss-mats, whereas species with highly ramified frontoclypeus structures (such as *D. discolor*, *D. romanicus*) inhabit fast flowing microhabitats (Graf, unpubl. data). Also, all filtering carnivores (except *C. nebulicola*) develop 4 distinct cephalic setae of presumably sensory function that might aid in exploration and detection of an ideal hydrological niche. Discreteness of hydraulic microhabitats was demonstrated in *Brachycentrus occidentalis*, a species that employs a similar filtering mode as the carnivorous Drusinae (Wetmore et al., 1990). Therefore, we assume that the degree of ramification represents adaptation to a certain niche, and is directionally proportional to hydraulics at the most often used microhabitat in carnivorous Drusinae. The modification of the head capsule and frontoclypeus may thus allow these taxa to optimize their foraging strategies under specific flow conditions (Wallace and Merrit, 1980). The preference for optimal hydrological conditions may explain why some of these species like *D. discolor* (Graf, 1997; Lavandier, 1992) or *D. muelleri* (Graf et al., 2005) accumulate in high densities similar to other non-annulipalpian Trichoptera (e.g., *Allogamus auricollis*: Alp, 2006; Geddes, 1981; Graf et al., 1992; Reichholf, 1995; *Brachycentrus maculatus*: Nielsen, 1943; *Brachycentrus subnubilus*: Burmeister, 1991; Gunn, 1985; Majecki et al., 1997; *Limnocentropus himalayanus*: Wiggins, 1969).

4.4. Ecology, endemism and potential threats of filtering carnivorous Drusinae

The findings of the present study highlight the significance of European mountain ranges as centres of freshwater biodiversity. Ecologically, larvae of all filtering carnivores exploit similar microhabitats: fast-flowing stretches in hypocrenal to metarhithral regions of cold mountain brooks (Graf et al., 2008; Graf and Schmidt-Kloiber, 2011). The disjunct distribution of adequate habitats due, e.g. to their prevalence at higher elevations (e.g. Pauls et al., 2006, 2009) or as a consequence of regional karstification (Previšić et al., 2009, 2014b)), and a putatively low dispersal capacity (e.g. Müller-Peddinghaus, 2011) presumably facilitated allopatric speciation of filtering carnivorous Drusinae even at small geographic scale leading to the present distribution patterns. Distribution patterns of filtering carnivorous Drusinae can be grouped into widespread species (*D. discolor*, *D. chrysotus*, *C. nebulicola*), regional endemics (*D. muelleri*, *D. romanicus*), and micro-endemics (*D. siveci*, *D. macedonics, D. meridionalis* stat. nov., *D. krpachi D. puskasi*, *D.* sp. nov. Valchiusella II) (Fig. 5). The collection of the micro-endemic *D.* sp. nov. Valchiusella II is remarkable as it demonstrates the presence of yet unknown species in a supposedly well-surveyed area, the Western Alps. The recent description of *D. krpachi* and *D. puskasi* increases the number of endemic Western Balkan Drusinae from 30 to 32, out of a total of now 40 Drusinae species known from this region (Graf et al., 2008; Graf and

Schmidt-Kloiber, 2011; Oláh, 2010, 2011; Oláh and Kovács, 2013; Previšić et al., 2014a; Vitecek et al., 2015, submitted; Ibrahimi, pers. comm.).

Endemic and micro-endemic freshwater species are particularly vulnerable to climate change and (anthropogenic) habitat alteration (Bálint et al., 2011; Conti et al., 2014; Hering et al., 2009; Tierno de Figueroa et al., 2010). Currently, construction of hydropower dams poses one of the greatest threats to freshwater biodiversity, especially in emerging economies (Zarfl et al., 2014). Many regional and micro-endemic Drusinae occur on the territory of such emerging economies, and are particularly threatened by increasing hydropower plant construction in the Western Balkans (Freyhof, 2012; Schwarz, 2012). Small hydropower plants fed by damming of small cold-water mountain streams are especially problematic for highly diverse highland caddisflies such as Drusinae (Previšić et al., 2014a; Vitecek et al., submitted) or *Chaetopteryx* species (Kučinić et al., 2013). The recent description of *D. krpachi* and *D. puskasi* (Vitecek et al., submitted) thus accentuates the exigency of biodiversity research in Eastern and Southern Europe in combination with the instigation of adequate conservation measures. Also, continuation of the presently prevailing energy policy will likely result in the loss of known and unknown biodiversity.

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Figure captions

Figure 1. Results of phylogenetic inference. B/MCMC species tree analysis for 42 species (107 terminal taxa) based on 3805bp-long sequence from 6 loci (mtCOI5-P + mtCOI3-P + 16SmrDNA + CADH + 28S). Majuscules and numbers at branches indicate clades referred to in the text. For a detailed description of the analytical processes, see Materials and Methods, section *Molecular dataset and phylogenetic inference*. Bold branches indicates posterior probabilities ≥95%.

Figure 2. Adult male apomorphies of Drusinae feeding types: a, generic male hind wing of a filtering carnivorous *Drusus* spp. and *Cryptothrix nebulicola*; b, generic male hindwing of an omnivorous shredder; c, position of the abdominal sternite V gland duct opening in filtering carnivorous and omnivorous shredder Drusinae (arrow), and formation of the male abdominal sternite V in *D. chrysotus* of *C. nebulicola*; d, generic male hindwing of a scraping grazer; e, position of the male abdominal sternite V gland duct opening and formation of male abdominal sternite V in scraping grazer Drusinae (*D. biguttatus*). Venation patterns depicted in A, and the lateral position of the male abdominal sternite V gland as illustrated in C are autapomorphic characters of filtering carnivorous Drusinae. Del. Vitecek.

Figure 3. Characteristics of Drusinae larvae. a, generic habitus (lateral view) of a filtering carnivorous *Drusus* spp. larva developing flocculent hair on head and prothorax (e.g., *D. discolor*), arrows 1-5 indicate larval autapomorphies of this group (1, forked setae dorsal the lateral line; 2, antennae not based on carinae; 3, a modified head capsule with a sharp bend of the frontoclypeus; 4, toothed mandibles; 5, filtering bristles on the 1st abdominal sternite and legs); b, generic habitus (ventral view) of a filtering carnivorous *Drusus* spp. larva developing flocculent hair on head and prothorax, arrows 5 indicate filtering bristles on the 1st abdominal sternite and legs; c, head and prothorax of a scraping grazer *Drusus* spp. (*D. serbicus*) showing the typical rounded head capsule, carinae and toothless mandibles; d, head of *D. chrysotus* showing the typical modification of the head capsule of this species; e, head of *D. muelleri* showing the typical modification of the head capsule of this species; f, lateral view of the thorax and head of *D. muelleri* showing the typical filtering bristles on the first abdominal sternite; g, Mid-leg of *D. muelleri* showing the typical filtering bristles at legs of filtering carnivorous Drusinae; h, lateral view of the head capsule of *D. discolor*, showing the flocculent hair as typical for some filtering carnivore *Drusus* spp.; i, lateral view of *Drusus* sp. nov. Valchiusella II, showing the typical cephalic setation of this taxon. Illustration a del. Vitecek; Photographs d-h by W. Lechthaler, Vienna.

Figure 4. Frontal view of larval heads of filtering carnivorous Drusinae illustrating the species-specific head and frontoclypeus morphology. a, *Cryptothrix. nebulicola*; b, *Drusus muelleri* and *D.* sp. Valchiusella I; c, *D. chrysotus*; d, *D. discolor*; e, *D. siveci*; f, *D. meridionalis* stat. nov.; g, *D. romanicus*; h, *D. krpachi*; i, *D. macedonicus*. Heads rotated, vertex to bottom. Left halves of heads stippled to provide a slightly more realistic view, right halves simplified line drawings. Del. Vitecek.

Figure 5. Distribution of filtering carnivorous Drusinae. Within filtering carnivorous Drusinae, several regional and micro-endemics occur concentrated in the Carpathians and the Balkans (*D. muelleri*, *D. romanicus*, *D. siveci*, *D. macedonics, D. meridionalis* stat. nov., *D. krpachi*, *D. puskasi*), opposed to widespread species (*D. discolor*, *D. chrysotus*, and *C. nebulicola*). *Drusus* sp. nov. Valchiusella II is a micro-endemic of the Western Alps.

Supplementary material

Figure A. 1. B/MCMCMC phylogenetic inference of Drusinae based on a 43 species (107 terminal taxa) 3805bp-long concatenated sequence from 6 loci (mtCOI5-P + mtCOI3-P + 16SmrDNA + CADH + 28S). Majuscules and numbers at branches indicate clades referred to in the text. Bold branches indicate posterior probabilities ≥95%. For details on the analytical methods used, see Materials and Methods, section *Molecular dataset and phylogenetic inference*.

Figure A. 2. B/MCMCMC phylogenetic inference of Drusinae based on morphological data. A, phylogenetic inference based on a purely morphological dataset; B, phylogenetic inference based on combined molecular and morphological dataset. Bold branches indicate posterior probabilities ≥95%.

For details on the analytical methods used, see Materials and Methods, section *Molecular dataset and phylogenetic inference*.

Appendix A: Morphological characters

MC001. Spinuliferous area on tergite VIII in dorsal view squarish: 0 = absent, 1 = present
MC002. Spinuliferous area on tergite VIII in dorsal view ± trilobate: 0 = absent, 1 = present
MC003. Spinuliferous area on tergite VIII in lateral view with a distinct hump: 0 = absent, 1 = present
MC004. Spinuliferous area on tergite VIII in lateral view with a slight hump: 0 = absent, 1 = present
MC005. Spinuliferous area on tergite VIII in dorsal and caudal view distinctly tripartite: 0 = absent, 1 = present
MC006. Spinuliferous area on tergite VIII in dorsal view ± oval: 0 = absent, 1 = present
MC007. Spinuliferous area on tergite VIII with a distinct dorsal protrusion: 0 = absent, 1 = present
MC008. Spinuliferous area on tergite VIII shield-shaped: 0 = absent, 1 = present
MC009. Spinuliferous area on tergite VIII with 2 protruding ventral lobes: 0 = absent, 1 = present
MC010. Spinuliferous area on tergite VIII with a caudal indentation in dorsal view: 0 = absent, 1 = present
MC011. Spinuliferous area on tergite VIII with a slight dorsal protrusion: 0 = absent, 1 = present
MC012. Spinuliferous area on tergite VIII in dorsal view U-shaped, closed caudally surrouding a weakly sclerotized, concave centre: 0 = absent, 1 = present
MC013. Spinuliferous area on tergite VIII in dorsal view U-shaped, closed cranially, surrounding a weakly sclerotized, concave centre, caudally with a median indentation: 0 = absent, 1 = present
MC014. Spinuliferous area on tergite VIII in dorsal view with 2 small Lateral protrusions: 0 = absent, 1 = present
MC015. Spinuliferous area on tergite VIII ± mushroomshaped in dorsal and caudal view, in lateral view with a ± distinct step: 0 = absent, 1 = present
MC016. Spinuliferous area on tergite VIII reduced, only a small number of dark spines remaining: 0 = absent, 1 = present
MC017. Spinuliferous area on tergite VIII in dorsal view ± triangular: 0 = absent, 1 = present
MC018. Spinuliferous area on tergite VIII in dorsal view as 2 distinct rodlike protrusions connected by a thin band of dark spines: 0 = absent, 1 = present
MC019. Spinuliferous area on tergite VIII in lateral, dorsal and caudal view distinctly tripartite, in lateral view high: 0 = absent, 1 = present
MC020. Spinuliferous area on tergite VIII composed of 2 ± semicircular caudally fused areas: 0 = absent, 1 = present
MC021. Spinuliferous area on tergite VIII a band with 2 round medial cranial protrusions: 0 = absent, 1 = present
MC022. Spinuliferous area on tergite VIII with 2 laterocaudal lobes, the general shape resembling a trapezium with rounded incisions: 0 = absent, 1 = present
MC023. Spinuliferous area on tergite VIII 2 laterocaudal lobes: 0 = absent, 1 = present
MC024. Spinuliferous area on tergite VIII 2 distinct lateraocaudal lobes, thinnly connected: 0 = absent, 1 = present
MC025. Spinuliferous area on tergite VIII as ± oval, caudally concentrated loose scatter of dark spines: 0 = absent, 1 = present
MC026. VIIIth tergite dorsally with a distinct indentation, not including the spinuliferous area: 0 = absent, 1 = present
MC027. VIIIth tergite with a distinct cranial incision: 0 = absent, 1 = present
MC028. Membraneaous, weakly scerotized area laterocaudal on the VIIIth tergite: 0 = absent, 1 = present
MC029. Superior appendages elongated caudally: 0 = absent, 1 = present
MC030. Superior appendages oval with dorsadly curved tips: 0 = absent, 1 = present
MC031. Superior appendages medially concave: 0 = absent, 1 = present
MC032. Superior appendages distinctly elongated (length:width 5:1), roundish in diameter, proximally slightly concave: 0 = absent, 1 = present
MC033. Superior appendages distinctly elongated (length:width 4:1), roundish in diameter, proximally distinctly concave: 0 = absent, 1 = present
MC034. Superior appendages ± oval in lateral view, ± oval in diameter, length:width 3.1, proximally slightly concave: 0 = absent, 1 = present
MC035. Superior appendages ± oval in lateral view, ± oval in diameter, length:width 2.5:1, proximally slightly concave: 0 = absent, 1 = present
MC036. Superior appendages ± triangular in lateral view: 0 = absent, 1 = present
MC037. Superior appendages ± oval in lateral view: 0 = absent, 1 = present
MC038. Superior appendages ovally squarish in lateral view: 0 = absent, 1 = present
MC039. Superior appendages outline in lateral view irregular: 0 = absent, 1 = present
MC040. Superior appendages in lateral view directed dorsaldly, dorsal and ventral outline each with a protuberance: 0 = absent, 1 = present
MC041. Superior appendages dorsal and ventral halfs seemingly converging, medially distinctly concave: 0 = absent, 1 = present
MC042. Superior appendages seemingly stalked, proximally slender: 0 = absent, 1 = present

MC043. Superior appendages flat (length:height<0.5): 0 = absent, 1 = present
MC044. Superior appendages in lateral view ± round, somewhat elongated: 0 = absent, 1 = present
MC045. Superior appendages round: 0 = absent, 1 = present
MC046. Superior appendages in lateral view with a dorsal protuberance: 0 = absent, 1 = present
MC047. Superior appendages in lateral view ventradly directed: 0 = absent, 1 = present
MC048. Superior appendages with a sharp dorsolateral, dorsomedial and ventral keel: 0 = absent, 1 = present
MC049. Superior appendages tip ± triangulate: 0 = absent, 1 = present
MC050. Superior appendages distinctly concave dorsally: 0 = absent, 1 = present
MC051. Superior appendages ± rectangular in lateral view: 0 = absent, 1 = present
MC052. Superior appendages in dorsal view with a triangular protrusion: 0 = absent, 1 = present
MC053. Superior appendages in dorsal view ± rectangular: 0 = absent, 1 = present
MC054. Superior appendages in with Y-shaped. with a dorsal and Ventral portrusion: 0 = absent, 1 = present
MC055. Superior appendages dorsal and Ventral protrusions of equal length: 0 = absent, 1 = present
MC056. Superior appendages thin, irregularly shaped: 0 = absent, 1 = present
MC057. Intermediate appendages separate, one half on either side, with various modifications: 0 = absent, 1 = present
MC058. Intermediate appendages ± an isoceles trapezium in caudal view: 0 = absent, 1 = present
MC059. Intermediate appendages in caudal view ± triangular with a fused dorsal portion: 0 = absent, 1 = present
MC060. Intermediate appendages in caudal view ± triangular: 0 = absent, 1 = present
MC061. Intermediate appendages with 2 distinct tips. 1 proximal - round and rough, 1 distal - smooth hook: 0 = absent, 1 = present
MC062. Intermediate appendages robust: 0 = absent, 1 = present
MC063. Intermediate appendages in lateral view with a long and flat rough round dorsal tip: 0 = absent, 1 = present
MC064. Intermediate appendages in lateral view with a high round dorsal tip: 0 = absent, 1 = present
MC065. Intermediate appendages in lateral view with a distinct notch and a bicuspid, rough tip: 0 = absent, 1 = present
MC066. Intermediate appendages in lateral view with a long, broad , rough tip: 0 = absent, 1 = present
MC067. Intermediate appendages in lateral view with a ± triangular tip: 0 = absent, 1 = present
MC068. Intermediate appendages in lateral view with a distinct, long tip, projecting dorsocaudally: 0 = absent, 1 = present
MC069. Intermediate appendages in lateral view with a triangular, caudadly directed tip and a distinct bulge: 0 = absent, 1 = present
MC070. Dorsal tips of intermediate appendages in Lateral, dorsal and caudal view with stout, ± triangular, caudoLateral projections on a robust base: 0 = absent, 1 = present
MC071. Dorsal tips of intermediate appendages in lateral view distinctly hook-shaped: 0 = absent, 1 = present
MC072. Dorsal tips of intermediate appendages in Lateral and dorsal view small hooks: 0 = absent, 1 = present
MC073. Dorsal tips of intermediate appendages in dorsal view wedgeshaped, caudadly converging: 0 = absent, 1 = present
MC074. Dorsal tips of intermediate appendages in dorsal view fused to an oval structure: 0 = absent, 1 = present
MC075. Dorsal tips of intermediate appendages in lateral view with a pointed, hook-like tip, in dorsal view tips fused, arching dorsadly: 0 = absent, 1 = present
MC076. Dorsal tips of intermediate appendages in dorsal view 2 separate laterad tips: 0 = absent, 1 = present
MC077. Dorsal tips of intermediate appendages in dorsal view 2 separate parallel tips: 0 = absent, 1 = present
MC078. Dorsal tips of intermediate appendages in in lateral view with 2 rough tips. 1 curved dorsoposteriad, 1 central, rounded, in dorsal view adjacent, parallel: 0 = absent, 1 = present
MC079. Dorsal tips of intermediate appendages in dorsal view ± triangular, parallel: 0 = absent, 1 = present
MC080. Dorsal tips of intermediate appendages fused, forming a stout plate: 0 = absent, 1 = present
MC081. Dorsal tips of intermediate appendages as a flat, stout plate dorsally with 2 bulbous protrusions: 0 = absent, 1 = present
MC082. Dorsal tips of intermediate appendages in lateral view with a distinct rounded dorsad protrusion: 0 = absent, 1 = present
MC083. Dorsal tips of intermediate appendages in dorsal view triangularly excised medially: 0 = absent, 1 = present
MC084. Dorsal tips of intermediate appendages 2 flat hooks: 0 = absent, 1 = present
MC085. Dorsal tips of intermediate appendages 2 large, rounded hooks: 0 = absent, 1 = present
MC086. Dorsal tips of intermediate appendages in dorsal view 2 distinct knobs, ±parallel: 0 = absent, 1 = present
MC087. Dorsal tips of intermediate appendages 2 distinct ± triangular protrusions in Lateral and dorsal view, with 2 median bulbous protrusions in dorsal view: 0 = absent, 1 = present
MC088. Intermediate appendages reduced to a membraneous structure: 0 = absent, 1 = present
MC089. Membraneous intermediate appendages ventrally open, with rounded dorsal and ventral protuberances: 0 =

absent, 1 = present
MC090. Membraneous intermediate appendages Ventrally closed: 0 = absent, 1 = present
MC091. Membraneous intermediate appendages Ventrally fused: 0 = absent, 1 = present
MC092. Intermediate appendages in lateral view rounded, bulbous, with a small dorsal bulge, in caudal view broad, in dorsal view broadly triangular: 0 = absent, 1 = present
MC093. Intermediate appendages in caudal view ± lozenge-shaped: 0 = absent, 1 = present
MC094. Intermediate appendages dorsally open: 0 = absent, 1 = present
MC095. Intermediate appendages developing 2 long hooks, situated ±medially in caudal view: 0 = absent, 1 = present
MC096. Intermediate appendages in dorsal view with a distinct rounded median bulge: 0 = absent, 1 = present
MC097. Intermediate appendages in lateral view bulging out Ventrally: 0 = absent, 1 = present
MC098. Dorsal tips of intermediate appendages in lateral view 2 pointed protrusions: 1 proximal, small; 1 distal, slender, long, ± caudad, somewhat curved ventradly, in dorsal view the latter parallel, pointed: 0 = absent, 1 = present
MC099. Dorsal tips of intermediate appendages in lateral view ± triangular with a rounded ridge and a proximal pointed protrusion, in dorsal view both lateradly curved: 0 = absent, 1 = present
MC100. Dorsal tips of intermediate appendages in lateral and caudal view regularly rounded, in caudal view distinctly protuberant: 0 = absent, 1 = present
MC101. Dorsal tips of intermediate appendages in dorsal view parallel with 1 proximal and 1 distal rounded bulge: 0 = absent, 1 = present
MC102. Dorsal tips of intermediate appendages in dorsal view parallel with 1 proximal and 1 distal rounded bulge, the latter bigger, separated by a rounded indentation with round edges: 0 = absent, 1 = present
MC103. Dorsal tips of intermediate appendages in dorsal view parallel with 1 proximal and 1 distal rounded bulge, the latter bigger, separated by a rounded indentation with pointed basal edges: 0 = absent, 1 = present
MC104. Lateral ventral projections (‘base’) of intermediate appendages in dorsal view evenly rounded : 0 = absent, 1 = present
MC105. Dorsal tips of intermediate appendages in dorsal and lateral view with a distinct rounded protrusion based on a robust, round structure: 0 = absent, 1 = present
MC106. Dorsal tips of intermediate appendages in lateral view with a distinct indentation separating a base and protrusion: 0 = absent, 1 = present
MC107. Dorsal tips of intermediate appendages in lateral view distinctly distinctly dorsadly directed, higher than the ventral border of tergite VIII: 0 = absent, 1 = present
MC108. Inferior appendages complex, with several facettes: 0 = absent, 1 = present
MC109. Inferior appendages bilobate: 0 = absent, 1 = present
MC110. Inferior appendages in lateral view ± triangular: 0 = absent, 1 = present
MC111. Inferior appendages in lateral view ± conical: 0 = absent, 1 = present
MC112. Inferior appendages in lateral view long: 0 = absent, 1 = present
MC113. Inferior appendages in lateral view short, robust: 0 = absent, 1 = present
MC114. Inferior appendages in lateral view blunt, bulbous: 0 = absent, 1 = present
MC115. Inferior appendages in lateral view blunt, with a concave Ventral line: 0 = absent, 1 = present
MC116. Inferior appendages in lateral view rounded: 0 = absent, 1 = present
MC117. Inferior appendages in lateral view with a protruding median tip: 0 = absent, 1 = present
MC118. Inferior appendages reduced: 0 = absent, 1 = present
MC119. Inferior appendages conical, short: 0 = absent, 1 = present
MC120. Caudal tips of inferior appendages bent medially: 0 = absent, 1 = present
MC121. Inferior appendages with a median lobe (delimited by a longitudinal Ventral incision): 0 = absent, 1 = present
MC122. Inferior appendages in dorsal and ventral view with a median tip and a distinct notch: 0 = absent, 1 = present
MC123. Inferior appendages in dorsal and ventral blunt: 0 = absent, 1 = present
MC124. Inferior appendages in dorsal and ventral view slender, with an indistinct median tip and a shallow notch: 0 = absent, 1 = present
MC125. Inferior appendages in lateral view proximally wide, distally slender: 0 = absent, 1 = present
MC126. Inferior appendages in lateral view distally distinctly straitened: 0 = absent, 1 = present
MC127. Inferior appendages in dorsal and ventral view with a distinct, pointed median tip : 0 = absent, 1 = present
MC128. Inferior appendages proximally with a dorsal triangular protrusion: 0 = absent, 1 = present
MC129. Inferior appendages in ventral view with a slight groove: 0 = absent, 1 = present
MC130. Inferior appendages in lateral view proximally bulbous, wide, tapering distinctly and curved dorsadly: 0 = absent, 1 = present
MC131. Inferior appendages in lateral view with a sharp dorsal tip: 0 = absent, 1 = present

MC132. Inferior appendages in ventral view with a distinct, sharpely delimited groove: 0 = absent, 1 = present
MC133. Inferior appendages in lateral view blunt: 0 = absent, 1 = present
MC134. Inferior appendages in lateral view curved dorsadly, long, slender: 0 = absent, 1 = present
MC135. Inferior appendages in Ventral view with a long proximal part and comparatively short tips: 0 = absent, 1 = present
MC136. Inferior appendages in dorsal and Ventral view proximally wide with a short, slender, conical tip: 0 = absent, 1 = present
MC137. Inferior appendages tips in lateral view somewhat bulbous, distinctly rounded: 0 = absent, 1 = present
MC138. Inferior appendages ± bipartite with a well sclerotized dorsal part and a less sclerotized seemingly ventral part: 0 = absent, 1 = present
MC139. Dorsal part of bipartite inferior appendages prolonged caudally, medially covered with dark spines: 0 = absent, 1 = present
MC140. Inferior appendages tip in lateral view bicuspid, Laterally distinctly indented, slender and elongate in Ventral view: 0 = absent, 1 = present
MC141. Inferior appendages tip in lateral view somewhat bifurcated with 2 tips of unequal length, laterally shallowly and broadly indent, in dorsal view triangular, slender, each with 2 tips separated by small indentation: 0 = absent, 1 = present
MC142. Inferior appendages tips in lateral view turned dorsally: 0 = absent, 1 = present
MC143. Inferior appendages tip in lateral view with a deep and broad Lateral concavity, fIntermediate appendagesly bifurcated with 2 tips of ± equal length, in dorsal view broadly triangular with the tips separated by a distinct indentation.: 0 = absent, 1 = present
MC144. Inferior appendages tips in lateral view caudadly directed: 0 = absent, 1 = present
MC145. Inferior appendages tips in lateral view with a distinct rounded bulge: ventral portion of the dorsal part rotated dorsolateradly : 0 = absent, 1 = present
MC146. Dorsal tips of intermediate appendages developed to distinct, slender pincer-like structures: 0 = absent, 1 = present
MC147. Inferior appendages with a lateral setal brush: 0 = absent, 1 = present
MC148. Inferior appendages in lateral view with a sharp triangular protrusion and a ragged ventrocaudal outline, in dorsal, ventral and caudal view with a distinct, broad pincer-like structure: 0 = absent, 1 = present
MC149. Inferior appendages in lateral view with a high tip, curved dorsocaudadly: 0 = absent, 1 = present
MC150. Inferior appendages tips in caudal view dorsally flat: 0 = absent, 1 = present
MC151. Inferior appendages in lateral view rounded with a dorsocaudadly curved tip: 0 = absent, 1 = present
MC152. Inferior appendages median lobes rounded with a ± ragged medial outline: 0 = absent, 1 = present
MC153. Inferior appendages median lobes with 2 distinct tips (1 visible in lateral view) and a ±ragged medial outline: 0 = absent, 1 = present
MC154. Inferior appendages in lateral view proximally wide, medially slightly constricted, curved dorsdaly in the posterior third: 0 = absent, 1 = present
MC155. Inferior appendages in lateal view proximally wide, tapering towards the rounded tip: 0 = absent, 1 = present
MC156. Parameres smooth, without spines: 0 = absent, 1 = present
MC157. Parameres ventrad, somewhat twisted: 0 = absent, 1 = present
MC158. Parameres terminally bulbous with a high number of spines: 0 = absent, 1 = present
MC159. Parameres simple, rodlike, short (approximately half the lenght of the aedeagus), bearing 3 recumbent spines: 0 = absent, 1 = present
MC160. Parameres simple, rodlike, somewhat bulbous medially and distally: 0 = absent, 1 = present
MC161. Parameres simple, with a single dorsal thorn-like spine in the distal third: 0 = absent, 1 = present
MC162. Parameres simple, with several thorn-like spines on a common base in the distal third: 0 = absent, 1 = present
MC163. Parameres simple, with a single bulbously based thorn-like spine in the distal third: 0 = absent, 1 = present
MC164. Parameres simple, with a median hook-shaped tip bearing several smaller thorn-like spines: 0 = absent, 1 = present
MC165. Parameres simple, in dorsal view with 3 thorn-like spines in the distal third: 0 = absent, 1 = present
MC166. Aedeagus with inflatable structures: 0 = absent, 1 = present
MC167. Aedeagus simple & fully sclerotized: 0 = absent, 1 = present
MC168. Aedeagus in lateral view with a distinct terminal protuberance: 0 = absent, 1 = present
MC169. Parameres with a terminal concentration of spines: 0 = absent, 1 = present
MC170. Parameres with several ± evenly distributed thorn-like spines : 0 = absent, 1 = present
MC171. Parameres with spines on the majority of the dorsal surface: 0 = absent, 1 = present
MC172. Parameres with a 1 proximal row of 3 based thorn-like spines and 1 distal row of 2 based thorn-like spines: 0 =

absent, 1 = present
MC173. Parameres with a continuous row of 5 thorn-like spines, 2 large & bulbously based, the remaining 3 smaller: 0 = absent, 1 = present
MC174. Parameres terminally bulbous, with 6 thorn-like dorsal spines: 0 = absent, 1 = present
MC175. Parameres with a concentration of thorn-like spines in the mediodistal quarter: 0 = absent, 1 = present
MC176. Parameres with 1 subterminal spine and a concentration of smaller spines: 0 = absent, 1 = present
MC177. Parameres with several terminal spines and concentration of smaller spines: 0 = absent, 1 = present
MC178. Parameres with 1 large terminal spine and several smaller spines, forming a brush: 0 = absent, 1 = present
MC179. Parameres with a terminal concentration of ± equally sized thorn-like spines: 0 = absent, 1 = present
MC180. Parameres with 3 major concentrations of thorn-like spines and a terminal spine: 0 = absent, 1 = present
MC181. Parameres with 2 major concentrations of thorn-like spines and a terminal spine: 0 = absent, 1 = present
MC182. Parameres with 1 concentration of thorn-like spines in the distal third and a terminal spine: 0 = absent, 1 = present
MC183. Parameres fused at their bases: 0 = absent, 1 = present
MC184. Parameres a series of 6 bulbously based thorn-like spines and several smaller pines proximally the first bb spine: 0 = absent, 1 = present
MC185. Parameres with additional small spines: 0 = absent, 1 = present
MC186. Parameres with additional small spines in the distal third: 0 = absent, 1 = present
MC187. Parameres with 1 long thin subterminal spine, and a ± terminal concentration of spines: 0 = absent, 1 = present
MC188. Parameres broad: 0 = absent, 1 = present
MC189. Parameres with several long, arching spines: 0 = absent, 1 = present
MC190. Parameres sigmoidally curved, short: 0 = absent, 1 = present
MC191. Parameres sigmoidally curved, long: 0 = absent, 1 = present
MC192. Parameres with a single dorsad protrusion in the distal third, bearing several small spines: 0 = absent, 1 = present
MC193. Abdominal segment IX with a distinct Lateral bulge in the dorsal half: 0 = absent, 1 = present
MC194. Abdominal segment IX with a rounded wedge-shaped protrusion: 0 = absent, 1 = present
MC195. Abdominal segment IX with a sharp wedge-shaped protrusion in the dorsal half: 0 = absent, 1 = present
MC196. Abdominal segment IX with a sharp protrusion in the dorsal half: 0 = absent, 1 = present
MC197. Abdominal segment IX with a rounded protrusion in the dorsal half: 0 = absent, 1 = present
MC198. Abdominal segment IX dorsally wider than ventrally: 0 = absent, 1 = present
MC199. Abdominal segment IX dorsally as wide as ventrally: 0 = absent, 1 = present
MC200. Abdominal segment IX ventrally wider than dorsally: 0 = absent, 1 = present
MC201. Abdominal segment IX transversely dilated, in Ventral view distance from lateralmost point of segment IX to straight anteroposterior line originating from lateralmost poin of inferior appendages is approximately 10% of total width of segment IX on each side: 0 = absent, 1 = present
MC202. Abdominal segment IX in lateral view dilated rhombus-shaped: 0 = absent, 1 = present
MC203. Abdominal segment IX in caudal view with a distinct bulge on either side, approximately at the insertion of superior appendages: 0 = absent, 1 = present
MC204. Abdominal segment IX with a distinctly widened at the insertions of Superior appendages: 0 = absent, 1 = present
MC205. Abdominal segment IX ventrally bulging caudad, with a median mentral protrusion, embracing the proximal part of the inferior appendages: 0 = absent, 1 = present
MC206. Inferior appendages fused with adominal segment IX: 0 = absent, 1 = present
MC207. Inferior appendages partially fused with abdominal segment IX: 0 = absent, 1 = present
MC208. Male wings brachypterous: 0 = absent, 1 = present
MC209. Position of sternite V glands mediolateral: 0 = absent, 1 = present
MC210. Position of sternite V glands lateral: 0 = absent, 1 = present
MC211. Sternite V with a distinct ± triangular laterodorsal protrusion: 0 = absent, 1 = present
MC212. Sternite V with a rounded laterodorsal prolongation: 0 = absent, 1 = present
MC213. Male hindwing without the typical setal pouch in the anal field: 0 = absent, 1 = present
MC214. Male hindwing grazer type (A2 and A3 fused): 0 = absent, 1 = present
MC215. Male hindwing carnivore type (A2 and A3 straight, unfused): 0 = absent, 1 = present
MC216. Male hindwing shredder type (A1 aberrant, A2 & A3 distinctly shortened): 0 = absent, 1 = present
FC001. Female supragenital plate developed to a distinct caudad protuberance: 0 = absent, 1 = present
FC002. Female anal tube a real tube, ventrally closed: 0 = absent, 1 = present
LC001. Mandibles with terminal teeth: 0 = absent, 1 = present

LC002. Mandibles lacking terminal teeth, spoonshaped: 0 = absent, 1 = present
LC003. Forked setae present dorsally the Lateral line: 0 = absent, 1 = present
LC004. Antennae located on carinae: 0 = absent, 1 = present
LC005. Setae on abdominal ventrum I anteriadly directed, stout: 0 = absent, 1 = present
LC006. Setae on abdominal ventrum I in a single row: 0 = absent, 1 = present
LC007. Abdominal ventrum I with 2 sclerotization centers: 0 = absent, 1 = present
LC008. Abdominal ventrum I scleortization tends to concentrate: 0 = absent, 1 = present
LC009. Abdominal ventrum I with 1 slerotized plate: 0 = absent, 1 = present
LC010. Pronotum and head covered in wooly hair: 0 = absent, 1 = present
LC011. Head with setae/hair on the frontoclypeus and the surrounding elevated rim: 0 = absent, 1 = present
LC012. Short pale spines on the Pronotum: 0 = absent, 1 = present
LC013. Larval head evenly rounded: 0 = absent, 1 = present
LC014. Typical setation pattern on the larval head (sensu Wiggins): 0 = absent, 1 = present
LC015. Additional setae on larval heads: 0 = absent, 1 = present
LC016. Fields of spinules dorsally the eyes: 0 = absent, 1 = present
LC017. Larval head modified: frontally flat, with slightly elevated field of spinules and #18 setal bases: 0 = absent, 1 = present
LC018. Larval head modified: flat with two shallow grooves: 0 = absent, 1 = present
LC019. Larval head modified: flat with a slightly elevated rim: 0 = absent, 1 = present
LC020. Head and Pronotum coarsely granulated: 0 = absent, 1 = present
LC021. Larval head modified: frontally concave, with thin, distinctly elongated Lateral protrusions of the parietale: 0 = absent, 1 = present
LC022. Larval head modified: frontally concave, with a thin, dark ridge surrounding the central concavity; setae located on this ridge, additionally, several long setae on the frontoclypeus: 0 = absent, 1 = present
LC023. Larval head modified: frontally concave with a modified frontoclypeus and a ±thin ridge around the Ventral 3/4 of the head : 0 = absent, 1 = present
LC024. Frontoclypeus discolor type: 0 = absent, 1 = present
LC025. Frontoclypeus siveci type: 0 = absent, 1 = present
LC026. Frontoclypeus krpachi type: 0 = absent, 1 = present
LC027. Frontoclypeus macedonicus type: 0 = absent, 1 = present
LC028. Frontoclypeus romanicus type: 0 = absent, 1 = present
LC029. Frontoclypeus meridionalis type: 0 = absent, 1 = present
LC030. Prontoum with a distinct transversal groove: 0 = absent, 1 = present
LC031. Pronotum with a broad rounded ridge, extending laterally: 0 = absent, 1 = present
LC032. Pronotum with a rounded ridge: 0 = absent, 1 = present
LC033. Pronotum with a distinct, protuberant ridge: 0 = absent, 1 = present
LC034. Pronotum with a high ridge: 0 = absent, 1 = present
LC035. Pronotum with a distinct ridge: 0 = absent, 1 = present
LC036. Pronotum ridge medially indent: 0 = absent, 1 = present
LC037. Pronotum ridge deeply indent: 0 = absent, 1 = present
LC038. Pronotum ridge in dorsal view medially located: 0 = absent, 1 = present
LC039. Pronotum ridge caudally ragged, not rounded and smooth: 0 = absent, 1 = present
LC040. Pronotum ridge thin: 0 = absent, 1 = present
LC041. Pronotum ridge in frontal view wider than head width: 0 = absent, 1 = present
LC042. Pronotum ridge with 2 anteriad spines: 0 = absent, 1 = present
LC043. Pronotum evely rounded: 0 = absent, 1 = present
LC044. Pronotum with the slightest step in the dorsal profile: 0 = absent, 1 = present
LC045. Pronotum setation white, recumbent: 0 = absent, 1 = present
LC046. Pronotum with ventrolateral bulge: 0 = absent, 1 = present
LC047. Pronotum with a distinct step: 0 = absent, 1 = present
LC048. Pronotum setation long, blond: 0 = absent, 1 = present
LC049. Larval head in lateral view with a bulge in the ventral part of the frontoclypeus: 0 = absent, 1 = present
LC050. Caudal edge of pronotum with a slight indentation: 0 = absent, 1 = present
LC051. Metanotal sclerites short, wide (length:width approximately 1:5): 0 = absent, 1 = present
LC052. Carinae extending to the eyes: 0 = absent, 1 = present
LC053. Head and pronotum dark: 0 = absent, 1 = present
LC054. Head and pronotum yellow: 0 = absent, 1 = present

LC055. Head and pronotum with dark spots: 0 = absent, 1 = present
LC056. Head and pronotum chestnut: 0 = absent, 1 = present
LC057. Dorsal gill formula X/1-1/1-1/1-1/1-0: 0 = absent, 1 = present
LC058. Dorsal gill formula X/1-1/1-1/1-1/1-1: 0 = absent, 1 = present
LC059. Dorsal gill formula X/1-1/1-1/1-1/1-1/1-0: 0 = absent, 1 = present
LC060. Dorsal fill formula X/1-1/1-1/1-1/1-1/1-1/1-0: 0 = absent, 1 = present
LC061. Dorsal fill formula X/1-1/1-1/1-1/1-1/1-1/1-1: 0 = absent, 1 = present
LC062. Dorsal fill formula X/0-1/1-1/1-1/1-1/1-1/1-1: 0 = absent, 1 = present
LC063. Dorsal fill formula X/1-1/1-1/1-1/1-1/0-1/0-1: 0 = absent, 1 = present
LC064. Dorsal gill formula X/0-1/1-1/1-1/1-0: 0 = absent, 1 = present
LC065. Dorsal gill formula X/0-1/1-1/1-1/0-1: 0 = absent, 1 = present
LC066. Lateral gills formula X/0: 0 = absent, 1 = present
LC067. Lateral gill formula X/1-1/1-1/1-1/1-0: 0 = absent, 1 = present
LC068. Lateral gill formula X/1-1/1-1/1-1: 0 = absent, 1 = present
LC069. Lateral gill formula X/1-1/1-1/1-0: 0 = absent, 1 = present
LC070. Lateral gill formula X/1-0/1-0: 0 = absent, 1 = present
LC071. Lateral gill formula X/0-0/1-0/1-0: 0 = absent, 1 = present
LC072. Lateral gill formula X/0-0/1-0: 0 = absent, 1 = present
LC073. Lateral gill formula X/1-0: 0 = absent, 1 = present
LC074. Lateral gill formula X/0-1/0-1: 0 = absent, 1 = present
LC075. Lateral gill formula X/0-1/1-1/1-0: 0 = absent, 1 = present
LC076. Ventral gill formula X/1-1/1-1/1-1/0-1/0-1: 0 = absent, 1 = present
LC077. Ventral gill formula X/1-1/1-1/1-1/1-1/1-1/1-1: 0 = absent, 1 = present
LC078. Ventral gill formula X/0-1/1-1/1-1/1-1/1-1/1-1: 0 = absent, 1 = present
LC079. Ventral gill formula X/1-1/1-1/1-1/1-1/1-1/1-0: 0 = absent, 1 = present
LC080. Ventral gill formula X/0-1/1-1/1-1/1-1/1-1: 0 = absent, 1 = present
LC081. Lateral line formula III-VIII1/2: 0 = absent, 1 = present
LC082. Lateral line formula III-VII: 0 = absent, 1 = present
LC083. Lateral line formula III-VIII1/3: 0 = absent, 1 = present
LC084. Lateral line formula III1/3-VIII1/2: 0 = absent, 1 = present
LC085. Lateral line formula III1/2-VIII1/2: 0 = absent, 1 = present
LC086. Lateral line formula III2/3-VIII1/4: 0 = absent, 1 = present
LC087. Lateral line formula II-II1/2+III1/5-VIII1/2: 0 = absent, 1 = present
LC088. Lateral line formulae II1/2-VII: 0 = absent, 1 = present
LC089. Lateral line formula II1/2-VIII1/3: 0 = absent, 1 = present
LC090. Lateral line formula III-IX: 0 = absent, 1 = present

Notes:
1. Gill formulas are to be read as follows: segments are separated by slash, an “X” represents the first segment, that does not bear gills. Positions of gills are either anterior or posterior on each segment, separated by a hyphen. Example: A larva developing dorsal gills on anterior and posterior positions of abdominal segments II, III and IV has a dorsal gill formula of X/1-1/1-1/1-1.
2. Lateral line formulas are to be read as follows: roman numerals refer to the segment, latin numerals (as fractions) indicate the starting and ending point of the lateral line in caudal direction. Thus, the lateral line formula III1/3-VIII1/2 applies to caddisfly larvae in which the lateral line starts approximately at the border of the cranial and median third of the 3rd abdominal segment and continues approximately to the border of the cranial and caudal half of the 8th abdominal segment.

Appendix B
#NEXUS

begin data;
dimensions ntax=41 nchar=308;
format datatype=standard gap=- missing=N;
matrix
Cryptothrix_nebulicola 10000000000000000000000001111000000000000000000000000010010000000000000
00000000000000000000000000000000000010000000011000000000000000000000000000000000000100010000001000

0000000000000000000000110001000000000000110001010101010000000000000100000000000000000000000000000
000001000000000000000000000000000000000000

Drusus_muelleri 010000000000000000000000001100100000000000000000000
0000010001000000000100000000000000000000000000000000000000100000000011000000000000000000000000
0000000000001000001000000000000000000000000001010010000000000001000010101010110000000000001100000
0000010000000000000000000000001000000100000000010010000000000001000

Drusus_sp_nov_Valchiusella_I 0100000000000000000000000011001000000000000000000000000000100000100
0000010000000000000000000000000000000000100000000011000000000000000000000000000000000000000010
0000100000000000000000000000101001000000000000100001010101011000000000000001100000000001000000000
0000000000000000100000010000000001001000000000001000

Drusus_sp_nov_Valchiusella_III NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-
NN10101100001000000000010000000000000000000000000000000000001000001000100000000000100000001000000

Drusus_chrysotus 00101000000000000000000000011000000100000000000000000000000010
0000010000000010000000000000000000000000000000000000000000100000000011000000000000000000000000000000
0000001000010000000000000000000000000000100010100000000000110001010101011000000000000000100000000010
0000000000000000000000100001000000000100000010000000100000

Drusus_discolor 001001000000000000000000001101000001000000000000000000
0000010000100000000010000000000000000000000000000000000000000100000000011000000000000000000000000000
0000000000000100010000000000000000000000000000010001010000000000100001010101011000110000000000000110
00000100000000000000000000000100000100000000010000001000000100000

Drusus_siveci 000001000000000000000000001101000001000000000000000000
0000010000000000000000100000000000000000000000000000000000000100010000011000000000000000000000000000
0000000000000100010000000000000000000000000000010001101000000000100001010101011000110000000000000101
00000100000000000000000000000100010000000000010000001000000100000

Drusus_romanicus 000001000000000000000000001100010000000000000000000000
0000010000100000000000001000000000000000000000000000000000000110000000000010000000000000000000000000
0000000000000001001000000000000000000000000000010000001000000000100001010101011000110000000000000100
00100100000000000000000000001000000001000000000100000010000000100000

Drusus_meridionalis 100001000000000000000000001100010000000000000000000000
000001000010000000000001000000000000000000000000000000000000100000000000010000000000000000000000
0000000000000001000100000000000000000000000000010000001000000000100001010101011000110000000000000010
000010100000000000000000000000100010000000000001000001000000100000

Drusus_macedonicus 000001000000000000000000001100000100000000000000000000
0000010000000000000000010000000000000000000000000000000000010000000000110010000000000000000000000000
0000000000000100001000000000000000000000000000010010010000000000100001010101011000110000000000000100
01000100000000000000000000001000000100000100000000000001000000100000

Drusus_krpachi 000101000000000000000000001100000100000000000000000000
0000010000000010000010000000000000000000000000000000000000000000000010011001000000000000000000000000
0000000000000000101000000000000000000000000000010000010000000000100001010101011000110000000000000100
1000010000000000000000000000100010000000000000000000001000000(1)00000

Drusus_puskasi 0001010000000000000000000011000001000000000000000000
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Appendix C

Descriptions of larvae and a potentially new species of filtering carnivorous Drusinae

Cryptothrix nebulicola McLachlan (Fig. 4a)

Material examined: 2 5th instar larvae: Switzerland, Graubünden, Obersaxen; leg. Bohle; 1983. 3 5th instar larvae: Italy, Südtirol, Ahornach bei Sand in Taufens; leg. Bohle; 20.VII.1981.

Description. *Fifth instar larva.* Head dark brown, granulated; setation modified; head capsule in lateral view ± flat with a sharp bend of the frontoclypeus between the eye and the anteclypeus, in cranial view with 2 shallow, parallel, sagittal grooves. Pronotum dark brown, edges almost black, granulated; with 1 dorsal and 1 lateral indentation on either side in the anterior half; caudal edge a distinct rim. Mesonotum completely covered by 2 sclerites, dark brown with darker apodemes, caudal half with black edges; with 1 anterior indentation and 1 posterior bulge on either side. Metanotum with 3 pairs of sclerites; anteromedian sclerites ovoid, cranially dark, mediocaudally light brown, with 3-6 setae; posteromedian sclerites ± triangular, fawn, with 18-20 setae; lateral sclerites brown, cranially lighter, caudally almost black, with 16-20 anteriorly concentrated setae; with a transversal band of setae, linking lateral and posteriomedian sclerites. Abdomen white, with single-filamentous gills; abdomen I sternum with a single row of long, sturdy setae; lateral line continuously from abdomen III-VIII, with forked lamellae. Case simple, constructed of mineral particles.

Distribution: The species is a regional endemic of the Western Alps (Fig. 5).

Comment: For an illustration and re-description of the male genitalia, see Vitecek et al. (submitted)

Drusus muelleri McLachlan (Fig. 4b)

Material examined: 3 5th instar larvae: Switzerland, Furkapass; N46.5888, E8.4327; leg. Graf; 21.VII.2006.

Description. *Fifth instar larva.* Head dark red-brown, coarsely granulated; setation modified; head capsule in lateral view flat with a sharp bend of the frontoclypeus between the eye and the anteclypeus, in cranial view with a shallow indentation and an elevated rim. Pronotum red-brown, coarsely granulated; with 1 lateral indentation on either side in the anterior half and a distinct median hump; caudal edge as a distinct rim; edges dark. Mesonotum completely covered by 2 sclerites, light brown with darker apodemes and black edges; with 1 shallow anterior indentation and 1 faint posterior bulge on either side. Metanotum with 3 pairs of sclerites; anteromedian sclerites ovoid, fawn, cranial egde black, with 6-8 setae; posteromedian sclerites ± triangular, fawn, with 6-7 setae; lateral sclerites fawn, medially with a dark spot, with 10-12 setae; with a transversal band of setae linking posteriomedian sclerites, and setal groups between posteromedian and lateral sclerites. Abdomen white, with single-filamentous gills; abdomen I sternum with a single row of long, sturdy setae; lateral line as a short segment on abdomen II, continuously from abdomen III to 1st half of abdomen VIII, with forked lamellae dorsally the lateral line. Case simple, constructed of mineral particles, lacking particles of plant origin.

Distribution: This species is a regional endemic of the Western Alps (Fig. 5).

Comment: For an illustration and re-description of the male genitalia, see Vitecek et al. (submitted)

Drusus sp. Valchiusella I (AppFig. 1a-e)

Material examined: 1 male: Italy, Piedmont, Torino, Traversella, Fondo, Alpe Gias del Prete; leg. Vincon; 12.VII.2012. 1 male: Italy, Torino, Valchiusella; N45.568, E7.9986; leg. Graf; 20.VI.2014. 6 5th instar larvae: Italy, Torino, Valchiusella; N45.61983, E 8,0233; leg. Vitecek; 20.VI.2014.

Diagnosis. This putative new species differs from *D. muelleri* in the male terminalia by the following charactes: intermediate appendages in lateral view with a distinct notch and a bicuspid tip, in lateral, dorsal and caudal view with distinct black setae; inferior appendages in dorsal and ventral view with a more pronounced median tip, in ventral and lateral view wider than those of *D. muelleri* (see Vitecek et al., submitted).

Description. Adults. Habitus: dark; sternites and tergites dark brown; cephalic, thoracic and abdominal setation blond and dark brown; cephalic and thoracic setal areas pale; legs proximally dark brown, distally fawn; haustellum and intersegmental integument pale, whitish; wings translucent dark brown, with dark setae on the veins and blond setae on the membrane. Male maxillary palp trisegmented. Forewing length 14-15 mm, spurformula 133.

Male terminalia: tergite VIII brown, single setae present; spinate area in lateral view convex, in dorsal view ± trilobate, flanked by membraneous, less sclerotized areas bearing setae with distinct bases. Ninth abdominal segment with a rounded wedge-shaped protrusion in the dorsal half; dorsally wider than ventrally in caudal view; bearing stout setae in the dorsal half. Superior appendages in lateral view caudally distinctly elongated; ± round in diameter, length:height ~ 5:1; in dorsal view proximally slightly concave medially. Intermediate appendages in lateral view with a distinct notch and a bicuspid, rough tip dorsally, in dorsal view two separate, wedge-shaped tips, in lateral, dorsal and caudal view with distinct black setae; in caudal view ± triangular. Inferior appendages in lateral view conical; in ventral and dorsal view with a median tip and a distinct notch, in ventral view with a longitudinal groove delimiting a median lobe. Parameres simple, with a single dorsal thorn in the distal third.

Fifth instar larva. Maximum head width 1.29-1.4 mm. All characters as in larvae of *D. muelleri*

Distribution: This putative species is a micro-endemic of the Graian Alps.

Drusus sp. nov. Valchiusella II (Fig. 3i)

Material examined: 15 5th instar larvae: Italy, Piedmont, Torino, Traversella, Fondo; N45.5282, E7.67578; leg. Graf; 07.VI.2013.

Diagnosis. This new species exhibits a unique combination of larval characters, additionally, the status of *D.* Valchiusella II as sp. nov. is clearly supported by the fact that this taxon is genetically different to all other Drusinae, and assumes a basal position to all hair-bearing filtering carnivorous Drusinae in species tree analysis (Fig. 1, Clade 2).

Description. Adults unknown.

Fifth instar larva. Maximum head width 1.41-1.48 mm. Head red-brown, smooth; head capsule in lateral view with a sharp bend of the frontoclypeus between the eye and the anteclypeus, in cranial view distinctly indent with an elevated rim surrounding the central concavity; setation modified: head and frontoclypeus with numerous twisted setae (Fig. 3i). Pronotum red-brown, smooth, with a mediotransveral hump bearing several longer setae; caudal edge as a distinct rim; edges dark; prosternal horn present. Mesonotum completely covered by 2 sclerites, yellowish-brown, granulated with smooth apodemes, edges black; with 1 shallow anterior indentation and 1 faint posterior bulge on either side. Metanotum with 3 pairs of sclerites; anteromedian sclerites ovoid, yellowish-brown, cranial edge darker, with 7-8 setae; posteromedian sclerites ± triangular, fawn, with 5-6 setae and a light spot close the cranial tip; lateral sclerites fawn, medially with a dark spot, anterior half darker, with 6-8 setae; with a band of setae groups between posteriomedian sclerites, and setal groups between posteromedian and lateral sclerites. Abdomen white, with single-filamentous gills; abdomen I sternum with a single row of long, sturdy setae; lateral line continuously from 2nd half of abdomen III to 1st third of abdomen VIII, with forked lamellae dorsally the lateral line. Case simple, constructed of mineral particles.

Distribution: This species is a micro-endemic of the Graian Alps (Fig. 5).

Drusus chrysotus Rambur (Fig. 4c)

Material examined: 6 5th instar larvae: Austria, Ladinger Hütte; leg. Graf; 16.VI.2006.

Description. *Fifth instar larva.* Head brown, granulated with smooth patches on the vertex; head capsule in lateral view with a sharp bend of the frontoclypeus between the eye and the anteclypeus, in cranial view distinctly indent with a laterally elevated rim, spanning across the vertex; setation modified: as a sparse band of setae spanning between the eyes, 6 distinct long setae - 3 on either side (1 located close to the frontoclypeal suture, 2 ± cranial the eye). Pronotum brown, granulated, with a mediotransveral hump bearing 1 distinct short seta on either side in central anteriolateral position and a median indentation; caudal edge as a distinct rim; edges dark; prosternal horn absent. Mesonotum completely covered by 2 sclerites, yellowish-brown, with dark apodemes and edges; with 1 shallow anterior indentation and 1 faint posterior bulge on either side. Metanotum with 3 pairs of sclerites; anteromedian sclerites ovoid, yellowish-brown, cranial egde darker, with 5-7 setae; posteromedian sclerites ± triangular, fawn, with 5-6 setae and a light spot close the cranial tip; lateral sclerites fawn, medially with a dark spot, anterior half darker, with 6-8 setae; with a band of setae groups between posteriomedian sclerites, and setal groups between posteromedian and lateral sclerites. Abdomen white, with single-filamentous gills; abdomen I sternum with a single row of long, sturdy setae; lateral line continuously from 2nd third of abdomen III to 1st third of abdomen VIII, with forked lamellae dorsally the lateral line. Case simple, constructed of mineral particles and particles of plant origin.

Distribution: This species is widely distributed, occuring in and the Alpine arc, the Mittelgebirge and the northern part of the Dinaric Alps (Fig. 5).

Comment: For an illustration and re-description of the male genitalia, see Vitecek et al. (submitted).

Drusus discolor Rambur (Fig. 4d)

Material examined: 2 5th instar larvae: France, Mt. Canigou; N42.4864, E2.4139; leg. Graf; 12.VII.2012. 4 5th instar larvae: France, Aragnouet; N42.8234, E0.1682; leg. Graf; 21.VIII.2012. 3 5th instar larvae: France, St. Pierre de la Martin; N42.9597, E0.8290; leg. Graf; 22.VII.2012.

Description. *Fifth instar larva.* Head brown to dark brown fading to yellow at the occipital, smooth with rugged patches on the vertex, covered in flocculent hair (Fig. 3h); head capsule in lateral view with a sharp bend of the frontoclypeus between the eye and the anteclypeus, in cranial view distinctly indent with an elevated rim and a modified frontoclypeus: a median, ± oval bulge with a median indentation, a dorsal protrusion, a distinct dorsolateral protrusion, a mediolateral protrusion, and a long ventrolateral protrusion extending to the ventral rim on either side; setation modified: a band of bristles running along the rim and a dense fluff of hair centrally, 4 distinct long setae - 2 on either side (1 located close to the frontoclypeal suture, 1 ± cranial the eye). Pronotum brown to dark brown, smooth, with an anteriad transversal band of bristles located on a mediotransveral hump; laterally with 1 distinct indentation; caudal edge as a distinct rim; edges dark. Mesonotum completely covered by 2 sclerites, yellowish-brown with darker apodemes and edges; with 1 shallow anterior indentation and 1 faint posterior bulge on either side. Metanotum with 3 pairs of sclerites; anteromedian sclerites ovoid, fawn, cranial egde darker, with 3-5 setae; posteromedian sclerites ± triangular, fawn, with 8-9 setae; lateral sclerites fawn, medially with a dark spot, anterior half darker, with 6-10 setae; with setal groups between posteriomedian sclerites, and setal groups between posteromedian and lateral sclerites. Abdomen white, with single-filamentous gills; abdomen I sternum with a single row of long, sturdy setae; lateral line continuously from 1st third of abdomen III to 1st half of abdomen VIII, with forked lamellae dorsally the lateral line. Case simple, constructed of mineral particles and particles of plant origin.

Distribution: This species is one of the most wide-spread Drusinae species, covering all major European mountain ranges from the Carpathians to the Pyrenees (Fig. 5).

Comment: For an illustration and re-description of the male genitalia, see Vitecek et al. (submitted).

Drusus siveci Malicky (Fig. 4e,)

Material examined: 2 5th instar larvae: same site; leg. Previšić; 02.VI.2009. 1 5th instar larvae: Sutjeska NP, mouth of the Jabučica River; N43.2902, E18.6173; leg. Previšić; 02.VI.2009.

Description. *Fifth instar larva.* Maximum head width 1.33-1.39 mm. Head yellowish-brown to reddish-brown, fading to yellow at the occipital, smooth with rugged patches on the vertex, covered in flocculent hair (Fig. 3h); head capsule in lateral view with a sharp bend of the frontoclypeus between the eye and the anteclypeus, in cranial view distinctly indent with an elevated rim and a modified frontoclypeus: a median, ± oval bulge with a median indentation, a faint mediad dorsal protrusion enclosing a shallow indentation, a dorsolateral protrusion, a faint mediolateral protrusion, and a thin, long ventrolateral protrusion extending to the ventral rim on either side; setation modified: a band of bristles running along the rim and a dense fluff of hair centrally, 4 distinct long setae - 2 on either side

(1 located close to the frontoclypeal suture, 1 ± cranial the eye). Pronotum reddish brown to brown, smooth, with an anteriad transversal band of bristles located on a mediotransveral hump; laterally with 1 distinct indentation; caudal edge as a distinct rim; edges dark. Mesonotum completely covered by 2 sclerites, fawn to pale brown with darker apodemes and edges; with 1 shallow anterior indentation and 1 faint posterior bulge on either side. Metanotum with 3 pairs of sclerites; anteromedian sclerites ovoid, fawn to pale brown, cranial egde slightly darker, with 4 setae; posteromedian sclerites ± triangular, fawn to pale brown, with 5-7 setae; lateral sclerites fawn, medially with a dark spot, with 6 setae; with setal groups between posteriomedian sclerites, and setal groups between posteromedian and lateral sclerites. Abdomen white, with single-filamentous gills; abdomen I sternum with a single row of long, sturdy setae; lateral line continuously from 1st third of abdomen III to 1st half of abdomen VIII, with forked lamellae dorsally the lateral line. Case simple, constructed of mineral particles and particles of plant origin.

Distribution: This species is a micro-endemic of the Western Balkans (Fig. 5).

Comment: For an illustration and re-description of the male genitalia, see Vitecek et al. (submitted).

Drusus meridionalis stat. nov. Kumanski (Fig. 4f)

Material examined: 8 5th instar larvae: Bulgaria, Pirin Mountains, Razlog, Bankso; N41.7508, E23.4142; leg. Neu, Bálint; 08.VI.2009.

Description. *Fifth instar larva.* Maximum head width 1.31-1.34 mm. Head chestnut, fading to yellow at the occipital, smooth, with slight indentations on the rugged vertex, covered in flocculent hair; head capsule in lateral view with a sharp bend of the frontoclypeus between the eye and the anteclypeus, in cranial view distinctly indent with an elevated rim and a modified frontoclypeus: a median, ± squarish bulge increasing in elevation from dorsal to the inconspicuous dorsolateral protrusion, with a distinct mediolateral protrusion, and a broad, long ventrolateral protrusion extending to the ventral rim on either side; setation modified: a band of bristles running along the rim and a dense fluff of hair centrally, 4 distinct long setae - 2 on either side (1 located close to the frontoclypeal suture, 1 ± cranial the eye). Pronotum chestnut with darker apodemes, smooth, with an anteriad transversal band of bristles located on a mediotransveral hump; caudal edge as a distinct rim; edges dark. Mesonotum completely covered by 2 sclerites, fawn to pale brown with darker apodemes and edges; with 1 shallow anterior indentation and 1 faint posterior bulge on either side. Metanotum with 3 pairs of sclerites; anteromedian sclerites ovoid, fawn to pale brown, cranial egde slightly darker, with 6 setae; posteromedian sclerites ± triangular, fawn to pale brown, with 6 setae; lateral sclerites fawn, medially with a dark spot, anterior half darker, with 6-9 setae; with setal groups between posteriomedian sclerites, and setal groups between posteromedian and lateral sclerites. Abdomen white, with single-filamentous gills; abdomen I sternum with a single row of long, sturdy setae; lateral line continuously from 1st half of abdomen III to 1st half of abdomen VIII, with forked lamellae dorsally the lateral line. Case simple, constructed of mineral particles and particles of plant origin.

Distribution: This species is a micro-endemic of the Eastern Balkans (Fig. 5).

Comment: For an illustration and re-description of the male genitalia, see Vitecek et al. (submitted).

Drusus romanicus Murgoci and Botosaneanu (Fig. 4g)
Material examined: 8 5th instar larvae: Romania, Retezat, Cimpuliui Neog; N45.320, E 22.914; leg. Graf; 02.VI.2007.
Description. *Fifth instar larva.* Head reddish-brown to dark brown, fading to yellow at the occipital, smooth, with distinct indentations on the vertex, covered in flocculent hair (Fig. 3h); head capsule in lateral view with a sharp bend of the frontoclypeus between the eye and the anteclypeus, in cranial view distinctly indent with an elevated rim and a modified frontoclypeus: a high median, ± squarish bulge with a distinct dorsal protrusion, a distinct dorsolateral protrusion, a distinct mediolateral protrusion, and a broad, long ventrolateral protrusion extending to the ventral rim on either side; parietale distinctly indent on either side opposite the angle between the mediolateral and the ventrolateral protrusion; setation modified: a band of bristles running along the rim and a dense fluff of hair centrally, 4 distinct long setae - 2 on either side (1 located close to the frontoclypeal suture, 1 ± cranial the eye). Pronotum reddish-brown to dark brown, smooth, with an anteriad transversal band of bristles located on a mediotransveral hump; laterally with 1 distinct indentation; caudal edge as a distinct rim; edges dark. Mesonotum completely covered by 2 sclerites, fawn to pale brown with darker apodemes, edges black; with 1 shallow anterior indentation and 1 faint posterior bulge on either side. Metanotum with 3 pairs of sclerites; anteromedian sclerites ovoid, fawn to pale brown, cranial egde slightly darker, with 6 setae; posteromedian sclerites ± triangular, fawn to pale brown, with 7-8 setae; lateral sclerites fawn, medially with a dark spot, anterior half darker, with 12-14 setae; with setal groups between posteriomedian sclerites, and setal groups between posteromedian and lateral sclerites. Abdomen white, with single-filamentous gills; abdomen I sternum with a single row of long, sturdy setae; lateral line continuously from 1st half of abdomen III to 1st half of abdomen VIII, with forked lamellae dorsally the lateral line. Case simple, constructed of mineral particles and particles of plant origin.
Distribution: This species is a regional endemic of the Carpathians (Fig. 5).
Comment: For an illustration and re-description of the male genitalia, see Vitecek et al. (submitted).

Drusus krpachi Kučinić, Graf and Vitecek (Fig. 4h)
Material examined: 2 5th instar larvae: Mavrovo National Park, Korab Mts.; N41.77784, E20.58032; leg. Previšić; 05.VII.2010.
Description. *Fifth instar larva.* Maximum head width 1.18-1.2 mm. Head chestnut to brown, smooth; head capsule in lateral view with a sharp bend of the frontoclypeus between the eye and the anteclypeus, in cranial view distinctly indent with an elevated rim and a modified frontoclypeus: a median, ± circular bulge with a slight dorsolateral protrusion, a more distinct mediolateral protrusion, and a long ventrolateral protrusion extending to the ventral rim on either side; setation modified: a band of bristles running along the rim with only few hairlike-structures centrally, 4 distinct long setae - 2 on either side (1 located close to the frontoclypeal suture, 1 ± cranial the eye). Pronotum dark chestnut to

brown, smooth, with an anteriad transversal band of bristles located on a mediotransveral hump; caudal edge as a distinct rim; edges dark. Mesonotum completely covered by 2 sclerites, brownish-yellow with darker apodemes and edges; with 1 shallow anterior indentation and 1 faint posterior bulge on either side. Metanotum with 3 pairs of sclerites; anteromedian sclerites ovoid, brownish-yellow, with 3-4 setae; posteromedian sclerites ± triangular, brownish-yellow, with 3-4 setae; lateral sclerites brownish-yellow, medially with a dark spot, with 8-9 setae; with setal groups between posteriomedian sclerites and lateral sclerites. Abdomen white, with single-filamentous gills; abdomen I sternum with a single row of long, sturdy setae; lateral line lateral line continuously from 2nd half of abdomen III to 1st half of abdomen VIII, with forked lamellae dorsally the lateral line. Case simple, constructed of mineral particles and (few) particles of plant origin.
Distribution: This species is a micro-endemic of the Korab Mountains, Western Balkans (Fig. 5).
Comment: For an illustration and re-description of the male genitalia, see Vitecek et al. (submitted).

Drusus macedonicus Schmid (Figs. 4i)
Material examined: 12 5th instar larvae: same data. Macedonia, Pelister Mt., springs of Caparska reka; N41.003889, E21.167944; leg. Graf and Previšić; 07.VII.2010.
Description. *Fifth instar larva.* Maximum head width 1.15-1.35 mm. Head chestnut to brown, smooth; head capsule in lateral view with a sharp bend of the frontoclypeus between the eye and the anteclypeus, in cranial view distinctly indent with an elevated rim and a modified frontoclypeus: a median, ±shield-shaped dorsally fading bulge with a long ventrolateral protrusion on either side; setation modified: a band of bristles running along the rim with only few hairlike-structures centrally, 4 distinct long setae - 2 on either side (1 located close to the frontoclypeal suture, 1 ± cranial the eye). Pronotum chestnut, smooth, with an anteriad transversal band of bristles located on a mediotransveral hump; caudal edge as a distinct rim; edges dark. Mesonotum completely covered by 2 sclerites, fawn with darker apodemes and edges; with 1 shallow anterior indentation and 1 faint posterior bulge on either side. Metanotum with 3 pairs of sclerites; anteromedian sclerites ovoid, fawn, cranial egde darker, with 7-8 setae; posteromedian sclerites ± triangular, fawn, with 7-8 setae; lateral sclerites fawn, medially with a dark spot, with 12-14 setae; with setal groups between posteriomedian sclerites, and setal groups between posteromedian and lateral sclerites. Abdomen white, with single-filamentous gills; abdomen II sternum with a single row of long, sturdy setae; lateral line continuously from 2nd half of abdomen III to 1st half of abdomen VIII, with forked lamellae dorsally the lateral line. Case simple, constructed of mineral particles and (few) particles of plant origin.

Distribution: This species is a micro-endemic of the Pelister Mountains, Western Balkans (Fig. 5).
Comment: For an illustration and re-description of the male genitalia, see Vitecek et al. (submitted).

Key to 5th instar larvae of carnivorous Drusinae
Larvae of carnivorous Drusinae are easily recognized and differentiated from all other Drusinae larvae by the following combination of characters: (1) larval mandibles with terminal teeth; (2) absence

of carinae; (3) presence of filtering bristles on legs and the first abdominal sternite; (4) a modified head capsule that is characterized by a sharp bend of the frontoclypeus between the eye and the anteclypeus in lateral view, optionally with modified seta/a woolly layer of hair (Table 3). The latter character is found in larvae of *D. discolor*, *D. siveci*, *D. romanicus*, *D. meridionalis* stat. nov., *D. macedonicus*, *D. krpachi*, and *D.* sp. nov. Valchiusella II. Larvae of filtering carnivorous Drusinae differ distinctly in the shape of the headcapsule and the modifications of the frontoclypeus, and can be easily identified using Table 3 in combination with the following key:

Carnivorous Drusinae 1 (without hair/setae on head capsule)…(1)

Carnivorous Drusinae 2 (with hair/setae on head capsule)…(2)

(1) Head flattened in lateral view, with 2 shallow grooves around frontoclypeus and a rounded vertex in frontal view (Fig. 4a)…*Cryptothrix nebulicola* (regional endemic of the Western Alps)

Head distinctly flattened in lateral and frontal view (Fig.4b), lateral line with a break from abdominal segment II to abdominal segment III…*Drusus muelleri* (regional endemic of the Western Alps) and *Drusus* sp. Valchiusella I (micro-endemic of the South-Western Alps)

Head distinctly concave in frontal view (Fig.4c), prosternal horn lacking…*Drusus chrysotus* (widely distributed, occuring in and well beyond the Alpine arc)

(2) Head and frontoclypeus covered in setae (Fig. 3i)…*Drusus* sp. nov. Valchiusella II (micro-endemic of the South-Western Alps)

Head, frontoclypeus and prothorax covered in flocculent hair (head: Fig. 3h)…(3)

(3) [Characters are only visible after removal of hair on the frontoclypeus]

Frontoclypeus structure as in Fig 4d: with a dorsal protrusion, a distinct dorsolateral protrusion, a mediolateral protrusion, and a long ventrolateral protrusion extending to the ventral rim on either side 4 distinct protrusions on either side …*Drusus discolor* (widespread, covering all major European mountain ranges from the Carpathians to the Pyrenees)

Frontoclypeus structure as in Fig 4e: with a faint mediad dorsal protrusion enclosing a shallow indentation, a dorsolateral protrusion, a faint mediolateral protrusion, and a thin, long ventrolateral protrusion extending to the ventral rim on either side …*Drusus siveci* (micro-endemic of the Western Balkans)

Frontoclypeus structure as in Fig 4f: with an inconspicuous dorsolateral protrusion, with a distinct mediolateral protrusion, a broad, long ventrolateral protrusion extending to the ventral rim on either side, and a slight indentation on each parietale…*Drusus meridionalis* stat. nov. (micro-endemic of the Eastern Balkans)

Frontoclypeus structure as in Fig 4g: with a distinct dorsal protrusion, a distinct dorsolateral protrusion, a distinct mediolateral protrusion, a broad, long ventrolateral protrusion extending to the ventral rim on either side, and a distinct indentation on each parietale…*Drusus romanicus* (regional endemic of the Carpathains)

Frontoclypeus structure as in Fig 4h: with 3 protrusions on either side: a slight dorsolateral protrusion, a more distinct mediolateral protrusion, and a long ventrolateral protrusion extending to the

ventral rim on either side …*Drusus krpachi* (micro-endemic of the Western Balkans)

Frontoclypeus structure as in Fig 4i: with a long ventrolateral protrusion on either side …*Drusus macedonicus* (micro-endemic of the Western Balkans)

Table 1. PCR primers and PCR cycling conditions.

Fragment	Primers & Primer Concentration		PCR Cycling conditions	Taq Kit	Additional Reagents
mtCOI5-P	HCO2198 & LCO1490 (Folmer et al. 1994)	0.25 µM	5‘ 95°C, 5 x (30‘‘ 95°C, 1‘ 44°C, 1‘ 72°C), 15x (30‘‘ 95°C, 30‘‘ 48°C, 1‘ 72°C), 20 x (30‘‘ 95°C, 30‘‘ 50°C, 1‘ + (10‘‘ * n) 72°C)	peqGOLD HotTaq	-
mtCOI3-P	Jerry & S20 (Pauls et al. 2006)	0.25 µM	5‘ 95°C, 35 x (45‘‘ 95°C, 30‘‘ 45°C, 45‘‘ 72°C), 5‘ 72°C	peqGOLD HotTaq	-
16SrDNA	Lepto-F & Lepto-R (Malm & Johanson 2008)	0.75 µM	3‘ 95°C, 35 x (30‘‘ 95°C, 30‘‘ 52°C, 40‘‘ 72°C), 5‘ 72°C	peqGOLD HotTaq	4 mg BSA
WG	WGbDrrev (5’-ACCCTCTCCCGCARCACTTGAG) & WGbDrfwd (5’-CTTGCTGGATGCGTCTGCC)[1]	0.5 µM	5‘ 95°C, 35 x (45‘‘ 95°C, 45‘‘ 60°C, 90‘‘ 72°C), 7‘ 72°C	Qiagen Hotstar Taq plus Master mix	-
CADH	1028r-ino & 743nF-ino (Johanson & Malm 2010)	0.25 µM	5‘ 95°C, 35 x (45‘‘ 95°C, 30‘‘ 50°C, 45‘‘ 72°C), 5‘ 72°C	peqGOLD HotTaq	-
D2 (28SnrDNA)	D1-3up1 (5’-CGAGTAGCGGCGAGCGAACGGA) & D3-TRIC-DN (5’-ATTCCCCTGACTTCGACCTGA)[2]	0.25µM	3‘ 95°C, 35 x (45‘‘ 95°C, 45‘‘ 60°C, 60‘‘ 72°C), 5‘ 72°C	peqGOLD HotTaq	2 mg BSA, 5% DMSO

1: unpublished primer sequence by M. Bálint
2: unpublished primer sequence by K. Kjer

Table 2. Substitution models used in phylogenetic analysis.

Fragment	unpartitioned	codon position 1	codon position 2	codon position 3
mtCOI5-P	GTR+G+I	TN93+G	TN93+G	HKY
mtCOI3-P	GTR+G+I	TN93+G+I	K2+G	HKY
16SrDNA	T92+G	-	-	-
WG	T92+G	T92	JC+G	JC
CADH	T92+G+I	HKY+G	TN93	T92
D2 (28SnrDNA)	T92+G+I	-	-	-

Table 3. Alternative topologies concerning the placement of *D.* sp. nov. Valchiusella II.

Hypothesis	Alternative topology	Relative frequency
T1	*Drusus chrysotus* + D. sp. nov. Valchiusella II	0,01
T2	*D. chrysotus* + *D.* sp. nov. Valchiusella II + *D. muelleri* + *D.* sp. Valchiusellae I	0,01
T3	*D. chrysotus* + *D. puskasi* + *D. krpachi* + *D. macedonicus* + *D. siveci* + *D.* sp. Bucegi + *D. romanicus* + *D. discolor* + *D. meridionalis* stat. nov.	0,19
T4	*D.* sp. nov. Valchiusella II + *D. puskasi* + *D. krpachi* + *D. macedonicus* + *D. meridionalis* stat. nov.	8,24
T5	*D.* sp. nov. Valchiusella II + *D. siveci* + *D.* sp. Bucegi + *D. romanicus* + *D. discolor*	38,84
T6	*D.* sp. nov. Valchiusella II + *D. siveci* + *D.* sp. Bucegi + *D. discolor*	2,01
T8	*D.* sp. nov. Valchiusella II + *D. romanicus*	7,73
T9	*D.* sp. nov. Valchiusella II + *D. meridionalis* stat. nov.	3,15
T10	*D.* sp. nov. Valchiusella II + *D. puskasi* + *D. krpachi* + *D. macedonicus*	4,49

Table 4. Synopsis of characters separating 5th instar Drusinae larvae according to feeding types.

Mandibles with terminal teeth	**Legs & first abdominal sternite filter bristles**	**Forked lamellae dorsal the lateral line**	**Antennae located on carinae**	**Head**	**Head covered in setae**	**Head covered in flocculent hair**	
no	absent	absent	yes (Fig. 2c)	round (Fig. 2c)			Drusinae grazer
yes (Fig. 2a, c)	absent	present (Fig. 3a, arrow 1)	no	round			*Drusus franzi*/ *D. alpinus*[b]
yes	present (Fig. 3a,b,j)	present	no	modified[a] (Fig. 3a,c,d,e,i)	x (Fig. 3i)		*Drusus* sp. nov. Valchiusellae II
yes	present	present	no	modified			Carnivorous Drusinae 1
yes	present	present	no	modified		x (Fig. 3h)	Carnivorous Drusinae 2

a: in lateral view with a distinct step between the labrum and the frontoclypeus surface.
b: omnivorous shredders

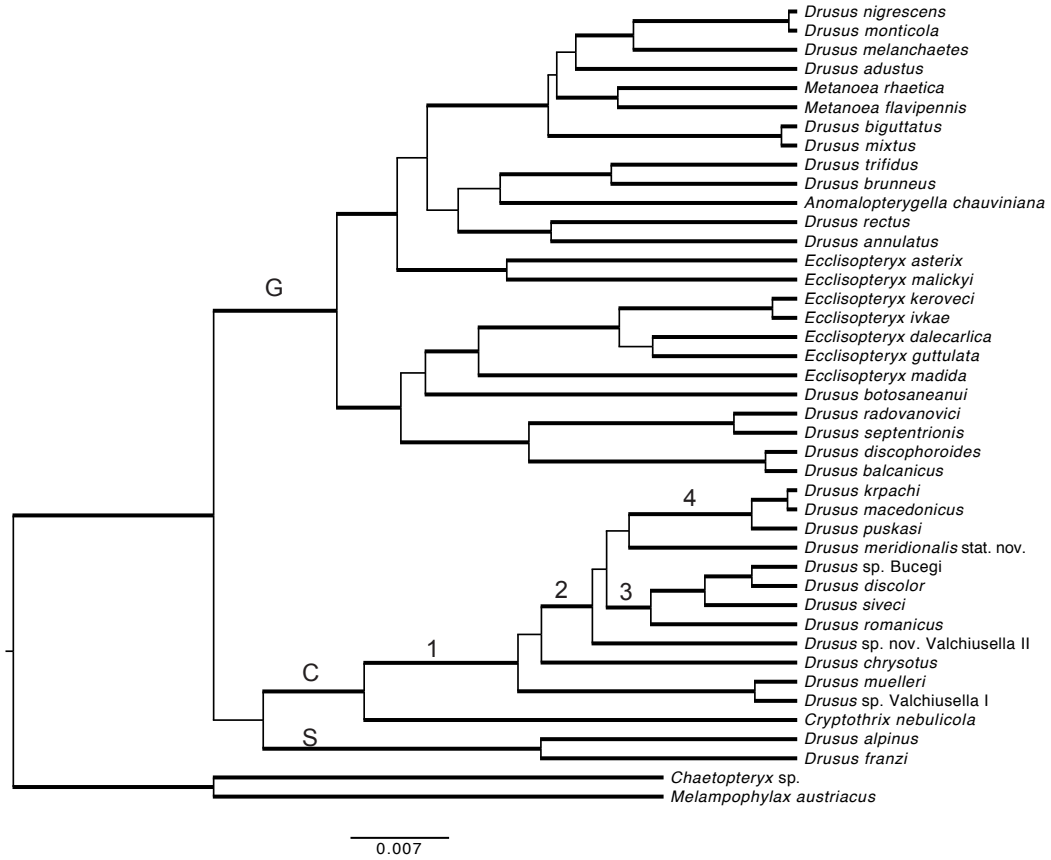


Figure 1

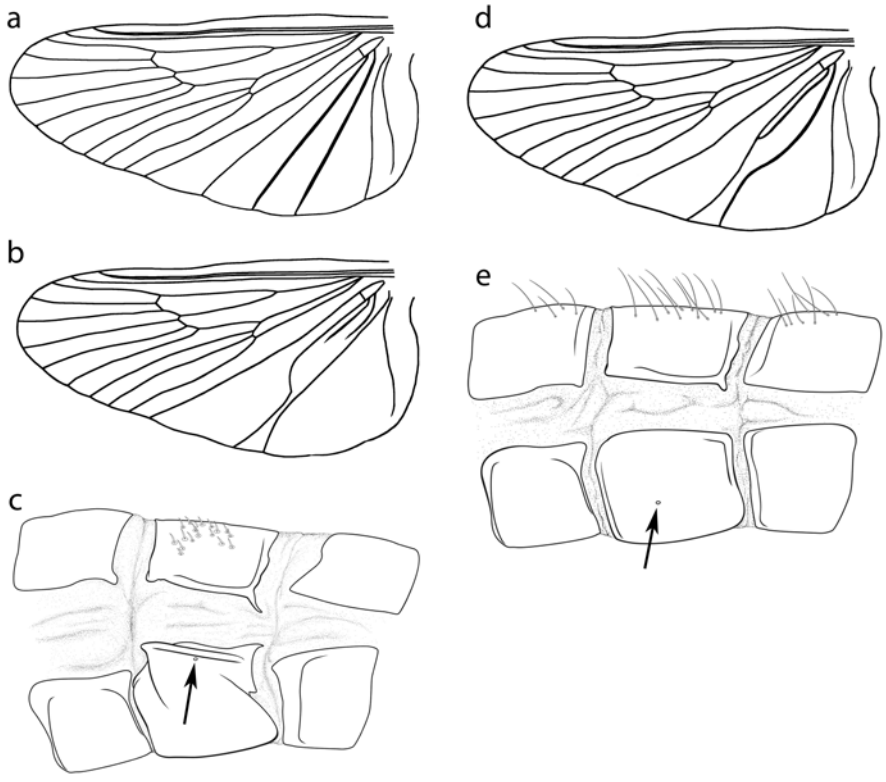


Figure 2

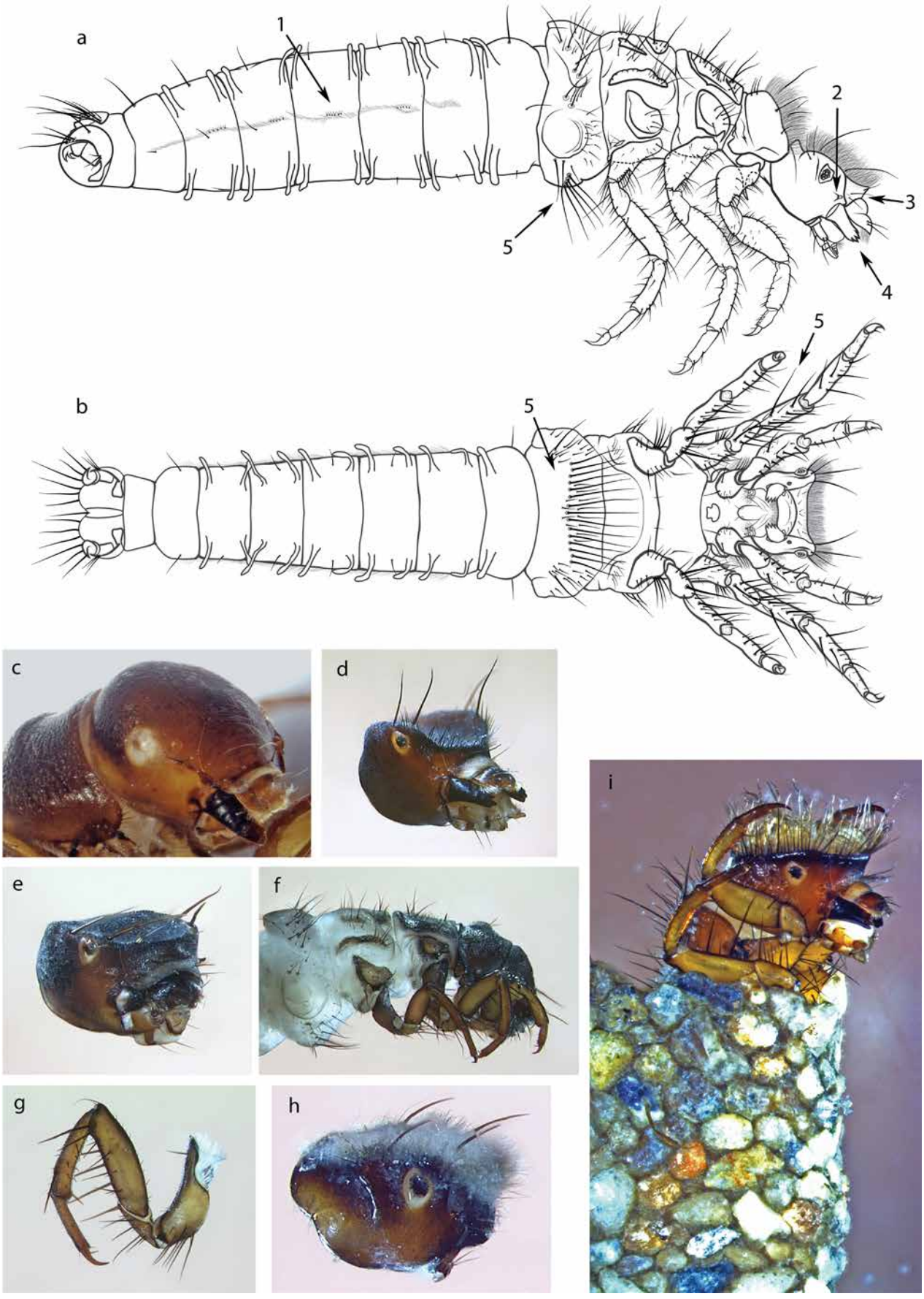


Figure 3

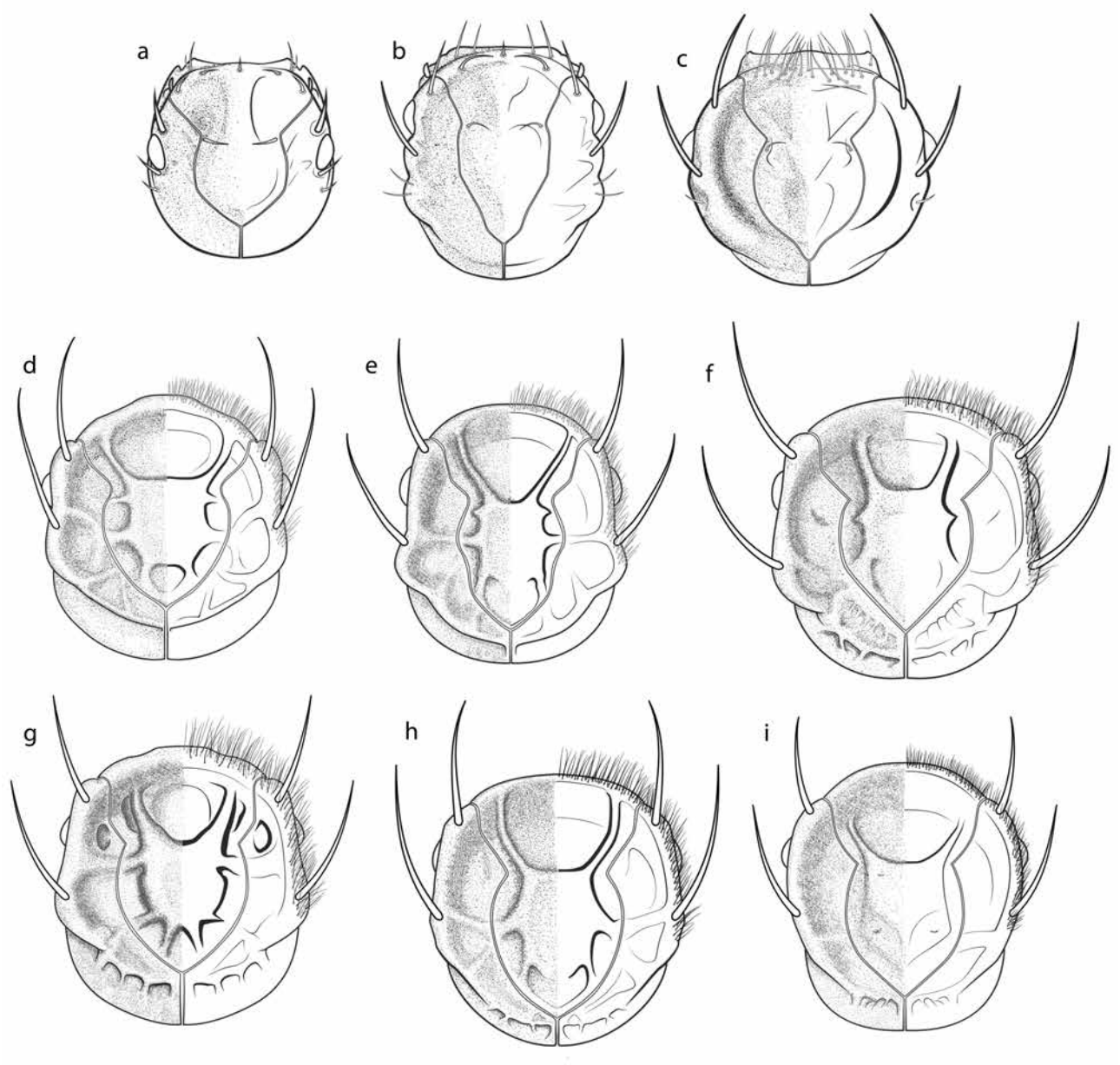


Figure 4

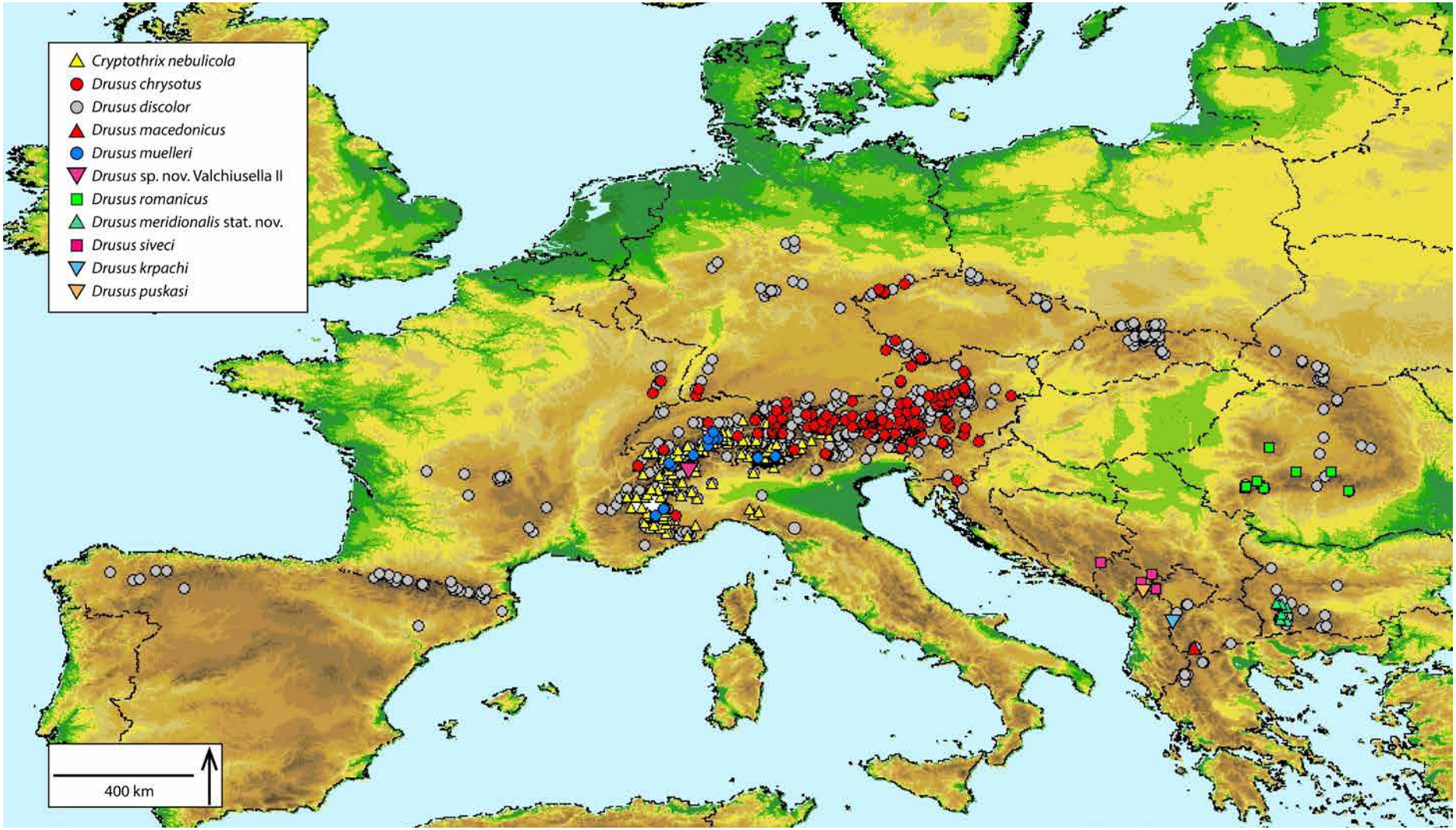
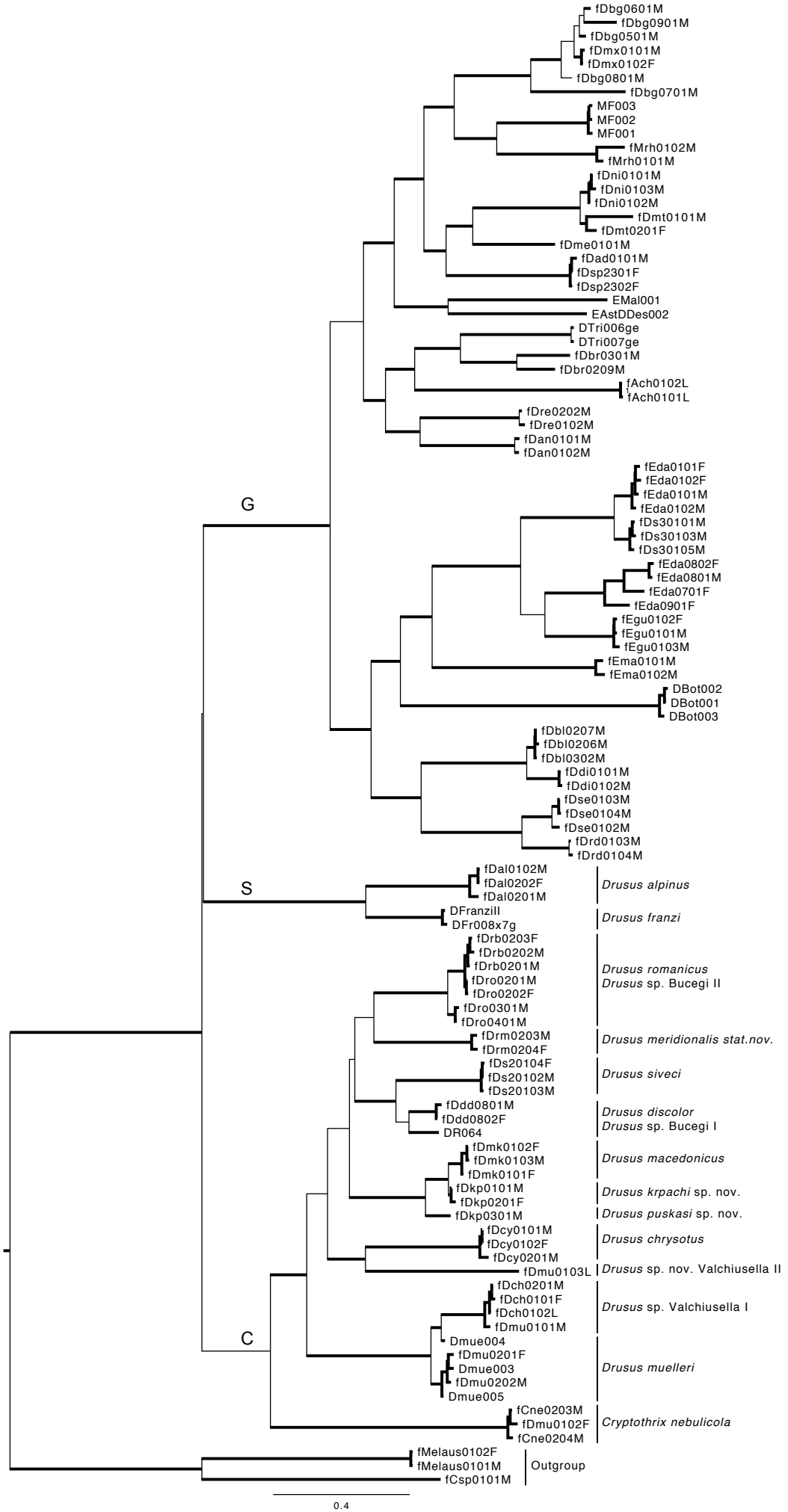

Cryptothrix nebulicola
Drusus chrysotus
Drusus discolor
Drusus macedonicus
Drusus muelleri
Drusus sp. nov. Valchiusella II
Drusus romanicus
Drusus meridionalis stat. nov.
Drusus siveci
Drusus krpachi
Drusus puskasi
400 km

Figure 5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MF002
MF001
fMrh0102M
fMrh0101M
fDni0101M
fDni0103M
fDni0102M
fDmt0101M
fDmt0201F
fDme0101M
fDad0101M
fDsp2301F
fDsp2302F
EMal001
EAstDDes002
DTri006ge
DTri007ge
fDbr0301M
fDbr0209M
fAch0102L
fAch0101L
fDre0202M
fDre0102M
fDan0101M
fDan0102M
G
fEda0101F
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S
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Drusus alpinus
DFranzilI
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Drusus franzi
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fDro0401M
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Drusus sp. Bucegi II
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Drusus meridionalis stat.nov.
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fDs20102M
fDs20103M
Drusus siveci
fDdd0801M
fDdd0802F
DR064
Drusus discolor
Drusus sp. Bucegi I
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fDmk0101F
Drusus macedonicus
fDkp0101M
fDkp0201F
Drusus krpachi sp. nov.
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Drusus chrysotus
fDmu0103L
Drusus sp. nov. Valchiusella II
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fDch0101F
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fDmu0101M
Drusus sp. Valchiusella I
C
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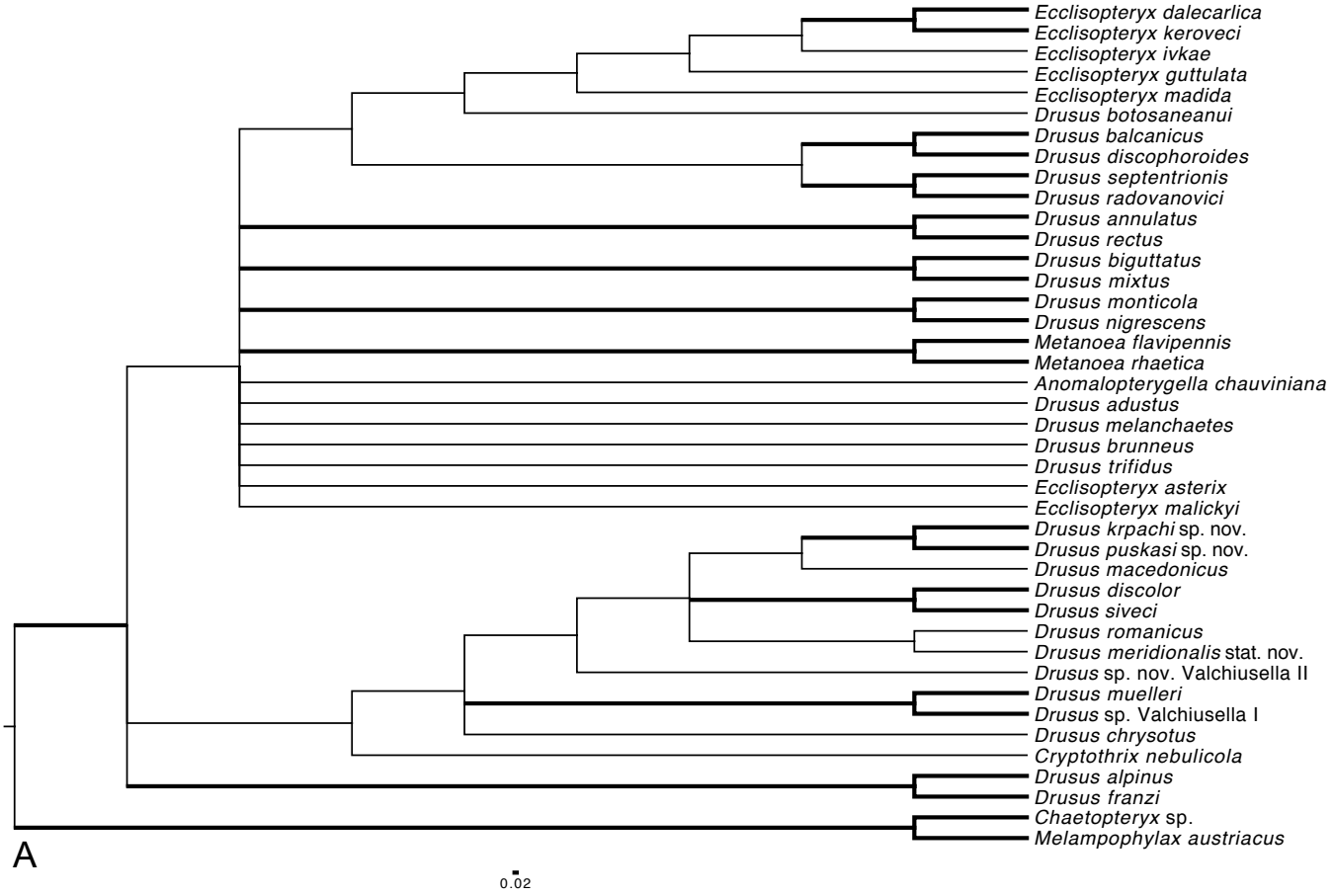
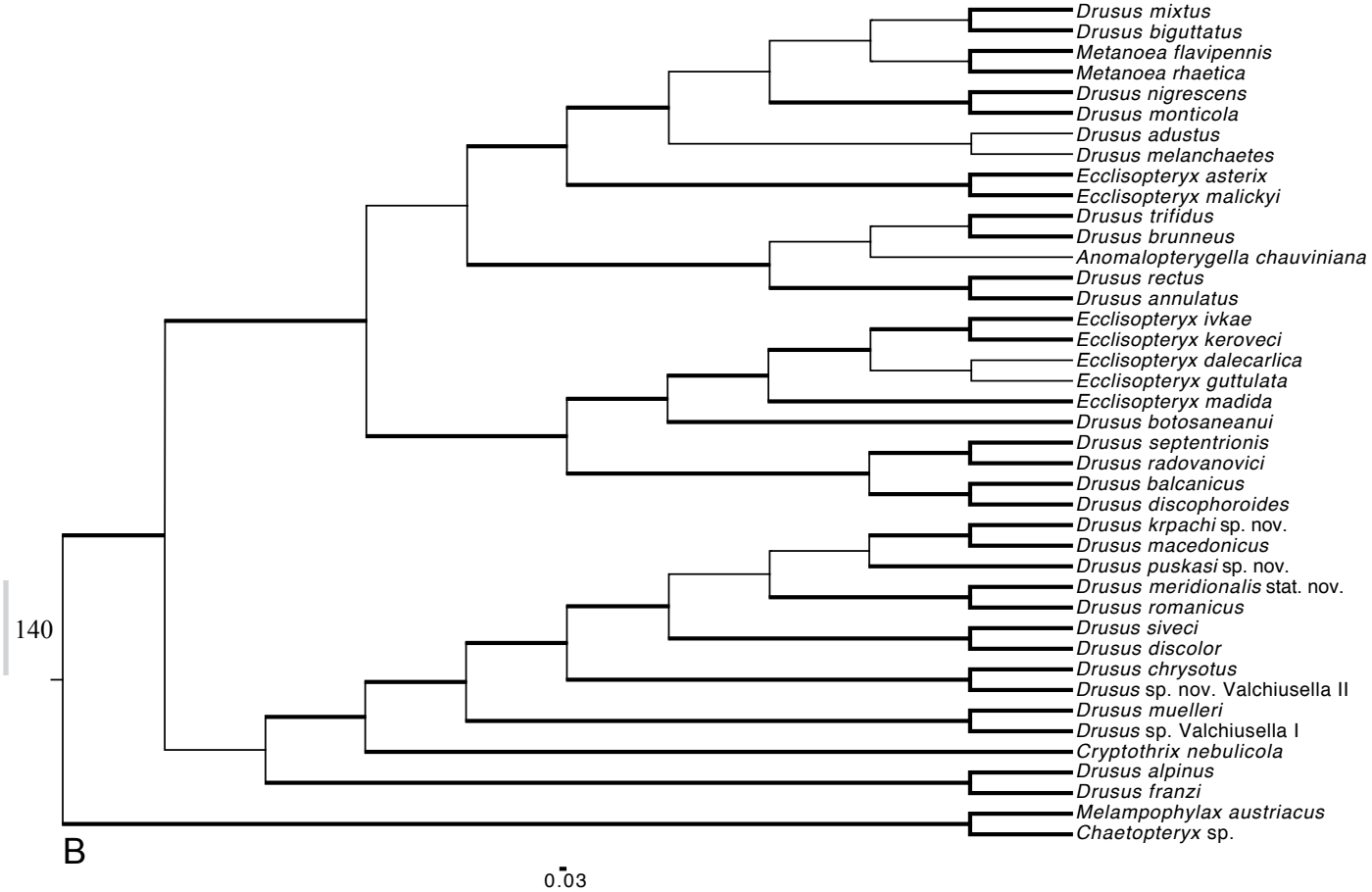
fDmu0201F
Dmue003
fDmu0202M
Dmue005
Drusus muelleri
fCne0203M
fDmu0102F
fCne0204M
Cryptothrix nebulicola
fMelaus0102F
fMelaus0101M
fCsp0101M
Outgroup
0.4

Figure A. 1


Ecclisopteryx dalecarlica
Ecclisopteryx keroveci
Ecclisopteryx ivkae
Ecclisopteryx guttulata
Ecclisopteryx madida
Drusus botosaneanui
Drusus balcanicus
Drusus discophoroides
Drusus septentrionis
Drusus radovanovici
Drusus annulatus
Drusus rectus
Drusus biguttatus
Drusus mixtus
Drusus monticola
Drusus nigrescens
Metanoea flavipennis
Metanoea rhaetica
Anomalopterygella chauviniana
Drusus adustus
Drusus melanchaetes
Drusus brunneus
Drusus trifidus
Ecclisopteryx asterix
Ecclisopteryx malickyi
Drusus krpachi sp. nov.
Drusus puskasi sp. nov.
Drusus macedonicus
Drusus discolor
Drusus siveci
Drusus romanicus
Drusus meridionalis stat. nov.
Drusus sp. nov. Valchiusella II
Drusus muelleri
Drusus sp. Valchiusella I
Drusus chrysotus
Cryptothrix nebulicola
Drusus alpinus
Drusus franzi
Chaetopteryx sp.
Melampophylax austriacus
A
0.02


Drusus mixtus
Drusus biguttatus
Metanoea flavipennis
Metanoea rhaetica
Drusus nigrescens
Drusus monticola
Drusus adustus
Drusus melanchaetes
Ecclisopteryx asterix
Ecclisopteryx malickyi
Drusus trifidus
Drusus brunneus
Anomalopterygella chauviniana
Drusus rectus
Drusus annulatus
Ecclisopteryx ivkae
Ecclisopteryx keroveci
Ecclisopteryx dalecarlica
Ecclisopteryx guttulata
Ecclisopteryx madida
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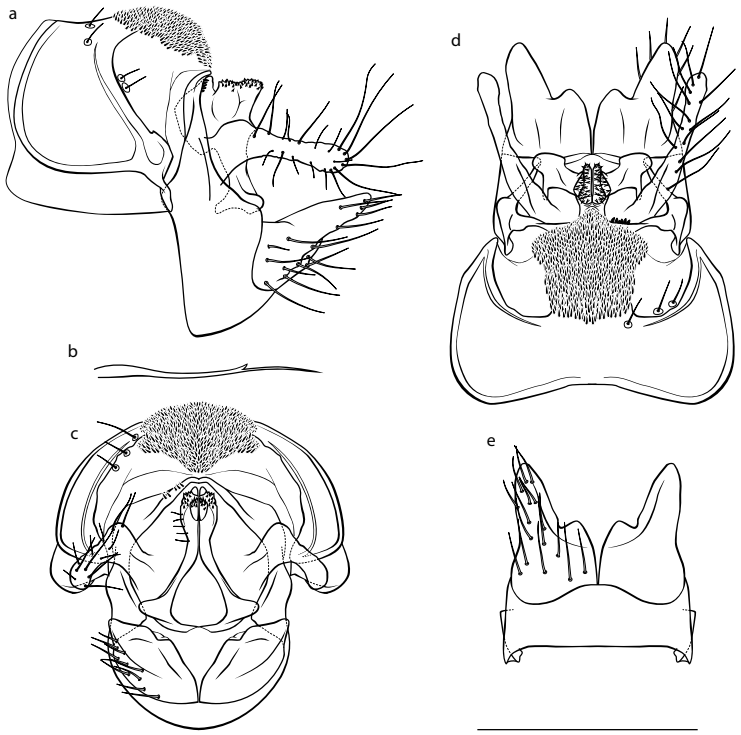
Drusus romanicus
Drusus siveci
Drusus discolor
Drusus chrysotus
Drusus sp. nov. Valchiusella II
Drusus muelleri
Drusus sp. Valchiusella I
Cryptothrix nebulicola
Drusus alpinus
Drusus franzi
Melampophylax austriacus
Chaetopteryx sp.
140
B
0.03

Figure A. 2



AppFig. 1

Diversity of Trichoptera in Europe

The last census of European Trichoptera tallied 1497 taxa (Graf et al. 2008). The descriptions of *Ecclisopteryx ivkae*, *Ecclisopteryx keroveci*, *Drusus crenophylax*, *Drusus krpachi*, *Drusus puskasi*, *Wormaldia sarda*, *Anisogamus waringeri*, *Consorophylax vinconi* (Chapters I, II, III; Appendix) do not significantly increase the number of European Trichoptera species but rather provide another piece of information on the European Trichoptera fauna, and its habitat preference and distribution. Further, these descriptions demonstrate the significance of alpha-taxonomic studies in Europe, and the need to train a new generation of scientists capable of performing this work. Particularly the availability of fast and easy-to-use molecular methods should promote 'classical' taxonomic knowledge, as both methods rely on each other to improve knowledge on biodiversity.

Interestingly, all newly described species (inculding the stonefly *Isoperla claudiae* (Appendix)) were found in renown hot–spots of Trichoptera diversity: alpine spring areas, habitats that are greatly endangered by global change. Spring habitats represent a unique ecotone and harbour high biodiversity, including high numbers of endemic or specialized taxa, the so-called crenobionts (e.g., Mori & Brancelj 2006, Barquín & Scarsbrook 2008, Witt et al. 2006, Botosaneanu 1998). Particularly the high habitat heterogeneity conferring diverse microhabitats, the refugial character, the relative physico-chemical consistency, and the hydrologic permanence of spring habitats are discussed to enhance spring biodiversity (cf. Danks & Williams 1991, Botosaneanu 1995, Erman & Erman 1995, Botosaneanu 1998, Vinson & Hawkins 1998, Cantonati et al. 2006, Mori & Brancelj 2006, Staudacher & Füreder 2007, Bottazzi et al. 2011). Further, glacial Dinodal refugia of European Trichoptera identified by Malicky (2000, 2006) comprise high numbers of stenochorous crenal species, suggesting a particular significance of spring habitats for specation in glacial refugia (cf. Botosaneanu 1995).

Springs are also among the most threatened aquatic habitats in Europe. Spring habitats are directly affected by livestock (over-)grazing, mining, pollution by pesticides or fertilizers, and, most recently, fracking (cf. Kaufmann & Krueger 1989, Strand & Merritt 1999, Barquín & Scarsbrook 2008 and references therein, Vidic et al. 2013). Water extraction for tourism, agriculture and hydroelectricity are key drivers of habitat degradation in (alpine) spring areas (cf. Barquín & Scarsbrook 2008, Zarfl et al. 2014). Additionally, human activities associated with forestry (construction of roads, timber rafting) and increasing water extraction for human consumption (either directly or via irrigated crops) or ski tourism (snowmaking) severely threaten springs thorughout Europe (cf. Fischer 1992, Puigdefábregas & Mendizabal 1998, de Santa Olalla Mañas et al. 1999, Trombulak & Frissell 2000, Gössling et al. 2012). Despite recent efforts, spring habitats in Europe remain underinvestigated, as demonstrated by the recent descriptions of several crenobiont taxa (e.g., references in Cantonati et al. 2012, General Introduction; Glöer & Pešić 2006, Glöer & Pešić 2008, Pešić & Glöer 2012).

European spring habitats have been shown to be of great significance for local and regional biodiversity. However, small-scale speciation of crenobionts and crenophilic species has not yet been studied. The Drusinae are typical members of crenal communities (Graf et al. 2008), and additionally feature a highly complex evolutionary history

shaped by historic climate and geography which potentially enhanced speciation within the group (Previšić et al. 2009, Previšić et al. 2014). Thus, the Drusinae represent an ideal model taxon to study biogeographic differentiation of discrete lineages (cf. Pauls et al. 2006, Previšić et al. 2014, Geismar et al. 2015).

Small-scale differentiation of crenobionts could be studied in the putative new species *Drusus* sp. Valchiusella (discussed in Chapter IV) and its closest congeners. Morphologically, this taxon exhibits only minute differences to *D. muelleri*, but molecular data suggest a more distinct delineation of *D. muelleri* and *D.* sp. Valchiusella. Further, distribution of *D. muelleri*, the putative new taxon, and the lost species *D. chapmani* (McLachlan 1901, Malicky 2005) are insufficiently known, but indicate a range of the group restricted to the Western Alps (with distribution data from Switzerland, Western Austria, Northern Italy, Liechtenstein and South-Eastern France). Assuming taxon sampling covering the whole range of the group, one could easily assess morphological and molecular variance of the taxa in relation to geographical distance, and potential dispersal barriers. Such a study would further allow to estimate population size, to model dispersal events, and also to estimate age of lineages provided information on the geological age of habitats is available.

Distribution, Conservation and Ecology of Drusinae

Distribution patterns of Drusinae in Europe are insufficiently known. An approximation of the general distribution is depicted in Figure 3 (General Introduction). Anatolia, the Caucasus, and the Balkans are undersampled. Collections performed in 2012 by R. Godunko in the Caucasus suggest high densities of *D. caucasicus* throughout the area. Additionally, species occurence data as indicated by Malicky (2004) concerning Anatolian Drusinae species are not represented in the current geodata. The significance of correct and complete georeferenced collection data covering the range of a species is evident when comparing locations of planned hydropower plants in the Western Balkans with distribution of Drusinae. Co-occurence of planned hydropower plants or hydropower plants under construction and Drusinae is common, and usually leads to local extinction of the latter at the site (A. Previšić, personal communication; but see Freyhof 2012, Schwarz 2012, Zarfl et al. 2014). Construction of hydropower plants poses one of the greatest threats to aquatic biodiversity, particularly in so-called emerging economies. In the Western Balkans damming of small cold-water mountain streams for hydroelectricity threatens many regional and micro-endemics such as Drusinae.

Results and species descriptions presented in this thesis indicate that currently prevailing energy policy will most likely result in loss of known and unknown aquatic diversity in Europe, particularly in the Western Balkans.

Ecologically, Drusinae were found to comprise three distinct feeding types: epilithic grazers, omnivorous shredders, and filtering carnivores. Compared to Pauls et al. (2008), monophyly of the feeding groups is confirmed, but grouping of feeding types was found to differ. A close relationship of grazers and shredders (as indicated in Pauls et al. 2008) is not returned as the most probable tree topology in species tree analysis (Chapter IV). Until a full revision of Drusinae is achieved, neither of the two results should be favoured. Intriguingly, complexity of larval head capsule

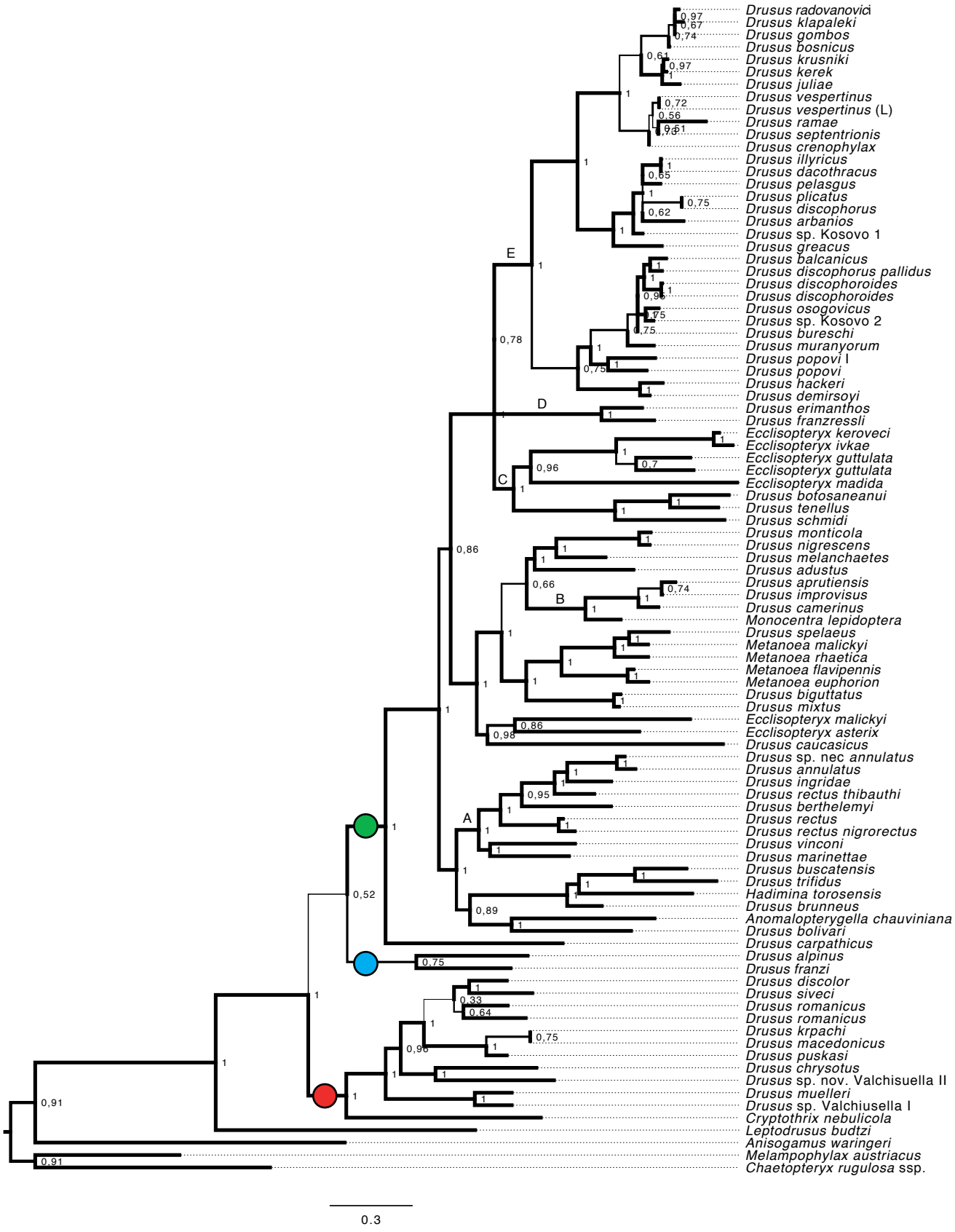
modifications within filtering carnivorous Drusinae seems to follow an evolutionary pattern: species that are basal in species tree topology exhibit simpler frontoclypei compared to derived species. But as relationships between filtering carnivores is not fully resolved, hypotheses about direction of evolution are not satisfactorily rejectable. Nevertheless, modifications of head capsules as observed in filtering carnivorous Drusinae potentially represent adaptations to optimally exploit the feeding niches of the species. The characteristic head capsule formations as observed in the filtering carnivores may support maintenance of the stereotypical filtering posture by alleviating hydrological stress. However, although a number of studies focussed on hydrological niche adaptations in aquatic insects (e.g., Statzner and Holm 1982, 1989; Statzner 1988), no study so far has focussed on morphological adaptations to passive filter-feeding in Trichoptera. A study on hydrological niche differentiation intersected with variation of head capsule morphology in filtering carnivorous Drusinae potentially might serve to directly assess ecological (hydrological) constraints on evolutionary patterns.

Systematics – current status

Systematically, results are not yet stringent. Although the status of the genus *Cryptothrix* is questionable based on species-tree analysis, no systematic changes could be effected. Other genera within Drusinae were demonstrated to be paraphyletic (e.g., *Drusus*, *Ecclisopteryx*), and should be revised as well. Thus, systematic changes should be undertaken in a revision dealing with the whole subfamily.

Currently, datasets are prepared to perform comprehensive phylogenetic analyses of Drusinae. Preliminary B/MCMCMC inference was performed in MrBayes (Ronquist et al. 2012) using a 6 loci, 3804 bp single specimen dataset comprising 94 terminal taxa, implementing the respective substitution models for each partition (see Chapter IV). 28S mRNA and 16SrRNA were not partitioned. Tree space was sampled every 10000^{th} generation for $10x10^6$ generations in 4 separate runs with 12 chains each. The last 500 trees of each run were manually combined and analysed in TreeAnnotator (Drummond and Rambaut 2013). Average standard deviations of split frequencies and MCMC parameters as examined using Tracer (Rambaut et al. 2014) did not indicate convergence of runs, and results are at best preliminary. To present, preliminary results of phylogenetic inference (Figure 4)

Figure 4 (next page). Preliminary results (maximum clade credibility tree) of Bayesian inference based on a 6 loci, 3804 bp dataset. Tree space was sampled every 10000th generation for 10x106 generations in 4 separate runs with 12 chains each. *Leptodrusus budtzi* is recoverd as basal sister to the rest of Drusinae ('true Drusinae'). True Drusinae are recovered as three distinct lineages, filtering carnivores (marked red), omnivorous shredders (marked blue), and scraping grazers. (marked green). Within scraping grazers, several well-supported clades are recovered, that additionally are geographically distinct. Clade A comprises representatives of Drusinae that occur, with the exception of *D. annulatus*, in Western Europe, and are taxonomically particularly rich in the Pyrenees. Apennine species are recovered as monophyletic clade B, and are morphologically characterized by the presence of scales on male hindwings. Clade C comprises representatives of Drusinae that exhibit spines on larval heads and pronota, and dilated segment IX in males. *Drusus erimanthos* and *D. franzressli* are species occurring in the Hellenic Balkans that typically develop translucent abdominal sternites V in males. Clade E contains species occurring in Western Europe, comprising species of the Western Balkan *bosnicus*-group and several Eastern Balkan species.


Drusus radovanovici
Drusus klapaleki
Drusus gombos
Drusus bosnicus
Drusus krusniki
Drusus kerek
Drusus juliae
Drusus vespertinus
Drusus vespertinus (L)
Drusus ramae
Drusus septentrionis
Drusus crenophylax
Drusus illyricus
Drusus dacothracus
Drusus pelasgus
Drusus plicatus
Drusus discophorus
Drusus arbanios
Drusus sp. Kosovo 1
Drusus greacus
Drusus balcanicus
Drusus discophorus pallidus
Drusus discophoroides
Drusus discophoroides
Drusus osogovicus
Drusus sp. Kosovo 2
Drusus bureschi
Drusus muranyorum
Drusus popovi I
Drusus popovi
Drusus hackeri
Drusus demirsoyi
Drusus erimanthos
Drusus franzressli
Ecclisopteryx keroveci
Ecclisopteryx ivkae
Ecclisopteryx guttulata
Ecclisopteryx guttulata
Ecclisopteryx madida
Drusus botosaneanui
Drusus tenellus
Drusus schmidi
Drusus monticola
Drusus nigrescens
Drusus melanchaetes
Drusus adustus
Drusus aprutiensis
Drusus improvisus
Drusus camerinus
Monocentra lepidoptera
Drusus spelaeus
Metanoea malickyi
Metanoea rhaetica
Metanoea flavipennis
Metanoea euphorion
Drusus biguttatus
Drusus mixtus
Ecclisopteryx malickyi
Ecclisopteryx asterix
Drusus caucasicus
Drusus sp. nec annulatus
Drusus annulatus
Drusus ingridae
Drusus rectus thibauthi
Drusus berthelemyi
Drusus rectus
Drusus rectus nigrorectus
Drusus vinconi
Drusus marinettae
Drusus buscatensis
Drusus trifidus
Hadimina torosensis
Drusus brunneus
Anomalopterygella chauviniana
Drusus bolivari
Drusus carpathicus
Drusus alpinus
Drusus franzi
Drusus discolor
Drusus siveci
Drusus romanicus
Drusus romanicus
Drusus krpachi
Drusus macedonicus
Drusus puskasi
Drusus chrysotus
Drusus sp. nov. Valchisuella II
Drusus muelleri
Drusus sp. Valchiusella I
Cryptothrix nebulicola
Leptodrusus budtzi
Anisogamus waringeri
Melampophylax austriacus
Chaetopteryx rugulosa ssp.
A
B
C
D
E
0.3

suggest monophyletic epilithic grazers and filtering carnivores. Omnivorous shredders are not well supported, and are returned as unsupported sister to epilithic grazers. *Leptodrusus budtzi* is recovered as basal sister to Drusinae, potentially indicating that this species is not part of 'real' Drusinae.

Filtering carnivores comprise *Cryptothrix nebulicola*, but relationships between species are not resolved. Interestingly, a similar tree topology as presented in Chapter IV was recovered: species with complex head capsule morphology seemingly are derived within filtering carnivores.

Within scraping grazers, several well-supported clades are reconstructed. These are geographically well-defined evolutionary lineages that also share morphological characteristics in adult and larval stage. *Drusus annulatus*, *D.* sp. nec *annulatus*, *D. ingridae*, *D. rectus*, *D. rectus thibaulti*, *D. rectus nigrorectus*, *D. berthelemyi*, *D. vinconi*, and *D. marinettae* are comprised in clade A (Figure 4). These species occur with the exception of *Drusus annulatus* predominately in Western Europe (Figure 5), and exhibit similarities in the formation of the male terminalia, particularly of superior and intermediate appendages.

Species of clade B (Figure 4) are inhabitants of the Apennine peninsula (Figure 6), and typically develop male hindwings bearing scales at minimum in the bristle pouch of the male hindwing.

Larval and adult morphology suggest a close relationship of *Ecclisopteryx keroveci*, *E. ivkae*, *E. dalecarlica*, *E. guttulata*, *E. madida*, *D. botosaneanui*, *D. schmidi* and *D. tenellus*. Larvae of this clade exhibit a high number of additional setae of unknown function on heads and pronota. Adult males develop a laterally dilated segment IX, a unique feature in Drusinae. These species are also recovered as a monophyletic entity in the prelim-

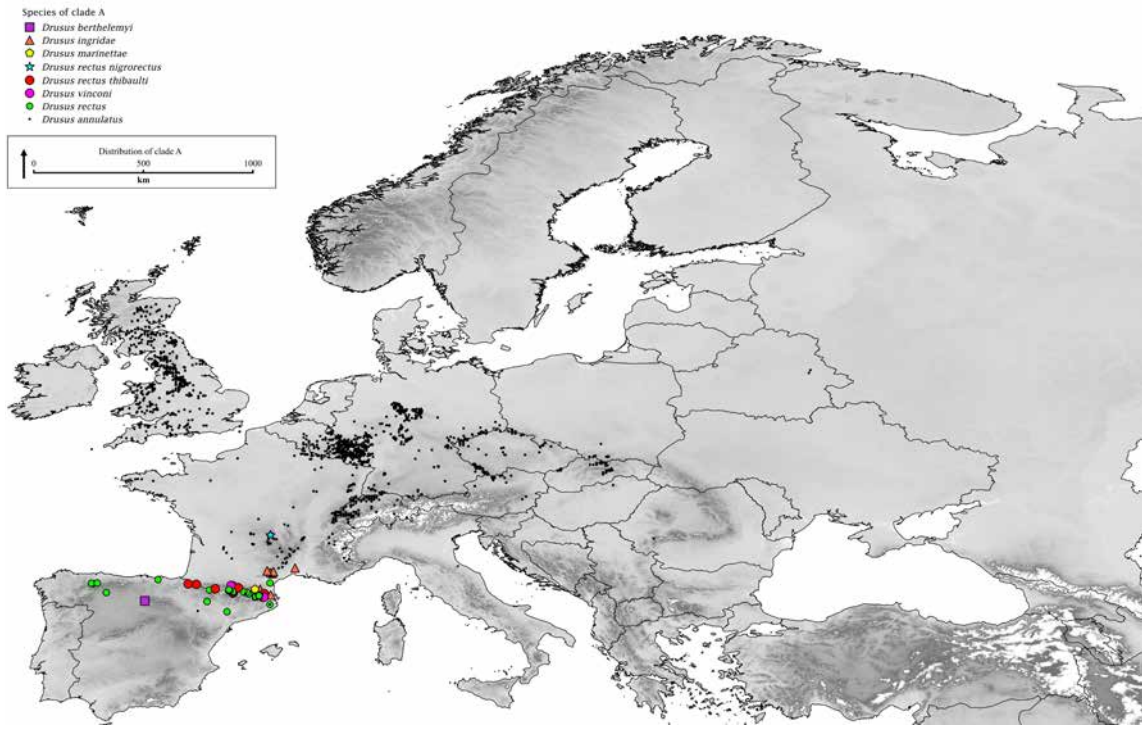


Figure 5. Distribution of species of clade A. *Drusus annulatus* is a widespread species, occurring throughout Europe. Species like *D. berthelemyi* or *D. marinettae* are endemics of the Pyrenees or the Iberian Peninsula. *Drusus ingridae* inhabits the foothills of the Massif Central and the Pyrenees.

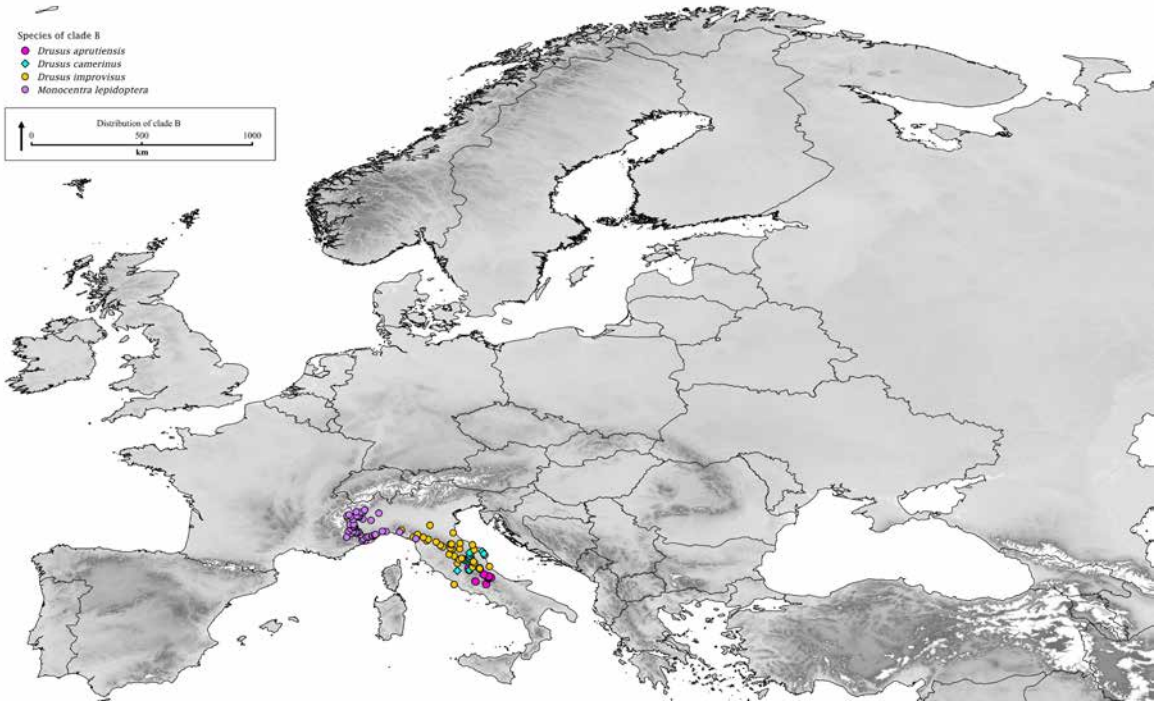


Figure 6. Distribution of species of clade B. *Monocentra lepidoptera* is the northernmost species of Apennine Drusinae, whereas *D. camerinus*, *D. improvisus*, and *D. aprutiensis* occur South of the Padan Plain.

inary concatenated B/MCMCMC analysis (clade C, Figure 4). Species of the group are distributed over entire Europe, with *E. dalecarlica* as the most widely distributed species (Figure 7). However, highest taxonomic richness in this group is recorded in the Balkans.

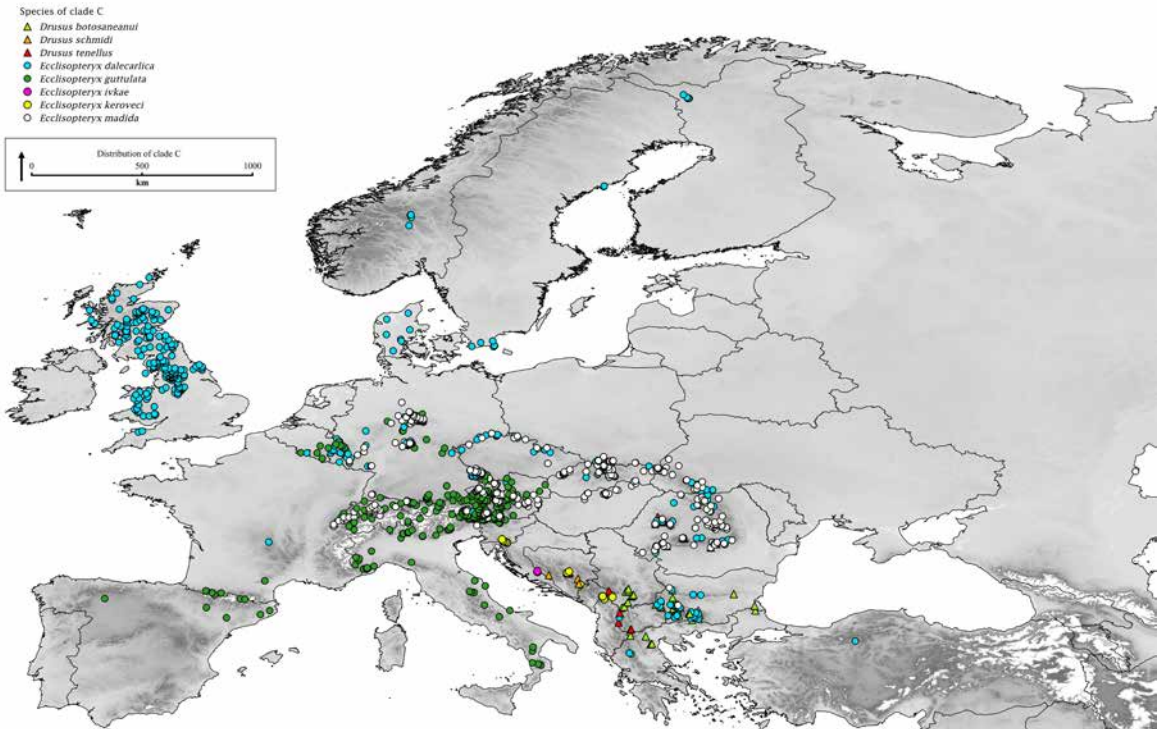


Figure 7. Distribution of species of clade C. Several widespread species of the genus *Ecclisopteryx* occur throughout Europe, taxonomic richness is highest in the Balkan Peninsula.

Drusus erimanthos and *D. franzressli* are characterised in the adult stage in exhibiting a pigmentless, translucent 5th abdominal sternite. Bayesian inference also suggests a close relationship of these species (clade D, Figure 4).

Clade E comprises a large number of species from the Balkans and Asia Minor, that typically develop protuberant concentrations of setae on abdominal tergites. This clade contains the *bosnicus*-group (Marinković-Gospodnetić 1976), in which larvae exhibit a field of spinules around each eye. Species of the *bosnicus*-group occur exclusively in the Balkans, and speciation patterns in this group have been linked with karstification (Previšić et al. 2014) (Figure 8).

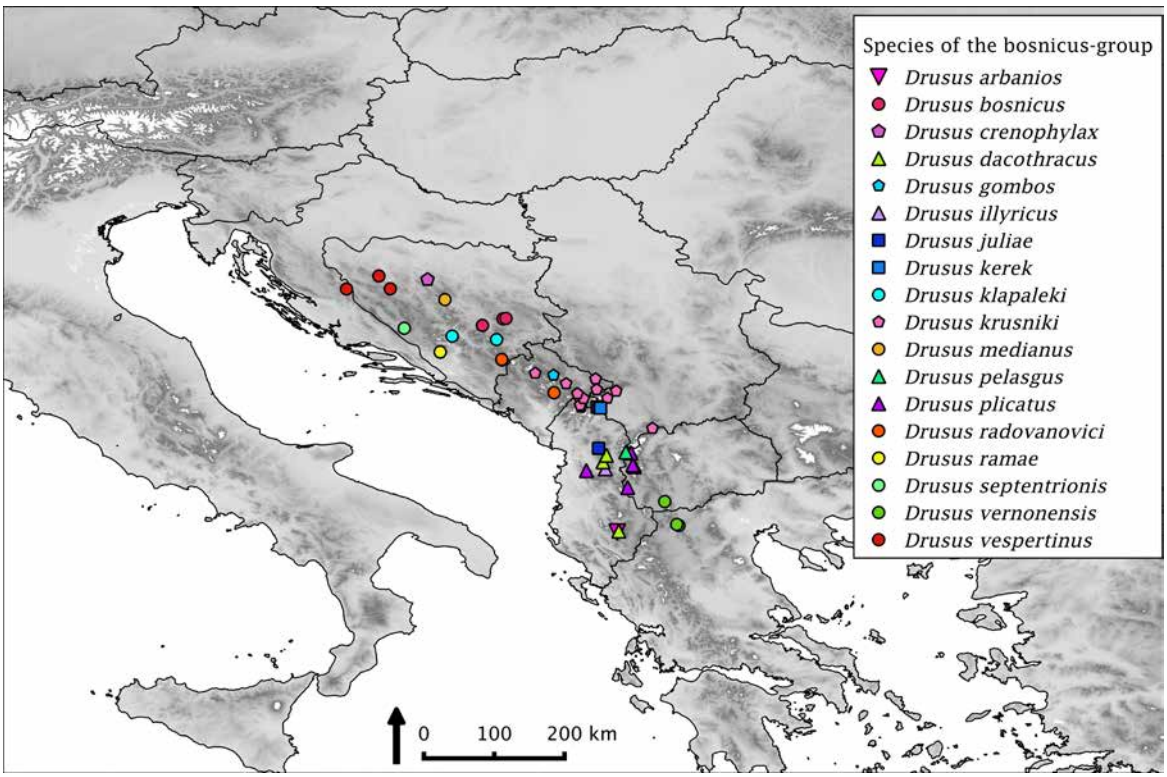


Figure 8. Distribution of species of the bosnicus-group. The *bosnicus*-group comprises several micro-endmic species that occur in the Westen Balkans. Note the interesting distribution patterns of *Drusus plicatus*, *D. pelasgus*, *D. dacothracus*, *D. illyricus*, and *D. arbanios*, that are morphogically almost undistinguishable, and occur in the same region with little or no geographical barriers.

Systematics – an outlook

Taxonomically, the subfamily Drusinae currently comprises several paraphyletic genera that do not reflect evolutionary history of the group. As feeding groups consistently are recovered as monophyletic in phylogenetic analysis, taxonomy should reflect this. In the interest of taxonomic stability it would be preferential to establish a minimal number of new taxa to ease application of new taxonomy. Thus, I suggest to establish three systematic partitions: i) the filtering carnivores [most likely to be summarized in the genus *Raptodrusus* nom. nud. as the type species for the genus *Drusus* is *D. annulatus* Rambur (Schmid 1956 mistakenly identified *D. discolor* as type species for the genus *Drusus*, referring to Stephens 1835 - erroneously as 1836 - but in this work only *Drusus annulatus* is mentioned as proper *Drusus*—thus, the genus name *Drusus* is taken by a scraping grazer)], ii) the omnivorous shredders [in a separate genus reflecting the species' feeding ecology, possibly as either *Fractodrusus* nom. nud., *Dàknodrusus* nom. nud., or *Trogodrusus* nom. nud.], iii) the epilithic grazers [most likely as genus *Drusus* with several subgenera for monophyletic and geographically distinct clades such as *Ecclisopteryx* — or as several genera comprising monophyletic and geographically distinct clades]. The proposed changes in nomenclature might be considered as violation of the International Code of Zoological Nomenclature [particularly of Articles 23.1 & 23.2], but will promote presicion and consitency by reducing the probability of the necessity to further conduct nomenclatural acts in the group. Interestingly, the genus *Leptrodrusus* is sister to all other Drusinae, indicating an ancestral state, and could be excluded from the subfamily.

Further opportunities

Filtering carnivores could be used as models to assess hydrological constraints on caddisfly larvae, and microhabitat specificity. Head capsule morphology potentially reflects limitations in hydrological microhabitats, and thus might serve to study evolutionary adaptations to ecological constraints.

Further, the comparatively high number of new species identified in the subfamily Drusinae since Schmid's seminal monograph potentially renders species distribution patterns in this group a general proxy of diversity of aquatic invertebrates. High diversity of Drusinae and high numbers of Drusinae endemics might indicate high diversity in other groups, or high rates of endemism in an area. Occurrence of Drusine might reliably predict diversity of other taxa inhabiting montane to alpine habitats, and could be used to identify areas of particular conservational interest.

The clade comprising (*Drusus buscatensis* + *Drusus trifidus*) + *Hadimina torosensis*) +*Drusus brunneus*) could be target of an evolutionary development study, as *H. torosensis* exhibits male and female genitalia unique among Drusinae, and is nested within the clade. Activity patterns of developmental genes could be assessed in an experimental setting, and compared between the species. Further, genomic and transcriptomic studies might enable quantification of genetic differences that determine differences in morphology.

Finally, data at hand could be used to estimate areas in which speciation within Drusinae occurred. This approach would also permit to assess refugial areas of Drusinae during the glaciation periods. Refugial areas of European Trichoptera have been proposed by Malicky (2000, 2006), and could be tested using Drusinae as model-taxon.

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Appendix I — Acknowledgments

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Appendix I — Zusammenfassung

Trichoptera stellen eine Ordnung holometaboler Insekten dar, die gemeinsam mit ihrer Schwesterngruppe der Lepidoptera die Überordnung Amphiesmenoptera bildet. In Europa erreicht die Familie der Limnephilidae die größte Artenvielfalt der 23 hier vorkommenden Köcherfliegenfamilien. Die Limnephilidenunterfamilie der Drusinae umfasst im Moment acht Gattungen von denen fünf monospezifisch sind. Des Weiteren beinhaltet die Unterfamilie der Drusinae einen hohen Anteil regionaler und Mikro-Endemiten.

Die erste systematische Bearbeitung der Gruppe behandelte 37 der 41 damals bekannten und in sieben Gattungen eingeteilten Arten und nutzte vor Allem Flügelmerkmale und Merkmale des männlichen Genitalapparates. Schlussendlich führte dies zur Einführung von sechs Artengruppen in der Gattung *Drusus* und zur Hypothese einer vornehmlich linearen Diversifizierung der Drusinae mit der Gattung *Drusus* an abgeleitetster Position und mehreren basalen Abtrennungen der anderen Gattungen.

Diese Erkenntnisse, insbesondere die Artengruppen- und Gattungskonzepte, wurden allerdings durch die Ergebnisse einer ersten molekulargenetischen Phylogenie der Unterfamilie verworfen. Mehrere Gattungen inklusive der größten Gattung *Drusus* wurden unter Einschluss der monospezifischen Gattungen als paraphyletisch wiedergegeben. Die Ergebnisse der auf molekularen Daten basierenden phylogenetischen Rekonstruktion wurden auch durch die unterschiedlichen Ernährungstypen der Drusinae-Larven und die damit zusammenhängenden morphologischen Merkmale unterstützt, indem alle durch molekular-genetische Methoden aufgefundenen monophyletischen evolutiven Linien jeweils einem einzigen Ernährungstyp zuzuordnen sind. Drusinae beinhalten drei unterschiedliche evolutive Linien, welche die Ernährungstypen der Larven widerspiegeln: i) filtrierende Karnivore, ii) omnivore Zerkleinerer, und iii) schabende Weidegänger.

Morphologisch ist jede dieser Gruppen im Larvenstadium gut definiert. Filtrierende Karnivore unterscheiden sich von Vertretern der anderen Gruppen durch die Ausbildung von Filterborsten und abgewandelten Kopfkapseln, wohingegen omnivore Zerkleinerer keine Abwandlungen der Kopfkapseln und keine Filterborsten aufweisen. Schabende Weidegänger zeichnen sich durch zahnlose Mandibeln aus — im Gegensatz zu filtrierenden Karnivoren und omnivoren Zerkleinerern, die zahnbewehrte Mandibeln ausbilden.

Der Großteil der Drusinae bevorzugt kalte, quellnahe, alpine bis montane Habitate der Europäischen und Eurasischen Gebirge. Die Unterfamilie umfasst eine hohe Anzahl an Endemiten und Mikro-Endemiten, besonders in den Balkangebirgen. Dabei weisen die stark disjunkten Verbreitungsmuster der Gruppe und insbesondere einiger Arten auf separates Überdauern verschiedener evolutiver Linien in geographisch getrennten Refugialräumen während mehrerer Eiszeiten hin. In Kombination mit der als gering eingeschätzten Verbreitungsfähigkeit der Drusinae könnte die Isolation von Populationen während der Eiszeiten zu der hohen Anzahl endemischer Drusinae-Arten beigetragen haben. Innerhalb der Drusinae kann von vornehmlich uni- bis semivoltinen Reproduktionszyklen ausgegangen werden, wobei die Flugzeiten vor allem von Frühling bis Herbst reichen. Ernährungsformen der Drusinae sind, wie bereits angeführt, divers, und umfassen omnivore Zerkleinerer, filtrierende Karnivore und schabende Weidegänger.

Das Ziel der Studie ist die Revision der Drusinae basierend auf molekularen und morphologischen

Daten, die von allen verfügbaren Entwicklungsstadien erhoben werden. Zusätzlich wurde die taxonomische Diversität der Drusinae mittels klassischer morphologischer und molekularer Methoden erhoben. Um die Anwendbarkeit der gewählten Methoden zu prüfen, wurden die filtrierenden karnivoren Drusinae in einer exemplarischen Studie die zur Klärung der Position der monospezifischen Gattung *Cryptothrix* betragen sollte bearbeitet.

Die ersten beiden in dieser Arbeit vorgelegten Studien befassen sich mit der taxonomischen Vielfalt der Drusinae, und beschreiben zwei neue Arten aus der Gattung *Ecclisopteryx*, eine neue Art der schabenden Weidegänger aus der Gattung *Drusus*, und zwei neue Arten von filternden karnivoren Drusinae. Die neuen Arten sind Mikroendemiten des westlichen Balkans und unterscheiden sich von ihren nächsten Verwandten in Form und Struktur der männlichen und weiblichen Terminalia, sowie in der Morphologie des Larvenstadiums.

Ergebnisse eines molekulargenetischen Datensatzes lassen auf eine nahe Verwandtschaft der beiden neuen *Ecclisopteryx*-Arten mit *E. dalecarlica*, *E. guttulata* und *E. madida* schließen. In einer einem Bayesischen Ansatz folgenden phylogenetischen Analyse, basierend auf einem 3-loci (mtCOI + nuWG + 16SrRNA) 1249 bp Datensatz, sind die beiden neuen Arten gut unterstützt und klar voneinander und den anderen *Ecclisopteryx*-Arten abgetrennt. Interessanterweise sind die Weibchen der neuen Arten besonders informativ, die Bestimmung der Männchen gestaltet sich schwieriger und beruht auf den Unterschieden in der Form der unteren Genitalanhänge. Die Larven der neuen Arten sind durch ihre jeweils einzigartigen Merkmalskombinationen leicht zu identifizieren.

Die neue Art *Drusus crenophylax* ist ein Mitglied der *bosnicus*-Gruppe und ähnelt in der Morphologie der männlichen Genitalien *D. vernonensis* und *D. discophorus*. Larven der neuen Art *D. crenophylax* unterscheiden sich von den anderen Arten der *bosnicus*-Gruppe in der Form des Kopfes und der Carinae sowie der Pronotalbeborstung. Die neue Art ist ein weiterer Mikroendemit des westlichen Balkans.

The neuen Arten *Drusus krpachi* und *Drusus puskasi* gehören der Klade der filtrierenden karnivoren Drusinae *sensu* Pauls et al. (2008) an und sind morphologisch *D. macedonicus* am ähnlichsten. Die neuen Arten unterscheiden sich von dieser Art in der Form der männlichen Genitalien, und sind Mikroendemiten des westlichen Balkans.

Filtrierende karnivore Drusinae weisen im Larvenstadium Abwandlungen der Kopfkapsel unklarer Funktion auf. Um zu prüfen ob die Komplexität der Kopfkapselabwandlungen innerhalb der filtrierenden Karnivoren einem evolutionären Muster steigender Komplexität folgt, wurden die Verwandtschaftsbeziehungen zwischen den Arten unter Nutzung von 108 terminalen Taxa rekonstruiert. In einer *species tree*-Analyse, basierend auf einem 6-loci (2 loci mtCOI + 16SrRNA + nuWG + CAD + 28S) 3805 bp, Datenset wurden Arten mit einer komplexeren Kopfkapselabwandlung als abgeleitet dargestellt. Zusätzlich rekonstruiert die *species tree*-Analyse filtrierende Karnivoren (die Gattung *Cryptothrix* miteingeschlossen) + omnivore Zerkleinerer als Schwesterngruppe der schabenden Weidegänger. Die Ergebnisse legen nahe, die monospezifische Gattung *Cryptothrix* einzuziehen und in die Gattung *Drusus* zu inkludieren. Zusätzlich wurden in dieser Studie eine neue Arten der filtrierenden Karnivoren identifiziert, und der Status von zwei weiteren potentiellen neuen Arten diskutiert. Die neue Art ist besonders auffällig, da sie eine möglicherweise basal zu der *dis-*

color-Gruppe *sensu* Pauls et al. (2008) stehende Merkmalskombination aufweist. Allerdings sind die Verwandtschaftsbeziehungen zwischen den Arten nicht vollständig aufgelöst, daher können keine Schlussfolgerungen bezüglich der Evolutionsrichtung gemacht werden.

Eine Revision der gesamten Unterfamilie ist im Moment in Erarbeitung begriffen. Dazu werden Ergebnisse phylogenetischer Rekonstruktion, Verbreitungsdaten monophyletischer Linien und morphologische Merkmale integriert werden, um eine für Drusinae allgemein gültige natürliche Nomenklatur zu erstellen, und damit die Verwandtschaftsbeziehungen zwischen Arten und Artengruppen zu berücksichtigen.

Appendix I — Abstract

Trichoptera are holometabolous insects and sister to Lepidoptera, and together with this order forms Amphiesmenoptera. In Europe, Limnephilidae are the most taxon-rich family of 23 known families, and contains a number of subfamilies. The limnephilid subfamily Drusinae currently comprises eight genera, of which five are monotypic. Further, Drusinae contain a high proportion of regional and and micro-endemic species.

The first systematic treatment of the group comprised 37 of 41 known species in seven genera, and focussed primarily on wing venation and male genitalia characters. Ultimately, this treatment lead to the establishment of six species groups in the genus *Drusus*, and suggested a rather linear diversification within Drusinae culminating in the most derived genus *Drusus*, with all other genera split at a more basal position.

However, a first molecular phylogeny of the group rejected most of the original grouping concepts but rather suggested three distinct evolutionary lineages. Some genera including the genus *Drusus* were recovered as paraphyletic, with the monotypic genera nested within. Feeding ecology of Drusinae larvae and the corresponding morphology were found to corroborate results from molecular phylogeny. Each evolutionary lineage recovered in phylogenetic analysis was found to exclusively comprise one feeding type. Drusinae thus comprise three distinct evolutionary lineages that reflect feeding ecology of larvae: i) filtering carnivores, ii) omnivorous shredders, and iii) scraping grazers. Morphologically, each group was found to be well defined in larval stage. Filtering carnivores differ from the other groups in exhibiting filter bristles and modified head capsules, whereas omnivorous shredders do not develop modified head capsules and lack filter bristles. Scraping grazers are characterized by toothless mandibles—opposed to filtering carnivores and omnivorous shredders that exhibit thoothed mandibles.

The majority of Drusinae is cold-stenotopic, and prefers cold alpine to montane habitats in European mountain ranges. The subfamily is rich in endemics and micro-endemics, particularly in the Balkans. Highly disjunct distribution patterns of the group and some species indicate geographically distinct survival of lineages during several glaciation periods. In combination with the low estimated dispersal capacity of Drusinae, isolation of populations during glacial periods might have contributed to the high number of endemic Drusinae species. Uni- to semivoltine reproductive cycles have been frequently found in Drusinae, with flight and emergence periods ranging from spring to autumn. As discussed above, feeding ecology of Drusinae larvae is diverse, and comprises omnivorous shredders, filtering carnivores and scraping grazers.

Objective of the study was to revise Drusinae based on molecular and morphological data obtained from all available lifestages. Moreover, taxonomic richness of Drusinae was assessed using standard taxonomic and molecular tools. Further, to test adequacy of methods employed a subgroup of Drusinae, the filtering carnivores, were treated in an exemplary study aiminig to resolve the position of the monotypic genus *Cryptothrix*.

The first three studies deal with taxonomic richness in Drusinae. Two new species of *Ecclisopteryx,* a new species of scraping grazing *Drusus* from the Western Balkans, and two new species of filtering carnivorous Drusinae are described. The new species are micro-endemics of the Western Balkans and differ from their congeners in male and female terminalia as well as in larval morphology.

Genetic data suggest a close relationship of the two new *Ecclisopteryx* species and *E. dalecarlica*, *E. guttulata* and *E. madida*. Both species are supported in phylogenetic inference under a Bayesian framework based on a concatenated 3-loci (mtCOI + nuWG + 16SrRNA), 1249 bp dataset. Particularly females of the new species are distinct from the females of other *Ecclisopteryx* species, and are easily recognized. Males are not as conspicuous as females, but can be distinguished by the shape of the inferior appendages. Larvae exhibit a unique combination of characters that enables their identification.

The new species *Drusus crenophylax* belongs to the *bosnicus*-group and is morphologically most similar to *D. vernonensis* and *D. discophorus*. Larvae of *D. crenophylax* differ from the most similar *bosnicus*-group species majorly in the shape of the head, the carinae and the setation of the pronotum. This new species is a micro-endemic of the Western Balkans.

The new species *Drusus krpachi* and *Drusus puskasi* belong to the filtering carnivore clade sensu Pauls et al. (2008) and are morphologically most similar to *D. macedonicus*. They differ from the latter species in the shape of the male genitalia, and are micro-endemics of the Western Balkans.

Filtering carnivorous Drusinae exhibit head capsule modifications of unknown function in the larval stage. To test whether complexity of head capsule modifications follows an evolutionary pattern of increasing complexity within filtering carnivores, relationships between taxa were inferred using 108 terminal taxa. In a species tree analysis based on a 6-loci (2 loci mtCOI + 16SrRNA + nuWG + CAD + 28S) 3805 bp dataset, species with a higher complexity of head capulse modifications were recovered as derived. Additionally, *species tree*-analysis suggests filtering carnivores (including *Cryptothrix*) + omnivorous shredders as sister to scraping grazer Drusinae. These results suggest to abolish the genus *Cryptothrix* and to include the species in the genus *Drusus*. Additionally this study identified a new species of filtering carnivores, and discusses the status of another two potentially new species. One of the new species is particular in exhibiting a seemingly basal character to the *discolor*-group *sensu* Pauls et al. 2008. However, as relationships between species of filtering carnivores were not resolved, no conclusions concerning the direction of evolution can be made.

Currently, a revision of the whole subfamily is in progress. Results of phylogenetic inference, distribution data of lineages and morphological characters will be used to implement nomenclatural changes reflecting relationships between species, species groups, and feeding types.

Appendix I — Curriculum vitae

Curriculum Vitae - Simon Vitecek

Education and Zivildienst:

04/2013-04/2014: visiting PhD student (intermittent, 9 month total) at the BiK-F, Frankfurt am Main, Germany, with Dr. Steffen Pauls, data generation and data analysis for the FWF project "The Drusinae (Insecta: Tichoptera) in a world of global change".

03/2013-04/2013: visiting PhD student at the University of Minnesota, Minneapolis and St. Paul, USA, with Dr. Ralph Holzenthal, special training in scientific entomological illustration.

since 01/03/2012: PhD student (supervised by Dr. Johann Waringer & Dr. Wolfram Graf, tutored by Dr. Steffen Pauls) in the FWF project "The Drusinae (Insecta: Tichoptera) in a world of global change", PI Dr. Johann Waringer.

since 11/2011: enrolled as PhD student at the University of Vienna

02/2011-11/2011: Zivildienst (compulsory paid community service): work as paramedic, assistance in education of paramedics at the Red Cross Vienna

11/2010: Diploma/equiv. MSc in Biology/Zoology at the University of Vienna, Austria. Supervisor: Univ.-Doz. Dr. Sylvia Anton. Thesis title: *Post- mating inhibition of sex pheromone responses in a male moth: in search for a brain and/or sex gland factor.*

10/2009-04/2010: Student researcher (stage M2) for Diploma Thesis at the INRA Versailles-Grignon, Paris, France, with Dr. Sylvia Anton and Dr. Christophe Gadenne

04/2007-11/2010: Main Study of Biology/Zoology at the University of Vienna, Austria (major subjects: evolutionary biology, neurobiology, taxonomy and systematics)

10/2005-04/2007: Basic Study of Biology at the University of Vienna, Austria.

Teaching experience, skills and interests

Teaching experience
Practicum: Biodiversity of Stream Ecosystems, summer terms 2014, 2015. University of Vienna
Practicum: Introduction to biodiversity and ecosystem research, winter term 2014. University of Vienna
Practicum: Digital illustration for Biologists, summer term 2015. University of Vienna
Practicum: Kenntnis Mitteleuropäischer Lebensgemeinschaften, summer term 2015. University of Vienna

Computer skills
Phylogenetics
– standard software (e.g., MrBayes, BEAST, TNT, MEGA, R)
Spatial data
– QGIS, R, GRASS
Statistics
– R (preferred)
– SPSS, Statistica, PRIMER, Canoco, ...
Scientific Illustrations and limited Graphic Design
– Adobe InDesign, Adobe Illustrator, Adobe Photoshop

Languages skills
German (fluent), English (fluent), French (intermediate), Bahasa Indonesia (basic)

Major scientific interests
All aspects of entomology focussing on (aquatic) insects with particular interests in phylogeography, phylogenetics, biodiversity, taxonomy, ecology, physiology, and developmental biology.
Biodiversity research
Tropical Ecology
Food webs

Additional Skills
PADI Advanced Open Water Diver
Drivers Licence B
Mountaineer/Climber

Publications (peer-reviewed, *: as equally contributing first author)

2015

Kučinić M, Previšić A, Graf W, Mihoci I, Šoufek M, Stanić-Koštroman S, Lelo S, Vitecek S, Waringer J. 2015. Larval description of *Drusus bosnicus* Klapálek 1899 (Trichoptera: Limnephilidae), with distributional, molecular and ecological features. Zootaxa 3957(1):085-097.

Vitecek S, Previšić A, Kučinić M, Bálint M, Keresztes L, Waringer J, Pauls S, Malicky H, Graf W. 2015. Description of a new species of *Wormaldia* from Sardinia and a new *Drusus* species from the Western Balkans (Trichoptera, Philopotamidae, Limnephilidae). ZooKeys 496:85-103. doi: 10.3897/zookeys.496.9169

Graf W, Vitecek S, Previšić A, and Malicky H. 2015. New species of Limnephilidae (Insecta: Trichoptera) from Europe: Alps and Pyrenees as harbours of unknown biodiversity. *Zootaxa* 3911(3):381–395.

Waringer J, Graf W, Bálint M, Kučinić M, Pauls SU, Previšić A, Keresztes L, Ibrahimi H, Živić I, and Bjelanović K. 2015. Larval morphology and phylogenetic position of *Drusus balcanicus*, *D. botosaneanui*, *D. serbicus* and *D. tenellus* (Trichoptera: Limnephilidae: Drusinae). *Euopean Journal of Entomology* 112(2):000–000.

2014

Graf W, Konar M, Murányi D, Orci KM, and Vitecek S. 2014. A new species of *Isoperla* (Insecta, Plecoptera) from the Karawanken, with considerations on the Southern Limestone Alps as centers of endemism. *ZooKeys* (448):27-36.

Pogoreutz C, Vitecek S, and Ahnelt H. 2014. First record of *Satyrichthys laticeps* and second record of S*atyrichthys kikin*geri (Teleostei: Peristediidae) from the Maldives Archipelago (Indian Ocean). *Marine Biodiversity Records* 7:e61.

Previšić* A, Graf* W, Vitecek* S, Kučinić M, Balint M, Keresztes L, Pauls SU, and Waringer J. 2014. Cryptic diversity of caddisflies in the Balkans: The curious case of *Ecclisopteryx* species (Trichoptera: Limnephilidae). *Arthropod Systematics & Phylogeny* 72(3):309-329.

2013

Duportets L, Maria A, Vitecek S, Gadenne C, and Debernard S. 2013. Steroid hormone signaling is involved in the age-dependent behavioral response to sex pheromone in the adult male moth Agrotis ipsilon. *General and Comparative Endocrinology* 186:58–66.

Vitecek S, Maria A, Blais C, Duportets L, Gaertner C, Dufour M-C, Siaussat D, Debernard S, and Gadenne C. 2013. Is the rapid post-mating inhibition of pheromone response triggered by ecdysteroids or other factors from the sex accessory glands in the male moth *Agrotis ipsilon*? *Hormones and Behavoir* 63(5):700–708.

Pogoreutz C, Vitecek S, and Ahnelt H. 2013. A new species of *Satyrichthys* (Teleostei: Peristediidae) from the Maldives Archipelago (Indian Ocean). *Zootaxa* 3694(2):153–160.

Waringer J, Graf W, Balint M, Kučinić M, Pauls SU, Previšić A, Keresztes L, and Vitecek S. 2013. The larvae of *Drusus franzressli* Malicky 1974 and *Drusus spelaeus* (Ulmer 1920 (Trichoptera: Limnephilidae: Drusinae) with notes on ecology and zoogeography. *Zootaxa* 3637(1):1-16.

Waringer J, Graf W, Balint M, Kučinić M, Pauls SU, Previšić A, Keresztes L, and Vitecek S. 2013. The larva of *Drusus vinconi* Sipahiler, 1992 (Trichoptera, Limnephilidae, Drusinae). *ZooKeys*(317):69-80.

2012

Deisig N, Kropf J, Vitecek S, Pevergne D, Rouyar A, Sandoz J-C, Lucas P, Gadenne C, Anton S, and Barrozo R. 2012. Differential interactions of sex pheromone and plant odour in the olfactory pathway of a male moth. *PLoS ONE* 7(3):e33159.

Publications (other)

2010

Vitecek S. 2010. Post-mating inhibition of sex pheromone responses in a male moth: search for a brain and/or sex gland factor. *Diploma thesis, University of Vienna*.

Posters/Talks

2015

Vitecek, S; Graf, W; Kučinić, M; Previšić, A; Bálint, M; Keresztes, L; Waringer, J; Pauls, S: The bland, the bald, the beautiful: Evolution of filtering carnivorous Drusinae (Limnephilidae, Trichoptera). Talk, DGaaE congress, Frankfurt am Main, 02.-05.03.2015.

2014

Vitecek, S; Graf, W; Kučinić, M; Previšić, A; Bálint, M; Keresztes, L; Waringer, J; Pauls, S: Revision of the carnivore subgroup within Drusinae (Limnephilidae). Talk, DGL congress, Magdeburg, 29.09.-02.10.2014.

Vitecek, S; Graf, W; Previšić, A; Kučinić, M; Keresztes, L; Bálint, M; Pauls, S; Waringer, J: A preliminary revision of the carnivore subgroup within Drusinae (Limnephilidae). Poster, SIL-Austria congress "Alpine Limnology", WasserCluster Lunz, 12.-14.02.2014.

Vitecek, S; Graf, W; Kučinić, M; Previšić, A; Keresztes, L; Bálint, M; Waringer, J; Pauls, S: Molecular methods and comparative morphology - an unholy union serving the greater good: a case of cryptic species and carnivorous Drusinae (Trichoptera:Limnephilidae). Talk, ScienceDay 2014, University of Vienna, 01.07.2014.

Awards/Grants/Stipends

02/2010: Leistungsstipendium der Universität Wien (Stipend for Performance Scholarship), Rank: 21.

01/2010: Kurzfristiges Auslandstipendium/KWA (Short-term grant abroad), awarded for the stay at the INRA Versailles.

Appendix II — further publications

In the course of this thesis I contributed to some publications that are not part of my PhD-thesis and shall not be treated as such – those publications do not formally constitute a part of my thesis. They are merely provided at request of my supervisors to document my scientific activities, and those of the people I have had to honor to work in.

The topics range from neurobiology to taxonomy of caddisfly larvae and Plecoptera, including the description of a new species; the papers are chronologically ordered.

Note: *Satyrichthys kikingeri* is considered a junior synonym of *S. laticeps* by T. Kawai (2014)*. However, the features of the latter species seem to be rather variable, and the range of the species is quite large. Also, diagnostic features are not easily assessed. Molecular methods could potentially clarify this situation.

*: [Kawai, T. 2014: Satyrichthys kikingeri Pogoreutz, Vitecek & Ahnelt, 2013, a junior synonym of Satyrichthys laticeps (Schlegel, 1852) (Actinopterygii: Teleostei: Peristediidae). Zootaxa 3900 (1): 135-140. doi: 10.11646/zootaxa.3900.1.9.]

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PLoS one

Differential Interactions of Sex Pheromone and Plant Odour in the Olfactory Pathway of a Male Moth

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Abstract

Most animals rely on olfaction to find sexual partners, food or a habitat. The olfactory system faces the challenge of extracting meaningful information from a noisy odorous environment. In most moth species, males respond to sex pheromone emitted by females in an environment with abundant plant volatiles. Plant odours could either facilitate the localization of females (females calling on host plants), mask the female pheromone or they could be neutral without any effect on the pheromone. Here we studied how mixtures of a behaviourally-attractive floral odour, heptanal, and the sex pheromone are encoded at different levels of the olfactory pathway in males of the noctuid moth *Agrotis ipsilon*. In addition, we asked how interactions between the two odorants change as a function of the males' mating status. We investigated mixture detection in both the pheromone-specific and in the general odorant pathway. We used a) recordings from individual sensilla to study responses of olfactory receptor neurons, b) *in vivo* calcium imaging with a bath-applied dye to characterize the global input response in the primary olfactory centre, the antennal lobe and c) intracellular recordings of antennal lobe output neurons, projection neurons, in virgin and newly-mated males. Our results show that heptanal reduces pheromone sensitivity at the peripheral and central olfactory level independently of the mating status. Contrarily, heptanal-responding olfactory receptor neurons are not influenced by pheromone in a mixture, although some post-mating modulation occurs at the input of the sexually isomorphic ordinary glomeruli, where general odours are processed within the antennal lobe. The results are discussed in the context of mate localization.

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Introduction

Most animals rely on olfactory cues to find their mating partner, food and shelter. For reproduction, the olfactory system faces the challenge of extracting salient odorant information emitted by sexual partners (pheromones) from an abundant background of general odorants. In the moth's natural environment, males are attracted by a female-emitted sex pheromone blend (containing several components), and could either ignore or use background general odours as additional cues to locate a potential mate. Indeed, in several moth species, the behavioural response of males to sex pheromones is enhanced by host plant odours [1]. This seems to reflect a strategy to optimize mating, since females often call when situated on a host plant. The simultaneous presence of a pheromone and a plant odour may result in interactions between these odour classes, which can either lead to suppression (masking) or enhancing (synergy) of the response to one odour by the other.

Detection of sex pheromones and general odours in animals is usually accomplished by two distinct olfactory pathways. In mammals, pheromone information is mainly processed by the accessory olfactory system, while the main olfactory system codes more general odours, *e.g.* food or shelter related odours [2]. In insects, such as moths, pheromone information is transmitted by specialized olfactory receptor neurons (ORNs) to the macroglomerular complex (MGC), a male-specific part of the primary olfactory processing centre, the antennal lobe (AL). Plant odour information is transferred by general ORNs to sexually isomorphic ordinary glomeruli (OG) [3]. Whereas in mammals both sub-systems seem to participate in mate recognition [2], very little is known about how both sub-systems contribute to pheromone and plant odour recognition in moths.

Pheromone-plant odour interactions may occur at different processing levels in the olfactory system. Olfactory mixture perception has already been studied at the peripheral level (vertebrates: *e.g.* [4,5]; invertebrates: *e.g.* [6–11]) and at the central level (vertebrates: *e.g.* [12,13]; invertebrates: *e.g.* [14–18]). However, most of these studies investigated coding of mixtures composed of odorants from the same contextual origin (*i.e.* mixtures of either

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general odorants or single pheromone components). Very few studies have focussed on the coding of mixtures of pheromones (reproduction cues) and general odours (*i.e.* food, predator, social, host cues) in the central nervous system (e.g. vertebrates [19,20], and invertebrates [21,22]). As for vertebrates, the coding of these two types of odour cues was generally believed to occur in two separate pathways of the insect olfactory system. However, unusual representations of plant odours and pheromones were recently observed in tortricid moths: in *Grapholita molesta*, pheromone processing seems to occur in OG rather than in the MGC [23] and in *Cydia pomonella* there is no clear segregation between the pheromone and the general odour sub-systems in the AL, both odour classes being represented in both the MGC and in OG [24].

In males of the noctuid moth *Agrotis ipsilon*, a transient post-mating inhibition of behavioural and central nervous responses to sex pheromone has been observed [21,25]. This plasticity prevents newly-mated males from orientating towards females and mating until the next night, allowing them to refill their sex glands for a potential new ejaculate. After mating, a strong decrease in sex pheromone sensitivity is observed up to the MGC [26]. Plant-odour processing, on the other hand, is much less affected by mating status. Behavioural responses to plant odours, such as a linden flower extract, observed in wind tunnel experiments remain stable after mating. Further, response thresholds of peripheral and central OG neurons to heptanal, a behaviourally attractive component of linden flower [27], are not modified [21,26]. However, an increase in calcium response intensity and in the firing response of OG neurons to heptanal is observed, originating probably from pre-synaptic modulation at ORN axon terminals [26]. Thus, pheromone and plant odour processing seem to be modulated differentially depending on the mating status. This plasticity allows mated males to transiently block their central pheromone responses after mating and to increase non-pheromonal odour detection, probably allowing more efficient localization of food sources in a natural environment [26].

Interestingly, the addition of plant odour (linden flower extract) enhanced the response of virgin males to sex pheromone, which in turn inhibited the response of mated males to plant odour both at the behavioural and central nervous level (within OG) [21].

Here we used a multi-level approach to investigate pheromone-plant odour interactions in the olfactory pathway of virgin and mated *A. ipsilon* males. In a first step, we tested the behavioural response of virgin males to heptanal using wind tunnel experiments to confirm previous reports of its attractiveness in the field also in the laboratory [27]. We analysed responses to a sex pheromone blend/plant odour (heptanal) mixture a) in pheromone-specific and heptanal-responding ORNs; b) globally in the AL by recording the calcium signal elicited within the two sub-systems of the AL, the MGC and the OG; and c) in projection neurons (PNs), branching in the MGC and leaving the AL towards higher order brain centres. We compared these data with previously described odour interactions within OG glomeruli [21]. The plant odour heptanal strongly suppressed the response of pheromone-specific ORNs (Phe-ORNs) to the sex pheromone blend. This effect was confirmed for all the different levels of the pheromone-specific olfactory pathway we investigated, independently of the mating status. Conversely, there was no modulation of heptanal-sensitive receptor neurons (Hep-ORNs) by the pheromone when presented together in a mixture with heptanal. In addition, mated males showed higher response intensities to heptanal and mixtures in calcium imaging recordings from OG than virgin males.

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Results

Behavioural responses of virgin males to heptanal

To confirm the behavioural attractiveness of heptanal, a volatile emitted by linden flowers, we performed wind tunnel experiments using virgin sexually mature *A. ipsilon* males. Best responses (32% males responding with an oriented flight) were obtained with 100 μg heptanal (Figure 1).

Pheromone-ORN responses are reduced by heptanal

Phe-ORNs housed in long trichoid sensilla on the antennae showed a typical excitatory response to the sex pheromone blend, but no response to heptanal or to the solvent. Spiking activity to a mixture of pheromone and heptanal was strongly reduced compared to the response evoked by the pheromone alone (Figure 2A).

We first analysed the effect of the mating status on the response of Phe-ORNs to pheromone, heptanal and their mixture at two doses (100 and 1000 μg) (Figure 2B). Phe-ORNs of virgin and mated insects displayed no significant differences in the spike response frequencies to the stimuli tested (3-way RM ANOVA, mating factor: $F_{1,29} = 0.25$, $p = 0.62$) (Figure 2B), but significant differences were found with respect to the other two factors: odour and doses tested (3-way RM ANOVA, odour factor: $F_{1,29} = 100.3$, $p = 0.00001$; dose factor: $F_{1.6,45.9} = 21.9$, $p = 0.00001$). As the mating status of males did not interplay in the differences observed, we further analysed only data from virgin males. Similar statistical differences as before were detected by performing a 2-way RM ANOVA with odours and doses as the two main factors (RM factors) (2-way RM ANOVA, odour factor: $F_{1,13} = 48.5$, $p = 0.00001$; dose factor: $F_{1.8,23.3} = 9.5$, $p = 0.001$) (Figure 2B). Addition of heptanal to the pheromone significantly reduced the firing rate of Phe-ORNs at all tested doses (simple effects, 1-way RM ANOVA $F_{1.6,20.5} = 14.9$, $p = 0.00001$, Tukey test, $p<0.005$ in all cases, i.e. 1–1000 μg of mix *vs.* phe). Heptanal doses between 1 and 100 μg (Figure 2B, green) evoked no significant responses with respect to the solvent (simple effects, 1-way RM ANOVA: $F_{1.4,18.9} = 4$, $p = 0.04$, Tukey test: 1–100 μg of hept vs. sol, $p>0.9$ in all comparisons). However, a high dose of 1000 μg of heptanal elicited a significant response in Phe-ORNs as compared to the solvent (Tukey test: 1000 μg of hep *vs.* sol, $p = 0.006$). Thus,

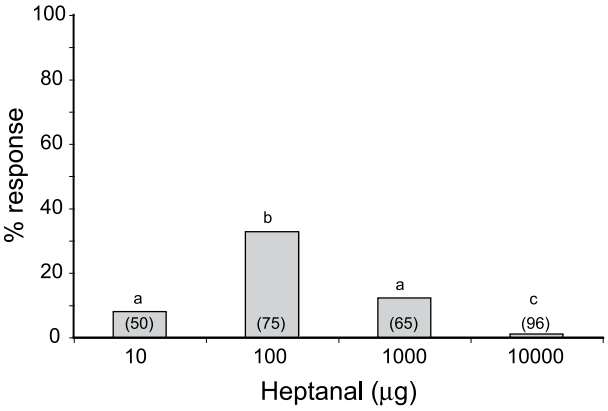

Figure 1. Behavioural responses of virgin *A. ipsilon* males to heptanal. The proportion of males showing an oriented flight towards the stimulus source was highest at a dose of 100 μg heptanal. Numbers in brackets represent the numbers of tested males. Bars with same letters are not statistically different (chi-square-test, $p<0.05$).
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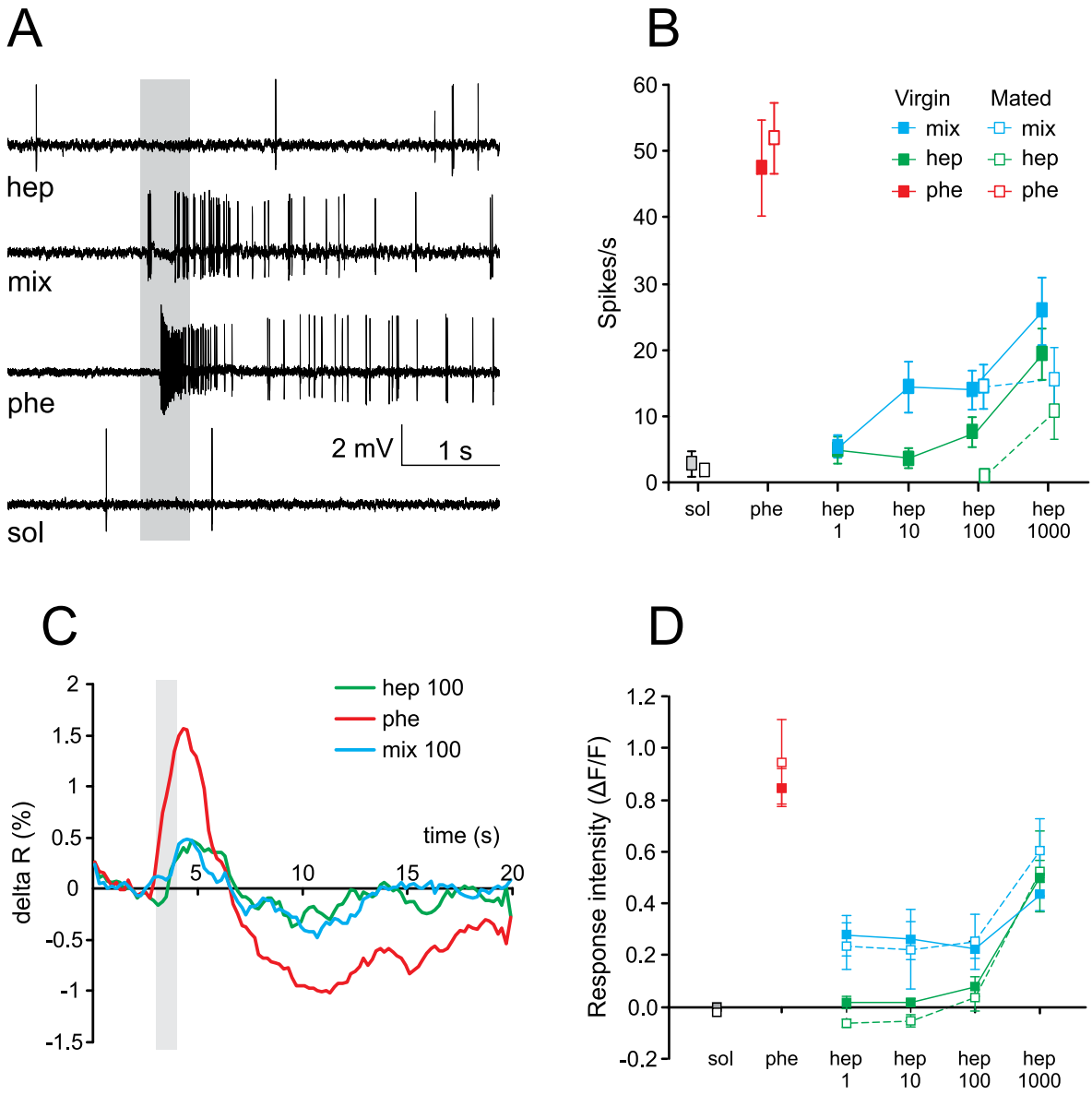

Figure 2. Pheromone-responding ORNs and MGC calcium responses in virgin and mated males. A) Typical single sensillum recordings showing an olfactory receptor neuron (ORN) excitatory response to the pheromone (10 ng), no response to heptanal (100 µg) and a reduced response to the pheromone/heptanal mixture in a virgin male. Solvent = hexane. The grey bar indicates the duration of the stimulus (0.5 s). **B**) Mean spike frequency of Phe-ORNs to the sex pheromone (10 ng), heptanal at different doses, and their mixture in virgin (n = 14) and mated (n = 22) males. Solvent (sol) refers to pooled data of stimulations with hexane and mineral oil. Phe-ORNs show a decreased firing frequency to the pheromone by the addition of heptanal at all doses tested. Phe-ORNs do not respond to heptanal as single odour except for the highest dose tested (1000 µg). No differences were found between virgin and mated males. **C**) Time course of odour-evoked calcium activity in the MGC of one mated male. The grey bar indicates the duration of the stimulus (1 s). **D**) Mean calcium responses in the MGC of virgin (n = 9) and mated (n = 8) males to sex pheromone (10 ng), different doses of heptanal and their mixtures. Stimulation with heptanal in the mixture strongly reduced the response intensity to pheromone at any dose of heptanal tested. Heptanal only induced calcium response for the highest dose tested (1000 µg). No differences were found between virgin and mated males. Hep: heptanal; mix: pheromone/heptanal mixture; phe: pheromone; sol: solvent.
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pheromone responses of Phe-ORNs were reduced by addition of heptanal at doses, irrespectively if they elicited or not responses on their own (which was the case only for the highest dose).

Pheromone-evoked calcium responses in the MGC are reduced by heptanal

Odour–evoked calcium responses were typical biphasic signals with a fast fluorescence increase followed by a slow decrease before returning to baseline (Figure 2C). The maximum intensity of the response to odour stimuli appeared around 1 s after stimulation onset, whereas the minimum was found around 5 s after odour onset. Controls (hexane, mineral oil, clean air) did not activate the AL (Figure 3B).

The sex pheromone induced high calcium responses in the MGC, and no responses to heptanal alone were observed, apart for the highest heptanal dose (Figure 2D, 3A, B). MGC calcium

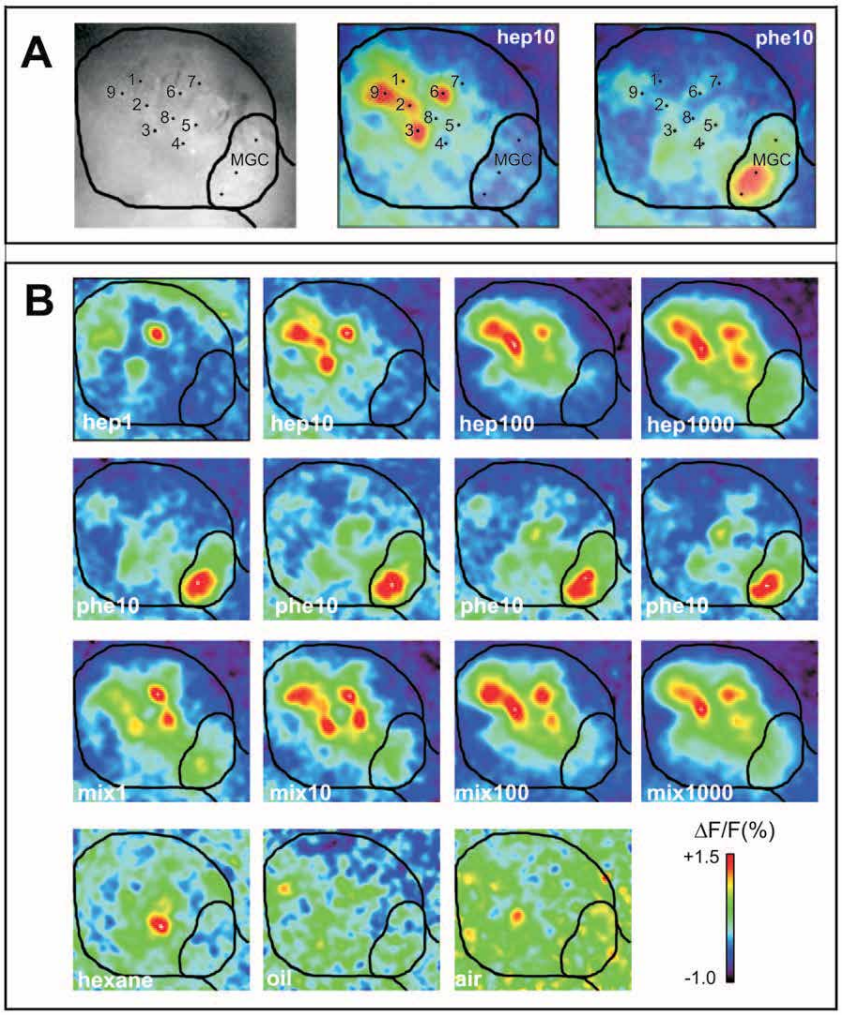

Figure 3. Odour-evoked calcium signals in the antennal lobe. A) Example of an anatomical staining of a right antennal lobe (AL) with the outline of the entire AL and MGC. Two activity maps obtained in response to heptanal (10 µg) (hep10) and to the pheromone blend (10 ng) (phe10) are shown with the outline of the AL. Numbers next to dots indicate the position of the nine analysed ordinary glomeruli (1–9), as well as three analysed locations within the MGC, for which activity was pooled. **B**) Activity signals obtained in a mated male stimulated with four doses of heptanal (hep) (1–1000 µg), four presentations of pheromone (phe) at 10 ng, and the respective pheromone/heptanal mixtures (mix). All maps are scaled to the same minimum/maximum.
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responses did not vary with mating status (3-way RM ANOVA, mating factor: $F_{1,15} = 0.0026$, $p = 0.96$), but did with odour (3-way RM ANOVA, odour factor: $F_{1,15} = 43.4$, $p = 0.000009$) and with the heptanal dose both as single odour or in the mixture (3-way RM ANOVA, dose factor: $F_{2.3,34.6} = 28.3$, $p = 0.0000001$) (Figure 2D). As for electrophysiological responses of Phe-ORNs, the addition of heptanal to the pheromone, at any of the heptanal doses tested, strongly reduced MGC response intensity (simple effects, 1-way RM ANOVA: $F_{2.7,44.4} = 29.4$, $p = 0.000001$, Tukey test for phe vs. 1–1000 µg of mix, $p<0.0001$ in all cases) (Figure 2D). Similarly to single sensillum recordings, we only observed a calcium response to heptanal in the MGC for the highest dose (1000 µg) (simple effects, 1-way RM ANOVA: $F_{1.4,23.2} = 35.5$, $p = 0.000001$, Tukey test, 1–100 µg hep *vs.* sol, $p>0.7$; 1000 µg *vs.* sol or any other hep dose, $p<0.0001$ in all cases) (Figure 2D). Concluding, as in Phe-ORN recordings, pheromone-induced responses in the MGC were reduced by addition of heptanal at doses, which did not elicit responses on their own, with the exception of the highest dose.

Heptanal-ORN responses are not affected by pheromone

Extracellular recordings from individual Hep-ORNs housed in short sensilla trichodea on the antennae revealed excitatory responses to heptanal and to the pheromone/heptanal mixture (Figure 4A). No responses were detected when the pheromone blend alone or the solvent were presented (Figure 4A). The spiking rate of Hep-ORNs was not significantly different between virgin and mated males (3-way RM ANOVA, mating factor: $F_{1,24} = 0.63$, $p = 0.43$). Moreover, no difference in Hep-ORN firing rate was observed between responses to heptanal and to the pheromone/heptanal mixture (3-way RM ANOVA, odour factor: $F_{1,24} = 0.18$, $p = 0.67$) (Figure 4A, B). However, the Hep-ORN spike frequency significantly increased with the dose of heptanal in both groups (3-way RM ANOVA, dose factor: $F_{1.2,29.9} = 14.2$, $p = 0.00032$) (Figure 4B). Thus, Hep-ORNs responded in a dose dependent

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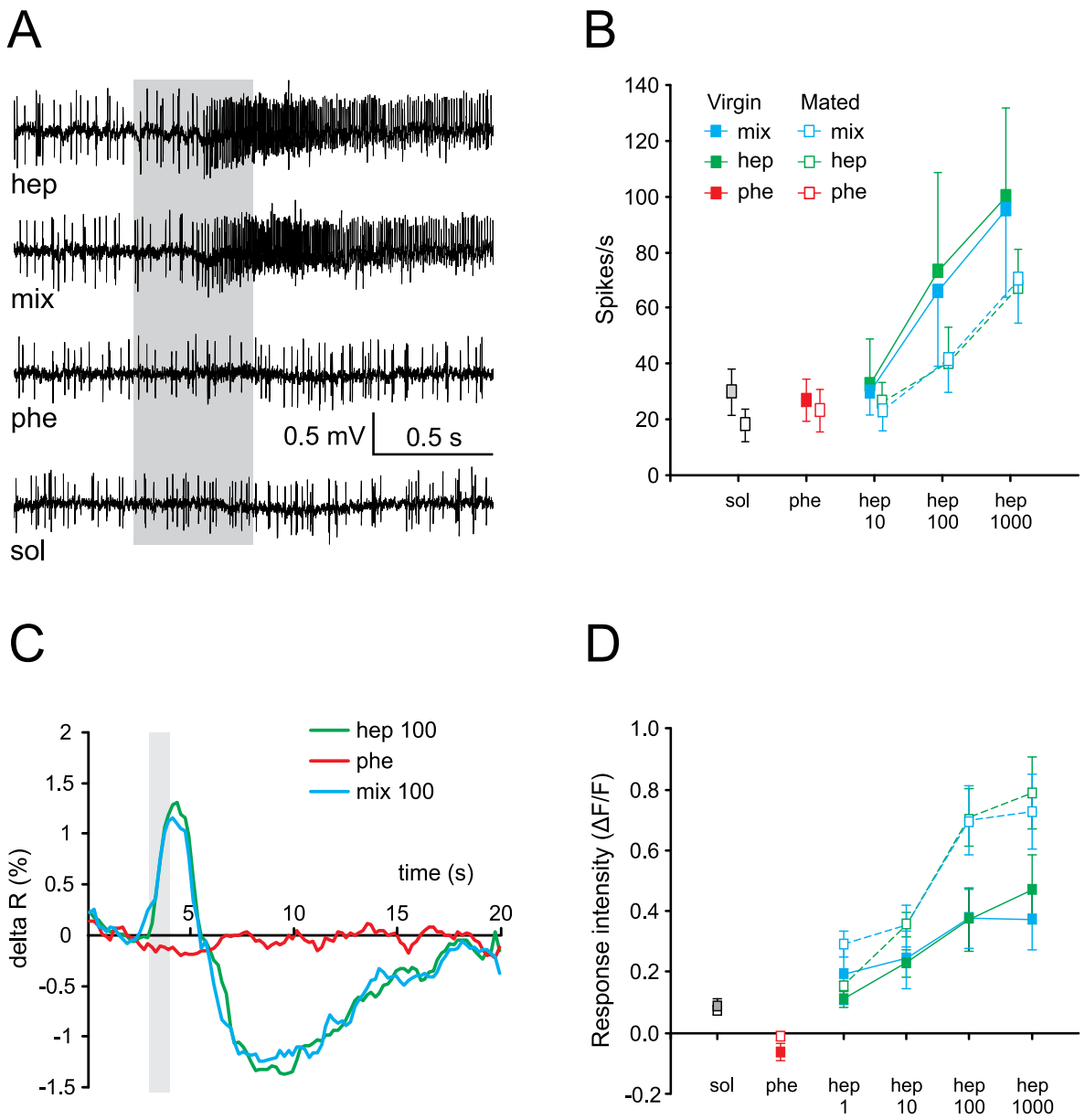

Figure 4. Heptanal-sensitive ORNs and OG calcium-evoked responses in virgin and mated males. A) Typical recording showing an excitatory response to heptanal (100 µg), no response to the pheromone (10 ng) and the solvent (mineral oil), and excitation to the pheromone/heptanal mixture in a virgin male. The grey bar indicates the duration of the stimulus (0.5 s). **B**) Mean spike frequency of Hep-ORNs to pheromone (10 ng), heptanal at different doses, and their mixture in virgin (n = 13) and mated (n = 13) males. Hep-ORNs show dose-dependent response to heptanal, but no response to the pheromone and solvent. The addition of pheromone in the mixture does not modify the response of Hep-ORNs to heptanal at any dose tested. No differences were detected between virgin and mated males. **C**) Time course of odour-evoked calcium activity in the OG. The grey bar indicates the duration of the stimulus (1 s). **D**) Mean calcium responses in the OG to pheromone (10 ng), heptanal at different doses, and their mixture in virgin (n = 9) and mated (n = 8) males. Stimulation with pheromone induced no response. Heptanal-induced responses increased with the dose and were significantly higher in mated than in virgin males, although it was not different from mixture responses. Hep: heptanal; mix: pheromone/heptanal mixture; phe: pheromone; sol: solvent.
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manner to heptanal, and their responses were neither affected by the addition of pheromone, nor by a change in mating status.

Heptanal-evoked calcium responses in OG are not affected by sex pheromone

OG calcium responses to heptanal or to the pheromone/heptanal mixture were biphasic signals and their time course was comparable to responses observed in the MGC (Figure 2C, 4C). In the OG, no calcium responses to sex pheromone blend stimulation were detected (Figure 3A, B and 4C).

Across all tested stimuli and doses, response intensities were significantly different between virgin and mated males (3-way RM ANOVA, mating factor, $F_{1,15} = 4.6$, $p = 0.048$) and dose-dependent in both groups (3-way RM ANOVA, dose factor,

$F_{1.9,29.2} = 39.7$, $p = 0.0000001$) (Figure 4D). Mated males showed higher responses particularly for doses of 100 and 1000 µg of heptanal alone and for the corresponding pheromone/heptanal mixtures than virgin males (simple effects, 1-way ANOVA: $F_{1,32} = 10.8$, $p = 0.002$ for 100 µg and $F_{1,32} = 9.2$, $p = 0.004$ for 1000 µg) (Figure 4D). The analysis revealed no significant differences in OG response intensity between heptanal and the pheromone/heptanal mixture in both groups (3-way RM ANOVA, odour factor: $F_{1,15} = 0.49$, $p = 0.49$) (Figure 4D). Thus dose-dependent calcium responses to heptanal in OG were not modified by the addition of sex pheromone and were higher in mated than in virgin males.

Pheromone responses in MGC PN neurons are reduced by heptanal

We describe here the analysis of the most common type of response pattern (97% of the recorded neurons) to the sex pheromone blend in MGC PN neurons, consisting of an excitatory followed by an inhibitory phase [28]. MGC PN response thresholds are lower in virgin than in mated males (*e.g.* [21]). We thus stimulated virgin males with a lower dose of pheromone (1 ng instead of 10 ng used in all other experiments) to avoid too strong responses. MGC PN responses were significantly different between the odours tested, *i.e.* solvent, heptanal, pheromone and pheromone/heptanal mixture (1-way RM ANOVA: $F_{3,45} = 49.9$, $p = 0.00001$ for virgin males and $F_{3,33} = 24.8$, $p = 0.00001$ for mated males) (Figure 5B). MGC PNs did not respond to heptanal (Figure 5A, B) as spike frequency did not differ between heptanal and solvent presentation, independently of mating state (Tukey test, hep vs sol: $p = 0.99$ for virgin males and $p = 0.8$ for mated males). In virgin males, addition of heptanal to 1 ng of the sex pheromone blend caused a reduction of the response (Tukey test, phe *vs.* mix, $p = 0.001$) (Figure 5B). The same type of effect was found in mated males when heptanal was added to 10 ng of the pheromone (Tukey test, phe vs. mix, $p = 0.04$) (Figure 5B). Thus, at the level of MGC PNs, addition of heptanal reduces the response to the pheromone. This effect was observed independently of the mating status. The effect of mating on the sensitivity of PNs could, however, not be directly compared statistically here, as different doses of pheromone were used for virgin and mated males.

Discussion

Using complementary methodological approaches, our study examines the coding of a sex pheromone/heptanal mixture at different levels of the olfactory pathway in an insect. In addition, we evaluated the effect of mating status on the processing of such a mixture in mature *A. ipsilon* males. We show that mixtures composed of the pheromone and the plant odour heptanal are differentially detected and processed by the olfactory pathway: heptanal strongly suppresses pheromone detection, but conversely the pheromone does not affect heptanal detection. The pheromone response suppression caused by heptanal starts at the periphery (the antennae) and persists throughout the output of the pheromone-specific part of the AL, the MGC. Heptanal detection, on the other hand, does not seem to be modulated by the pheromone, at least up to the input to the OG. Mating status did not affect mixture processing in the pheromone-specific antennal and AL pathway (Phe-ORN, input and output of the MGC), but induced a plasticity of responses to heptanal and to the pheromone/heptanal mixture at the input to the OG. Further, we confirmed that heptanal, which was used as a plant odour throughout this study, is behaviourally attractive to *A. ipsilon* males

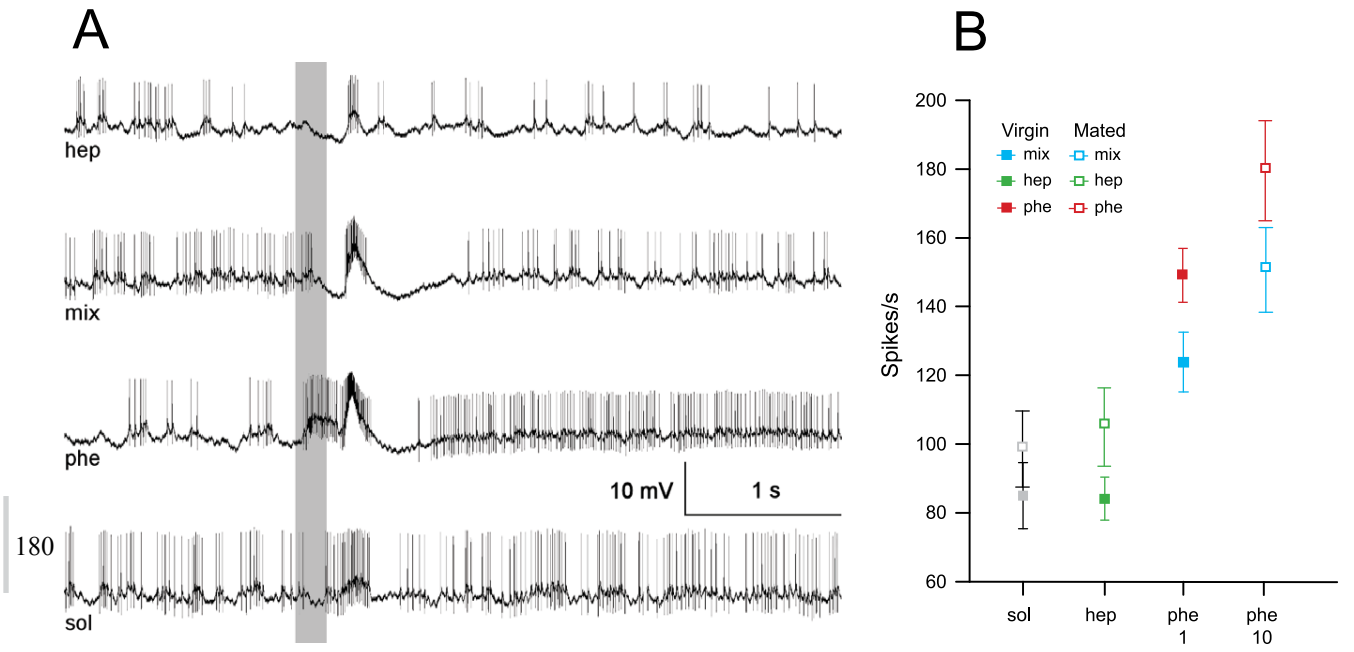

Figure 5. Responses of AL PNs within the MGC of virgin and mated males. A) Typical responses of a pheromone-sensitive PN in a virgin male, showing an excitatory response to pheromone (1 ng), no response to heptanal (100 µg) and the solvent (hexane), and a reduced firing rate during excitation to the pheromone/heptanal mixture. The grey bar indicates the duration of the stimulus (0.2 s). **B**) Spike frequency of PNs during the excitatory period to the pheromone (1 ng in virgin and 10 ng in mated males), heptanal (100 µg) and the pheromone/heptanal mixture in virgin (n = 17 neurons) and mated (n = 15 neurons) males. Spike frequencies of PNs do not differ between heptanal and solvent. Hep: heptanal; mix: pheromone/heptanal mixture; phe: pheromone; sol: solvent.
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in the wind tunnel, the level of response being similar to that obtained after stimulation with a linden flower extract [21].

Pheromone-plant odour interactions at the peripheral level

Our results show that addition of heptanal strongly inhibits the responses of Phe-ORNs to the sex pheromone. Neither inhibitory nor excitatory responses (unless very high doses were used) were obtained in these neurons upon heptanal stimulation alone. Different forms of interactions have been found at the peripheral level after stimulation with mixtures of plant odours and pheromone. In *Helicoverpa zea*, linalool and a green leaf volatile were found to increase the response of Phe-ORNs when presented simultaneously with the main pheromone component [11]. On the contrary, a decrease of Phe-ORN response induced by the addition of plant odours has been found in various moth species such as *Antheraea pernyi* [29], *Adoxophyes orana* [30], *Bombyx mori* [6], and *Spodoptera littoralis* [31]. The suppressive effect of plant odours on Phe-ORN responses, as observed in our and the above cited studies, might originate from non-competitive inhibition of pheromone and plant volatile compounds for olfactory receptors or other actors involved in signal reception, or could be due to an inhibition of the transduction pathway as proposed for *B. mori* [32]. Our results show that the lowest dose of heptanal used (1 µg) was enough to reduce the firing rate of Phe-ORNs to the pheromone. Further experiments are necessary to determine the threshold dose of heptanal needed to produce this suppression effect. Even 1000 µg of heptanal in the mixture evoked a significant reduction of the pheromone response in spite of the fact that when presented alone, it elicited significant firing rates in Phe-ORNs. In contrast to the suppressive effect of heptanal on pheromone responses in Phe-ORNs, Hep-ORNs not only did not respond to the pheromone alone but their responses were not modified by the presence of sex pheromone in the mixture. This suggests the existence of different peripheral interactions between plant odour and pheromones depending on the ORN type.

Pheromone-plant odour interactions at the AL level

Our calcium imaging experiments revealed a compound signal response consisting mainly of ORN responses [33,34]. Calcium responses measured at the level of the MGC were consistent with those obtained from single sensillum recordings of Phe-ORNs (Figure 6). Thus, the suppressive interactions observed within the MGC largely originate from interactions at the peripheral level. In addition, as shown by our PN recordings, this effect does not seem to be modified within the AL, *i.e.* input = output (Figure 6). Interestingly, the type of interactions we found between sex pheromone and heptanal in *A. ipsilon* is different from that found in the silkmoth *B. mori*, in which sex pheromone responses in PNs of the MGC are enhanced by the host plant odour cis-3-hexen-1-ol [22]. There might thus either be different effects of different plant volatiles on sex pheromone detection, or different interaction effects in different moth species, depending on the natural context.

In contrast to the pheromonal pathway, we found no mixture interactions in the heptanal pathway, at least at its input level. Indeed, responses to heptanal were not affected by the addition of pheromone, neither in Hep-ORN recordings nor in odour-evoked calcium responses of OG. However it should be noted that in

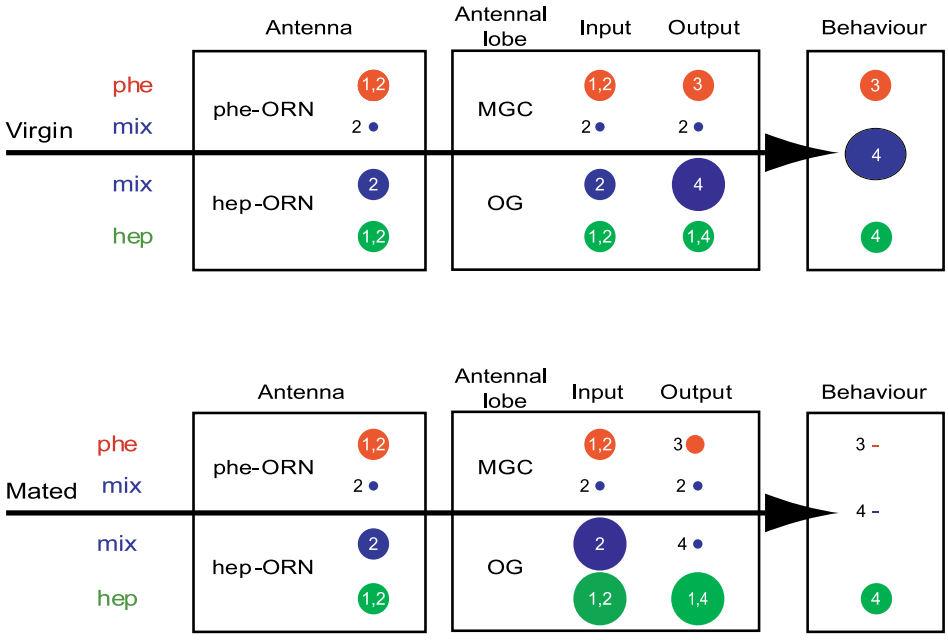

Figure 6. Sex pheromone-plant odour interactions in the olfactory pathway of virgin and mated *A. ipsilon* males. Whereas pheromone sensitivity decreases drastically in AL output neurons after mating, heptanal sensitivity seems to increase already at the AL input level. Synergistic behavioural responses to odour mixtures in virgin males are correlated with enhanced antennal lobe responses. Likewise inhibitory behavioural responses to mixtures of pheromone and plant odour in mated males match inhibitory interactions within ordinary glomeruli of the antennal lobe. Pheromone reception and antennal lobe processing, on the other hand are inhibited by heptanal, independently of mating state. This might serve to improve temporal resolution of discontinuous stimuli, which are common in a natural environment. AL: antennal lobe; hep: heptanal; MGC: macroglomerular complex; mix: heptanal/pheromone mixture; OG: ordinary glomeruli; ORN: olfactory receptor neuron; phe: pheromone. Size of disks indicates response strength. Dash means no response. Numbers refer to previously published data: (1) Barrozo et al., 2011 [26] (2) This paper. (3) Gadenne et al., 2001 [25]. (4) Barrozo et al., 2010 [21].
doi:10.1371/journal.pone.0033159.g006

virgin *A. ipsilon*, a synergistic effect of mixtures of sex pheromone and heptanal has been demonstrated in OG output neurons, in correlation with a synergistic behavioural effect (Figure 6) [21]. Contrary to interactions in the pheromonal pathway, which appear at the periphery, interactions in the heptanal pathway would be the product of AL processing. Although the neural mechanisms leading to mixture interactions within the AL still need to be unveiled, they may mainly originate from lateral interactions through inhibitory or excitatory local interneurons [35,36].

Odour interactions as a function of mating state

We did not find any differences in mixture interactions at the input or output of the AL pheromone pathway and in Hep-ORNs between virgin and mated males. However, OG calcium responses (this paper) and heptanal-responding OG PNs [21] were modulated by mating status. OG PNs of virgin males showed enhanced responses to the mixture compared to heptanal alone, while mated males exhibited reduced responses to the mixture compared to heptanal alone (Figure 6) [21]. These mixture interactions observed in OG PNs were correlated with the behavioural synergism or inhibition to mixtures observed in virgin and newly-mated males, respectively (Figure 6) [21]. It is important to state here that the reduction of pheromone detection by plant odours has been described in several moth species [6,29,30,32]. In recent studies on the peripheral and central olfactory system, this inhibition has been shown to improve pulse resolution of pheromone stimuli [31,37,38] an important feature to allow orientation towards a naturally intermittent pheromone signal [39].

Although reciprocal modulation of heptanal and sex pheromone processing is clear in our model insect, our results show that interaction mechanisms occur at different levels in moths. Whereas the modulation of pheromone responses by heptanal is essentially happening in the periphery, probably due to competition for the olfactory receptors, considerable mating-dependent plasticity and signal processing of mixtures occurs within the OG at the AL level (Figure 6).

Conclusions

The ecological importance of the co-occurrence of different classes of odours involved in different behavioural contexts is evident in the natural environment of an insect. The present study is a first step to better understand how a male moth processes crucial information cues for reproduction (sex pheromone) in a complex odorous environment (plant odours), and how the reproductive state might modulate its response. Our data, showing reciprocal modulation of the two types of stimuli, give some indications about how a male moth can process cues originating simultaneously from a mating partner and a plant odour background, ultimately leading to an appropriate behavioural response.

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Materials and Methods

Insects

Adult males and females of the noctuid moth, *A. ipsilon* Hufnagel, were reared in the laboratory, and behavioural and physiological experiments were performed as described previously [21,25]. Briefly, 5-day old sexually mature virgin and mated males were used for experiments during the 8-hour scotophase. Newly-mated males were obtained by pairing virgin 5-day-old males and 3-day-old sexually mature females before the onset of the scotophase. Newly–mated males were prepared for calcium imaging or electrophysiological recordings within one to two hours after the end of copulation, and females were dissected to confirm the presence of the male spermatophore.

Odour Stimulation

For electrophysiological experiments, odour stimulations with sex pheromone blend and heptanal were performed as described previously [21,40]. Briefly, the behaviourally active pheromone blend consisting of (*Z*)7-dodecen-1-yl acetate, (*Z*)9-tetradecen-1-yl acetate and (*Z*) 11-hexadecen-1-yl acetate at a ratio 4:1:4 [41], and the behaviourally attractive plant odour, heptanal [27], were used in all experiments. Ten ng of the pheromone blend diluted in hexane were used in ORN recordings and imaging experiments because they elicit a clear response [25]. For AL intracellular recordings 1 ng and 10 ng doses of the pheromone were used for virgin and mated males respectively, because of a higher sensitivity of central neurons in virgin males [21]. Four doses of heptanal (1, 10, 100 and 1000 µg each diluted in 10 µl of mineral oil, resulting in concentrations of 1/10.000 to 1/10 volume/volume) were used for electrophysiological recordings and imaging experiments. All compounds were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France) and 10 µl of stimulus solution were applied on a piece of filter paper (0.5×2 cm, Fisherbrand, Fisher Bioblock, Illkirch, France) introduced in a Pasteur pipette. The solvents hexane and mineral oil applied on a filter paper were used as control stimuli. When stimulating with mixtures, two filter papers were inserted into a glass pipette; then the pheromone (diluted in hexane) and heptanal (diluted in mineral oil) were added separately on the filter papers. This procedure avoided interactions between the two odour solutions, but allowed simultaneous application of the two stimuli in the same air puff. To exclude potential absorption of the pheromone in mineral oil or changes in the airflow due to a second filter paper in the pipette, we carried out control experiments under exactly the same experimental conditions as in the main experiments, in which we stimulated Phe-ORNs with: 1- a single filter paper with pheromone (phe), 2- one filter paper with pheromone and a second clean filter paper (phe+cfp), 3- one filter paper with pheromone and a second filter paper with mineral oil (phe+oil), 4- a single filter paper with mineral oil (oil). Phe-ORN spike response frequencies did not change significantly when a second clean filter paper or a filter paper with mineral oil was inserted in the pipette with respect to the pheromone alone (Tukey test $p>0.05$), but as expected, responses were significantly lower when only mineral oil was used (Tukey test, oil vs. phe, phe+cfp, phe+oil, $p<0.0001$ in all cases) (1-way ANOVA for RM $F_{3,24} = 60.3$, $p = 0.00001$) (Fig. S1). Pipettes were left under a fumehood for 30 min to allow evaporation of hexane before use. Antennal stimulation was done with a stimulus controller (CS55, Syntech, Kirchzarten, Germany) as described before [40]. Stimulation lasted for 0.5 s for ORN recordings, 1 s for calcium imaging recordings and 0.2 s for PN recordings.

Single sensillum recordings of ORNs

Preparation of animals and recordings were performed as described earlier [26,40]. Briefly, recordings from pheromone sensilla were carried out according to the tip recording technique [42]. Recordings from plant odour sensilla were carried out with electrolytically sharpened tungsten wires. Sensilla were selected randomly along the stem in the middle part of the antenna. Responses for both sensillum types were calculated as the frequency of APs during the last 0.3 s period of the stimulation time (0.5 s) and mean responses and standard deviation were calculated for each stimulus in virgin and mated males.

Calcium imaging

Animals were mounted individually in Plexiglas chambers and the head was fixed. The brain capsule was opened, glands and trachea removed, and then 20 µl dye solution (50 µg Calcium Green 2-AM dissolved with 50 µl Pluronic F-127, 20% in dimethylsulfoxide, Molecular Probes, Eugene, OR, USA) was bath-applied for a minimum of 1 hour, before being washed with Ringer. For recordings, a T.I.L.L. Photonics imaging system (Martinsried, Germany) was coupled to an epifluorescent microscope (Olympus BX-51WI, Olympus, Hamburg, Germany) equipped with a 10× (NA 0.3) water immersion objective. Signals were recorded using a 640×480 pixel 12-bit monochrome CCD camera (T.I.L.L. Imago, cooled to −12°C). Each animal was subjected to up to three series of olfactory stimulations with interstimulus intervals of 80 s. Identification of individual glomeruli was done by superposing activity maps using Adobe Photoshop (Version CS2). Raw data analysis was done using custom–made software written in IDL (Research Systems Inc., Colorado, USA) and Visual Basic (Microsoft Excel) according to previous work [8]. Briefly, after noise filtering and bleaching correction, relative fluorescence changes ($\Delta F/F$) were calculated as $(F-F_0)/F_0$ (F_0 = reference background). For each glomerulus, the time course of $\Delta F/F$ was calculated by averaging 25 pixels (5×5) at the centre of each glomerulus. Nine ordinary glomeruli (OG) were identified in all preparations (named 1–9) and average signals from the 9 glomeruli were calculated for each stimulus. For the MGC, due to its important size three locations were analysed and their data pooled, as they were not significantly different (Figure 3A).

Intracellular recordings of AL neurons

Preparation, intracellular recordings and response analysis of AL neurons from virgin and newly-mated males were performed as described previously [21]. PNs were randomly impaled within the array of the MGC. Data were recorded and analysed using Autospike 32 software (Syntech, The Netherlands). Numbers of APs elicited by a stimulus during the excitatory phase of the response (starting 0.2 s after the onset of stimulation and lasting about 0.4 s) were determined and mean response frequencies and standard deviations for PNs were calculated for each stimulus for virgin and mated males.

Behavioural tests

Behavioural tests were performed in a wind tunnel as described previously [21]. Briefly, 5-day-old mature virgin males were exposed during mid-scotophase in a 2 m-long wind tunnel and their response was quantified to evaluate attractiveness of the stimulus. Males were transferred before the onset of scotophase from their rearing chamber into the wind tunnel room. For stimulation, heptanal diluted in mineral oil was used at four doses (10; 100; 1000; 10000 µg in 10 µl). Stimuli were dispensed on a filter paper and placed in the airflow upwind to the release site in the wind tunnel on a vertical holder. Each experimental male was tested only once to one stimulus and at a single dose, and then the animal was discarded. Assays were performed during 3 min, and partial flight, complete flight and landing on the pheromone source were considered as an oriented response [43,44]. The proportion of males performing an oriented flight was analysed.

Statistical Analysis

For electrophysiological experiments, data were analysed using 3-way repeated measures (RM) ANOVAs including 3 main factors: one fixed factor for mating status, and two RM factors for odour and dose. A 2-way RM ANOVA was carried out by excluding the mating factor of the analysis but keeping the RM factors odour and dose. Odours and doses were always considered as RM since they were presented one after the other in the same preparation. For the analysis, the pheromone as single odour was considered as the first point of the curve of mixtures, as it corresponds to a case of mixture without heptanal (*i.e.* phe+hep 0 µg). Responses to hexane and mineral oil were pooled and considered one data point: 'solvent'. Further, solvent was included as the first point in the curve of heptanal (*i.e.* hep 0 µg) (Figs. 2B, D and 4B, D). When interactions among factors were significant, the simple effects were analysed by means of 1-way ANOVA with or without the RM factor, and then followed by Tukey test for post-hoc comparisons if necessary.

Virgin and mated males were analysed separately in Figure 5B by means of a 1-way RM ANOVA (main factor: odour, with four levels: solvent, heptanal, pheromone and the pheromone/heptanal mixture). In this experiment, virgin and mated males were stimulated with different doses of pheromone as single odour or in the mixture (i.e. 1 ng of pheromone for virgin and 10 ng for mated, see above) and therefore data were not comparable.

Statistical assumptions of homogeneity of variance (Levene's test, Box M), normality and sphericity (Mauchly's test) were checked. Violation of sphericity was overcome by using Greenhouse-Geisser correction for the degrees of freedom (df) if necessary, thus the df were not integer numbers.

For behavioural experiments, statistical differences ($p<0.05$) of responses to the different doses of heptanal were evaluated using a chi-square test.

Supporting Information

Figure S1 Responses of pheromone-responding ORNs stimulated with control stimuli. Stimulation with pipettes containing one filter paper with pheromone elicited responses, which were not significantly different from responses to pipettes containing one filter paper with pheromone (1 ng/10 µl, phe) and a second clean filter paper (cfp) or a second filter paper with mineral oil (10 µl, oil). Stimulation with mineral oil (10 µl) alone did not induce any ORN response (n = 9 for each stimulus type). For statistical analysis see text. The box represents the interquartile range (IQR) of the data, the horizontal line inside the box represents the median. The whiskers show the range of the remaining sample. (EPS)

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Author Contributions

Conceived and designed the experiments: ND JK SV AR JCS PL CG SA RBB. Performed the experiments: ND JK SV DP AR RBB. Analyzed the data: ND JK SV DP AR JCS PL CG SA RBB. Wrote the paper: ND JK SV JCS PL CG SA RBB.

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Steroid hormone signaling is involved in the age-dependent behavioral response to sex pheromone in the adult male moth *Agrotis ipsilon*

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ABSTRACT

In most animals, including insects, male reproduction depends on the detection and processing of female-produced sex pheromones. In the male moth, *Agrotis ipsilon*, both behavioral response and neuronal sensitivity in the primary olfactory center, the antennal lobe (AL), to female sex pheromone are age- and hormone-dependent. In many animal species, steroids are known to act at the brain level to modulate the responsiveness to sexually relevant chemical cues. We aimed to address the hypothesis that the steroidal system and in particular 20-hydroxyecdysone (20E), the main insect steroid hormone, might also be involved in this olfactory plasticity. Therefore, we first cloned the nuclear ecdysteroid receptor EcR (*AipsEcR*) and its partner Ultraspiracle (*AipsUSP*) of *A. ipsilon*, the expression of which increased concomitantly with age in ALs. Injection of 20E into young sexually immature males led to an increase in both responsiveness to sex pheromone and amount of *AipsEcR* and *AipsUSP* in their ALs. Conversely, the behavioral response decreased in older, sexually mature males after injection of cucurbitacin B (CurB), an antagonist of the 20E/EcR/USP complex. Also, the amount of *AipsEcR* and *AipsUSP* significantly declined after treatment with CurB. These results suggest that 20E is involved in the expression of sexual behavior via the EcR/USP signaling pathway, probably acting on central pheromone processing in *A. ipsilon*.

1. Introduction

In most animals, the transition from juvenile to adult state is marked by the acquisition of a new behavioral repertoire that is critical for survival and success during adulthood. Some adult behaviors such as those related to reproductive, social and cognitive functions emerge as results of changes in the structural and functional organization of the nervous system (Cooke, 2011; Nelson and Guyer, 2011; Withers et al., 2008). This neural and behavioral plasticity is at least in part influenced by endogenous signals, especially hormonal factors (Elekonich and Robinson, 2000; Jarriault et al., 2009; Schulz et al., 2009; Woodley and Baum, 2003) and the mechanisms underlying this endocrine regulation are still under intense investigation.

In arthropods, ecdysteroids, through the main active form 20-hydroxyecdysone (20E), are known as key regulators of post-embryonic development by coordinating the processes of molting and metamorphosis. During the transition to the adult stage, the 20E signaling pathways participate in lysis and remodeling of neural circuits to reshape the nervous system for the emergence of adult functions (Hewes, 2008; Truman and Riddiford, 2002). In addition to its developmental role, this morphogenetic hormone exerts crucial actions throughout adult life, by regulating not only some physiological and cognitive functions, i.e., reproduction, lifespan, memory formation (Ishimoto and Kitamoto, 2011; Simon et al., 2003) but also the expression of behavioral patterns essential for sexual interactions in various insect species. For example, disruption of steroid hormone biosynthesis affected the display of olfactory-driven behaviors in the fruit fly *Drosophila melanogaster* (Freeman et al., 1999).

In insects, most 20E effects appear to be mediated by a dimeric complex composed of two proteins belonging to the nuclear receptor superfamily, the ecdysone receptor (EcR) and the ultraspiracle (USP), a homologue of the vertebrate retinoid X receptor (Spindler et al., 2009). Once bound to its receptor complex, 20E stimulates the expression of EcR and USP followed by the induction of early genes encoding transcription factors, such as E75, E78, HR3, HR4

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and FTZ-F1, that amplify the original hormonal signal through the induction of batteries of secondary genes (Nakagawa and Henrich, 2009). It has been reported the existence of different EcR and USP isoforms that are believed to direct tissue- and stage-specific responses to 20E (Bender et al., 1997; Schubiger et al., 1998). For example, the *D. melanogaster* EcR gene encodes three proteins, *DmEcRA*, *DmEcR-B1*, *DmEcR-B2*, (Talbot et al., 1993) and two USP isoforms are present in *Manduca sexta*, *MsUSP-1* and *MsUSP-2* (Jindra et al., 1997).

In contrast to the intensive studies on the identification of ecdysteroid genomic signaling pathways in the context of metamorphosis and reproduction (Beckstead et al., 2005; Cruz et al., 2008), considerably less attention has been paid to those involved in behavioral and neural plasticity in adults. In the house cricket *Acheta domesticus*, the expression of EcR and USP was associated to 20E effects on neural proliferation and differentiation in the adult mushroom bodies, which are the main sites of multisensory integration (Cayre et al., 2000). More recently, gene knockout experiments performed in *D. melanogaster* showed that EcR is needed in the establishment of neural circuits required for the performance of male courtship behaviors (Dalton et al., 2009; Ganter et al., 2007).

In noctuid moths, males use female-emitted pheromones to find their mating partners. 20E is known to be present in hemolymph and reproductive tissues throughout the adult life in males (Bigot et al., 2012; Polanska et al., 2009). The exposure to methoxyfenozide, a 20E mimetic, or the administration of exogenous 20E was reported to modify the responsiveness of males to pheromone cues (Bigot et al., 2012; Hoelscher and Barrett, 2003). In the noctuid moth *Agrotis ipsilon*, the behavioral and central nervous responses to female sex pheromone are known to be age- and hormone-dependent (Anton and Gadenne, 1999; Gadenne and Anton, 2000; Gadenne et al., 1993). Newly emerged males are sexually immature and do not respond behaviorally to the female-produced sex pheromone. Three to five days after emergence, males become sexually mature and are highly attracted by sex pheromone (Gadenne et al., 1993). This increase in pheromone response with age is the consequence of an increasing biosynthesis of Juvenile Hormone (JH), which, in turn, enhances the sensitivity of neurons in the primary olfactory centre, the antennal lobe (AL) (Anton and Gadenne, 1999; Duportets et al., 1998; Gadenne and Anton, 2000).

In our study, we explored the role of the ecdysteroid signalling system in this age-dependent olfactory plasticity in *A. ipsilon* males. Thus, *A. ipsilon* EcR (*AipsEcR*) and USP (*AipsUSP*) were cloned, and their tissue- and age-related expression profiles were determined at transcriptional and protein levels. Further, doses of exogenous 20E or curcubitacin B (CurB), an antagonist of the 20E/EcR/USP complex (Dinan et al., 1997), were injected in young immature or older mature males, and the effects of these treatments were tested on both the expression levels of *AipsEcR* and *AipsUSP* proteins in the AL, and the sex pheromone behavioral responses. Our results demonstrate that 20E regulates the behavioral responsiveness to sex pheromone, probably through a signaling pathway involving the EcR/USP heterodimer, which could act on central processing of the pheromone signal in the ALs of *A. ipsilon* males.

2. Material and methods

2.1. Insects and tissue collection

Adults of *A. ipsilon* originated from a laboratory colony established in Bordeaux and transferred to Versailles. The colony is based on field catches in southern France and wild insects are introduced each spring. Insects were reared on an artificial diet (Poitout and Bues, 1974) in individual cups until pupation. Pupae were sexed, and males and females were kept separately in an inversed light/dark cycle (16 h light:8 h dark photoperiod) at 22 °C. Newly emerged adults were removed from the hatching containers every day and were given access to a 20% sucrose solution *ad libitum*. The day of emergence was considered as day-0.

For expression studies, the tissues (legs, wings, brains, ALs, antennae, thoraces and abdomens) were dissected under Ringer's solution in mid-scotophase (3–5 h after lights off), when males respond maximally to the sex pheromone. The tissues were immediately flash-frozen in Eppendorf vials kept in liquid nitrogen and stored at −80 °C until further treatment. For the collection of ALs, brains were first dissected, ALs were then cut from the protocerebrum with a pair of fine scissors.

2.2. RNA isolation and cDNA synthesis

Total RNAs were extracted with TRIzol reagent (Gibco BRL, Paisley, UK) according to the manufacturer's instructions, and were quantified by spectrophotometry at 260 nm. Single-stranded cDNAs were synthesized from total RNAs (5 μg) with 200 U of SuperScript II Reverse Transcriptase (Invitrogen). The reaction contained a dNTP mix, RNase OUT, Oligo(dT) primer and sterile water to a final volume of 20 μL. The mix was heated to 65 °C for 5 min before adding the enzyme and then incubated for 1 h at 42 °C.

For 5′ and 3′ rapid amplification of cDNA ends (RACE) PCR, cDNAs were synthesized from 1 μg of 5-day-old male brain RNA at 42 °C for 1.5 h using the SMART™RACE cDNA Amplification Kit (Clontech) with 200 U of M-MuLV Reverse Transcriptase RNase H (Finnzymes), 5′- or 3′-CDS-primer and SMART II oligonucleotide.

2.3. Cloning of A. ipsilon EcR and USP

Partial cDNAs encoding putative EcR and USP were identified from a *Spodoptera littoralis* male antennal EST library (Legeai et al., 2011) by local TBLASTN analysis in BioEdit against public databases (GenBank, Tremble). Two pairs of DNA primers (EcRdir1, EcRrev1) and (USPdir1, USPrev1) were designed from A/B and D regions of *S. littoralis* EcR and USP, and PCRs were carried out with 200 ng of brain cDNA with 2.5 units of High Expand Fidelity DNA polymerase (Boerhinger Mannheim). 0.2 μM of primer pairs EcRdir1 (5′-GCCGGAGGATGTCCCTCGGCGC-3′), EcRrev1 (5′-GCCCCACAGCTCCAGGCCGCC-3′) or USPdir1 (5′-CGGCGATGCTAGACGGCTTGCG-3′), USPrev1 (5′-CGCTCCTCTTGGACTGCCTCCC-3′) were added thereafter with 0.2 mM dNTP. Following an initial denaturation at 94 °C for 5 min, the thermal amplification procedure included 35 cycles of denaturation at 94 °C for 30 s, annealing at 64 °C for 1 min, elongation at 72 °C for 1 min and then final elongation at 72 °C for 10 min.

The 5′ and 3′ regions of the corresponding cDNA were obtained by 5′- and 3′-RACE (SMART RACE cDNA Amplification Kit) following manufacturer's instructions. For 5′-RACE, we used 2.5 μL 5′-RACE-ready cDNA with a specific reverse primer EcR5′-RACE (5′-CTCGTTTTCTGATTTCGGTGTCGT-3′) for *AipsEcR* or USP5′-RACE (5′-ACTTAGGCGGAACATTGCTGTCTG-3′) for *AipsUSP* and Universal Primer Mix (UPM, Clontech) as the forward anchor primer. The 3′-RACE amplification was carried out with UPM as the reverse primer and a specific forward primer EcR3′-RACE (5′-TGTGACACGCTCGCAGA CAT-3′) for *AipsEcR* or USP3′-RACE (5′TGTGACACGCTCGCAGACAT-3′) for *AipsUSP*. Touchdown PCR was performed using hot start as follows: after 1 min at 94 °C, five cycles of 30 s at 94 °C and 3 min at 60 °C, then five cycles of 30 s at 94 °C, 30 s at 59 °C and 3 min at 72 °C, then 25 cycles of 30 s at 94 °C, 30 s at 58 °C and 3 min at 72 °C, then 10 min at 72 °C.

PCR products were purified by agarose gel electrophoresis (NucleoSpin® Extract II, Macherey-Nagel GmbH & Co. KG, Düren, Germany) and cloned into pCRII-Topo plasmid (Invitrogen,

Carlsbad, CA, USA). After colony isolation, DNA minipreps were prepared (NucleoSpin® Plasmid DNA Purification, Macherey-Nagel GmbH & Co. KG, Düren, Germany) and correct insertion was determined by restriction enzyme analysis. The DNA clone containing the proper insert was sequenced by GATC Biotech SARL, Marseille, France. By merging the overlapping sequences obtained from the 5′- and 3′-RACE, two putative full-length cDNAs of 1707 bp and 1900 bp were generated and named *AipsEcR* and *AipsUSP*, respectively.

2.4. PCR and qPCR

PCR was performed on 100 ng of cDNA preparations from various tissues with 1.25 units of High Expand Fidelity DNA polymerase (Boerhinger Mannheim). The pair of specific primers qEcRdir1 (5′-GCATCACGTATAGCATGGCGCAAT-3′) and qEcRrev1 (5′-GCCGTTAATACTCGAAGCTGGTGA-3′) or qUSPdir1 (5′-AGACAGAGCGTCGGGCAAACATTA-3′) and qUSPrev1 (5′-TCCTGTACCGCTTCCCTCTTCAT-3′) were added at 0.2 μM with 0.2 mM dNTP. Following an initial denaturation at 94 °C for 5 min, the thermal amplification procedure included 30 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s, elongation at 72 °C for 30 s and then final elongation at 72 °C for 10 min. Amplification products of 199 bp length of *AipsEcR* and 203 bp length of *AipsUSP* were loaded on 1.5% agarose gels and visualized with SYBR Safe. The expression of ribosomal gene RpL8 (accession number JX975720) was analyzed as an internal control for RNA quantity and quality, using a specific primer pair Ai-rpL8dir (5′-CCAGTTTGTCTACTGCGGCAA-3′) and Ai-rpL8rev (5′-GCTTAACCCTAGTACGCTTGGCA-3′).

Real time qPCR was performed on cDNA preparations using the ICycler iQ™ Real-Time PCR Detection System (Bio-Rad) according to manufacturer's instructions. Three independent cDNA preparations were made for each sample. Each 12 μL reaction consisted of 6 μL Absolute Blue SYBR Green fluor (Thermo Scientific, Waltham, MA, USA), 4 μL cDNA (25 ng/μL), 2 μL of pairs of primers qEcRdir1 and qEcRrev1 or qUSPdir1 and qUSPrev1 at 10 μM. PCR conditions were 35 cycles of 95 °C for 30 s, 65 °C for 30 s, 72 °C for 30 s. Fluorescence measurements over a 55–95 °C melting curve confirmed the presence of a single-specific peak and the absence of primer-dimer peaks for the two primer pairs. Each run included a negative control (water) and a fivefold dilution series of pooled cDNA (from all conditions). The fivefold dilutions series were used to construct a relative standard curve to determine the PCR efficiencies and for further quantification analysis. In all experiments, the primers gave amplification efficiencies of 90–100%. Each reaction was performed in three technical replicates with at least five independent biological replicates using the technical platform of The Integrative Biology Institute (Pierre et Marie Curie University, France). Expression levels were analyzed with ICycler iQ software and geNORM Visual Basic application for Microsoft Excel as described by Vandesompele (Vandesompele et al., 2002). The cycle threshold values (Ct-values) were determined for the candidate gene and the reference gene RpL8 which exhibited the most stable expression levels as a function of age among other tested control genes, RpL13, GAPDH and β-actin. The average Ct-value of each triplicate reaction was used to normalize the candidate gene expression level to the geometric mean of RpL8 level in Q-Gene software (Simon, 2003).

2.5. Protein extraction and western analysis

Tissues were homogenized in 10 vol (w/v) of lysis buffer: 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 0.5% Triton X-100, 0.5% SDS, and protease inhibitors (1:200, Sigma). The extracted proteins were quantified according to the Bradford method (Bradford, 1976) using serum albumin as standard. The samples with equal protein contents were adjusted to an equal volume with 50 mM Tris (pH 6.8), containing 2% SDS, 8% glycerol and 2% 2-mercaptoethanol with Bromophenol Blue as a marker, and boiled for 5 min. Twenty mg of proteins were loaded in each lane and separated on 7.5% SDS-polyacrylamide resolving gel in a miniprotean vertical gel electrophoresis apparatus (Bio-Rad). Proteins were then transferred to nitrocellulose membranes in a mini trans-blot electrophoretic (Bio-Rad) using 120 mA current for 1 h. The membranes were then probed overnight at 4 °C with the following primary antibodies: polyclonal rabbit anti-EcR-B1 and anti-USP-1 raised against the peptide motifs DTLADMRRRW and TMSVTALINW of the lepidopteran EcR-B1 and USP-1 (1:4500, 1:6000, Proteogenix) respectively and monoclonal rabbit anti-β-tubulin (1:80000, Sigma). Membranes were then washed three times with TTBS buffer: 0.2% Tween-20, 150 mM NaCl, 25 mM Tris (pH 7.5), followed by incubation with peroxidase-conjugated secondary antibody goat anti-rabbit IgG (1:1000, Roche) for 1 h at room temperature. Protein bands were visualized by Enhanced Chemiluminescence (ECL) (Lumi-Light^{Plus} Western Blotting Kit, Roche). The relative amounts of immunoreactivity were quantified using Image Gauge (Fujifilm) and Image ProPlus (Media Cybernetics) software, and the ratio of *AipsEcR* and *AipsUSP* to β-tubulin was calculated for each sample.

2.6. Chemicals

For wind tunnel experiments, we prepared an artificial pheromone blend containing (Z)-7-dodecenyl acetate (Z7–12:Ac), (Z)-9-tetradecenyl acetate (Z9–14:Ac), and (Z)-11-hexadecenyl acetate (Z11–16:Ac) (Sigma–Aldrich, Saint-Quentin Fallavier, France) at a ratio 4:1:4 (Gemeno and Haynes, 2000; Picimbon et al., 1997). 20-Hydroxyecdysone was a gift from Pr. René Lafont (Pierre et Marie Curie University, Paris, France) and the antagonist of the 20E/EcR/USP complex, CurB, was purchased from Sigma–Aldrich. Stock solutions of 20E and CurB were prepared in ethanol at a concentration of 10^{-2} M, then stored at −20 °C. For experiments, the stock solutions were diluted to 10^{-5} M in a NaCl (145 mM) solution.

2.7. Injection treatments

Day-0 males or day-5 males (0.2 ± 0.02 g body weight) were anesthetized with carbon dioxide and received an injection of 2 μL 10^{-5} M 20E or CurB respectively in the abdomen before the onset of scotophase. After 1- and 2-days post-injection delay for 20E-injected males and 6 h for CurB-injected males, the amount of *AipsUSP* and *AipsEcR* proteins in AL was determined by Western blot, and the behavioral response to sex pheromone of injected males was tested in wind tunnel experiments at the middle of scotophase. Control experiments were performed by injection of 2 μL NaCl. At the concentration of 10^{-5} M, 20E has been previously reported to affect the responsiveness of male to sex pheromone in several species of moths (Bigot et al., 2012; Hoelscher and Barrett, 2003) as well as CurB was shown to efficiently inhibit the *in vitro* formation of 20E/EcR/USP complex (Dinan et al., 1997).

2.8. Wind tunnel experiments

Experiments were performed under red light illumination using a 2 m long flight tunnel in mid-scotophase as described previously (Barrozo et al., 2010; Gadenne et al., 2001). Environmental conditions during the bioassay were held constant: 22 °C, 50 ± 10% relative humidity and a wind speed of 0.3 ms^{-1}. A cage containing a single experimental male was introduced in the wind tunnel. After a few minutes during which the male was allowed to adjust to the airflow, a filter paper containing the stimulus was placed 160 cm

upwind from the cage. Ten ng of artificial sex pheromone blend were used for stimulation. The behavior of the male was observed during 3 min and partial flight, complete flight toward the pheromone source as well as landing on the pheromone source were counted as an oriented response (Jarriault et al., 2009). The general flight activity of the males was also accounted by addition of oriented and random flights.

2.9. Bioinformatic

For the bioinformatic study of insect EcR and USP orthologs, the protein sequences were aligned using ClustalW in BioEDit 7.0. Accession numbers for EcR and USP protein sequences retrieved from GenBank are as follows: *D. melanogaster* EcR-A, USP (*DmEcR-A*, *DmUSP*): NP_724456, NM_057433; *M. sexta* EcR-B1, USP-1 (*MsEcR-B1*, *MsUSP-1*): AAA86699, U44837; *Tenebrio molitor* USP (*TmUSP*): AJ251542; *Choristoneura fumiferana* EcR-A (*CfECR-A)*: AAC61596; *Plodia interpunctella* EcR-B1, USP-2 (*PiEcR-B1*, *PiUSP-2*): AY489269, AY619987; *Bombyx mori* USP-1 (*BmUSP-1*): NM_001173375 and *Spodoptera litura* EcR-B1 (*SlEcR-B1*): ABX79143.

2.10. Statistical analysis

For behavioral assays, statistical differences ($P \leqslant 0.05$) were evaluated among groups (NaCl versus CurB, and NaCl versus 20E) using a chi-square-test. For *AipsEcR*, *AipsUSP* expression studies, statistical differences were analyzed by comparing means using student's *t*-test and one-way ANOVA followed *post hoc* by the Tukey test.

3. Results

3.1. Identification and comparison of the primary structure of AipsEcR and AipsUSP

Taking in account the highly conserved protein sequences of steroid nuclear receptors, two pairs of primers for EcR and USP were designed from *S. littoralis* sequences and used to amplify two partial cDNA fragments of expected size 221 and 318 bp, from RT-PCR reactions performed with total RNA of *A. ipsilon* brains. The remaining 5′ and 3′ ends of the two fragments were then obtained by developing a RACE/PCR-based strategy. The nucleic acid sequences for the 5′- and 3′-RACE reaction products were assembled with the original fragment to generate two full-length cDNA sequences named *AipsEcR*, *AipsUSP* and deposited in the GenBank under the accession numbers JQ731607 and JQ731606, respectively. The *AipsEcR* cDNA of 3597 bp length contains an open reading frame (ORF) of 1767 bp, a 384 bp 5′-untranslated region (5′-UTR) and a 1446 bp 3′-UTR, with a polyadenylation signal upstream of the poly(A) tail (Fig. 1S). The *AipsUSP* cDNA of 2182 bp length is composed of a 1398 bp ORF, a 271 bp 5′-UTR and a 513 bp 3′-UTR (Fig. 2S). Using biosciences software, the *AipsEcR* and *AipsUSP* ORFs were translated into 589 and 466 amino acid sequences, predicting 66 kDa and 52 kDa proteins respectively as determined with MWCALC application (Infobiogen).

The analysis of deduced amino acid sequence of *AipsEcR* and *AipsUSP* revealed the presence of a transactivating (A/B) domain, a DNA-binding (C) domain, a hinge (D) domain and a ligand-binding (E/F) domain (Figs. 1S and 2S). By comparing the full protein sequence of *AipsEcR* and *AipsUSP* with those of EcR and USP identified in various insect species, a high percentage of amino acid identity was observed in the C, D and E/F regions, with more than 87% between *AipsEcR*, *AipsUSP* and EcR, USP isoforms isolated from *M. sexta* (*MsEcR-B1*, *MsUSP-1*) and *P. interpunctella* (*PiEcR-B1*, *PiUSP-2*) (Table 1). Overall in the A/B region, *AipsEcR* has high amino acid identity with *SlEcR-B1* (85%), *PiEcR-B1* (67%), *MsEcR-B1* (66%), also similarity (as amino acid identity) between *AipsUSP* and *MsUSP-1* (83%), *BmUSP-1* (80%) is high (Table 1).

3.2. Tissue-related expression patterns of AipsEcR and AipsUSP

To determine the tissue distribution of *AipsEcR* and *AipsUSP*, their expression amounts were determined by RT-PCR in antenna, brain, AL, thorax, leg, wing and abdomen of 5-day-old male. *AipsEcR* and *AipsUSP* cDNA fragments of expected size (199 bp and 203 bp) were co-amplified at different levels in all tested tissues (Fig. 1). AipsEcR was detected in lower amounts, and more in the brain and thorax as compared with others tissues, than AipsUSP (Fig. 1).

3.3. Age-related expression patterns of AipsEcR and AipsUSP

To assess the possible involvement of *AipsEcR* and *AipsUSP* in the age-related olfactory plasticity, the expression of the corresponding genes was quantified in ALs of 1- to 5-day-old males by real time PCR and Western blot. Expression of *AipsEcR* and *AipsUSP* genes was found to be age-dependent at transcriptional and protein levels in the AL (Fig. 2). Transcriptional activity of *AipsEcR* and *AipsUSP* genes increased significantly from day-1 to day-3 after emergence, then its level remained practically stable until day-5 (Fig. 2A and B). Regarding protein patterns, *AipsEcR* and *AipsUSP* were detected at day-1 and day-2, reached peak expression at day-3 and day-4, respectively, and maintained this level until day-5 (Fig. 2C and D). The amounts of AL *AipsEcR* and *AipsUSP* proteins in 4- or 5-day-old sexually mature males were 1.5 to 2 times higher than in 1- or 2-day-old sexually immature males.

3.4. Effects of CurB and 20E treatments on behavioral responses to sex pheromone, and expression of AipsEcR and AipsUSP in ALs of A. ipsilon males

In order to demonstrate a functional link between the 20E/EcR/USP complex and the olfactory system, we manipulated the circulating 20E titer by injecting exogenous 20E, or by blocking the complex by injecting CurB into males of different ages. Subsequently, effects of treatment on the flight behavior towards the conspecific sex pheromone and the expression level of *AipsEcR* and *AipsUSP* proteins in the AL were examined.

Injections of 20E into sexually immature 0-day-old males ($N = 30$) had no significant effect on both their general flight activity (67%) ($\chi^2 = 0.48$; $P = 0.485$) and their oriented response to sex pheromone (20%) ($\chi^2 = 0.05$; $P = 0.819$) when they were tested only 24 h after injection as compared with those of NaCl-treated control males ($N = 28$) (75% and 14% respectively) (Fig. 3A). Moreover, the 20E-injected 1-day-old males showed a slight increase in the levels of *AipsEcR* and *AipsUSP* proteins in ALs compared to NaCl-treated males (Fig. 3B and C).

In contrast, 20E-injected 0-day-old males tested 48 h after injection ($N = 68$) showed a significant increase in the oriented response (46%) ($\chi^2 = 6.27$; $P = 0.012$), although there was no significant effect on the general flight activity (78%) ($\chi^2 = 0.0045$; $P = 0.946$), as compared to NaCl-treated males ($N = 71$) (24% and 77% respectively) (Fig. 3D). 20E-injected males showed much higher levels of *AipsEcR* and *AipsUSP* proteins in ALs compared to NaCl-injected males (Fig. 3E and F). NaCl-injected males did not differ from control untreated males in respect to flight responses and protein levels (data not shown). Moreover, 20E administration did not affect the behavioral response and the amounts of *AipsEcR* and *AipsUSP* in ALs in 5-day-old specimens, probably because sexually mature males have already reached a high level in both the

Table 1
Comparison of amino acid sequences of A/B, C, D, E/ F regions between *AipsEcR*, *AipsUSP* and homologues.

Receptor	A/B region		C region		D region		E/F region	
	Identity	Length*	Identity	Length*	Identity	Length*	Identity	Length*
AipsEcR	100	185	100	66	100	97	100	220
SlEcR-B1	85	187	98	66	82	92	94	223
PiEcR-B1	67	142	98	66	91	91	90	222
MsEcR-B1	66	146	98	66	89	91	92	224
DmEcR-A	32	234	94	66	39	100	70	221
CfEcR-A	30	112	89	66	81	91	87	224
AipsUSP	100	112	100	66	100	26	100	235
MsUSP-1	83	112	98	66	100	26	88	232
BmUSP-1	80	113	98	66	98	26	84	232
PiUSP-2	49	60	98	66	100	26	91	233
DmUSP	33	103	97	66	54	35	48	246
TmUSP	31	86	94	66	50	37	49	217

* Length is expressed as total number of amino acids constituting the region. Identity of *AipsEcR* or *AipsUSP* compared to sequences of other species' EcR or USP is expressed as a percentage of equal amino acids. For abbreviations and accession numbers of EcR and USP sequences, see material and methods, and results.

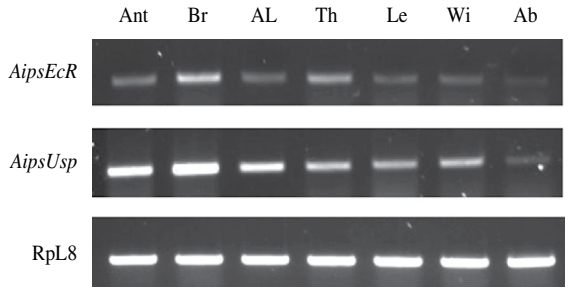

Fig. 1. Tissue-related *AipsEcR* and *AipsUSP* expression in *A. ipsilon* males. One hundred nanograms of total RNAs from various tissues of 5-day-old-males were analyzed by RT-PCR using pairs of *AipsEcR* and *AipsUSP* specific primers. RNA templates were extracted from antenna (Ant), brain (Br), antennal lobe (AL), wing (Wi), leg (Le), thorax (Th), and abdomen (Ab). For RT-PCR controls, specific primers for the *A. ipsilon* RpL8 gene encoding a ribosomal protein were used.

sensitivity to sex pheromone and the 20E inducibility of EcR/USP complex (data not shown).

CurB administration into 5-day-old sexually mature males ($N = 53$) resulted in a significant decrease of the oriented response (19%) only 6 h after injection as compared to NaCl-treated males ($N = 48$) (52%) ($\chi^2 = 12.27$; $P = 0.00045$) (Fig. 4A). In contrast, the general flight activity of CurB-injected males (91%) was not statistically different from that of NaCl-treated males (98%) ($\chi^2 = 2.43$; $P = 0.118$) (Fig. 4A). CurB-injected males showed lower levels of *AipsEcR* and *AipsUSP* proteins as compared to NaCl-treated males (Fig. 4B and C).

4. Discussion

4.1. High sequence identity of EcR and USP between A. ipsilon and other insect species

The deduced amino acid sequence of *AipsEcR* and *AipsUSP* exhibit the structural hallmarks of the nuclear receptor superfamily with the presence of A/B, C, D and E/F domains (McEwan, 2009; Nakagawa and Henrich, 2009). The C region of *AipsEcR* and *AipsUSP* contains two Cys2-Cys2 type zinc finger motifs that are required for the recognition and binding of specific hormone response elements (HREs) in target genes (Beato et al., 1995). The E region is known to be essential for ligand binding, transcriptional activation or repression, nuclear translocation and dimerization process (Truss and Beato, 1993). It has been demonstrated that the USP protein forms a heterodimeric complex with the EcR protein for binding to the ecdysone response element (EcRE) sequence and transactivation (Perera et al., 2005). The helix-turn-zipper motif, which seems to be critical for receptor dimerization (Maksymowych et al., 1992) is also found in *AipsEcR* and *AipsUSP*.

It is well established that all EcR or USP isoforms share DNA- and ligand-binding domains, but each has its own isoform-specific segment in the N-terminal region of A/B domain, which contains a transactivating domain (Hu et al., 2003). The predicted sequences of A/B domains of *AipsEcR* and *AipsUSP* exhibit significant amino acid identities with the corresponding region of *MsUSP-1* and B-1 isoform of other insect EcR orthologs, despite differences in the domain length. Taken together, these data indicate that *AipsEcR* is a B1 type isoform and *AipsUSP* is a homologue of *M. sexta* USP-1.

4.2. Ubiquitous distribution of AipsEcR and AipsUSP

Our results reveal that *AipsEcR* and *AipsUSP* genes are differentially co-expressed throughout the whole body, including cephalic, thoracic, abdominal regions and extending to the locomotor appendages and the antennae. This differential expression could result from a tissue-specific transcriptional regulation of *AipsEcR* and *AipsUSP* genes. Furthermore, the ubiquitous tissue distributions observed for *AipsEcR* and *AipsUSP* are in concordance with those described in other insects, especially in the noctuid moth *S. littoralis* (Bigot et al., 2012) and in the fruit fly *D. melanogaster* where EcR and USP are present in both reproductive, digestive, excretory and nervous systems (Carney and Bender, 2000; Dalton et al., 2009). Therefore, it is highly probable that the 20E/EcR/USP complex exerts a multifunctional role in the adult moth *A. ipsilon*, an interpretation supported by recent work demonstrating that many aspects of adult life are linked to EcR/USP function and activity in *D. melanogaster* (Carney and Bender, 2000; Dalton et al., 2009; Ishimoto and Kitamoto, 2011; Simon et al., 2003).

Interestingly, *AipsEcR* and *AipsUSP* gene expression was localized in the antennae and in the brain, especially in ALs, which are considered as the primary olfactory center. These molecular data suggest that the olfactory processing regions are a substrate of steroid signaling system in *A. ipsilon*. Similar studies performed in the male *S. littoralis* provided evidence for the expression of EcR and USP in the pheromone-sensitive olfactory sensilla (Bigot et al., 2012). Also, *in situ* hybridization and quantitative RT-PCR experiments revealed the preferential expression of steroid-regulated genes like E75, FTZ-F1, BR-C, EcR, E74 and HR38 in the mushroom bodies of *Apis mellifera* (Takeuchi et al., 2007; Velarde et al., 2009; Yamazaki et al., 2006). Moreover, vertebrate steroid receptors were

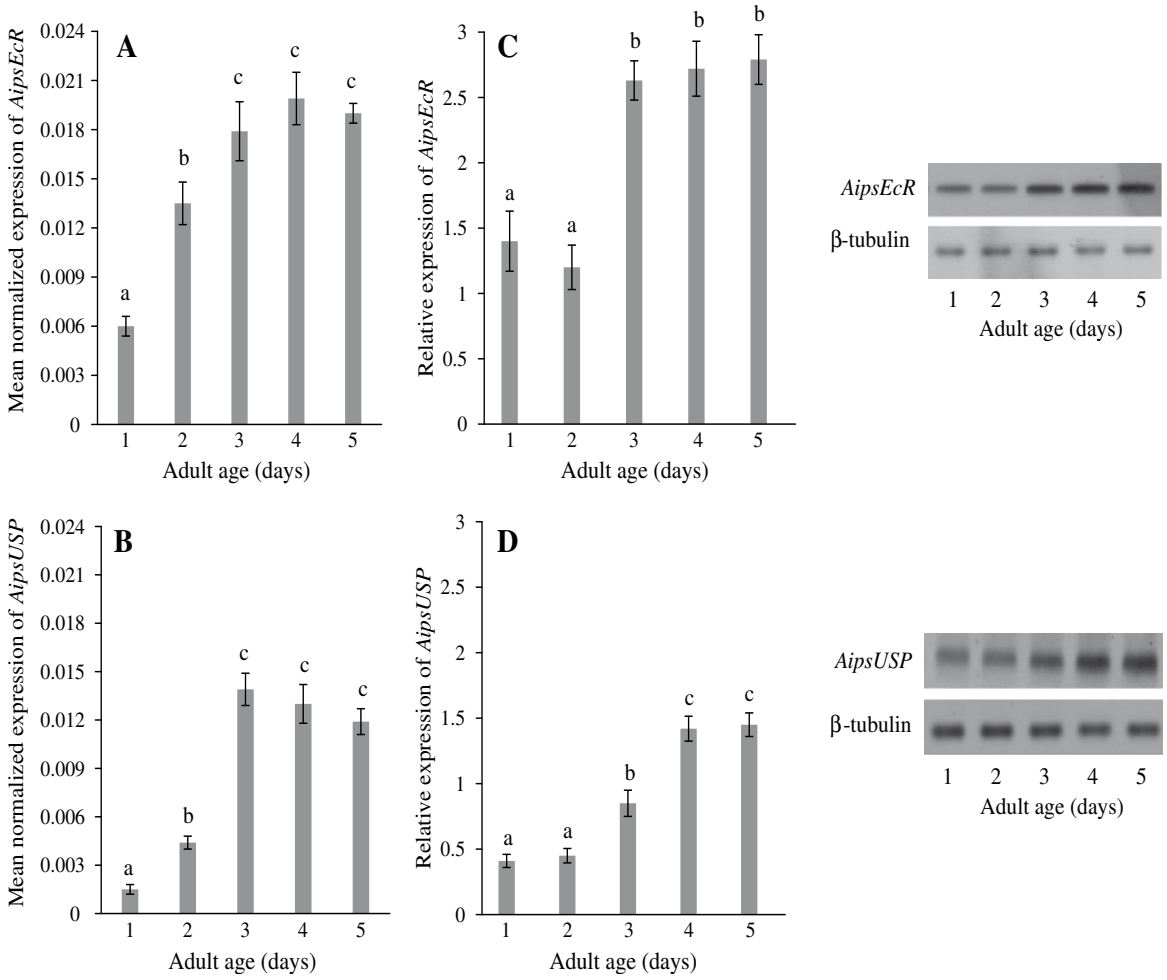

Fig. 2. Age-related *AipsEcR* and *AipsUSP* transcriptional activity and protein level from ALs of 1- to 5-day-old *A. ipsilon* males. (A and B) Transcriptional activity of *AipsEcR* and *AipsUSP* respectively. The synthesized cDNAs from ALs were amplified by real-time qPCR using pairs of *AipsEcR* and *AipsUSP* specific primers. (C and D) Protein levels of *AipsEcR* and *AipsUSP* respectively. The extracted proteins from ALs were analyzed by Western blot with the antibody anti-EcR-B1 and anti-USP-1. For real-time qPCR experiments, the control used was the *A. ipsilon* RpL8 gene expression of which was previously analyzed to be invariant considering the age of males. For Western blot, the control used was rabbit β tubulin protein. Bars represent means ± SD ($n = 6$ repetitions). Bars with same letters are not significantly different (ANOVA, Tukey *post hoc* test; $P \leqslant 0.05$).

shown repeatedly to be expressed in the sensory neurons of the accessory and vomeronasal olfactory systems associated with the detection of pheromonal cues, as well as in distinct areas of the olfactory bulbs (OBs), the first-order relay stations for olfactory information (Alekseyenko et al., 2006; Maruska and Fernald, 2010; Moffatt, 2003).

4.3. Age-dependent increase of AipsEcR and AipsUSP expression in ALs

192 Our results show age-dependent transcriptional activity and protein levels of *AipsEcR* and *AipsUSP* in ALs, which are concomitant with the age-dependent increase of behavioral responses to sex pheromone. These observations led us to hypothesize that this age-dependent expression of *AipsEcR* and *AipsUSP* might be linked to the neuronal plasticity that occurs in ALs during the establishment of sexual behavior in the male adult moth *A. ipsilon* (Anton and Gadenne, 1999). In different social insect taxa, previous studies indicated that there is a correlation between the reshaping of some brain areas and the expression amount of several steroid-regulated genes during the caste differentiation within the colony. For instance, in adult worker honeybees and ants, EcR and HR38 receptors display a differential expression associated with the restructuring of neural circuits of the mushroom bodies that accompanies the behavioral switch from nurse to forager (Nemoto and Hara, 2007; Yamazaki et al., 2006). Moreover, numerous sex steroid receptor subtypes exhibit fluctuations in their expression levels according to sex, reproductive and social state in the OBs of mammals and teleost fishes. This molecular plasticity appears to be critical in fine-tuning olfactory responsiveness to behaviorally relevant cues (Maruska and Fernald, 2010; Moffatt, 2003).

4.4. Ecdysone signaling is involved in the behavioral maturation of sex pheromone response

In young immature males, 20E has the ability to highly increase the behavioral responsiveness to sex pheromone and its action is associated with the 20E inducibility of *AipsEcR* and *AipsUSP* in ALs. In contrast, CurB, an antagonist of the 20E/EcR/USP complex, reduced these behavioral and molecular responses in older mature males. Taken together, these results provide evidence that 20E controls the expression of *A. ipsilon* male sexual behavior, probably through the induction of EcR/USP signaling pathway in AL that might act on the central processing of pheromonal information. Such a mode of action has been reported in mammals, for instance in rat and mice where sex steroids, like 17-β-oestradiol and dihydrotestosterone, were able to facilitate the neuronal responses to

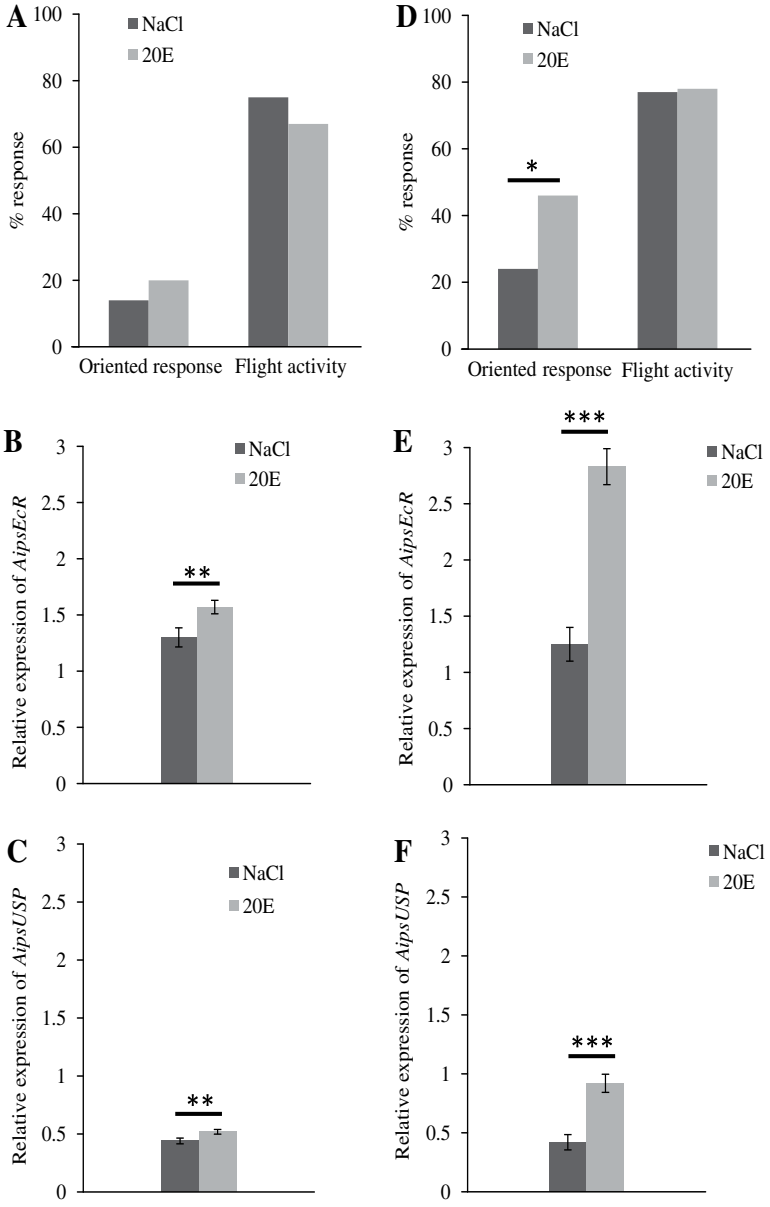

Fig. 3. Effect of 20-hydroxyecdysone (20E) on the oriented response and general flight activity towards the sex pheromone, and protein expression of *AipsEcR* and *AipsUSP* in the antennal lobe of *A. ipsilon* males. (A) Effect of 20E on the behavioral response of 1-day-old males. (B–C) Protein levels of *AipsEcR* and *AipsUSP* respectively in 20E-injected 1-day-old males. 0-day-old sexually immature males received an injection of 20E or NaCl. The percentage of oriented response and flight activity as well as the amount of *AipsEcR* and *AipsUSP* proteins were evaluated 24 h after injection. (D) Effect of 20E on the behavioral response of 2-day-old males. (E–F) Protein levels of *AipsEcR* and *AipsUSP* respectively in 20E-injected 2-day-old males. 0-day-old sexually immature males received an injection of 20E or NaCl. The percentage of oriented response and flight activity as well as the amount of *AipsEcR* and *AipsUSP* proteins were evaluated 48 h after injection. For Western blots, the control used was rabbit β tubulin protein. Asterisk indicates a significant difference among groups for behavioral assays (χ^2 test; $P \leqslant 0.05$) and protein levels of *AipsEcR* and *AipsUSP* (student's *t*-test; $P \leqslant 0.05$). Number of tested males in (A) and (D) are given in Section 3.4. Bars in (B), (C), (E), and (F) represent means ± SD ($n = 6$ repetitions).

female volatile odor within the accessory olfactory bulb of males (Yoshikage et al., 2007).

In adult insects, recent data also highlighted the key role of steroid signaling machinery in the control of physiological and behavioral functions. In *D. melanogaster*, reduced EcR levels were shown to induce changes in lifespan and deficiencies in male courtship memory formation and recall (Ishimoto and Kitamoto, 2011; Simon et al., 2003). EcR expression in *fruitless*-expressing olfactory receptor neurons was also found to be necessary in the establishment of neural circuitry required for the initiation of wild type male courtship behavior (Dalton et al., 2009). In addition, an increased 20E circulation was shown to affect the olfactory responsiveness of the male moth *S. littoralis* to sex pheromone via the induction of EcR, USP and E75 genes (Bigot et al., 2012).

The transduction of 20E signaling can be modulated by JH via its action on the expression of EcR, USP and several ecdysone primary-response genes, including HR3, E75 and BR-C (Dubrovsky et al., 2004; Henrich et al., 2003; Siaussat et al., 2004; Zhou et al., 1998). It has been previously reported that JH controls the maturation of behavioral and central nervous responses to sex pheromone in male *A. ipsilon* (Anton and Gadenne, 1999; Gadenne et al., 1993; Jarriault et al., 2009). We recently showed that JH action might be transduced through the induction of the Krüppel homolog 1 transcription factor (Kr-h1) in *A. ipsilon* (Duportets et al., 2012). Kr-h1 also appeared to function as a key regulator in mediating the interaction between the JH and 20E signaling pathways governing developmental plasticity of MB neurons during fruit fly metamorphosis and caste transitions in adult honey bees (Grozinger and

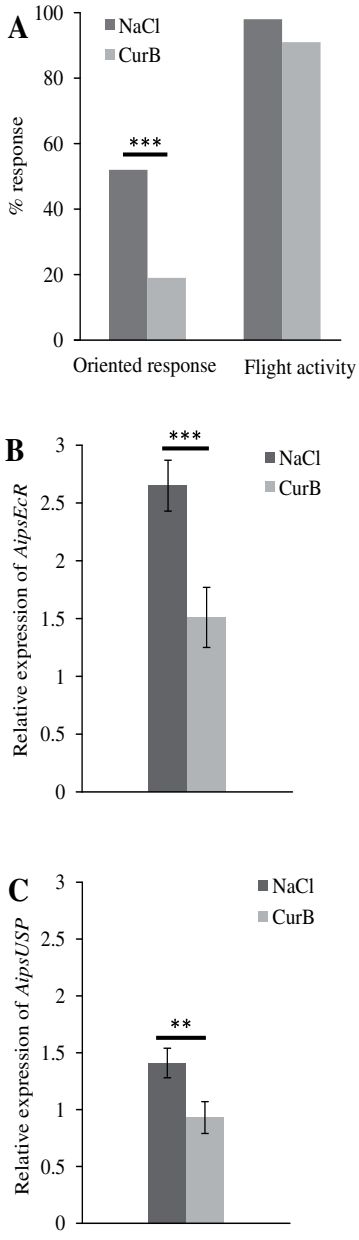

Fig. 4. Effect of cucurbitacin B (curB) on the oriented response and general flight activity towards the sex pheromone, and protein expression of *AipsEcR* and *AipsUSP* in the antennal lobe of *A. ipsilon* males. (A) Effect of curB on the behavioral response of 5-day-old males. (B–C) Protein levels respectively of *AipsEcR* and *AipsUSP* in curB-injected 5-day-old males. 5-day-old sexually mature males received an injection of curB or NaCl. The percentage of oriented response and flight activity as well as the amount of *AipsEcR* and *AipsUSP* proteins were evaluated 6 h after injection. For Western blots, the control used was rabbit β tubulin protein. Asterisk indicates a significant difference among groups for behavioral assays (χ^2 test; $P \leqslant 0.05$) and protein levels of *AipsEcR* and *AipsUSP* (student's *t*-test; $P \leqslant 0.05$). Number of tested males in (A) are given in Section 3.4. Bars in (B) and (C) represent means ± SD ($n = 6$ repetitions).

Robinson, 2007; Hewes, 2008; Pecasse et al., 2000; Shi et al., 2007). Therefore, more specific research is needed to identify regulatory interactions and protein partners for EcR, USP in order to obtain a better understanding of the interplay among JH and 20E signaling during the age-dependent plasticity of olfaction in *A. ipsilon*.

Besides their well-known genomic effects, the ecdysteroids are suspected to control some physiological processes through extra-nuclear pathways (Champlin and Truman, 2000; Iga et al., 2010). Indeed, a G Protein-coupled receptor (GPCR) activated by both 20E and dopamine able to induce rapid non genomic effects has recently been identified in the adult *D. melanogaster* central nervous system (Srivastava et al., 2005). Experiments are now in progress to explore the possible involvement of a GPCR signaling in the olfactory plasticity in *A. ipsilon*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2013.02.024.

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Is the rapid post-mating inhibition of pheromone response triggered by ecdysteroids or other factors from the sex accessory glands in the male moth *Agrotis ipsilon*?☆

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ABSTRACT

In many animals, male copulation is dependent on the detection and processing of female-produced sex pheromones, which is generally followed by a sexual refractory post-ejaculatory interval (PEI). In the male moth, *Agrotis ipsilon*, this PEI is characterized by a transient post-mating inhibition of behavioral and central nervous responses to sex pheromone, which prevents males from re-mating until they have refilled their reproductive tracts for a potential new ejaculate. However, the timing and possible factors inducing this rapid olfactory switch-off are still unknown. Here, we determined the initial time delay and duration of the PEI. Moreover, we tested the hypothesis that the brain, the testis and/or the sex accessory glands (SAGs) could produce a factor inducing the PEI. Lastly, we investigated the possible involvement of ecdysteroids, hormones essential for development and reproduction in insects, in this olfactory plasticity. Using brain and SAG cross-injections in virgin and newly-mated males, surgical treatments, wind tunnel behavioral experiments and EIA quantifications of ecdysteroids, we show that the PEI starts very shortly after the onset of copulation, and that SAGs contain a factor, which is produced/accumulated after copulation to induce the PEI. Moreover, SAGs were found to be the main source of ecdysteroids, whose concentration decreased after mating, whereas it increased in the haemolymph. 20-Hydroxyecdysone (20E) was identified as the major ecdysteroid in SAGs of *A. ipsilon* males. Finally, 20E injections did not reduce the behavioral pheromone response of virgin males. Altogether our data indicate that 20E is probably not involved in the PEI.

Introduction

In many animal species, male reproduction is dependent on the detection and processing of female-produced sex pheromones. Responses to such pheromones depend not only on their chemical properties as signals, but also on environmental conditions and the physiological state of the receiver (Kolb and Whishaw, 1998; Meinertzhagen, 2001). Male reproductive success depends both on the ability to locate and copulate with a female, and to effectively transfer an ejaculate (Dewsbury, 1982). Contrarily to females, in which mating induces drastic long-lasting physiological and behavioral changes (Flanagan-Cato et al., 2006; Gillott, 2003; Huck et al., 1987), males can often remate after a variable time delay. However, remating in males is limited by the number of ejaculates they can deliver and the time required to replenish depleted reserves. Therefore, newly-mated males should delay the risk-taking and energy-consuming search for new sexual partners. By avoiding unsuccessful reproduction, an individual may increase both its probability of surviving to the next reproductive opportunity and the amount of energy available to undergo a next reproductive event. Although males of many vertebrate and few invertebrate species are also known to enter a post-ejaculatory refractory interval (PEI) (Aversa et al., 2000; Fischer and King, 2008; Phillips-Farfán and Fernández-Guasti, 2009; Reddy and Guerrero, 2000; Ureshi and Sakai, 2001), the mechanisms that lead to this sexual abstinence are far from being understood.

In the noctuid moth, *Agrotis ipsilon*, evidence is accumulating that the modulation of pheromone responses occurs through neuronal plasticity (Anton et al., 2007). In this species, we previously showed

☆ Sex accessory glands and post-mating olfactory inhibition in a moth.

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that newly-mated males are no longer attracted to sex pheromone, and that the response to pheromone is restored during the next night (Gadenne et al., 2001). This plasticity is not only seen at the behavioral level, but is accompanied by a decrease in the sensitivity of pheromone-specific neurons within the primary olfactory centre, the antennal lobe (AL): most neurons have much higher pheromone response thresholds after mating (Barrozo et al., 2010a; Gadenne et al., 2001). This olfactory switch-off is restricted to the responses to sex pheromone as newly-mated males still respond to plant odors (Barrozo et al., 2010a). This transient olfactory plasticity thus allows newly-mated males to enter a PEI in order to replenish their sex accessory glands (SAGs) for a potential new female encounter and copulation (Duportets et al., 1998).

The fast change in neuron sensitivity leading to this transient olfactory switch-off following mating could involve a down- or up-regulation of neuroregulatory peptides, biogenic amines or hormones such as juvenile hormone (JH) and ecdysteroids. Although biogenic amines have been shown to influence pheromone sensitivity in animals (including insects), we recently showed that octopamine and serotonin are probably not involved in the transient post-mating olfactory switch off in *A. ipsilon* males (Barrozo et al., 2010b). Although JH is necessary to elicit high sensitivity to sex pheromone in males during adult maturation, the positive effect of this hormone is rather slow (1–2 days) (Gadenne and Anton, 2000), and therefore it is rather unlikely that this hormone is involved in the fast post-mating switch off (hours or less). Moreover, no change in JH biosynthetic activity following mating in males was observed (Duportets et al., 1998).

In many vertebrates, steroids are known to control male sexual behavior by affecting the detection of sensory signals (Hull and Dominguez, 2007). In male mice, sex steroids control the processing of female odors, by modulating pheromone-induced immediate early genes in the accessory olfactory system (Yoshikage et al., 2007) and the expression of pheromone receptor genes in the vomeronasal organ (Alekseyenko et al., 2006). In male hamsters, gonadal steroids regulate behavioral responses to sex pheromones by acting on the medial preoptic nucleus (Swann, 1997). In a cichlid fish, reproductive status was found to modulate the expression of sex steroid receptors in the olfactory bulb (Maruska and Fernald, 2010).

In insects ecdysteroids have essential roles in coordinating developmental transitions such as larval moulting and metamorphosis, and also reproduction events, through their major active form: 20-hydroxyecdysone (20E) (Spindler et al., 2009). Indeed, 20E has been detected in the haemolymph and reproductive tissues of species of various taxa, including moths (reviewed in Brown et al., 2009). Although ecdysteroids are known to be present throughout life in both male and female adults, their functions in male physiology and behavior are not fully understood. In male *Drosophila melanogaster*, 20E mediates courtship behaviors (Dalton et al., 2009; Ganter et al., 2011), courtship memory and memory and sleep through the action of its receptors (Ishimoto and Kitamoto, 2011; Ishimoto et al., 2009). In the noctuid moth *Spodoptera littoralis*, 20E was recently shown to control the peripheral detection of pheromone (Bigot et al., 2012).

Here, we studied the time characteristics of the PEI in *A. ipsilon*. Moreover, we tested the hypothesis that factors present in the brain or reproductive tissues could induce the observed PEI. Lastly, we investigated the possible involvement of ecdysteroids in this olfactory plasticity.

Materials and methods

Insects

Adult males and females of *A. ipsilon* Hufnagel (Lepidoptera: Noctuidae) originate from a laboratory colony in Bordeaux. Wild insects are introduced into the colony each spring. The animals were reared on an artificial diet in individual cups until pupation. Pupae were sexed and males and females were kept separately in an inversed light/dark cycle (16 h light: 8 h dark photoperiod; scotophase starts at 10 am until 6 pm) at 22 °C, 50% relative humidity. Newly emerged adults were removed every day and were given access to a 20% sucrose solution *ad libitum*. The day of emergence was considered as day-0.

Mating experiments

Mating experiments were performed as previously described (Barrozo et al., 2010a). Briefly, virgin 5-day-old sexually mature males and virgin 3-day-old sexually mature females were individually paired in cylindrical plastic containers before the onset of scotophase in a room under the same inverted light/dark cycle and temperature as cited above. Observations of the pairs were performed to detect the onset and the end of the copulation. In our photoperiod conditions, copulation occurs between 12:30 and 17:30 (mean onset and end of copulation at 14:10 ± 0:56 and 16:03 ± 0:55 respectively; $n = 270$) and lasts between 1 and 2 h (mean duration of copulation: 1 h 53 min ± 28 min; $n = 270$) (Fig. 1). Once copulation had ended, newly-mated males were removed from the observation room. For behavioral tests, they were transferred to the wind tunnel room, and females were dissected to check for the presence of the spermatophore, in order to confirm that mating was successful.

Time onset of PEI was analyzed by testing the behavioral pheromone response of males, which had been allowed to copulate for various time durations (Fig. 1). After the onset of copulation, males *in copula* were manually delicately separated from the females at different time intervals (5, 15 or 30 min) throughout copulation. For the 30-min copulation assays, visual observations of the onset of copulation were performed every 10 min with a red lamp. For the 5 and 15-min copulation assays, observations were performed every 5 min. As males were separated at different times after the onset of copulation, we chose to wait 2 h after the separation time in order to test the behavioral response of mated males at a time, which exceeded the naturally occurring mating duration of 113 min (Fig. 1).

Our previous results showed that mated males did not respond to the sex pheromone when they were tested within 1 h after the end of copulation (Gadenne et al., 2001). To analyze the duration of PEI within the scotophase, we therefore extended the time-window between the end of copulation and the test time by testing the behavioral pheromone response of newly-mated males at least 2 h after the end of copulation, and up to the end of the scotophase (Fig. 1). We thus mainly used males that had started and ended copulation early during the scotophase in order to test a long time-window before the return of the photophase. Control experiments were performed by testing the behavioral response of virgin males to sex pheromone up to the end of the scotophase.

Surgical treatments

Castrated males were obtained by removing the testes of last instar larvae. Larvae were immobilized on a dissecting pad under Ringer's saline, and a dorso-lateral incision was performed in the 4th abdominal segment. Although noctuid larvae can theoretically be sexed according to external structures (Hinks and Byers, 1973), we found it difficult in *A. ipsilon* larvae, and therefore chose to dissect larvae at random. In the case of a male larva, the testes were quickly removed. The wound was dried with a tissue, allowed to heal naturally, and the larva was transferred to the rearing medium. Sham-operated larvae were obtained by performing an incision and gently touching the testes, without extracting them.

Adult males lacking SAGs were obtained by removing the Herold gland (Herold, 1815), i.e. the imaginal disc for the seminal vesicles, SAGs, common duct, and external genitalia of the adult male

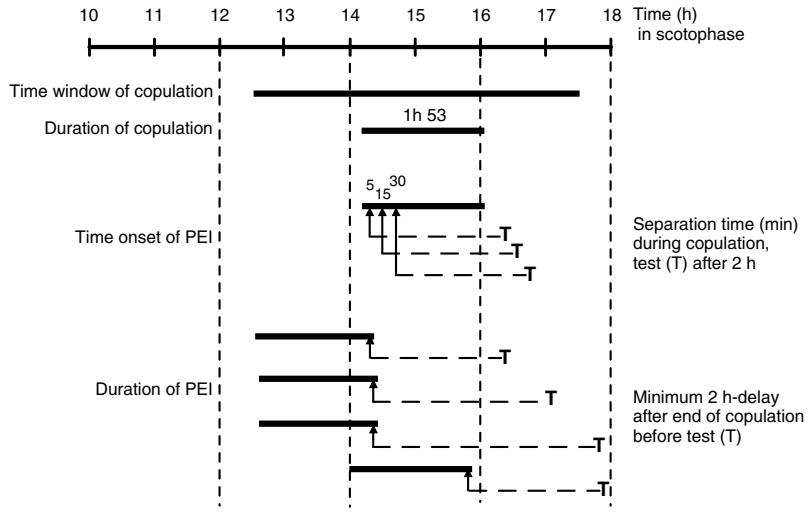

Fig. 1. Schematic representation of mating experiments performed for the study of the initiation and duration of the post-ejaculatory interval inducing the post-mating olfactory switch off in *A. ipsilon* males. The time window of copulation starts at 12:30 and ends at 17:30. The main duration of copulation is 1 h 53 min. Time onset of the PEI was studied by separating males and females in copula after 5, 15 and 30 min. Behavioral tests were performed 2 h after separation. Duration of the PEI was studied by testing mated males at least 2 h after the end of copulation and up to the end of the scotophase.

(Verson and Bisson, 1896) as described previously (Shirk et al., 1983). Briefly, larvae were immobilized on a dissecting pad under Ringer's saline, and a small opening was performed on the 9th abdominal sternite. The Herold gland was localized and quickly removed. The wound was dried with a tissue, and the larva was transferred to the rearing medium. Sham-operated larvae were obtained by performing an incision and gently touching the Herold gland, without extracting it.

Mortality of castrated and SAG-deprived animals did not differ from overall mortality in the rearing (ranging from 40 to 50% between larval and adult stage).

The behavioral response to sex pheromone of 5-day-old castrated and Herold gland-deprived adults was tested in wind tunnel experiments and compared to that of 5-day-old sham-operated adults. Directly after the behavioral tests, castrated and Herold gland-deprived males were checked for the effectiveness of surgery (absence of testes or SAG, respectively).

Tissue collections, extraction, and injections

Brains, testes, and SAGs were dissected from 5-day-old mated and virgin males in the second half of the scotophase, when this species shows the highest reproductive activity (Gadenne et al., 1993; Xiang et al., 2010). Tissues of newly-mated males were dissected out within 1 h following the end of mating. Dissections were conducted in Ringer's solution (8.76 g NaCl; 0.441 g $CaCl_2H_2O$; 0.224 g KCl; 2.29 g TES Buffer in 1 l ultrapure H_2O; +NaOH for pH 6.9; 8.55 g sucrose). In virgin males, the fused SAGs appear thick and strongly reddish colored, whereas SAGs of newly-mated males are thin and translucent.

For cross-injection experiments, tissues were then immediately placed in Eppendorf tubes in liquid nitrogen and stored at –80 °C. Ringer's solution was then added to the frozen tissues: 1 µL for each brain, and 2 µL for each SAG. Tissues were then allowed to thaw on ice and subsequently homogenized with a tissue homogenizer Polytron (Model P200, Fisher Scientific SAS, Illkirch, France) five times on ice for 1 s each. SAGs from virgin males were further centrifuged at 4 °C at 1500 rpm for 10 s, and the supernatant was collected for injections. For injections into the abdomen, each male received a dose of 2 brain-equivalents (2 µL of crude solution) or a dose of 1 SAG-equivalent (1 SAG represents the two fused SAGs from one male; 2 µL of supernatant/4 µL of crude solution). All injections were performed 1 h prior to the behavioral test. Both virgin and mated males were injected with brain and SAG extracts. Virgin males were injected with tissues from mated males, and with tissues from virgin males as controls. Mated males were injected with tissues of virgin males. Untreated (non injected) virgin and mated males served as controls.

For ecdysteroid quantification, fresh organs (brains, testes, SAG) were weighed on an analytical balance (Mettler H54AR, Viroflay, France) as batches of 5 to 10 organs, then stored at −18 °C in methanol (Merck, Semoy, France) (250 µL/10 organs). They were then homogenized with a Polytron (ProScientific Inc, Oxford CT, USA), centrifuged (10 min, 10,000 rpm) and the supernatant was collected. The residue was reextracted with methanol (250 µL) and the pooled supernatants were dried under vacuum in a SpeedVac Concentrator (Eppendorf, Hamburg, Germany). Haemolymphatic ecdysteroid quantifications were also performed in virgin, and in mated males as soon as copulation was terminated (0 h post-mating) and 1 h after the end of copulation (1 h post-mating). To collect haemolymph, the top of an insect's head was cut, and the whole animal was placed in a 0.2 mL tube with a hole in the bottom. This tube was then placed inside a 1.5 mL Eppendorf vial and centrifuged at 500 rpm for 10 min. Haemolymph pooled from 5 insects (10–50 µl/insect were collected) was suspended in ten volumes of methanol (Merck, France) and centrifuged at 10,000 rpm for 10 min. The supernatant was collected and dried as described above, samples were then stored at −80 °C until use.

For high-pressure liquid chromatography (HPLC) analysis, ecdysteroids were extracted from whole virgin males. First, wings were removed, and animals were cut into pieces with scissors, then ground in a glass-Teflon homogenizer (5 males in 5 mL methanol). All the extracts were stirred during 3 h then sonicated and centrifuged (15 min, 4000 rpm). The pellets were resuspended in methanol for a second extraction. All supernatants were pooled and dry-evaporated. After the methanol phase had evaporated, a partition with chloroform/water (1:2, v/v) was performed twice. The aqueous phase was purified on a Sep-Pak C_{18} cartridge (according to Lafont et al., 1982): a polar fraction was first eluted with 5 mL of 30% methanol and free ecdysteroids were then eluted with 5 mL of absolute methanol. Only this fraction was further analyzed by HPLC. The same purification steps were used for a large batch of SAGs (64) from 5-day old virgin males, to analyze their ecdysteroid content by HPLC.

HPLC analyses of ecdysteroids

Ecdysteroids were analyzed on a Beckman apparatus (System Gold, Fullerton, CA, USA), with UV detection at 250 nm, at a flow rate of 1 mL min^{-1} using two HPLC systems: a normal-phase system (NP-HPLC) using a silica column (250 mm × 4.6 mm i.d., Hypersil, Zorbax silica 5 μm, AIT Chromato, Le Mesnil Le Roi, France) and dichloromethane/propan-2-ol/water (125:30:1.5, v/v/v) as solvent; and a reverse-phase system, using a Spherisorb ODS2 column (250 mm × 4.6 mm, C18 5 μm, AIT) and methanol:water (50:50,v/v) as solvent. Fractions were collected every 42 s, evaporated until dry and resuspended in EIA buffer for ecdysteroid quantification (see below). The retention times of immunoreactive fractions were compared to the following reference ecdysteroids: 20E, ecdysone (E), and 2-deoxyecdysone (2dE). All ecdysteroids were generous gifts from Pr René Lafont (UPMC, Paris).

Quantification of ecdysteroids

Ecdysteroids were quantified with an enzyme immunoassay (EIA) adapted from the method described by Porcheron et al. (1989), by using goat anti-rabbit IgG (Jackson Immunoresearch Lab, West Grove, PA, USA) and 2-succinyl-20E coupled to peroxidase as enzymatic tracer. The enzymatic activity was measured using orthophenylenediamine (Sigma Aldrich, Saint-Quentin Fallavier, France) as substrate. For quantification in haemolymph, we used a polyclonal anti-20E antiserum AS4919 (Porcheron et al., 1976, 1989), which displays the same cross-reactivity towards E and 20E (Porcheron et al., 1989). For ecdysteroid quantification in other tissues, the polyclonal anti-ecdysone antiserum L2 (generous gift from Dr. M. De Reggi, Marseille) was used because of its great sensitivity (De Reggi et al., 1992). L2 displayed the highest affinity towards E (10.7 pg yielding 50% maximum binding), and recognized 20E fivefold less than E in our experimental conditions (antiserum L2 and enzymatic tracer used respectively at 1/50,000 and 1/100,000 initial dilution). Dried samples were resuspended in EIA buffer solution. In routine experiments, calibration curves were generated with 20E (ranging from 7.5 pg to 1920 pg) and results were given as 20E equivalents.

Statistical differences in ecdysteroid titers between physiological states were assessed by pairwise comparisons using either Student's t-test or a Mann–Whitney U-test, according to whether prerequisites for the use of parametrical testing were met (Sokal and Rohlf, 1995).

20E injection treatments

20-Hydroxyecdysone was a gift from Pr. R. Lafont (UPMC, Paris, France). Stock solutions of 20E were prepared in ethanol at a concentration of 10^{-2} M, then stored at −20 °C. For experiments, the stock solutions were diluted to 10^{-5} M in a NaCl (145 mM) solution.

Five-day-old virgin males (0.2 ± 0.02 g body weight) were anesthetized with carbon dioxide and received an injection of 2 μL 10^{-5} M 20E (approximately 10 ng) in the abdomen 6 h (at 9 am) or 2 h (at 1 pm) before the behavioral test. The behavioral response to sex pheromone of injected males was then tested during the same day in wind tunnel experiments in the second half of scotophase. Control experiments were performed by injection of 2 μL NaCl solution. At the concentration of 10^{-5} M, 20E has been previously reported to affect the responsiveness of males to sex pheromone in several species of moths (Bigot et al., 2012; Hoelscher and Barrett, 2003).

Wind tunnel experiments

Experiments were performed using a 2 m long wind tunnel under the same inverted photoperiod as for the mating experiments as previously described (Barrozo et al., 2010a,b; Gadenne et al., 2001). Experiments started 4 h after lights off (14:00) and lasted up to lights on (18:00). Environmental conditions during the bioassay were held constant: 22 °C, 50 ± 10% relative humidity, wind speed of 0.3 m s^{-1}. A cage containing a single experimental male was introduced in the wind tunnel. After 30 s during which the male adjusted to the airflow, a filter paper containing the stimulus was placed 160 cm upwind from the cage.

Pheromone stimulation was performed with an artificial pheromone blend containing (Z)-7-dodecen-1-yl acetate (Z7-12:OAc), (Z)-9-tetradecen-1-yl acetate (Z9-14:OAc), and (Z)-11-hexadecen-1-yl acetate (Z11-16:OAc) (Sigma Aldrich, Saint-Quentin Fallavier, France) at a ratio of 4:1:4 (Gemeno and Haynes, 1998; Picimbon et al., 1997). 10 ng of pheromone blend were used for all behavioral tests as this dose was shown to give the best behavioral results with sexually mature virgin males (Barrozo et al., 2010b).

The behavior of the moth was observed during 3 min, and oriented partial flight (at least 1/2 of the distance), complete flight (up to the source without landing), and landing on the pheromone source were considered as oriented response (Jarriault et al., 2009). Oriented as well as random flights were counted altogether in order to quantify the general flight activity of insects. Both untreated virgin males (which are expected to orient towards the pheromone) and treated experimental males were tested in the wind tunnel during each experimental day.

Statistical differences between groups of copulation-length manipulated and cross-injected experimental males were evaluated using a $R \times C$ test of independence by means of a G-test and applying the Williams's correction (Sokal and Rohlf, 1995). In addition, individual *post hoc* comparisons were carried out and the experimentalwise error rate was adjusted by using the Dunn-Šidák method (Sokal and Rohlf, 1995). Statistical differences between groups of NaCl- and 20E-injected males were evaluated by means of a G-test and applying the Williams's correction (Sokal and Rohlf, 1995). Statistical differences between groups of surgically treated experimental males were assessed by means of 3 × 2 χ^2-tests (Sokal and Rohlf, 1995).

Results

Time onset of PEI

Males were tested in the wind tunnel for their behavioral response to sex pheromone. In virgin males, the general flight activity was high (roughly 90%), and 55% of them performed an oriented response to pheromone (Fig. 2). None of the newly-mated males that were left to copulate normally (average duration 113 ± 28 min) showed an oriented response (Fig. 2, control).

Males manually separated from females during copulation were tested 2 h later. Males separated 30 min and 15 min after the onset of copulation did not show any oriented behavioral response (Fig. 2). Twenty-two percent of the males that were separated from their partners 5 min after the onset of copulation showed an oriented response, however still statistically different from the responses of virgin males ($G = 14.13$; $df = 1$; $P \leq 0.001$) (Fig. 2). The general flight activity of mated males tested after the different copulation durations was statistically different from that of virgin males ($G = 20.79$; 69.39; 56.75; 52.03; $df = 1$; $P \leq 0.001$ between virgin and durations of 5 min; 15 min; 30 min; and control copulation, respectively) (Fig. 2). However, general flight activity was significantly higher in males that were mated for 5 min as compared with males mated for 15 min ($G = 15.05$; $df = 1$; $P \leq 0.001$), 30 min ($G = 8.28$; $df = 1$; $P \leq 0.01$), and control males ($G = 7.2$; $df = 1$; $P \leq 0.01$), but still differed from that of virgin males ($G = 20.79$, $df = 1$; $P \leq 0.001$) (Fig. 2).

Altogether these results show that the initiation of olfactory switch-off occurs a few minutes after the onset of copulation.

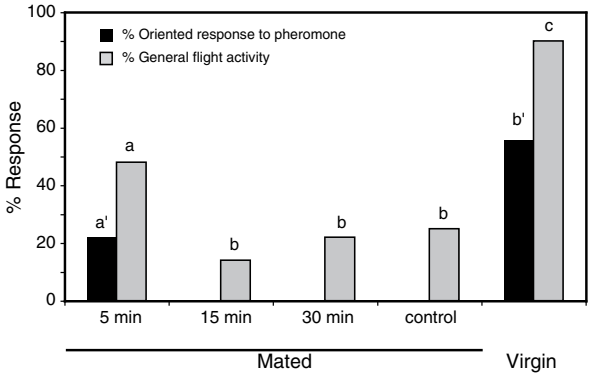

Fig. 2. Effects of mating duration on pheromone response in *A. ipsilon* males. Day-5 males were allowed to copulate with day-3 mature females, and then forced apart from the females at different times (5, 15, 30 min) after the onset of copulation (n = 50, 50, and 56 males respectively). They were then tested for their response to sex pheromone in a wind tunnel 2 h later. A control group of males (n = 50) was left to copulate without separation (mean copulation time: 113 min), and they were tested 1 h after the end of copulation (control). Virgin males (n = 66) were also tested in the wind tunnel. Bars with the same letters are not statistically different (G-test, $P \leq 0.05$).

Duration of PEI

Not a single mated male out of 31 tested at least 2 h after the end of copulation responded to the pheromone, independently of the delay between the end of copulation and the behavioral test.

This result shows that the PEI lasts at least the whole scotophase, i.e. the period of activity, after copulation.

Effects of tissue cross-injection on pheromone response of virgin and mated males

Tissues of virgin and mated males were injected into mated and virgin experimental males respectively, and their response to an artificial pheromone blend was quantified in a wind tunnel (Fig. 3). A general $R \times C$ log likelihood test of independence (G-test) revealed significant differences between groups (Gadj = 232.26, df = 7, $P \leq 0.001$). More precisely, control mated males did not show any oriented responses as compared with control virgin males (Gadj = 63.17, df = 1, $P \leq 0.001$) (Fig. 3). Also, compared with virgin control males, mated males did not respond to pheromone after the injection of brain (M + vBr) (Gadj = 72.62, df = 1, $P \leq 0.001$) or SAG (M + vSAG) (Gadj = 64.61, df = 1, $P \leq 0.001$) extracts from virgin males (Fig. 3).

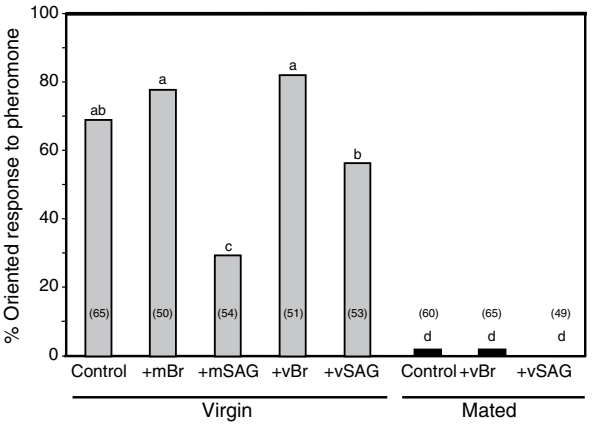

Fig. 3. Effects of brain/SAGs injections on the behavioral response of *A. ipsilon* male moths to sex pheromone. Brains (Br) and sex accessory glands (SAGs) from virgin (vBr/vSAG) and mated males (mBr/mSAG) were injected into mated and virgin males respectively. vBr and vSAG were also injected into virgin males as controls. See Materials and methods section for details. Number in brackets represent the number of tested males. Grey bars: virgin males, black bars: mated males. Bars with the same letters do not differ statistically (G-test, $P \leq 0.05$).

Responses of virgin males injected with brain extracts from virgin males (V + vBr) or mated males (V + mBr) did not differ from that of control virgin males (Gadj ≤ 2.41, df = 1, n.s.) (Fig. 3). The response of virgin males injected with SAG extracts from virgin males (V + vSAG) was not different from that of control virgin males (Gadj = 1.93, df = 1, n.s.) (Fig. 3). However, the response of virgin males injected with SAG extracts of mated males (V + mSAG) was significantly lower than that of virgin control males (Gadj = 18.14, df = 1, $P \leq 0.001$) (Fig. 3).

These results show that the behavioral pheromone response of mated males could not be restored by any of the performed tissue injections. On the contrary, the response of virgin males injected with SAG extracts from mated, but not virgin, males was significantly reduced.

Effects of mating on ecdysteroid levels in brain and reproductive tract

In brains of both virgin and mated males, ecdysteroids were below the EIA detection levels. In testes, ecdysteroid levels were very low in virgin 5-day old males (10.4 ± 0.8 ng 20E equivalents/organ) and below the EIA detection limit in newly-mated males (Fig. 4A). In SAGs of virgin males, ecdysteroid levels were significantly higher per animal (103 ± 37 ng 20E equivalents) than in SAGs of post-mated males (8.8 ± 0.9 ng/animal) (Mann–Whitney U-test; df = 20, P = 0.00021) (Fig. 4B).

Effects of mating on haemolymph ecdysteroid levels

Ecdysteroid levels were quantified in the haemolymph of virgin and mated males, 0 h and 1 h after mating (Fig. 5). Compared with virgin males, ecdysteroid titers were significantly higher in newly-mated males at 0 h (t-test; df = 21, P = 0.038) and 1 h (t-test; df = 27, P = 0.026) after the end of copulation (Fig. 5).

Identification of ecdysteroids in A. ipsilon males

Using HPLC separation of ecdysteroids followed by EIA, we analyzed the nature of ecdysteroids present in whole virgin 5-day old males and their SAGs. In NP-HPLC, the major immunoreactive peak in both samples co-migrated with reference 20E (Figs. 6A and B). Two other immunoreactive smaller peaks were detected in whole animals, co-migrating with 2dE and E, respectively (Fig. 6A). The fractions corresponding to each of the three ecdysteroid peaks from whole animals were pooled, purified and further separately analyzed

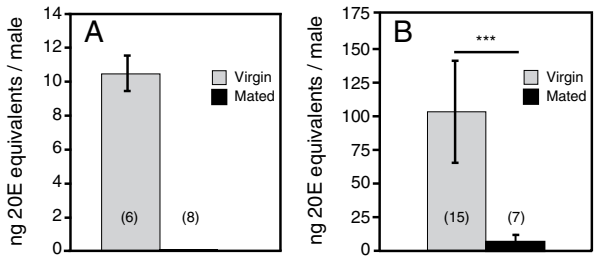

Fig. 4. Ecdysteroid titers in testes (A) and sex accessory glands (SAGs) (B) as a function of mating status in *A. ipsilon* males. Quantification by EIA, expressed as ng 20E equivalents per male (mean ± SD). Ecdysteroid titers were under detection limits in testes from mated males (A). Numbers in brackets represent the number of repetitions of 10 organs. Asterisks indicate a statistical difference between groups (Mann–Whitney U-test, $P \leq 0.05$).

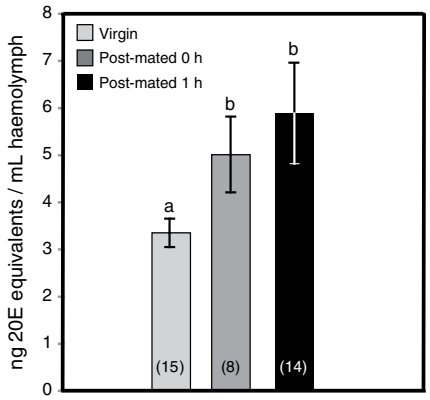

Fig. 5. Ecdysteroid titers in the haemolymph as a function of mating status in *A. ipsilon* males. Ecdysteroids were quantified by EIA, and titers expressed as ng 20E equivalents per mL haemolymph. Ecdysteroid levels are lowest in virgin males (grey bar), and increased in males straight after (0 h) or 1 hour (1 h) after the end of copulation. Bars (mean ± SD) with the same letters are not statistically different (t-test, $P \leq 0.05$). Numbers in brackets represent the number of repetitions of 5 pooled males.

with a RP-HPLC system followed by EIA. Each peak gave rise to an immunoreactive compound co-migrating with the same ecdysteroid as observed in NP-HPLC (data not shown). This confirms the presence of 20E, 2dE and E in *A. ipsilon* males. The same ecdysteroids were also detected in SAGs, with 20E being predominant (Fig. 6B).

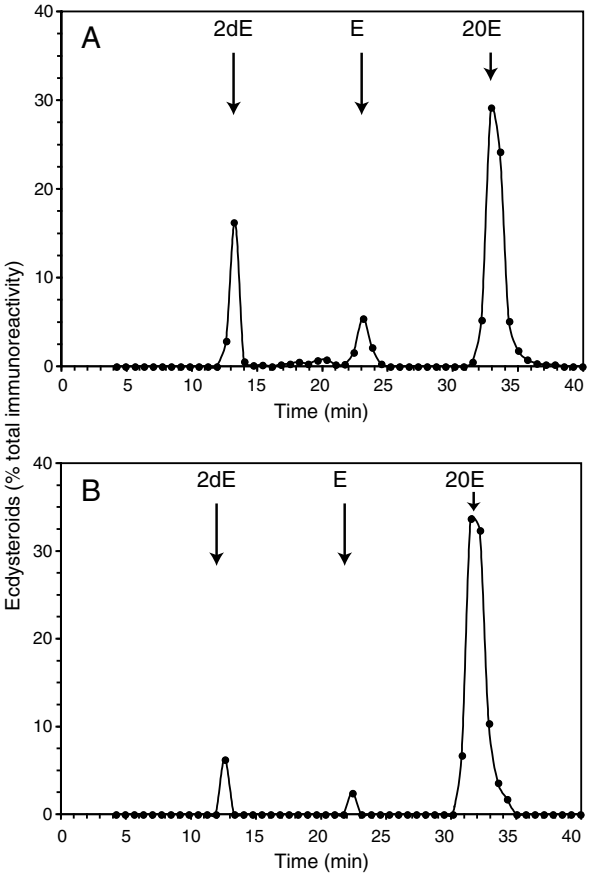

Fig. 6. Normal-phase HPLC-EIA analysis of free ecdysteroids in 5-day old virgin males of *A. ipsilon*. (A) Whole animals, (B) SAGs. HPLC conditions : Silica column Zorbax Sil; solvent, dichloromethane/propan-2-ol/water (125:30:1.5, v/v/v); flow rate of 1 mL min^{-1}. Arrows indicate retention times of ecdysteroid standards: 20E, 20-hydroxyecdysone; E, ecdysone; 2dE, 2-deoxyecdysone. Ecdysteroids were quantified by EIA in 0.7-min fractions as 20E equivalents, and are expressed as percentage of total immunoreactivity.

Effects of 20E injection on pheromone response in sexually mature virgin males

There was no statistical difference in the oriented responses between 20E- and NaCl-injected 5-day-old virgin males to sex pheromone when injections were performed 6 h ($G = 0.040$, $df = 1$, $P = 0.841$) or 2 h ($G = 0$; $df = 1$; $P = 1$) before the behavioral test (Fig. 7).

Effects of testes or SAG deprivation on pheromone response

We analyzed the behavioral response of virgin males (responding to the pheromone) lacking testes or SAGs. There was no statistical difference in the proportion of testes-deprived males ($n = 34$), sham-operated ($n = 36$), and control males ($n = 73$) responding to an artificial sex pheromone blend ($\chi^2 = 3.96$, $df = 2$, $P = 0.13$) (Fig. 8A).

Similarly, there was no statistical difference in the proportion of SAG-deprived males ($n = 47$), sham-operated ($n = 26$), and control males ($n = 77$) responding to an artificial sex pheromone blend ($\chi^2 = 1.01$, $df = 2$, $P = 0.602$) (Fig. 8B).

Discussion

Although the post-mating effect on female receptivity has been studied in detail in many insect species including moths (review in Gillott, 2003), very little is known on the effects of mating on the sexual inhibition in male insects. Since a few years, we have focused our studies on the PEI, characterized by a fast and transient inhibition of pheromone responses, in *A. ipsilon* males. Results of the present study allow us to extend our knowledge on i) the timing of this PEI, ii) the tissues involved in the PEI, and iii) the possible role of ecdysteroids.

The inhibition of the pheromone response starts very early after the onset of copulation and lasts up to the end of the scotophase

When copulating males were forced apart from the females only 15 min after the onset of copulation, we found that their olfactory system was already switched off (no response to pheromone in the wind tunnel). Post-hoc dissection of females showed an absence of spermatophore in their reproductive tracts. Only a whitish milky

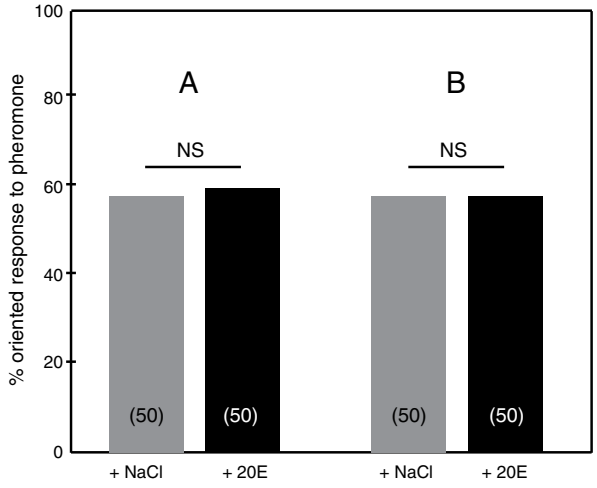

Fig. 7. Effects of 20E injections on pheromone responses in virgin *A. ipsilon* males. 20E or NaCl was injected in males 6 h (A) or 2 h (B) before the behavioral test. Numbers in brackets represent the number of tested males. G-test, $P \leq 0.05$. See Materials and methods section for details.

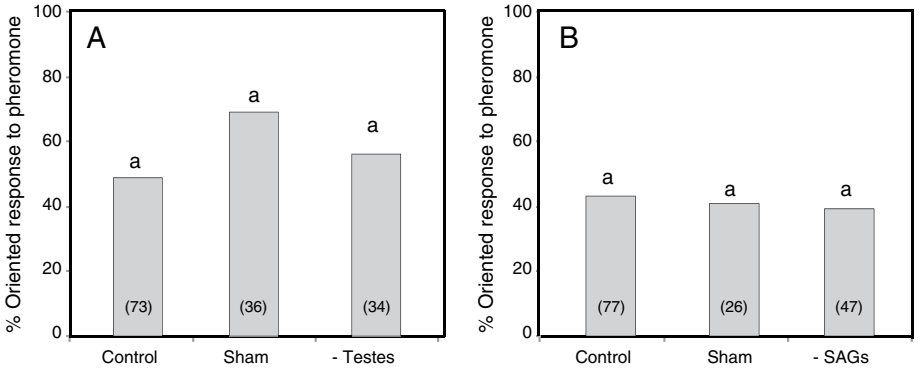

Fig. 8. Effects of testes (A) and sex accessory gland (SAGs) deprivation (B) on pheromone responses in *A. ipsilon* males. For each experiment, bars with the same letters are not statistically different (χ^2 test, $P \leq 0.05$). Numbers in brackets represent the number of tested males. See Materials and methods section for details.

liquid was seen in the tracts. Similarly, the absence of a spermatophore was observed in females of *Heliothis zea* (Raina, 1989) and *Spodoptera litura* (Seth et al., 2002), when males were separated after a few minutes of copulation (5–15 min). In contrast to the lack of response after 15–30 min copulation, males that were forced apart from females after 5 min of copulation still showed a positive behavioral response to sex pheromone. When we performed detailed observations of copulation behavior, we sometimes noticed copulation attempts that were not successful: males and females went apart after few seconds or minutes of copulation. The very same males successfully mated afterwards during a second attempt. We hypothesize that the olfactory switch off in *A. ipsilon* males takes place only after the copulation has been secured (males and females are really in copula), i.e. when the formation of a spermatophore is engaged. This does not seem to be the case in *Nasonia* wasps, in which the post-mating behavioral switch in females has been shown to be independent of any genital contact (Ruther et al., 2010). However, the observed delay of the PEI in *A. ipsilon* would allow males which have failed to start a true copulation to be still responsive to another female for a possible new copulation attempt.

Although a few studies have shown a post-mating decrease of sexual responsiveness in males of insect species (Fischer and King, 2008; Lachmann, 2000; Norville et al., 2010; Reddy and Guerrero, 2000; Sakai et al., 1995; Ureshi and Sakai, 2001), information on the latency of this PEI is scarce.

In contrast, the delay of the post-mating decrease in sexual receptivity has been described in insect females. In the parasitic wasp *Nasonia vitripennis*, mated females cease to respond to the male pheromone as soon as 5 min after the end of copulation (Ruther et al., 2007). In *Ceratitis capitata*, mated females start to respond to fruit odor and stop to respond to the male pheromone, but no information on the latency is given (Jang, 1995).

In *A. ipsilon* the male PEI lasts up to the end of the scotophase: no behavioral response was observed between the end of copulation and the end of the scotophase. We previously showed that males were unable to remate within the same scotophase (Gadenne et al., 2001). Altogether these results show that as soon as the male has engaged in copulation (i.e., presumably started formation of a spermatophore) and up to the end of scotophase, its pheromone-specific olfactory system is switched off.

The inhibition of pheromone response is induced by a putative factor produced in the SAGs after or during copulation

The behavioral response of virgin males to pheromone is decreased after injection of SAGs of mated donor males. Contrarily, the response of mated males could not be restored by injection of SAG extracts of virgin donor males. This could be due to a factor produced in the SAGs, inducing the inhibition of pheromone response observed in mated males. Surprisingly, although we assumed that this SAG factor would act at the central nervous level to induce the PEI, injection of brain extracts from mated males had no effect on virgin males. However we cannot exclude the possibility that the quantity of brain extract injected (2-brain equivalent) was not sufficient to elicit an effect in virgin males.

Our data show that surgical deprivation of virgin males from either their testes or SAGs did not reduce their responsiveness towards sex pheromone. Moreover, we show that soon after onset of copulation, males presumably engaged in spermatophore production ceased to respond to sex pheromone. This lead us to hypothesize that a factor is newly or over-produced in SAGs after mating (rather than decreased) to induce the PEI. Unfortunately we could not confirm the role of this SAG factor in the induction of the PEI by testing their behavioral response after mating, because SAG-deprived males cannot copulate, as they lack the external genitalia.

It was found in Drosophila and moths that SAGs contain a peptide, named sex peptide, which is transferred into the female during copulation, switching off its pheromone production (reviewed in Kubli, 2003). Recently this peptide has been shown to act in females at the central nervous level through its receptors in order to induce the cascade of events leading to the switch off of pheromone production (Rezával et al., 2012).

Although we have not yet searched for its existence and possible role in this moth, previous data showed that haemolymph titers of sex peptide were similar in virgin and mated males of *H. zea* (Raina, 1989), thus reducing the probability that this peptide could indeed act in the male itself and induce the PEI.

Other factors such as ecdysteroids (*D. melanogaster*: Harshman et al., 1999; *Anopheles gambiae*: Pondeville et al., 2008) and JH (*Heliothis virescens*: Park et al., 1998; *Apriona germari*: Tian et al., 2010) are also produced in male SAGs and transferred to females during copulation. JH is probably not involved in the PEI, as we previously showed that levels of JH biosynthesis did not change in males following mating (Duportets et al., 1998).

20E is present in SAGs but probably not involved in the induction of the pheromone-specific post-mating inhibition in A. ipsilon males

Our analysis of ecdysteroids in *A. ipsilon* revealed 20E as the major form. No ecdysteroids were detected in brains of virgin males. This is not surprising, as brains of most insect species appear to be incapable of ecdysteroid synthesis (Warren et al., 1999). However, brains from honeybee workers cultured *in vitro* could release high amounts of 20E, but did not store it (Yamazaki et al., 2011). In vitro incubation

of brains from *A. ipsilon* males did not allow us to detect any ecdysteroid in the culture medium (data not shown). Presence of 20E has been described in adult testes of different insect species (reviewed in Brown et al., 2009). However, in *A. ipsilon* testes, ecdysteroid levels were low in virgin males and not detectable in post-mated males. They were 10 times higher in SAGs of virgin males. These organs have been shown as a site of ecdysteroid secretion in *Blattella germanica* (Gillott and Ismail, 1995) and *A. gambiae* (Pondeville et al., 2008). Levels of 20E in SAGs were significantly lower in post-mated males than in virgin males. In mosquito males, SAG levels of 20E also decrease after mating, and it was shown that 20E is transferred during mating to the female (Pondeville et al., 2008). This could also be the case in *A. ipsilon*. Haemolymph titers of 20E increased significantly after mating, suggesting that copulation could induce a shift of the hormone from the SAGs into the haemolymph. However, the post-mating 20E increase in the haemolymph could also originate from other tissues that we have not identified so far. More generally, there is a lack of knowledge in the endocrinology of reproduction in male moths (De Loof, 2006).

Given that injection of mated male SAGs containing low levels of 20E modifies the behavior of virgin males, it is unlikely that 20E alone induces the observed post-mating inhibition. Moreover, the injections of 20E in *A. ipsilon* virgin males performed at two different times before the test did not induce the expected decrease in their behavioral response, in opposition to recent results in *S. littoralis* (Bigot et al., 2012). Although the quantity of 20E injected in virgin males (10 ng) was higher than that in the haemolymph (3–6 ng/mL haemolymph: approximately 0.25 ng/male), we cannot exclude the possibility that low levels of another form of ecdysteroids are involved in the PEI of *A. ipsilon*.

Work is now in progress to identify the putative factor(s) produced/accumulated in the SAGs during mating, which could elicit the very fast olfactory switch-off at the central nervous level. Transcriptomic and peptidomic comparative profiles of both SAGs and brains from mated and virgin males might elucidate the modulators at the origin of the switch off.

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A new species of *Satyrichthys* (Teleostei: Peristediidae) from the Maldives Archipelago (Indian Ocean)

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Abstract

A new species of the genus *Satyrichthys*, *Satyrichthys kikingeri* **sp. nov.**, is described from the Rasdhoo Atoll, Maldives Archipelago. The new species is placed in a group of *Satyrichthys* with at least three lip barbels and unequal parietal bones. It differs from its congeners in the combination of the following characters: (1) 3/3 lip and 1/0 chin barbels, (2) 15 fin rays in the second dorsal fin, 13 fin rays in the anal fin, (3) 25 bony plates in the dorsal, 29 in the upper lateral and 20 in the lower lateral rows, (4) 21st to 28th bony plates in the upper lateral row with forward directed spines and (5) parietal bones unequal in size on midline. *Satyrichthys kikingeri* **sp. nov.** is the first *Satyrichthys* species reported from the Republic of the Maldives.

Key words: Peristediidae, *Satyrichthys kikingeri* **sp. nov.**, Indian Ocean, Maldives

Introduction

The scorpaeniform family Peristediidae (armored sea robins) is characterized by (1) a body entirely enclosed by four rows of spinous bony plates (scutes) on each side; (2) a large bony head with spines and ridges; (3) each first infraorbital (lachrymal) anteriorly extending in distinct rostral projections; (4) barbels on the lower jaw, and (5) pectoral fins with the two ventral most fin rays free and enlarged (Miller 1974; Miller & Richards 2002; Richards 1984, 1999; Kawai 2008, 2013).

About 33 peristediid species in six genera (*Gargariscus* Smith 1917, *Heminodus* Smith 1917, *Paraheminodus* Kamohara, 1958, *Peristedion* Lacépède 1801, *Scalicus* Jordan 1923 and *Satyrichthys* Kaup 1873) are known, all from deep waters of tropical and temperate oceans (Kawai 2008, 2013). The distribution of most species is only fragmentarily known and the phylogeny and relationship of peristediid fishes was poorly understood (Kamohara 1957, Miller 1974, Chen & Shao 1988, Kawai *et al*. 2004a). Kawai (2008) revised the genus-level classification of the armored sea robins in a comprehensive study on the phylogeny of peristediid fishes and separated the two closely related genera *Satyrichthys* and *Scalicus*.

The genus *Satyrichthys* is diagnosed as follows: (1) no teeth in upper and lower jaws; (2) lateral margin of the head smooth; (3) the ventral row of bony plates on caudal peduncle extending posteriorly, separating the posterior bony plates of the lower lateral series; (4) only the posterior lip and chin barbels branched, and (5) fewer than 20 soft rays in dorsal and anal fin (Kawai 2008, 2013).

In his recent review of the genus *Satyrichthys*, Kawai (2013) recognizes seven species: *S. clavilapis* Fowler, 1938, *S. laticeps* (Schlegel 1848), *S. longiceps* (Fowler 1943), *S. milleri* Kawai, 2013, *S. moluccense* (Bleeker 1850), *S. rieffeli* (Kaup 1859) and *S. welchi* (Herre 1925). One specimen of an undescribed species of the genus *Satyrichthys* was collected by one of us (C. P.) in the Maldives in 2012. We provide a description of the new species.

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Material and methods

The Maldives archipelago is located in the tropical Indian Ocean, south-west of India (Anderson *et al.* 2011). It stretches north–south from about 7°N to about 0.5°S, and consists of 22 atolls arranged in two parallel rows which are separated by an Inner Sea basin (Gischler 2006; Anderson *et al.* 2011), forming the central and largest part of the Chagos-Laccadive Ridge (Anderson 1998). The archipelago rises steeply from the Indian Ocean seabed. The outside atoll rim reef slope drops to about 30–50 m, slopes gently for about half a kilometer to 125–170 m depth, finally dropping to abyssal depths (Anderson 1998). Between the double-chain of atolls in the central Maldives, bottom depths range between 200–500 m, while outside the atolls, the reef slopes drop steeply to the Indian Ocean seabed, at about 2,000–3,000 m (Anderson *et al.* 2011). The archipelago stretches North-South for about 1,000 km and East-West for up to 150 km.

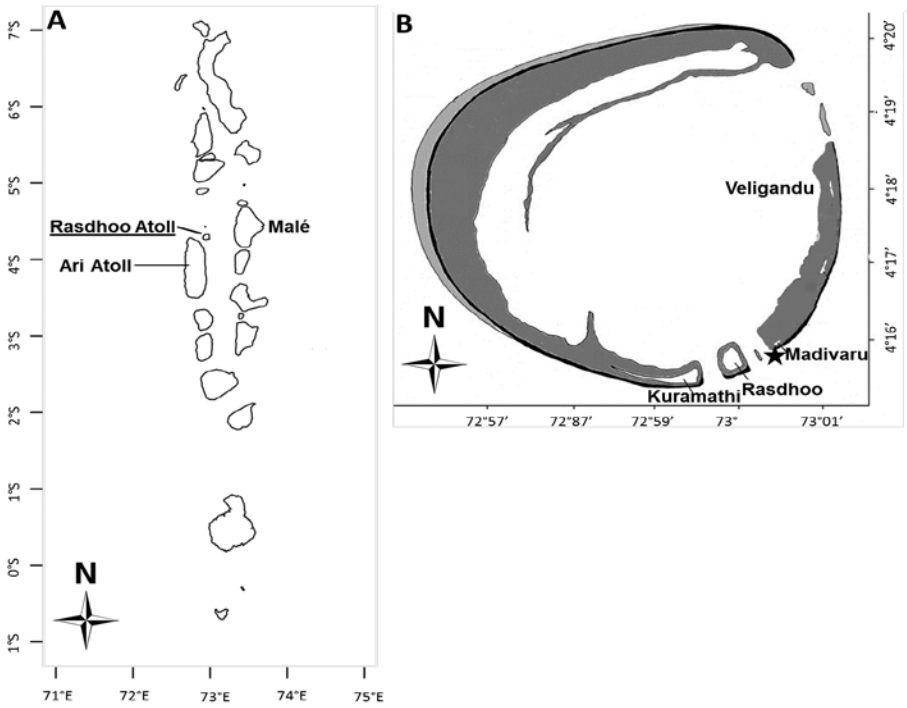

FIGURE 1. Collection site of *Satyrichthys kikingeri*. (A) Location of Rasdhoo Atoll, Ari Atoll in the Maldives Archipelago. Adapted from Anderson *et al.* (2011). (B) Rasdhoo Atoll, North Ari Atoll, Maldives. The collection site off Madivaru in Madivaru Channel is marked with a filled asterisk. Adapted from Gischler (2006).

On 4 May 2012, one specimen of *Satyrichthys kikingeri* **sp. nov.** was collected off the uninhabited sand and rubble island Madivaru in the Madivaru channel (Fig. 1), Rasdhoo Atoll, Maldives, floating dead on the surface. Rasdhoo is a small ring-shaped atoll of 9.25 km in diameter (Gischler 2006) and belongs to the administrative district of North Ari (Alifu Alifu) Atoll.

208 In the systematics of the Peristediidae we follow Kawai (2008, 2013) and of the genus *Satyrichthys* (*sensu* Kawai 2013). Morphometrics and meristics follow Kawai *et al*. (2004b). Grouping of barbels (lip and chin barbels respectively) follow Miller (1974) and Kawai (2013). Nomenclature of head spines follows Miller (1967). Morphometric characters were taken with an electronic caliper to the nearest 0.1 mm. The standard and head lengths are abbreviated as SL and HL, respectively. Counts and measurements were made twice, the data are presented as mean. The holotype is deposited in the Ichthyological Collection of the Naturhistorisches Museum Wien (NMW 96546), Austria.

***Satyrichthys kikingeri* sp. nov.**
Kikinger's robust-armed gurnard
(Figs. 1, 2; Tables 1, 2)

Holotype. NMW 96546, 442 mm SL with rostral projections, 428 mm without; Maldives Archipelago, Rasdhoo Atoll, Madivaru Channel, 4°15'52''N, 73°0'1.6''E, collected May 4, 2012.

Diagnosis. A *Satyrichthys* with (1) three lip and 0/1 chin barbels, (2) bony plates in the upper lateral row of the caudal peduncle with forward directed spines, (3) parietal bones unequal in size on midline, (4) no dusky spots on head, trunk, tail or fins.

Description. Measurements, proportional values and counts of the holotype are provided in Table 1 and Table 2 respectively.

Body fusiform, tapering, covered with bony plates arranged in four longitudinal rows. All plates with posteriorly directed spines except the last five plates in the dorsal and ventral rows; 21st to 28th plates in upper lateral row with doubled spines, the anterior forward-oriented. First dorsal fin originating between first and second bony plates of the dorsal row. The two ventral free rays of the pectoral fin are thickened and the dorsal free ray longer than the ventral ray. Caudal fin small, emarginate with posterior lobe longer than ventral lobe (Fig. 2).

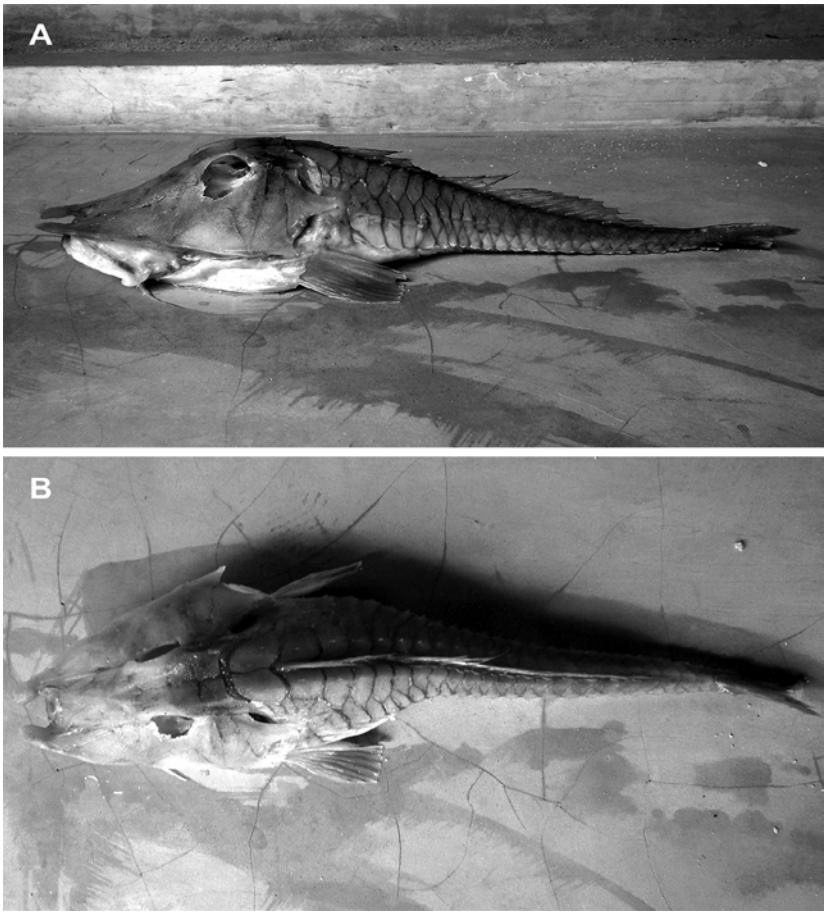

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FIGURE 2. Holotype of *Satyrichthys kikingeri*, NMW 96546, 442 mm SL with rostral projections, 427.7 mm without rostral projections; Maldives Archipelago. (A) Lateral view. (B) Dorsal view.

Head large, depressed, with expanded edges. Rostral projections short, flat and wide with rounded tips pointing inwards. Distance between rostral projections less than interorbital distance at the base (Fig. 2B). Mesethmoid with one tiny, barely discernible mesethmoid spine. Frontal with one small supraorbital spine located dorsally of posterior quarter of orbit. Lateral ridge of parietal ends in a distinct parietal spine. Posttemporal ends in a weak

spine. Nuchal plate (first plate of dorsal row) bearing a small nuchal spine dorsal of the second bony plate of the upper lateral row. Opercle with two conspicuous opercular spines, upper slightly shorter than lower. Preopercular ridge ending posteriorly in a single short preopercular spine; extending anteriorly on the infraorbitals 2 and 3 to about dorsal of the angle of the mouth, and separated from the ridge extending posteriorly from the rostral projections (infraorbital 1) by a short gap.

Mouth large, inferior. Lower jaw not reaching below orbit. Premaxilla, dentary, vomer, palatine toothless. Isthmus between gill membranes wide. Three barbels on lower lip. Anterior two barbels short and unbranched, posteriormost barbel long and branched. One small single barbel on left side of chin. Formula for barbels: 3/3 - 1/0 (the slash separates barbels of the left and the right mandible) (Fig. 3).

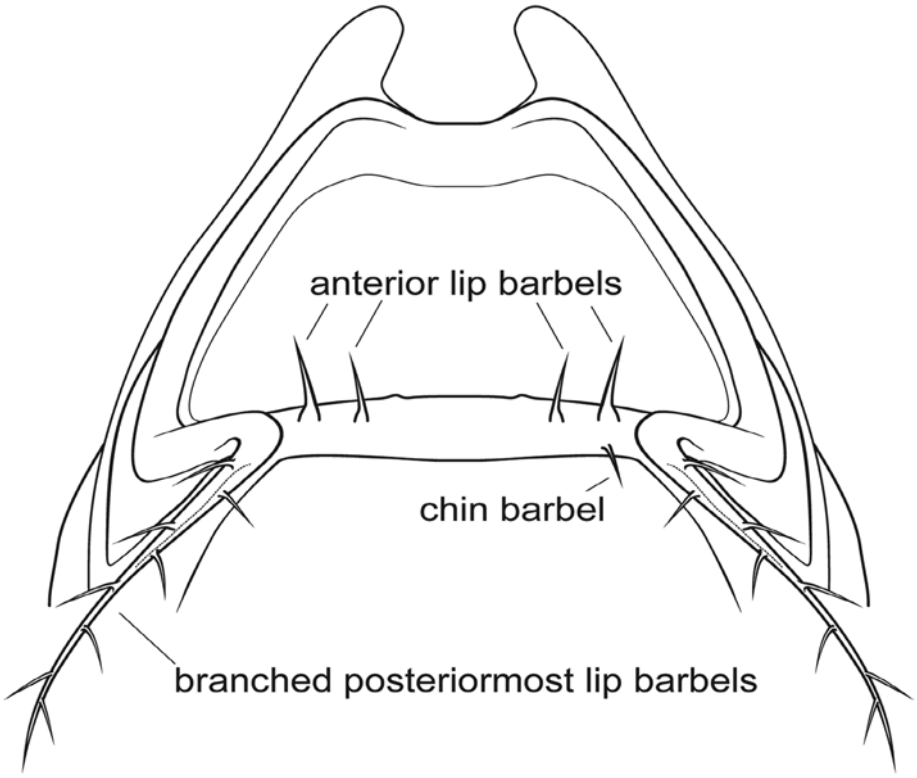

FIGURE 3. Illustration of the ventral side of the lower jaw, showing barbel arrangement of the holotype of *Satyrichthys kikingeri*, NMW 96546, 442 mm SL with rostral projections, 427.7 mm without rostral projections. Maldives Archipelago.

Coloration. Description of coloration is based on pictures taken shortly after collecting the dead specimen and after preservation. Head, body, and fins uniformly bright red orange after collection, ventral surfaces white. Color of specimen preserved in 4 % seawater-buffered formaldehyde pale fawn. No body markings have been identified before or after preservation.

210 **Etymology.** Named for the marine biologist and conservationist Dr Reinhard Kikinger who headed the previous biostation and current EcoCentre at Kuramathi Island Resort in Rasdhoo Atoll, Maldive Islands, from 1999-2012.

Distribution. Known only from Rasdhoo Atoll in the Maldives Archipelago, depth unknown.

Remarks. *Satyrichthys kikingeri* **sp. nov.** differs from its congeners in the combination of the following characters: (1) 3/3 lip and 0/1 chin barbels, (2) 15 fin rays in the second dorsal fin, 13 fin rays in the anal fin, (3) 25 bony plates in the dorsal, 29 in the upper lateral and 20 in the lower lateral and ventral rows, (4) 21st to 28th bony plates in the upper lateral row with forward directed spines, (5) parietal bones unequal in size on midline and (6) shape of emarginated caudal fin with dorsal lobe longer than ventral lobe.

TABLE 1. Morphometric measurements for the holotype of *Satyrichthys kikingeri*.

Morphometric characters	length [mm]	% in SL	% in HL
Standard length	427.7	100.0	
Body depth	97.1	22.7	54.4
Body width	99.2	23.2	55.7
Head length	194.9	41.7	100
Head depth	97.8	22.9	54.9
Head width	134.2	31.3	75.3
Distance from snout to origin of first dorsal fin	196.2	41.2	98.8
Distance from snout to origin of anal fin	284.3	59.6	143.1
Distance from snout to anus	280.1	58.8	141.0
Distance from anus to origin of caudal fin	172.3	40.3	96.7
Snout length	95.1	22.2	53.4
Rostral Projection length	24.2	5.7	13.5
Longest barbel length	52.3	12.2	29.3
Upper jaw length	70.4	16.5	39.5
Lower jaw length	69.9	16.3	39.2
Orbital diameter	32.6	7.6	18.3
Interorbital width	33.9	7.7	19.0
Preopercular spine length	25.1	5.9	14.1
Pectoral fin length	80.2	18.8	45.0
Length of dorsal detached pectoral fin ray	54.1	12.6	30.4
Length of ventral detached pectoral fin ray	41.9	9.8	23.5
Pelvic fin length	71.8	16.8	40.3
Length of first dorsal spine	49.3	11.5	27.2
Caudal fin length	52.2	12.2	29.3
Caudal peduncle length	43.3	10.1	24.3
Caudal peduncle depth	18.1	4.2	10.2
Length of base of first dorsal fin	56.4	13.2	31.6
Height of first dorsal fin	48.0	11.2	26.9
Length of base of second dorsal fin	148.2	34.7	83.2
Heigth of second dorsal fin	26	6.1	14.6
Length of base of anal fin	137.5	32.1	77.2
Height of anal fin	29.1	6.8	6.3

Similar species. An important character in separating species of the genus *Satyrichthys* is the number of lip and chin barbels (Miller 1964, Kawai 2008, 2013). With only one or two lip barbels *S. longiceps*, *S. clavilapis* and *S. rieffeli* differ from all other *Satyrichthys* species which are characterized by at least 3 lip barbels on both sides of the lower jaw.

With three lip barbels *S. kikingeri* is most similar to *S. laticeps*, *S. milleri*, *S. moluccense* and *S. welchi* (Kawai 2013) but is easily separated from *S. milleri* and *S. welchi*.

S. kikingeri differs from *S. milleri* in the number of lip and chin barbels (3/3 and 1/0 vs. 4–5/4–5 and 4/4), number of anal fin rays (13 vs. 15–17), number of preopercular spines (1 vs. 1–2) and the shape of posterior spines of the dorsal row (doubled vs. single)

S. kikingeri differs from *S. welchi* in the number of lip and chin barbels (3/3 and 1/0 vs. 4/4 and 3/3), the number of dorsal (15 vs. 17–19) and anal (13 vs. 17–18) fin rays, the size of the parietal bones (different size of both parietals vs. same size) and in the coloration of the dorsal fins (uniformly colored vs. dusky spots).

TABLE 2. Meristic characters for the holotype of *Satyrichthys kikingeri*.

Meristic characters	total number
Dorsal fin rays	VII + 15
Anal fin rays	13
Pectoral fin rays (including two free rays)	15
Pelvic fin rays	I + 5
Caudal fin rays	12
Bony plates in dorsal row	25
Bony plates in upper lateral row	29
Bony plates in lower lateral row	20
Bony plates in ventral row	20
Bony plates before anus	2
Bony plates in upper lateral row with forward directed spines	8 (21st–28th)
Upper gill rakers	5
Lower gill rakers including the one at angle	17
Lip barbels (left/right)	3/3
Chin barbels (left/right)	1/0
Longest lip barbel bearing X/X flagella (left/right)	8/8
Branchiostegal rays	6

S. kikingeri is obviously close to *S. moluccense* and *S. laticeps* but distinguishable on meristic and morphometric characters (Tables 1, 2).

S. kikingeri and *S. moluccense* have the same number of lip barbels in common and differ slightly in the number of chin barbels (1/0 vs. 0 or 2/0 or 2). Both species differ in the number of second dorsal (15 vs. 17–18) and anal (13 vs. 17–19) fin rays, number of bony plates in the upper lateral (29 vs. 31–34) and dorsal (25 vs. 26–28) rows, the size of the parietal bones (different size of both parietals vs. same size) and in body proportions (%HL / %SL) (Table 1, Kawai 2013), e.g. body depth (54.5% vs. 39.1–51.1% / 22.7 vs. 16.2–20.8%), body width (55.7% vs. 32.6–44.4% / 23.2% vs. 13.3–17.7%), head depth (54.9% vs. 35.8–49.9% / 22.9% vs. 16.7–20.3%) or distance from snout to anus (141% vs. 113.8–128.3% / 58.8% vs. 46.4–52.6%).

S. kikingeri and *S. laticeps* have in common that the parietal bones are of different sizes. Both species differ in the number of lip and chin barbels (3/3 and 1/0 vs. 4–5/3–4 and 2–5/2–4) and number of anal fin rays (13 vs. 14–17). It should be noted that the typical number of lip barbels for *S. laticeps* is obviously 4/4. Kawai (2013) investigated 53 specimens and found only 1 specimen with 5 lip barbels on the left and one specimen with 3 lip barbels on the right side. Both species have similar body proportions (%SL) (Table 1, Kawai 2013) but differ e.g. in the width of body (23.2% vs. 12.4–20.3%), distance from snout to anus (58.8% vs. 46.2–53.6%) or caudal peduncle depth (4.2% vs. 2.2–3.4%).

Four unidentified specimens of *Satyrichthys* were collected in 1991 at the North Male Atoll (Maldives) in a depth of 180 m (Anderson *et al*. 1992). The largest and the smallest of these were deposited at the Bernice Pauahi Bishop Museum at Honolulu, Hawaii (BPBM 34965) (Adam *et al*. 1998). Their taxonomic status is discussed elsewhere. The whereabouts of the two other specimens is unknown. These four specimens document the first record of this genus for the Republic of the Maldives. Adam *et al*. (1998) report *Satyrichthys investigatoris* from the Maldives, a species now placed in the genus *Scalicus* (Kawai 2008).

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Ecology. The single specimen of *S. kikingeri* was found floating dead on the surface. Therefore no ecological data are associated with this sample and the exact depth inhabited by this species is unknown. Dead *Satyrichthys* sp. specimens are occasionally found on the beaches of Maldivian islands (Adam *et al*. 1998). Rasdhoo Atoll is located north-west of North Ari Atoll, west of the deep inner sea basin dividing the Maldives Archipelago into two north-south oriented chains of atolls (Anderson *et al.* 2011) (Fig. 1). Given the lifestyle of other known Peristediidae, the appearance of the specimen, and the availability of appropriate habitat, it is likely that *S. kikingeri* is a deep-water form.

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The larvae of *Drusus franzressli* Malicky 1974 and *Drusus spelaeus* (Ulmer 1920) (Trichoptera: Limnephilidae: Drusinae) with notes on ecology and zoogeography

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Abstract

Water quality monitoring is greatly dependent on identification tools for aquatic and semi-aquatic insects. Species-level identification improves resolution and precision of water quality assessment and requires comprehensive keys. With the aim of increasing the suitability of Drusinae for such applications, this paper gives a description of the hitherto unknown larvae of *Drusus franzressli* Malicky 1974 and *Drusus spelaeus* (Ulmer 1920). Information on the morphology of the larvae is given and the most important diagnostic features are illustrated. In the context of already available keys, the larvae of *D. franzressli* and *D. spelaeus* key together with *Metanoea flavipennis* (Pictet 1834), *M. rhaetica* Schmid 1956, *D. improvisus* McLachlan 1884, *D. nigrescens* Meyer-Dür 1875 and *Ecclisopteryx malickyi* Moretti 1991. These species are easily separated by differences in larval morphology (dorsal outline and sculpturing of pronotum, presence/absence of lateral gills at 2nd and 3rd abdominal segments, start of lateral fringe) and their distribution ranges. *Drusus franzressli* is endemic to the Hellenic western Balkans whereas *D. spelaeus* is endemic to the western Alps (Grenoble area). In addition, ecological characteristics are briefly discussed.

Key words: 5th instar larva, description, identification, distribution

Introduction

Caddisflies are considered primary indicator taxa for monitoring water quality (Barbour *et al*. 1999; Barbour & Yoder 2000; AQEM consortium 2002; Graf *et al*. 2002; Hering *et al*. 2006). This also fully applies to the subfamily Drusinae in which larvae are restricted to water quality classes I or I–II and are used as bioindicators (sensitive species) (Moog *et al*. 2002; Graf *et al*. 2002).

Unfortunately, no comprehensive and integrated effort has been made to complete the available keys to larval Drusinae. The recent taxon Drusinae, considered a tribe of subfamily Limnephilinae by Vshivkova *et al*. (2007), comprises 97 species restricted to Eurasian mountain ranges from the Caucasus in the east to the Iberian Peninsula in the south-west (Graf *et al*. 2008; Kučinić *et al*., 2011a; Malicky 2004, 2005a; Olah 2010, 2011). From this large inventory, larvae from only 41 species (42%) have been described so far and included in keys (Botosaneanu 1959; Décamps & Pujol 1975; Despax 1927; Graf *et al*. 2011; Kučinić *et al*. 2008, 2010, 2011a, b; Moretti & Pirisinu 1981; Moretti 1983; A. Previšić, W. Graf & M. Kučinić unpublished data; Sipahiler 2002; Szczesny 1978; Vieira-Lanero 2000; Vieira-Lanero *et al*. 2005; Waringer *et al*. 2008, 2010; Waringer & Graf 2011).

In the present paper we take a further step at completing the larval taxonomy of subfamily Drusinae by

providing descriptions of the larvae of *Drusus franzressli* Malicky 1974 and *D. spelaeus* (Ulmer 1920) with the latter species originally described as *Metanoea spelaea*.

Material and methods

Adults and larvae were collected by M. Bálint in Greece and W. Graf in the western Alps using a hand net and kick sampling at the following locations: *Drusus franzressli*: springs and torrent in and east of the village of Vargiani (38.64° N, 22.43° E, 900–980 m a.s.l.), approximately 10 km north of Amfissa, Phocis Prefecture, Greece, 12 May 2012 (leg. M. Balint). *Drusus spelaeus*: Gorge du Furon, stream cave outlet, Bruyant Engins (45.15° N, 05.17° E, 1012 m a.s.l.) and at the surroundings of Grotte Choranche (45.07°N, 5.40°E), both Département Isère, Rhône-Alpes, France, 7 and 8 July 2012 (leg. W. Graf).

The material intended for sequencing was transferred to 100 % alcohol, the material for morphological analyses in pure 70% ethanol in order to keep the specimens more flexible. The larvae were studied and photographed using a Nikon SMZ 1500 binocular microscope with DS-Fi1 camera and NIS-elements D 3.1 image stacking software for combining 8 to 42 frames in one focused image.

Species affiliation was based on two lines of evidence:

1) The species were collected close to their *loci typici* where other Drusinae species are lacking or larvae were clearly different from the species in question and by collecting adults of both sexes at the same sites as the larvae;

2) We used molecular data from two gene regions to confirm conspecificity of the larvae and adults. We followed the methods outlined by Pauls *et al*. (2006, 2008) to generate sequence data for mitochondrial cytochrome *c* oxidase I (mtCOI, 541 base pairs (bp)) and nuclear wingless (nWG, 472 bp) gene regions. For *D. franzressli* one larva was analysed alongside two males and two females from the Vargiani springs in Greece (Table 1); the other larva was kept as a reference for further morphological studies. For *D. spelaeus* we analysed four males, two females and two larvae from two localities (Table 1). Although we targeted both genes for all specimens we experienced sequencing issues and despite repeated PCR amplification and sequencing trials, the two putative *D. spelaeus* larvae were each only successfully sequenced for one of the two genes. We performed Bayesian (B/MCMC) phylogenetic analysis independently for each gene. B/MCMC inference of the mtCOI gene region was based on 187 individuals representing 59 Drusinae species. B/MCMC inference of the nWG gene region was based on 140 individuals representing 51 Drusinae species and the same outgroups as in mtCOI. We performed two parallel runs with 4 chains each. Tree space was searched for 5.000.000 generations, the first 2.500.000 generations were deleted as burn-in.

Deposition of voucher specimens (sampling locations are given in Table 1): The two 5th instar larvae of *D. franzressli* and the seventeen 5th instar and one 4th instar larvae of *D. spelaeus* are deposited in the collection of J. Waringer (Vienna, Austria). All this material is from the same location as the larvae used for DNA extraction. Comparative material of *Metanoea rhaetica* Schmid 1956 (seven 5th instar larvae), *Metanoea flavipennis* (Pictet 1834) (ten 5th instar larvae), *Drusus improvisus* (McLachlan 1884) (ten 5th instar larvae), *Drusus nigrescens* Meyer-Dür 1875 (five 5th instar larvae), *Drusus biguttatus* (Pictet 1834) (seven 5th instar larvae), and *Drusus camerinus* Moretti 1981 (two 5th instar larvae) is in the collection of J. Waringer (Vienna, Austria).

Results

Description of the fifth instar larva of *Drusus franzressli*

Association of Adult and Larva. All putative *D. franzressli* specimens carried the same unique haplotype of mtCOI and varied by 0–2 bp (Ø 0.64 bp) in nWG. Conspecificity was further supported by the fact that all specimens of *D. franzressli* formed a clearly distinct monophyletic clade compared with all other known Drusinae species (S.U. Pauls, unpublished data). The clade was strongly supported with high posterior probability (pp) support values of 1.0 and 0.97 for mtCOI and nWG, respectively.

TABLE 1. Material used for genetic association of larvae and adults of *Drusus franzressli* and *D. spelaeus*.

Taxon	Locality	Latitude	Longitude	Elevation	Stage	Collector	Collection Date	COI	WG
D. franzressli	Greece, Phocis county, Vargiani springs and torrent in the village	38.64163°N	22.42525°E	900 m asl	Male	Dányi, Kontschán & Murányi	08.04.2009	KC684460	KC684448
D. franzressli	Greece, Phocis county, Vargiani springs and torrent East of the village	38.64169°N	22.42737°E	980 m asl	Female	Bálint	07.05.2012	KC684461	KC684449
D. franzressli	Greece, Phocis county, Vargiani springs and torrent East of the village	38.64169°N	22.42737°E	980 m asl	Male	Bálint	07.05.2012	KC684462	KC684450
D. franzressli	Greece, Phocis county, Vargiani springs and torrent East of the village	38.64169°N	22.42737°E	980 m asl	Female	Bálint	07.05.2012	KC684463	KC684451
D. franzressli	Greece, Phocis county, Vargiani springs and torrent East of the village	38.64169°N	22.42737°E	980 m asl	Larva	Bálint	07.05.2012	KC684464	KC684452
D. spelaeus	France, Rhône-Alpes, Drome, Bruyant Engins SW of St Antoine l'Abbaye	45.146556°N	5.17086°E	1012 m asl	Male	Graf	07.07.2012	KC684465	KC684453
D. spelaeus	France, Rhône-Alpes, Drome, Bruyant Engins SW of St Antoine l'Abbaye	45.146556°N	5.17086°E	1012 m asl	Male	Graf	07.07.2012	KC684466	KC684454
D. spelaeus	France, Rhône-Alpes, Drome, Bruyant Engins SW of St Antoine l'Abbaye	45.146556°N	5.17086°E	1012 m asl	Male	Graf	07.07.2012	KC684467	KC684455
D. spelaeus	France, Rhône-Alpes, Drome, Bruyant Engins SW of St Antoine l'Abbaye	45.146556°N	5.17086°E	1012 m asl	Female	Graf	07.07.2012	KC684468	KC684456
D. spelaeus	France, Rhône-Alpes, Drome, Bruyant Engins SW of St Antoine l'Abbaye	45.146556°N	5.17086°E	1012 m asl	Larva	Graf	07.07.2012	-	KC684457
D. spelaeus	France, Rhône-Alpes, Drome, Bruyant Engins SW of St Antoine l'Abbaye	45.146556°N	5.17086°E	1012 m asl	Larva	Graf	07.07.2012	KC684469	-
D. spelaeus	France, Rhône-Alpes, Isere, Parc Naturel de Vercours, Grotte Choranche	45.07221°N	5.39727°E		Male	Graf	08.07.2012	KC684470	KC684458
D. spelaeus	France, Rhône-Alpes, Isere, Parc Naturel de Vercours, Grotte Choranche	45.07221°N	5.39727°E		Female	Graf	08.07.2012	KC684471	KC684459

Biometry. Body length of final instar larva ranging from 10.4 to 10.9 mm, head width from 1.25 to 1.33 mm (n= 2).

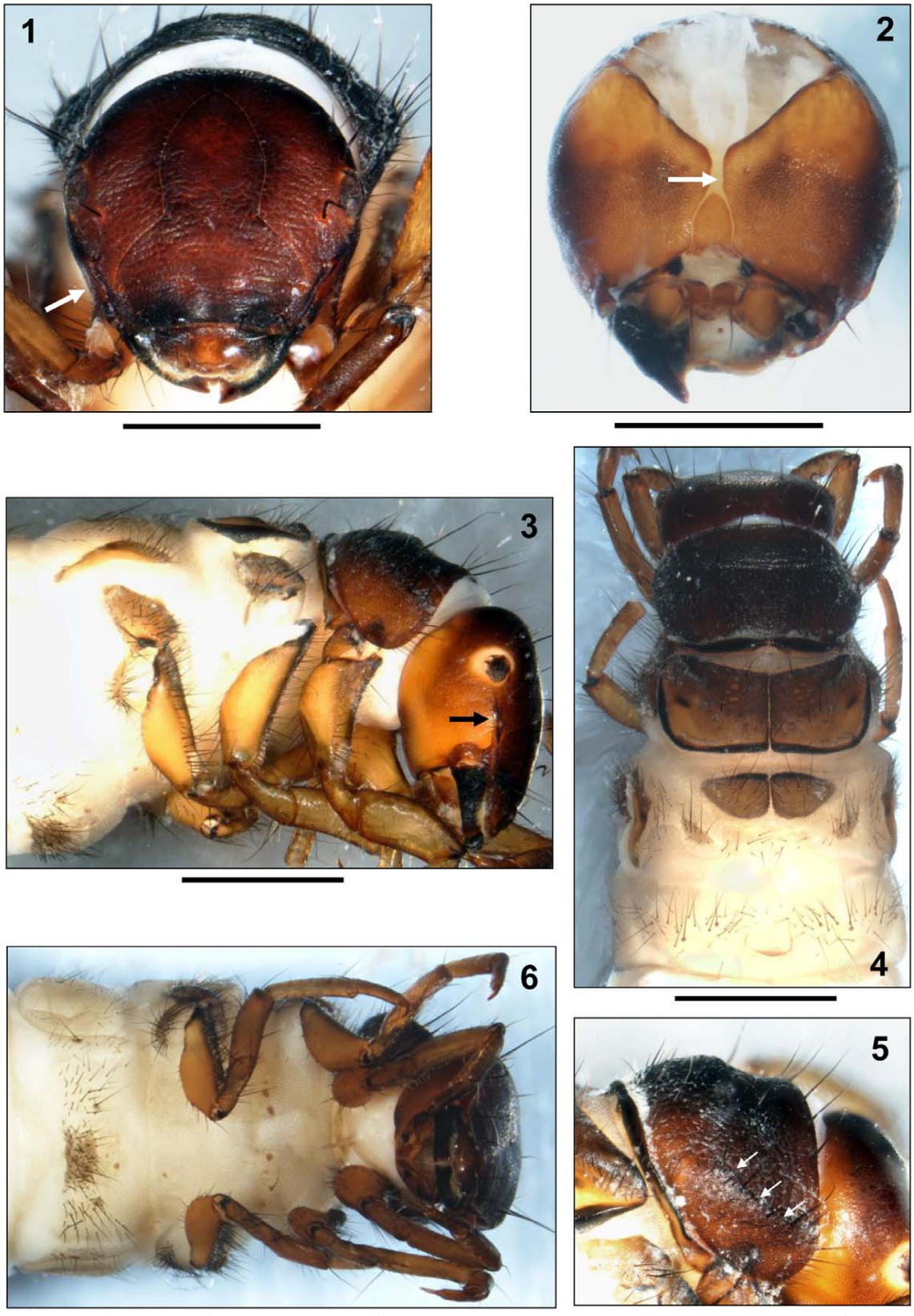
Head. Head capsule coarsely granulated, almost circular in shape and hypognathous (Figs. 1, 3), dorsally with blackish brown coloration and blackish muscle attachment spots. Ventral parietalia sections, submentum, maxillolabial sclerites and premandibular areas yellowish brown (Figs. 2, 3). Yellowish-white ring around each eye (Fig. 3). In lateral view, head capsule with carina (approximately 0.07 mm wide) extending from anterior eye margin to frontolateral corner of frontoclypeus (Figs. 1, 3, arrows). Head capsule with complete set of 18 pairs of primary setae (nomenclature by Wiggins 1998) and lacking any additional spines or spinule areas known from other Drusinae larvae (e.g., *Ecclisopteryx* spp., *Drusus trifidus* McLachlan 1868, *D. bosnicus* Group). Frontoclypeus bell-shaped, with narrow central constriction (Fig. 1). Antennae located at dorsal surface of lateral carina and halfway between eye and anterior head margin (Fig. 3), each consisting of 1 short cylindrical base and 1 short flagellum. At each parietal, 10 dorsal and 2 ventral primary setae present, with primary setae numbered 2, 3, 9 and 14 long and conspicuous (Figs. 1, 3). Each side of frontoclypeus with 6 primary setae, 3 of them along anterior border. Labrum dark to yellowish brown, with setal brush and primary setae numbered 1–3 at anterolateral margins; on dorsal area, setation consisting of primary setae numbered 4–6 (Fig. 1). Ventral apotome bell-shaped, yellowish brown, postgenal suture approximately 55% of apotome length (Fig. 2). Blackish brown mandibles lacking terminal teeth along edges as well as lacking ridges in central concavity (Figs. 1, 3, 6).

Thorax. Pronotum chestnut brown, very coarsely granulated (Figs. 3–5); its posterior margin thickened and darkly striped (Fig. 5). Pronotal transverse groove at end of anterior 3rd lacking. Dorsal profile in lateral view with posterior 2/3rds of pronotum rounded, this curvature creating distinct step leading down to anterior, lower section of pronotum (Figs. 3, 5). Each side with distinct lateral ridge extending almost whole length of pronotum (Fig. 5, arrows). Two setal rows along anterior border of pronotum: (1) Dense fringe of short, curved, fine, yellow setae; (2) widely-spaced, continuous row of long, straight, dark setae meeting at anterior pronotal midline (Figs. 3–5); in total, 40–50 dark setae of varying lengths distributed over each pronotal half. In addition, pronotal surface covered by high number of tiny, pale, recumbent setae; spines present in other Drusinae (e.g., *D. trifidus*) lacking. Prosternite light brown, narrow, spindle-shaped; prosternal horn present (Fig. 6).

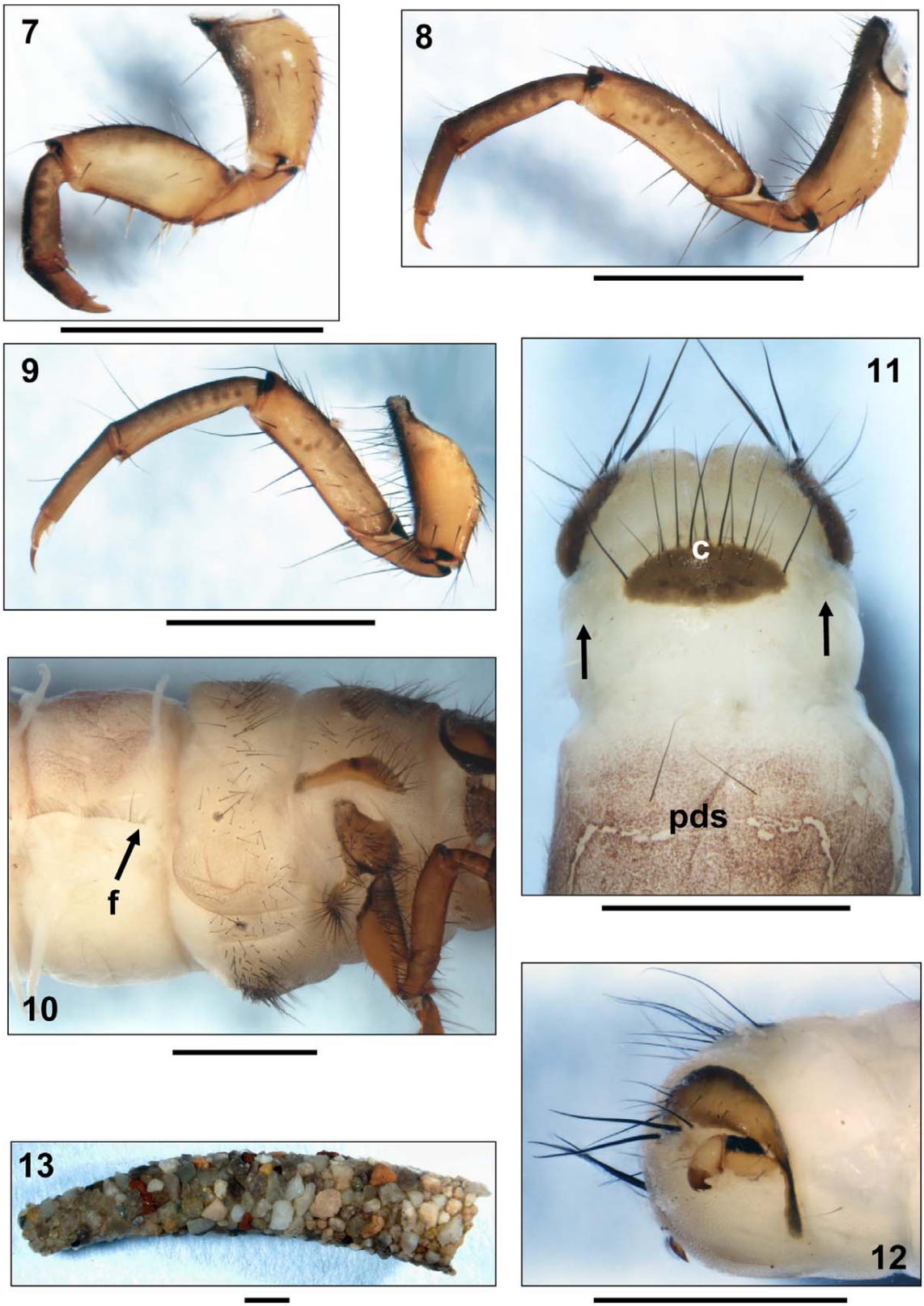
Mesonotum completely covered by 2 blackish brown sclerites with whitish muscle attachment spots and yellowish brown posterior and lateral sections; their lateral and posterior margins darkly sclerotized (Fig. 4). Counts for mesonotal setae are as follows (nomenclature sensu Wiggins 1998): anterior setal group *sa*1: 11–15, posterior group *sa*2: 25–30, lateral group *sa*3: 20–25.

Metanotum partially covered by 3 pairs of dark to yellowish brown sclerites. Anterior metanotal sclerites (*sa*1, sensu Wiggins 1998) very large, broadly triangular, strongly tapering laterally and almost in close median contact; each with black anterior margin; approximately 25 setae per sclerite (Fig. 4). Row of setae present between small posteromedian sclerites (*sa*2, sensu Wiggins 1998); 10–15 setae per sclerite. Small setal group present between each lateral (*sa*3, sensu Wiggins 1998) and posteromedian sclerite (*sa*2); *sa*3 sclerites with approximately 30 setae per sclerite, concentrated anteriorly (Figs. 3, 4). Legs light brown with numerous setae on coxae, trochanters and femora; tibiae and tarsi with only small number of setae; on all femora several proximodorsal setae present (Figs. 7–9). Coxa, femur and tibia of each foreleg wider than those of mid- and hind legs. Setae present only at proximal sections of fore- and midtrochanters. Additional setae present at both anterior and posterior faces of all femora; ventral trochanteral brush present at distal sections of fore- and midtrochanters. Rows of minute spines lacking along ventral edges of all femora; forefemora each with 3 yellow and 1 dark ventral-edge setae, midfemora each with 3 dark and hind femora each with 4 dark ventral edge setae. Dorsal setae only at distal third of mid- and hind tibiae (Figs. 7–9).

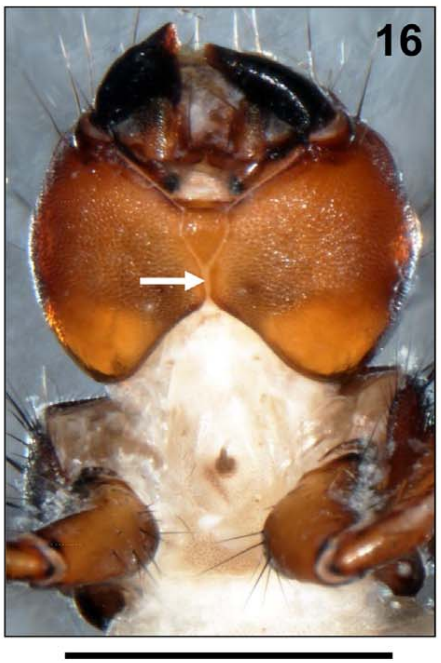
Abdomen. First abdominal segment with 1 dorsal and 2 lateral fleshy protuberances (Figs. 4, 10). Setal areas *sa*1, *sa*2 and *sa*3 (sensu Wiggins 1998) fused, thereby creating continuous transverse row of setae anterior to dorsal protuberance until dorsal section of each lateral protuberance. Sharply delimited basal sclerites present for about 50% of these setae; without setal group posterior to dorsal protuberance (Figs. 4, 10). Posterior sclerites lacking at lateral protuberances (Fig. 10). In front of each lateral protuberance, continuous band of anterolateral setae linking with each dorsal and ventral *sa*3 setal group (Fig. 10). On 1st abdominal sternum, setal areas *sa*1, *sa*2 and *sa*3 fused, creating continuous field of setae, with center originating at large sclerotized plate. In addition, two areas of fused basal sclerites situated at each anterolateral corner of central plate (Fig. 6). On 8th abdominal dorsum, two long posterodorsal setae (pds) present (Fig. 11, pds). Only 1 posterolateral seta present on each half of 9th abdominal dorsum (Fig. 11, arrows).

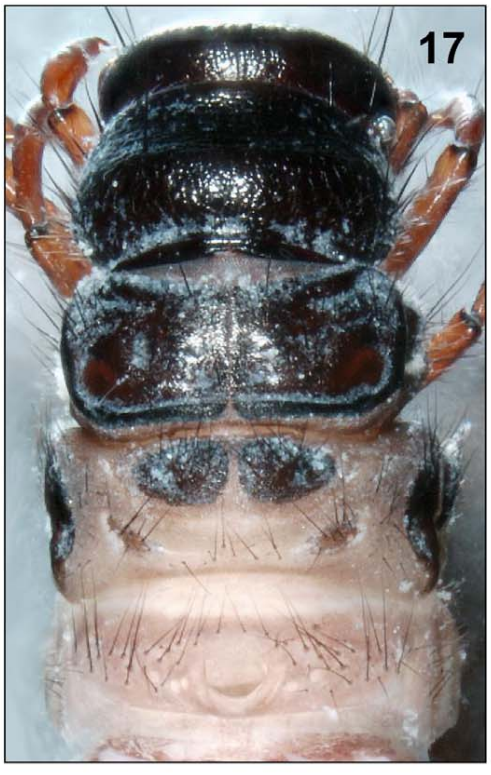


FIGURES 1–6. *Drusus franzressli* Malicky 1974, 5th instar larva. 1, head, dorsal view (arrow: lateral carina); 2, head, ventral view (arrow: postgenal suture); 3, head and thorax, right lateral view (arrow: lateral carina); 4, head, thorax and first abdominal segment, dorsal view; 5, pronotum, right lateral view (arrows: lateral ridge); 6, head, thorax and first abdominal segment, ventral view. Scale bars: 1 mm.

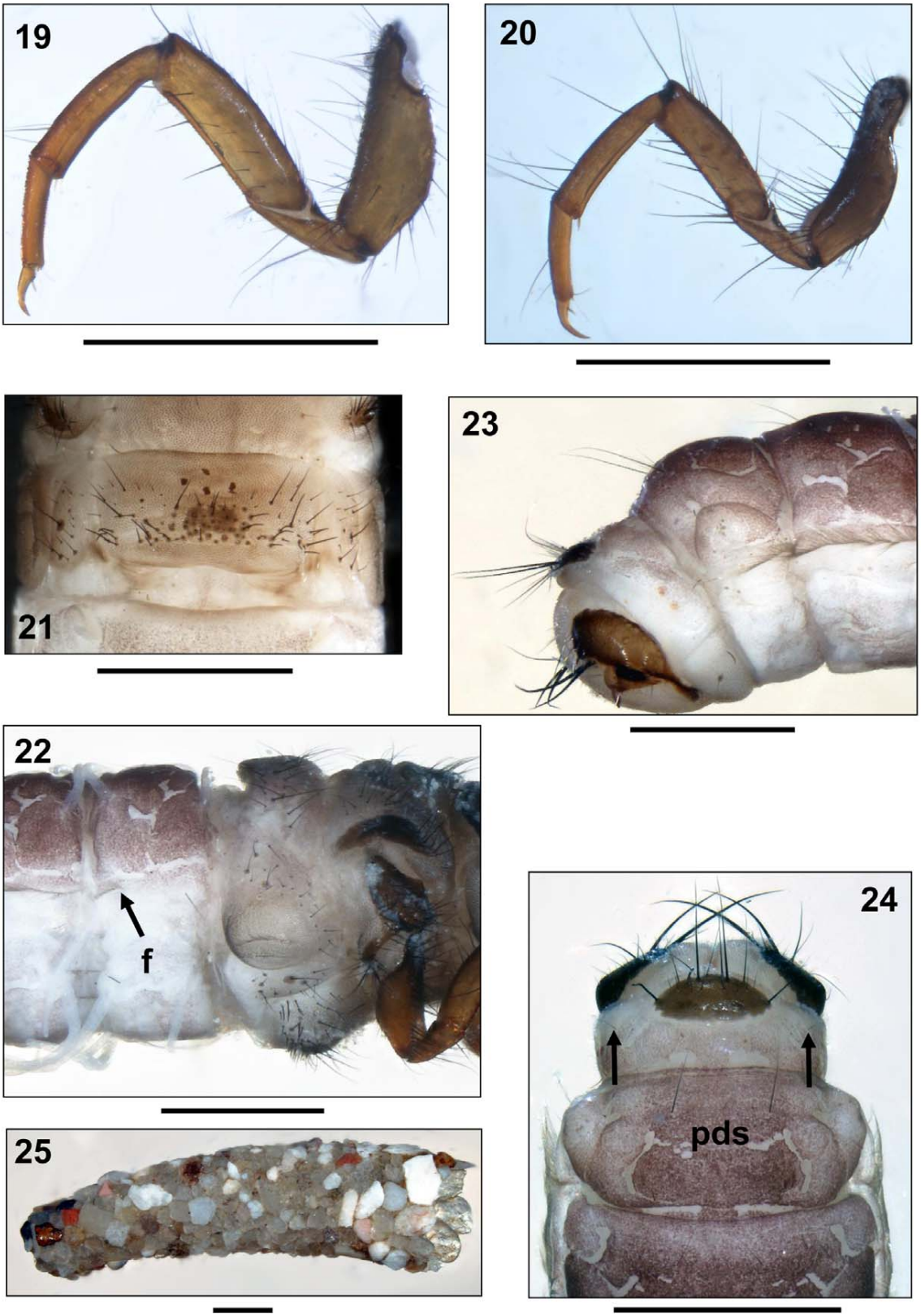


FIGURES 7–13. *Drusus franzressli* Malicky 1974, 5th instar larva. 7, right fore leg, anterior view; 8, right mid leg, anterior view; 9, right hind leg, anterior view; 10, metathorax and 1st and 2nd abdominal segments, right lateral view (f: start of lateral fringe at 2nd segment); 11, 8th and 9th abdominal terga, dorsal view (arrows: posterolateral setae; pds: posterodorsal setae; c: position of c setae); 12, tip of abdomen, right lateral view; 13, larval case, right lateral view. Scale bars: 1 mm.

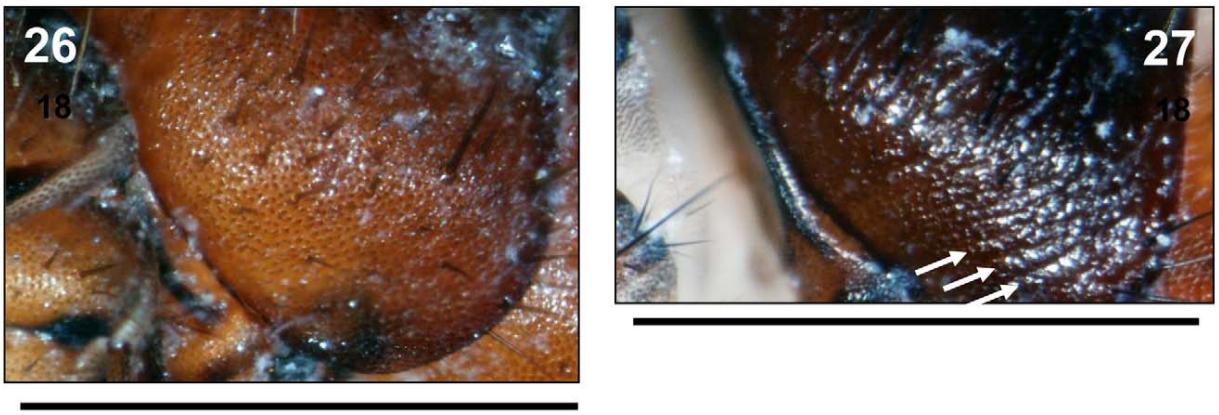




FIGURES 14–18. *Drusus spelaeus* (Ulmer 1920), 5th instar larva. 14, head, dorsal view; 15, head and prothorax, right lateral view; 16, head and prothorax, ventral view (arrow: postgenal suture); 17, head, thorax and 1st abdominal segment, dorsal view; 18, right fore leg, anterior view. Scale bars: 1 mm.



FIGURES 19–25. *Drusus spelaeus* (Ulmer 1920), 5th instar larva. 19, right mid leg, anterior view; 20, right hind leg, anterior view; 21, 1st abdominal sternum, ventral view; 22, metathorax and 1st, 2nd and 3rd abdominal segments, right lateral view (f: start of lateral fringe at 2nd segment); 23, tip of abdomen, right lateral view; 24, 8th and 9th abdominal terga, dorsal view (arrows: posterolateral setae; pds: posterodorsal setae); 25, larval case, right lateral view. Scale bars: 1 mm.



FIGURES 26–27. Drusinae, 5th instar larvae, lateral part of pronotum. 26, *Metanoea flavipennis* (Pictet 1834), right lateral view; 27, *Drusus spelaeus* (Ulmer 1920), right lateral view (white arrows: ribbed structure created by adjacent granuli). Scale bars: 1 mm.

Morphological separation of fifth instar larvae of *Drusus franzressli* and *D. spelaeus* from other European Trichoptera

A summary of morphological features for the identification of limnephilid and Drusinae larvae was provided by Waringer (1985). Within the framework of the limnephilid key by Waringer & Graf (2011) and Waringer *et al.* (2010), *Drusus franzressli* and *D. spelaeus* are separable from other species by the following features:

- gills consisting of single filaments only; dorsal gills present (Figs. 10, 22);
- metanotum covered by three pairs of small sclerites (Figs. 4, 17);
- mandibles spoon-shaped (terminal teeth and central cavity ridges lacking; Figs. 1, 3, 15);
- head capsule without groups of additional spines or spinules (Figs. 1–3, 14, 15);
- anterior-row setae present near dorsal midline of pronotum (Figs. 4, 14);
- dorsal-edge setae restricted to distal 3rd of mid- and hind tibiae (Figs. 9, 19);
- center of first abdominal sternum with one or two large sclerotized patches or concentrations of fused basal sclerites of setae (Figs. 6, 21).

In the key by Waringer *et al.* (2011), *Drusus franzressli* and *D. spelaeus* key together with *D. improvisus* (Waringer *et al.* 2008), *D. nigrescens* (Waringer *et al.* 2007), *Metanoea flavipennis* (Waringer *et al.* 2000) and *M. rhaetica* (Waringer 1985); *Ecclisopteryx malickyi* Moretti 1991 (Graf *et al.* 2011) also keys with these species. These species are easily separated by differences in dorsal profile and sculpturing of the pronotum (e.g., Figs. 5, 15, 26, 27), setation at the center of the anterior pronotal border (e.g., Figs 6, 21), presence/absence of lateral gills on the 2nd and 3rd abdominal segments, beginnings of the abdominal lateral fringe (e.g., Figs. 10, 22) and distribution (Table 2).

Phenology, habitat and distribution

Our last instar larval samples of *D. franzressli* were collected on 7 May, phenologically fitting the reported short emergence period occurring mainly in spring; adults were sampled from April to June (Malicky 2005b). According to Malicky (2005b), this is an indication for a univoltine, stenochronous life cycle as observed in a number of Greek caddisfly species (e.g., *Drusus erimanthos* Malicky 1992: on the wing in April*; Allogamus pertuli* Malicky 1974: on the wing in late autumn and winter; Malicky 2005b). With respect to longitudinal zonation patterns, M. Bálint observed *D. franzressli* larvae from the spring to 500 m downstream of the spring, indicating that the species is restricted to (karstic) springs and the hypocrenal and epirhithral regions of small streams (Graf *et al.*, 2008). Our

TABLE 2. Synopsis of characters separating the currently known Drusinae larvae (5th instars) which share the following group morphomatrix: spoon-shaped mandibles; lack of additional head spines or spinules; anterior-row setae present near dorsal pronotal midline; dorsal gills present; dorsal edge setae restricted to distal third of mid and hind tibiae; basal sclerites of setae at first abdominal sternum fusing to sclerotized plates or multilobed patterns.

Species/character	Dorsal outline of pronotum / median incision present?	Pronotal sculpturing	Sclerotization at first abdominal sternum	Posterolateral gills present at 2nd and 3rd abdominal segment?	Start of lateral fringe	Distribution
Drusus nigrescens	high ridge / yes	coarsely granulated, ribbed	multilobed sclerotized pattern	yes	last third III	western alpine
Drusus franzressli	low central ridge / no	coarsely granulated	central plate	no	first third II	hellenic western Balkans
Ecclisopteryx malickyi	high ridge / no	coarsely granulated	multilobed sclerotized pattern	yes	last third III	southern alpine
Drusus improvisus	evenly rounded, high profile / no	coarsely granulated, ribbed	multi-lobed sclerotized pattern	yes	last third II	Apennines
Metanoea flavipennis	evenly rounded, low profile / no	finely granulated	central plate	yes	last third II	western alpine
Metanoea rhaetica	evenly rounded, low profile / no	finely granulated	central plate	no	last third II	eastern alpine
Drusus spelaeus	evenly rounded, low profile / no	coarsely granulated, ribbed	central plate or multi-lobed sclerotized pattern	yes	last third II	western alpine

sampling location was a brook of 1–3 m width, approximately 0.1 m depth and with current velocities of 0.5–1 ms^{-1}. The shaded stream bed consisted of limestone with a few large stones and some gravel, deeply eroded into the limestone bedrock. The only macrophyte cover (50–60%) consisted of water mosses.

Drusus franzressli is endemic to the Hellenic western Balkans and restricted to the mountains of Central Greece. In addition to our sampling sites, adults are reported from Vardousia, Panetolikon and Pendayi from 520 to 1600 m a.s.l., with some sites being well over the treeline (Malicky 2005b; Zobodat 2011).

The fifth and fourth instar larvae of *D. spelaeus* were collected on 7 July 2012. The emergence period of this species is mainly in summer, but also in autumn (Graf *et al*. 2008; Zobodat 2011). As its name implies, the adults of *D. spelaeus* are associated with caves, an ecological trait also well known from a number of other caddisfly species (e.g., Malicky & Winkler 1974; Moretti & Cianficconi 1982). In fact, our sampling site of the larvae of *D. spelaeus* was situated in the immediate vicinity of a cave stream outlet at Bruyant Engins near Grenoble in France. *Drusus spelaeus* is a widely distributed endemic of the western Alps in France.

Discussion

With respect to male genital morphology (e.g., large and pointed inferior appendages), *Drusus franzressli* is close to *D. graecus* (McLachlan 1876) which was considered as an isolated Drusinae species by Schmid (1956).

According to Schmid (1956), *D. spelaeus*, belongs to the *D. mixtus* Group, the largest and most heterogenous subgroup of the genus *Drusus*. Besides *D. spelaeus*, Schmid (1956) included *D. mixtus* (Pictet 1834), *D. biguttatus*, *D. improvisus*, *D. brunneus* Klapálek 1898, *D. trifidus* and *D. bolivari* (McLachlan 1880) in the subgroup. Establishing the phylogenetic position of *D. franzressli* and *D. spelaeus* has not yet been completed. For example, Waringer *et al*. (2008, 2011) tested the validity of the *D. mixtus* Group based on five species, but found no evidence for monophyly of this morphologically heterogenous group.

The three-gene-phylogeny (mtCOI, mtLSU, nuWG) of the hitherto sequenced 57 Drusinae species (Pauls *et al*. 2008, Pauls *et al*. unpublished data) also raised questions concerning the validity of other species groups *sensu* Schmid (1956) and even the genera, both of which were based on adult genital morphology: *Drusus* is clearly polyphyletic with *Anomalopterygella*, *Ecclisopteryx* and *Metanoea* nested within; *Ecclisopteryx* is not monophyletic, whereas *Metanoea* is monophyletic.

In addition to epilithic grazers, such as *Drusus franzressli* and *D. spelaeus*, carnivorous filterers (e.g., *Drusus muelleri* McLachlan 1868) with serrated mandible edges and filtering bristles, and omnivorous generalists with teeth on mandible edges (e.g., *D. alpinus* (Meyer-Dür 1875)) were defined by three well-supported clades in our phylogeny (S.U. Pauls *et al*. unpublished data).

Regarding the evolution of feeding type, either a progression from ancestral omnivorous shredders (e.g., *Drusus alpinus*) to both filtering carnivores (e.g., *D. chrysotus* (Rambur 1842)) and epilithic grazers (e.g., *D. franzressli*, *D. spelaeus*) or a progression from filtering carnivores to omnivorous shredders and epilithic grazers are plausible based on the phylogeny by Pauls *et al*. (2008). Based on the fact that most limnephilids are known to be shredders, ancestral character state reconstructions show that the first scenario seems to be more likely (Pauls *et al*. 2008). Serrated-mandibles-with-teeth appears to be the ancestral state, which is maintained in the carnivorous filterers and omnivore generalist shredders. The spoon-shaped grazer mandible, as in the larvae of *D. franzressli* and *D. spelaeus*, appears to be derived, having lost the teeth on the mandible edge. As pointed out by Weaver & Morse (1986), feeding specialisation in Trichoptera may have opened opportunities to colonise new ecological niches and could have strongly promoted diversification. This is supported by our yet-unpublished data (S.U. Pauls *et al*. unpublished data), where the majority of Drusinae are found among the putatively derived grazers, and additional larval identifications and associations continue to support the clear segregation into three feeding-type-associated clades and the much greater diversity of epilithic grazers than shredders or carnivorous filterers (e.g., Waringer *et al*. 2008; Graf *et al*. 2011; A. Previšić, W. Graf & M. Kučinić unpublished data).

With few exceptions, all Limnephilidae are shredders (Graf *et al*. 2002). Other feeding types are only found in the Drusinae and sporadically among other genera (*Allogamus*, *Annitella*, *Melampophylax* and *Micropterna*). Beyond Drusinae, evolutionary progressions from omnivorous shredders to epilithic grazers may also have occurred with, for example, *Allogamus antennatus* (McLachlan 1876) and *A. mendax* (McLachlan 1876) leading to *A. pertuli* Malicky 1974 (Waringer *et al*. 2012); *Annitella obscurata* (McLachlan 1876) and *A. thuringica* (Ulmer

1909) leading to *A. apfelbecki* (Klapálek 1899) (Waringer *et al*. 2009); *Melampophylax melampus* (McLachlan 1876) leading to *M. mucoreus* (Hagen 1861) and *M. nepos* (McLachlan 1880); and *Micropterna sequax* McLachlan 1875 and *M. lateralis* (Stephens 1837) leading to *M. testacea* (Gmelin 1789). Considering this high number of potentially derived grazers, changes in feeding ecology may be responsible for much of the diversification within Limnephilidae.

There are many examples of endemic caddisfly species limited to a single or very few mountain ranges, thereby creating fragmented montane sky-island populations. This makes such groups ideal models for studying evolutionary processes like speciation and diversification. The alpine chain or the Pyrenees are hot spots for endemism, with *D. spelaeus* providing a fine example for an endemic restricted to the Grenoble area in the western Alps. Whereas the number of endemic Trichoptera species in the Pyrenees is up to 24 (= 10%) (Graf *et al*. 2008), the number of endemic caddisfly species in Greece is up to 72, yielding a proportion of 24% when compared with the overall Greek inventory of approximately 300 species. The corresponding percentages for the Apennine Peninsula are approximately 15%, the Iberian Peninsula 26% and for Asia Minor 31%. In Greece, the Cyclades and Crete have the highest share of endemic species. On the Greek mainland, however, there are no significant concentrations of endemic species in distinct mountain ranges; here, most endemic species are widely spread over the mountains of Central Greece, which also applies to *D. franzressli* (Malicky 2005b).

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The larva of *Drusus vinconi* Sipahiler, 1992 (Trichoptera, Limnephilidae, Drusinae)

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Abstract

This paper describes the previously unknown larva of *Drusus vinconi* Sipahiler, 1992. Information on the morphology of the 5th larval instar is given, and the most important diagnostic features are illustrated. In the context of existing identification keys the larva of *D. vinconi* keys together with *D. annulatus* (Stephens, 1837), *D. biguttatus* (Pictet, 1834), *D. ingridae* Sipahiler, 1993, *Hadimina torosensis* Sipahiler, 2002 and *Leptodrusus budtzi* (Ulmer, 1913). These species differ in the contours of the pronotum in lateral view, the presence/absence of the pronotal transverse groove, the shape of the median notch of the pronotum (in anterior view), pronotal sculpturing, presence/absence of the lateral carina of the head capsule, the number of proximo-dorsal setae on the mid-and hind femora, where the lateral fringe starts on the abdomen, and in geographic distribution. With respect to zoogeography, *Drusus vinconi* is a (micro-) endemic of the Western Pyrenees. The species prefers stony substratum in springs and springbrooks of the montane and subalpine region (Graf et al. 2008; Sipahiler 1992, 1993). As a grazer, the larvae of *D. vinconi* feed on biofilm and epilithic algae.

Keywords
Drusus vinconi, 5th instar larva, description, identification, distribution

Introduction

Extant Drusinae currently comprise 99 species. Thirty species are reported from the Alpine chain, another 34 species are known from the Balkan Peninsula (including many endemics). A total of 17 species have been described from south and southwestern Europe (Apennine, Iberia, Corsica, Pyrenees, southern France), and 18 species and 2 subspecies are known from Asia Minor and the Caucasus (Graf et al. 2008; Ivanov 2011; Malicky 2004, 2005; Oláh 2010, 2011; Sipahiler 2005;). However, the larvae of only 41 species (41%) have been described so far and included in keys (Botosaneanu 1959; Décamps and Pujol 1975; Despax 1927; Graf et al. 2011; Kučinić et al. 2008, 2010, 2011a, b; Moretti & Pirisinu 1981; Moretti, 1983; Previšić et al. 2009; Sipahiler 2002; Szczesny 1978; Vieira-Lanero 2000; Vieira-Lanero et al. 2005; Waringer et al. 2008; Waringer & Graf 2011). To improve our knowledge of larval Drusinae taxonomy, we provide the description of the larva of *Drusus vinconi* Sipahiler, 1992 based on larval material collected in the Département Pyrénées-Atlantiques of the French Midi-Pyrénées region.

Material and methods

Hand nets were used to collect larvae and adults of *Drusus vinconi* in and beside a small stream about 7 km SW of the ski area Arette La Pierre Saint Martin, Département Pyrénées-Atlantiques, Midi-Pyrénées, France (42°57'17.67"N, 0°49'26.91"W) on 23 July 2012 (leg. W. Graf). The material was preserved in 90% ethanol. A Nikon SMZ 1500 binocular microscope with DS-Fi1 camera and NIS-elements D 3.1 image stacking software for combining 8–50 frames in one focused image were used to study and photograph the larvae.

Species affiliation was enabled by the fact that putative *Drusus vinconi* larvae were collected close to their *locus typicus* where the only other Drusinae larvae present, *D. discolor* (Rambur, 1842), are clearly different from the species in question by their dense hair cover on head and pronotum. In addition, adults of both sexes of *D. vinconi* were collected at the same sites as the unknown larvae.

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Deposition of voucher specimens: 2 5th instar larvae of *D. vinconi* are deposited in the collection of J. Waringer (Vienna, Austria) and 2 5th instar larvae and 1 male and 1 female in the collection of W. Graf (Vienna, Austria). Comparative material of other Drusinae included the following: *Drusus annulatus* (Stephens, 1837), 9 5th instar larvae; *Drusus biguttatus* (Pictet, 1834), 5 5th instar larvae; *Drusus ingridae* Sipahiler, 1993, 1 5th instar larva; *Leptodrusus budtzi* (Ulmer, 1913), 1 5th instar larva (all taxa: collection of J. Waringer, Vienna, Austria).

Results

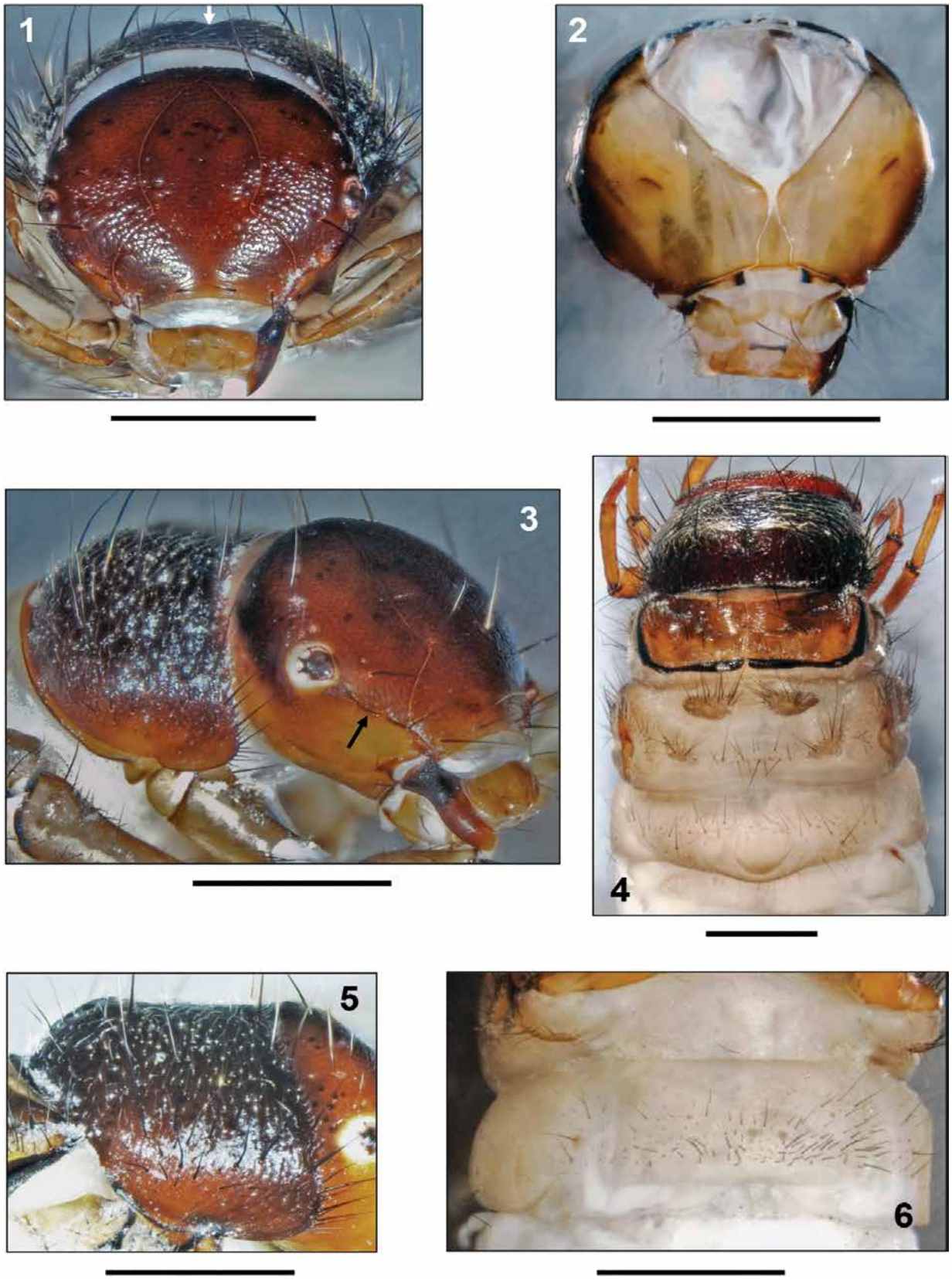
Description of the 5th instar larva of *Drusus vinconi*

Biometry. Body length of 5th instar larvae ranging from 9.7 to 10.8 mm, head width from 1.76 to 1.90 mm (n = 2).

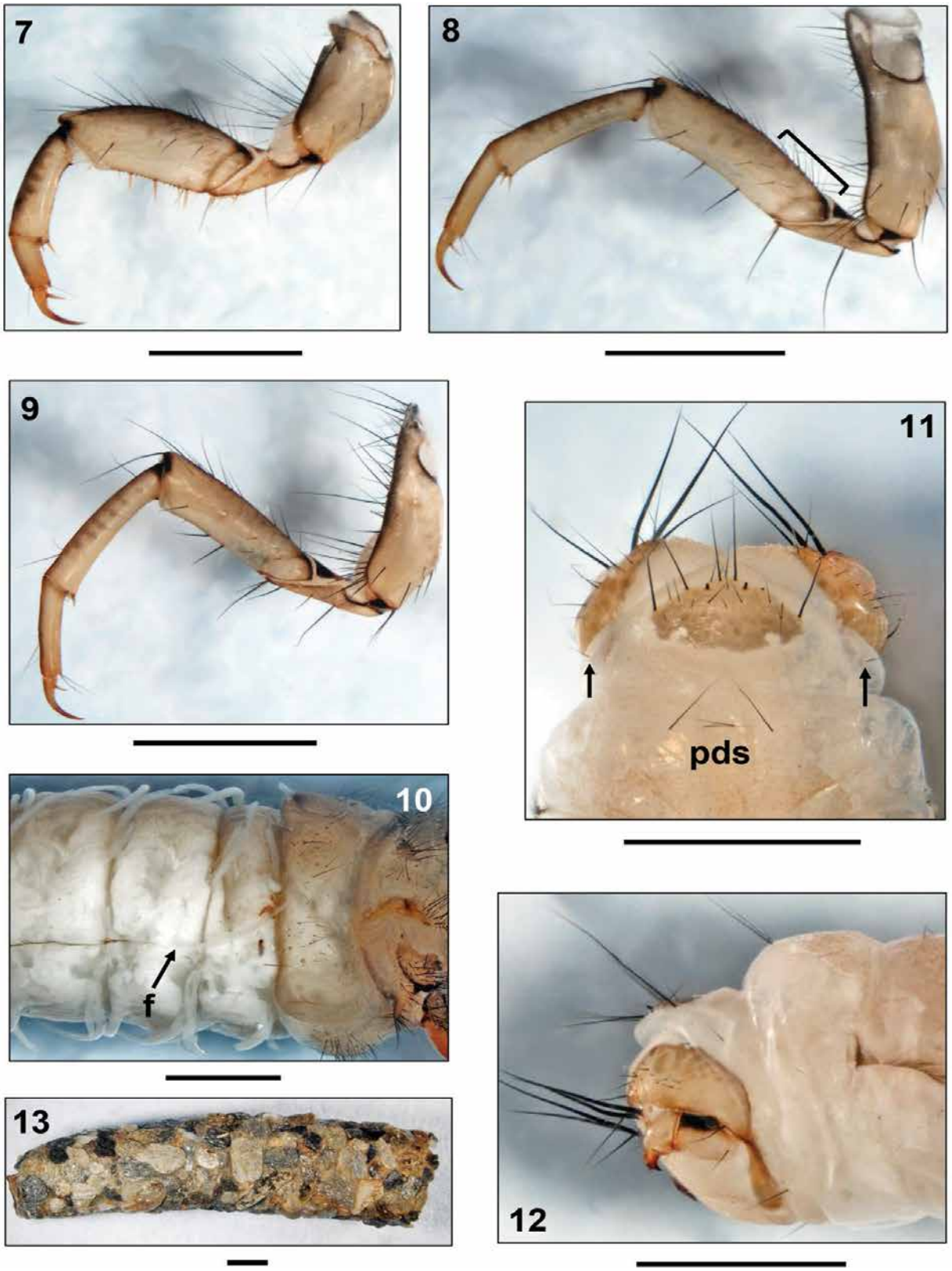
Head. Head capsule coarsely granulated, almost circular in shape, hypognathous (Figs 1, 3), dorsally chestnut to black brown, with blackish muscle attachment spots. Ventral parietalia sections, submentum, maxillolabial sclerites and premandibular areas yellowish (Figs 2, 3). Eyes surrounded by whitish ring (Fig. 3). In lateral view, head capsule bearing carina which extends from anterior eye margin to anterior corner of frontoclypeus (Fig. 3, black arrow). Complete set of 18 pairs of primary setae on head capsule (nomenclature *sensu* Wiggins 1998); no additional spines or spinule areas as known from other Drusinae larvae (e.g., *Ecclisopteryx* spp., *Drusus trifidus* McLachlan, 1868, most of the *D. bosnicus* group except *D. ramae* Marinković-Gospodnetić, 1971) present. Frontoclypeus bell-shaped, with narrow median constriction (Fig. 1). Antennae located dorsally on central section of lateral carinae (Fig. 3), each consisting of 1 short cylindrical base and 1 prominent lateral seta. On each parietal, 10 dorsal and 2 ventral primary setae present (Figs 1, 3). Each side of frontoclypeus bearing 6 primary setae, 3 of them along anterior border. Labrum yellowish brown, anterolateral margins with setal brush and primary setae 1–3; dorsally, setation consisting of primary setae 4–6 (Fig. 1). Yellow ventral apotome funnel-shaped with postgenal suture reaching approximately 29% of apotome length (Fig. 2). Black brown mandibles (sometimes brownish on distal half; Fig. 3) spoon-shaped, lacking terminal teeth along edges as well as ridges in central concavity (Figs 1, 3).

Thorax. Pronotum chestnut brown and very coarsely granulated, with adjacent series of granuli creating ribbed structures (Figs 3, 4). Posterior margin thickened and darkly striped; no pronotal transverse groove at end of anterior 3rd (Fig. 5). In lateral view, dorsal profile of pronotum low, with posterior 2/3rds being evenly rounded (Fig. 5). Along anterior pronotal border 2 setal rows present, including: i) dense fringe of short, curved, fine, yellow setae, ii) continuous row of widely-spaced long, straight, dark setae meeting at pronotal midline (Figs 1, 3, 4, 5). Each pronotal half bearing in total 35–45 dark setae of varying lengths. In addition, pronotal surface covered by high number of tiny, pale, curved, recumbent setae (Fig. 5); no spines as present in other Drusinae (e.g., *D. trifidus*). Prosternite inconspicuous, pentangular in shape, pale yellow, with light brown posterior border. Prosternal horn present (Fig. 3).

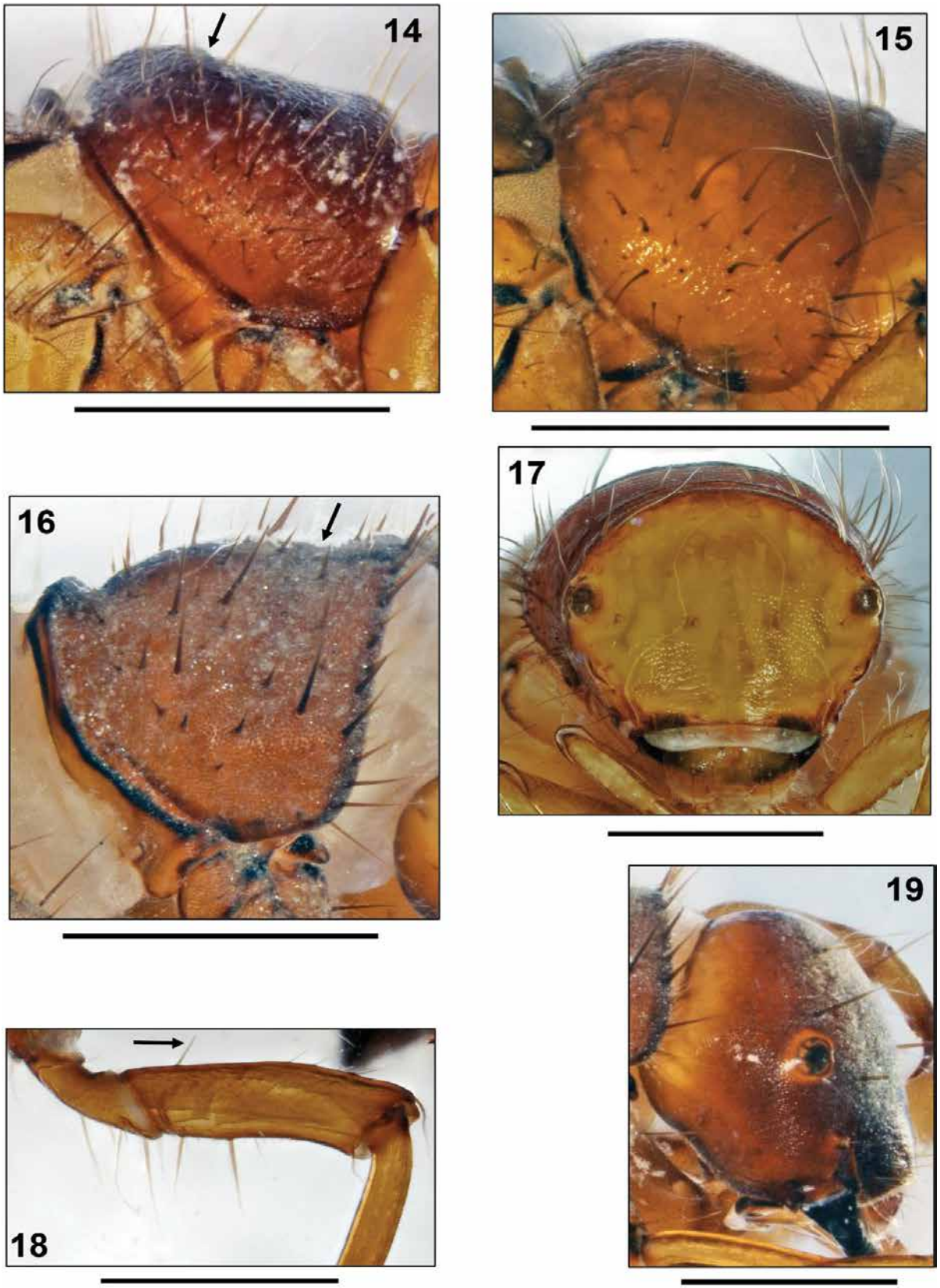
Mesonotum completely covered by 2 yellow brown to dark brown sclerites with anterolateral sections bearing darkest coloration. Median to dark brown muscle attachment spots present, lateral and posterior margins darkly sclerotized (Fig. 4). Counts for mesonotal setae (nomenclature *sensu* Wiggins 1998): anterior setal group *sa1*: 8–15,



Figures 1–6. *Drusus vinconi* Sipahiler, 1992, 5th instar larva. **1** Head, dorsal view (arrow: median notch) **2** Head, ventral view **3** Head and prothorax, right lateral view (arrow: lateral carina) **4** Head, thorax and abdominal segment I, dorsal view **5** Pronotum, right lateral view **6** Abdominal sternum I, ventral view. Scale bars: 1 mm.



Figures 7–13. *Drusus vinconi* Sipahiler, 1992, 5th instar larva. **7** Right fore leg, anterior view **8** Right mid leg, anterior view (bracket: proximodorsal setae) **9** Right hind leg, anterior view **10** Metathorax and 1st 4 abdominal segments, right lateral view (f: start of lateral fringe at segment III) **11** Abdominal segments VIII-IX, dorsal view (arrows: posterolateral setae; pds: posterodorsal setae) **12** Apex of abdomen, right lateral view **13** Larval case, right lateral view. Scale bars: 1 mm.



Figures 14–19. 14–16 Pronota of 5th instar larvae, right lateral views. **14** *Drusus annulatus* (Stephens, 1837) (arrow: dorsal profile angled) **15** *Drusus biguttatus* (Pictet, 1834) **16** *Leptodrusus budtzi* (Ulmer, 1913) (arrow: transverse groove) **17** *D. biguttatus*, head of 5th instar larva, frontal view. **18–19** *L. budtzi*, 5th instar larva **18** Left midleg, posterior view (arrow: proximodorsal seta) **19** Head, right lateral view. Scale bars: 1 mm.

posterior group *sa2*: 25–30, lateral group *sa3*: 30–35 (Fig. 4). In addition, small number of tiny, pale, curved, recumbent setae present.

Metanotum partially covered by 3 pairs of yellowish grey sclerites (Fig. 4). Anterior metanotal sclerites (sclerites of setal area 1, *sa1*, *sensu* Wiggins 1998) very large, ovoid, tapering laterally. Medially, the 2 sclerites strongly divergent, widely spaced; their median separation nearly as high as their length along the longitudinal body axis (Fig. 4). Posteromedian sclerites (sclerites of setal area 2, *sa2*, *sensu* Wiggins 1998) small, triangular, with approximately 20 setae per sclerite, framing row of setae (Fig. 4). Lateral sclerites (sclerites of setal area 3, *sa3*, *sensu* Wiggins 1998) with approximately 25–30 setae concentrated in cranial section (Fig. 10). Groups of setae present between *sa2* and *sa3* (Fig. 4).

Legs light brown with numerous setae on coxae, trochanters, and femora; tibiae and tarsi sparsely setose. Femora with several proximodorsal setae (e.g. Fig. 8, black bracket), and with setation on anterior and posterior faces; fore femora with 4, mid and hind femora with 3 yellow ventral-edge setae; no minute spines along ventral edges present. Foreleg coxa, femur and tibia wider than those of mid- and hind legs. Fore and mid trochanters with setae only on proximal sections; fore trochanters additionally with distal ventral trochanteral brush. Mid- and hind tibiae with dorsal setae only on distal 3rd (Figs 8, 9).

Abdomen. Abdominal segment I with 1 dorsal and 2 lateral fleshy protuberances (Figs 4, 10). Continuous transverse row of setae present anterior of dorsal protuberance (comprising fused setal areas *sa1*, *sa2*, *sa3*, *sensu* Wiggins 1998), stretching laterally from dorsal sections of lateral protuberances; posterior of dorsal protuberance, another row of setae present (Fig. 4). All these setae with small basal sclerites. Lateral protuberances without posterior sclerites (Fig. 10). Anterior of each lateral protuberance a continuous band of anterolateral setae connected to each dorsal and ventral *sa3* setal group (Fig. 10). Abdominal sternum I with fused setal areas *sa1*, *sa2* and *sa3*, creating continuous field of setae, therein occurs pair of central large basal sclerites with irregular borders and small number of randomly distributed basal sclerites of smaller diameter (Fig. 6). Abdominal dorsum VIII with 2 long and 2 short posterodorsal setae (pds) (Fig. 11 pds); only 1 posterolateral seta present on each half of abdominal dorsum IX (Fig. 11, arrows). Abdominal dorsum IX bearing beige pentangular sclerite with 8 long and several short setae (Fig. 11). Beige anal prolegs are of limnephilid type with medium brown anal claws, each with 1 small accessory hook (Fig.12).

All gills as single filaments (Fig. 10). Dorsal gills present at most from abdominal segments II-VII (presegmental positions). Ventral gills present from segment II (presegmental) to segment VII (postsegmental). In lateral row, gills present on segments II-III only (ventrolateral position). Lateral fringe extends from anterior border of segment III (Fig. 10 f) to middle of segment VIII.

Case. Larval case 8.5–12.1 mm long (n= 2), curved, conical (width at anterior opening 2.9–3.2 mm, at posterior opening 1.9–2.2 mm), consisting of mineral particles (sand grains of mixed size; Fig. 13).

Morphological separation of 5th instar larvae of *Drusus vinconi* from other European Trichoptera

Within the framework of the larval key by Waringer and Graf (2011), *Drusinae* larvae are separated from other Trichoptera species by the following features:

- sclerites present on pro-, meso- and metanota; mesontum completely covered by 2 sclerites in close contact separated by a straight suture; metanotum incompletely sclerotized by 6 sclerites (Fig. 4);
- prosternal horn present (Fig. 3);
- fleshy protuberances at abdominal segment I present dorsally and ventrally (Figs 4, 10);
- gills consisting of single filaments only (Fig. 10);
- transverse groove lacking at the anterior 3rd of the pronotum (Fig. 5) except in *Leptodrusus budtzi* (Fig. 16).

Within the subfamily Drusinae, *D. vinconi* is characterised by the following set of morphological details:

- mandibles spoon-shaped (Figs 1, 3);
- head capsule without additional spines or spinules (Fig. 1);
- anterior-row setae present near dorsal pronotal midline (Figs 1, 3);
- dorsal gills present (Fig. 10);
- dorsal edge setae restricted to distal 3rd of mid and hind tibiae (Figs 8, 9);
- basal sclerites of setae on abdominal sternum I separated (Fig. 6);
- pronotum evenly rounded (Fig. 5).

At this position in the key, *Drusus vinconi* appears together with *D. annulatus*, *D. biguttatus* (Pictet, 1834), *D. ingridae*, *Hadimina torosensis* Sipahiler, 2002 and *Leptodrusus budtzi*. These species are easily distinguished by differences in dorsal profile, presence/absence of the lateral carina on the head capsule, number of proximo-dorsal setae on mid-and hind femora, origin of abdominal lateral fringe, and geographic distribution (Table 1).

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Discussion

Drusus vinconi is a (micro-)endemic of the Western Pyrenees. Its *locus typicus* is situated at the ruisseau de Chousse, a tributary of the Vert d'Arette, near the Serre de Benou, at 1300 m a.s.l. At this site *D. discolor* was the only other Drusinae species. Larvae of *D. discolor* are clearly different from *D. vinconi* larvae by their dense hair cover on the head and pronotum.

Table 1. Synopsis of characters separating the currently known Drusinae larvae (5th instars) which share the following morphomatrix: spoon-shaped mandibles; lack of additional head spines or spinules; anterior-row setae present near dorsal pronotal midline; dorsal gills present; dorsal edge setae restricted to distal third of mid and hind tibiae; basal sclerites of setae at first abdominal sternum separated; pronotum evenly rounded. Data for *Hadimina torosensis* were taken from Sipahiler (2002).

Species / character	Dorsal outline of pronotum (lateral view)	Pronotal transverse groove at end of anterior 3rd present?	Pronotum with median notch (anterior view)?	Pronotal sculpturing / cover of procumbent pale setae	Head capsule with lateral carina?	More than one proximo- dorsal seta on mid-and hind femora?	Start of lateral fringe	Distribution
Drusus annulatus	angled (Fig. 14)	no	no	coarsely granulated / sparse	yes	yes	first third III	widespread
Drusus biguttatus	evenly rounded, high profile (Fig. 15)	no	no (Fig. 17)	coarsely granulated / sparse	yes	yes	last third II	widespread
Drusus ingridae	evenly rounded, low profile	no	no	coarsely granulated / sparse	yes	yes	first third III	Pyrenees, Massif Central
Drusus vinconi	evenly rounded, low profile (Fig. 5)	no (Fig. 5)	yes (Fig. 1)	coarsely granulated / dense	yes (Fig. 3)	yes (Figs. 8, 9)	first third III (Fig. 10)	Pyrenees
Hadimina torosensis	evenly rounded, high profile	no	?	? / ?	yes	yes	first third II	Asia Minor
Leptodrusus budtzi	evenly rounded, low profile (Fig. 16)	yes (Fig. 16)	no	finely granulated / sparse (Fig. 16)	no (Fig. 19)	no (Fig. 18)	last third II	Corsica, Sardinia, Mallorca

Adults of *D. vinconi* are morphologically close to *D. monticola* McLachlan, 1876. Differences exist in the structure of the male intermediate appendages which are triangular, and in the preanal appendages which are long and ovoid in *D. vinconi*. The female is characterised by a very short median scale (Sipahiler 1992).

The species was abundant in a small, stony stream near the ski area Arette La Pierre St Martin in the Département Pyrénées-Atlantiques of the Midi-Pyrénées region, France. *Drusus vinconi* is a rheophilic species inhabiting springs and springbrooks where it can be observed on the surface of boulders and large stones (Graf et al. 2008). According to its mouthpart anatomy, *D. vinconi* is a grazer, feeding exclusively on epilithic algae and biofilm. Records exist from montane and subalpine sites situated well above 800 m a.s.l. (Sipahiler 1992, 1993). Adults fly in June and July.

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RESEARCH ARTICLE

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A new species of *Isoperla* (Insecta, Plecoptera) from the Karawanken, with considerations on the Southern Limestone Alps as centers of endemism

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Abstract

A new species of the genus *Isoperla* (Plecoptera, Perlodidae), belonging to the *oxylepis* species-group is described, and the male mating call is characterized. Its range falls within a small region of the Southern Limestone Alps which is well known to be one endemism-centre of aquatic insects.

Keywords

Isoperla, new species, endemism, Austria, Slovenia, Southern Alps

Introduction

The genus *Isoperla* consists of about 150 species (DeWalt et al. 2011, Baumann and Lee 2009, Murányi 2011, Szczytko and Stewart 1979, Zwick and Surenkhorloo 2005) and covers the Holarctic and Oriental regions. In Europe 56 species are known so far (Graf et al. 2009, Murányi 2011), of which ten occur in Austria (Graf 2010). *Isoperla* is a morphologically difficult genus, especially the *grammatica* and *tripartita* species groups that both exhibit high variability, and requires further resolution. A synthesis of zoogeographical, morphological, molecular, and possibly behavioural data will be required to get full knowledge on the diversity of this highly interesting genus.

Recently a series of specimens were collected from the Karawanken Alps in southern Austria and the nearby Kamnik Alps in northern Slovenia deviating from all hitherto known species. In this paper we provide morphological descriptions of males, females and the larva. Additionally we illustrate drumming signals of one male.

Material and methods

Adult specimens were collected using sweep nets, larvae were collected by handpicking from cobbles (mesolithal), the dominant substrate type. Collected specimens were stored in 70% ethanol. Morphological characteristics of male terminalia were examined in KOH-treated, cleared specimens. Comparative material from the authors' collections enabled the identification of the new species.

Vibratory signal recordings were made using a small, dynamic speaker (SAL YD78) as a vibration transducer. The speaker was connected to the microphone input socket of a solid state, digital recorder (Zoom H4n). The examined specimen was placed on the diaphragm of the speaker. To prevent the specimen from escaping the speaker was covered by a sheet of hobby glass. During the recordings ambient air temperature was measured using a P 300W thermometer. Vibration recordings were analysed and oscillograms produced using the software Adobe Audition 1.5 (Adobe Systems Incorporated, San Jose, California, USA). Drumming signal terminology follows Stewart and Sandberg (2006) and Murányi et al. (2014).

Results

***Isoperla claudiae* Graf & Konar, sp. n.**
http://zoobank.org/50F79ECE-AD68-4DD1-BD7F-643D25205189
Figs 1–3

Type material. Holotype: 1 male, Austria, Carinthia, Dolintschitschach brook southeast of Feistritz ob Bleiburg (46°32'6"N, 14°45'52"E), 600m a.s.l., 30.5.2014, leg.

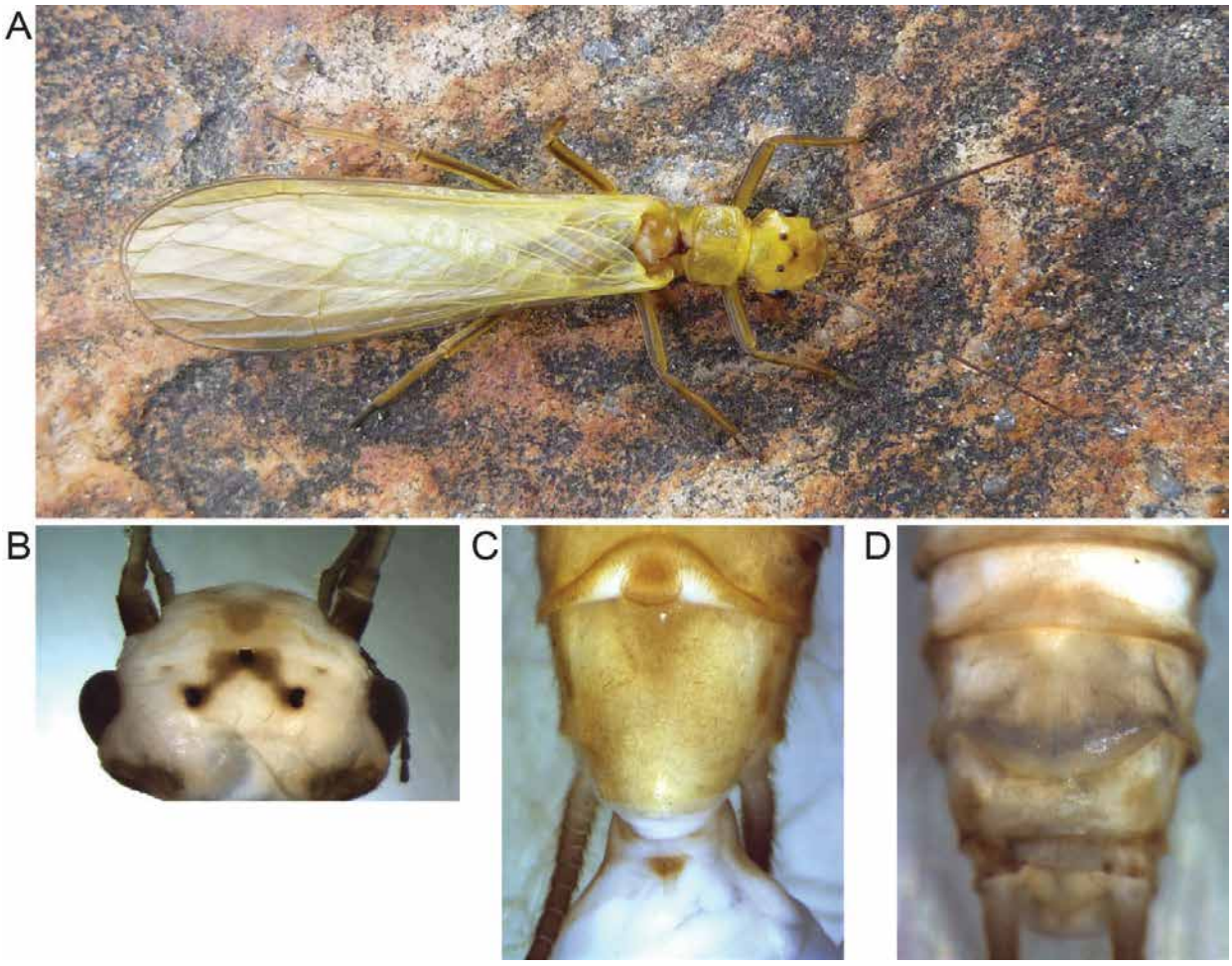

Figure 1. *Isoperla claudiae* sp. n. **A** habitus **B** colouration of the head of *I. claudiae* **C** ventral view of the male abdomen with extruded penis **D** ventral view of the female abdomen.

W. Graf; Paratypes: 3 males, 2 females, same place, date and collector. The holotype is deposited at the Linzer Landesmuseum, Linz, Austria, paratypes are stored in the first author's collection.

Other material. 1 male (drumming call examined), 1 female (HNHM: PLP4333), Slovenia, Upper Carniola, Kamnik municipality, Kamnik Alps, small forest brook S of Podvolovljek Pass (46°16.250'N 14°41.325'E), 980m a.s.l., 09.07.2013, leg. D. Murányi, I. Sivec.

Type locality. Austria, Carinthia, Feistriz ob Bleiburg, Dolintschitschach brook.

Etymology. The species is named in honour of the second author's wife Claudia.

Diagnosis. An *Isoperla* exhibiting the following combination of characters: (1) a small medial penial armature in the form of an equilateral triangle, lacking lateral penial armatures; (2) yellow head and pronotum with a small horseshoe-like brown marking connecting the occelli.

Description. Medium-sized species, macropterous. Body length: holotype 10.5 mm, allotypes 11–12 mm; forewing length: holotype 12 mm, paratypes 12–14 mm. Primary colouration yellow, head and pronotum mostly yellow with dark brown

markings; pilosity short. Primary colouration of the head yellow, with a dark horseshoe-like brown patch connecting the three ocelli (Fig. 1A, B). Occiput with indistinct rugosities but with brown patches laterally. Eyes normal sized. Scape brown, pedicel and the following antennomeres brown; palpi greyish to light brown. Pronotum yellow with a delicate brownish marking at the posterior margin, trapezoidal, edges angled; rugosities hardly visible and yellow. Anterior part of the mesonotum yellow, remaining portions brown; metanotum medially dark brown, laterally and anterior of the insertion of wings whitish. Wings yellowish, particularly the anterior half; venation mostly whitish to yellow, costa and apical part of radii brown. Ventral surface of thorax pale, meso- and metabasisternum inconspicous, furcasternites and furcal pits pale. Femora brown dorsally and yellow ventrally. Tibiae brownish dorsally, pale ventrally; tarsi brown.

Male abdomen (Fig. 1C): 1st to 7th tergite dorsally brown (with some tiny pale spots) with increasing laterally whitish areas towards the apex, 8th to 10th tergite mostly yellowish with small brown medial patches and medially interrupted anterior stripes up to T9, T10 pale without markings. Laterally and ventrally all segments whitish to yellow, lacking dark markings. Pilosity on segment posterior ends short and inconspicuous. Ventral lobe of sternite VIII yellow, slightly longer than wide, its posterior margin strongly convex with long marginal pilosity. Sternite IX yellowish. Paraprocts brown, regularly curved in caudal with with blunt tips; cerci light brown, apically dark brown.

Penis (Fig. 2): Divided into four lobes and a basal section in extruded position. Medial penial armature located on the medial lobe adjacent to the ventral lobe, lateral penial armatures lacking. The medial penial armature resembles an equilateral triangle of 130 μm width and 97 μm length formed by slightly brownish coloured scales that are relatively blunt and short and vary in length (4.98–6.26 μm). The median basal area is sparsely covered by shorter scales. The medial penial armatures are connected distally by a narrow band of colourless scales with an area densely covered by smaller triangular scales. Similar scales are located proximal to the medial armatures. Their length varies between 4.4 and 7.2 μm. With the exception of the medial armatures the central area of the ventral penis is bald. Lateral portions of lateral lobes covered by dense scales similar to the ones on medial and ventral lobes, being denser at the connection to the ventral lobe.

Female abdomen (Fig. 1D): 1st to 7th tergite dorsally brown with increasing laterally whitish areas towards the apex, 8th to 10th tergite mostly yellowish with small brown medial patches. Laterally and ventrally all segments entirely whitish with dark markings reduced to delicate brownish lines at the posterior end of sternites. Subgenital plate covers most of sternite VIII width and half of sternite IX length, posterior margin rounded semicircularly. Sternite X and paraprocts yellowish; cerci generally brownish, the first segment being pale.

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Larva (Fig. 3): Body length of the matured larva: 13 mm. General colour brown but with pale markings. Pilosity dense, pronotal, posterior tergal and cercal fringes short and acute; swimming hairs lacking. Head dark brown with two yellow spots

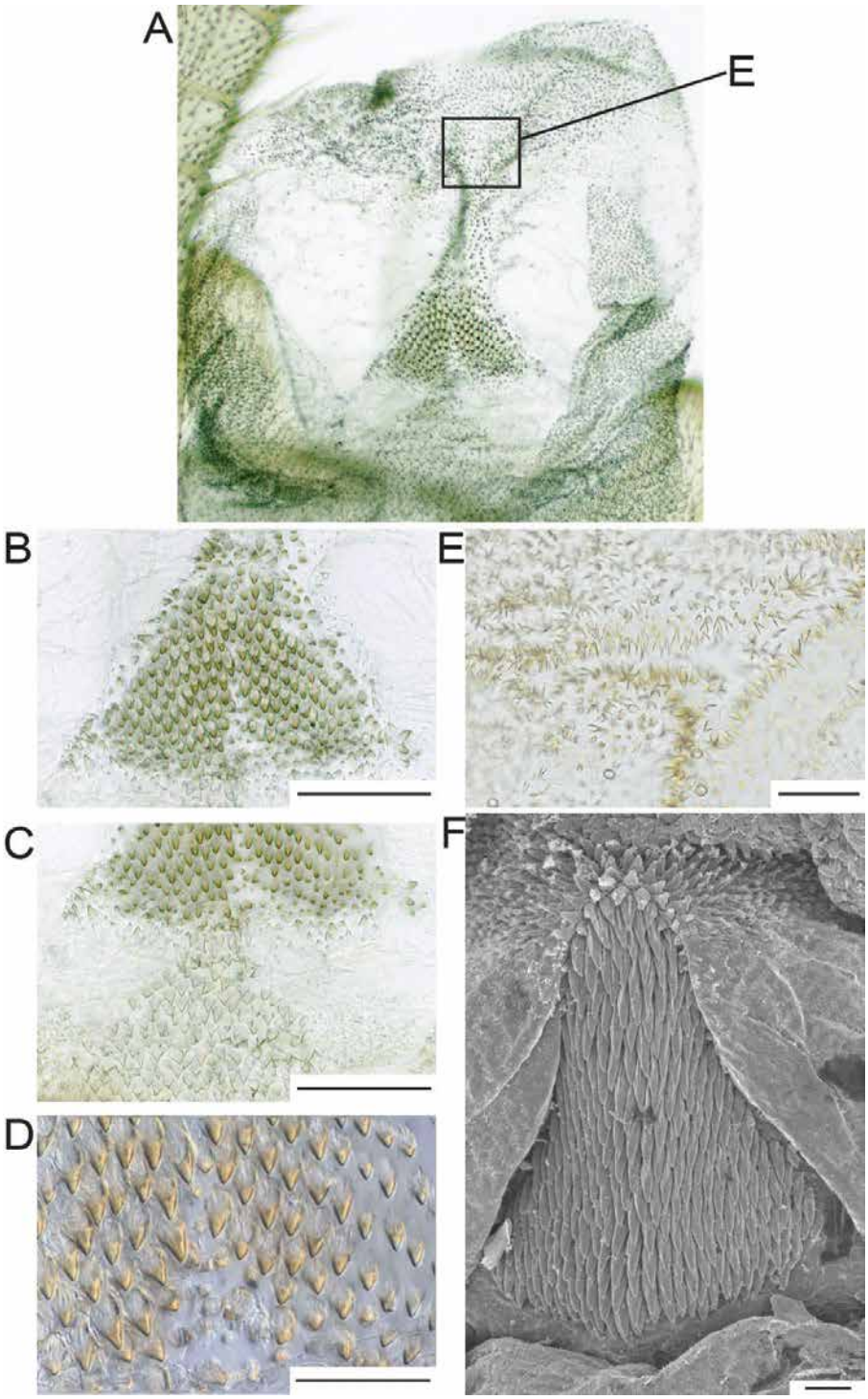

Figure 2. Penis of *Isoperla claudiae* sp. n. **A** ventral view of the extruded penis **B** medial penial armature, scale bar 50 μm **C** medial penial armature, scale bar 50 μm **D** scales of the medial penial armature, scale bar 20 μm **E** scales found caudally the medial penial armature, scale bar 20 μm **F** medial penial armature of *I. orobica*, scale bar 200 μm. Photographs **A**–**E** by W. Lechthaler, Vienna.

anterior to the M-line, two posterior to the M-line, one around the median occellus and one laterally to the each posterior ocellus. Two large pale spots laterally on the occipit (Fig. 3A). M-line distinct, tentorial callosities hardly visible; eyes normal

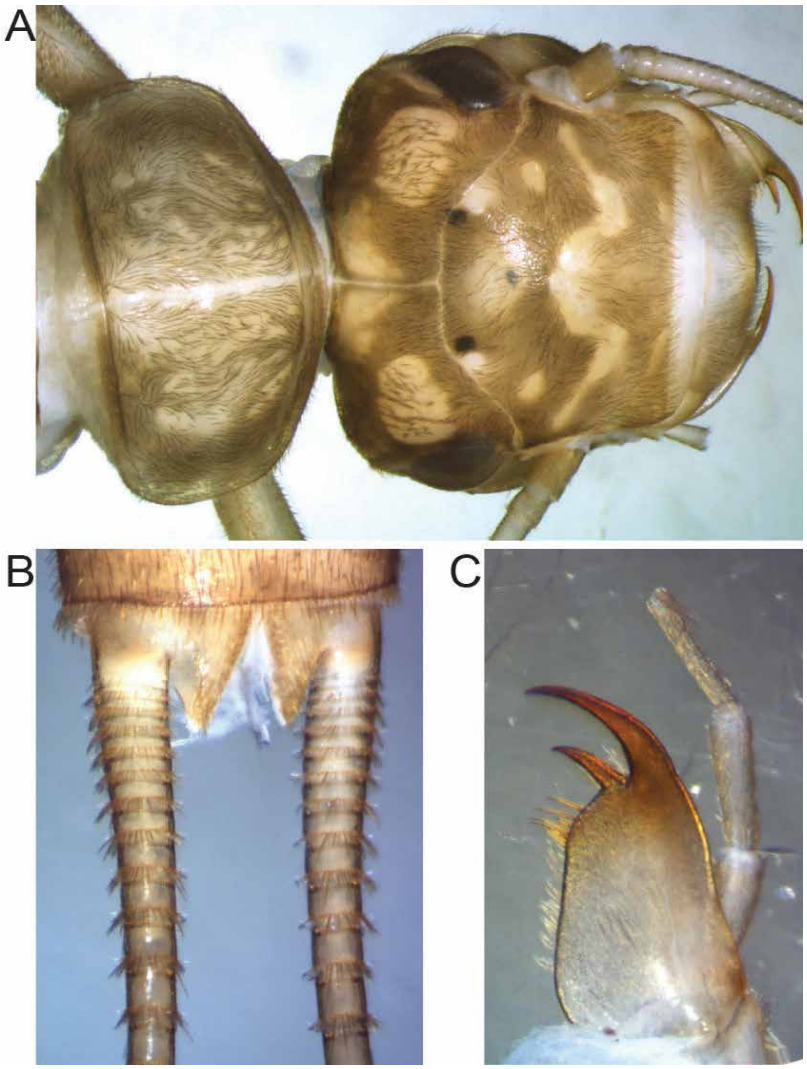

Figure 3. Larval characters of *I. claudiae* sp. n. **A** mature larva of *I. claudiae* sp. n. in dorsal view, head and pronotum **B** ventral view of the abdomen end of *I. claudiae* sp. n. **C** lacinia of *I. claudiae* sp. n.

sized. Scape and pedicel pale, the following antennomeres light brown; palpi yellowish, mouthparts light brown. Lacinia triangular, with 6 strong setae beneath the two apical teeth, thin hairs present all along the inner margin; galea with scattered setae on the whole surface (Fig. 3C). Pronotum rectangular with rounded corners, twice as wide as long, brown but with a narrow medial pale stripe along the medial
250 suture and a marbled impression due to several medial pale areas, lateral parts uniformly brown, margins laterally pale. Mesonotum and metanotum mostly brown but with a pale, marmoreal pattern; wingpads brownish. Ventral surface of thorax pale, furcasternites and furcal pits inconspicuous. Legs uniformly pale. Abdominal tergites brown with a pair of roundish pale spots laterally to a median, darker area. The spots are increasing in size towards the entirely pale last tergite. Ventral surface of abdomen pale brown, the distal segments darker. Paraprocts brown; cerci light brown with dense circumferential rows of bristles of varying length at the end of each segment.

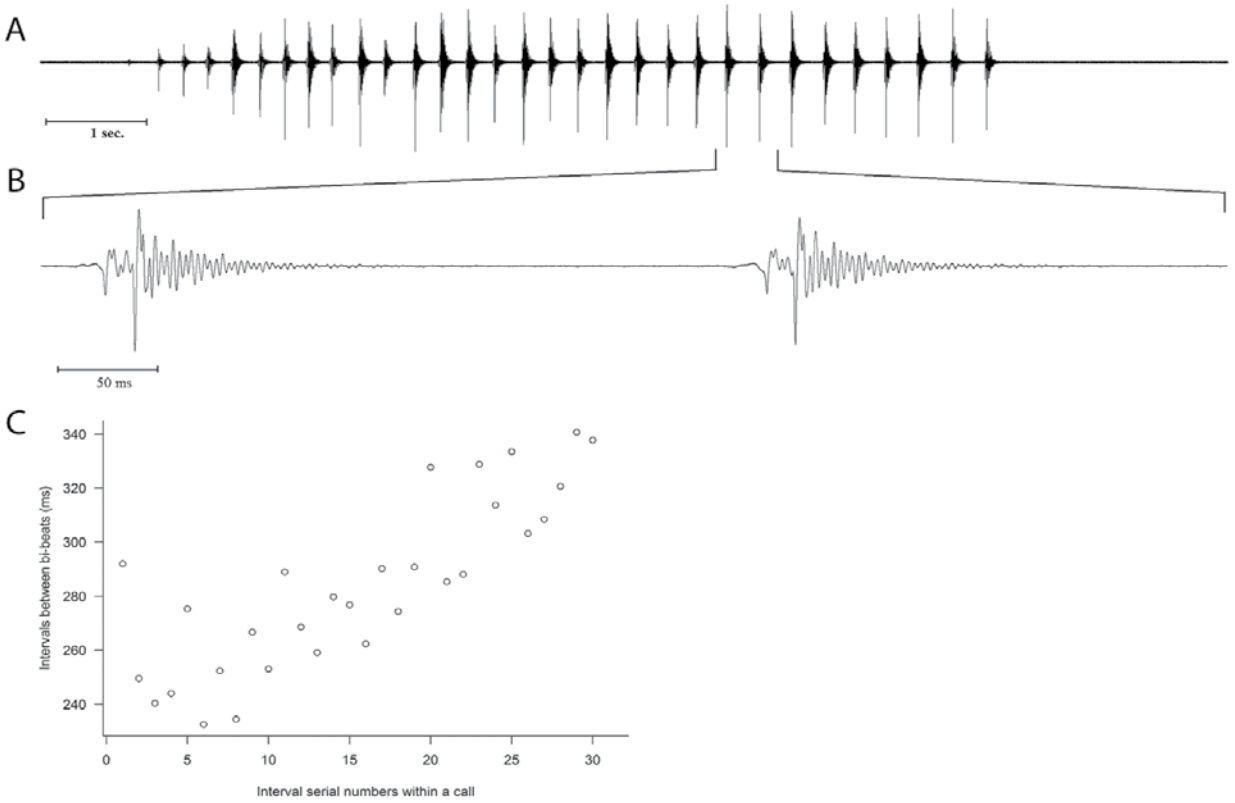

Figure 4. Oscillograms showing the drumming call of an *I. claudiae* sp. n. male (ambient air temperature 24.2 °C). **A** oscillogram showing rhythm and amplitude variation patterns of a call **B** a faster oscillogram of two bi-beats from the second half of the call **C** variation of interval duration between bi-beats during the call presented in **A**. Inter-beat intervals were measured from the amplitude peak of one bi-beat to the amplitude peak of the next bi-beat (measured on the same call presented in **A**).

Ecology and distribution. The species was collected in a small spring-brook at 535 m a.s.l. in the Karawanken, and a small forest brook at 980 m a.s.l. in the Kamnik Alps (Southern Limestone Alps).

Preliminary description of the male drumming call. Since only one signal from a single male could be recorded we cannot give any information on the variation range of the signal parameters in this species. The aim of this preliminary description is only to report the basic features of the signal, but even that should be treated with some caution since we cannot be sure whether or not the recorded signal shows some deviant features.

As it is observable in (Fig. 4A) the male call is a sequence of bi-beats. After an initial crescendo the peak amplitude of bi-beats fluctuate around a constant value. In bi-beats the first beat is of lower amplitude (missing in the low amplitude initial part of the call and sometimes hardly detectable even in the main part of the signal), the second one is of higher amplitude and followed by a long, decaying wave train (Fig. 4B). Inter beat interval within bi-beats varied between 8–20 ms during the call. The interval between bi-beats (or single beats at the initial part) gradually increased during the sequence (except for a short initial part of the sequence where inter beat interval decreases) varying between 230–350 ms (Fig. 4C).

Discussion

Relationships

The new species can be attributed to the *oxylepis* species-group sensu Murányi (2011), currently comprising *I. oxylepis oxylepis* (Despax), *I. oxylepis balcanica* Raušer, *I. bosnica* Aubert, *I. orobica* Ravizza and *I. submontana* Raušer. These species develop similarly shaped medial penial armatures and scales of penial armatures, lack real lateral armatures, and develop dense, uncoloured scales on each lobe.

Isoperla claudiae sp. n. is most similar to *I. orobica*, a species restricted to the southwestern Alps, but can be easily distinguished from the latter species as the scales of the medial penial armature are shorter in *I. claudiae* sp. n., a higher density of uncoloured scales on the penis in *I. claudiae* sp. n., as well as yellow, hardly visible rugosities of the pronotum in *I. claudiae* sp. n.

The male drumming call of *I. claudiae* sp. n. is clearly different from the drumming call of *I. oxylepis*, which is the only species of the *I. oxylepis* species-group, where published information regarding the vibratory signals is available (Rupprecht 1969, 1983). Amongst the European species of *Isoperla* the male call of *I. claudiae* sp. n. is most similar to that of *I. rivulorum* (Pictet) (Rupprecht 1969, Tierno de Figueroa and Sánchez-Ortega 1999, Tierno de Figueroa et al. 2000, 2002, 2011, Tierno de Figueroa and Luzón-Ortega 2002, Luzón-Ortega et al. 2010), but the beat group repetition period seems to be longer in this species (230–350 ms, 24.2 °C, Fig. 4C) than in *I. rivulorum* (Luzón-Ortega et al. 2010) reported 103–163 ms at 20 °C), and *I. rivulorum* frequently produces 3 beats per beat group.

The Southern Limestone Alps as centers of endemism

The southern slopes of the Alps from the Ligurian Prealps in the southwest to the Pohorje Mountains in the east are densely covered by microendemic species. Concentrations of endemic species in the south and south-eastern Alps are well known among Trichoptera species (Malicky 1983, 2000); regarding Plecoptera *Leuctra dylani* Graf, *L. juliettae* Vinçon & Graf, *L. muranyii* Vinçon & Graf and *Protonemura bipartita* Consiglio are restricted to small areas from the Bergamo prealps to the Lessinian Alps; the
252 apterous *L. istenicae* Sivec and *Siphonoperla ottomoogi* Graf are nested as microendemics in southeastern refugia referred to as the Steirische Randgebirge.

The western alpine slopes (Biellese, Graian and Cottian Alps) are another area of alpine endemism where a high diversity within the genus *Leuctra* is found (Ravizza Dematteis and Ravizza 1988; Vinçon and Ravizza 1998), and their distribution patterns are associated with the presence of nunataks during the Würm glaciation (Ravizza and Ravizza Dematteis 1993, 1994). The hitherto known range of *Isoperla claudiae* sp. n. fits well in this hot-spot of biodiversity and supports the Dinodal theory of Malicky (1983, 2000), which suggests glacial species-specific refugia

within the Alps based on distribution patterns of endemic caddisfly species. Most of these microendemic species are stenoecious elements of springs and small streams in medium altitudes.

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First record of *Satyrichthys laticeps* and second record of *Satyrichthys kikingeri* (Teleostei: Peristediidae) from the Maldives Archipelago (Indian Ocean)

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Four specimens of the peristediid genus Satyrichthys *were sampled with bottom long-lines from a depth of 180 m during the VA 'Farumas' Reef Fish Survey off North Malé Atoll, Maldives, in 1999. The largest and smallest of these specimens were deposited at Bernice Pauahi Bishop Museum (BPBM 34965, standard length (SL) 278.25 mm; BPBM 41160, SL 413.65 mm). Based on measurements and meristic counts, BPBM 34965 is characterized as* Satyrichthys laticeps *and BPBM 41160 is characterized as* Satyrichthys *cf.* kikingeri. Satyrichthys laticeps *is not only recorded for the first time for the Republic of the Maldives, but also complements distribution data on the species in the north-eastern Indian Ocean.* Satyrichthys kikingeri*, hitherto known only from a single specimen collected in 2012 from Rasdhoo Atoll in the Maldives, is now documented by a second specimen.*

Keywords: *Satyrichthys*, Peristediidae, armoured searobin, armoured gurnard, new record, distribution, zoogeography, Republic of the Maldives

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INTRODUCTION

Currently eight species are recognized in the scorpaeniform genus *Satyrichthys*, widely distributed in deep waters of the temperate and tropical Indian and Pacific Oceans (Miller, 1974; Heemstra, 1982; Richards, 1984, 1999; Kawai, 2013; Pogoreutz *et al.*, 2013). Three of these species are documented exclusively from the Indian Ocean (*S. kikingeri* Pogoreutz *et al.*, 2013) or for both oceans (*S. laticeps* (Schlegel, 1852) and *S. milleri* Kawai, 2013), the latter only from the eastern-most Indian Ocean (Andaman Sea). The other five species are known from the Pacific Ocean (Kawai, 2013; Pogoreutz *et al.*, 2013). *Satyrichthys kikingeri* and *S. laticeps* are the only species occurring in the Indian Ocean west of Sri Lanka (Kawai, 2013).

Before the description of *S. kikingeri* only four specimens of the genus, not determined to the species level, were reported from the Maldives (Anderson *et al.*, 1992; Pogoreutz *et al.*, 2013). [*Satyrichthys investigatoris* (Alcock, 1898), recorded by Adam *et al.* (1998), is now placed in the genus *Scalicus* (Kawai, 2013).] Two of these specimens were deposited at the Bernice Pauahi Bishop Museum (BPBM) at Honolulu (Hawaii) (Adam *et al.*, 1998). The aims of this paper are to identify these two individuals of *Satyrichthys* spp. to species level and to highlight the importance of these records for the sampling area.

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MATERIALS AND METHODS

The Maldives archipelago is located in the tropical Indian Ocean, south-west of India. It stretches north–south from about 7°N to about 0.5°S (Anderson *et al.*, 2011). Between the double-chain of atolls in the central Maldives, depths range between 200 and 500 m, while outside the atolls, the reef slopes drop to abyssal depths (Anderson *et al.*, 2011).

In 1991, four specimens determined as *Satyrichthys* sp. were sampled with bottom long-lines from a depth of 180 m at 'Site 2' during the VA 'Farumas' Reef Fish Survey off Giraavaru Island located at North Malé Atoll, Maldives (Anderson *et al.*, 1992; Adam *et al.*, 1998; Figure 1). The largest and the smallest of these specimens were preserved in 50% isopropanol and deposited at the BPBM at Honolulu (Hawaii) (Adam *et al.*, 1998) (Figure 2).

All morphometric measurements were made twice with an electronic caliper to the nearest 0.1 mm. Data for each specimen are presented as mean percentage of respective standard length (% SL). Morphometrics and meristics follow Pogoreutz *et al.* (2013). Both specimens were characterized as *Satyrichthys* in the combination of the following characters: (1) no teeth in both oral jaws; (2) lateral margin of the head smooth; (3) the posterior bony plates of both lower lateral series separated by the ventral row; (4) only the posterior lip

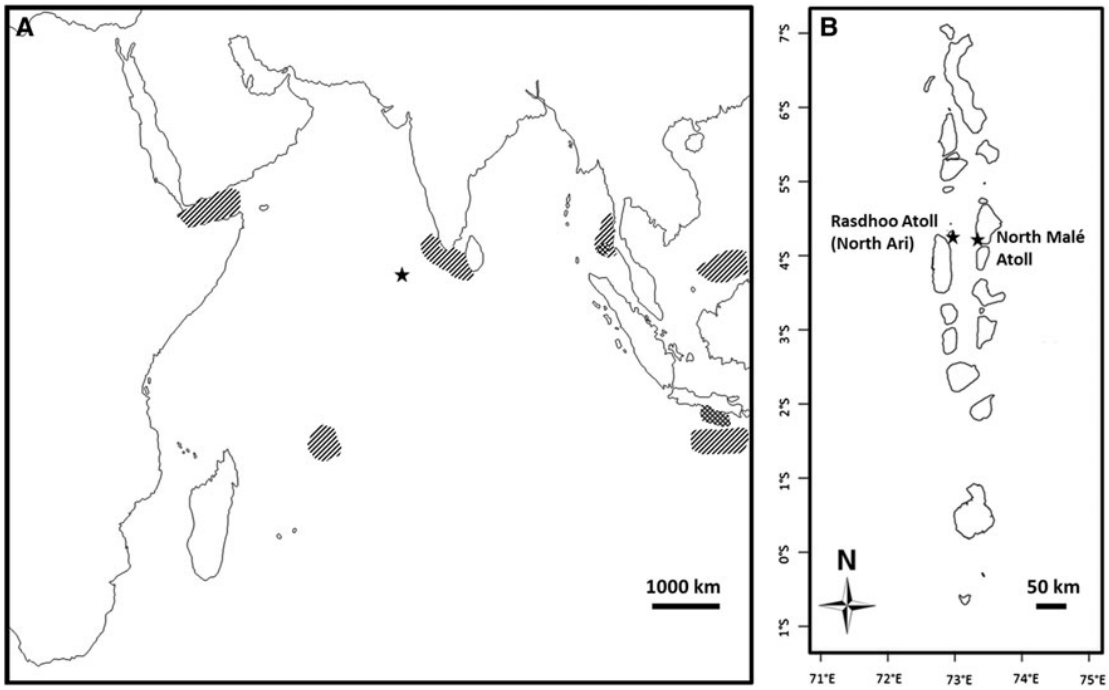

Fig. 1. Distribution of *Satyrichthys* spp. in the Indian Ocean (A), and collection sites of *Satyrichthys* specimens in the Maldives archipelago (Rasdhoo Atoll: *S. kikingeri*, holotype, NMW 96546, for details see Pogoreutz *et al.* (2013); North Malé Atoll, 'Site 2' of the VA 'Farumas' Reef Fish Survey, collected from a depth of 180 m in 1999: S. *laticeps*, BPBM 34965, SL 278.25 mm; *S.* cf. *kikingeri*, BPBM 41160, SL 413.65 mm; for details see Anderson *et al.* (1992) and Adam *et al.* (1998)) (B). Ruled and checked areas mark the distribution of *S. laticeps* and *S. milleri*, respectively, based on information on collection sites of museum specimens examined by Miller (1974) and Kawai (2013). Collection sites of the holotype of *S. kikingeri* (Pogoreutz *et al.*, 2013) and the two specimens discussed in the present publication are marked with asterisks. (B) Adapted from Pogoreutz *et al.* (2013). SL, standard length.

barbels are branched; and (5) less than 20 soft rays in dorsal and anal fins (Kawai, 2013). The sex of both specimens is unknown. Specimens were identified to species level based on: (1) size of frontal spine; (2) size of post-temporal spine; (3) size of parietal bones; (4) shape, spacing and number of gill rakers; (5) number and location of lip and chin barbels; (6) length, branching and number of flagellae of posteriormost lip barbel; and (7) presence of forward directed spines on bony plates in the upper lateral row of the caudal peduncle.

RESULTS

The smaller specimen (BPBM 34965, SL 278.25 mm) is determined as *S. laticeps* in the combination of the following characters: (1) frontal with a distinct supraorbital spine; (2) post-temporal ends in a conspicuous, well-developed spine; (3) parietal bones unequal in size on midline; (4) gill rakers elongate, slender and closely spaced; (5) four lip and three chin barbels on the left and right side; (6) posteriormost lip barbel long and branched, bearing 8/8 flagellae on the anterior and posterior side respectively; and (7) bony plates in the upper lateral row to the caudal peduncle with forward directed spines. Measurements (in mm) and count data are within the range given by Kawai (2013) (Table 1).

The larger specimen (BPBM 41160, SL 413.65 mm) is determined as *S. kikingeri* in the combination of the following characters: (1) frontal with small supraorbital spine; (2) post-temporal ends in a weak spine; (3) parietal bones unequal in size on midline; (4) gill rakers short, rounded and widely spaced; (5) three lip barbels on each side and one chin barbel on the left side (vs none on the right side); (6) posteriormost lip barbel long and branched, bearing 8/8 flagellae on the anterior and posterior side respectively; and (7) bony plates in the upper lateral row of the caudal peduncle with forward directed spines. Measurements (in mm) and count data of specimen are provided in comparison to holotype (Pogoreutz *et al.*, 2013) (Table 1).

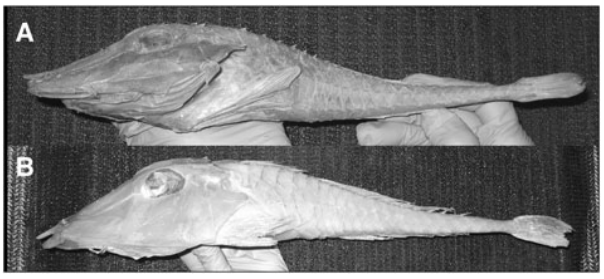

Fig. 2. Lateral views of two peristediids collected from a depth of 180 m at 'Site 2' during the VA 'Farumas' Reef Fish Survey off North Malé Atoll, Maldives, and deposited at Bernice Pauahi Bishop Museum at Honolulu, Hawaii (Anderson *et al.*, 1992; Adam *et al.*, 1998): (A) *Satyrichthys laticeps* (BPBM 34965, SL 278.25 mm), a first record for the Maldives archipelago; (B) *Satyrichthys* cf. *kikingeri* (BPBM 41160, SL 413.65 mm), a second record for this species. SL, standard length.

DISCUSSION

The number of lip barbels, which is an important diagnostic feature for the genus *Satyrichthys* (Kawai, 2008), allows separation of species within this genus into two groups: those with 2 barbels, and those with 3 or 4 barbels on the lip of the lower jaw (Kawai, 2013). To date only species with three or more

Table 1. Comparison of measurements and counts among specimens of *Satyrichthys kikingeri*, *S. laticeps* and deposited *Satyrichthys* spp. specimens caught during the VA 'Farumas' Reef Fish Survey off North Malé Atoll, Maldives, in 1991. Holotype counts and measurements of *S. kikingeri* are taken from Pogoreutz *et al.* (2013). Counts and measurements of holotype and other specimens of *S. laticeps* are taken from Kawai (2013). %SL, percentage of standard length; d, damaged; N, sample size.

Morphometric characters	**Holotype *Satyrichthys kikingeri***	**BPBM 41160 (*S.* cf. *kikingeri*)**	**Holotype *Satyrichthys laticeps***	**Range *S. laticeps* (N = 49)**	**BPBM 34965 (*S. laticeps*)**
Standard length	427.7	413.65	230.0	89.0–487	278.25
Measurements (% SL)					
Body depth	22.7	15.3	16.6	15.4–23.7	19.9
Body width	23.2	19.2	13.9	12.4–20.3	20.2
Head length	41.7	39.6	39.0	37.9–45.8	39.5
Head depth	22.9	17.6	16.3	15.9–24.1	17.7
Head width	31.3	27.0	28.1	24.4–40.2	d
Distance from snout to origin of first dorsal fin	41.2	37.8	38.5	37.2–43.7	38.0
Distance from snout to origin of anal fin	59.6	55.2	54.2	51.8–59.6	54.5
Distance from snout to anus	58.8	50.4	48.4	46.2–53.6	51.0
Snout length	22.2	19.7	20.6	19.9–23.1	20.2
Rostral projection length	5.7	d	9.5	6.3–12.4	d
Longest barbel length	12.2	10.1	19.5	11.4–26.7	15.0
Upper jaw length	16.5	14.7	16.0	14.8–17.9	15.6
Lower jaw length	16.3	12.1	15.0	14.4–18.1	17.0
Orbital diameter	7.6	8.8	8.2	7.0–11.9	8.0
Interorbital width	7.7	8.1	7.2	6.8–10.4	6.9
Preopercular spine length	5.9	7.8			7.9
Pectoral fin length	18.8	d	18.1	14.7–26.2	22.0
Length of dorsal detached pectoral fin ray	12.6	13.1	14.9	13.2–21.6	17.6
Length of ventral detached pectoral fin ray	9.8	11.5			13.0
Pelvic fin length	16.8	13.2	20.6	16.8–22.5	18.3
Length of first dorsal spine	11.5	8.3	11.1	0.8–14.4	10.6
Caudal fin length	12.2	12.4			15.6
Caudal peduncle length	10.1	12	11.1	9.6–13.3	13.2
Caudal peduncle depth	4.2	2.5	2.8	2.2–3.4	2.9
Meristic characters					
Dorsal fin rays	VII + 15	VI + 15	VII + 17	VI–VIII + 16–18	VII + 15
Anal fin rays	13	14	17	16–18	15
Pectoral fin rays (including detached ones)	15	16	15	14–17	15
Caudal fin rays	12				18
Bony plates in dorsal row	25	24	26	23–27	23
Bony plates in upper lateral row	29	30	32	29–32	29
Bony plates in lower lateral row	20	19	21	16–22	18
Bony plates in ventral row	20	20	22	19–22	19
Bony plates before anus	2	2	2	2	2
Upper gill rakers	5	3	4	4–6	5
Lower gill rakers including one at angle	17	20	17	15–22	17
Lip barbels (left/right)	3/3	3/3	4/4	4–5/3–4	4/4
Chin barbels (left/right)	1/0	1/0	3/3	2–5/2–4	3/3

lip barbels are known from the Indian Ocean: *S. kikingeri* (3 barbels); *S. laticeps* (4 barbels); and *S. milleri* (4 barbels). From these three species, *S. laticeps* and *S. kikingeri* appear to be closely related but differ in the combination of meristic and morphometric characters, discussed in detail in Pogoreutz *et al.* (2013).

The two *Satyrichthys* specimens sampled in 1991 off North Malé Atoll were identified as *S. laticeps* and *S.* cf. *kikingeri* based on morphometric and meristic characters. *Satyrichthys laticeps* represents the first record of this species for the Republic of the Maldives. The second specimen of *S.* cf. *kikingeri* shows minor meristic and some morphometric differences compared with the larger type specimen. However, the majority of count data are close or identical to those of the holotype (Pogoreutz *et al.*, 2013). As demonstrated by Kawai (2013) for *S. laticeps*, the range of body proportions in *Satyrichthys* species is obviously wide. These few differences may possibly be size or sex dependent. Therefore, until the variability of this species is better understood, we treat this specimen as *S.* cf. *kikingeri*.

All species of *Satyrichthys* are deep-water inhabitants of little commercial value. Therefore, distribution of most species is insufficiently documented (Richards, 1984, 1999; Kawai, 2008, 2013). *Satyrichthys laticeps* is the only species known to occur in the entire Indo-Pacific range of the genus (Kawai, 2013). Nevertheless, records of *S. laticeps* (mostly of synonyms) from the western (Saya de Malha Bank), northern (Arabian Sea) and eastern (Sri Lanka) Indian Ocean indicate a patchy distribution (Kawai, 2013). The present record from the Maldives complements data on the range of this species, suggesting a distribution in suitable habitats across the whole Indian Ocean. Still, the question arises how a benthic

er form such as *S. laticeps* managed to cross the dis-
m the Asian mainland to Maldivian waters. We
arval dispersal, as in many other marine teleosts, to
at importance for this group. Unfortunately, pelagic
ration (PLD) and dispersal potential of peristediids
known, and what little is known remains still
g. Bottari *et al.* (2010) mention a PLD for the
armoured sea-robin *Peristedion cataphractum* (L.
a 'few months'. However, ocean currents are com-
own as powerful agents of passive fish larval disper-
en & Sponaugle, 2009). As the Maldives archipelago
to currents changing seasonally with the monsoons
n *et al.*, 2011), there might be a potential highway for
d larval dispersal.
hough *Satyrichthys* sp. sometimes can be found dead
pon beaches (Adam *et al.*, 1998), or floating on the
ce in the Maldives (Pogoreutz *et al.*, 2013), there
published records of this genus for the Maldives
go until recently (Kawai, 2013; Pogoreutz *et al.*,
his is surprising, considering the depth range of
m of the sea floor between atolls (Anderson *et al.*,
nd therefore availability of potentially suitable
'o date, there are two species of *Satyrichthys* recorded
area, *S. kikingeri* (Pogoreutz *et al.*, 2013) and *S. lati-*
sent study). Despite the current taxonomic efforts
013; Pogoreutz *et al.*, 2013), the exact range of this
emains unknown. However, *Satyrichthys* larvae
e ocean currents as highways for dispersal, be more
listributed than previously acknowledged, and
higher diversity in the Maldives archipelago than
y thought.

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New species of Limnephilidae (Insecta: Trichoptera) from Europe: Alps and Pyrenees as harbours of unknown biodiversity

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Abstract

New species are described from the genera *Consorophylax* and *Anisogamus* (Trichoptera, Limnephilidae, Limnephilinae, Stenophylacini). Additionally the larva of the genus *Anisogamus*, and the larval stages of *Anisogamus waringeri* **sp. nov.** and *A. difformis* (McLachlan 1867) are described. The new species *Consorophylax vinconi* **sp. nov.** is a microendemic from the Southern Alps and differs from its congeners in the shape of the parameres, which are distinctly straitened in the distal quarter in the new species. The new species *Anisogamus waringeri* **sp. nov.** represents the second species in the hitherto monospecific genus *Anisogamus*. Compared to *Anisogamus difformis*, the male of *A. waringeri* **sp. nov.** has more-slender superior appendages; a more-rounded basal plate of the intermediate appendages, lacking pointed protuberances; and parameres shorter than the aedaegus, proximally with one dorsal and several ventral tines. Further, the two species are disjunctly distributed in the European mountain ranges (*A. difformis*: Alps, *A. waringeri* **sp. nov.**: Pyrenees). Larvae of species in the genus *Anisogamus* are characterized by the lack of a dorsal protuberance on abdominal segment I, a unique feature among Eurasian Limnephilidae. *Anisogamus difformis* and *A. waringeri* **sp. nov.** larvae differ in pronotum shape. The discovery of two new species demonstrates the significance of taxonomic studies in Europe, and the importance of adequate training for young scientists in order to assess an incompletely described biodiversity under threat of extinction.

Key words: endemism, species description, *Consorophylax*, *Anisogamus*, caddisflies

Introduction

Both the Alps and the Pyrenees are centres of biodiversity in Europe. Particularly patterns of plant, vertebrate and terrestrial invertebrate diversity in European alpine ecosystems have been extensively studied (e.g., Wohlgemuth 2002; Nagy *et al.* 2003; Iserbyt *et al.* 2008; Huemer 2011). Increasingly, aquatic invertebrates (and EPT-taxa in particular) have become the focus of attention in both the Alps and the Pyrenees (e.g., Sipahiler 1999, 2000; Graf 2005; Graf *et al.* 2008a; Malicky 2004, 2008; Brown *et al.* 2009). The genus *Consorophylax* Schmid 1955 currently comprises seven cold-stenotherm species (Malicky 2004, 2008). Larvae of the genus prefer crenal to epirhithral segments of alpine to montane springs and brooks, and mainly behave as shredders (Graf *et al.* 2008b). 263
Consorophylax species show a complex distribution pattern, with several microendemics and two widespread species inhabiting the majority of the Alpine arc. In particular, the southern slopes of the Alps can be identified as centres of species richness in the genus, as microendemics have been found exclusively on the southern slopes of both the Western and Eastern Alps (Kimmins & Botosaneanu 1967; Graf *et al.* 2008b).

The genus *Anisogamus* McLachlan 1874 is currently represented by a single species, *A. difformis* (McLachlan 1867). The species is known predominantly from the Alps, but has also been recorded in the Pyrenees. As the larva was hitherto not described, ecological parameters of adult collection points indicated a cold-stenotherm species

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with a preference for the crenal and epirhithral segments of alpine to montane springs and brooks (Graf *et al.* 2008b).

During extensive collections in 2012 and 2013, WG recovered several male adult and larval specimens of putative *A. difformis* from the Pyrenees. Also, undescribed *Consorophylax* specimens were collected in 2012 by Gilles Vinçon, Grenoble, France. Using a classical taxonomic approach we were able to identify the specimens as new species, *A. waringeri* **sp. nov.** and *C. vinconi* **sp. nov.** Additionally, genetic sequence data were used to delineate species of *Anisogamus* and to affiliate the larva of *A. waringeri* **sp. nov.** with its identifiable adult.

In this paper, we describe the two new species of these genera *Consorophylax* and *Anisogamus* (Limnephilidae, Limnephilinae, Stenophylacini), the larval stage of the genus *Anisogamus*, and the larvae of *A. difformis* and *A. waringeri* **sp. nov.** We also provide a re-description of *A. difformis*.

Material and methods

Adult specimens were collected using sweep nets, larvae were collected by handpicking. Collected specimens were stored in 70% and 96% EthOH, for morphological and molecular analyses, respectively. Larvae of *A. difformis* were fixed and stored in formaldehyde for a prolonged period, preventing molecular analysis of those specimens. Morphological characteristics of male genitalia were examined in KOH-treated, cleared specimens. Comparative material from the authors' collections enabled the identification of new species. Nomenclature of male genitalia follows Nielsen (1957, for *Stenophylax stellatus* (Curtis 1834), synonym of *Potamophylax latipennis* Curtis 1834). Larval morphological features are named following Wiggins (1998) and Waringer & Graf (2011), nomenclature of primary setae and setal areas follows Wiggins (1998). Illustrations were prepared according to Thomson & Holzenthal (2010): Briefly, pencil drawings of the cleared specimens were produced using a camera lucida mounted on a compound microscope, and digitally edited and "inked" with Adobe Illustrator (v. 16.0.4, Adobe Systems Inc.)

In addition to morphological features, molecular genetic sequence data were compared in order to delineate two *Anisogamus* species and to support larval association in *Anisogamus* **sp. nov.** DNA extraction and amplification of the 658-bp-long "barcode region" of the mitochondrial cytochrome oxidase *c* subunit I (mtCOI) using primers LCO-1490 and HCO-2198 (Folmer *et al.* 1994) and the 541-bp-long fragment of the same gene using primers S20 and Jerry (Simon *et al.* 1994; Pauls *et al.* 2006) were performed as outlined by Pauls *et al.* (2008); Previšić *et al.* (2009) and Gibson *et al.* (2010). Sequences were edited manually using the program Geneious R7 (Biomatters 2014) and aligned using MAFFT (Katoh & Standley 2013). Sequences were deposited in GenBank under Accession nos: KP174658-KP174663. Inter- and intraspecific genetic distances (uncorrected *p*-distances) were calculated in Mega 4.0.1 (Tamura *et al.* 2007).

Consorophylax vinconi sp. nov. Graf & Malicky

Figs. 1A–D, 2

Holotype. 1 male: Italy, Torino, Valchiusella, Fondo, Burdeiver brook (45°30'59.60''N 7°39'09.72''E), 1800–1900 m a.s.l., 01.ix.2012, leg. G. Vinçon. Holotype deposited in the Biologiezentrum des Oberösterreichischen Landesmuseums, Linz, Austria. Paratype. 1 male, same collection date, in coll. Malicky.

Diagnosis. The new species is a *Consorophylax* most similar to *C. piemontanus* Botosaneanu 1967 (in Kimmins & Botosaneanu 1967), but exhibiting (1) parameres that are distinctly constricted in the distal quarter, with terminal spines, and (2) inferior appendages that are slightly bifurcated in lateral view and with a sharp median tip in caudal view. *Consorophylax piemontanus* has parameres tapered, lacking terminal spines; inferior appendages are not bifurcate in lateral view and with a rounded median tip in caudal view.

Description. General appearance light brown (in alcohol), tergites and sternites light brown; cephalic and thoracic setal areas cream-coloured; cephalic, thoracic and abdominal setation light brown; legs light brown; haustellum and intersegmental integument cream-coloured; wings light brown, translucent, setation on veins and membrane light brown, length of each forewing 15 mm. Male maxillary palps each trisegmented, tibial spur fomula 2,3,4.

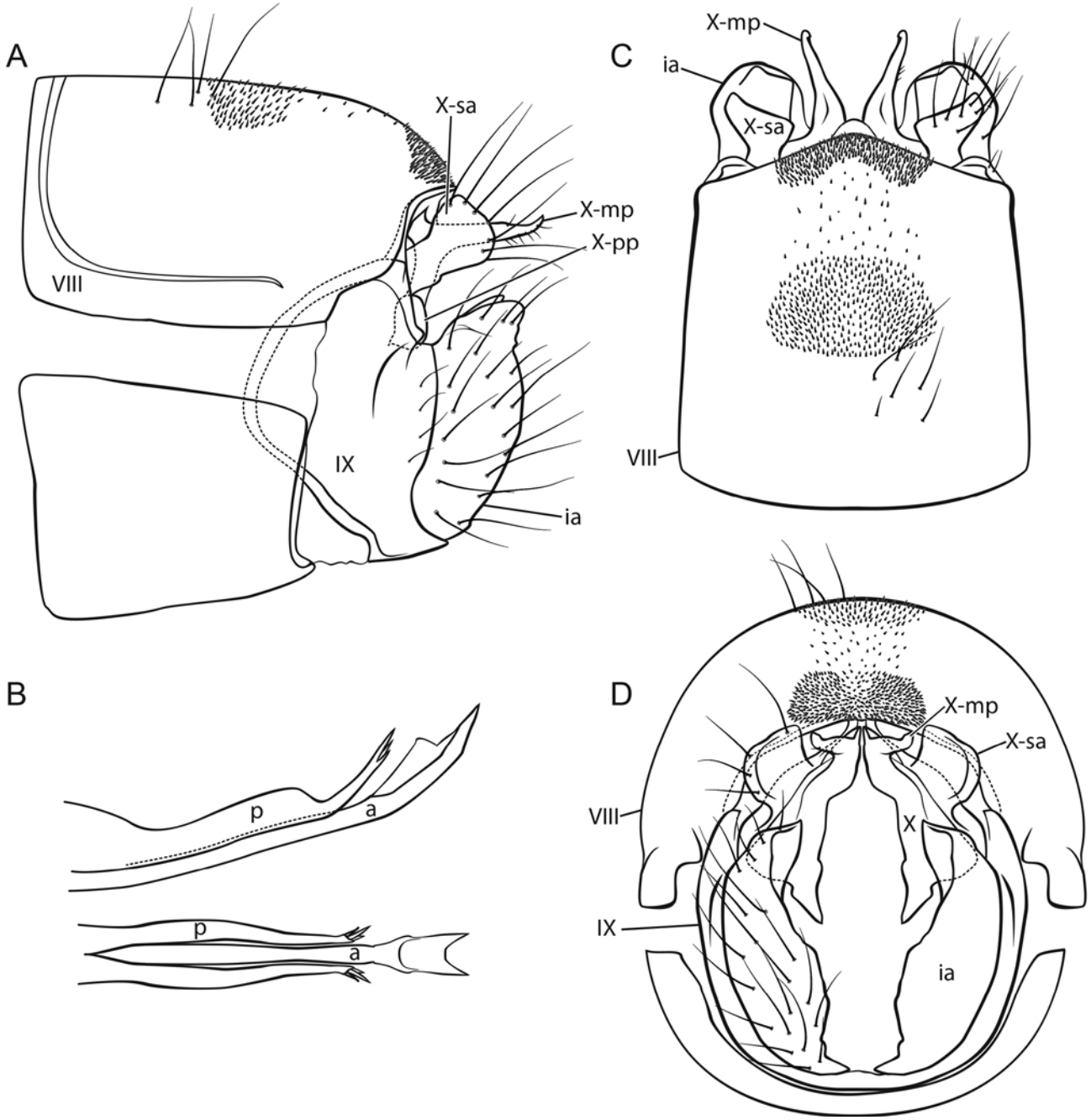

FIGURE 1. Male genitalia of *Consorophylax vinconi* **sp. nov.** A, left lateral; B, phallic apparatus, left lateral (upper) and dorsal; C, dorsal; D, caudal. Abbreviations: VIII = abdomen VIII; IX = abdomen IX; X = segment X; X-sa = lateral process of segment X ('superior appendage'); X-mp = median process of segment X ('intermediate appendage'); X-pp = posterior process of segment X; ia = inferior appendage; p = paramere; a = aedeagus.

Male genitalia (Figs. 1A–D). Tergite VIII (VIII) light brown, with median circular area of spines extending to bilobed caudal area of spines. Dorsal third of segment IX (IX) reduced to narrow transverse bridge, ventral 2/3rds broad, with distinct triangular anterior protuberance in lateral view. Lateral processes of segment X ("superior appendages", X-sa) in lateral view capitate, with small proximal ventral bulge; in dorsal view subtriangular; in caudal view subrectangular, medially concave. Sclerite of segment X (X) divided into clearly separate vertical plates on either side of phallocrypt and between lateral processes, each bearing 1 long subhorizontal, median process ("intermediate appendage", X-mp) from dorsal end, in lateral and dorsal views this process forming distinctly tapering caudad rod with sharp tip curved dorsad; posterior process on each sclerite of segment X (X-pp) in lateral view forming rounded bulge visible between lateral processes of segment X and inferior appendages (ia), 265

in caudal view ventral part of each half of segment X subtriangular with rounded posterior process directed somewhat laterad and median process pointed toward viewer. Inferior appendages in lateral view broad, stout, each with dorsal portion directed somewhat caudad and consisting of median and lateral tip; in dorsal view stout; in caudal view dorsal portion with sharp median tip and blunt lateral tip. Aedeagus (a) slender, in lateral view curved dorsad, in dorsal view tip bifurcate. Parameres (p) in lateral view basally broad, each abruptly constricted in distal quarter to form slender, dorsally curved tip bearing 3-4 small spines.

Female, pupa, larva, and egg unknown.

Etymology. Named for the French entomologist Gilles Vinçon.

Distribution & biogeography of *Consorophylax* species. The genus *Consorophylax* has a strictly European alpine distribution. Most species are restricted to small areas, like *C. carinthiacus* Malicky 1992 (Karnische Alpen, southern Alps), *C. corvo* Malicky 2008 (Piemont, western Alps), *C. delmastroi* Malicky 2004 (Piemont, western Alps), *C. montivagus* (McLachlan 1867) (Koralpe, Saualpe, southeastern Alps), *C. piemontanus* (Piemont, western Alps) (Fig. 2). *Consorophylax styriacus* Botosaneanu (in Kimmins & Botosaneanu 1967) (eastern Alps) and *C. consors* (McLachlan 1880) (western Alps) have a slightly broader distribution within the Alpine arc (Fig. 2). Although we do not know the exact distribution range, *C. vinconi* **sp. nov.** is possibly another microendemic species within the genus. *Consorophylax vinconi* **sp. nov.** is most similar to *C. piemontanus*, which was described from Avigliana, some 13 km west of Torino. Interestingly, the type locality of *C. vinconi* **sp. nov.** is about 56 km north-northeast from the type locality of *C. piemontanus*. This supports the interpretation of *C. vinconi* **sp. nov.** as an alpine microendemic.

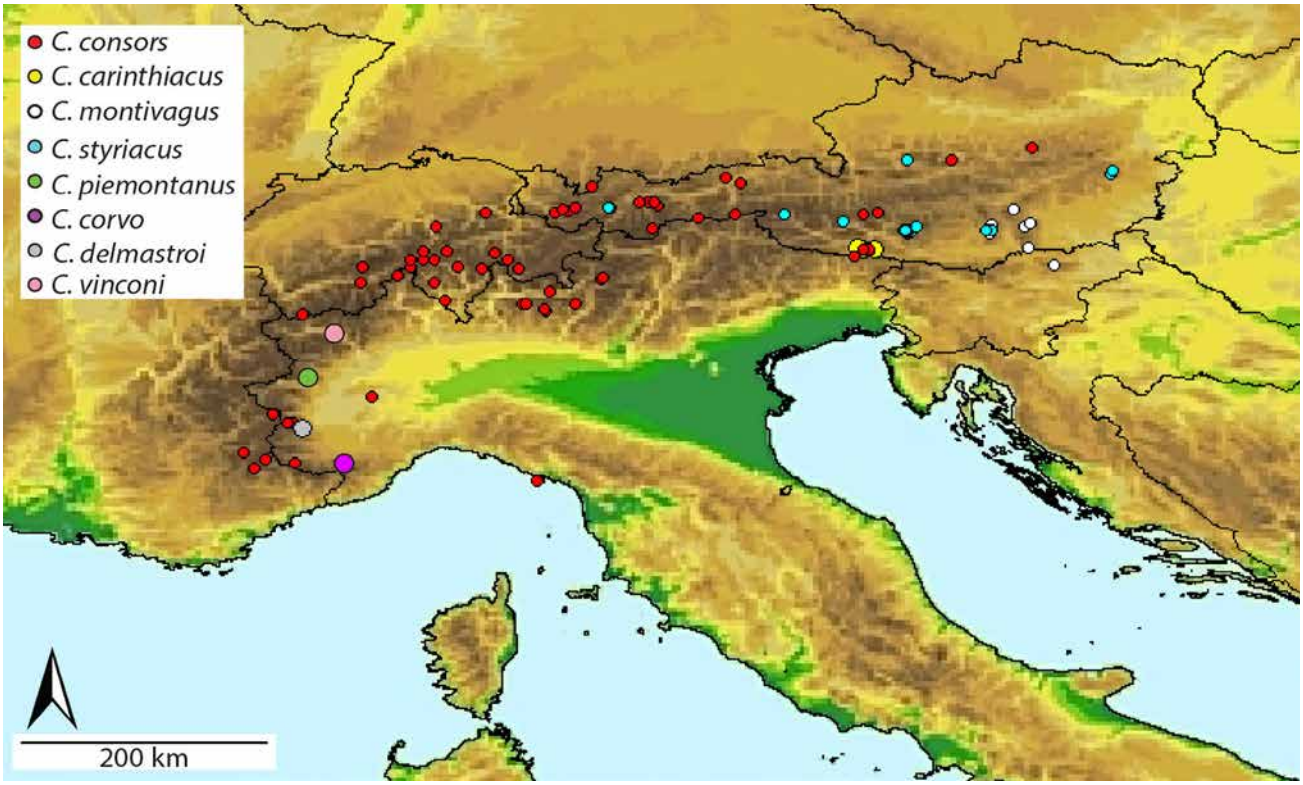

FIGURE 2. Known distributions of *Consorophylax* species.

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***Anisogamus waringeri* sp. nov. Graf & Vitecek**
Figs. 3A–D, 4A–D

Holotype. 1 male, France, Pyrenées-Orientales, Mont Canigou, Col de Jou, Refuge de Mariailles (42°29'6.21"N 002°24'48.31"E) 12.vii.2012, leg. W. Graf. Holotype deposited in the Biologiezentrum des Oberösterreichischen Landesmuseums, Linz, Austria. Paratypes: 8 males, same location, 12.vii.2012, 13.vii.2013, leg. Graf.

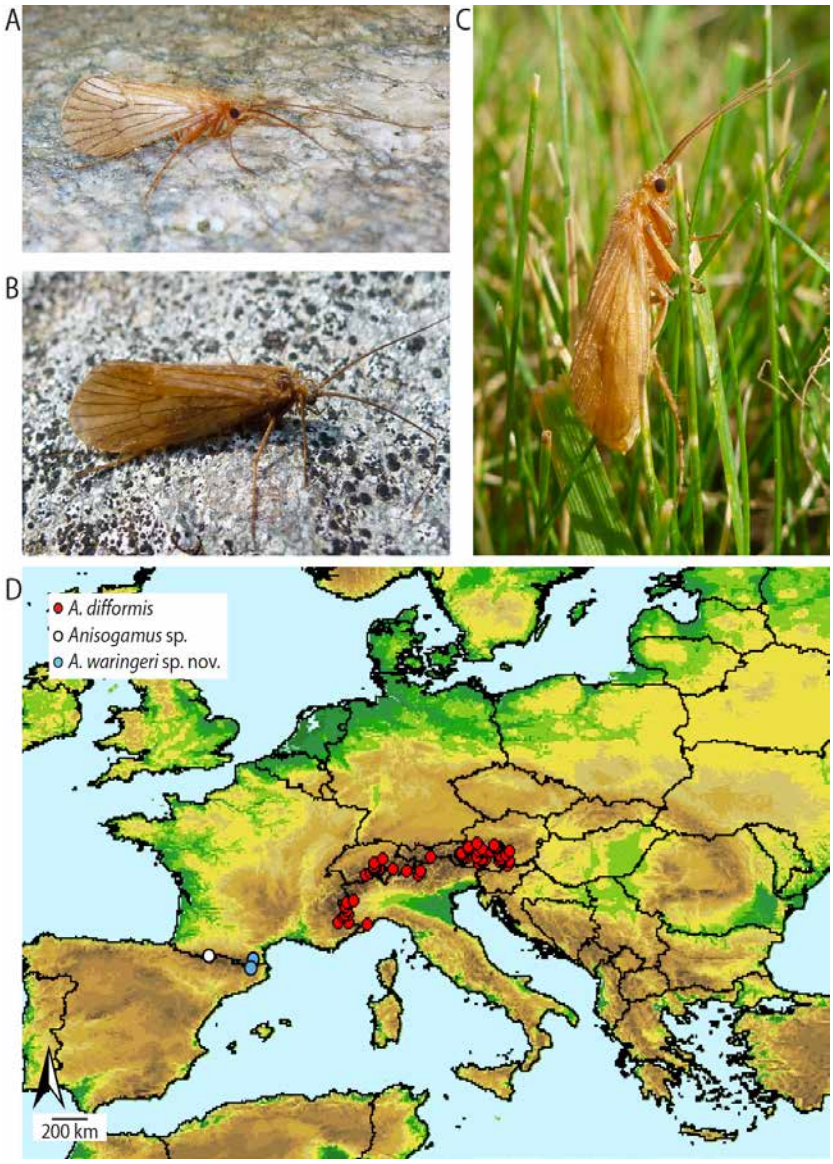

FIGURE 3. Habitus images and known distributions of *Anisogamus* species. A, male *A. waringeri* **sp. nov.**, right lateral; B, male *A. difformis*, right lateral; C, shorter-winged female of *A. waringeri*, right lateral; D, map of the known geographical distributions of *Anisogamus* species. Records of *Anisogamus* sp. from the Pyrenees are those of Décamps (1967). *Anisogamus waringeri* **sp. nov.** was recorded from the Pyrenees by Menéndez & González (2009) as *A. difformis* (pers. comm. M. A. González).

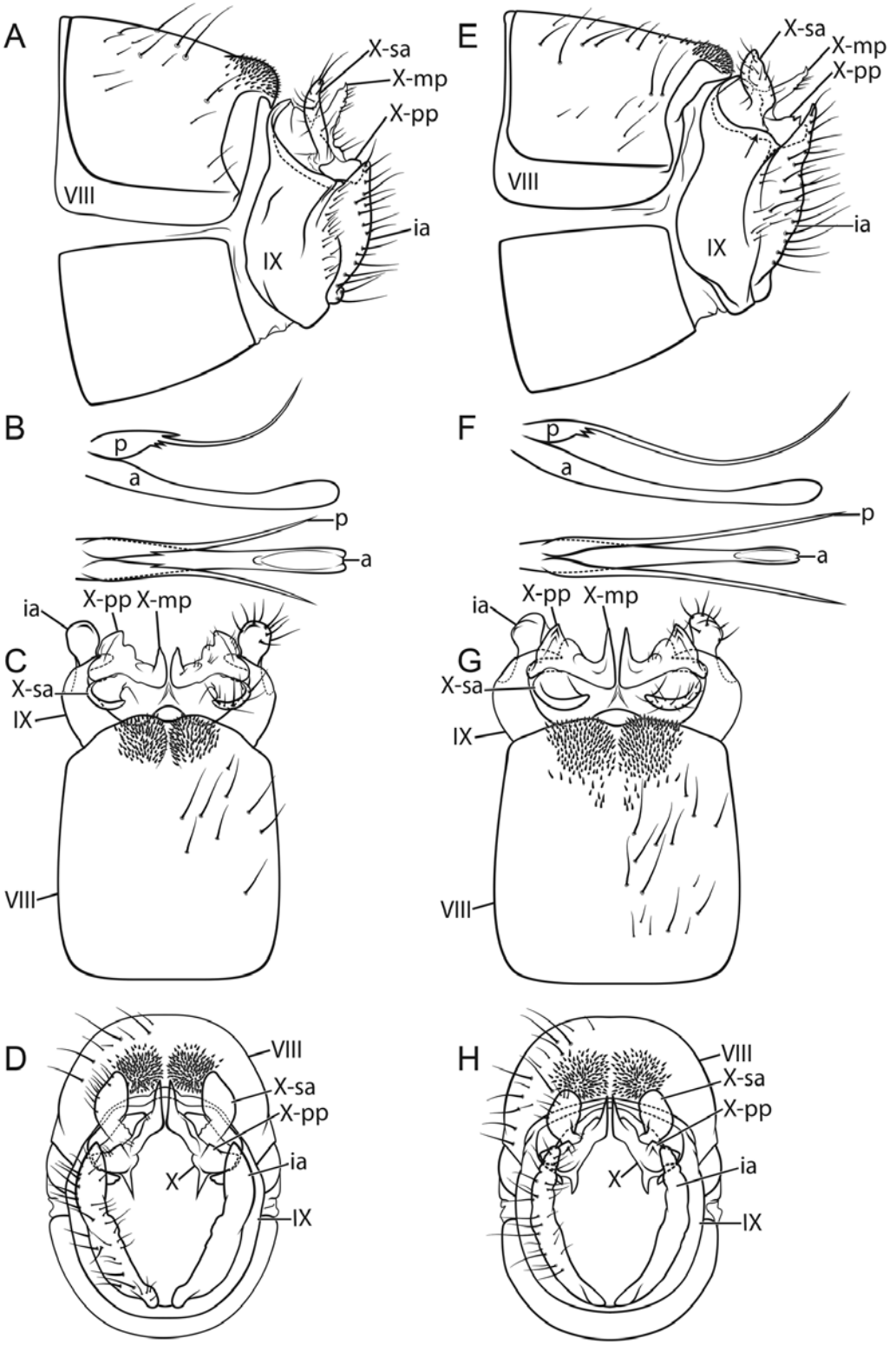

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FIGURE 4. Male genitalia of *Anisogamus* species. A–D, *A. waringeri* **sp. nov.**: A, left lateral; B, phallic apparatus, left lateral (upper) and, dorsal; C, dorsal; and D, caudal. E–H, *A. difformis*: E, left lateral; F, phallic apparatus, left lateral (upper) and dorsal; G, dorsal; and H, caudal. Abbreviations as in Fig. 1.

Diagnosis. *Ansiogamus waringeri* **sp. nov.** has the following combination of male genitalia characters: (1) lateral processes of segment X ("superior appendages") slender, tall; (2) median processes of segment X ("intermediate appendages") with tips projecting dorsad, posterior processes of segment X in lateral view forming a rounded hump, discernible in caudal and dorsal view as a sharp ridge; (3) general appearance of inferior appendages stout, in lateral view stout with a short tip, in caudal view pointed; and (4) parameres shorter than the aedeagus, in dorsal and lateral views with a dorsomesal tine. The male differs distinctly from that of *A. difformis* (McLachlan 1867) which exhibits the following combination of male genitalia characters: (1) lateral processes of segment X ("superior appendages") stout, wide; (2) median processes of segment X ("intermediate appendages") with tips projecting posterodorsad, posterior process of segment X in lateral view with 2 sharp projections, discernible in caudal and dorsal view as distinct, sharp tips; (3) general appearance of inferior appendages slender, in lateral view slender with a long tip, in caudal view rounded; (4) parameres longer than the aedeagus, lacking a dorsal tine.

Description. General appearance (Fig. 3A): yellow to fawn, tergites and sternites yellow to fawn; cephalic and thoracic setal areas cream-coloured; cephalic, thoracic and abdominal setation yellow; legs yellow to fawn; haustellum and intersegmental integument cream-coloured; wings yellow, translucent, with dark veins, setation on veins and membrane yellow. Male maxillary palps each trisegmented, spur formula 1,3,4.

Male genitalia (Figs. 4A–D). Tergite VIII yellow to fawn, with pair of suboval spinate areas clearly separated medially. Abdomen IX in lateral view lacking dorsad bulge. Lateral processes of segment X ("superior appendages") in lateral view slender, angled dorsad, in dorsal view posteriorly concave, in caudal view suboval, somewhat converging medially. Segment X clearly separated into pair of subtriangular vertical plates on either side of phallocrypt and between its lateral processes in caudal view, each with dorsal end bearing 1 long median process ("intermediate appendage") projecting nearly dorsad in lateral view; sclerites of segment X in lateral and caudal views each with rounded posterior process, this posterior process in dorsal view rounded subtriangular with sharp dorsal ridge. Inferior appendages partially fused with abdominal segment IX, in lateral view each stout with short, stout dorsal tip, this tip in caudal view pointed; in dorsal and caudal views inferior appendages stout. Aedeagus in lateral view distally bulbous, in dorsal view distal part semimembraneous. Parameres shorter than aedeagus, evenly curved dorsad; each with proximal part bulbous, bearing 2–3 ventral tines and distinct dorsomesal tine (best seen in dorsal view).

Female, pupa, and egg unknown.

Etymology. Named for Johann Waringer, Austrian entomologist.

***Anisogamus difformis* (McLachlan 1867)**

Figs. 4E–H.

Material examined. 7 males; Austria, Carinthia, Saualpe, Ladinger Hütte, 28–29.vi.2012, leg. W. Graf. 7 males, 3 females: Italy, Torino, Fondo, Gias del Prete (45°31'13.37"N 007°39'40.59"E) 12.vii.2012, leg. G. Vinçon. 1 male: France, Provence Alpes-Côte d'Azur (44°20'16.4"N 006°47'27.9"E) 29.vii.2012, leg. W. Graf. 4 males: Austria, Carinthia, Saualpe, Offener Hütte, 30.vi.2006, leg. W. Graf. 9 males: Austria, Carinthia, Saualpe, Ladinger Alm, 29.vi.2012, leg. W. Graf. 27 males: Austria, Carinthia, Saualpe, Ladinger Hütte, 15.vii.2006, leg. W. Graf.

Type locality. Austria, Carinthia, Saualpe.

Description. General appearance (Fig. 3B) yellow to fawn, sclerites yellow to fawn; cephalic and thoracic setal areas pale cream-coloured; cephalic, thoracic and abdominal setation yellow; legs yellow to fawn; haustellum and intersegmental integument pale cream-coloured; wings yellow, translucent, with dark veins, setation on veins and membrane yellow. Male maxillary palps each trisegmented. Spur formula 1,3,4.

Male genitalia (Fig. 4E–H). Tergite VIII yellow to fawn, with pair of suboval spinate areas separated medially. Abdomen IX in lateral view with dorsal bulge (Fig. 4E, arrow). Lateral processes of segment X ("superior appendages") in lateral view stout dorsal lobes, in dorsal view posteriorly concave, in caudal view suboval, somewhat converging medially. Segment X clearly separated into pair of subtriangular vertical plates on either side of phallocrypt and between its lateral processes in caudal view, each with dorsal end bearing 1 median process ("intermediate appendage") projecting posterodorsad in lateral view; sclerites of segment X in lateral, dorsal and caudal views each with 2 pointed protuberances on rounded posterior processes, these posterior processes in dorsal

view rounded subtriangular. Inferior appendages partially fused with abdominal segment IX, in lateral view slender with acute dorsal tip, this tip in caudal view rounded; in dorsal and caudal views inferior appendages slender, delicate. Aedeagus in lateral view distally bulbous, in dorsal view distal part semimembraneous. Parameres longer than aedeagus, evenly curved dorsad; each with proximal part bulbous, bearing 2–3 ventral tines.

Description of the larvae of the genus *Anisogamus*

Note: Photographs were obtained for larval specimens of *A. difformis* and *A. waringeri*. The description of larval characters refers to both species, if not stated otherwise. Larvae of *A. waringeri* were compared with larvae collected close to the type locality of *A. difformis* in Carinthia and morphological differences are described.

Larvae of the family Limnephilidae typically share the following set of morphological characters (Waringer & Graf 2011):

- sclerites present on pro-, meso-, and metanota;
- mesonotum completely covered by 2 sclerites, separated by unbranched longitudinal suture;
- metanotum incompletely sclerotized with 3 pairs of small sclerites in anteromedian, posteromedian and lateral positions [only 5 sclerites in *Hydatophylax* spp., metanotal sclerites reduced to just setal groups in some species];
- prosternal horn present [absent from *Drusus chrysotus* Rambur 1842];
- antennae situated halfway between eyes and anterior head margin;
- fleshy protuberances present laterally and dorsally on abdominal segment I.

In the genus *Anisogamus*, only lateral protuberances are present on abdominal segment I and the dorsum is completely covered by setae without a protuberance (Fig. 5A, triangle). To our current knowledge, this is the major character to distinguish larvae of the genus *Anisogamus* from all other limnephilid genera, which develop 3 protuberances on abdominal segment I. Ignoring the strikingly different morphology of abdominal segment I, recent larval determination keys focusing on Central European Chaetopterygini and Stenophylacini (Waringer & Graf 2011, 2013) key the larvae of *Anisogamus* spp. together with a group species in which larvae develop more than one posterolateral seta on each side of abdominal segment IX (*Allogamus ligonifer* McLachlan 1862, *Potamophylax* spp., *Platyphylax frauenfeldi* Brauer 1857, *Acrophylax zerberus* Brauer 1867 and *Psilopteryx psorosa* Kolenati 1860). By examining morphological characters listed by Waringer *et al.* (2013; table 1), *P. psorosa* remains the only taxon similar to *Anisogamus* spp. larvae. The ventral-edge setae on mid- & hind femora in *Anisogamus* have contrasting color, while all ventral-edge setae in *Psilopteryx* are dark (Fig. 5C, D).

Descriptions of the fifth instar larvae of *Anisogamus difformis/waringeri* sp. nov.

Material. *Anisogamus waringeri* **sp. nov.**: Four 5th instar larvae, France, Pyrenées orientales, Mont Canigou, Col de Jou, Refuge de Mariailles (42°29'6.21"N, 002°24'48.31"E), 13.vii.2013, leg. W. Graf. *Anisogamus difformis*: Five 5th instar larvae, Austria, Carinthia, Gleinalpe, Hochalm, 21.vi.2014, leg. W. Graf.

Biometry. *Anisogamus waringeri* **sp. nov.**: Body length of final instar larva (fixed in alcohol) 12.6–13.7 mm, head width 1.56–1.68 mm (n=3). *Anisogamus difformis*: Body length of final instar larvae (fixed in formaldehyde) 10–12.5 mm, head width 1.52–1.68 mm (n=5).

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Head. Head capsule finely granulated with microspinules, slightly elongated in shape, hypognathous (Fig. 6A), with medium brown coloration. Muscle attachment spots on frontoclypeus and parietalia dark brown and distinct (Fig. 6A). Yellowish ring present around each eye (Fig. 6A). Head capsule with 17 pairs of primary setae, lacking seta #18 and any additional setation: Each parietal bearing 10 dorsal and 1 ventral primary setae, of which #9, 14, and 17 long and conspicuous. Further, in *A. difformis* lengths of setae #13 and 16 less than half as long as #15, whereas setae #13 and 16 more than half as long as #15 in *A. waringeri* **sp. nov.** Frontoclypeus bell-shaped with a narrow central constriction, each side bearing 6 primary setae, #5 long and conspicuous (Fig. 6B). Labrum medium

brown; with setal brush and primary setae #1–3 at anterolateral margins; on dorsal area, setation consisting of primary setae #4–6. Antennae situated halfway between eyes and anterior head margin (Fig. 6A,B), short, each consisting of 1 short cylindrical base and 1 short flagellum. Ventral apotome narrow and elongate, medium brown, postgenal suture approximately 45–50% of apotome length. Each black mandible with 5 terminal teeth along its edge; in addition, ridges present in central concavity.

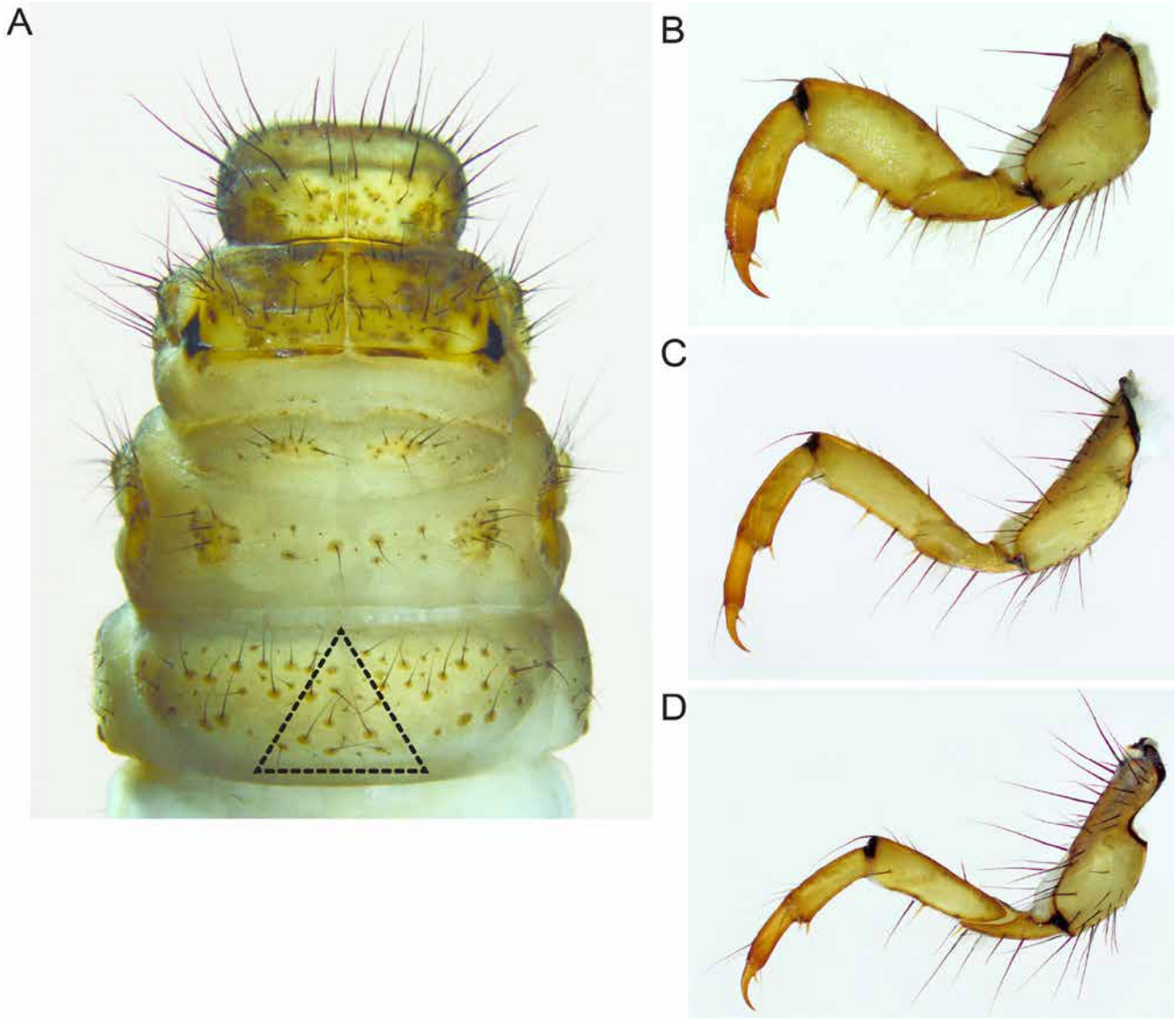

FIGURE 5. Larva of *A. waringeri* **sp. nov.** A, thorax and abdominal dorsum I, with the usual position of a dorsal protuberance marked with a dotted triangle; B, right prothoracic leg, anterior; C, right mesothoracic leg, anterior; D, right metathoracic leg, anterior.

Thorax. Pronotum medium to yellowish brown with dark brown muscle attachment spots and with finely granulate surface (Fig. 5A), its posterior and lateral margins thickened and darkly striped (Fig. 5A). Pronotal transverse groove at end of anterior 3rd (Figs. 6B, C, arrows); in profile, posterior 2/3rds very slightly rounded (Fig. 6C,D). Pronotal transverse groove much shallower in *A. waringeri* **sp. nov.** (Fig. 6D, arrow), more pronounced in *A. difformis* (Fig. 6C, arrow). Along anterior border three setal rows present: (1) dense fringe of short, curved, fine, yellow setae; (2) widely-spaced, continuous row of intermediate curved, pale yellow setae; and (3) widely-spaced, continuous row of long, straight, dark setae present. Following row (3), row of pale yellow (*A. difformis*) or dark (*A. waringeri* **sp. nov.**) setae present (Fig. 6E,F, arrows). Further, in *A. difformis*, dark and pale/yellow setae present on pronotum, whereas in *A. waringeri* **sp. nov.**, only dark setae present. Including anterior setal rows (2) and (3), 40–50 setae of varying lengths distributed over each pronotal half in *A. waringeri* **sp. nov.**

(Fig. 7A), and 30–40 in *A. difformis*. Central prosternite inconspicuous; prosternal horn present. Mesonotum completely covered by 2 medium brown sclerites, their posterior margins darkly sclerotized (Fig. 5A). Metanotum partially covered by 3 pairs of medium brown sclerites. Anterior metanotal sclerites (sclerites of setal area 1, *sa*1) narrowly elliptical; distance between them greater than their length (Fig. 5A). Row of setae present between posteromedian sclerites (sclerites of setal area 2, *sa*2); setae lacking between each lateral (sclerites of setal area 3, *sa*3) and posteromedian sclerite (*sa*2) (Fig. 5A). Legs yellow to fawn with numerous setae on coxae, trochanters and femora; tibiae and tarsi with only small number of setae; all femora each with only 1 proximodorsal seta present (Figs. 5B–D). Coxa, femur and tibia of each foreleg much wider than those of mid- and hind legs. Additional setae lacking from anterior and posterior faces of all femora; ventral trochanteral brush at distal section of each trochanter present on all legs. Rows of minute spines present along ventral edges of femora; pairs of ventral-edge setae pale on forefemora, but pale and dark on mid- and hind femora, respectively (Figs. 5B–D).

Abdomen. Abdominal segment I with 2 lateral fleshy protuberances, lacking dorsal protuberance (Fig. 5A, triangle). Dorsal setal areas *sa*1 and *sa*2 fused, creating continuous band of setae completely spanning dorsum (Fig. 5A); setal area *sa*3 separate, small, covering dorsal section of each lateral protuberance (Fig. 7B, circle); 1 large, oval sclerite on posterior surface of lateral protuberance, lacking setae but bearing 2 holes (Fig. 7B). Ventral setal areas *sa*1 and *sa*2 fused to form continuous field of setae, many on basal sclerites of differing sizes, these sclerites sometimes fused in some specimens; setal areas *sa*1 not fused centrally leaving median bald patch (Fig. 7C, circle); setal areas *sa*3 separate, covering area below each lateral protuberance (Fig. 7B). On abdominal dorsum VIII, number of posterodorsal setae typically 8–16, with 4–6 long setae and 4–10 remaining setae short. Each half of abdominal dorsum IX with 1–3 posterolateral seta(e) (Fig. 7D, arrow). Further, pair of long stout setae present on abdominal venter IX (Fig. 7D, arrow). Median brown sclerite on abdominal tergum IX semicircular; along its posterior border, 7–8 long and several shorter setae present, 1 long seta having position of central intermediate *c* setae. Anal prolegs of limnephilid type, light brown, with medium brown muscle attachment spots. Anal claws medium brown, each with 1 small accessory hook. All gills single filaments. Dorsal gills present at most from segment II (presegmental position) to segment V (postsegmental position). Ventral gills ranging from segment II (presegmental position) to segment VII (postsegmental position). Lateral gills present from segment II (presegmental) to segment III (postsegmental position) in *A. waringeri* **sp. nov.**, lacking in *A. difformis*. Lateral fringe extending on each side from last 3rd of abdominal segment III to end of abdominal segment VIII, forked lamellae above lateral line absent.

Case (Fig. 7E). Larval case of mineral particles and leaf particles; length 13.1–14.8 mm in *A. waringeri* **sp. nov.** (n= 3), 11.7–12.9 mm in *A. difformis* (n=3); almost straight, slightly bend, tapering somewhat posteriorly (width at anterior opening 3.5–3.9 mm and at posterior opening 2.6–3.0 mm in both species).

Differential diagnosis of *Anisogamus* larvae. Larvae of *A. waringeri* **sp. nov.** and *A. difformis* differ quite distinctly in the following characters: (1) The pronotal transverse groove in lateral view is deep in *A. difformis*, shallow in *A. waringeri* **sp. nov.**; (2) the pronotal setation is dark and pale/yellow in *A. difformis*, only dark in *A. waringeri* **sp. nov.**; (3) lateral gills are lacking in *A. difformis*, present from segment II (presegmental) to segment III (postsegmental position) in *A. waringeri* **sp. nov.**

Anisogamus species delimitation and larval affiliation

An analysis of the genetic distance of mtCOI between two *Anisogamus* males clearly supports differentiation of *A. difformis* and *A. waringeri* **sp. nov.** as separate species. Uncorrected *p*-distances recorded in both fragments of the mtCOI gene (i.e., 8.2% and 9.6%, in the "barcode region" and the "S20-Jerry region", respectively; Table 1), are in line with interspecific distances commonly recorded in Limnephilidae (e.g., Graf *et al.* 2005, Previšić *et al.* submitted) and other caddisfly families (e.g., Hydropsychidae; Pauls *et al.* 2010). Haplotypes of the "barcode region" of the adult male of *A. waringeri* **sp. nov.** and the hitherto unknown larva collected at the same locality differed in a single base pair, whereas haplotypes of the "S20-Jerry region" were completely identical in these two specimens (Table 1). Since these values are well within the intraspecific variability of mtCOI usually observed in caddisflies (e.g., Pauls *et al.* 2009, 2010; Previšić *et al.* 2009, 2014) data at hand enable confident affiliation of the larva and the male of *A. waringeri* **sp. nov.**

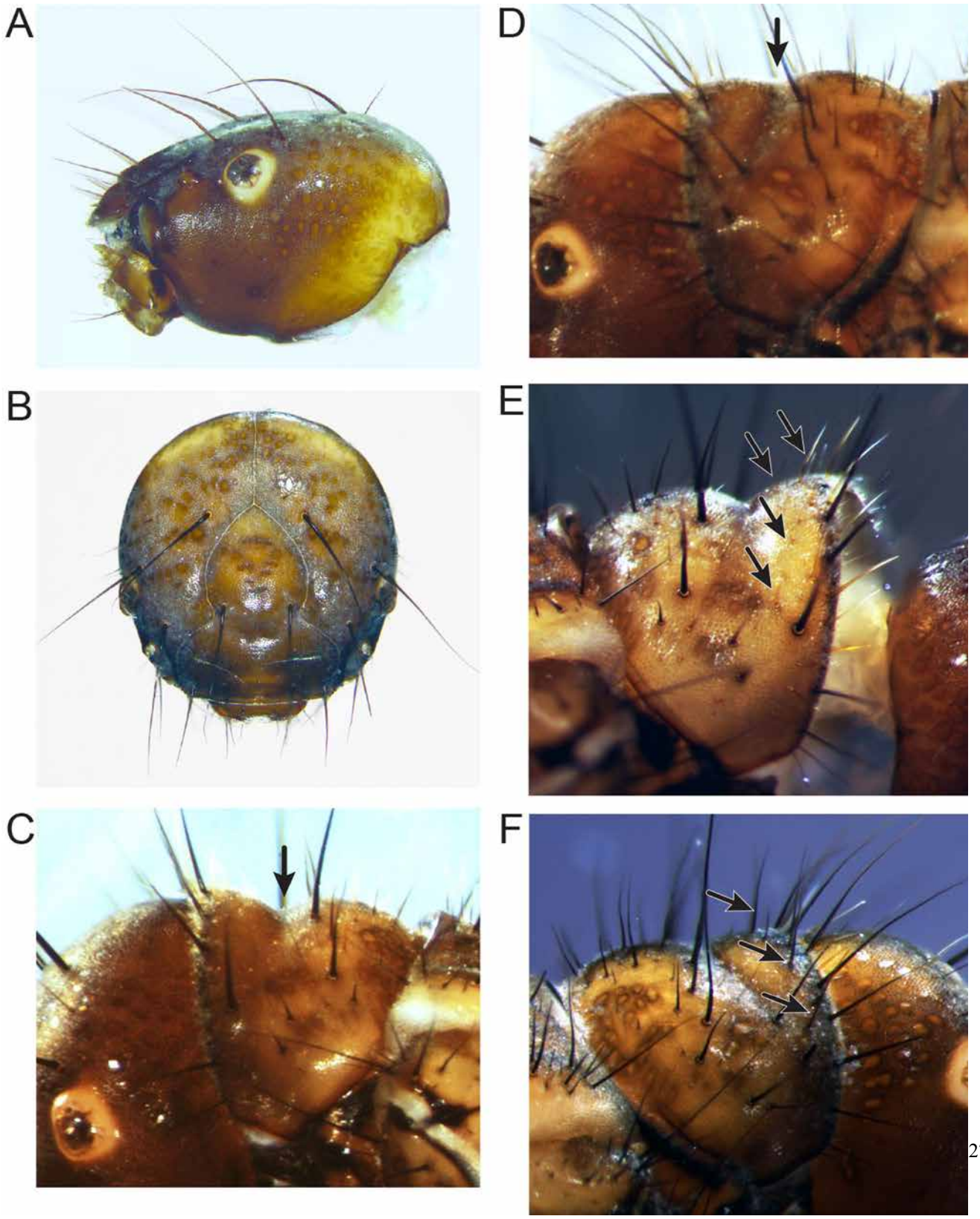

FIGURE 6. Larval characters of *A. waringeri* **sp. nov.** and *A. difformis*. A, *A. waringeri* **sp. nov.** head, left lateral; B, *A. waringeri* **sp. nov.** head, frontal; C, *A. difformis* pronotum, left lateral; D, *A. waringeri* **sp. nov.** pronotum, left lateral; E, *A. difformis* pronotum, right lateral (arrows indicating row of pale yellow setae); F, *A. waringeri* **sp. nov.** pronotum, right lateral (arrows indicating row of all-dark setae).

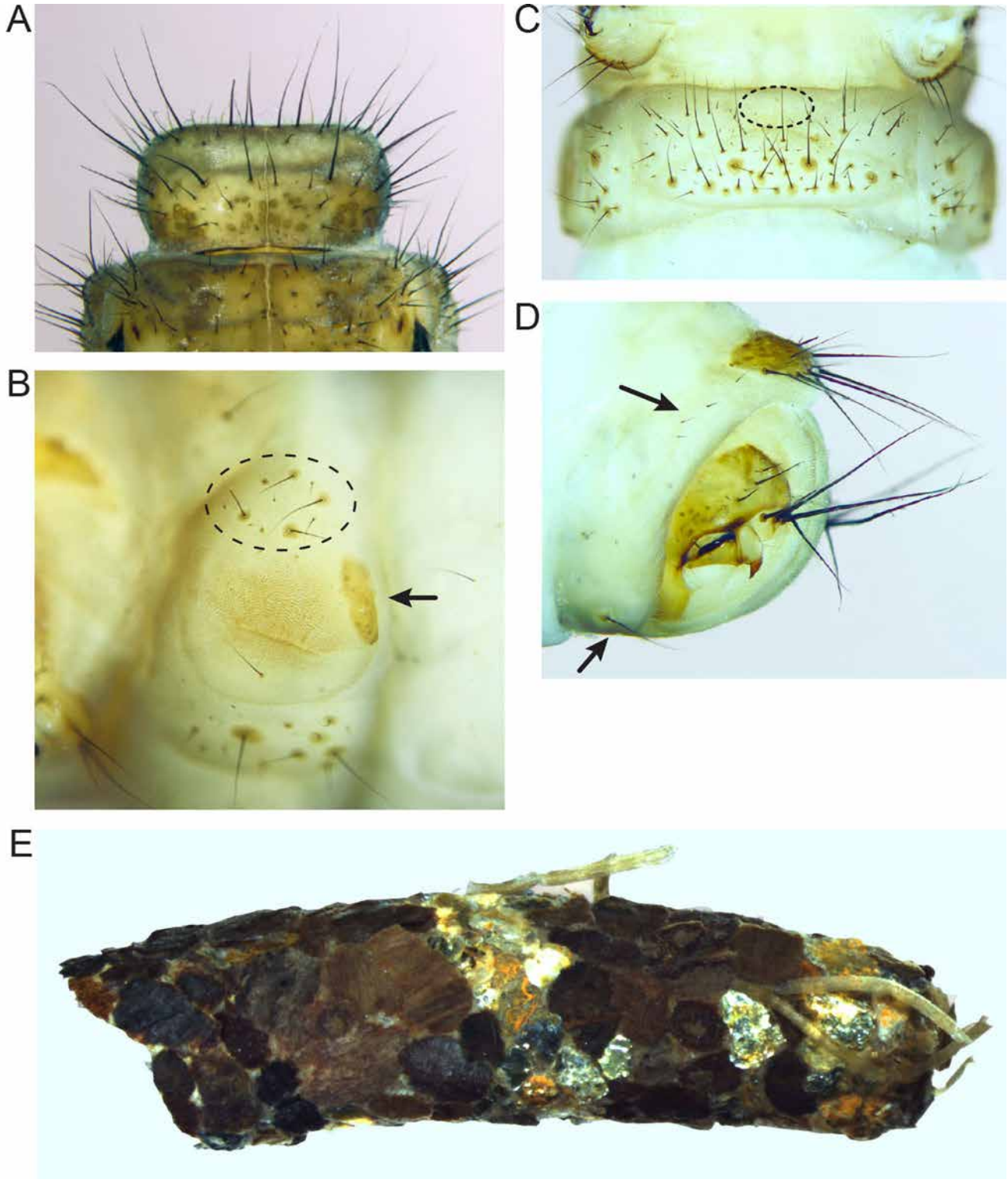

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FIGURE 7. Larval characters of *A. waringeri* **sp. nov.** A, pronotum, dorsal; B, left lateral protuberance, left lateral (dotted circle indicating setal area *sa*3, arrow indicating associated sclerite); C, abdominal sternum I, with the separated setal areas 1 (*sa*1) marked with a dotted circle; D, left side of abdominal segments IX and X and left anal proleg and claw, left lateral (upper arrow indicating posterolateral setae of abdominal dorsum IX, lower arrow indicating long stout seta on abdominal venter IX); E. larval case of *A. waringeri* **sp. nov.**

TABLE 1. Inter- and intraspecific genetic distances of two mitochondrial cytochrome oxidase I (mtCOI) gene fragments recorded for *Anisogamus* species. Values below diagonal in second and third columns indicate number of nucleotide differences and above diagonal uncorrected pairwise distances (*p*) (shown as percents), respectively. Abbreviations are used to denote life stages; IM/M = adult male, L = larva.

Species	Stage	Specimen codes	mtCOI "barcode"		
			Andiff01	Ansp01	fAns0101L
Anisogamus difformis	IM/M	Andiff01		8.2	8.2
Anisogamus waringeri	IM/M	Ansp01	54		0.2
Anisogamus waringeri	L	fAns0101L	54	1	

TABLE 1. (Continued)

Species	mtCOI "S20-Jerry"			GenBank Access. No.	
	Andiff01	Ansp01	fAns0101L	"barcode"	"S20-Jerry"
Anisogamus difformis		9.6	9.6	KP174661	KP174658
Anisogamus waringeri	52		0	KP174662	KP174659
Anisogamus waringeri	52	0		KP174663	KP174660

Distribution & biogeography of *Anisogamus* species. The genus *Anisogamus* was established by McLachlan in 1874 based on the species *A. difformis*, and its type locality is situated in the Eastern Alps (Austria, Carinthia, Saualpe, Stelzing (Kimmins 1949)). Collated distribution data for *A. difformis* suggest a panalpine presence of the species (Fig. 3D).

Specimens of *A. waringeri* were collected at the Col de Jou, Mont Canigou, Pyrenées-Orientales, France. At a location close by, Décamps (1967) found putative *A. difformis* to be present (but very rare) in the valley of the Neste d'Aure at 1600 m a.s.l. and in the tributaries of the Têt river at 1100 m a.s.l. Specimens of *A. waringeri* **sp. nov.** were collected in the watershed of the Têt river, whereas the Neste d'Aure is some 125 km west of the recent collection points. Menéndez & González (2009) recorded *A. difformis* from the eastern Prepyrenees (Girona, Setcades), some 20 km south of the type locality of *A. waringeri* **sp. nov.**, and were re-identified by M. A. González as *A. waringeri* **sp. nov.** (pers. comm. M. A. González). From the same area, *Stenophylax nurianus* was described by Navás (1917), illustrating a specimen similar to the genus *Anisogamus*, but the type specimen is lost (pers. comm. M. A. González), and the description and the figure itself do not allow certain identification. Further, this species was proposed by Schmid (1949) to be a synonym of *A. difformis*, based on his own collection and identification of 2 putative *A. difformis* specimens. Thus, we consider *Stenophylax nurianus* a *nomen dubium* in concordance with Malicky (2005), justifying the description of *A. waringeri* **sp. nov.** We further conclude that *A. waringeri* **sp. nov.** is the single representative of the genus *Anisogamus* in the Pyrenees.

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Larval description of *Drusus bosnicus* Klapálek 1899 (Trichoptera: Limnephilidae), with distributional, molecular and ecological features

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Abstract

In this study we present morphological, molecular and ecological features of the last instar larvae of *Drusus bosnicus* with data about distribution of this species in Bosnia and Herzegovina. We also included the most important diagnostic features enabling separation of larvae of *D. bosnicus* from larvae of the other European Drusinae and Trichoptera species.

Key words: Drusinae, 5th instar larva, identification, morphology, Bosnia and Herzegovina

Introduction

The Balkan Peninsula is one of the most interesting centres of diversity for different animal groups in Europe (*e.g.*, Gottstein-Matočec *et al*. 2002; Džukić & Kalezić 2004; Kryštufek 2004; Bedek *et al.* 2006; Bilandžija *et al.* 2013) including Trichoptera (*e.g.*, Kumanski & Malicky 1999; Malicky 2005; Oláh 2010, 2011). In this region (Albania, Bosnia and Herzegovina, Bulgaria, Croatia, Kosovo, Macedonia, Montenegro, Serbia, Greece) 40 species from the Eurasian genus *Drusus* (family Limnephilidae) have been recorded (Schmid 1956; Radovanović 1942; Krušnik 1987; Kumanski 1988; Malicky 2004, 2005; Oláh 2010, 2011; Oláh & Kovács 2013). Except *Drusus botosaneanui* Kumanski 1968, *Drusus discolor* (Rambur 1842), *Drusus biguttatus* (Pictet 1834) and *Drusus tenellus* (Klapálek 1898), all other *Drusus* species are endemic to this region. *Drusus tenellus* and *D. botosaneanui* also occur in Romania, *D. biguttatus* and especially *D. discolor* are widespread throughout Europe. *Drusus* larvae usually inhabit springs and the crenal section of mountain streams and rivers with low water temperature.

Within the family Limnephilidae, one of the most interesting groups is the *Drusus bosnicus* Group (Schmid 1956) with numerous endemic species described from the Balkan Peninsula (*e.g.*, Radovanović 1942; Marinković-Gospodnetić 1976). All species of the *Drusus bosnicus* Group share some morphological and behavioural features like the shape of male genitalia, the dark coloration of adults and diurnal activity. The first species described from this group at the end of the 19th century was *Drusus bosnicus* Klapálek 1899 (Fig. 1), from the type locality at the spring of the River Bosna situated in Sarajevo, central Bosnia and Herzegovina (Fig. 2). Recently, seven newly described species from Albania (Oláh 2010, 2011) mostly belonging to the *Drusus bosnicus* Group. The main

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distribution area of the *Drusus bosnicus* Group is the Balkan Peninsula with more than 20 described species (*e.g.*, Radovanović 1942; Marinković–Gospodnetić 1976; Kumanski 1988). So far, larvae of 5 species of the *Drusus bosnicus* Group from the Balkan Peninsula have been described: *Drusus klapaleki* Marinković-Gospodnetić 1971a, *Drusus medianus* Marinković-Gospodnetić 1976, *Drusus radovanovici* Marinković-Gospodnetić 1971a, *Drusus ramae* Marinković-Gospodnetić 1971a, and *Drusus septentrionis* Marinković-Gospodnetić 1976 (Kučinić *et al*. 2008, 2010, 2011a, 2011b).

In this study we present morphological features of the last instar larvae of *Drusus bosnicus*, combined with molecular and ecological notes and distribution data on this species. We also present the most important diagnostic features enabling separation of larvae of *D. bosnicus* from larvae of the other European Drusinae species.

Material and methods

Fieldwork and sampling. The studied material of *D. bosnicus* comprises 10 larvae and one adult, collected on 17 May 2008, all of them from the spring of the Paljanska Miljacka River (Fig. 3), situated 15 kilometres southeast of Sarajevo, and two adults of *D. bosnicus* collected at the spring of the Bosna River situated in Sarajevo. Larvae were collected by handpicking and adults with an entomological net. Collected specimens were stored in containers with 80 and 96% EtOH, for morphological and molecular analysis, respectively. All collected specimens are deposited in the Faculty of Science in Zagreb, Croatian Natural History Museum in Zagreb (Croatia) and the Institute of Hydrobiology and Aquatic Ecology Management in Vienna (Austria). Larval morphological features were analysed according to Wiggins (1996) and Kučinić *et al*. (2010, 2011a, 2011b). Systematic presentation follows Morse (2015). We use *Drusus adustus* McLachan 1867 as a valid name (syn. *Drusus destitutus* Kolenati 1848). In this paper, we include species *Leptodrusus budtzi* (Ulmer 1913), but it is possible that this species does not belong to Drusinae (J. Waringer, personal communication, 2015).

DNA extraction and PCR amplification. One adult male and two larvae of *D. bosnicus* from the spring of the Paljanska Miljacka River were genetically analysed in order to support the association of the larvae with the adults. Additionally we included two adult males from the spring of the Bosna River. Association was based on a 541-bp-long fragment of the mitochondrial cytochrome oxidase I (mtCOI) gene and sequences were taken from Kučinić *et al.* (2010), Previšić *et al.* (2009) and Previšić *et al.* (2014b). Intraspecific *p*-distances were calculated in Mega 4.0.1 (Tamura *et al.* 2007).

Electronic microscopy, macrophotography and measuring. Morphological observations of samples were accomplished using a Tescan TS 5136 variable pressure scanning electron microscope (SEM). Samples were mounted with graphitic adhesive tape on the SEM stub and coated with carbon. The samples were examined by SEM operating in secondary electron (SE), or back-scattered (BSE) mode at an accelerating voltage of 20 kV, running current of 110 pA and variable pressure of 30 Pa to $5x10^{-1}$ Pa, sometimes pressure was increased to 10 Pa to eliminate sample charging.

Macrophotographing and measurements of larvae and larval cases were accomplished using a Leica Wild MZ8 stereomicroscope and Olympus SP-500 UZ digital camera, processed with computer programme Olympus Quick Photo Camera 2.2. In larvae of *D. bosnicus* the following features were measured: head width, total body length, length of the anterior-median metadorsal sclerites, their width in the widest median part and distance between them, and also the length of the posterior metadorsal sclerites (N=10). The following characters of cases were measured: total length, width of the anterior part, and width of the posterior part.

Description of fifth instar larva of *Drusus bosnicus*

Case and Larva. Case constructed completely of mineral particles (Fig. 4), slightly curving, total length 9.50–13.60 mm, width of anterior part 2.92–3.76 mm, width of posterior part 1.66–2.62 mm (N=10). Overall body shape eruciform (Fig. 5), total length without case 8.50–13.34 mm (N=10).

Head. Head capsule hypognathous, width 1.57–1.77 mm (N=10) (Figs 5–8). In lateral view, posterior part of head capsule (vertex) flat with slight median concavity (Figs 5–8). Head brown, dorsally darker and laterally lighter, with granular surface sculpturing (Fig. 9). Genae of parietals reddish-brown to yellowish with lighter ring

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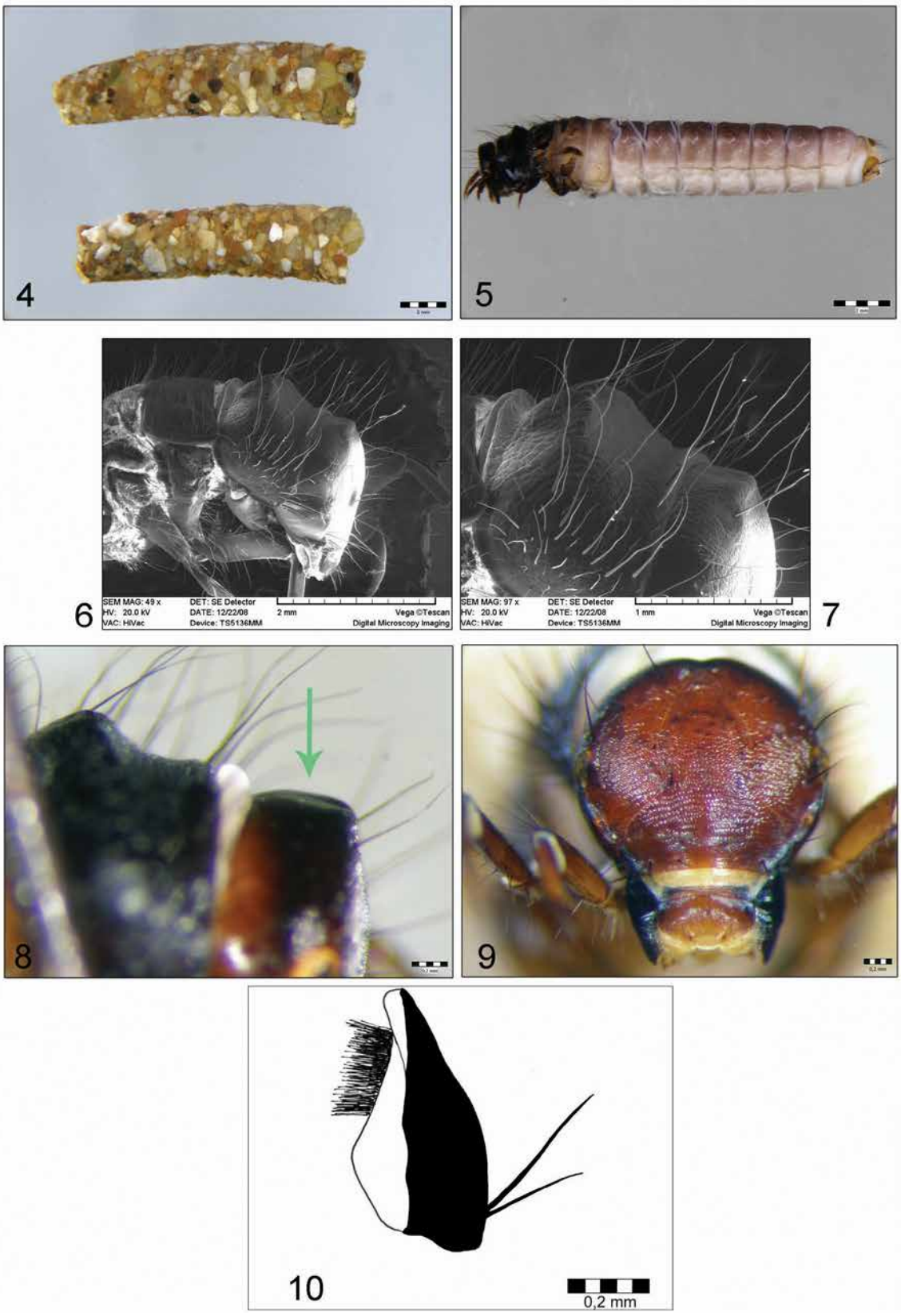
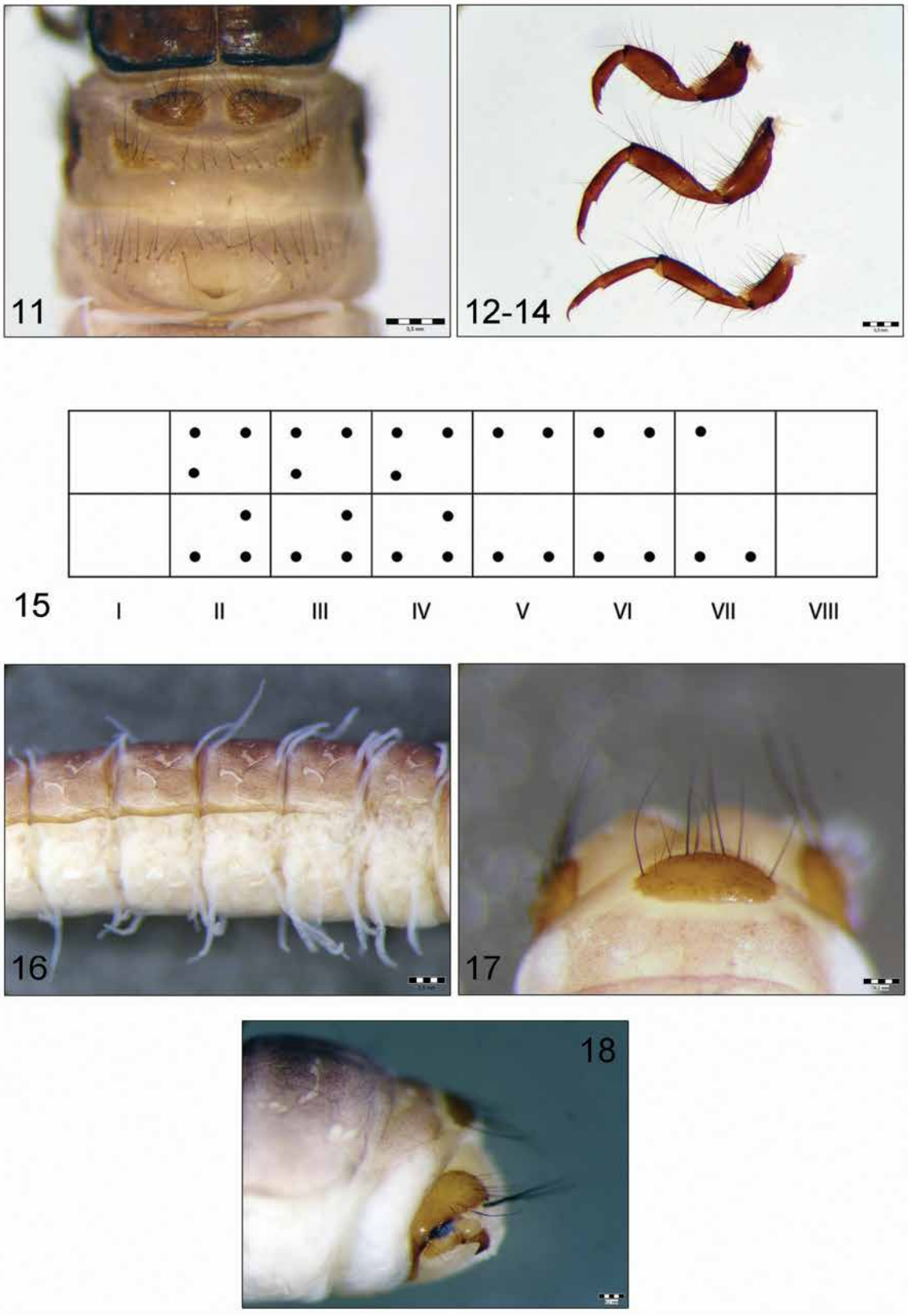
FIGURES 1–3. 1, *Drusus bosnicus* Klapálek 1898, adults in copula; 2, Bosnia and Herzegovina with distribution of *D. bosnicus*; 3, spring of the Paljanska Miljacka River, site where larvae and adults of *Drusus bosnicus* Klapálek 1898 were collected.



FIGURES 4–10. *Drusus bosnicus* Klapálek 1898. 4, case, right lateral; 5, larva, left lateral; 6 head, pronotum and mesonotum, right lateral; 7, head and pronotum, right lateral; 8 head, right lateral; 9, head, frontal; 10, left mandible, ventral.



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FIGURES 11–18. *Drusus bosnicus* Klapálek 1898. 11, thorax (pronotum, mesonotum and metanotum), dorsal; 12, left foreleg, posterior; 13, left midleg, posterior; 14, left hind leg, posterior; 15, abdominal gill diagram: position of filaments on left side of abdominal segments I-VIII, with ● = gill present; 16, abdomen with gills, right lateral; 17, abdominal segment IX, dorsal; 18, left anal proleg, left lateral.

around each eye (Fig. 5). Dorsum of head capsule with dark muscle attachment spots (Fig. 9). Distinct area with small number of spinules (small spines) positioned laterally on each side of head capsule in areas between setae 13 and 16, but some specimens without visible spinules. Frontoclypeal apotome bell-shaped with narrow central region (Fig. 9). Antennae short, brown to dark brown, each positioned on small, noticeable prominences. Labrum symmetrical, brown posteriorly to yellowish anteriorly (Fig. 9), with setal brush at anterolateral margins (Fig. 9) and thin primary setae on dorsal surface. Mandibles black, distally reddish (Fig. 9); typical for grazers, mesal margin of each mandible with setal brush (Fig. 10); two setae present laterobasally on each mandible (Fig. 10). Labium and maxillae light-brown (yellowish). Maxillary palps 5-segmented.

Thorax. Pronotum dark brown to black (Fig. 5) with granular sculptured surface. Posterior margin rounded, both posterior and lateral margins thick and darkly sclerotized. Anterior part (50–60%) of pronotum slightly concave and rising, posterior part with prominent median hump (Figs. 5, 7–8). Pronotum bearing dark setae, especially laterally and on anterior margin, some of them long and conspicuous (Figs. 5–8). Dorsal (posterior part) and lateral regions of pronotum with white recumbent setae (Fig. 8); in some specimens, pronotum with small number of white recumbent setae. Prosternal horn present.

Mesonotal sclerites brown, lighter than pronotum, with dark setae and uneven rugous surface (Figs. 5–6, 11). Posterior and lateral margins thick and darkly sclerotized (Fig. 11).

Metanotum with 3 pairs of dorsal sclerites (Fig. 11). Anteromedian (*sa*1) sclerites elongated, triangular with rounded apices, inter-sclerite distance less than width of either sclerite (Fig. 11), covered by setae, mainly anteriorly, color similar to mesonotum. Length of anteriomedian sclerites 0.51–0.65 mm (mean 0.59 mm); width of anteriomedian sclerites 0.26–0.34 mm (mean 0.30 mm); distance between anteromedian sclerites 0.07–014 mm (mean 0.10 mm). Posteromedian (*sa*2) sclerites smaller and lighter than *sa*1 sclerites (Fig. 11), triangularly or irregularly ellipsoid and with many setae. Length of posteromedian sclerites 0.32–0.51 mm (mean 0.45 mm). Group of setae present on membranes between *sa*2 sclerites and between *sa*2 and *sa*3 sclerites. Lateral (*sa*3) sclerites longitudinally prolonged, sickle-shaped, lighter brown with dark median region, and group of setae anteriorly.

Legs yellow-brown to dark brown or black, with dark ventral and dorsal margins (Figs. 5, 12–14). Coxae and femora of all legs with dark setae on both dorsal and ventral edges (Figs. 12–14). Trochanters of all legs without setae on dorsal margins (Figs. 12–14). Ventral margin on distal part of each foreleg trochanter with row of fine, yellowish setae (trochanteral brush). Midleg trochanters with few, fine, yellowish setae apicoventrally. Foreleg coxae and femora wide compared to those of mid- and hind legs (Figs. 12–14). Additional setae present on anterior and posterior faces of all femora. Mid- and hind legs similar in shape and size (Figs. 13–14), with slender coxae, trochanters, and femora. Setae on dorsal edges of tibiae present only distally in all legs (Figs. 13–14). Tarsi each with claw and basal seta, and tibial spurs light brown, almost yellowish (Figs. 12–14).

Abdomen. Abdominal segment I with well-developed dorsal (Fig. 11) and lateral protuberances. Numerous setae present anterior and lateral to dorsal proturbance. Distinction between dorsal setal areas *sa*1 and *sa*2 not possible (Fig. 11). Ventrally with numerous setae, some of them with small sclerites at bases.

Single filamentous gills present on segments II–VII (Figs. 15–16). Dorsal pre-segmental gills present on segments II–VII; dorsal post-segmental gills present on segments II–VI, ventral pre- and post-segmental gills present on segments II–VII (Fig. 15). Lateral pre- and post-segmental gills present on segments II–IV (Fig. 16). On abdominal segment VIII, four posterodorsal setae present. Lateral fringe extending from last third of segment II to segment VIII (Fig. 16); in some specimens, only few setae forming lateral fringe on segment II or setae not visible.

Segment IX bearing irregular, semicircular, light brown dorsal sclerite, generally with 8 long, dark setae on posterior margin and several shorter, lighter setae on posterior half of sclerite (Fig. 17). Anal prolegs typical of limnephilids (Fig. 18), each with longitudinally prolonged lateral sclerite, sickle-shaped, yellowish, with setae mainly in posterior part and 2 large, dark setae at posterior end (Fig. 18). Anal claws and accessory hook brown to dark brown (Fig. 18).

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Discussion

Association of *Drusus bosnicus* larvae and adults. COI haplotypes of adult males and larvae from the spring of the Paljanska Miljacka River were either completely identical or differed in one nucleotide position (Table 1).

Overall, intraspecific variability recorded in *D. bosnicus* (Table 1) is in line with the observed variability of the same COI fragment in populations of some other *Drusus* species (*e.g.*, Pauls *et al.* 2009; Previšić *et al.* 2009), including species of the *D. bosnicus* Group (*e.g.*, Kučinić *et al.* 2008). However, the association of larvae and adults are not completely reliable based solely on comparisons of sequences of a single gene from one specimen each (*e.g.*, Zhou *et al.* 2007). In addition to the molecular genetic data, association of adults and larvae of *D. bosnicus* are further supported by:

Species distribution data on the *Drusus bosnicus* Group in Bosnia and Herzegovina, in which all species have restricted ranges and generally show allopatric distribution (Marinković-Gospodnetić 1979; Kučinić *et al.* 2008, 2011a, 2011b; Previšić *et al.* 2009),

Presence of *D. bosnicus* as exclusive *Drusus* species in the Paljanska Miljacka River spring (Marinković-Gospodnetić 1979).

TABLE 1. Intraspecific uncorrected pairwise distances (*p*) shown as percentage and number of nucleotide differences (in brackets) of the mitochondrial cytochrome oxidase I (mtCOI) gene recorded in *D. bosnicus*. Abbreviations are used to denote life stages; IM(M) = adult male, L = larva.

Sampling site	Specimen code	Stage	DbsIM1	DbsIBIM2	DbsVMIM1	DbsVML1	DbsVML2	GenBank accession nos
Bosna spring	DbsIM1	IM(M)						FJ002689
Bosna spring	DbsIBIM2	IM(M)	0.2% (1)					KC881518
Paljanska Miljacka spring	DbsVMIM1	IM(M)	0.0% (0)	0.2% (1)				GQ470609
Paljanska Miljacka spring	DbsVML1	L	0.0% (0)	0.2% (1)	0.0% (0)			GQ470610
Paljanska Miljacka spring	DbsVML2	L	0.2% (1)	0.4% (2)	0.2% (1)	0.2% (1)		GQ470611

Morphological separation of *Drusus bosnicus* larvae from other European Trichoptera larvae. In Europe, larvae of numerous different species of Trichoptera have been described (*e.g.*, Urbanič *et al.* 2003a, 2003b; Graf *et al.* 2011; Sáinz-Bariáin & Zamora-Muñoz 2012; Waringer *et al.* 2012a, 2012b) including larvae of species in the genus *Drusus* (*e.g.*, Lepneva 1966; Decamps & Pujol 1975; Szczesny 1978; Waringer 1987; Wallace *et al.* 1990; Waringer & Graf 1997; Waringer *et al*. 2000, 2007a, 2007b, 2011; Graf *et al*. 2005, 2009; Kučinić *et al*. 2008). The larvae of the subfamily Drusinae share common morphological features such as:

- a fully sclerotised pronotum and mesonotum;
- metanotum with six sclerites;
- additional setae present on anterior and posterior faces of mid- and hind leg femora;
- gills with one filament;
- slightly curved case; etc.

Distinctive features of *D. bosnicus* larvae are the specific shape of the head, pronotum, and anteromedian sclerites, enabling separation from other known larvae of European Trichoptera. Also, larvae of *D. bosnicus* differ from other larvae of the *Drusus bosnicus* Group in the shape of the head, except for *D. ramae* larvae. *Drusus bosnicus* and *D. ramae* exhibit similar head shapes in lateral view, but differ in pronotal shape (Figs. 19–21). 285
Larvae of *D. ramae* have two very prominent and widely separated humps posteriorly on the pronotum (Figs. 20–21), whereas the pronotum in *D. bosnicus* larvae has a ridge in this position (Figs. 6–7, 19).

The following features clearly distinguish larvae of *D. bosnicus* from other described Drusinae larave (Lepneva 1966; Hickin 1967; Decamps & Pujol 1975; Szczesny 1978; Sedlák 1980; Waringer 1987; Wallace *et al.* 1990; Pitsch 1993; Urbanič *et al.* 2003c; Graf *et al.* 2005, 2009; Waringer & Graf 1997; Waringer *et al.* 2000, 2007a, 2007b, 2008a, 2008b, 2010, 2013a, 2013b, 2015; Vieira-Lanero *et al.* 2005; Kučinić *et al.* 2008, 2010, 2011a, 2011b; Previšić *et al.* 2014a):

- *Anomalopterygella chauviniana* (Stein 1874), *D. bolivari* (McLachlan 1880) and *D. brunneus* Klapálek 1898, have a different shape of the posterior part of the pronotum than the larva of *D. bosnicus*. These species have a very pronounced mid-dorsal concavity in the dorsal hump which is lacking in *D. bosnicus*.
- *Cryptothrix nebulicola* McLachlan 1867, *D. alpinus* (Meyer-Dür 1875), *D. chrysotus* (Rambur 1842), *D. discolor*, *D. franzi* Schmid 1956, *D. muelleri* McLachlan 1868, and *D. romanicus* Murgoci & Botosaneanu 1954 have teeth along the mesal margin of each mandible which are lacking in *D. bosnicus*.
- In *Drusus adustus*, *D. alpinus*, *Drusus annulatus* (Stephens 1837), *D. biguttatus*, *D. camerinus* Moretti 1981, *D. balcanicus* Kumanski 1973, *D. croaticus* Marinković-Gospodnetić 1971b, *D. franzressli* Malicky 1974 (in Malicky & Kumanski 1974), *Drusus franzi*, *D. improvisus* McLachlan 1884, *D. klapaleki*, *D. medianus*, *D. mixtus* (Pictet 1834), *Drusus monticola* McLachlan 1876, *D. nigrescens* Meyer-Dür 1875, *D. rectus* McLachlan 1868, *D. septentrionis*, *D. trifidus* McLachlan 1868, *D. vinconi* Sipahiler 1992, and *Ecclisopteryx malickyi* Moretti 1991 the head vertex is evenly rounded. In *D. bosnicus* this part of the head is flat with a slight median concavity.
- *Drusus alpinus*, *D. aprutiensis* Moretti 1981, *D. camerinus* Moretti 1981, *D. croaticus*, *D. franzi* Schmid 1956, and *D. mixtus* lack prominent, long median setae dorsally on the anterior border of the pronotum; *D. bosnicus* larvae have long, median setae in this part of the pronotum.
- *Drusus biguttatus, D. croaticus, D. ingridae* Sipahiler 1993, *D. vinconi* Sipahiler 1992, and *Hadimina torosensis* Sipahiler 2002 have an evenly rounded pronotum in lateral view. The pronotum of *D. bosnicus*, in lateral view, has a prominent median hump.
- *Drusus botosaneanui*, *Drusus tenellus*, *Drusus schmidi* Botosaneanu 1960, *Ecclisopteryx dalecarlica* Kolenati 1848, *E. guttulata* (Pictet 1834), *E. ivkae* Previšić, Vitecek & Graf 2014, *E. keroveci* Previšić, Vitecek & Graf 2014, and *E. madida* (McLachlan 1867) have prominent spines on the head and/or pronotum; *D. bosnicus* lacks these prominent spines on the head and pronotum.
- *Drusus discolor* and *D. romanicus* have hair-like structures on the head and pronotum; these structures are absent in *D. bosnicus*.
- *Drusus carpathicus* Dziedzielewicz 1911 lacks dorsal gills on the abdomen; *D. bosnicus* has such gills.
- In *Drusus adustus* and *D. melanchaetes* McLachlan 1876, setae along the whole length on the dorsal side of mid- and hind-leg tibiae are present; in *D. bosnicus* such setae are present only distally.
- *Drusus franzressli*, *D. spelaeus* (Ulmer 1920), *Metanoea rhaetica* Schmid 1956 and *M. flavipennis* (Pictet 1834) have a large median sclerotized patch on abdominal sternum I which is lacking in *D. bosnicus*.
- *Drusus radovanovici* has numerous thin, long, yellow (yellowish) setae on the dorsal and lateral parts of the pronotum. Such long setae are lacking in *D. bosnicus*.
- In *Drusus serbicus* Marinković-Gospodneti 1971b, the dorsal profile of the pronotum in lateral view has an annular crest that is highest at its dorsal centre and gradually declining laterally. This crest is lacking in *D. bosnicus*.
- *Drusus trifidus* has numerous light spines on the pronotum which are lacking in *D. bosnicus*.
- *Ecclisopteryx asterix* Malicky 1979 has ovoidal metanotal anteromedian sclerites (metanotal *sa*1 sclerites) which are more triangular in *D. bosnicus.*
- *Leptodrusus budtzi* has a rounded pronotum in lateral view with a transverse rim in its anterior part. The pronotum of *D. bosnicus* does not have this shape and lacks a rim.

Distribution, protection status, and feeding ecology of *Drusus bosnicus*. According to literature data (Marinković-Gospodnetić 1979) *Drusus bosnicus* was found in central Bosnia and Herzegovina at three localities: the Bosna River spring, the Paljanska River spring, and the Mokračka Miljacka River spring. The distance between springs of the Paljanska and Mokračka Miljancka Rivers is 21 kilometres, and the distance between these springs and the spring of the Bosna River is about 34 kilometres. This species thus occupies the smallest area known in the *Drusus bosnicus* Group, except for *D. ramae* (Kučinić *et al.* 2010).

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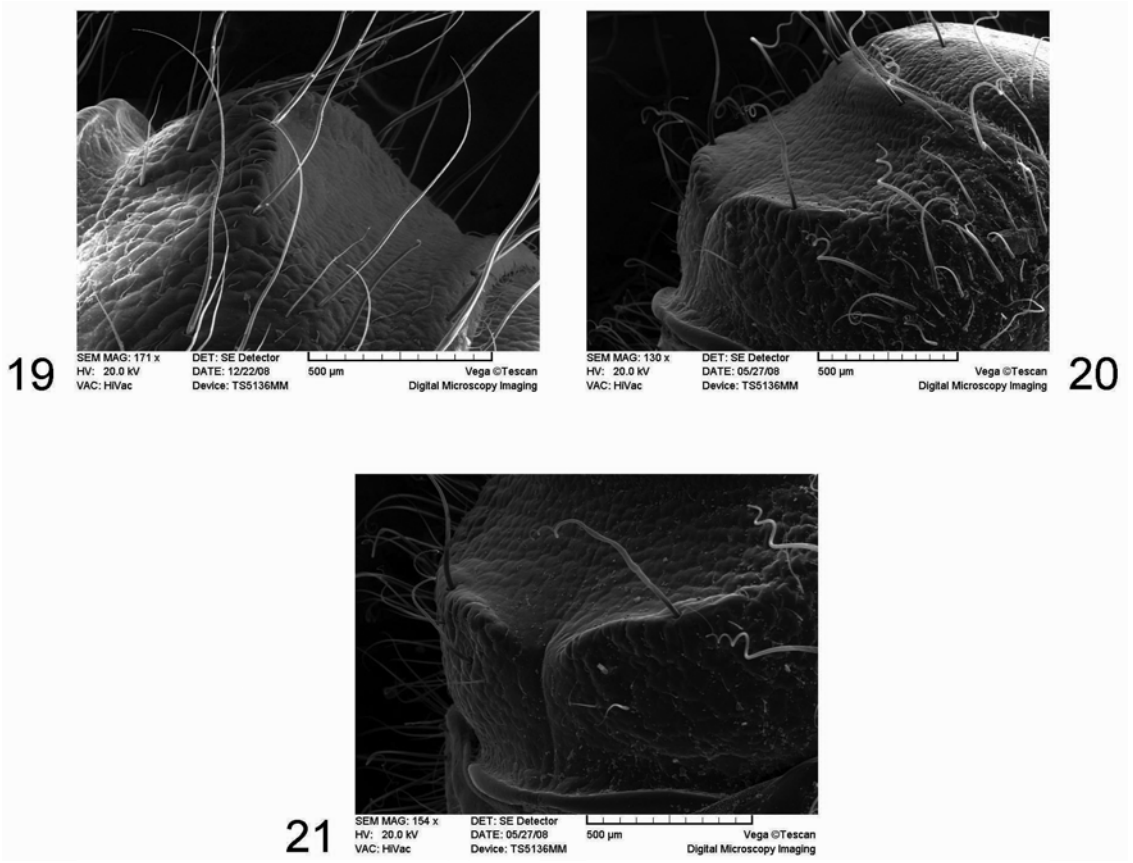
In our investigation of caddisflies in Bosnia and Herzegovina, we found *D. bosnicus* in all three localities listed in literature (Marinković-Gospodnetić 1979), but not elsewhere (Stanić-Koštroman 2009). Considering the locations of these springs, the spring of the River Bosna is situated in Sarajevo and two other springs are located near villages, it is very important to protect them. A spring of the Paljanska Miljacka River is used as a source for drinking water, but the habitat was not considerably disturbed, so aquatic fauna is preserved, including species of the population of *D. bosnicus* (Kučinić *et al*. 2010). The other two springs are natural, at the moment without visible negative anthropogenic influence.



FIGURES 19–21. Pronotum. 19, *Drusus bosnicus* Klapálek 1898, right lateral; 20–21, *Drusus ramae* Marinković-Gospodnetić 1971a: 20, right lateral; 21, right dorsolateral.

According to morphological features of the mandibles, *D. bosnicus* belongs to the group of grazers, like larvae of the other species that form the *Drusus bosnicus* Group from the Balkan Peninsula (Kučinić *et al.* 2008, 2010, 2011a, 2011b). Larvae of these species are feeding mainly on epilithic algae in the periphyton of mosses, cobbles, pebbles and gravel. This consistent feeding ecology of all species from the *Drusus bosnicus* Group in Bosnia and Herzegovina is possibly a result of the monophyletic origin of the species (Marinković-Gospodnetić 1976; Kučinić *et al.* 2011a). The taxa of the *Drusus bosnicus* Group were separated in different periods during the Pleistocene, probably from one ancestral species (Marinković-Gospodnetić 1976; Previšić *et al.* 2009; Kučinić *et al.* 2011a, 2014). Recent molecular study showed that two different species from the *Drusus bosnicus* Group inhabit the spring of the Bosna River (Kučinić *et al.* 2014; Previšić *et al*., 2014b*).* This is possibly an indication of a more complex distribution pattern and evolutionary history of the *Drusus bosnicus* Group than previously recognised (*e.g*., Kučinić *et al.* 2011a, 2014). So far, we have not found adults or last instar larvae of this possibly new species in this spring.

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Larval morphology and phylogenetic position of *Drusus balcanicus*, *D. botosaneanui*, *D. serbicus* and *D. tenellus* (Trichoptera: Limnephilidae: Drusinae)

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Abstract. In a recent 3-gene phylogeny of the trichopteran subfamily Drusinae Banks 1916, molecular data clearly correlated with the morphology and feeding ecology of larvae. The largest of three main groups, the Drusinae grazer clade, exhibits an unusual larval feeding ecology for Limnephilidae, and is the most diverse group. In this paper we describe four previously unknown Drusinae larvae included in this clade: *Drusus balcanicus* Kumanski, 1973 (micro-endemic to Eastern Balkans), *Drusus botosaneanui* Kumanski, 1968 (Dinaric Western Balkans, Hellenic and Eastern Balkan, Asia Minor), *Drusus serbicus* Marinković-Gospodnetić, 1971 (micro-endemic to Dinaric Western Balkans), and *Drusus tenellus* (Klapálek, 1898) (Carpathians, Dinaric Eastern Balkans). Characteristically, the larvae of these species have toothless mandibles typical of the Drusinae grazer clade. Larvae and adults were unambiguously associated using a phylogenetic analysis based on two mitochondrial [mtCOI, mtLSU (=16S) rDNA] and two nuclear genes (nuWG, nuCAD). In addition, information on the morphology of the larvae is given and the diagnostic features necessary for identification are illustrated.

INTRODUCTION

Geographically the Drusinae Banks, 1916 are restricted to Eurasian mountain ranges from Iran and the Caucasus in the East to the Iberian Peninsula in the south-west. Three quarters of the known species are endemic to a single or very few mountain ranges, making the group an ideal model for studying evolutionary processes like speciation and diversification (Schmid, 1956; Kumanski, 1973; Marinković-Gospodnetić, 1971a, b, 1976; Sipahiler, 2002; Malicky, 2005). As cold-water adapted aquatic insects that occur as fragmented montane sky-island populations, Drusinae are also very sensitive to global change and their species are among the most threatened by climate warming. The taxon currently comprises eight genera (*Drusus* Stephens, 1837, *Monocentra* Rambur, 1842, *Ecclisopteryx* Kolenati, 1848, *Cryptothrix* McLachlan, 1867, *Metanoea* McLachlan, 1880, *Leptodrusus* Schmid, 1955, *Anomalopterygella* Fischer, 1966, and *Hadimina* Sipahiler, 2002) and more than hundred species (Malicky, 2004, 2005; Graf et al., 2008; Kučinić et al., 2011a; Oláh, 2010, 2011; Oláh & Kovács, 2013; Previšić et al., 2014). Unfortunately, larvae of only 45 species are described (references in Waringer et al., 2013a, b). In this paper we improve the knowledge of the larval taxonomy of Drusinae by presenting descriptions of the hitherto unknown larvae of *Drusus balcanicus* Kumanski, 1973, *D. botosaneanui* Kumanski, 1968, *D. serbicus* Marinković-Gospodnetić, 1971, and *Drusus tenellus* (Klapálek, 1898). The putative larvae of these four species were associated with co-occurring adults using molecular data from four gene regions and following the methods outlined by Pauls et al. (2006, 2008).

Table 1. PCR primers and PCR cycling conditions.

Fragment	Primers	Primer concentration	PCR cycling conditions	Taq Kit	Additional reagents
COI-5P (barcode region)	HCOI, LCOI (Folmer et al., 1994)	0.25 µM	5′95°C, 5× (30″95°C, 1′44°C, 1′72°C), 15× (30″95°C, 30″48°C, 1′72°C), 20× (30″95°C, 30″50°C, 1′ + (10″ * n) 72°C), 5′72°C	peqGOLD HotTaq	–
COI-3P	Jerry, S20 (Pauls et al., 2006)	0.25 µM	5′95°C; 35× (45″95°C, 30″45°C, 45″72°C); 5′72°C	peqGOLD HotTaq	–
16SrDNA	Lepto-F, Lepto-R	0.75 µM	3′5°C, 35× (30″95°C, 30″52°C, 40″72°C), 5′72°C	peqGOLD HotTaq	4 µg BSA
WG	WGbDrrev 5′-ACCCTCTCCCGCARCACTTGAG-3′ WGbDrfwd 5′-CTTGCTGGATGCGTCTGCC-3′	0.5 µM	5′95°C, 35× (45″95°C, 45″60°C, 90″72°C), 7′72°C	Qiagen Hotstar Taq Plus Master mix	–
CAD	1028r-ino, 743nF-ino (Johanson & Malm 2010)	0.25 µM	5′95°C, 35× (45″95°C, 30″50°C, 45″72°C), 5′72°C	peqGOLD HotTaq	–

As caddisfly larvae are important indicator taxa for monitoring water quality (Barbour et al., 1999; Barbour & Yoder, 2000; AQEM consortium, 2002; Graf et al., 2002; Hering et al., 2006) and are frequently used as bioindicators (sensitive species) (Moog et al., 2002; Graf et al., 2002), the newly-described larvae will improve resolution of ecological assessment procedures. Further, larval morphology is also seen as an important tool in phylogenetics and taxonomy (van Emden, 1957; Meier & Lim, 2009; Minoshima et al., 2013). The descriptions of the four new Drusinae larvae will, therefore, also increase our present knowledge of the phylogenetic structure of the Drusinae grazer clade sensu Pauls et al. (2008).

MATERIAL AND METHODS

Species collection

Adults and larvae of *Drusus serbicus*, *D. botosaneanui*, *D. balcanicus*, and *D. tenellus* were collected by hand on the Balkan Peninsula (for locations see Material examined).

The material intended for sequencing was placed in pure 96% alcohol and that for morphological analyses in pure 70% ethanol in order to keep the specimens more flexible.

Morphological study

Morphological terminology, including setal nomenclature, follows Wiggins (1998). The larvae were described in terms of the set of morphological characters for Drusinae defined by Waringer & Graf (2011). Larvae were studied and photographed using a Nikon SMZ 1500 binocular microscope with DS-Fi1 camera and NIS-elements D 3.1 image stacking software, which combine 8 to 42 frames in one focused image. The two 5th instar larvae of *D. balcanicus* and the three larvae of *D. botosaneanui*, *D. serbicus* and *D. tenellus* are deposited in the collection of J. Waringer (Vienna, Austria).

For SEM microscopy, two fifth instar larvae of *Drusus serbicus* were air dried, gold coated using a BAL-TEC SCD 005 sputter coater and examined using a JEOL JSM-6390lv scanning electron microscope.

Comparative material of other Drusinae species included the following (all larvae preserved in pure 70% ethanol): *Drusus franzressli* Malicky, 1974 (two 5th instar larvae), *D. spelaeus* (Ulmer, 1920) (five 5th instar larvae), *D. schmidi* Botosaneanu, 1960 (six 5th instar larvae), *D. improvisus* (McLachlan, 1884) (eight 5th instar larvae), *D. nigrescens* Meyer-Dür, 1875 (five 5th instar larvae), *D. rectus* McLachlan,1868 (six 5th instar larvae), *D. brunneus* Klapálek, 1898 (one 5th instar larva), *D. bosnicus* Klapálek, 1899 (one 5th instar larva), *D. radovanovici* Marinković-Gospodnetić, 1971 (one 5th instar larva), *D. septentrionis* Marinković-Gospodnetić, 1976 (two 5th instar larvae), *D. trifidus* McLachlan, 1868 (three 5th instar larvae), *Ecclisopteryx dalecarlica* Kolenati, 1848 (one 5th instar larva), *E. guttulata* (Pictet, 1834) (three 5th instar larvae), *E. madida* (McLachlan,1867) (one 5th instar larva), *Hadimina torosensis* Sipahiler, 2002 (one 5th instar larva), *Metanoea rhaetica* Schmid, 1956 (seven 5th instar larvae), and *M. flavipennis* (Pictet, 1834) (ten 5th instar larvae). This material is deposited in the collection of J. Waringer (Vienna, Austria).

Molecular study

We used phylogenetic analysis to associate the larvae. We inferred phylogenetic trees based on molecular sequence data from two nuclear and two mitochondrial genes of the four target species and those previously published for 43 other species of Drusinae (Supplementary table S1). We extracted DNA from larval and adult specimens using the DNEasy Blood & Tissue Kit (Qiagen) following the manufacturer's protocol. PCRs were carried out in 10 µl of solution. PCR procedures and primers are listed in Table 1. PCR products were sequenced on an ABI 3177XL capillary sequencer at the Biodiversity and Climate Research Laboratory Centre. Sequences were edited in Geneious vR7 (biomatters). Sequences were aligned using the Muscle-plugin in Geneious vR7.

We inferred phylogenetic trees for each locus separately using a Bayesian/MCMC analysis implemented in MrBayes v3.2.1 (Ronquist et al., 2012). Nucleotide substitution models were selected using the Bayesian Information Criterion in the model test module of MEGA v5.2 (Tamura et al., 2011). In the protein coding genes, nucleotide substitution models were identified separately for each codon position (see Table 2 for codon-specific selected models). We did not partition the 16SrDNA fragment. All model estimations were performed using all sites, i.e. including the gaps in LSU. The B/MCMC analysis was based on 2 parallel runs with six chains each that explored tree space for 10 million generations. Phylogenetic trees were based on 15,000 trees (2 × 7,500) following a 25% burn-in phase. We assessed the parameter files in Tracer Version 1.4.6 (Drummond & Rambaut, 2007) to determine if each run had reached stationarity. We used the

Table 2. Characteristics of the molecular data sets used in the phylogenetic analysis and larval-adult associations.

Locus	*N* sequences	Length (bp)	Variable sites (*N* / %)	Substitution model by codon position (1st / 2nd / 3rd)	Average standard deviation of split frequencies after 25 mio & 100 mio generations
COI	187	1210*	533 / 44	TN93+G / HKY+G / GTR+I+G	0.014–0.007
LSU	197	362	162 / 44	T92+G	0.011–0.005
CAD	163	848	644 / 76	HKY+G / HKY / K2+G	0.008–0.004
WG	181	352	147 / 42	JC+G / JC / T92	0.008–0.005

* 11 Ns were added between the two fragments of mtCOI.

Table 3. Results of larval associations based on phylogenetic reconstruction for each of the four loci. The 5th instar larvae of the target species are deposited in the collection of J. Waringer (Vienna, Austria). m – males, f – females, l – larvae, pp – posterior probability.

Species	CAD			COI			WG			LSU		
	pp	Association criterion	*N* (m/f/l)	pp	Association criterion	*N* (m/f/l)	pp	Association criterion	*N* (m/f/l)	pp	Association criterion	*N* (m/f/l)
D. serbicus	1.0	monophyly; identical haplotypes	4/1/2	1.0	monophyly; identical haplotypes	4/1/2	0.98	monophyly; identical haplotypes	4/1/2	0.99	identical haplotypes	4/1/2
D. tenellus	1.0	monophyly	1/1/1	1.0	monophyly	1/1/2	1.0	sister clades	1/1/2	0.98	monophyly (by exclusion to *D. botosaneanui*)	1/1/2
D. botosaneanui	1.0	monophyly; larvae nested within males	3/0/7	1.0	monophyly	3/1/3	0.89	monophyly; identical haplotype to ♀	0/1/4	1.0	identical haplotype	3/1/4
D. balcanicus	1.0	monophyly; identical haplotypes	5/0/2	0.9	monophyly; identical haplotypes	6/1/2	0.82	identical haplotypes	6/1/2	0.97	identical haplotype	6/1/2

average standard deviation of split frequencies between runs after 2,500,000 generations if both runs reached the same optimality space.

RESULTS

Identification of the larvae

The putative conspecific larvae always clustered in monospecific clades with adults (Table 3, supplementary Figs S2a–d). There are, however, some weaknesses in the resolution of the larval association clades. In the WG phylogeny, clades including *D. botosaneanui* and *D. balcanicus* were not significantly supported (pp < 0.95). For COI and CAD all sequences of *D. balcanicus* are basal to a highly supported clade for *D. discophoroides* Kumanski, 1979 (pp = 1.0), but are not grouped in supported clades. For LSU there is a similar situation regarding *D. tenellus*. However, these topological inconsistencies only insignificantly weaken the overall associations of adults with larvae, which are further supported by identical haplotypes in all species except *D. tenellus*.

Drusus serbicus Marinković-Gospodnetić, 1971a

Material examined. 3 ex. of fifth instar, Golija Mt, spring Ilinac (Serbia), 43°20′00″N, 20°16′55″E, 1500 m a.s.l., 22 June 2013, leg. Kučinić, Bjelanović, Živić.

General morphology. Larva eruciform, head and sclerotized parts chestnut to blackish brown, nonsclerotised parts whitish. Body length 9.8–10.8 mm, head width 1.33–1.43 mm.

Head. Head capsule coarsely granulated, almost circular in shape and hypognathous (Figs 1A–C), dorsally with blackish muscle attachment spots. Ventral parietalia sections, submentum, maxillolabial sclerites and premandibular areas medium to orange brown (Figs 1C, D). Yellowish-white ring around each eye (Fig. 1C). In lateral view, head capsule with carina (0.40–0.45 mm long and approximately 0.04 mm wide) starting a short distance from anterior margin of eye and extending to frontomedian corner of frontoclypeus (Fig. 1C, arrow).

Head capsule with complete set of 18 pairs of primary setae and lacking any additional spines or bristles known to occur in other Drusinae larvae (e.g., *Ecclisopteryx* spp., *Drusus trifidus*). However, posterior to each eye, there is a spinule area surrounding the bases of setae 15 and 16 (diameter 0.13–0.18 mm; Figs 1E, F; white ovals). Such spinule areas occur in most members of the *Drusus bosnicus* Group sensu Marinković-Gospodnetić (1971a), e.g., *Drusus klapaleki* Marinković-Gospodnetić, 1971b. Frontoclypeus bell-shaped, with narrow central constriction (Figs 1A, B).

Antennae arise on dorsal rim of lateral carina and halfway between eye and anterior head margin (Fig. 1E, arrow), each consisting of 1 short cylindrical base and 1 short flagellum. On each parietale there are 10 dorsal and 2 ventral primary setae, with primary setae 5, 9 and 14 long and conspicuous (Figs 1B, C, E). Six primary setae on each side of frontoclypeus, 3 of them along anterior border. Labrum medium to light brown, with setal brush and primary setae 1–3 on anterolateral margins; on dorsal area, setation consists of primary setae 4–6 (Figs 1A, E).

Ventral apotome elongated triangular, medium to light brown, postgenal suture approximately 55% of apotome length (Fig. 1D). Blackish brown to dark brown mandibles lacking terminal teeth along edges as well as lacking ridges in central concavity (Figs 1D, E).

Thorax. Pronotum black brown to chestnut brown, very coarsely granulated (Figs 1C, 2A, B); its posterior margin thickened and darkly striped (Fig. 2C). Pronotal transverse groove on end of anterior third lacking. Dorsal profile in lateral view with annular crest highest at dorsal centre and gradually declining laterally (Figs 2B, C). With semicircular step between crest centre and posterior pronotal rim (Fig. 2A, between arrows). In anterior view, pronotal crest with a dorsocentral notch (Fig. 1A, black arrow). Two setal rows along anterior border of pronotum: (1) Dense fringe of short, curved, fine, yellow setae; (2) continuous row of long, widely-spaced, straight, dark setae meeting on anterior pronotal midline (Figs 2A, B); in total, 80–90 dark setae of varying lengths distributed over each pronotal half (Figs 1C, 2A, C). In addition, pronotal surface covered by high number of tiny, pale, recumbent setae (Fig. 2D); spines present in some other Drusinae (e.g., *Ecclisopteryx dalecarlica*) are absent. Pentangular prosternite light brown with medium brown posterior rim; prosternal horn present.

Mesonotum completely covered by 2 medium to yellowish brown sclerites with fine granulation except along posterior border and on lateral half of anterior border; their lateral and posterior margins with black sclerotization (Fig. 2E). Counts for mesonotal setae are as follows: Anterior setal group *sa*1: 15–18, posterior group *sa*2: 15–20, lateral group *sa*3: 28–30.

Metanotum partially covered by 3 pairs of medium to dark brown sclerites. Anterior metanotal sclerites (*sa*1)

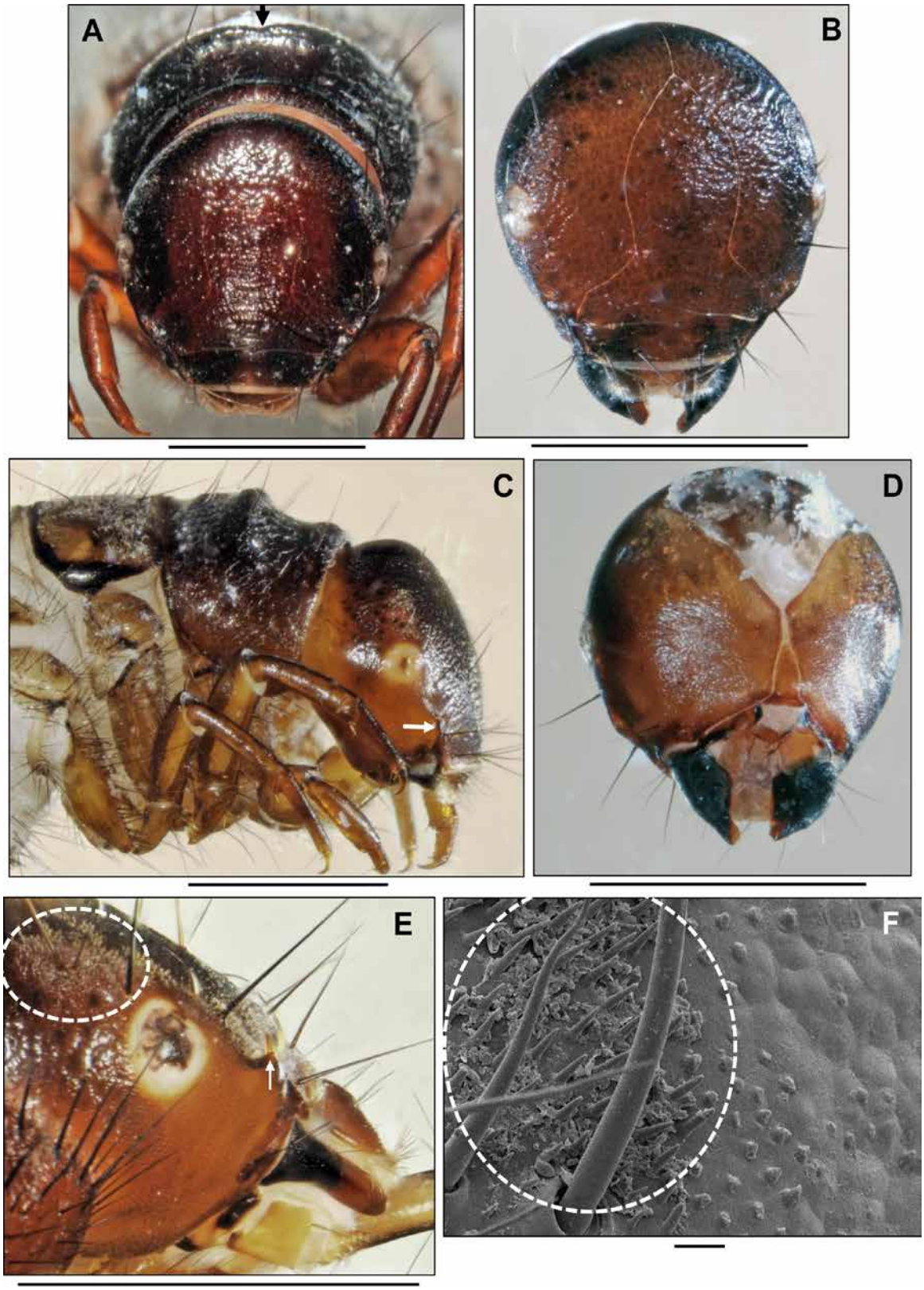

Fig. 1. *Drusus serbicus* (5th instar larva). A – head and pronotum, frontal view (arrow: median notch); B – head, frontal view; C – head, pro- and mesothorax, right lateral view (arrow: lateral carina); D – head, ventral view; E–F – head, dorsolateral view, details of spinule area (white ovals) (arrow in E: antenna). Scale bars: A – E = 1 mm; F = 0.02 mm.

very large, broadly ovoid, strongly tapering laterally, each with black anterior margin; separated by less than own length (Fig. 2E). Approximately 15 setae per sclerite (Fig. 2E). Row of setae present between small posteromedian sclerites (*sa*2); each sclerite bears 14–17 setae. Small setal group present between each lateral (*sa*3) and posteromedian sclerite (*sa*2); each *sa*3 with approximately 25–30 setae, concentrated anteriorly (Fig. 2E). Legs orange brown with

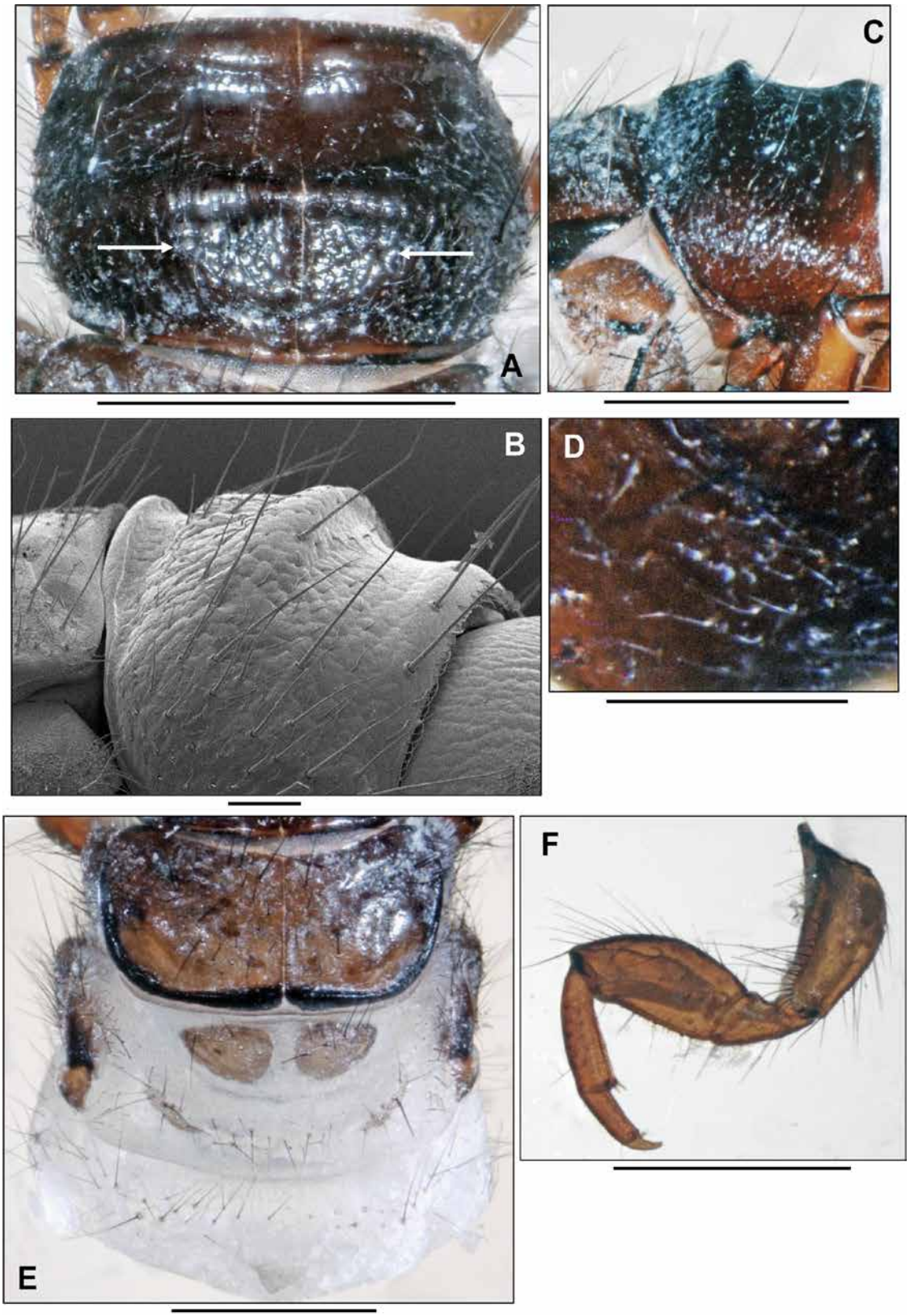

Fig. 2. *Drusus serbicus* (5th instar larva). A–C – pronotum, dorsal and right lateral view (arrows: semicircular step posterior of pronotal annular crest); D – pronotum, central posterior region, showing white recumbent setae; E – mesonotum, metanotum and 1st abdominal segment, dorsal view; F – left fore leg, posterior view. Scale bars: A, C, E–F = 1 mm; B = 0.2 mm; D = 0.5 mm.

setae numerous on coxae, trochanters and femora, sparse on tibiae and tarsi (Figs 2F, 3A, B). All femora with several proximodorsal setae. Coxa, femur and tibia of each foreleg wider than those of mid- and hind legs. Setae present on proximal parts of trochanters of all three pairs of legs. Additional setae present on both anterior and posterior faces

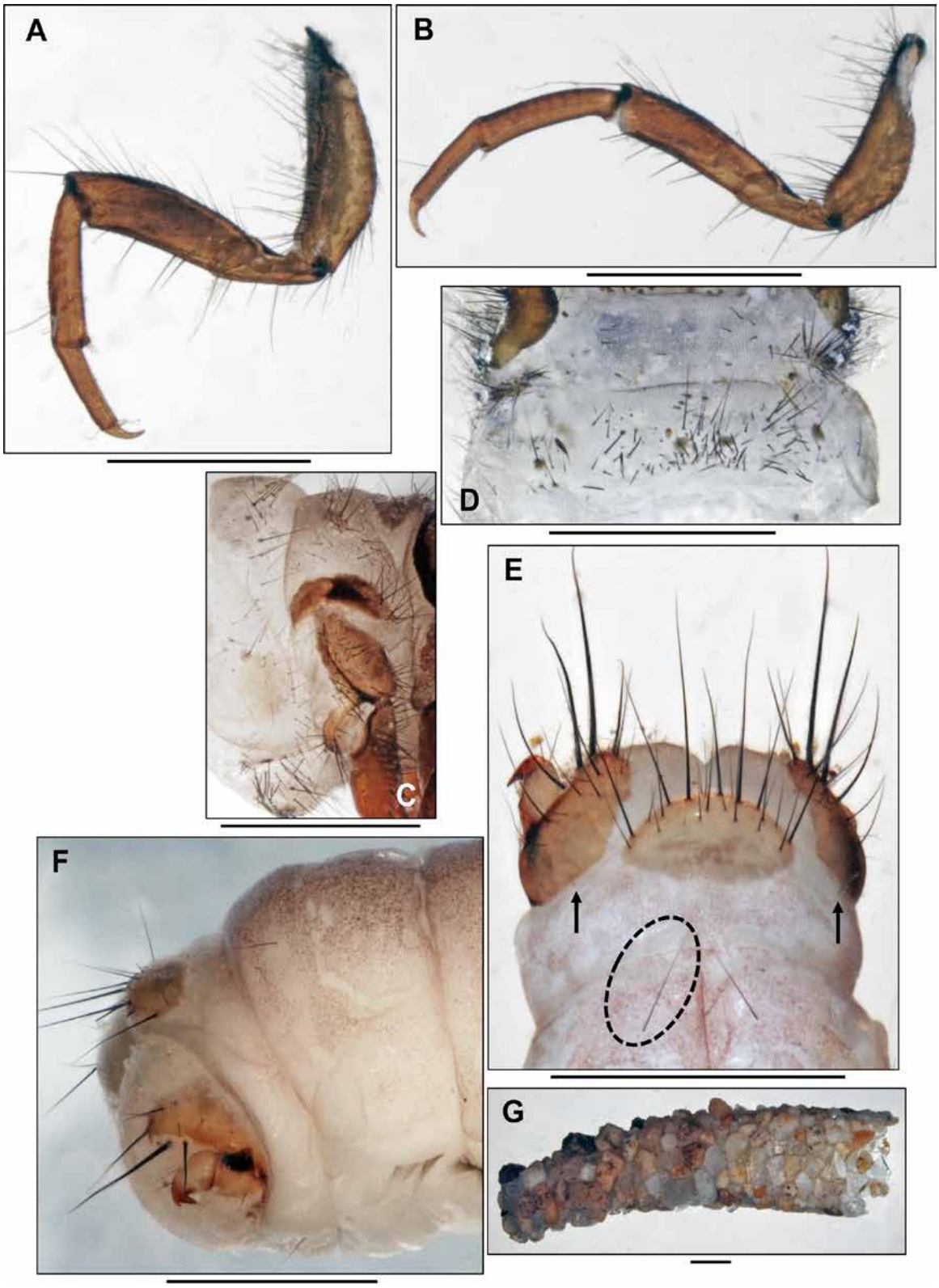

Fig. 3. *Drusus serbicus* (5th instar larva). A – left mid leg, posterior view; B – left hind leg, posterior view; C – metathorax and 1st abdominal segment, right lateral view; D – 1st abdominal sternum; E – abdominal segments VIII–IX, dorsal view (arrows: posterolateral setae; dotted oval: posterodorsal setae); F – apex of abdomen, right lateral view; G – case, right lateral view. Scale bars: A–G = 1 mm.

of all femora; ventral trochanteral brush present on fore and mid leg. Ventral edges of fore femora each with 5 yellow setae, of mid- and hind femora each with 4 dark setae. Dorsal setae only on distal third of mid and hind tibiae (Figs 2F, 3A, B).

Abdomen. First abdominal segment with 1 dorsal and 2 lateral fleshy protuberances (Figs 2E, 3C). Dorsal setal areas *sa*1, *sa*2 and *sa*3 fused, thereby creating continuous transverse row of setae anterior to dorsal protuberance, which extends to the dorsal section of each lateral protu-

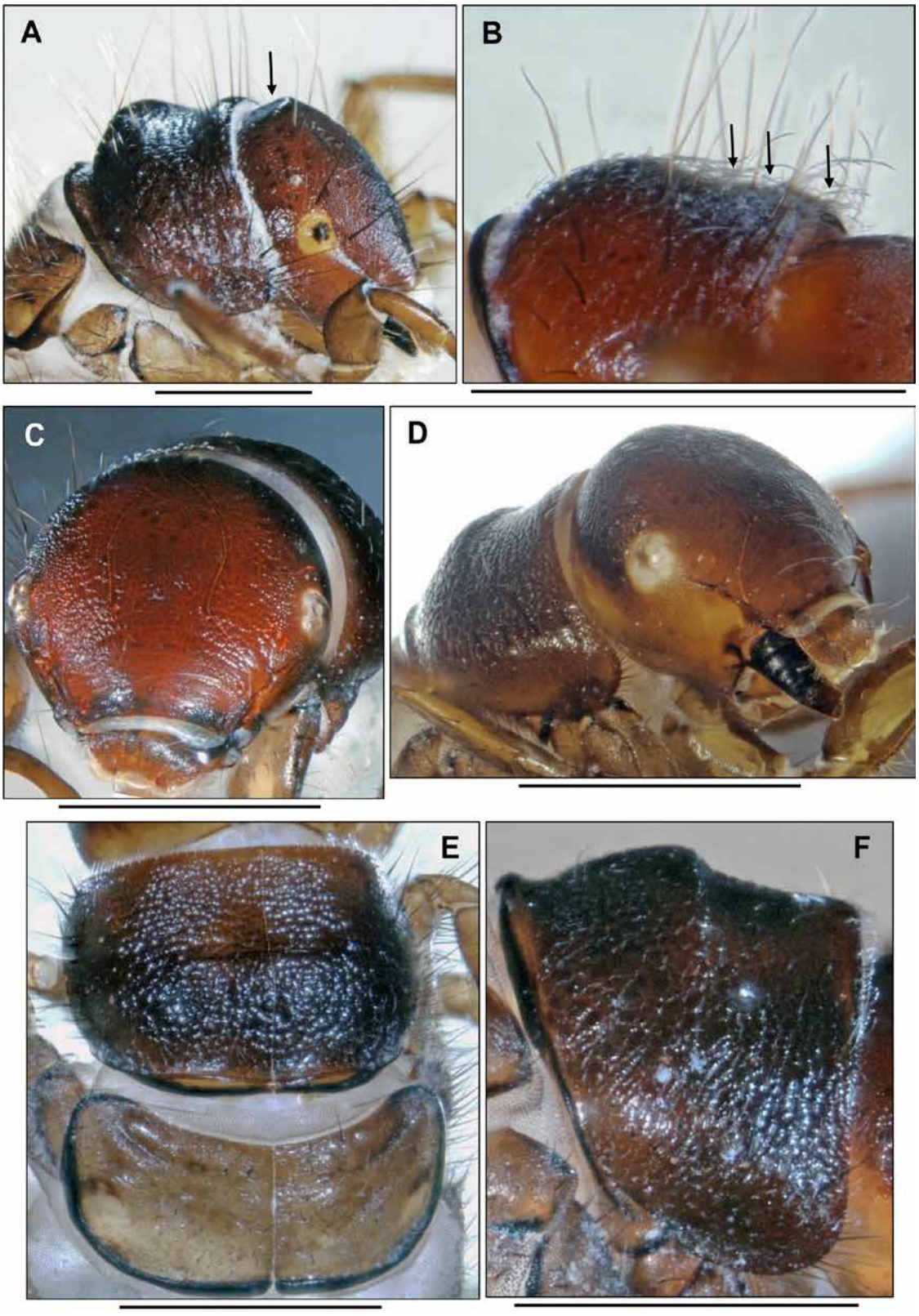

Fig. 4. A – *Drusus bosnicus* (5th instar larva), head and pronotum, right lateral view (arrow: vertex flattened); B – *Drusus radovanovici* (5th instar larva), pronotum, right lateral view (arrows: thin long yellowish setae); C–F – *Drusus balcanicus* (5th instar larva): C – head, frontal view; D – head and pronotum, right anterolateral view; E – pro- and mesonotum, dorsal view; F – pronotum, right lateral view. Scale bars: A–F = 1 mm.

berance. Sharply delimited basal sclerites present in about 30% of these setae; without setal group posterior to dorsal protuberance (Fig. 2E). Lateral protuberances lacking posterior sclerites (Fig. 3C). A continuous band of anterolateral setae present in front of each lateral protuberance, linking each dorsal and ventral *sa*3 setal group (Fig. 3C). First

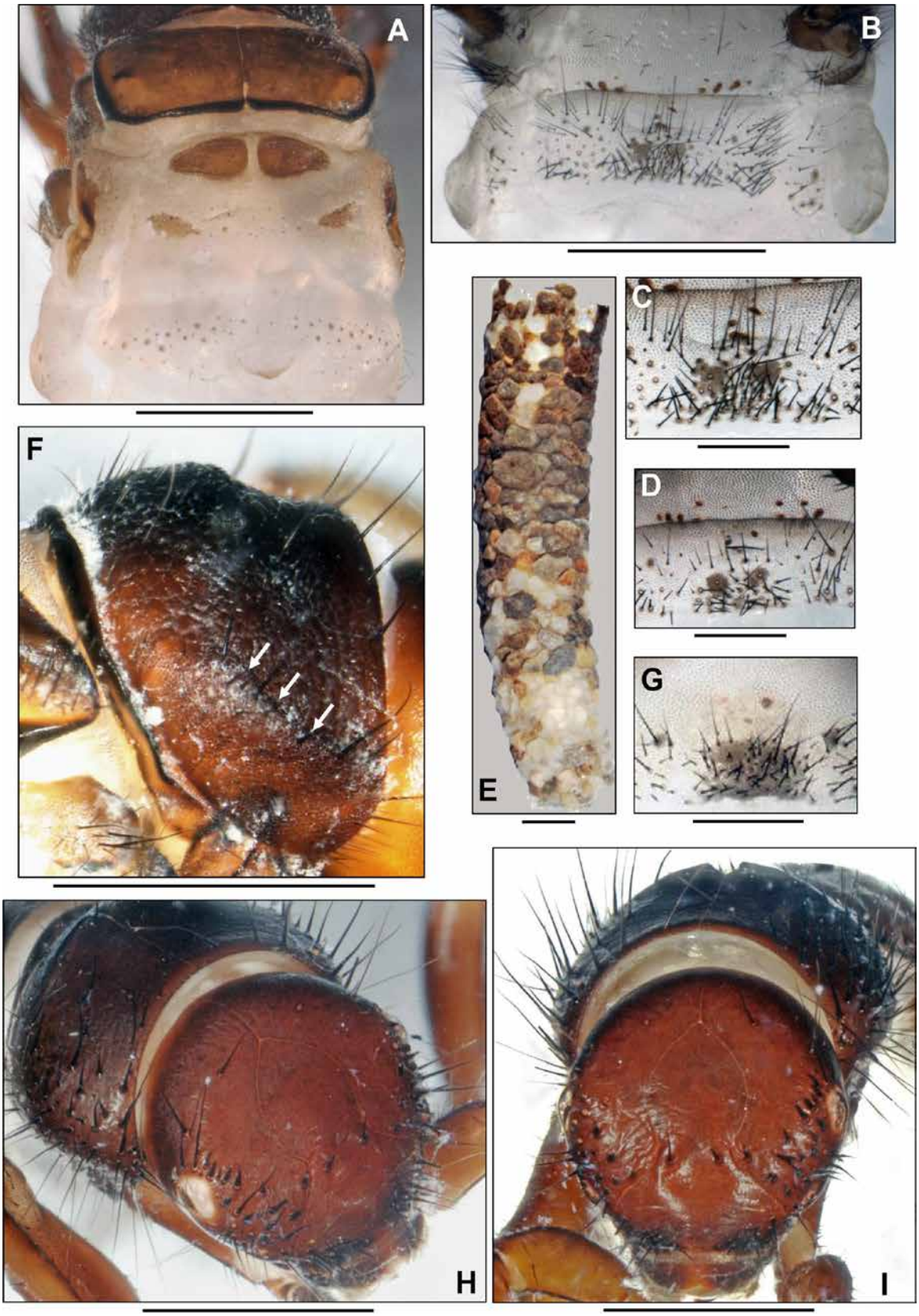

Fig. 5. A–E – *Drusus balcanicus* (5th instar larva): A – mesonotum, metanotum and 1st abdominal dorsum, dorsal view; B – 1st abdominal sternum, ventral view; C,D – details of central areas of 1st abdominal sternum, ventral view; E – case, right lateral view. F, G – *Drusus franzressli* (5th instar larva): F – pronotum, right lateral view (arrows: lateral ridge); G – detail of central area of 1st abdominal sternum, ventral view. H, I – *Drusus botosaneanui* (5th instar larva): H – head and pronotum, dorsolateral view; I – head and pronotum, frontal view. Scale bars: A, B, E, F, H, I = 1 mm; C, D, G = 0.3 mm.

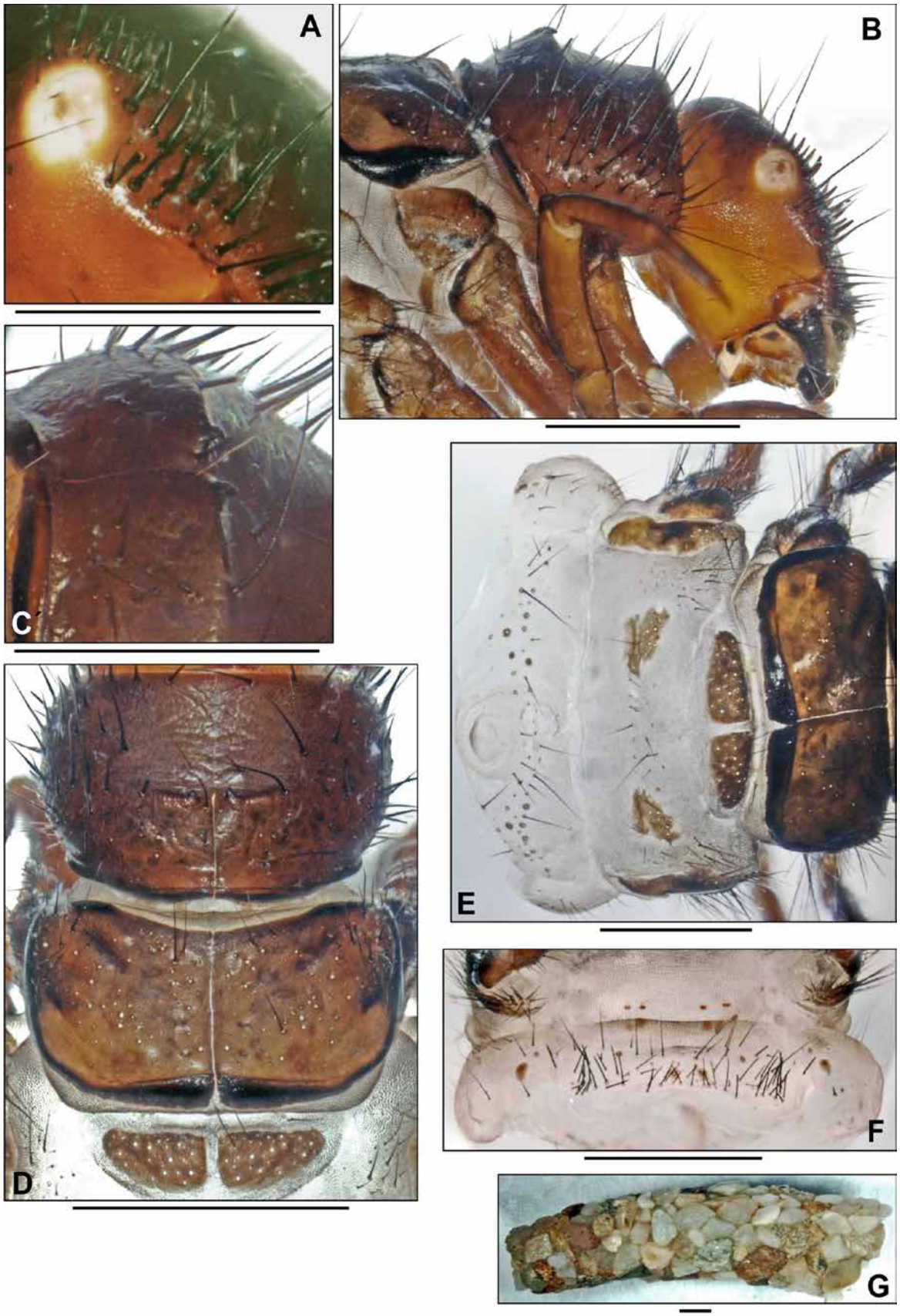

Fig. 6. *Drusus botosaneanui* (5th instar larva). A – head, detail of spines and bristles on right parietale; B – head, pro- and mesonotum, right lateral view; C – detail of central ridge on pronotum showing median notch flanked by anteriorly directed hooks; D – pronotum, mesonotum and anterior part of metanotum, dorsal view; E – mesonotum, metanotum and 1st abdominal segment, dorsal view; F – 1st abdominal sternum, ventral view; G – case, right lateral view. Scale bars: B, D–G = 1 mm; A, C = 0.5 mm.

abdominal sternum with ventral setal areas *sa*1, *sa*2 and *sa*3 fused, creating continuous field of setae; basal sclerites of setae on the central area of the first abdominal sternum mostly small and inconspicuous except for four larger basal sclerites near midline and immediately ventral to the lateral protuberances. Basal sclerites never fuse with one another (Fig. 3D). Eighth abdominal dorsum bears two to four long posterodorsal setae (pds) (Fig. 3E, dotted oval). Only 1 posterolateral seta present on each half of 9th abdominal dorsum (Fig. 3E, arrows).

All gills single filaments. Dorsal gills present at most on the 2nd (presegmental position) to the 7th segment (presegmental position). Ventral gills on the 2nd (postsegmental) to 8th segment (presegmental). Lateral gills lacking. Lateral fringe extending from posterior third of 2nd to middle of 8th abdominal segment; in addition, a prominent seta surrounded by a small number of isolated lateral fringe setae on anterior border of 2nd segment. Light brown sclerite on 9th abdominal tergum semicircular (Fig. 3E); 7–8 long and several shorter setae present along its posterior border, 1–2 of the long setae take the position of central intermediate *c* setae (Fig. 3E). Anal prolegs of limnephilid type, light to medium brown, with light muscle attachment spots. Anal claws medium brown, each with 1 small accessory hook (Fig. 3F).

Case. Larval case 10.2–10.5 mm long (n = 3), curved, conical (width at anterior opening 2.45–2.7 mm and at posterior opening 1.7–2.3 mm), consisting of mineral particles (sand grains of mixed sizes; Fig. 3G).

Habitat. This species inhabits the epirhithral section of oxygen-rich streams with high to moderate currents, but is also encountered near the source (hypocrenal region) down to the metarhithral zone. *Drusus serbicus* is a grazer feeding on epilithic biofilms and associated algae.

Key to larvae of species of *Drusus* of the grazer clade having spinule areas on head capsules

As in the other species in the Drusinae grazer clade, the mandibles are spoon-shaped (lack terminal teeth and ridges in central cavity; Fig. 1E). The larva of *Drusus serbicus* is similar to six Drusinae species from the Balkan Peninsula, which have a small field of spinules (= small spines approximately 0.03 mm long) posterior to their eyes (Figs 1E, F). Based on the recent detailed descriptions of Kučinić et al. (2008, 2010, 2011a, b, in press) and unpublished data of Previšić et al., *D. serbicus* is integrated into the following dichotomous key:

1 Head with flat vertex (Fig. 4A)..... ..... *Drusus bosnicus* Klapálek, 1899
– Vertex evenly rounded (e.g., Fig. 1C) 2
2 Pronotum with thin, long, yellow setae (Fig. 4B) ....*Drusus radovanovici* Marinković-Gospodnetić, 1971b
– Pronotum without thin, long, yellow setae 3
3 Pronotum with numerous short, white, recumbent setae (Fig. 2D) 4
– Pronotum without numerous short, white, recumbent setae... .....*Drusus septentrionis* Marinković-Gospodnetić, 1976
4 Dorsal pronotal hump smoothly rounded .... *Drusus medianus* Marinković-Gospodnetić, 1976
– Dorsal pronotal hump with distinct ridge (e.g., Fig. 2 A–C).. 5
5 Anterior metanotal sclerites elongate and triangular (width / length ratio ≥ 2.0) .... *Drusus vespertinus* Marinković-Gospodnetić, 1976
– Anterior metanotal sclerites broadly triangular (width / length ratio < 2.0; e.g., Fig. 2E)..... 6
6 In lateral view, posterior side of dorsal ridge gently descends .....*Drusus klapaleki* Marinković-Gospodnetić, 1971b
– In lateral view, dorsal ridge annular, posterior side sharply descending (Figs 2A–C) ....*Drusus serbicus* Marinković-Gospodnetić, 1971a

***Drusus balcanicus* Kumanski, 1973**

Material examined. 2 ex. of fifth instar, Troyan Pass brook, south side (Bulgaria), 42°47′21″N, 24°37′05″E, 1450 m a.s.l., 12 June 2013, leg. Keresztes, Torok, Kolocsar.

General morphology. Larva eruciform, head and sclerotized parts dark brown, nonsclerotised parts whitish. Body length 11.0–11.2 mm, head width 1.27–1.30 mm.

Head. Head capsule granulated, roundish (Figs 4C, D). Labrum dark brown, with setal brush (Fig. 4D). Ventral apotome yellowish to light brown and with postgenal suture approximately 70–75% of apotome length. Head capsule lacking any additional spines, bristles or areas of spinules.

Thorax. Pronotum with adjacent series of granuli creating ribbed structures (Fig. 4F). Dorsal profile in lateral view with posterior half of pronotum rounded, this curvature creates a distinct step leading down to anterior, lower part of pronotum (Fig. 4F). Lateral ridge lacking. In total, 35–40 dark setae of varying lengths distributed over each pronotal half. Prosternite very light and indistinct.

Mesonotum completely covered by 2 medium brown to yellowish sclerites with dark brown muscle attachment spots (Fig. 4E). Their anterolateral corners, lateral and posterior margins darkly sclerotized. Number of setae in anterior setal group *sa*1 30–40, in posterior group *sa*2 25–30 and in lateral group *sa*3 25–30.

Anterior *sa*1 metanotal sclerites triangular, with approximately 25–30 setae per sclerite (Fig. 5A). Setal counts for both posteromedian *sa*2 sclerites and lateral *sa*3 sclerites are 15–20 setae per sclerite. Legs light brown. All other details as in *D. serbicus*.

Abdomen. Centre of 1st abdominal sternum with large or medium concentrations of fused basal sclerites of setae, creating multilobed patterns of sclerotized areas positioned mostly posterior of the two largest basal sclerites (Figs 5B–D).

Dorsal gills present at most on 2nd (presegmental position) to the 5th segment (presegmental position). Ventral gills on the 2nd (presegmental) to 7th segment (presegmental). Lateral gills lacking. Lateral fringe, details of 9th abdominal sclerite and of anal prolegs as in *D. serbicus*.

Case. Larval case 10.6–10.7 mm long (n = 2), curved, slightly conical. Width at anterior opening 2.5–2.6 mm and at posterior opening 1.8–1.9 mm, consisting of mineral particles (sand grains of mixed size; Fig. 5E).

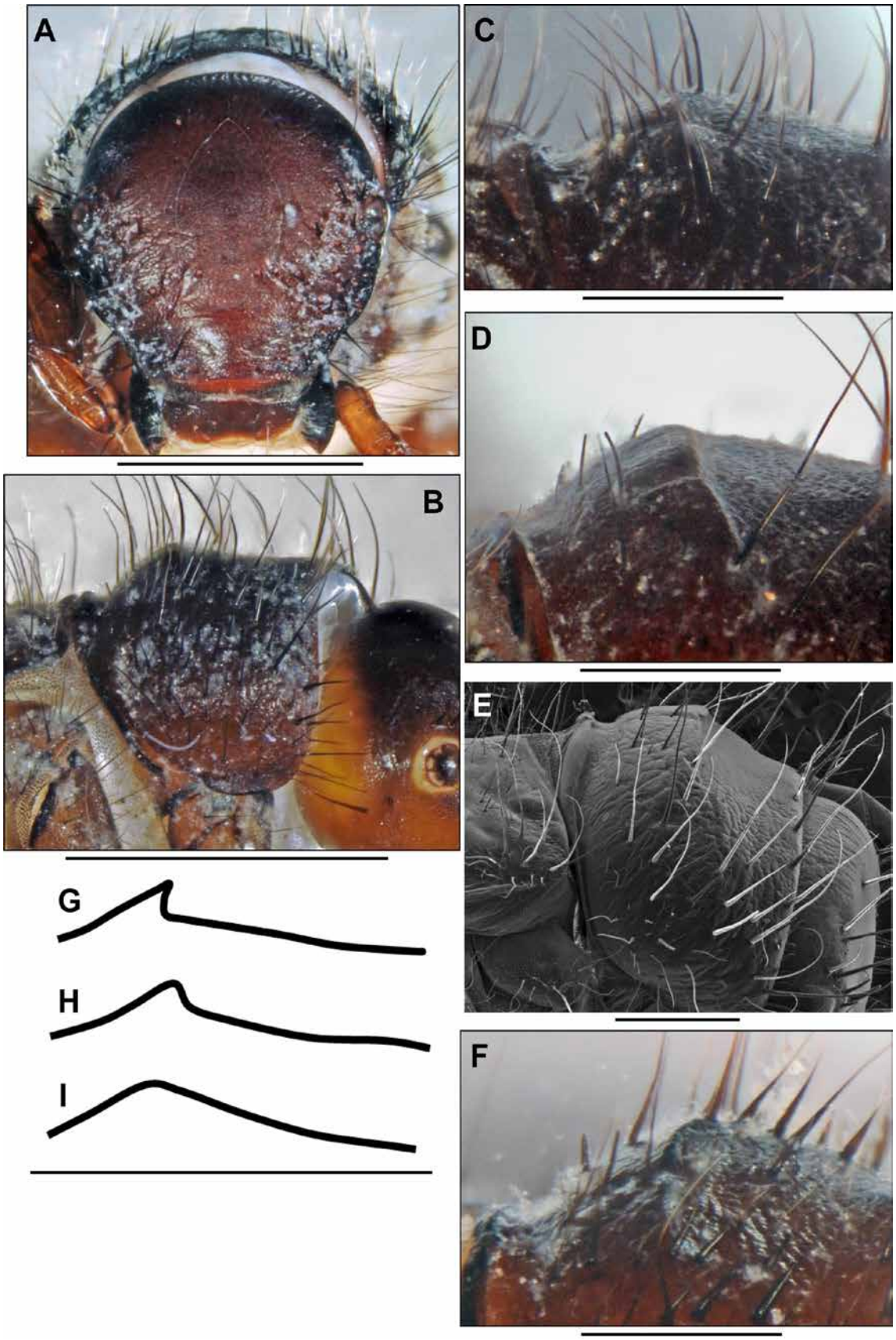

Fig. 7. A–C, *Drusus tenellus* (5th instar larva): A – head and pronotum, frontal view; B – head and pronotum, right lateral view; C – detail of central notch, right dorsolateral view. D, E – *Drusus schmidi* (5th instar larva): D – detail of central notch, right dorsolateral view; E – head, pro- and mesonotum, right lateral view. F – *Ecclisopteryx madida* (5th instar larva), detail of central notch, right dorsolateral view. G–I – schematic cross sections (right lateral view) of central pronotal ridges (5th instar larvae): G – *Ecclisopteryx madida*; H – *Drusus schmidi*; I – *Drusus tenellus*. Scale bars: A, F–I = 1 mm; B–E = 0.5 mm.

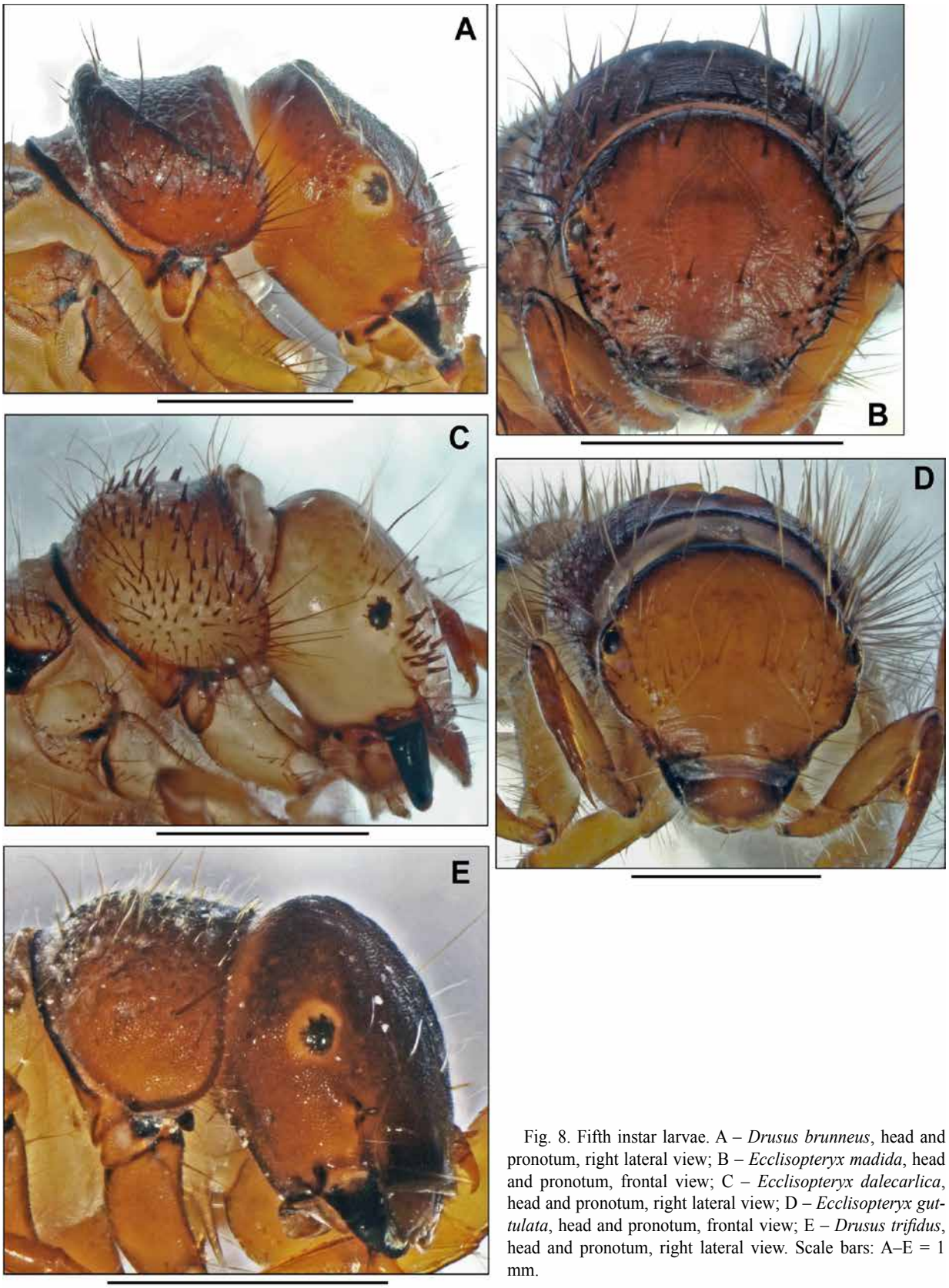

Fig. 8. Fifth instar larvae. A – *Drusus brunneus*, head and pronotum, right lateral view; B – *Ecclisopteryx madida*, head and pronotum, frontal view; C – *Ecclisopteryx dalecarlica*, head and pronotum, right lateral view; D – *Ecclisopteryx guttulata*, head and pronotum, frontal view; E – *Drusus trifidus*, head and pronotum, right lateral view. Scale bars: A–E = 1 mm.

Habitat. *Drusus balcanicus* is confined to oxygen-rich headwaters of streams and to springs. Data logger records over a full year revealed arithmetic means of water temperatures for typical habitats of *D. balcanicus* (e.g., springs below the Troyan pass, Bulgaria) of 6.65°C (range 1.08–15.44°C). This species grazes epilithic algae.

Table 4. Synopsis of the characters separating the currently known Drusinae larvae (5th instars) with spoon-shaped mandibles and no spinule areas, additional bristles or spines on the head capsule (i.e., only the standard set of 18 pairs of primary setae is present).

Species	Dorsal gills present?	Basal sclerites of setae on first abdominal sternum fused to sclerotized plates or in multilobed patterns?	Anterior-row setae present near dorsal pronotal midline?	Dorsal edge setae on entire length of mid- and hind tibia?	Pronotum evenly rounded?	References
Drusus carpathicus	no	no	yes	no	yes	Szczesny (1978)
Drusus improvisus *Drusus rectus* *Drusus spelaeus* *Metanoea flavipennis* *Metanoea rhaetica*	yes	yes	yes	no	yes	Table 5 (present paper)
Drusus balcanicus *Drusus franzressli* *Drusus nigrescens* *Ecclisopteryx malickyi*	yes	yes	yes	no	no	Table 5 (present paper)
Drusus camerinus	yes	yes	no	no	yes	Waringer et al., 2008a
Drusus melanchaetes *Drusus adustus*	yes	no	yes	yes	yes	Graf (1993) Waringer et al. (2008b)
Anomalopterygella chauviniana *Drusus bolivari* *Drusus monticola* *Drusus ramae*	yes	no	yes	no	no	Urbanič et al. (2003) Vieria-Lanero et al. (2005) Kučinić et al. (2010) Waringer et al. (2010) Waringer & Graf (2011)
Drusus annulatus *Drusus biguttatus* *Drusus ingridae* *Drusus rectus* *Drusus vinconi* *Ecclisopteryx asterix* *Hadimina torosensis* *Leptodrusus budtzi*	yes	no	yes	no	yes	Moretti & Pirisinu (1981) Sipahiler (2002) Urbanič et al. (2003) Waringer et al. (2013b)
Drusus aprutiensis *Drusus camerinus* *Drusus croaticus* *Drusus mixtus* *Drusus trifidus*	yes	no	no	no	yes	Kučinić et al. (2008) Waringer et al. (2008a, 2010, 2011)

Diagnosis of species of *Drusus* of the grazer clade lacking areas of spinules or additional spines on head capsule

Mandibles are spoon-shaped (Fig. 1E). The larva of *Drusus balcanicus* lacks spinule areas and additional bristles and spines on its head capsule (Fig. 4C; Table 4). Dorsal gills present; basal sclerites of setae on the first abdominal sternum fused to sclerotized plates or form multilobed patterns (Fig. 5B); anterior row of setae present near the dorsal pronotal midline (Fig. 4C); mid and hind legs with dorsal edge setae restricted to distal third of tibiae. It shares these features with *Drusus franzressli*, *D. improvisus*, *D. nigrescens* Meyer-Dür, 1875, *D. rectus*, *D. spelaeus*, *Ecclisopteryx malickyi* Moretti, 1991, *Metanoea flavipennis* and *M. rhaetica*. Due to the presence of a low central ridge on the pronotum, the larva of *D. balcanicus* is similar to that of *Drusus franzressli* (Table 5), but the latter also has a distinct lateral ridge (Fig. 5F, arrows) which is absent in *D. balcanicus* (Fig. 4F). In addition, the basal sclerites of the central setae on the first abdominal sternum are fused into a large, uniform central plate (Fig. 5G) in *D. franzressli* and form a multilobed sclerotized pattern in *D. balcanicus* (Figs 5B–D).

***Drusus botosaneanui* Kumanski, 1968**

Material examined. 2 ex. of fifth instar, Gornje Lukovo polje (Macedonia), 41°52′03″N, 20°42′00″E, 1642 m a.s.l., 31 May 2012, leg. Kučinić, Krpač.

General morphology. Larva eruciform, head and sclerotized parts dark brown, nonsclerotised parts whitish. Body length 10.3–10.5 mm, head width 1.26–1.30 mm.

Head. Head capsule roundish, dark brown with lighter orange areas around foramen occipitale and a smooth surface sculptured by shallow wrinkles (Figs 5H, I). Each parietale with 20–25 long bristles and short, strongly tapering spines plus a standard set of 12 primary setae, mostly anterior and dorsal to the eye. Also on frontoclypeus there are 12–18 long bristles and short, strongly tapering spines, plus standard set of 6 pairs of primary setae, mostly on anterolateral corners (Figs 5H, I).

Ventral apotome orange, broadly bell-shaped; postgenal suture approximately 60–66% of apotome length.

Thorax. Pronotum dark brown to blackish brown. Pronotal surface relatively smooth, sculptured by shallow wrinkles (Figs 6C, D). Dorsal profile in lateral view with low ridge not elongated laterally. Ridge gently ascending from posterior pronotal border with a distinct step leading down to anterior, lower 2/3 of pronotum (Figs 6B, D). In anterior view with deep central notch flanked by two an-

Table 5. Synopsis of characters separating the currently known Drusinae larvae (5th instars), which share the following group morphomatrix: Spoon-shaped mandibles; lack of additional head bristles, spines or spinule areas; setae of anterior row present near dorsal pronotal midline; dorsal gills present; setae on dorsal edge restricted to distal third of mid- and hind tibiae; basal sclerites of setae on first abdominal sternum fusing into sclerotized plates or multilobed patterns.

Species	Dorsal outline of pronotum / median incision present?	Pronotal sculpturing	Sclerotization on 1st abdominal sternum	Posterolateral gills present on 2nd and 3rd abdominal segment?	Start of lateral fringe	Distribution	References
Drusus nigrescens	high ridge / yes	coarsely granulated, ribbed	multilobed sclerotized pattern	yes	last third III	western alpine	Waringer et al. (2007)
Ecclisopteryx malickyi	high ridge / no	coarsely granulated	multilobed sclerotized pattern	yes	last third III	southern alpine	Graf et al. (2011)
Drusus franzressli	low central ridge / no	coarsely granulated	central plate	no	first third II	Hellenic Western Balkans	Waringer et al. (2013a)
Drusus balcanicus	low central ridge / no	coarsely granulated, ribbed	multilobed sclerotized pattern	no	first third II	Eastern Balkans	present paper
Drusus improvisus	evenly rounded, high profile / no	coarsely granulated, ribbed	multi-lobed sclerotized pattern	yes	last third II	Apennines	Waringer et al. (2008a)
Drusus rectus	evenly rounded, low profile/no	coarsely granulated, ribbed	multilobed sclerotized pattern	yes	last third III	Pyrenees, Massif central	unpubl. data
Drusus spelaeus	evenly rounded, low profile / no	coarsely granulated, ribbed	central plate or multi-lobed sclerotized pattern	yes	last third II	western alpine	Waringer et al. (2013a)
Metanoea flavipennis	evenly rounded, low profile / no	finely granulated	central plate	yes	last third II	western alpine	Waringer et al. (2000)
Metanoea rhaetica	evenly rounded, low profile / no	finely granulated	central plate	no	last third II	eastern alpine	Waringer (1985)

teriorly directed hooks (Figs 6B–D). In total, 60–75 long dark bristles and short, strongly tapering spines are distributed over each pronotal half. Prosternite light brown, with medium brown anterolateral corners, trapezoidal in shape and tapering posteriorly.

Mesonotal sclerites dark brown to blackish brown with lateral and posterior margins darkly sclerotized. There are 15–25 setae in anterior setal group *sa*1, 17–35 in posterior group *sa*2 and 18–25 in lateral group *sa*3 (Fig. 6D).

Dark brown anterior *sa*1 metanotal sclerites ovoid, with approximately 25–30 setae per sclerite (Fig. 6D). Setal counts on medium brown posteromedian *sa*2 sclerites are 15–20 and on lateral *sa*3 sclerites 17–25 setae per sclerite, respectively; the latter sclerites medium brown with black brown markings (Fig. 6E). Legs medium to dark brown. All other details as in *D. serbicus*.

Abdomen. Posterior sclerite present on lateral protuberances. On 1st abdominal sternum, ventral setal areas *sa*1, *sa*2 and *sa*3 fused, creating continuous field of 70–100 setae; basal sclerites of setae in the central area of the first abdominal sternum mostly small and inconspicuous except for four larger basal sclerites near midline and immediately ventral to the lateral protuberances (Fig. 6F). Eighth abdominal dorsum with 2 to 4 long and 4 short posterodorsal setae (pds). Only 1 posterolateral seta is present on each half of 9th abdominal dorsum. Light to medium brown sclerite on 9th abdominal tergum semicircular, with 10 long and several shorter setae along its posterior border, 2 of the long setae in the position of central intermediate *c* setae.

Dorsal gills present at most on 2nd (presegmental position) to 7th segment (postsegmental position). Ventral gills on 2nd (presegmental) to 7th segment (postsegmental). Dorsolateral gills on 2nd (presegmental) to 4th segment (presegmental) and ventrolateral gills on 2nd (postsegmental) to 4th segment (postsegmental). Lateral fringe extending from beginning of 3rd to first third of 8th abdominal segment.

Case. Larval case 10.2–10.4 mm long (n = 2), curved, conical. Width at anterior opening 3.1–3.3 mm and at posterior opening 1.9–2.2 mm. Case consists of mineral particles (sand grains of mixed size; Fig. 6G).

Habitat. *Drusus botosaneanui* inhabits springs and the upper regions of the headwaters of streams as well as midstream regions of rivers at 655 to 1450 m above sea level (Ibrahimi et al., 2012). Mean annual water temperatures of the sites inhabited by *D. botosaneanui* (e.g., tributary of Beli Iskar, Bulgaria) were 5.66°C (annual range 0.08–14.38°C). This species grazes on biofilms and epilithic algae.

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Drusus tenellus (Klapálek, 1898)

Material examined. 3 ex. of fifth instar, Mavrovo, Lukovo Pole (Macedonia), 41°42′03″N, 20°39′52″E, 1665 m a.s.l., 3 July 2010, leg. Previšić; 2 ex. of fifth instar, Murinska Rijeka (Montenegro), 42°39′18″N, 19°52′48″E, 878 m a.s.l., 5 July 2012, leg. Previšić.

General morphology. Larva eruciform, head and sclerotized parts dark brown, nonsclerotised parts whitish. Body length of final instar larva 10.7–12.9 mm, head width 1.24–1.34 mm.

Table 6. Synopsis of characters separating the currently known Drusinae larvae (5th instars) with spoon-shaped mandibles and with additional bristles and spines on the head capsule (in addition to the standard set of 18 pairs of primary setae).

Species	Pronotum with ridge extending laterally to the anterior pronotal margin?	Dorsal outline of pronotum / deep median incision present?	Number of setae, spines and bristles on the frontoclypeus	Colour of pronotal setae, spines and bristles	References
Drusus brunneus	yes	high ridge / no	>20	black brown	Szczesny (1978)
Drusus botosaneanui	no	low central ridge / yes	>20	black	present paper
Ecclisopteryx guttulata	no	low central ridge / yes	12–16	black brown	Szczesny (1978); Pitsch (1993); Waringer & Graf (2011)
Ecclisopteryx madida[1]	no	low central ridge / no	>20	black brown	Szczesny (1978); Pitsch (1993); Waringer & Graf (2011)
Drusus schmidi[1]	no	low central ridge / no	>20	black	Kučinić et al. (unpubl. data)
Drusus tenellus[1]	no	low central ridge / no	>20	black	present paper
Ecclisopteryx keroveci[2] *Ecclisopteryx ivkae*[2]	no	low central ridge / no	12	black brown	Previšić et al. (2014)
Drusus trifidus	no	evenly rounded / no	12	pale yellow	Szczesny (1978)
Ecclisopteryx dalecarlica	no	evenly rounded / no	12–16	black brown	Szczesny (1978); Pitsch (1993); Waringer & Graf (2011)

[1] In *E. madida*, the pronotal ridge is rather sharp and almost concave anteriorly (Figs 7F, G; 8B), in *D. schmidi* there is a distinct step (Figs 7D, E, H), and in *D. tenellus* the anterior section of the ridge gently slopes down to the anterior part of the pronotum (Figs 7B, C, I). [2] In *E. keroveci*, the number of additional spines on each parietale is 12–20, in *E. ivkae* 1–7.

Head. Head capsule roundish, dark brown with lighter orange areas around foramen occipitale and with smooth surface sculptured by shallow wrinkles (Figs 7A, B). Setation as in *D. botosaneanui*. Ventral apotome orange, narrow and parallel-sided; postgenal suture approximately 60–66% of apotome length.

Thorax. Pronotum dark brown to blackish brown. Pronotal surface coarsely granulated with adjacent series of granuli creating ribbed structures (Fig. 7B). Dorsal profile in lateral view with low ridge not elongated laterally. Joint between the posterior and anterior sides of ridge smooth and lacking distinct step; anterior side gently sloping down to anterior part of pronotum (Figs 7B, C, 7I). Central notch very shallow, flanking anteriorly directed hooks absent (Figs 7A, C). In total, 60–75 long dark bristles and short, strongly tapering spines scattered over each pronotal half. Prosternite light brown, with medium brown anterolateral corners, trapezoidal in shape and tapering posteriorly.

Mesonotal sclerites dark brown to blackish brown with lateral and posterior margins darkly sclerotized. Number of setae in anterior setal group *sa*1 15–25, in posterior group *sa*2 17–35 and in lateral group *sa*3 18–25.

Dark brown anterior *sa*1 metanotal sclerites ovoid, with 15–20 setae. Setal counts for medium brown posteromedian *sa*2 sclerites are 15–20 setae per sclerite and lateral *sa*3 sclerites 17–25, respectively; the latter sclerites medium brown with black brown markings. Legs medium to dark brown. All other details as in *D. serbicus*.

Abdomen. Posterior sclerite absent. First abdominal sternum as in *D. botosaneanui*. On 8th abdominal dorsum, there are 2 to 4 long and 4 short posterodorsal setae (pds). Only 1 posterolateral seta on each half of 9th abdominal dorsum. Sclerite on 9th abdominal tergum as in *D. botosaneanui*.

Dorsal gills present at most on 2nd (presegmental position) to 7th segment (postsegmental position). Ventral gills on 2nd (presegmental) to 7th segment (postsegmental). Dorsolateral gills on 2nd (presegmental) to 4th (presegmental) and ventrolateral gills on 2nd (postsegmental) to 4th segment (postsegmental). Lateral fringe extending from beginning of 3rd to first third of 8th abdominal segment.

Case. Larval case 11.0–11.5 mm long (n= 3), curved, conical. Width at anterior opening 2.7–3.0 mm and at posterior opening 1.9–2.0 mm. Case consists of mineral particles (sand grains of mixed size).

Habitat. *D. tenellus* prefers the epi- and metarhithral zone of oxygen-rich streams with high to moderate currents at altitudes >1450 m a.s.l. Mean annual water temperature for sites inhabited by *D. tenellus* (e.g., Strežimirska reka, Mavrovo, Macedonia) were 6.87°C (annual range 5.11–8.54°C). This species is a grazer of biofilms and epilithic algae.

Diagnosis of species of *Drusus* of the grazer clade with additional spines on head capsule

Mandibles are spoon-shaped (Fig. 1E). *Drusus botosaneanui* and *D. tenellus* belong to the group of Drusinae

species, which, in addition to their standard set of 18 pairs of primary setae, have short, thick spines or long, tapering bristles on each parietale (and frontoclypeus in some species): *Drusus brunneus*, *D. schmidi*, *D. trifidus*, *Ecclisopteryx dalecarlica*, *E. guttulata*, *E. ivkae* Previšić, Graf & Vitecek, 2014, *E. keroveci* Previšić, Graf & Vitecek, 2014 and *E. madida* (Table 6). Because the number of frontoclypeal setae is >20 (Figs 5I, 7A) and the dorsal ridge (Figs 6B, 7B) does not extend to the anterolateral corners of the pronotum as in *D. brunneus* (Fig. 8A), *D. botosaneanui* and *D. tenellus* key out with *Ecclisopteryx madida* and *Drusus schmidi*. *D. botosaneanui* can be easily separated from the other three species by the deep central notch in its pronotal ridge, flanked by two anteriorly directed hooks (Figs 6B–D). In *Drusus schmidi*, *D. tenellus* and *Ecclisopteryx madida* the central notch is very shallow (Figs 7A, C–F, 8B) and without hooks. These species can be separated by the profile of the central pronotal ridge: in *E. madida*, the ridge is rather sharp and almost concave anteriorly (Figs 7F, G, 8B), in *D. schmidi* there is a distinct step (Figs 7D, E, H), and in *D. tenellus* the anterior side of the ridge gently slopes toward the anterior part of the pronotum (Figs 7B, C, I).

DISCUSSION

Previous studies that associated adults and larvae in caddisflies have used COI (e.g., Waringer et al., 2008b; Graf et al., 2009), COI & WG (Waringer et al., 2013a), COI & 28S rDNAs (Zhou et al., 2007), or COI, WG & LSU sequences (Previšić et al., 2014). The additional use of WG, LSU and CAD did not bring additional information to our 1200 bp long COI sequences. However, the use of unlinked nuclear markers provides independent support for the sorting of mitochondrial lineages, which could also result from historical isolation of presently admixed populations (e.g., Elbrecht et al., 2014). It is thus advisable to use both nuclear and mitochondrial markers for life stage associations. The nuclear genes we used, WG and CAD, proved sufficiently variable to discern species in this study. The level of variation is similar in WG and even higher in CAD compared with COI. Of the two genes we used, CAD performed somewhat better in our study, but both seem suitable for associating life stages of caddisflies.

All four species described in the present paper belong to the largest group of epilithic grazers, which lack terminal teeth on their mandibles (Figs 1D, E, 4D). Based on the presence or absence of bristles and setae in addition to the standard set of 18 pairs of primary setae on the larval head capsule, the grazer clade is separated into three subgroups:

Subgroup 1 with an area of spinules posterior to each eye (Figs 1E, F; white ovals). Such spinule areas occur in members of the *Drusus bosnicus* Group. Marinković-Gospodnetić (1971a) assigned *D. bosnicus*, *D. klapaleki*, *D. plicatus* Radovanović, 1942, *D. radovanovici* and *D. ramae* Marinković-Gospodnetić, 1971b to the *D. bosnicus* Group based on similarity of main structures of the male genitalia. Later, *D. krusniki* Malicky, 1981, *D. medianus*, *D. septentrionis* and *D. vespertinus* were added to this group (see discussion in Kučinić et al., 2011a). Of these species, the spinules are absent in *D. ramae*. They are present in the hitherto unknown larva of *D. serbicus*.

In subgroup 2 of the grazer clade, additional spines and bristles are present on the parietalia and/or the frontoclypeus; this is the case in *Ecclisopteryx dalecarlica*, *E. guttulata*, *E. ivkae*, *E. keroveci*, *E. madida*, *Drusus brunneus*, *D. schmidi*, *D. trifidus*, and the hitherto unknown larvae of *D. botosaneanui* and *D. tenellus* (Figs 5H, I, 6A, B, 7A). In this group, *D. trifidus* has only 0–2 additional spines per parietale.

Finally, *D. balcanicus* belongs to the largest subgroup of the grazer clade, in which only the standard set of primary setae is present on the head capsule (Figs 4C, D).

The spines that define the subgroup 2 (e.g., Figs 5H, I, 7A) have a length of 0.4 mm or more in *Ecclisopteryx guttulata* and are one magnitude longer than the spinules in *Drusus serbicus* and associated species measuring up to 0.03 mm. These morphological traits are in line with distinct differences in downstream distribution patterns: species with additional spines on their head capsule (morphological features summarized in Table 6) are most abundant in the epi- and metarhithral section, whereas Drusinae species without spines or with spinules are only found in spring or spring brook sections (eucrenal-hypocrenal). Statzner & Higler (1985) have shown that the eucrenal and hypocrenal sections of streams (source and springbrooks) are frequently characterized by relatively low hydraulic stress. The hypocrenal-epirhithral transition zone is followed by a section with high hydraulic stress, which, after the next zone of transition at the break-point of the slope, is then followed by a zone of lower hydraulic stress (Statzner & Higler, 1985). As Drusinae larvae face into the current (own observation), the presence of spines on the head capsule of this species group summarized in Table 6 may be associated with their presence in such hydrologically high-stress sections within the stream continuum. Videler (1995) has shown that small irregularities in the scales of fish can reduce shear stress in the boundary by a maximum of 10% compared with the shear stress of a smooth surface, a mechanism based on the impedance of cross flow under well-defined conditions. The function of roughness probably reduces total drag by generating premature turbulence and by boundary layer thinning, despite an increased friction over the surface (Videler, 1995).

The adults and larvae of *Drusus serbicus*, *D. botosaneanui* and *D. balcanicus* were sampled in the months of May and June in 2012 and 2013, and of *D. tenellus* in July 2010. This is in accordance with the reported spring flight period of *D. serbicus* as the type and paratype specimens were collected on 30 May 1970 (Marinković-Gospodnetić, 1971a). *Drusus balcanicus* is also a spring and early summer species with a rather short flight period, whereas it is longer in *D. botosaneanui*, which is on the wing from spring to autumn (Graf et al., 2008). A prolonged flight period has also been recorded for *D. tenellus*, for which adults are still being collected in the first week of October (Oláh & Kovács, 2013).

With respect to distribution, *D. balcanicus* is a species (micro-) endemic to the eastern Balkan Peninsula where it is restricted to the Stara Planina and Vitosha, whereas *D. serbicus* is (micro-) endemic to the Dinaric Western Balkans and restricted to the Dinaric Alps. *Drusus tenellus* has a wider range, with records from the Carpathians and the Dinaric Eastern Balkans. The distribution of *D. botosaneanui* is even wider, covering the Dinaric Western Balkans, the Hellenic and Eastern Balkans as well as Asia Minor.

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Supplementary files:

S1 (http://www.eje.cz/2015/037/S01.pdf). Specimen and sequence information for the data used in the present study.

S2 (http://www.eje.cz/2015/037/S02.pdf). Phylogenetic trees used for determining the larval associations of *D. balcanicus*, *D. botosaneanui*, *D. serbicus*, and *D. tenellus*. Shown are the 50% majority rule consensus trees based on B/MCMC phylogenetic inferences for a) mitochondrial COI; b) mitochondrial LSU; c) nuclear WG; and d) nuclear CAD datasets. Posterior probabilities above 0.94 are shown on the supported nodes. The specimen level topology is shown for those taxa that were subject to life stage associations (highlighted in grey boxes; specimen codes as in supplementary file S1). Species-clades were collapsed for the other taxa for clarity.