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DISSERTATION

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Disentangling the evolutionary history of *Veronica* (Plantaginaceae) in southeastern Europe

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Abstract

Southeastern Europe is a centre of European biodiversity, but very little is known about factors causing the observed richness. Here, we contribute to fill this gap by reconstructing spatio-temporal diversification, evolution and hybridization patterns of three groups within *Veronica* (Plantaginaceae) exhibiting different habitat requirements. To this end, we use ploidy level estimation, molecular markers (Amplified Fragment Length Polymorphism [AFLP], plastid and nuclear DNA sequences) and morphometry.

Veronica saturejoides, *V. thessalica* and *V. erinoides* constitute a group of closely related alpine taxa endemic to the Balkan Peninsula. The phylogeny inferred from nuclear DNA sequence data and AFLPs support the monophyly of *V. saturejoides*. In contrast, plastid DNA regions suggest a closer relationship of *V. saturejoides* subsp. *saturejoides* to *V. thessalica*, most likely indicating introgression from *V. thessalica* into *V. saturejoides* subsp. *saturejoides*.

In the cytologically variable and taxonomically intricate complex of woodland taxa within the *Veronica chamaedrys* group, diploid and tetraploid cytotypes are widespread. Diploids predominate on the southern Balkan Peninsula; most tetraploids are of independent autopolyploid origin. Two of the identified plastid lineages coincide with geographically distinct AFLP clusters, but genetic groups are not congruent with current taxonomy. Altogether, the genetic data suggest forest refugia on the southern-most Balkan Peninsula (Greece), in Bulgaria, Istria (Croatia and Slovenia) and maybe the southeastern Carpathians (Romania).

Veronica subgen. *Pseudolysimachium* sect. *Pseudolysimachion* has its evolutionary centre in southeastern Europe, where *V. barrelieri, V. orchidea* and *V. spicata* grow in grasslands mainly. Several geographically restricted entities have been described within the species. Hybridization played a major role in the formation of this highly variable section on the diploid as well as on the tetraploid level and most intraspecific taxa are of hybridogenic origin.

All three groups show the importance of gene flow and polyploidization in the diversification of taxa in southeastern Europe.

Zusammenfassung

Südosteuropa ist ein Zentrum europäischer Biodiversität. Über Faktoren, die zur Entstehung dieses Artenreichtums geführt haben, ist allerdings noch wenig bekannt. Diese Arbeit beschreibt anhand dreier Gruppen der Gattung *Veronica* (Plantaginaceae) die räumlich-zeitlichen Veränderungen sowie Evolution und Hybridisierungsmuster dieser Gruppen in Südosteuropa. Zu diesem Zwecke verwenden wir Ploidiestufenbestimmung, molekulare Marker (Amplified Fragment Length Polymorphism [AFLP], plastide bzw. nukleäre DNS-Sequenzen) und morphometrische Messungen.

Veronica saturejoides, *V. thessalica* und *V. erinoides* bilden eine Gruppe nah verwandter alpiner Arten, die am Balkan endemisch ist. Eine auf DNS-Sequenzen aus dem Kerngenom und auf AFLPs aufbauende Phylogenie unterstützt die Monophylie von *V. saturejoides*. Im Gegensatz dazu weisen DNS-Plastidenregionen auf eine nähere Verwandtschaft von *V. saturejoides* subsp. *saturejoides* und *V. thessalica* hin, was auf Introgression von *V. thessalica* zu *V. saturejoides* subsp. *saturejoides* hindeutet.

Innerhalb der zytologisch variablen und taxonomisch komplexen Gruppe von *Veronica chamaedrys*, deren Taxa in verschiedenen Waldlandschaften vorkommen, sind sowohl di- als auch tetraploide Zytotypen weit verbreitet. Auf der südlichen Balkanhalbinsel herrschen Diploide, die meinsten tetraploiden sind mehrmals durch Autopolyploidisierung entstanden. Zwei der beschriebenen Plastidenlinien decken sich mit geographisch voneinander getrennten AFLP-Gruppen, die genetischen Gruppen stimmen allerdings nicht mit der derzeitigen Taxomomie überein. Insgesamt deuten die genetischen Daten auf glaziale Waldrefugien auf der südlichsten Balkanhalbinsel (Griechenland), in Bulgarien, Istrien (Kroatien und Slowenien) und möglicherweise auch in den südöstlichen Karpaten (Rumänien) hin.

Veronica subg. *Pseudolysimachium* sect. *Pseudolysimachion* hat ihr Diversitätszentrum in Südosteuropa, ihre Arten *V. barrelieri, V. orchidea* und *V. spicata* kommen in Graslandschaften vor. Eine frühe auf Kreuzungsversuchen basierende Hypothese, dass Hybridisierung bei der Sippenbildung dieser hochvariablen Sektion eine große Rolle gespielt hat, wird durch unsere genetischen Daten unterstützt. Etliche der auf der Balkanhalbinsel endemischen intraspezifischen Taxa sind tatsächlich solche Hybridformen, die es sowohl auf di- als auch auf tetraploider Ebene gibt.

Alle drei Gruppen unterstreichen die Bedeutung von Hybridisierung und Polyploidisierung in der Evolution von Pflanzenarten in Südosteuropa.

Introduction

One of the major European biodiversity hotspots is southeastern Europe, in particular the Balkan Peninsula (Horvat & al., 1974; Kryštufek & Reed, 2004; Turrill, 1929). The Balkan Peninsula harbors about 6530 plant species of which approximately one-third are endemic (Horvat & al., 1974; Polunin, 1980). Species-level diversity is also very high among animals, for example within the herpetofauna (Džukić & Kalezić, 2004). An important factor causing this diversity is the highly varied topography of the Balkan Peninsula, ranging from sea level to high mountain areas, which leads to climatic diversity and natural fragmentation of habitats (Reed & al., 2004).

Satellite image of southeastern Europe

During the cold stages of the Pleistocene, in which the climate was drier (Frenzel, 1992) and more continental (Horvat & al., 1974) than at present, European forest vegetation retreated to three main refugial areas, the Iberian, Apennine and Balkan Peninsulas (Comes & Kadereit, 1998; Hampe & al., 2003; Hewitt, 2000; Magri & al., 2006; Médail & Diadema, 2009; Petit & al., 2003; Taberlet & al., 1998). In contrast to the Iberian and Apennine Peninsulas, the refugium on

the Balkan Peninsula lacks an east-west oriented barrier to the North – as is the case with the Pyrenees and Alps – and range expansions towards the North were thus easily possible. Pleistocene glaciation was restricted to high mountains of the Balkan Peninsula (Turrill, 1929), e.g., Pindus (Hughes & al., 2007) or Prokletije (Milivojević & al., 2008). The environment was relatively stable during these cold stages (Tzedakis & al., 2002), but each taxon responded independently to fluctuations and communities were not stable over time (Taberlet & al., 1998).

Little is, however, known about the processes behind the patterns of biodiversity. Such processes may include divergence in phases of allopatric isolation as well as subsequent introgressive hybridization or polyploidisation. Introgression may on the one hand blur species boundaries in hybrid zones if isolating factors are lacking (Hardig & al., 2000) (Raudnitschka & al., 2007), which may also be the case if populations differentiated in phases of allopatry, e.g., during restriction to different refugia, and subsequently came into secondary contact (e.g. Petit & al., 1999). On the other hand, hybridization may enhance speciation when it is followed by ecological selection and spatial isolation (Buerkle & al., 2000; Rieseberg & al., 2003). The outcome of hybridization can be strongly modified by polyploidy which is a major force in plant evolution and diversification (Ramsey & Schemske, 2002; Soltis & al., 2009; Wendel, 2000). Polyploidization after hybridization may reduce gene flow via the triploid block (Köhler & al., 2010), but gene flow may still be unidirectionally possible from diploid parents to their polyploid hybrids (Chapman & Abbott, 2010). While hybridization can lead to allopolyploids, which may differ conspicuously from their diploid progenitors in morphology and physiology, autopolyploids arise from the crosses within or between populations of a single species (Ramsey & Schemske, 1998). Although autopolyploids may be more difficult to distinguish on the basis of morphology alone (Levin, 1983, 2002), it has recently been shown that they often co-exist with their diploid parental populations (Husband & Sabara, 2004; Kolář & al., 2009; Kron & al., 2007). The elevated biodiversity observed on the Balkan Peninsula may thus depend on various factors, such as topography and climatic history, and processes, as for example hybridization or polyploidy, which may act in varying degrees at different time levels.

An excellent group for studying patterns of diversification and evolution on the Balkan Peninsula is the genus *Veronica* (Plantaginaceae sensu APG III, 2009). Two thirds of its European species occur on the Balkan Peninsula, several as endemics, exhibiting different habitat requirements – from forest to steppe habitats, growing from sea level to the alpine region (Albach, 2006). To elucidate evolutionary patterns within the genus, three species groups of

different subgenera were chosen occupying different habitats – the alpine region, woodlands and grasslands.

V. saturejoides subsp. *kellereri*

V. vindobonensis

V. barrelieri

Veronica saturejoides, *V. erinoides* and *V. thessalica* constitute a group of closely related alpine taxa which are endemic to the Balkan Peninsula. *V. saturejoides* occupies the largest area and is divided into three geographically separated subspecies, one occurring from Bosnia to Montenegro, the second one is restricted to Mt. Munella in northern Albania, and the third one is restricted to western Bulgaria. *Veronica erinoides* is found in southern Greece, whereas *V. thessalica* is distributed from northern Greece to Macedonia, southern Serbia and northern Albania. All three species grow on dry habitats on calcareous rocks and are of similar habit. *V. saturejoides* and *V. erinoides*, though distinct species, differ only little in morphology (Contandriopoulos & Quézel, 1965, Fischer, 1970a); *V. thessalica*, however, differs remarkably in the structure of the inflorescence (Fischer, 1970a). Their taxonomic relationship has never been tackled with molecular methods and are explored in **Paper 1**.

The *V. chamaedrys* group is a widespread element of woodlands, growing at forest margins and in open forests. In southeastern Europe the group comprises *V. ch.* subspp. *chamaedrys*, *chamaedryoides* and *micans* as well as *V. krumovii, V. orbelica* and *V. vindobonensis* (Albach et al., 2004). *Veronica ch.* subsp. *chamaedryoides, V. krumovii* and *V. orbelica* (taxonomy and species delimitation of the latter two was, however, still not studied in detail) are restricted to that area, whereas the other two taxa are widespread. Apart from morphological differences, these

taxa exhibit also different ploidy levels. While the nominal taxon is mainly tetraploid with some exceptions only (Fischer, 1970b, 1973a; Fischer, 1973b; Mirek & Fischer, 1986), the other taxa were suggested to be exclusively diploid (Fischer, 1970b; Mirek & Fischer, 1986; Peev, 1972; Strid & Franzén, 1984). The association of ploidy level and taxonomy, as well as potential differentiation due to isolation in forest refugia are tested in **Paper 2**.

The grassland species *V. barrelieri, V. orchidea* and *V. spicata* have their centre of diversity in southeastern Europe (Trávniček, 1998). As the delimitation of species is often not straightforward, several species have been recognized (Härle, 1932). In addition, numerous intraspecific taxa have been described (Albach & Fischer, 2003). The high diversity of the group was explained by Härle (1932), who undertook extensive crossing experiments, with ongoing hybridization between the taxa, as morphological boundaries between species could not be drawn. Fischer (1974), in contrast, on the basis of extensive herbarium studies concluded that hybridization was less frequent in nature than observed by Härle. Additionally, the taxa differ cytologically; whereas *V. barrelieri* and *V. orchidea* are mostly diploid (Graze, 1933), *V. spicata* is mostly tetraploid in southeastern Europe; diploids occur only in disjoint mountain ranges (Graze, 1933; Trávniček & al., 2004). The impact of polyploidy and hybridization in this group, which has never been elucidated with molecular methods, is explored in **Paper 3**.

All three species groups have been explored morphologically (e.g. Fischer, 1974) and karyologically (e.g. Graze, 1933; Contandriopoulos & Quézel, 1965; Trávniček & al., 2004) in the past. Traditional cytotaxonomy, however, is often based on a few chromosome counts only and may underestimate the complexity of cytotype distribution patterns (e.g. Kolar & al., 2009). Recent advances in flow cytometry allow to screen a large number of individuals in a relatively short time period. Additionally, molecular methods can be applied to get deeper insights into evolutionary processes. Amplified Fragment Length Polymorphism (AFLP) is a highly resolving PCR-based method which involves restriction of the DNA and ligation of double-stranded adapters, followed by selective PCR amplification of sets of the restriction fragments (Vos & al., 1995) which may then be separated and visualized on an automated sequencer. AFLPs patterns, which are nearly entirely nuclear-derived (Bussell & al., 2005), are rapidly changing through time. Plastid DNA (cpDNA) sequence data, on the other hand, may elucidate older patterns than AFLPs due to their slow mutation rate. In contrast to nuclear DNA, cpDNA is maternally inherited in *Veronica* as in most other Angiosperms (Zhang & al., 2003). Plastid DNA gene sequences have been used to resolve phylogenies at higher taxonomic levels, whereas

sequencing of non-coding regions is applied at lower taxonomic levels as they are assumed to be under fewer functional constraints (Shaw & al., 2007) to consequently exhibit accelerated mutation rates. In contrast, nuclear ribosomal DNA exhibits an elevated rate of sequence evolution; on higher taxonomic levels rRNA genes are employed, whereas at lower taxonomic level internal and external intergenic spacers are analyzed (Small & al., 2004). Previous molecular analyses of the three species groups studied yielded only poor resolution (Albach, 2006).

Altogether, we assess the role of allopatric differentiation, polyploidy and hybridization in affecting biodiversity in southeastern Europe. To this end we apply ploidy level estimation, molecular markers (AFLPs, plastid and nuclear DNA sequences) and morphometry and focus on the following questions: (1) Which evolutionary scenario can be assumed for the three species *V. saturejoides*, *V. thessalica* and *V. erinoides* based on taxonomic relationships (2) Are polyploids within the *V. chamaedrys* group of auto- or allopolyploid origin; did they origin once or multiple times? Does the phylogeographical pattern reflect putative forest refugia? To which extent is the current taxonomy reflected in the molecular patterns and morphometry? (3) What is the role of polyploidy and hybridization between *V. barrelieri*, *V. orchidea* and *V. spicata*? How is the current taxonomy reflected in patterns inferred from molecular data and morphometry?

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Phylogenetics and differentiation of *Veronica* subgenus *Stenocarpon* on the Balkan Peninsula

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ABSTRACT

The Balkan Peninsula is considered the most important refugium for species during the Pleistocene glaciations and today harbours about 2000 endemic species, but we know surprisingly little about the evolution of taxa in this region. *Veronica saturejoides*, *V*. *thessalica* and *V*. *erinoides* are a group of closely related alpine taxa endemic to the Balkan Peninsula. Here, we analyze four DNA regions (the nuclear chalcone synthase intron (CHSi) and ribosomal ITS region and the plastid *rpoB-trnC* spacer and *trnL-trnL-trnF* region) and AFLP fingerprints to provide a phylogenetic hypothesis for the relationships among these taxa. Additionally, we analyze leaf morphological characters used to distinguish the three subspecies of *V*. *saturejoides*. The analyses support the distinction of the three subspecies based on previously intuitively suggested characters. Nuclear chalcone synthase intron data indicate that the southern taxa are genetically much more diverse than the more northern *V*. *saturejoides* subsp. *saturejoides*. Phylogenetic relationships inferred from this region and AFLP fingerprints support the monophyly of *V*. *saturejoides*. In contrast, plastid DNA regions suggest a closer relationship of *V*. *saturejoides* subsp. *saturejoides* to *V*. *thessalica*. The most likely scenario involves introgression of *V*. *saturejoides* subsp. *saturejoides* from *V*. *thessalica*.

ADDITIONAL KEYWORDS: AFLP, Balkan Peninsula, chalcone synthase intron, glacial refugia, ITS, leaf morphology, phylogeography, *rpoB-trnC* spacer, *trnL-trnL-trnF*-region,

INTRODUCTION

The Balkan Peninsula is the hotspot of biodiversity in Europe for animals (Dzukic & Kaleciz, 2004; Krystufek & Reed, 2004) and plants. About 6530 plant species occur there with about one third of them endemic (Horvat, Glavac & Ellenberg, 1974; Polunin, 1980). Possible factors possibly responsible for this species richness are the high topographic and climatic diversity of the Peninsula (Krystufek & Reed, 2004). This is especially true for the montane to alpine regions of the Balkan Peninsula, which comprise about 70% of the area (Reed, Krystufek & Eastwood, 2004). Phylogeographic analyses of montane to alpine species have resolved in much detail possible refugia and intraspecific structure in alpine species of the Alps (Schönswetter *et al*., 2005), but such analyses are scarce for the Balkans (Zhang, Comes & Kadereit, 2001; Frajman & Oxelman, 2007; Stefanovic *et al*., 2008). The use of fossil pollen data (Tzedakis *et al*., 2002; Tzedakis, 2004) and DNA markers (Taberlet *et al*. 1998; Petit *et al*., 2003) have revealed major refugia for forest species in the Balkan Peninsula, but the intraspecific geographic structure for alpine species is unknown. Additional phylogeographical analyses of alpine species, for which fossils are generally lacking, are therefore needed to identify distribution patterns and phylogeographic relationships of regions on the Balkan Peninsula.

One complicating factor in phylogeographical analyses is gene exchange of the study group with related taxa. The Pleistocene has been shown to be a time of active evolution in alpine species of the Alps and secondary contact during the interglacials has been implied as a reason for hybrid speciation, in particular polyploid speciation (Stebbins, 1984; Gauthier, Lumaret & Bedecarrats, 1998; Brochman *et al*., 2004). Therefore, these processes also need to be considered in phylogeographical analyses of species from the Balkan Peninsula.

One genus with a complex evolutionary history in the Balkan Peninsula is *Veronica* (Albach 2006), a species-rich genus growing in various kinds of habitats from grasslands in the lowlands to forests and up to the alpine zone. Alpine species in the Balkan Peninsula belong either to *V*. subgenus *Veronica* or to *V*. subgenus *Stenocarpon*, which are only distantly related monophyletic groups in the genus *Veronica* (Albach *et al*., 2004, 2005). The alpine species of *V*. subg. *Veronica* present on the Balkan Peninsula are widespread across Europe and populations on the Balkan Peninsula possibly represent old refugial populations (Albach, Schönswetter & Tribsch, 2006), whereas the alpine species of *V*. subg. *Stenocarpon* on the Balkan Peninsula include three to four species endemic to the region and restricted to only a few localities (Fig. 1).

The term “locality” is here used for a given area of alpine habitat surrounded by non-alpine habitat at lower elevation, which can in the larger areas harbour several distinct populations. *Veronica erinoides* is found in the mountains of southern Greece. *Veronica thessalica* is scattered across alpine regions of northern Greece, Macedonia, southern Serbia and northern Albania. Finally, *V*. *saturejoides* is divided into three subspecies of which the type subspecies is distributed throughout the Dinaric Alps from northern Bosnia to Montenegro at altitudes above 1200 m. *Veronica saturejoides* subsp. *munellensis* was known only from Mount Munella in northern Albania, but here we report a second locality from northern Albania based on intensive investigation of herbarium specimens. All collections of this taxon are more than hundred years old and we currently do not know whether this is due to the inaccessibility of the localities or the extinction of the subspecies. Finally, *V*. *saturejoides* subsp. *kellereri*, which is often considered a separate species, *V*. *kellereri*, is restricted to the Pirin and Rila mountains in western Bulgaria. All three species are prostrate, highly branched herbs with small leaves and a dense terminal or pseudo-terminal inflorescence. They all occur on dry stony habitats on calcareous rocks. These similarities have led to some confusion about the taxon limits and misidentification in the older literature and in herbaria (e.g. Velenovsky, 1902). The current taxonomic concept is based mostly on the studies by Contrandriopoulos & Quézel (1965) and Fischer (1970). These studies provided good diagnostic characters for distinguishing *V*. *erinoides*, *V*. *thessalica* and *V*. *saturejoides* but only weak characters to distinguish the three subspecies of *V*. *saturejoides*. Therefore, a detailed quantitative analysis of the characters suggested to be diagnostic by Fischer (1970) and a search for other diagnostic characters seemed warranted. Unfortunately, the latter remained unsuccessful (von Sternburg, 2007). Furthermore, molecular characters are needed to give a reliable phylogenetic hypothesis about taxon limits, relationships between them and if possible within the taxa. Previous phylogenetic analyses of DNA sequence data (e.g. Albach, 2006) did not sample all species from the Balkan Peninsula. Nevertheless, Albach (2006) and Albach *et al*. (2005) demonstrated that within *V*. subg. *Stenocarpon* the Balkan endemic species *V*. *thessalica* and *V*. *saturejoides* are not closely related to *V*. *fruticulosa* from the Alps as would be expected from the biogeographical perspective but form an independent lineage together with *V*. *mampodrensis* from the Iberian Peninsula. Besides the deficiencies in taxon sampling, these studies lacked support for most relationships within subgenus *Stenocarpon*. Additional markers are thus required to resolve internal relationships.

Here, we provide a phylogenetic framework for studies on the Balkan endemic species of *V*. subgenus *Stenocarpon*. The subgenus includes 34 species, with seven occurring in Europe, one

in Mexico, two in Turkey, three in the Caucasus and more than half of them in restricted areas of Central Asia. All species of the subgenus are perennial and, as far as they have been investigated, diploid (2*n* = 16; Albach *et al*., 2008) that grow in alpine meadows and montane rocky habitats. Little to nothing is known about their breeding system and dispersal mechanisms, although vegetative reproduction by rerooting of broken-off branches is likely. Including all species of the subgenus is extremely difficult, especially since most of the species with the exception of *V*. *fruticans*, *V*. *fruticulosa*, *V*. *ciliata*, *V*. *macrostemon* and *V*. *densiflora* are rare plants with narrow to a small distributions. A comprehensive sampling of the subgenus is therefore beyond the scope of the present study. We focused on the European species and additionally sampled some additional non-European representatives. For the analysis, we investigated DNA sequence data from both the nuclear (chalcone synthase intron - CHSi, ITS) and plastid (*trnL-trnL-trnF*-region, *rpoB-trnC* spacer) genomes and AFLP fingerprints. The present study is the first to employ sequence data from the nuclear CHSi and the plastid *rpoB-trnC* region in phylogenetic analyses of *Veronica*. The intron of the chalcone synthase, one of the central genes in the flavonoid biosynthetic pathway (Ferrer *et al*., 1999), is the first nuclear low copy region to be employed in phylogenetic analyses of *Veronica*. Nuclear low copy genes are valuable phylogenetic markers, although it may be difficult to find appropriate markers due to the lack of universal primers (Sang, 2002). The data additionally allows preliminary insights into the patterns of genetic diversity in four of the five taxa considered here. Leaf size and shape have been measured for all three subspecies of *V*. *saturejoides* and *V*. *thessalica* to test if the taxa can be determined using the characters suggested by Fischer (1970). Additionally, we provide genome size estimates to evaluate the possibility of polyploidy being involved in the evolution of the group. The aim of this study was to test the boundaries of taxa from this subgenus in the Balkan Peninsula, detect possible patterns of hybridization and provide a phylogenetic framework for further investigations in the phylogeographical patterns of the species.

MATERIALS AND METHODS

PLANT MATERIAL

In the preparation of this study, we checked with our co-workers the occurrence of the taxa in more than 50% of all known localities of the taxa (Fig. 1). Plant material of *V*. *erinoides* (two out of seven known localities), *V*. *saturejoides* subsp. *saturejoides* (five out of 14 known localities) and subsp. *kellereri* (two populations from one of two known localities) and *V*. *thessalica* (three populations from two out of five known localities) was collected in the field and stored in silica gel for DNA analyses.

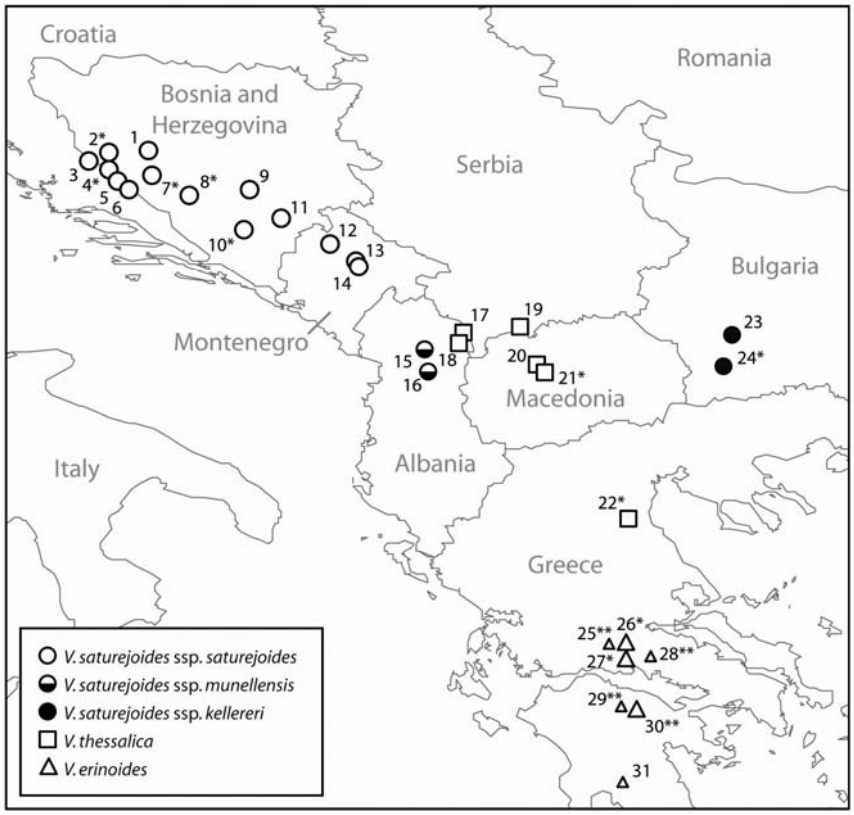

Figure 1: Distribution of *Veronica erinoides*, *V*. *saturejoides* and *V*. *thessalica* based on herbarium specimens investigated and literature. Smaller symbols indicate populations reported in the literature but not seen as herbarium specimens in this study. Numbers refer to localities as indicated in Table 2. Asterisks mark populations sampled in the DNA sequence analysis. Smaller circles indicate populations reported in the literature but not seen from herbarium specimens.

For morphological analyses 3–14 individuals were collected as herbarium specimens. *Veronica saturejoides* subsp. *munellensis* was not found during the field trips, because the localities in northern Albania are fairly inaccessible, and herbarium material was too old for DNA sequencing. Additional species of *V*. subgenus *Stenocarpon* were also included in the analyses. *Veronica prostrata* and *V*. *arvensis* were chosen as outgroups in the sequence analyses, because they belong to related subgenera (Albach, Martínez-Ortega & Chase, 2004; Albach *et al*., 2005) and amplification of CHSi in several other species of related subgenera failed (von Sternburg, 2007). Origin, voucher information and GenBank accession numbers for all sequences of this study are given in Table 1. In addition to the herbarium specimens collected for this study, herbarium specimens from various European herbaria (B, BM, C, E, G, HAL, K, SOM, W, WU, Z, ZT) were studied (Table 2). Together they comprise 137 specimens. Latitude and longitude of old herbarium specimens were determined using Fuzzy Gazetteer (Kohlschütter, 2005) and Google Earth.

DNA SEQUENCING

Total genomic DNA was extracted from silica gel dried leaf material using the NucleoSpin Plant Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's specifications. The chalcone synthase intron (CHSi) was amplified using primers CHS1F and CHS2RN (Strand, Leebens-Mack & Milligan, 1997) for all individuals. All other regions were amplified for one individual per population. ITS sequences were amplified using the primers ITS A (Blattner, 1999) and ITS4 (White *et al*., 1990) and include ITS1, 5.8S rDNA and ITS2. The *trnL-trnL-trnF* region was amplified with primers c and f of Taberlet *et al*. (1991) and includes the *trnL* intron, 3´ *trnL* exon and *trnL-trnF* spacer. The *rpoB-trnC* spacer was amplified using primers *rpoB* and *trnC-R* (Shaw *et al*., 2005). PCR reactions included 1 min at 94°C, 35 cycles of 18 sec at 94°C, 30 sec at 55°C and 1 min at 72°C with a final extension time of 8 min at 72°C with the exception of an annealing temperature of 60°C for the *rpoB-trnC* spacer.

PCR products were separated on 0.8 % TBE-agarose gels and if more than one fragment was present the one corresponding to the expected size was excised and cleaned using the QIAquick™ PCR purification and gel extraction kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocols. Sequencing reactions (10 µl) were carried out using two µl of the BigDye Terminator Cycle Sequencing mix (Applied Biosystems Inc.), 1 µl of the same primers used for the PCR and 1–5 µl of DNA. Both strands were sequenced on a 3730 DNA Analyzer (Applied Biosystems Inc.).

Table 1: Information on vouchers and GenBank accession numbers used in this study. For locality numbers see Fig. 1. Sequences used in the combined analysis are printed in bold.

Species	Country	Locality	No. of Indivi-duals	GenBank accession numbers				Voucher
				CHSi	ITS	*rpoB-trnC*	*trnL-trnL-trnF*	
V. ciliata	China	Qinghai	1	-	-	-	AF486385	Miehe *et al*. 98-33313, GOET
V. densiflora	Russia	Altai	1	-	AY741521	-	-	M. Staudinger s.n. , SALA
V. erinoides	Greece	26	1	-	AY741523	-	-	Hagemann, Scholz & Schmitz 461, SALA
V. erinoides	Greece	26	10	**EU282055**	**EU282103**	**EU282066**	**EU282091**	M. von Sternburg 007, WU
V. erinoides	Greece	27	9	**EU282054**	**EU282102**	**EU282068**	**EU282092**	M. von Sternburg 006, WU
V. fruticans	Austria	Carinthia, Hohe Tauern: Goldberggruppe	5	**EU282056**	**EU282107**	**EU282073**	**EU282083**	P. Schönswetter 7.7.2006, WU
V. fruticans	Spain	Cataluña, Lleida: Aiguestortes.	4	**EU282057** EU282058	**EU282108**	**EU282071**	**EU282084**	P. Schönswetter & B. Frajman 3.8.2006, WU
V. fruticans	Spain	Guadalajara, El Cardoso de la Sierra, Pico del Lobo	1	EU282059	-	-	-	M. M. Martínez-Ortega 1009, SALA
V. fruticans	Great Britain	Scotland	1	-	AY144462	-	-	V. Halcro 30, K
V. fruticulosa	Cultivated	New York Botanical Garden	1	-	AF313005	-	AF486393	L. Struwe 1408, WU
V. fruticulosa	Cultivated	Botanical Garden Bonn	1	**EU282060**	**AF313004**	**EU282076**	**AF486383**	D. C. Albach 71, BONN
V. lanosa	Pakistan	-	1	-	AY540868	-	-	Schickhoff 1377, GOET
V. macrostemon	Russia	Altai	1	**EU282063**	**AY741522**	**EU282082**	**AY486441**	M. Staudinger AL23-18, SALA
V. mampodrensis	Spain	Palencia	1	**EU282062**	**DQ227331**	**EU282077**	**DQ227337**	M. M. Martinez-Ortega et al. 713, SALA
V. monticola	Georgia	-	1	-	DQ227333 DQ227334	-	-	Iranisvili 26071983, WU
V. nummularia	Spain	Cataluña, Gerona: Tosses, Niu d'Aliga	1	**EU282061**	**DQ227335**	**EU282081**	**EU282094**	M. M. Martínez-Ortega & L. Delago 718, SALA
V. saturejoides subsp. *kellereri*	Bulgaria	24 - Mt. Vihren A	9	**EU282040** EU282041	**EU282096**	**EU282075**	**EU282086**	P. Schönswetter & B. Frajman 28.06.2006, WU
V. saturejoides subsp. *kellereri*	Bulgaria	24 - Mt. Vihren B	14	**EU282038** EU282039	**EU282095**	**EU28270**	**EU282085**	M. von Sternburg 29.06.2006, WU
V. saturejoides subsp. *kellereri*	Bulgaria	24	1	-	AY144461	-	AY486450	D. C. Albach 558, WU
V. saturejoides subsp. *saturejoides*	Bosnia-Hercegovina	4	5	**EU282042**	**EU282097**	**EU282072**	**EU282087**	B. Surina 19.07.2006, WU
V. saturejoides subsp. *saturejoides*	Bosnia-Hercegovina	2	3	EU282045	EU282100	Identical to above	Identical to above	B. Surina 18.07.2006, WU
V. saturejoides subsp. *saturejoides*	Bosnia-Hercegovina	7	5	EU282044	EU282099	Identical to above	Identical to above	B. Surina 20.07.2006, WU
V. saturejoides subsp. *saturejoides*	Bosnia-Hercegovina	8	10	EU282046	EU282101	Identical to above	Identical to above	B. Surina 05.07.2006, WU
V. saturejoides subsp. *saturejoides*	Bosnia-Hercegovina	10	5	EU282043	EU282098	Identical to above	Identical to above	B. Surina 22.07.2006, WU
V. saturejoides subsp. *saturejoides*	Bosnia-Hercegovina	12	4	Identical to above	Identical to above	FJ620683	Identical to above	B. Surina 9.7.2008, NHM Rijeka
V. thessalica	Macedonia	21	10	**EU282052** EU282053	**EU282106**	**EU282079**	**EU282088**	P. Schönswetter & B. Frajman 15.08.2006, WU
V. thessalica	Greece	22 A	8	**EU282048** EU282049	**EU282074**	**EU282074**	**EU282090**	M. von Sternburg 27.07.2006, WU
V. thessalica	Greece	22 B	5	**EU282050** EU282051	**EU282105**	**EU282067**	**EU282089**	P. Schönswetter & B. Frajman 17.08.2006, WU
V. thessalica	Greece	22 C	1	-	AF509792	-	AF513343	Raus & Rogl 5072, SALA
V. arvensis	Germany	Stromberg bei Bockenau		**EU282065**	**AF313002**	**EU282078**	**AF486380**	D. C. Albach 147, WU
V. prostrata	Austria	-		**EU282064**	-	**EU282080**	**EU282093**	D. C. Albach 860, MZJG
V. prostrata	Cultivated	Botanical Garden, Bonn		-	**EU282109**	-	-	D. C. Albach 67, BONN

Sequences were assembled and edited using Sequencher™4.7.2 (Gene Codes Corp., Ann Arbor, MI, USA). Assembled sequences were manually aligned prior to analysis. Indels were coded for the parsimony analysis with SeqState v. 1.32 (Müller, 2005) using the modified complex indel coding method (Müller, 2006), which was shown to be the best indel coding method available (Simmons, Müller & Norton, 2007).

SEQUENCE ANALYSIS

Datasets were analyzed separately and combined. Sequences from 16 individuals were sequenced for all four regions with maximum parsimony and maximum likelihood using PAUP v.4.0b10 (Swofford, 2002). Parsimony analyses included heuristic search with random taxon addition (100 replicates) and TBR branch swapping. Parsimony bootstrap support was estimated by analyzing 1000 replicates with the same search conditions but 10 replicates of random taxon addition and a tree limit of 50 per replicate. Maximum likelihood analyses involved models estimated by Modeltest v.3.6 (Posada & Crandall, 1998) based on the AIC and heuristic searches using stepwise taxon addition and TBR branch swapping. Likelihood bootstrap support was estimated by analyzing 700 replicates and the same search conditions as for the maximum likelihood analysis.

For all four datasets, analyses with specific constraints to test specific relationships in individual analyses (*V*. *saturejoides* subsp. *saturejoides* either constrained to be the sister to *V*. *thessalica* or sister to *V*. *saturejoides* subsp. *kellereri*) were analyzed identically to the standard analyses. Resulting topologies were compared with the results from the unconstrained analyses using the Templeton test as implemented in PAUP. Additionally, all most parsimonious trees from the single data sets were tested with the data set from all other single datasets.

AFLP GENERATION

The AFLP procedure followed Vos *et al*. (1995) with modifications. To test for reproducibility six replicates were included. Genomic DNA was digested with the two restriction endonucleases *Eco*RI and *Mse*I and ligated to double-stranded *Eco*RI and *Mse*I adaptors in one step at 37°C for 3 hours. The reaction mix for circa 0.5 µg template DNA contained 1.1µl T4 DNA ligase buffer (Promega, Mannheim, Germany), 1.1 µl 0.5 M NaCl, 0,55 µl BSA (1mg/ml, New England Biolabs, Frankfurt, Germany), 1.0 µl 50 µM *Mse*I-adapters (genXpress, Wiener Neudorf, Austria), 0.02µl *Mse*I (50U/µl, New England Biolabs), 1.0 µl 5 µM *Eco*RI-adapters (genXpress),

0.0625µl *Eco*RI (80U/µl, Promega), 0.2 µl T4 DNA ligase (3U/µl, Promega) and 1.53 µl ddH_2O. Ligated DNA fragments were diluted 10-fold. Preselective and selective amplifications were performed in a thermocycler (GeneAmp PCR System 9700, Applied Biosystems, Darmstadt, Germany) with PCR protocols following Vos *et al*. (1995). The reaction mix for preselective amplification contained 1.14 µl 10× RedTaq PCR reaction buffer (Sigma-Aldrich, Deisenhofen, Germany), 0.2 µl RedTaq PCR reaction mix (1U/µl, Sigma), 0.22 µl 10 mM dNTPs (Applied Biosystems), 0.58 µl 5 µM preselective primers (genXpress), 5,86µl ddH_2O and 2 µl diluted product of the restriction-ligation reaction. The PCR product was diluted 10-fold. The reaction mix for the selective amplification contained 1 µl 10× RedTaq PCR reaction buffer (Sigma), 0.2 µl RedTaq PCR reaction mix (1U/µl, Sigma), 0.22 µl 10 mM dNTPs (Applied Biosystems), 0.54 µl 5µM *Mse*I-primer (genXpress), 0.54 µl 1µM *Mse*I-primer (Applied Biosystems), 5,5 µl ddH_2O and 2 µl diluted product of the preselective amplification. The three primer combinations for the selective PCR were *Eco*RI (6-Fam)-ACA / *Mse*I-CAT; *Eco*RI (VIC)-AAG / *Mse*I-CTG and *Eco*RI (NED)-ACC / *Mse*I-CAA. The selective PCR product was purified using Sephadex G-50 Fine (GE Healthcare Bio-Sciences, Uppsala, Sweden) applied to a Multi Screen-HV plate (Millipore, Molsheim, France) in three steps (200 µl each) and packed at 600g for 1 min the first two times and 5 min at the last step. The same rotation speed was used for the 5 min centrifugation of the samples (5 µl of each selective PCR product). 1.2 µl of the elution product was combined with 9.9 µl HiDi formamide (Applied Biosystems) and 0.1 µl internal size standard GeneScan ROX (Applied Biosystems) and run on an ABI 3130x automated capillary sequencer. Raw AFLP data were collected and aligned with the internal size standard using ABI Prism GeneScan analysis software 3.7 (Applied Biosystems). Peaks (i.e. fragments) were scored manually in Genemarker (SoftGenetics, State College, Pennsylvania) as present (1) or absent (0) in a readable region of bands from 75 to 500 bp in length. Each peak superior to an intensity of signal of 1000 was selected and checked for each sample for the selective amplifications with 6FAM- and VIC-dyes. However, this limit was decreased to 500 for NED-dyes because of the low intensity of this amplification.

AFLP ANALYSIS

A neighbor-joining analysis including 1000 bootstrap replicates using Nei–Li distances (Nei & Li, 1979) was conducted in TREECON (Van de Peer & De Wachter, 1994). Additionally, two runs of 1000 bootstrap replicates were conducted using parsimony in PAUP4.0b10 (Swofford, 2002) using ACCTRAN character state transformation in heuristic searches with simple taxon addition and TBR branch swapping. The runs differed in their weighting schemes with one run weighting

gains 2:1 over losses and one with the reverse weighting. The strategy was shown to detect patterns of hybridization in the AFLP data set by Albach (2007). We used the model-based clustering based on a Bayesian Markov chain Monte Carlo (MCMC) approach as implemented in the program STRUCTURE 2.2 (Pritchard *et al.* 2000) to identify genetically homogeneous groups. An admixture analysis was run with uncorrelated allele frequencies. The appropriate number of groups (*K*) and the most likely assignment of each individual to a certain group were estimated performing 10 runs for each *K* value ranging from 1 to 9 and 1 million MCMC replicates (100 000 additional replicates as burn-in). The structure runs were performed at the Bioportal of the University of Oslo (www.bioportal.uio.no). The optimal number of clusters was determined according to Rosenberg *et al.* (2002) as implemented in the R-Skript in Structure 2.1-SUM (Ehrich *et al.*, 2006).

LEAF CHARACTERS

Leaf characters that have been used to distinguish subspecies of *V*. *saturejoides* were investigated and measured for 137 herbarium specimens of *V*. *saturejoides* and *V*. *thessalica* (Table 2). Measurements were either taken using the „Moticam 1000“ digital camera and the software „Motic Images Plus v2.0“ (Motic China group Co., Ltd) or under the binocular. The following characters were measured: leaf length, leaf width and position of widest point relative to the length (Fig. 8). For all characters the position of the leaf along the stem (upper, middle, lower position) and maturity of the stem (sterile, flowering, fruiting) was noted. Leaf indentation was measured but could not be quantified reliably. Leaf indumentum was scored qualitatively. Altogether 1029 leaves from 256 individuals were measured. Boxplots were generated using SPSS v15.0 (SPSS Inc.; Chicago, USA), which was also used to test for correlations among characters and significant differences in characters between taxa.

GENOME SIZE MEASUREMENTS

Genome sizes of *Veronica saturejoides* subsp. *saturejoides* (two individuals from Cvrasnica Planina; voucher: B. Surina 05.07.2006, WU), *V*. *saturejoides* subsp. *kellereri* (Pirin Mts., voucher: M. von Sternburg 2.7.2006, WU), *V*. *thessalica* (Mt. Olimbos; voucher: M. von Sternburg 27.7.2006, WU), and *V*. *erinoides* (Mt. Giona; voucher: M. von Sternburg 25.7.2006, WU) were estimated using flow cytometry on CyFlow ML (Partec GmbH, Münster, Germany) equipped with a green laser (Cobolt Samba 100mW) using *Pisum sativum* as internal standard

following the protocol of Baranyi & Greilhuber (1996) with propidium iodide staining. For the first, silica gel dried material was used, whereas living material was used for the other three taxa.

Table 2: Herbarium specimens measured in morphological analyses. Numbers at the beginning of the lines refer to locality numbers used in Fig. 1.

V. saturejoides subsp. *saturejoides*

Cultivated material
Edinburgh (Cult.) 04-05.1896; K H2006/01047-7 & E E32542

Bosnia and Hercegovina
1 – Mount Vitorog 44°45' N; 16°27' E: 1600 m; J. Stadlmann & F. Faltis 18.07.1904; WU 2242
2 – Šator Planina 44°9' N; 16°36' E: 1680 - 1840 m; E. Janchen 16.07.1904; WU 2436 / 1872 m; B. Surina 18.07.2006; WU
3 – Dinaric Mountains, Mount Dinara 44°3' N; 16°23'; E: unknown collector; E E32543 / 1680 m; I. Horvat 18.07.1930; WU
4 – Dinaric Mountains, Mount Troglav 43°56' N; 16°35' E: 1500 - 1600 m; E. Janchen & B. Watzel 04.07.1907; WU 2375 / 1900 m; B. Surina 19.07.2006; WU
7 – Dinaric Mountains, Mount Cincar 43°54' N; 17°3' E: 1600 - 2 m; F. Fiala 20.06.1893; B B100217748 / 1400 m; B. Surina 20.07.2006; WU
8 – Vran Planina 43°40' N; 17°29' E: J. Stadlmann, F. Faltis, E. Wibiral 25.07.1907; WU 2754
9 – Bjelašnica Mountains, Mount Hranisava 43°44' N; 18°8' E: 1800 m; G. Beck 06.1888; K H2006/01047-7 & EE32544 & GG86663 & W 3758, 5714, 15915 & B B100217746 & WU 1467, 1788 / K. Maly 18.06.1905; B B100217750 / Curcic 01.06.1898; W 8926 / Sagorski 18.06.1906; W 3607 / 1800 m; G. & M. A. Fischer 29.07.1972; WU
10 – Mount Velika Velež 43°17'36,8" N 18°4'44,3" E: 1640 m, B. Surina 22.07.2006, WU
11 – Lelija Planina 43°25' N; 18°29' E: J. A. Knapp 1869; G:G86656

Croatia
unknown locality: K. Maly; B: B100217751 & G:G86655 & W
3 – Dinaric Mountains, Mount Dinara 44°3' N; 16°23' E: K. Maly; W 11309
5 – Dinaric Mountains, Mount Prologh 43°50' N; 16°41' E: 1200 m; T. Pichler 18.07.1868; BM 68744, G 86660, 86664, 86666, W, WU 2804 / T. Pichler 07.1872; WU 1788
6 - Dinaric Mountains, Mount Kamešnica 43°45' N; 16°48' E: T. Pichler 06.1872; B B100217742, BM 68745, G G86654, G86662, G86665, K H2006/01047-2, W 15968, WU s.n., WU 2804 / 1500 m; T. Pichler 07.1870; G G86661, K H2006/01047-3, W 8682, WU s.n., WU 771 / 1200 - 1600 m; H. Handel-Mazzetti 04.07.1909; WU 2436

Montenegro
unknown locality: W. Dod; BM 68742
12 – Mount Durmitor 43°8' N; 19°2' E: A. Baldacci 08.1890; G G86657, W 5176, WU 1169
13 – Mount Sinjavina 42°57' N; 19°20' E: A. Baldacci 21.08.1891; G 86658, K H2006/01047-5
14 – Mount Gradište, near Kolašin 42°52' N; 19°21' E: J. Rohlena 07.1903; Z 39140

V. saturejoides subsp. *munellensis*
Albania
15 – Mount Munella 41°58' N; 20°6' E: A. Baldacci 30.06.1897; K H2006/01047-7, G 86659
16 – Mount Deja; 41°43' N; 20°9' E: 2100 m; Guiseppi; K H2006/01047-6

V. saturejoides subsp. *kellereri*

Bulgaria

23 – Rila Mountains, area of Ribni jesera 42°7’ N; 23°29’ E: W. Hilbig 06.08.1978; HAL 067565

24 – Pirin Mountains

unknown locality: Kellerer 1909; SOM 67911 / M. Antsev 19.08.1973; SOM 125269 / 1880 m; N. Andreev 15.06.1976; SOM 134175 / R. Taskova 05.07.1996; SOM: 153318

Kazana 41°46'12" N; 23°24'37" E: 2100 m; B. Kuzmanov 21.07.1980; B B100217745, G 86667 / 2380 m; B. Acktarov 07.08.1938; SOM 67913 / 2400 m; B. Acktarov 11.08.1938; SOM: 67915, 67917 / 2400 m; B. Kuzmanov 14.07.1972; SOM 128053 / 2500 m; B. Kuzmanov 10.08.1976; G 86668 / 2350 m; J. Röthlisberger 13.07.2001; Z 39139/ 2100-2900m; B. Frajman & P. Schönswetter 28.6.2006, WU / 2290m; M. von Sternburg 29.6.2006, WU / 2293 m; M. von Sternburg 2.7.2006, WU / 2302 m; M. von Sternburg, 2.7.2006, WU

Mount Bajuvi Dupki: Kellerer 05.07.1924; SOM 67909 / B. Acktarov 09.08.1939; SOM 67916

Mount Koncheto 41°47'34" N; 23°22'50" E: 2500 m;; F. Cernoch 17.07.1967; B 100217743

Mount Pirin 41°40’ N; 23°30’ E: J. K. Urumov 20.07.1915; SOM 67822, 67920

Mount Vihren 41°46’ N; 23°24’ E: Rev. & Mrs. H. P. Thompson 28.07.1933; K H2006/01047-1 / I. Horvat 24.08.1936; WU / G. Beck 20.08.1976; B100217744 / 2300 m; B. Acktarov 12.08.1938; SOM 67919 / 2600 m; B. Kitanov 10.07.1940; SOM 96252 / E. Jaeger 30.07.1961; HAL 074703 / D. Peev 18.07.1964; SOM 124413 / 2500-2700 m; unknown collector 07.1967 G 86669 / 2400 - 2700 m; H. Seitter 1967; ZT 12698 / 2200 m and 2914 m; G. & M. A. Fischer 27.07.1971; WU (six sheets) / N. Andreev 21.07.1973; SOM 134147 / 2200 m; R. Taskova 06.07.1996; SOM 153319 / 1965 m; D. Albach 22.06.2001; WU 4478/025663 / 2387 m, M. von Sternburg 29.6.2006, WU / 2322 m; M. von Sternburg 2.7.2006, WU

Okadenski Rid 41°48'33" N; 23°21'40" E: 2150 m; 24.07.1952; D. Jordanov & B. Kitanov; HAL 09350, SOM 67677, W 1953/4232

Razloski sycholol 41°47'7" N; 23°23'30" E: D. Peev 26.08.1964; SOM 128937 / 2040 m; V. Goranova 23.07.2004; SOM 161693 / 2200 m; S. Tsoneva 23.07.2004; W 2005 / 5929

V. thessalica

Albania

18 – Luma district, Galica Lums (=Djalica-e-Lumes) 42°0'57" N; 20°28'18" E: 2470 m; I. Dörfler 19.06.1918; B 100311205, C 52/2006, BM 67963, G 86670, W 1927/9372, ZT 12699 / 2200-2400 m; H. Zerny 19.06.1918; W 1958 / 25178

Macedonia

19 – Ljuboten, Mount Skardus 42°12'22" N; 21°6'20" E: J. Bornmüller 26.07.1918; B 100217747 / 2450 m; Rev. & Mrs. H. P. Thompson 17.06.1937; K H2006/01047-4

20 – Golsnica planina, Mount Pepeljak 41°48’ N; 21°20’ E: 2250-2300 m; J. Bornmüller 21.06.1918; B 100311204

21 – Mount Jakupica 41°43´16” N; 21°24´50” E: B. Frajman & P. Schönswetter 15.8.2006, WU

Greece

22 – Macedonia region, Prefecture Pieria, Mount Olimbos 40°5'0" N; 22°21'0" E: 2850 m; H. Handel-Mazzetti 15.07.1927; W 1927/19098 / 2850 m; O. Dibowski 07.07.1928; G 86678, W 1928/7018 / 2800 m; Metlesics 03.06.1958; W 1960/17730 / 2800-2850 m; Gneuser 26.07.1971; G 86677 / 2100m; Klaus, Kummert & Mück 07.06.1972; W 1973/01855 / 2700 m; A. Charpin & J. J. Lazare 19.09.1989; G 86680 / 2700m; M. von Sternburg 27.7.2006, WU / B. Frajman & P. Schönswetter 17.8.2006, WU

RESULTS

DNA SEQUENCE ANALYSIS

The chalcone synthase intron was sequenced for eight (out of 33) species of *V*. subgenus *Stenocarpon*. Sequencing was not possible from herbarium derived DNA, thus limiting our sampling within the subgenus. We tried DNA from a number of species outside the subgenus but were only successful twice (*V*. *arvensis* from *V*. subgenus *Chamaedrys* and *V*. *prostrata* from *V*. subgenus *Pentasepalae*). The CHSi-region is 575-637bp long in the ingroup and up to 770bp in the outgroup and includes 16 scorable indels (Table 3). When sequences within one population were identical, only one sequence has been used in the analyses. Four different alleles was found in *V*. *thessalica*. Two individuals out of 13 (15%) from one population in Greece were polymorphic at the position differentiating allele A and B and were, therefore, inferred to be heterozygotes. Sequences from all 28 investigated individuals of *V*. *saturejoides* subsp. *saturejoides* are identical. In *V*. *saturejoides* subsp. *kellereri* two widely divergent alleles were found. Eight out of 23 individuals were heterozygotes (35%). The analysis of the CHSi dataset resulted in 65 most parsimonious trees (Fig. 2, Table 3).The most parsimonious trees showed the two subspecies of *V*. *saturejoides* to be sister taxa (90% parsimony bootstrap support (PBS); 87% likelihood bootstrap support (LBS), with *V*. *erinoides* as sister to the pair (95 PBS, 64 LBS) and *V*. *thessalica* in a more distant position. The most likely tree had essentially the same topology as the most parsimonious trees (Fig. 2).

Table 3: Information on the four DNA datasets. * Note that for the CHSi data set one sequence per population was used in the analysis unless the population was polymorphic. The number, thus, reflects neither the number of haplotypes nor the number of sequences actually generated.

	CHSi	ITS	*Combined nuclear*	*rpoB-trnC*	*trnL-trnL-trnF*	*Combined plastid*	*Combined*
Number of sequences in separate analyses	28*	29	16	17	26	16	16
Average GC content	35.0%	56.6%	-	30.8%	35.0%	-	-
Characters in the alignment with/without outgroup	1148/649	768/763	1916/1412	1296/1262	925/924	2221/2186	4137/3598
Scored indels	16	28	44	32	16	48	92
Parsimony informative characters	49	97	80	44	52	57	137
Number of most parsimonious trees	65	405	2	12	10562	12	6
Tree length of most parsimonious trees	179	215	289	152	134	187	482
Consistency index	0.97	0.81	0.73	0.96	0.87	0.90	0.77
Retention index	0.97	0.87	0.80	0.94	0.88	0.93	0.82
Substitution model in maximum likelihood analysis	TrN+I	GTR+I+Γ	-	TVM+Γ	TVM+I	-	GTR+Γ
Number of most likely trees	1	2	-	1	1	-	
ln L of most likely tree	2340.00	2066.49	-	2398.77	1853.30	-	

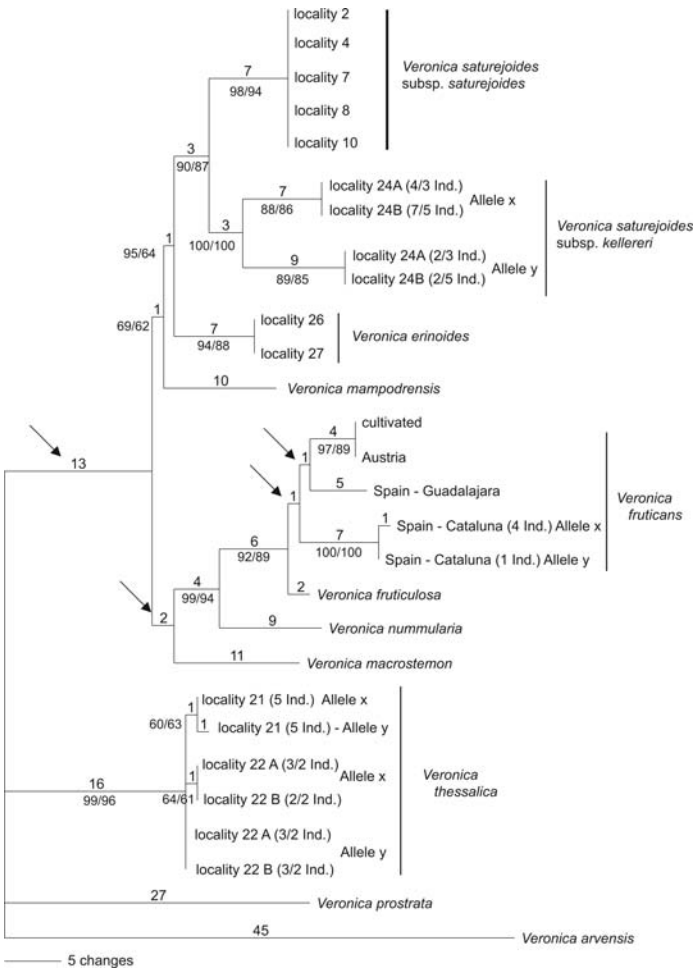

Figure 2: One random tree of the 65 most parsimonious trees of the analysis of the CHSi dataset. Arrows indicate branches not present in all most parsimonious trees and in the most likely tree. Numbers on the branches indicate parsimony branch lengths (above branch), parsimony bootstrap percentage (below branch, before slash) and likelihood bootstrap percentage (below branch, after slash). Only bootstrap values above 50% are indicated. Numbers in brackets after the names indicate number of individuals in which the specific allele has been found (homozygous/heterozygous individuals). See Table 1 for information on the localities.

The ITS analysis resulted in 405 most parsimonious trees (Fig. 3A, Table 3). The strict consensus tree of the most parsimonious trees is to a large extent unresolved, but *V. saturejoides* subsp. *kellereri* is not monophyletic in any of the individual trees. However, none of the most parsimonious trees shows a sister group relationship of *V. saturejoides* subsp. *saturejoides* to *V. thessalica* as in the plastid DNA analyses (see below). The most likely trees (Fig. 3B) do not differ from the most parsimonious trees in well supported relationships. Combining both nuclear datasets (results not shown) resulted in essential the same topology as that of the CHSi analysis. Support for the monophyly of *V. saturejoides* received 61% PBS, support for the clade including *V. macrostemon*, *V. nummularia*, *V. fruticans* and *V. fruticulosa* increased to 77% PBS.

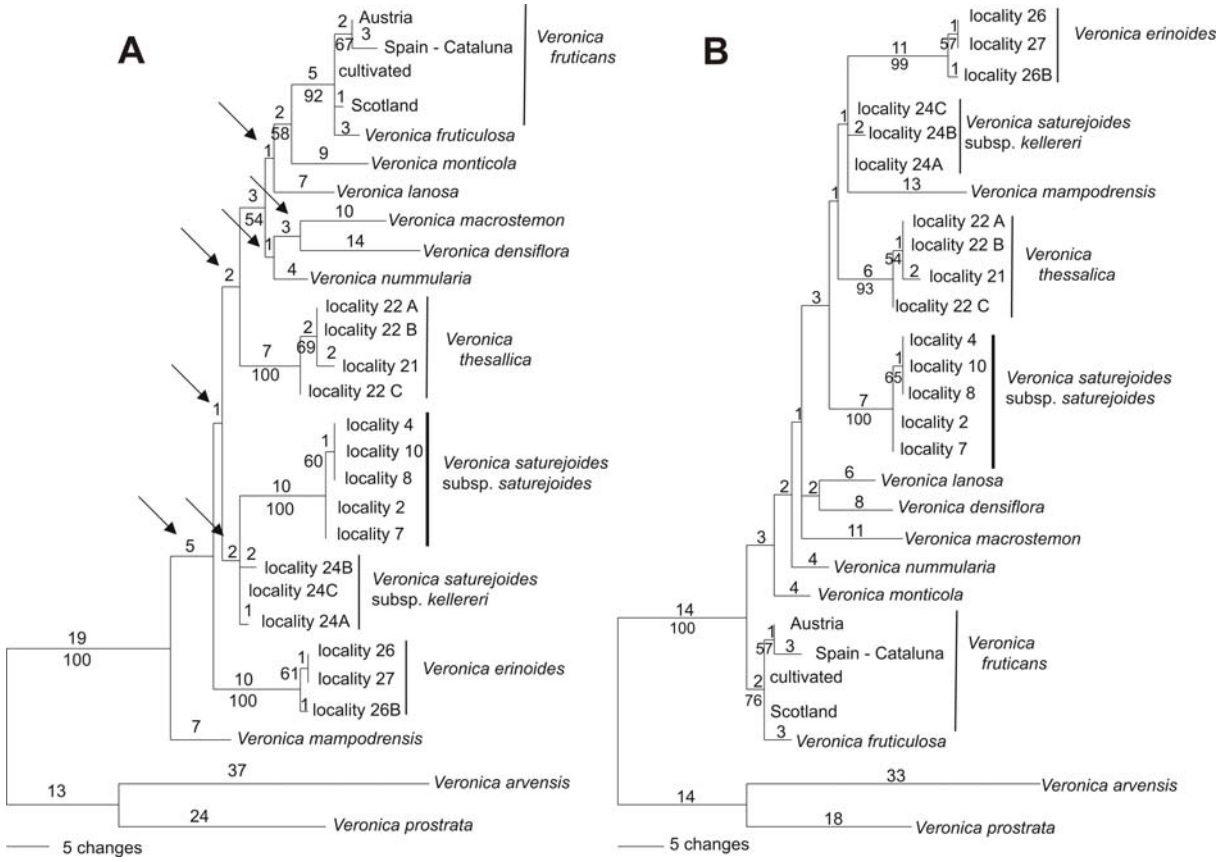

Figure 3: Results from the analysis of the ITS dataset. A) one random tree of the 405 most parsimonious trees. Arrows indicate branches not present in all most parsimonious trees. B) One of the most likely trees. The other tree does not differ in topology but in branch lengths. Numbers on the branches indicate parsimony branch lengths (above branch) and bootstrap percentage (below branch). Only bootstrap values above 50% are indicated. See Table 1 for information on the localities.

In contrast, the most parsimonious and most likely trees resulting from the analyses of the *rpoB-trnC* dataset (Fig. 4, Table 3) and from the analyses of the *trnL-trnL-trnF* dataset (Fig. 5, Table 3) identified a clade of *V*. *thessalica* and *V*. *saturejoides* subsp. *saturejoides* (*rpoB-trnC*: 100 PBS, 98 LBS; *trnL-trnL-trnF*: 82 PBS, 89 LBS) with *V*. *saturejoides* subsp. *kellereri* as sister to the pair (57 PBS) and *V*. *erinoides* as sister to these three (52 PBS) in the *rpoB-trnC* parsimony analysis. None of these relationships was found or contradicted by branches supported in the bootstrap analyses of the *trnL-trnL-trnF* dataset. The difference in the inference of the root between parsimony and likelihood already found with ITS was also found in the *rpoB-trnC* dataset, although here the root found in the parsimony analysis (Fig. 4A) is identical to that in the likelihood analysis of ITS (Fig. 3B) and vice versa.

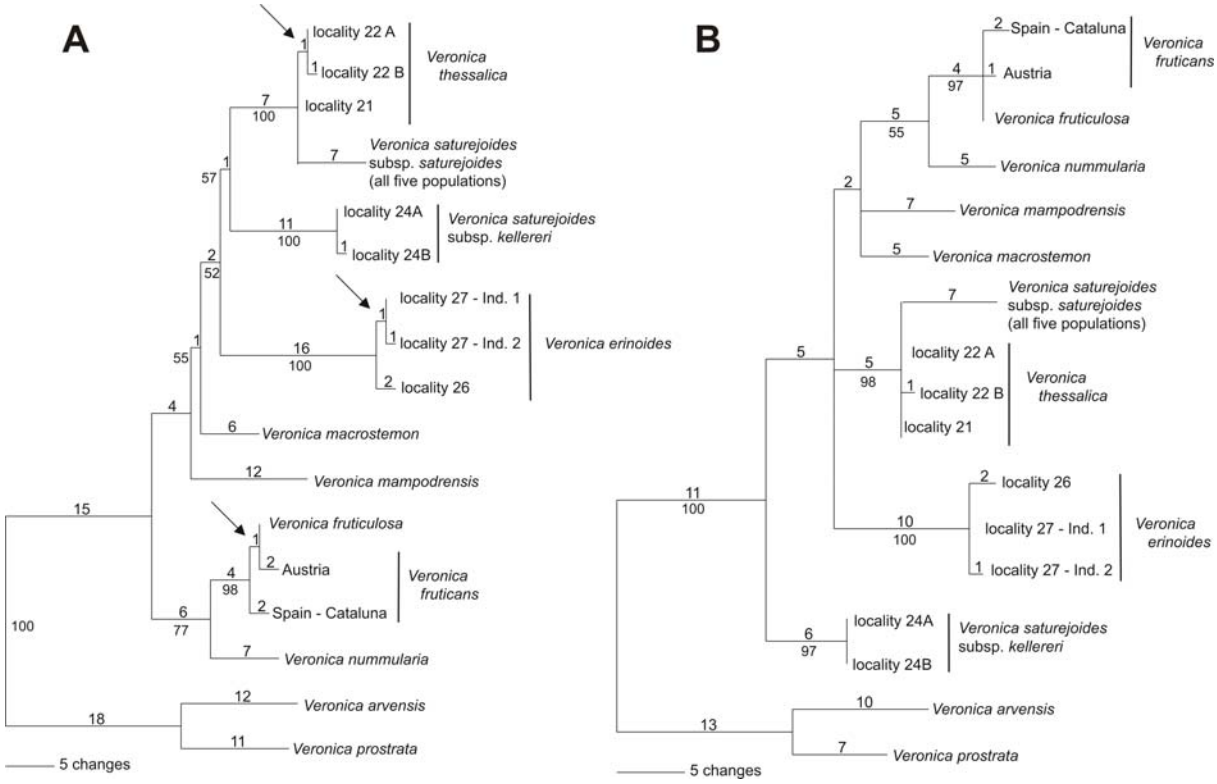

Figure 4: Results from the analysis of the *rpoB-trnC* dataset. A) one random tree of the 12 most parsimonious trees. Arrows indicate branches not present in all most parsimonious trees. B) most likely tree. Numbers on the branches indicate parsimony branch lengths (above branch) and bootstrap percentage (below branch). Only bootstrap values above 50% are indicated. See Table 1 for information on the localities.

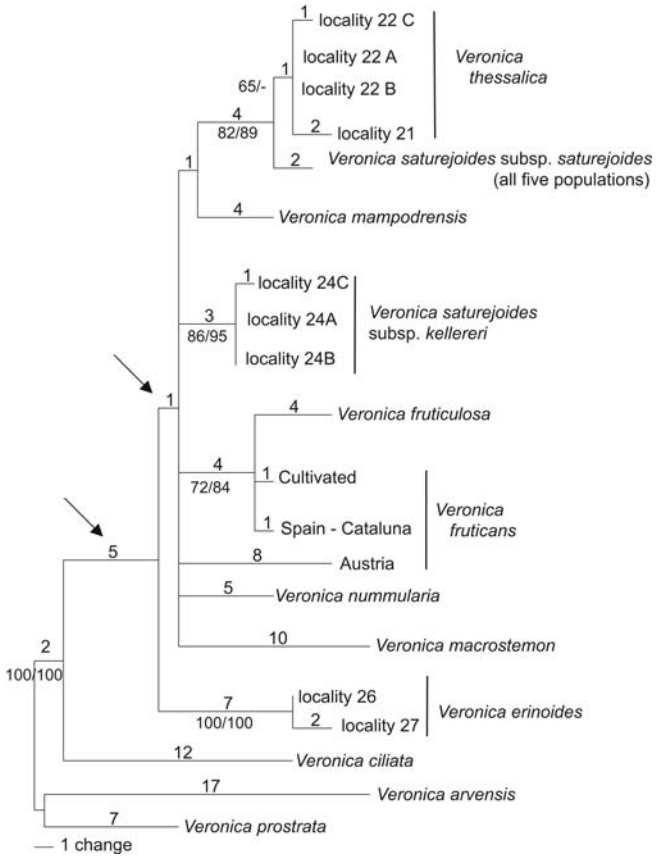

Figure 5: One random tree of the 10562 most parsimonious trees from the *trnL-trnL-trnF* dataset. Arrows indicate branches not present in all most parsimonious trees and in the most likely tree. Numbers on the branches indicate parsimony branch lengths (above branch), parsimony bootstrap percentage (below branch, before slash) and likelihood bootstrap percentage (below branch, after slash). Only bootstrap values above 50% are indicated. See Table 1 for information on the localities.

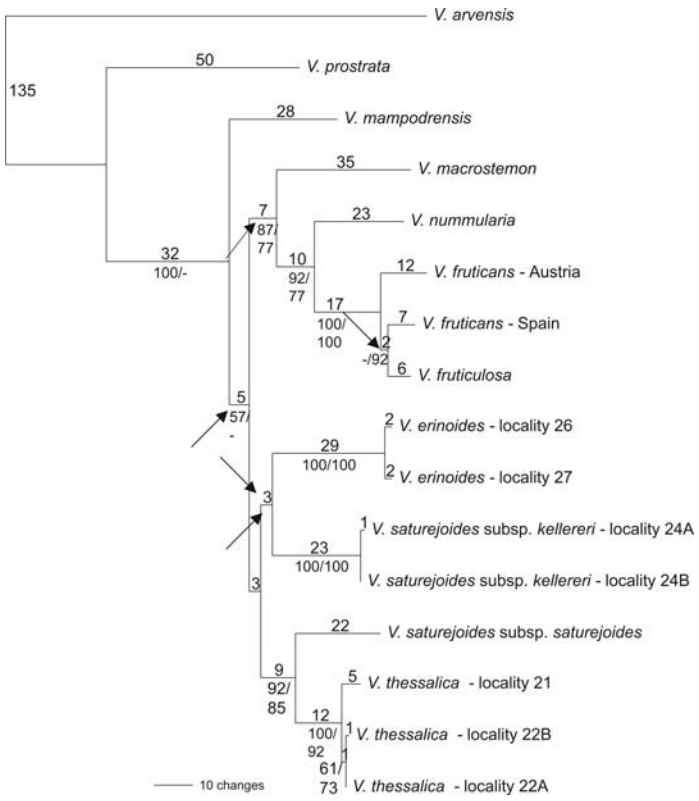

Figure 6: One of the most parsimonious trees from the combined analysis of CHSi, ITS, *rpoB-trnC* and *trnL-trnl-trnF*. Arrows pointing downwards indicate branches not present in all most parsimonious trees. Arrows pointing upwards indicate branches not present in the most likely tree. Numbers on the branches indicate parsimony branch lengths (above branch), parsimony bootstrap percentage (below branch, before slash) and likelihood bootstrap percentage (below branch, after slash). Only bootstrap values above 50% are indicated. See Table 1 for information on the localities.

The Templeton-tests revealed significant incongruence between the CHSi, ITS and plastid DNA topologies (Table 4). Asking more specifically whether the position of *V*. *saturejoides* subsp. *saturejoides* was responsible for this result, the tests revealed that the ITS and *trnL-trnL-trnF* datasets were not able to reject either position (sister to *V*. *saturejoides* subsp. *kellereri* or sister to *V*. *thessalica*), but CHSi and *rpoB-trnC* clearly rejected the alternative non-parsimonious topology.

Table 4: Results of the Templeton tests. sat-the – test for the sister-group relationship of *V*. *saturejoides subsp*. *saturejoides* and *V*. *thessalica*; sat-kel – test for the monophyly of *V*. *saturejoides*; significant tests (at 95%) are marked with an asterisk.

Topology →	CHSi	ITS	*rpoB-trnC*	*trnL-trnL-trnF*	sat-the	sat-kel
CHSi	-	<0.04*	<0.04*	<0.04*	0.02*	-
ITS	<0.001*	-	<0.001*	<0.24	0.52	-
rpoB-trnC	<0.003*	<0.04*	-	<0.48	-	0.01*
trnL-trnL-trnF	<0.04*	<0.03*	<0.31	-	-	0.18

The analysis of the combined dataset (Fig. 6) provided support for the relationship of two or more species in three cases. *Veronica fruticans* and *V*. *fruticulosa* are shown to be closely related and undifferentiable using these four DNA regions. Sister to these two species is *V*. *nummularia* (92 PBS, 77 LBS), as also seen in most separate analyses. Finally, monophyly of the clade consisting of *V*. *thessalica* and *V*. *saturejoides* subsp. *saturejoides* was as strongly supported (92 PBS, 85 LBS) as in the separate analyses.

AFLP ANALYSIS

Overall, 255 polymorphic bands were scored with a maximum of 85 fragments per individual. *Veronica erinoides*, *V*. *thessalica*, and both subspecies of *V*. *saturejoides* each had more than 30 private fragments supporting their distinctness from each other. Both subspecies of *V. saturejoides* combined had 14 private fragments with all other combinations of two of these four taxa having between two and seven private fragments. The neighbor-joining analysis (Fig. 7) further confirmed the four well-supported (100% BS) taxa and also found high support (98% BS) for the monophyly of *V*. *saturejoides* as in the nuclear analysis and moderate support (79% BS) for the sister group relationship of *V*. *saturejoides* and *V*. *erinoides*. The parsimony bootstrap

analyses supported the same relationships, albeit with lower percentages. No significant difference was seen between weighting schemes. In the admixture analysis excluding the outgroup, the optimal grouping at k = 4 corresponded to the four taxa revealed by all other molecular analysis. With one exception the admixture analysis grouped all samples with more than 99% probability in the appropriate cluster. The only exception, one sample of *V. saturejoides* subsp. *kellereri* grouped for 2.7% with *V. thessalica* at k=4. At k = 3 *V. thessalica* and *V. erinoides* grouped together.

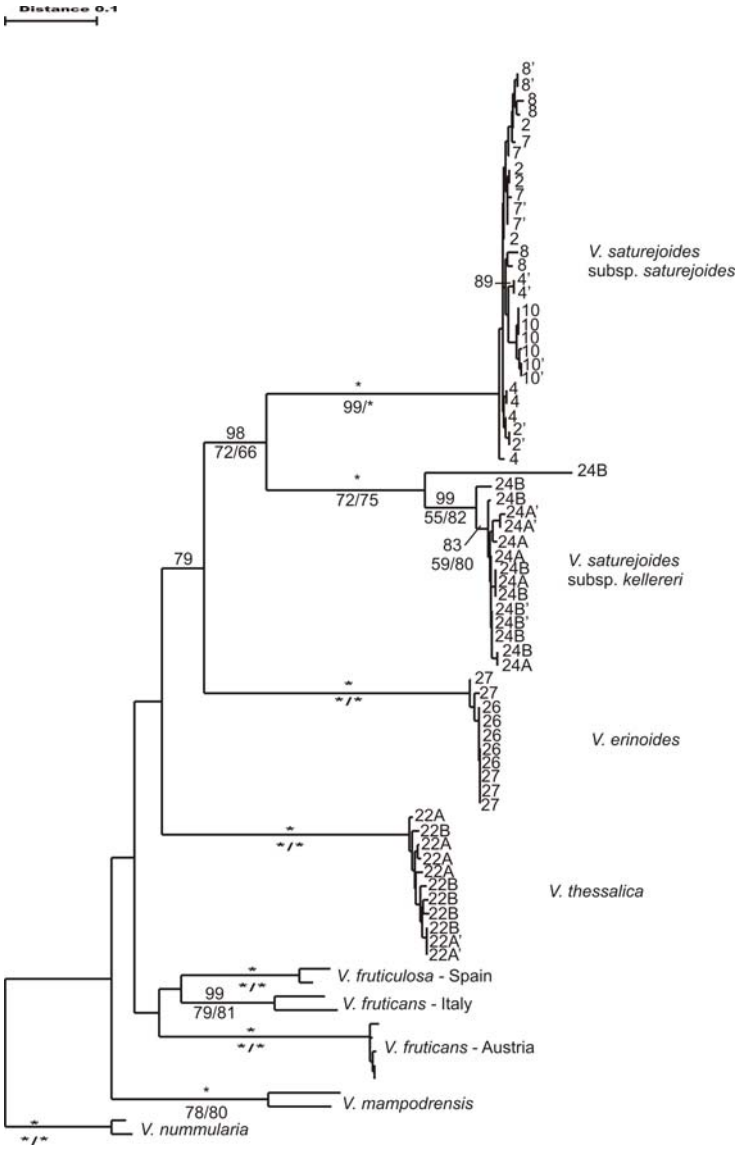

Figure 7: Neighbor-joining (NJ) tree based on the AFLP data set. Numbers at terminals refer to the localities in Fig. 1. Apostrophe after the number indicates AFLP replicates. Numbers on the branches indicate bootstrap support (NJ-values above the branch, parsimony 2:1/1:2-weighted below the branch).

MORPHOLOGICAL ANALYSES

The inspection of 137 herbarium specimens demonstrated differences in several leaf characters between the subspecies of *V*. *saturejoides* and *V*. *thessalica*. Leaf indumentum is distinct between the three subspecies and is the most stable character to differentiate the subspecies. Leaves of *V*. *saturejoides* subsp. *kellereri* are pubescent on the whole adaxial side, whereas those of *V*. *saturejoides* subsp. *saturejoides* is only pubescent on the leaf margin becoming denser towards the base. Leaves of *V*. *saturejoides* subsp. *munellensis* are sparsely but evenly pubescent on the adaxial side and the leaf margin. *Veronica thessalica* has glabrous leaves with some cilia at the base. The abaxial side is glabrous in all subspecies and *V*. *thessalica*. Leaf indentation is strongest in *V*. *saturejoides* subsp. *munellensis* (1-2mm) but variable within the subspecies. All leaf size characters are correlated at $p<0.001$ using Pearson correlation. Despite the overlap indicated in Fig. 8, subspecies differ significantly ($p<0.001$) in leaf size characters according to the Mann-Whitney-test except for the length between subsp. *kellereri* and subsp. *munellensis*. Leaves of *V*. *saturejoides* subsp. *kellereri* are relatively small (5.68mm ±1.29 mm) and elliptic to slightly obovate (widest point at 0.42±0.05 of its length; Fig. 8) in the lower parts of sterile shoots becoming roundish towards the top with the reverse tendency on fertile shoots (von Sternburg, 2007). Leaves of *V*. *saturejoides* subsp. *saturejoides* are longer (8.3 mm ±2.2 mm) and clearly obovate (widest point at 0.38±0.07 of its length; Fig. 8) with the same tendency within shoots and between sterile and fertile shoots (von Sternburg, 2007). Leaf shape in *V*. *saturejoides* subsp. *munellensis* is identical to that of *V*. *saturejoides* subsp. *saturejoides* (widest point at 0.39±0.06 of its length) but smaller (5.53mm ±1.45mm; Fig. 8). The best characters to differentiate the subspecies seems to be the width of the leaves (2.31mm±0.57mm in *V*. *saturejoides* subsp. *munellensis*, 3.17mm±0.82mm in subsp. *saturejoides* and 3.61mm±0.87mm in subsp. *kellereri*), which leads to leaves wider than long in *V*. *saturejoides* subsp. *kellereri* in contrast to the other subspecies. A difference in the shape of leaves of *V*. *saturejoides* subsp. *saturejoides* between populations could not be detected (von Sternburg, 2007). Finally, it should be noted that leaf shape should only be compared in plants from the native habitat, because plants exhibit large phenotypic plasticity under different climatic conditions (von Sternburg & Albach, pers. obs.).

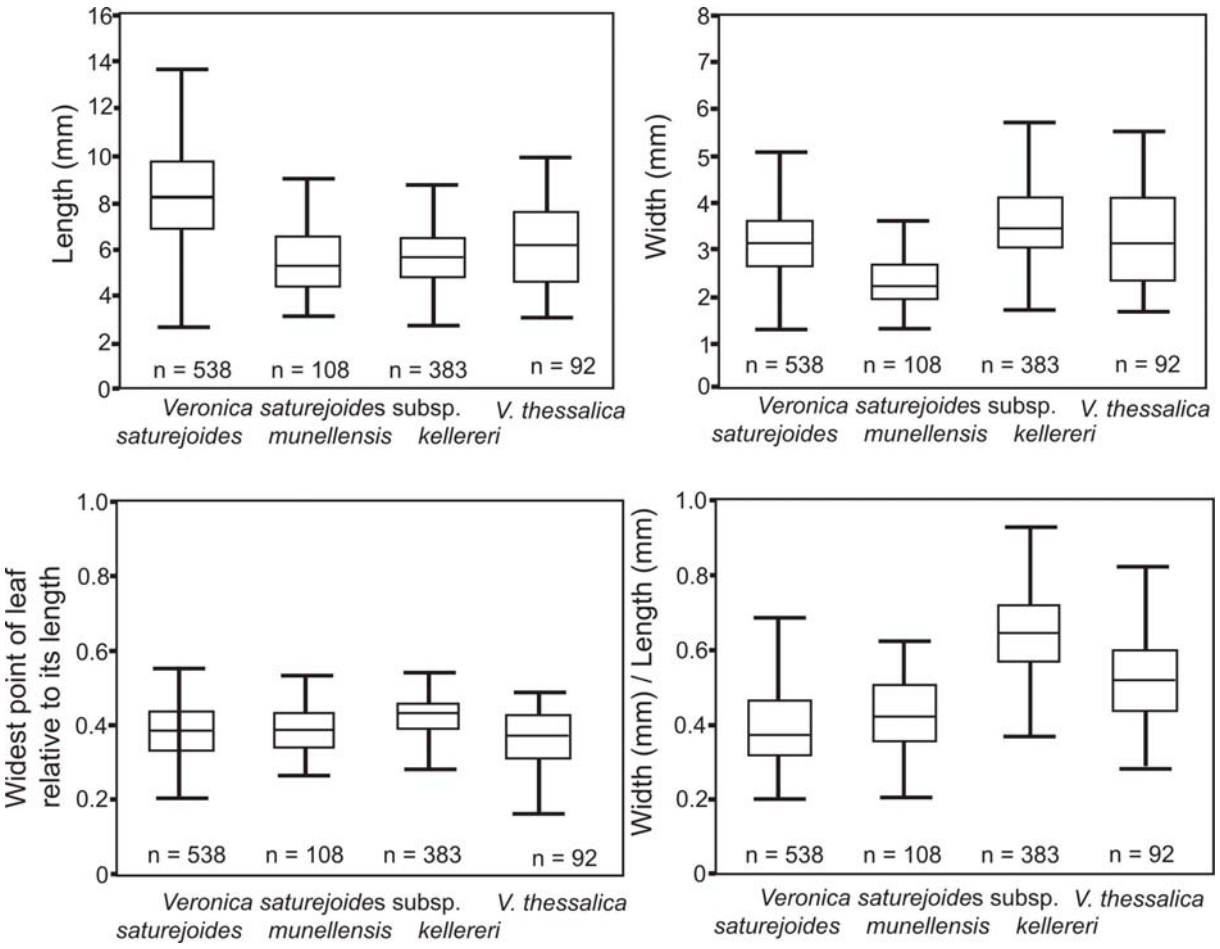

Fig. 8: Boxplot indicating variation in leaf morphological traits among the three subspecies of *Veronica saturejoides*. The box indicates the interquartile (25–75 %) range. The bar within the box indicates the mean value. Whiskers below and above the box indicate the whole range of values.

GENOME SIZE MEASUREMENTS

The estimations of genome sizes revealed all of the investigated species to have the same ploidy level. The two individuals of *Veronica saturejoides* subsp. *saturejoides* have 1C-values of 0,90 pg (CV: 8,2) and 0,84 pg (CV: 5,8), *V. saturejoides* subsp. *kellereri* 0,88 pg (CV: 3,5), *V. thessalica* 0,94 pg (CV: 3,2) and *V. erinoides* 0,75 pg (CV: 2,7).

DISCUSSION

MOLECULAR CHARACTERS

The chalcone synthase is a single copy gene in *Antirrhinum majus* L., many Brassicaceae and *Hippophae rhamnoides* (Wienand, Sommer & Schwarz, 1982; Koch, Haubold & Mitchell-Olds, 2000; Bartish, Kadereit & Comes, 2006) but may be found in a multigene family in other taxa (Durbin, McCaig & Clegg, 2000; Matsumura *et al*., 2005). It also seems to be a single copy gene in *V*. subgenus *Stenocarpon*. Most of our sequences did not show polymorphisms and those that did showed a clear additive pattern of alleles sequenced from other individuals, in which we did not find polymorphisms. Thus, the observed polymorphisms can all be explained by heterozygosity at a single locus. This intron has been proposed as a phylogenetically informative region by Strand *et al*. (1997) but in contrast to the gene (e.g., Koch, Haubold & Mitchell-Olds, 2001) has apparently only been used once in a phylogeographical analysis (Bartish *et al*., 2006). It has about the same number of nucleotides as the ITS region in *Veronica* but has only about half of the parsimony informative characters. Nevertheless, it is a valuable phylogenetic marker as indicated by its high consistency and retention indices (Table 3). Notably the region contains many long indels, especially between different subgenera. *Veronica arvensis* has one insertion of 194 bp relative to all other taxa analysed and *V*. *prostrata* has an insertion of 303 bp relative to most species of *V*. subgenus *Stenocarpon* (277 bp relative to *V*. *arvensis*, 353 bp relative to *V*. *saturejoides*). It will be necessary to have more sequences of CHSi from *Veronica* to detect whether large deletions occurred in the members of *V*. subgenus *Stenocarpon* or whether *V*. *arvensis* and *V*. *prostrata* independently evolved large insertions in the chalcone synthase intron. The variation is not surprising given the large range of intron size between less than 100bp and several kilobases reported so far (Wang *et al*., 2000). The CHSi is the first nuclear low copy DNA region employed in phylogenetic analyses of *Veronica*. It has been chosen based on an extensive survey of available primer for such DNA regions (von Sternburg, 2007). The difficulty to amplify the region in taxa from other subgenera of *Veronica* demonstrates that the available primers are not even universal in *Veronica*.

Three plastid DNA regions have previously been used in phylogenetic analyses of *Veronica*: *rbcL* (Wagstaff *et al*., 2002), *trnL-trnL-trnF* (e.g. Albach *et al*., 2004) and *rps16* intron (Albach & Chase, 2004). Although especially the latter two provided good support for subgenera, they are not variable enough to resolve relationships among closely related species. The *rpoB-trnC*-spacer is among the most informative plastid DNA regions (Shaw *et al*., 2005), although this

may not be universally true (Goodson, Santos-Guerra, & Jansen, 2006). Its length here (up to 1223 base pairs) is at the upper range reported (Shaw *et al*., 2005). In the combined analysis it contributed 34 potentially parsimony informative characters compared to 23 contributed by the *trnL-trnL-trnF* dataset (48% more). Furthermore, it seems to be better at resolving relationships based on the higher CI/RI-values (Table 3).

PHYLOGENETIC RELATIONSHIPS IN *V*. SUBGENUS *STENOCARPON*

Reconstructing the phylogeny of *V*. subgenus *Stenocarpon* has been problematic due to the short internal branches relative to the terminal branches (Albach *et al*., 2005; Albach, 2006). This has prevented the delimitation of clades within the subgenus and detection of the correct root of the phylogeny using nuclear ribosomal DNA and the plastid *trnL-trnL-trnF* region (Albach, 2006). The use of other markers both from the nuclear and plastid genome helped detect incongruences between the markers but did not provide a reliable phylogenetic hypothesis for the subgenus or a solution to the rooting problem. Using herbarium specimens for sequencing the chalcone synthase intron for further species of *V*. subgenus *Stenocarpon* has been unsuccessful and restricted the number of species in our analysis. The *rpoB-trnC* spacer has provided a useful test of the *trnL-trnL-trnF*-plastid phylogeny but did not resolve the phylogenetic question better. Using AFLP fingerprints in a phylogenetic analysis is limited by two factors. First, AFLP fingerprints require well preserved DNA, which excludes all but the freshest herbarium specimens from such an analysis. Second, homoplasy of bands becomes a problem with increasing genetic distance and, therefore, AFLPs should usually not be used across more distant taxonomic groups. The long branches leading to the terminal taxa and short branches connecting them (Fig. 7) suggest that increasing taxon sampling in an AFLP analysis is unlikely to resolve the relationships of the subgenus. The most important conclusion regarding the phylogeny of the subgenus is therefore the detection of incongruence between the nuclear (especially CHSi) and plastid DNA datasets. Incongruence between nuclear and plastid DNA datasets can have different reasons including hybridization, introgression, lineage sorting or paralogy. We can exclude the possibility that we have sequenced paralogous loci based on the fact that CHS seems to be single copy in *V*. subgenus *Stenocarpon* (see above) and that CHSi, ITS and AFLPs show congruent results with respect to the monophyly of *V*. *saturejoides*. This congruence also excludes ancient lineage sorting as a likely explanation for the observed relationships. Another explanation would be a hybrid origin of *V*. *saturejoides* subsp. *saturejoides* from *V*. *thessalica* and *V*. *saturejoides* subsp. *kellereri*. A hybrid origin could either be at the diploid level or involve polyploidy. Chromosome numbers are available for all taxa, but

V. *saturejoides* subsp. *saturejoides* and all species in the subgenus counted so far are diploid (Albach *et al*., 2008). The genome size measurements reported here also indicate that polyploidy is not involved in the evolution of the subgenus in the Balkan Peninsula. Although the sequence data is compatible with a diploid hybrid origin of *V*. *saturejoides* subsp. *saturejoides*, the AFLP results contradict such a conclusion. In a hybridization scenario, we would expect both parents to contribute at least some private markers to the progeny and the admixture analysis to reveal at least some contribution of one genome to the other. However, *V*. *thessalica* does not share more private markers with *V*. *saturejoides* subsp. *saturejoides* than with subsp. *kellereri* and the admixture analysis did not find any support for such a scenario. We therefore prefer introgression in *V*. *saturejoides* subsp. *saturejoides* as discussed in the next paragraph as an explanation for the incongruence.

EVOLUTION IN THE BALKAN PENINSULA

The results are compatible with either of two scenarios. Either *V*. *saturejoides* subsp. *saturejoides* introgressed into *V*. *thessalica* or *V*. *saturejoides* subsp. *saturejoides* was introgressed by the plastid of *V*. *thessalica*. The scenario of *V*. *saturejoides* subsp. *saturejoides* plastid DNA introgressing into *V*. *thessalica* cannot be excluded based on the phylogeny but is unlikely given the monophyly and diversity of plastid DNA sequences in *V*. *thessalica* and the monomorphy of the *V*. *saturejoides* subsp. *saturejoides* populations with respect to the plastid DNA sequences. Such a scenario would imply that the introgressed plastid DNA would have diversified in *V*. *thessalica* but not in *V*. *saturejoides* since the introgression event or the loss of diversity in the latter. A simpler scenario would involve the reverse pathway for the plastid DNA. The clear genetic differences between the two subspecies of *V*. *saturejoides* and lack of a significant number of shared private AFLP bands imply an older event with drastic range contraction before the cpDNA introgression event and many generations of backcrossing afterwards compatible with a northward extension of the distribution area.

Although we did not find a nuclear DNA marker to substantiate an influence of *V*. *thessalica* in *V*. *saturejoides* subsp. *saturejoides*, such an influence could be suggested by morphology and hidden in our analysis by the lack of the southernmost populations of *V*. *saturejoides* subsp. *saturejoides*, northernmost material of *V*. *thessalica* and any material of *V*. *saturejoides* subsp. *munellensis*. After the submission of the manuscript, material from Mt. Durmitor, northern Montenegro became available (Table 1). DNA sequences for three of the four markers were identical to that of more northern populations whereas the fourth, the *rpoB-trnC* spacer, differed

by a single substitution. Thus, also this population does not reveal an influence from *V. thessalica*. The morphological analyses indicate an intermediacy of *V*. *saturejoides* subsp. *munellensis* between the other two subspecies (Fig. 8). Leaf characters of *V*. *thessalica* have not been studied in as much detail, but the range of leaf shapes overlaps with that of *V*. *saturejoides* subspp. *saturejoides* and *munellensis* and the leaf indumentum resembles that of *V*. *saturejoides* subsp. *saturejoides*. An involvement of subsp. *munellensis* in some kind of genetic exchange between the taxa in the southern Dinaric Mountains is possible, but this hypothesis can currently not be tested.

While crucial material from northern Albania, northern Macedonia and southern Serbia is currently lacking, the analyses here still set the scene for a more intensive study of the southern Dinaric populations of taxa involved here. This involves a scenario in which *V*. *saturejoides* subsp. *saturejoides* was restricted to one or two smaller population in the northern part of its range during the Pleistocene or migrated there only recently. The separation of the two more western populations (4 – Mt. Troglav, 10 – Mt. Velika Vez) from the three more eastern populations (Fig. 7) suggests at least two Pleistocene populations, which could have occurred west and east of the Dinaric ridge.

The genetic homogeneity of different populations of *Veronica saturejoides* subsp. *saturejoides* spanning 300 km from northern to northern Montenegro is remarkable, especially considering the diversity found within populations of *V. thessalica* and *V*. *saturejoides* subsp. *kellereri*. The pattern of southern richness in haplotypes and northern purity for the group in the western Balkan Peninsula is also found in several species of *Edraianthus* (Stefanovic *et al*., 2008). Lack of morphological diversity (von Sternburg, 2007) parallels that of the genetic diversity and suggests that southern populations were not separated from the northern populations for a long time.

In the absence of a reliable method of dating divergence times in *Veronica* using molecular clocks due to the high substitution rate heterogeneity (Müller & Albach, unpubl.), we assume that *V*. *saturejoides* subsp. *saturejoides* originated in the south of its range, in the southern Dinaric mountains of southern Serbia, Montenegro and northern Albania, rather than towards the north based on the higher taxonomic diversity of the group in the south and the *rpoB-trnC* sequence from Mt. Durmitor that lacks a synapomorphic substitution present in all other individuals from subsp. *saturejoides*. A Pleistocene restriction to the southern Dinaric mountains has been shown for alpine animals (Sotiropoulos *et al*., 2007) and was inferred as the region of speciation in

Edraianthus (Stefanovic *et al*., 2008). The distribution of the different taxa in *V*. subgenus *Stenocarpon* is furthermore, despite the ecological differences, surprisingly similar to that of the different subgroups of *Fagus* (beech) detected in the southern Balkan Peninsula (Magri *et al*., 2006), which could indicate that montane forests and alpine plants were restricted to similar regions by colder and drier climate in the Pleistocene. Latitudinal and altitudinal migration from these refugia subsequently presented the opportunity for hybridization and potentially formation of new taxa (Frajman & Oxelman, 2007; Stefanovic *et al*., 2008; results presented here) similar to what has been found further north in other parts of Europe (Comes & Kadereit, 1998). Therefore, it seems to be worthwhile to study the alpine flora and fauna of the southern Dinaric Mountains in more detail to reveal the complexity of its evolution.

To conclude, the results presented here allow clear differentiation of four taxa on the Balkan Peninsula with a fifth (*V*. *saturejoides* subsp. *munellensis*) currently unavailable for DNA analysis. While the species rank for *V*. *thessalica* and *V*. *erinoides* is beyond doubt, the data allows both recognition of *V*. *saturejoides* subsp. *kellereri* at the subspecific and specific rank. We have given criteria for the distinction between these two ranks in a previous study of other species of *Veronica* (Martínez-Ortega *et al*., 2004). Most of these criteria (genetic cohesion, absence of or insignificant extant gene flow, long branches in phylograms or phenograms, high number of genetic autapomorphies) allow the recognition at the species rank. Furthermore, the non-monophyly of *V*. *saturejoides* in the plastid DNA-phylogenetic trees (Fig. 4, 5) seems to strongly argue for the species rank. However, we feel that leaf pubescence does not suffice as a clearly diagnosable morphological character (Fig. 8) and the pattern in the plastid DNA sequences can be best explained as interspecific introgression. Therefore, we feel that it is most appropriate to use the subspecific rank for *V*. *saturejoides* subsp. *kellereri*.

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Disentangling phylogeography, polyploid evolution and taxonomy of a woodland herb (*Veronica chamaedrys* group, Plantaginaceae) in southeastern Europe

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Abstract

Southeastern Europe is a centre of European biodiversity, but very little is known about factors causing the observed richness. Here we contribute to fill this gap by reconstructing the spatio-temporal diversification of the cytologically variable and taxonomically intricate complex of *Veronica chamaedrys* (Plantaginaceae), growing in open forests, forest edges and grasslands, with flow-cytometry, molecular markers (AFLPs, plastid DNA sequences) and morphometry. Our results show that both diploid and tetraploid cytotypes are widespread, but diploids predominate on the southern Balkan Peninsula. Plastid sequences suggest a first split into three main lineages in the mid Pleistocene and a continuous diversification during the last 0.4 my. Two of the identified plastid lineages coincide with geographically distinct AFLP clusters. Altogether, the genetic data suggest forest refugia on the southern-most Balkan Peninsula (Greece), in Bulgaria, Istria (Croatia and Slovenia) and maybe the southeastern Carpathians (Romania). Morphometric and genetic data show little congruence with current taxonomy.

Keywords: *Veronica chamaedrys*; Balkan Peninsula; Southeastern Europe; Polyploidy; Genome size; AFLP; Plastid DNA; Morphometrics

1. Introduction

It is long and widely acknowledged that southeastern Europe, and especially the Balkan Peninsula, is a centre of European biodiversity (Turrill, 1929). One early recognized factor is the refugial character of the Balkan Peninsula with a high proportion of relic taxa, even if in many cases the claimed relic status still needs to be confirmed. Much less is, however, known about diversification processes and their spatio-temporal patterns on the Balkan Peninsula especially at lower taxonomic levels, i.e., within and among closely related species. This is partly due to the fact that in molecular studies the Balkan Peninsula is often neglected, such as in large-scale phylogeographical studies, where only a few (e.g. Taberlet, 1994; Santucci et al., 1998; Trewick et al., 2002) or no samples were included (Dumolin-Lapègue et al., 1997; Petit et al., 2002). Detailed studies focussing on the Balkan Peninsula are few and mostly deal with vertebrates (Podnar et al., 2004; Kryštufek et al., 2007; Sotiropoulos et al., 2007; Ursenbacher et al., 2008), butterflies (Schmitt et al., 2006) or mountain plants (Frajman and Oxelman, 2007; Stefanović et al., 2008; Albach et al., 2009).

Southeastern Europe is recognized as a prime refugium for temperate European forest vegetation during the cold stages of the Pleistocene, together with the Iberian and Apennine Peninsulas (Comes and Kadereit, 1998; Taberlet et al., 1998; Gömöry et al., 1999; Hewitt, 2000; Hampe et al., 2003; Petit et al., 2003; Magri et al., 2006; Médail and Diadema, 2009). On the Balkan Peninsula, Pleistocene glaciation was restricted to the high massifs (Hughes et al., 2007; Milivojević et al., 2008), but as the climate was drier and more continental than at present (Horvat et al., 1974), survival of tree species was likely restricted to small areas with favourable conditions – “refugia within refugia” – as has been hypothesised for the Iberian Peninsula (Gómez and Lunt, 2007). These forest refugia were previously assumed to have been restricted to the southern tips of the Southern European peninsulas (Horvat et al., 1974; Hewitt, 2000), but recent studies found evidence for survival of tree species significantly further north than previously assumed (Stewart and Lister, 2001).

In southeastern Europe refugia have been suggested for numerous temperate tree species. Refugia of the beech (*Fagus sylvatica*) were suggested in Istria and adjacent areas on the Dalmatian coast, southern Bulgaria to northwestern Greece and maybe parts of the Carpathian arc (Magri et al., 2006). Possible refugia for the hornbeam (*Carpinus betulus*) were proposed in Romania and northern Greece (Grivet and Petit, 2003). Caucasian and European ash (*Fraxinus angustifolia* and *F. excelsior*) survived in at least two possible refugial areas, a western one in

the Dinaric Alps and an eastern one stretching from the Rhodopes to the Carpathians (Heuertz et al., 2006). The hypothesis of several unconnected forest refugia during the Pleistocene is also supported by the understory vegetation that is much more diverse, regionally differentiated, and richer in endemics than in forests in central and northern Europe (Meusel and Jäger, 1992; Willner et al., 2009). However, the hypothesis of multiple forest refugia in southeastern Europe has never been tested with a herbaceous species in a phylogeographic framework.

A major force in plant evolution and diversification is polyploidy (Ramsey and Schemske, 1998; Wendel, 2000; Ramsey and Schemske, 2002), which may in many cases be the result of secondary contact of populations differentiated in phases of allopatry, e.g., during restriction to different refugia (e.g. Petit et al., 1999). Polyploidy is recognised as an important mode of diversification by, for instance, promoting adaptation to new ecological niches or conferring reproductive isolation, which may eventually lead to speciation (Otto and Whitton, 2000). While allopolyploids may differ conspicuously from their diploid progenitors in morphology and physiology, autopolyploids that arise from the crosses within or between populations of a single species (Ramsey and Schemske, 1998) are often more difficult to distinguish on the basis of morphology alone (Levin, 1983; 2002). Recent cytogeographical studies not only indicate a higher incidence of autopolyploidy than previously thought, but also that autopolyploids often co-exist with their diploid parental populations (Husband and Sabara, 2004; Kron et al., 2007; Kolář et al., 2009). Despite the widely recognized importance of polyploidization in plant diversification and speciation, very little is known about its contribution to the high diversity on the Balkan Peninsula.

A good system to investigate diversification patterns on the Balkan Peninsula in the contexts of putative differentiation due to isolation in refugia and of polyploidy is the *Veronica chamaedrys* group (Plantaginaceae). Although it is widely distributed from western Europe to western Siberia, the Caucasus and Syria (Riek, 1935; Tutin et al., 1972) and has a rather broad ecological amplitude (Dale and Causton, 1992a; b; c; d), it is a characteristic and widespread element of southeastern European forest vegetation, growing at forest margins and open forests dominated by, e.g., oaks, hornbeam or beech, in grasslands, thickets and hedges (Tutin et al., 1972). This perennial herb is outbreeding (Goyder, 1983) with at least central European genotypes being self-incompatible (Albach, unpublished) and has the ability of clonal growth (Boutin and Harper, 1991). The *V. chamaedrys* group is a member of *V.* subg. *Chamaedrys* sect. *Chamaedrys* subsection *Chamaedrys* (Albach et al., 2008) and comprises *V. ch.* subspp. *chamaedrys*,

chamaedryoides and *micans* as well as *V. krumovii, V. micrantha, V. orbelica* and *V. vindobonensis* (Albach et al., 2004), whose phylogenetic relationships have not been resolved so far (Albach, 2006). With the exception of *V. micrantha,* which is endemic to the northwestern and central-western Iberian Peninsula (Benedí et al., 2009), all taxa occur in southeastern Europe and *V. ch.* subsp. *chamaedryoides, V*. *krumovii* and *V. orbelica* are restricted to that area. Apart from morphological differences concerning, among others, indumentum characters, these taxa do also differ karyologically. In particular, *V. ch.* subsp. *chamaedrys* is mainly tetraploid (Fischer, 1970; Fischer, 1973b; Mirek and Fischer, 1986) with only a few diploids recorded from southern Austria (Fischer, 1973a), whereas *V. ch.* subsp. *chamaedryoides* and subsp. *micans*, as well as *V. krumovii*, *V. orbelica* and *V. vindobonensis* were suggested to be exclusively diploid (Fischer, 1970; Peev, 1972; Fischer, 1973b; Fischer, 1974; Strid and Franzén, 1984; Mirek and Fischer, 1986). Since traditional cytotaxonomy, which is often based on a few chromosome counts only, may grossly underestimate the actual intricacy of polyploid complexes in general and of cytotype distribution patterns in particular (e.g. Suda et al., 2004), the association of ploidy level and taxonomy in the *V. chamaedrys* group remains to be tested, in particular if diploid and tetraploid taxa within the same genetic group are spatially segregated.

Here, we explore diversification patterns within the cytologically polymorphic *V. chamaedrys* group in southeastern Europe employing genetic (plastid sequences and AFLP fingerprints), ploidy level and morphometric data. Specifically, we want to assess the distribution of cytotypes in this region to test the hypothesis that polyploids are more frequent at higher latitudes, were range shifts of taxa due to climatic oscillations were more pronounced than in the South. We also want to infer mode and minimum number of polyploidization events to test (i) whether polyploids originated via autopolyploidy, as frequently observed in angiosperms (Otto, 2007), or via allopolyploidy, as possible after secondary contact of once geographically isolated diploid lineages (Petit et al., 1999), and (ii) whether polyploid cytotypes originated once or multiple times. Furthermore, we want to test whether the phylogeographical pattern of the woodland herb *V. chamaedrys* group agrees with those of tree species found in the same vegetation types. Finally, we want to assess whether and to which extent current taxonomy reflects genetically and/or morphometrically defined lineages.

2. Material and Methods

2.1. Plant material

In the summers of 2006 and 2007 the *V. chamaedrys* group was sampled in 121 sample sites (in the following referred to as "populations"). Leaf material was collected and immediately stored in silica gel. Voucher specimens are deposited at the Faculty Centre of Biodiversity, University of Vienna, Austria (herbarium WU; voucher numbers given in Table 1). Plants were determined by Manfred A. Fischer based on Fischer (1970; 1973b; 1974; 1991), Mirek and Fischer (1986) and Peev (1972; 1995) as well as on personal experience. A detailed description of taxa delimitation as used in our study (in particular subsuming *V. orbelica* and *V. ch.* subsp. *chamaedrys* var. *eglandulosa)* is given in Appendix 1, an overview over the characters used for determination in Appendix 2.

2.2. Flow cytometry

DNA ploidy levels were estimated for five individuals per population. Flow cytometry was conducted with silica gel dried material following the protocol of Baranyi and Greilhuber (1996) with propidium iodide staining using a CyFlow ML (Partec GmbH, Münster, Germany) equipped with a green laser (Cobolt Samba 532 nm, Cobolt AB, Solna, Sweden) and using *Pisum sativum* cultivar 'Kleine Rheinländerin' as internal standard.

2.3. Molecular methods

Total genomic DNA was extracted from silica gel dried tissue (c. 10 mg) of one individual per population. In 8 populations with mixed cytotypes, one individual per cytotype was analysed and will in the following be referred to with a separate population identifier: this concerns populations 38/39, 42/43, 45/46, 52/53, 78/79, 86/87, 95/96 and 108/109 (Fig. 1, Table 1). Extraction followed the CTAB-protocol of Doyle and Doyle (1987) with a few modifications: after precipitation with isopropanol and subsequent centrifugation, the DNA pellet was washed in 70% ethanol, dried at 37°C and re-suspended in TE-buffer. The quality of the extracted DNA was checked on 1% TAE-agarose gels.

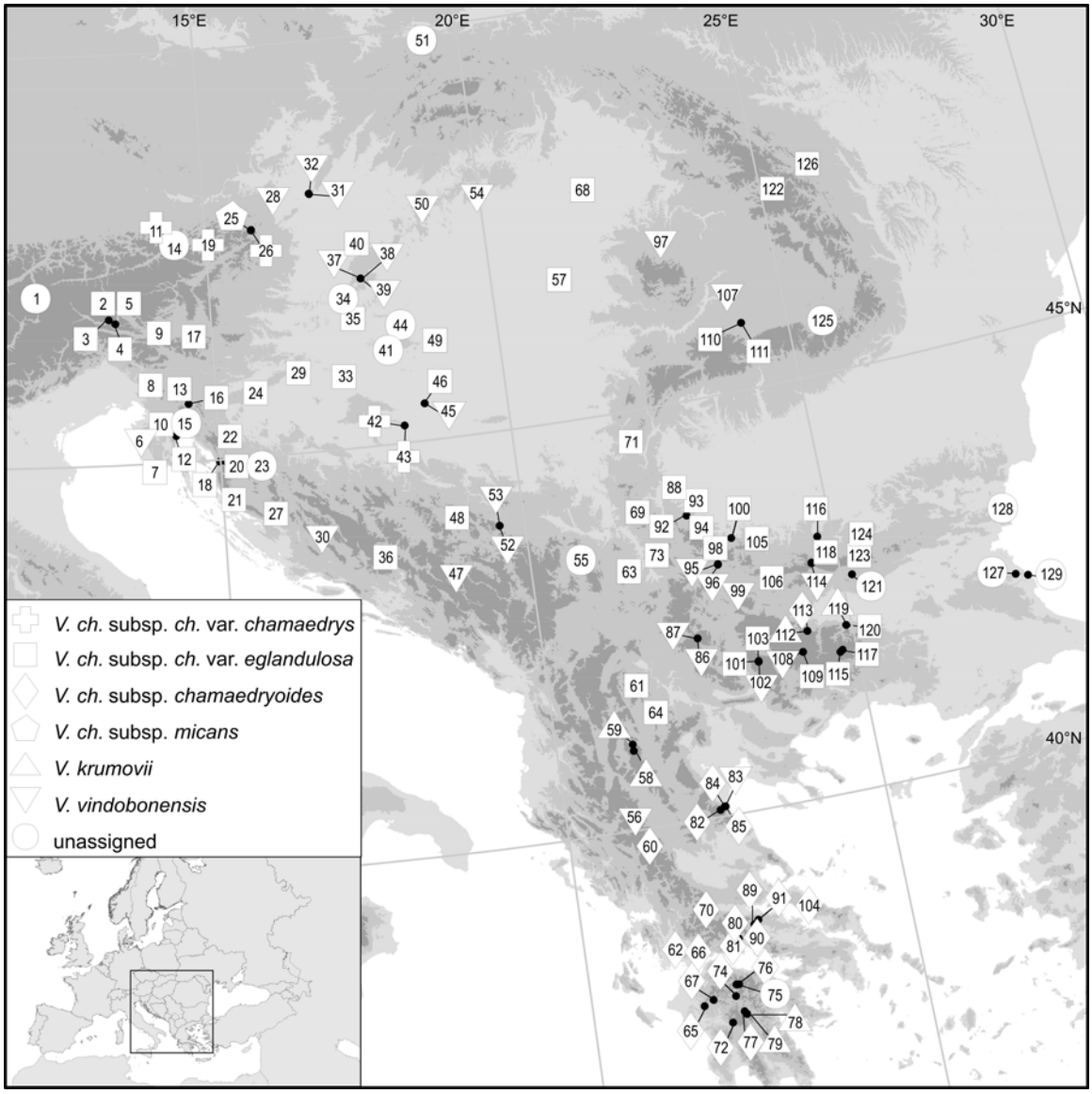

Fig. 1. Sampled populations of taxa of the *Veronica chamaedrys* group in southeastern Europe. Details of the collected populations are given in Table1.

Table 1. Population number, taxon, collection information, coordinates, ploidy, cpDNA haplotype and GenBank accession numbers of 129 sampled populations of the *Veronica chamaedrys* group from southeastern Europe. Populations that could not be assigned to a taxon are indicated with "–". cha, *V. chamaedrys* subsp. *chamaedrys* var. *chamaedrys* ; coi, *V. chamaedrys* subsp. *chamaedryoides*; egl, *V. chamaedrys* subsp. *chamaedrys* var. *eglandulosa*; kru, *V. krumovii*; mic, *V. chamaedrys subsp. micans;* kru, *V. krumovii;* vin, *V. vindobonensis.* In the column "GenBank accession no." the first number refers to the *trnH-psbA* spacer sequence, the second to the *rps16-trnK* spacer and the third to the *rpl32-trnL* spacer.

Pop. no.	Taxon	Locality (voucher number)*	Altitude (m a.s.l.)	Coordinates (E, N)	Ploidy	Haplo-type	GenBank accession no.
1	-	A: Mayerhofen (Bardy_ch1)	1650	11°54´56´´, 47°03´58´´	4*x*	h27	
2	egl	A: Obervellach (Bardy_ch2)	850	13°09´34´´, 46°56´18´´	4*x*	h33	
3	egl	A: Greifenburg (Bardy_ch3)	1525	13°11´53´´, 46°46´10´´	4*x*	h27	
4	egl	A: Lake Weißensee (Bardy_ch4)	1000	13°18´25´´, 46°43´08´´	4*x*	h28	
5	egl	A: Mt. Maltaberg (Bardy_ch5)	1250	13°30´52´´, 46°57´54´´	4*x*	h33	
6	vin	HR: Poreč (Bardy_ch6)	140	13°39´39´´, 45°15´31´´	2*x*	h23	
7	egl	HR: Pula (Bardy_ch7)	60	13°51´50´´, 44°53´51´´	2*x*	h23	
8	egl	SLO: Predmeja (Frajman & Schönwetter 11129)	970	13°52´31´´, 45°57´12´´	4*x*	h52	
9	egl	A: St. Egyden (Bardy_ch9)	640	14°04´10´´, 46°35´01´´	4*x*	h52	
10	egl	HR: Lanišće (Bardy_ch10)	470	14°05´49´´, 45°24´48´´	2*x*	h26	
11	cha	A: Sengsengebirge (Bardy_ch11)	1400	14°12´14´´, 47°48´27´´	4*x*	h27	
12	egl	HR: Opatija (Bardy_ch12)	270	14°16´05´´, 45°19´21´´	4*x*	h23	
13	egl	SLO: Pekel pri Borovnici (Frajman & Schönwetter 11113)	345	14°22´18´´, 45°53´26´´	4*x*	h53	
14	-	A: Rosenau am Hengstpaß (no voucher)	800	14°23´00´´, 47°42´00´´	4*x*	h27	
15	-	HR: Trstenik mire (no voucher)	965	14°27´29´´, 45°29´19´´	4*x*	h56	
16	egl	SLO: Vrhnika pri Ložu (Frajman 12022)	590	14°30´42´´, 45°41´69´´	4*x*	h23	
17	egl	A: Bad Eisenkappel (Bardy_ch17)	1600	14°40´44´´, 46°31´00´´	4*x*	h35	
18	egl	HR: Vratnik pass (Bardy_ch18)	750	14°59´9´´, 44°58´43´´	4*x*	h24	
19	cha	A: Mt. Hochschwab (Bardy_ch19)	1510	15°04´37´´, 47°35´19´´	4*x*	h33	
20	egl	HR: Žuta Lokva (Bardy_ch20)	440	15°06´41´´, 44°57´08´´	4*x*	h25	
21	egl	HR: Baške Oštarije (Frajman & Schönwetter 12063)	1150	15°10´40´´, 44°30´20´´	4*x*	h52	
22	egl	HR: Ogulin (Bardy_ch22)	330	15°11´12´´, 45°16´21´´	4*x*	h24	

Pop. no.	Taxon	Locality (voucher number)*	Altitude (m a.s.l.)	Coordinates (E, N)	Ploidy	Haplo-type	GenBank accession no.
23	-	HR: Lake Plitvička jezera (no voucher)	700	15°36′43′′, 44°52′29′′	4*x*	h24	
24	egl	HR: Zagreb (Bardy_ch24)	230	15°41′09′′, 45°46′42′′	4*x*	h55	
25	mic	A: Mt. Schneeberg (Bardy_ch25)	1600	15°49′58′′, 47°45′14′′	2*x*	h30	
26	cha	A: Mt. Schneeberg (Bardy_ch26)	1700	15°50′19′′, 47°45′27′′	4*x*	h24	
27	egl	HR: Gračac (Bardy_ch27)	560	15°52′08′′, 44°17′48′′	4*x*	h24	
28	vin	A: Mödling (Bardy_ch28)	300	16°16′40′′, 48°04′49′′	2*x*	h38	
29	egl	HR: Križevci (Bardy_ch29)	130	16°27′30′′, 45°58′38′′	4*x*	h23	
30	vin	BiH: Mt. Troglav (Bardy_ch30)		16°36′06′′, 43°56′07′′	2*x*	h39	
31	vin	A: Mt. Hundsheimer Berg (Frajman & Schönwetter 11096)	340	16°56′14′′, 48°08′21′′	2*x*	h55	
32	vin	A: Mt. Hundsheimer Berg (Englisch_ch32)	450	16°56′20′′, 48°07′57′′	2*x*	h43	
33	egl	HR: Bilo Gora (Bardy_ch33)	210	17°14′19′′, 45°52′36′′	4*x*	h23	
34	-	H: Nemesvita (Bardy_ch34)	190	17°21′56′′, 46°49′25′′	4*x*	h33	
35	egl	H: Balatonboglar (Bardy_ch35)	140	17°30′02′′, 46°34′16′′	4*x*	h34	
36	egl	BiH: Mt. Čvrsnica (Surina & Modrić_ch36)	1530	17°37′36′′, 43°39′06′′	4*x*	h54	
37	vin	H: Ajka (Bardy_ch37)	420	17°42′30′′, 47°02′45′′	2*x*	h43	
38	vin	H: Ajka (Bardy_ch38)	420	17°42′30′′, 47°02′45′′	2*x*	h39	
39	vin	H: Ajka (Bardy_ch39)	420	17°42′30′′, 47°02′45′′	4*x*	h55	
40	egl	H: Sokorópátka (Bardy_ch40)	230	17°42′33′′, 47°28′27′′	4*x*	h55	
41	-	H: Pécs (Bardy_ch41)	200	18°03′33′′, 46°08′32′′	4*x*	h56	
42	cha	HR: Slavonski Brod (Bardy_ch42)	170	18°11′35′′, 45°12′40′′	4*x*	h31	
43	cha	HR: Slavonski Brod (Bardy_ch43)	170	18°11′35′′, 45°12′40′′	2*x*	h55	
44	-	H: Dombóvár (Bardy_ch44)	220	18°19′27′′, 46°25′52′′	4*x*	h33	
45	vin	HR: Vladislavci (Bardy_ch45)	90	18°34′48′′, 45°26′58′′	2*x*	h37	
46	egl	HR: Vladislavci (Bardy_ch46)	90	18°34′48′′, 45°26′58′′	4*x*	h33	
47	vin	BiH: Maglić (Bardy_ch47)	1700	18°45′10′′, 43°17′52′′	2*x*	h29	
48	egl	SRB: Podromanija (Bardy_ch48)	1070	18°53′19′′, 44°01′27′′	4*x*	h6	
49	egl	H: Baja (Bardy_ch49)	140	18°53′20′′, 46°11′41′′	4*x*	h23	
50	vin	H: Esztergom (Bardy_ch50)	110	18°57′18′′, 47°47′32′′	2*x*	h47	
51	-	PL: Andrychow (no voucher)	348	19°20′15′′, 49°50′45′′	4*x*	h33	
52	vin	SRB: Kremna (Bardy_ch52)	780	19°34′40′′, 43°51′43′′	2*x*	h52	
53	vin	SRB: Kremna (Bardy_ch53)	780	19°34′40′′, 43°51′43′′	4*x*	h11	
54	vin	H: Gyöngyö (Bardy_ch54)	390	19°57′44′′, 47°50′19′′	2*x*	h50	
55	-	SRB: Mt. Kapaonik (Bardy_ch55)	1770	20°50′08′′, 43°18′55′′	4*x*	h5	

Pop. no.	Taxon	Locality (voucher number)*	Altitude (m a.s.l.)	Coordinates (E, N)	Ploidy	Haplo-type	GenBank accession no.
56	vin	GR: Smolikas (Frajman & Schönwetter 11638)	1400	21°03′48′′, 40°06′09′′	2*x*	h66	
57	egl	H: Békéscsaba (Bardy_ch57)	130	21°12′04′′, 46°43′6′′	4*x*	h32	
58	kru	MK: Nižepole - Orlove bari (Frajman & Schönwetter 11634)	1800-2150	21°12′35′′, 40°57′01′′	2*x*	h60	
59	kru	MK: Kopanke (Frajman & Schönwetter 11636)	1700-1890	21°12′45′′, 41°01′43′′	2*x*	h59	
60	coi	GR: Metsovo (Calvo & al. JC0881)	1618	21°13′39′′, 39°47′32′′	2*x*	h67	
61	egl	MK: Begovo pole (Frajman & Schönwetter 11698)	1800-2200	21°24′50′′, 41°43′16′′	4*x*	h1	
62	coi	GR: Kato Kerasovo (Bardy_ch62)	230	21°26′07′′, 38°31′06′′	2*x*	h76	
63	egl	SRB: Bojnik (Bardy_ch63)	480	21°35′36′′, 43°05′25′′	2*x*	h70	
64	egl	MK: Pletvar (Frajman & Schönwetter 11682)	1000	21°39′18′′, 41°21′58′′	2*x*	h64	
65	coi	GR: Hani Ponopoulou (Bardy_ch65)	620	21°39′47′′, 37°48′19′′	2*x*	h77	
66	coi	GR: Vlachomandra (Bardy_ch66)	200-230	21°42′06′′, 38°27′05′′	2*x*	h74	
67	coi	GR: Lampia (Bardy_ch67)	860-880	21°48′45′′, 37°51′47′′	2*x*	h78	
68	egl	H: Debrecen (Bardy_ch68)	130	21°52′02′′, 47°45′02′′	4*x*	h51	
69	egl	SRB: Mt. Rtanj (Frajman & Schönwetter 11374)	1000-1560	21°53′30′′, 43°46′30′′	4*x*	h9	
70	coi	GR: Aghios Georgios (Bardy_ch70)	630-640	21°55′24′′, 38°56′10′′	2*x*	h68	
71	egl	SRB: Dobra (Bardy_ch71)	140	21°58′36′′, 44°37′50′′	4*x*	h45	
72	coi	GR: Moní Philosóphou (Bardy_ch72)	680	22°02′35′′, 37°33′30′′	2*x*	h72	
73	egl	SRB: Mt. Suva planina (Bardy_ch73)	940	22°05′24′′, 43°13′45′′	4*x*	h12	
74	coi	GR: Klitoria (Bardy_ch74)	490	22°09′04′′, 37°51′47′′	2*x*	h79	
75	-	GR: Kalavrita (Aedo & al. CA14139bis*)	1980	22°11′22′′, 37°59′50′′	4*x*	h65	
76	coi	GR: River Styx (Bardy_ch76)	1250	22°13′42′′, 37°59′41′′	2*x*	h76	
77	coi	GR: Ostrakina (Bardy_ch77)	1500	22°14′29′′, 37°40′09′′	4*x*	h62	
78	kru	GR: Ostrakina (Bardy_ch78)	1350-1420	22°16′11′′, 37°38′00′′	4*x*	h62	
79	kru	GR: Ostrakina (Bardy_ch79)	1350-1420	22°16′11′′, 37°38′00′′	2*x*	h61	
80	coi	GR: Poulani (Bardy_ch80)	1110-1130	22°19′36′′, 38°43′17′′	2*x*	h76	
81	coi	GR: Amfissa (Bardy_ch81)	870-890	22°20′39′′, 38°31′54′′	2*x*	h76	

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82	coi	GR: Olimbos (Frajman & Schönwetter 11675)	1200-1500	22°24′08′′, 40°04′53′′	2*x*	h63	
83	vin	GR: Litochoro (Frajman & Schönwetter 11660)	580	22°28′44′′, 40°06′54′′	2*x*	h63	
84	coi	GR: Litochoro (Frajman & Schönwetter 11659)	580	22°28′44′′, 40°06′54′′	2*x*	h67	
85	coi	GR: Litochoro (Frajman & Schönwetter 11658)	370	22°29′28′′, 40°06′49′′	2*x*	h67	
86	vin	MK: Mt. Rujen (Frajman & Schönwetter 11708)	1800-2252	22°30′55′′, 42°09′29′′	2*x*	h13	
87	vin	MK: Mt. Rujen (Frajman & Schönwetter 11708)	1800-2252	22°30′55′′, 42°09′29′′	4*x*	h1	
88	egl	BG: Vidin vis Gradec (Bardy_ch88)	50	22°33′57′′, 43°59′51′′	4*x*	h4	
89	coi	GR: Amfiklia (Bardy_ch89)	280	22°36′41′′, 38°40′07′′	2*x*	h73	
90	coi	GR: Mt. Parnassos (Bardy_ch90)	1400	22°37′11′′, 38°30′18′′	2*x*	h73	
91	coi	GR: Regini (Bardy_ch91)	370	22°41′07′′, 38°42′43′′	2*x*	h73	
92	egl	BG: Belogradčik (Bardy_ch92)	450	22°41′11′′, 43°38′09′′	4*x*	h2	
93	egl	BG: Vidin (Bardy_ch93)	100	22°52′60′′, 43°47′35′′	4*x*	h1	
94	egl	BG: Montana (Bardy_ch94)	325	22°53′40′′, 43°27′33′′	4*x*	h57	
95	vin	BG: Godec (Bardy_ch95)	650	23°03′09′′, 42°59′32′′	2*x*	h36	
96	vin	BG: Godec (Bardy_ch96)	650	23°03′09′′, 42°59′32′′	4*x*	h15	
97	vin	RO: Fildu de Jos (Bardy_ch97)	350	23°04′55′′, 46°55′31′′	2*x*	h44	
98	egl	BG: Petrohan pass (Bardy_ch98)	1320	23°07′20′′, 43°07′15′′	4*x*	h20	
99	vin	BG: Mt. Vitosha (Bardy_ch99)	1650	23°17′00′′, 42°34′00′′	2*x*	h21	
100	egl	BG: Vraca (Bardy_ch100)	250	23°20′52′′, 43°16′14′′	4*x*	h3	
101	egl	BG: Mt. Vihren (Bardy_ch101)	2049	23°24′50′′, 41°45′27′′	4*x*	h58	
102	vin	BG: Bansko (Frajman & Schönwetter 11326)	1900	23°24′59′′, 41°45′36′′	2*x*	h13	
103	egl	BG: Mt. Vihren (Sternburg_ch103)	1900-1930	23°25′00′′, 41°45′00′′	4*x*	h14	
104	coi	GR: Manoudi (Bardy_ch104)	110	23°28′37′′, 38°46′12′′	2*x*	h75	
105	egl	BG: Vraca (Bardy_ch105)	600	23°45′39′′, 43°12′31′′	2*x*	h8	
106	egl	BG: Saranci (Frajman & Schönwetter 11275)	760	23°52′56′′, 42°42′47′′	4*x*	h17	
107	vin	RO: Valea Lunga (Bardy_ch107)	400	24°03′08′′, 46°08′31′′	2*x*	h49	
108	vin	BG: Batak (Bardy_ch108)	1450	24°08′44′′, 41°45′50′′	2*x*	h22	
109	egl	BG: Batak (Bardy_ch109)	1450	24°08′44′′, 41°45′50′′	4*x*	h1	
110	egl	RO: Sibiu (Bardy_ch110)	550	24°12′24′′, 45°48′36′′	4*x*	h48	

Pop. no.	Taxon	Locality (voucher number)*	Altitude (m a.s.l.)	Coordinates (E, N)	Ploidy	Haplo-type	GenBank accession no.
111	egl	RO: Sibiu (Bardy_ch111)	550	24°12´24´´, 45°48´36´´	4*x*	h48	
112	kru	BG: Peštera (Bardy_ch112)	800	24°16´47´´, 42°00´09´´	2*x*	h41	
113	coi	BG: Peštera (Bardy_ch113)	700	24°16´47´´, 42°00´09´´	2*x*	h69	
114	vin	BG: Trojanski pass (Bardy_ch114)	1550	24°33´57´´, 42°47´28´´	2*x*	h39	
115	egl	BG: Trojanski pass (Bardy_ch115)	1350	24°43´60´´, 41°40´29´´	4*x*	h1	
116	egl	BG: Loveč (Bardy_ch116)	200	24°44´38´´, 43°05´09´´	4*x*	h18	
117	egl	BG: Sokelovtsi (Bardy_ch117)	1570	24°46´25´´, 41°41´45´´	4*x*	h1	
118	egl	BG: Trojan (Bardy_ch118)	500	24°47´01´´, 42°54´34´´	4*x*	h17	
119	kru	BG: Manastir Sveta Petka (Bardy_ch119)	525	24°54´49´´, 41°58´42´´	2*x*	h42	
120	egl	BG: Manastir Sveta Petka (Bardy_ch120)	525	24°54´49´´, 41°58´42´´	4*x*	h1	
121	-	BG: Kazanlăk (no voucher)	450	25°10´33´´, 42°33´39´´	4*x*	h19	
122	egl	RO: Vatra Dornei (Bardy_ch122)	875	25°13´41´´, 47°19´52´´	4*x*	h51	
123	egl	BG: Šipka pass (Bardy_ch123)	1250	25°19´24´´, 42°44´52´´	4*x*	h16	
124	egl	BG: Veliko Tărnova (Bardy_ch124)	350	25°27´52´´, 43°00´25´´	4*x*	h7	
125	-	RO: Braşov (Bardy_ch125)	900-950	25°35´34´´, 45°38´02´´	2*x*	h46	
126	egl	RO: Gura Humorului (Bardy_ch126)	500	25°56´05´´, 47°32´06´´	4*x*	h40	
127	-	BG: Tsarevo (Albach_45)	247	27°45´58´´, 42°07´04´´	4*x*	h10	
128	-	BG: Goritsa (Albach_21)	70	27°49´13´´, 42°55´01´´	4*x*	h10	
129	-	BG: Ahtopol (Albach_52)	50	27°57´15´´, 42°04´15´´	4*x*	h71	

* A, Austria; BG, Bulgaria; BiH, Bosnia and Herzegovina; GR, Greece, H, Hungary; HR, Croatia; MK, FYR of Macedonia; RO, Romania; SLO, Slovenia; SRB, Serbia

The AFLP procedure followed Vos et al. (1995) with the modifications described in Schönswetter et al. (2009). Initially, selective primers were screened using 23 primer combinations. The five final primer combinations for the selective PCR (fluorescent dye in brackets) were *Eco*RI (6-FAM)-ACT/ *Mse*I-CTA, *Eco*RI (VIC)-ACC/ *Mse*I-CAA, *Eco*RI (NED)-ACC/ *Mse*I-CTT, *Eco*RI (6-FAM)-ACT/ *Mse*I-CAT, and *Eco*RI (VIC)-AGG/ *Mse*I-CTC. 5 µl each of 6-FAM, NED and VIC labelled selective PCR products were combined and purified using Sephadex G-50 Superfine (GE Healthcare Bio-Sciences, Uppsala, Sweden) applied to a Multi Screen-HV plate (Millipore, Molsheim, France). 1.2 µl of the elution product were mixed with 10 µl formamide (Applied Biosystems, Foster City, CA, USA) and 0.1 µl GeneScan 500 ROX (Applied Biosystems) and

run on an ABI 3130x automated capillary sequencer (Applied Biosystems). Twenty individuals were replicated to calculate the error rate and to allow non-reproducible fragments to be excluded from the analysis. Raw AFLP data were aligned with the internal size standard using ABI Prism GENESCAN 3.7.1 (Applied Biosystems), and imported into GENOGRAPHER 1.6.0 (available at http://hordeum.oscs.montana.edu/genographer) for scoring. The error rate was calculated as the ratio of mismatches (scoring of 0 vs. 1) over matches (1 vs. 1) in AFLP profiles of replicated individuals (Bonin et al., 2004).

Three regions of the plastid genome, the *trnH-psbA* spacer (primers *trnH*-F, *psbA*-R; Tate and Simpson, 2003), the *rps16-trnK* spacer (primers *rps16*x2F2, *trnK*(UUU)x1; Shaw et al., 2007) and the *rpl32-trnL* spacer (primers *rpl32*-F, trnL (UAG); Shaw et al., 2007), were sequenced from one individual per population. PCR conditions for all three regions were 5 min at 95°C followed by 30 cycles of 1 min at 95°C, 1 min at 50°C and 3 min at 65°C, followed by 7 min at 65°C. Reaction volumes of 24 µl included 8 µl REDTaq ReadyMix PCR Reaction Mix (Sigma-Aldrich, Vienna, Austria), 2 µl of each primer (10 µM), 8 µl of H_2O, and 4 µl of 1:10 diluted template DNA of unknown concentration. The PCR products were cleaned with Exonuclease I and Calf Intestine Alkaline Phosphatase (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. All reactions were carried out on a GeneAmp 9700 thermocycler (Applied Biosystems). BigDye Terminator chemistry (Applied Biosystems) was used according to the manufacturer's instructions for cycle sequencing following electrophoresis on a 3130xl Genetic Analyzer capillary sequencer (Applied Biosystems). Sequences were edited with SEQMAN II 5.05 (DNAStar, Madison, WI, USA) and manually aligned using BIOEDIT 7.0.4.1 (Hall, 1999). All sequences were deposited in GenBank (Table 1; accession numbers not yet available). Sequences from the three plastid regions were concatenated based on the assumption that the plastid forms a single linkage group.

2.4. Data analyses

Genetic structure of AFLP data was inferred using the population mixture analysis implemented in BAPS 5.2 (Bayesian Analysis of Populations Structure; (Corander et al., 2003); http://www.abo.fi./fak/mnf/mate/jc/software/baps.html). This program, which can handle dominant markers like AFLPs under the module "clustering with linked loci" (Corander and Tang, 2007), treats both the frequencies of the markers and the number of genetically divergent groups as random variables. Stochastic optimization is used to infer the mode of the posterior distribution. As our data set included diploid as well as tetraploids individuals, the following

strategy was adopted. A mixture analysis of the diploid individuals only was conducted with the maximal number of groups (K) set to 2 to 10. Each run was replicated 10 times and the results were averaged according to the resultant likelihood scores. Results of the mixture analysis of diploid individuals (excluding two individuals which could not be unambiguously assigned to a single gene pool, see Results) were used to define “training” clusters for subsequent assignment of tetraploid individuals (“admixture based on pre-defined populations”). Admixture coefficients were estimated using 500 iterations, and the significance of these coefficients was estimated by employing the simulation strategy described by Corander and Marttinen (2006) using 50 reference individuals and 10 iterations each. An Unweighted Pair Group Method with Arithmetic mean (UPGMA) tree was inferred based on Kullback-Leibler distances (Kullback and Leibler, 1951) among clusters as implemented in BAPS 5.2. A Neighbour-joining (NJ) analysis based on a matrix of Nei-Li distances (Nei and Li, 1979) including 2000 pseudo-replicates was conducted with TREECON 1.3b (Van de Peer and De Wachter, 1997). Using the program SPLITSTREE4 version 4.6 (Hudson and Bryant, 2006), a NeighbourNet (NNet) was constructed based on the same distance matrix. A principal Co-ordinate analysis (PCoA) based on a matrix of Jaccard distances among individuals was conducted using NTSYS-PC 2.0 (Rohlf, 1998).

Phylogenetic analysis was conducted using the approach implemented in BEAST 1.4.8 (Drummond and Rambaut, 2007), as this allows taking into account the genealogical uncertainty due to the stochastic nature of the coalescence process. As inversions introduce substitutional changes which actually are due to a structural mutation, these have been inverted prior to all analyses. The best-fit substitution model was determined using the Akaike Information Criterion (AIC) as implemented in MODELTEST 3.6 (Posada and Crandall, 1998). As the set of models until the cumulative Akaike weight exceeded 0.95 included often non-nested models with at least 2 substitution rates, we finally used a GTR+Γ model subsuming the proportion of invariable sites in the gamma distribution and using Jeffrey´s priors for the substitution model parameters.

Since a strict clock model was rejected (Bayes factors < -13; calculated with Tracer 1.4 available from http://tree.bio.ed.ac.uk/software/tracer/), rate evolution was modeled in a relaxed clock framework using a lognormal distribution (Drummond et al., 2006) with uniform distributions for mean and standard deviation of 0–100 and 0–10, respectively. Due to the lack of external calibrations, we used a strong prior on the substitution rate, derived from previously published substitution rates for plastid regions (Yamane et al., 2003; Smith et al., 2008), and modeled it with a normal distribution with a mean of 4×10^{-3} substitutions per site per million years and a wide standard deviation of half the mean. After initial analyses, the root was constrained to a maximum age of 10 million years. We used the Bayesian skyline plot (Drummond et al., 2005)

as the most general demographic model, as it also allows fluctuations in population size to be detected. Using different group intervals (m = 3, 5, 10) gave very similar results (absolute Bayes factors < 2.4), and only those with m = 3 are shown. Stationarity of the Markov chain, which was run for 3×10^7 generations with sampling every 1000^{th} generation, was determined using TRACER 1.4. The first 10% of sampled generations was discarded as burn-in, after which all effective sample size (ESS) values were greater than 290. A second run was conducted to confirm convergence of the Markov chain on the stationary distribution. All parameter estimates were based on these two runs combined (54,000 sampling points). A chronogram was constructed from the majority rule consensus tree calculated using PAUP 4.0b10 (Swofford, 2001) with node heights being the median values of the age estimates determined with FIGTREE 1.2.3. (available from http://tree.bio.ed.ac.uk/).

A statistical parsimony haplotype network was constructed using TCS 1.21 (Clement et al., 2000). For this analysis, insertions/deletions longer than one base pair as well as inversions were re-coded as single step mutations, and then sequence gaps were treated as a fifth character state. Mononucleotide repeats of varying length were excluded, since these are prone to homoplasy at larger geographic scales (Ingvarsson et al., 2003).

In order to compare the within-group differentiation of the lineages identified by BEAST, we calculated π, the mean number of pairwise differences (Tajima, 1983) and its variance (Tajima, 1993) with Arlequin 3.11 (Excoffier et al., 2005). Population expansions of the four cpDNA lineages were tested using mismatch distribution with a unimodal distribution indicating population expansion (Rogers and Harpending, 1992). Agreement between the observed and the expected distribution under a sudden-expansion model was tested in ARLEQUIN 3.11 (Excoffier et al., 2005) via the sum of squared differences, which, if significant at $p\leq0.05$, indicates deviation from the expansion model (Schneider and Excoffier, 1999). Significance was assessed via a non-parametric bootstrapping procedure with 10,000 replicates.

2.5. Morphometry

In 98 of the 129 sampled populations one or two flowering or fruiting individuals, whose phenological stage allowed scoring of more than half of the morphometric characters, were available for morphometric analysis. Five sampled locations (38/39, 42/43, 52/53, 78/79, 86/87) included two cytotypes that could not be distinguished in the morphometric analysis and were subsequently treated as polymorphic in ploidy level. One qualitative and nineteen quantitative characters (including one ratio), including those deemed diagnostic for intraspecific or specific taxa in the *V. chamaedrys* group, were scored. Unless stated differently, length characters were measured in mm, and densities in number per mm^2; leaf characters were scored on the leaf pair

subtending the lowermost inflorescences while the stem indumentum was determined from the internodium below the lowermost inflorescences. Qualitative characters were scored once, whereas quantitative characters other than leaf dimensions were measured five times and averaged. Of quantitative characters that were highly correlated ($p < 0.001$) only the one with the fewest missing characters was retained for the final analysis, thus resulting in twelve quantitative characters (Table 2; data matrix in Appendix 3).

Table 2. Morphological characters or ratios of characters employed in a morphometric analysis of 98 populations of the *Veronica chamaedrys* group from southeastern Europe.

Abbreviation	Morphological character or ratio
KG	Calyx hairs glandular (1) or not (0)
KHD	Density of hairs on the calyx lobes
KHL	Length of hairs on the calyx lobes
LLW	Length / maximum width of lamina
LP	Lenght of the petiole
LS	Length of the style
LT	Number of teeth on one half of the lamina
LTB	Vertical distance from first tooth to the base of the lamina (negative if lamina base is rounded to cuneate, positive if base is cordate)
LTLW	Length / width of lamina tooth at maximum width line
LUBD	Density of hairs on the upper side of the lamina next to the apex of the lamina
LUBL	Length of hairs on the upper side of the lamina next to the apex of the lamina
SRD	Density of hairs between the two opposite lines of hairs on the stem
SRL	Length of hairs on the two opposite lines of hairs on the stem

The seven excluded characters were: (1) the length of the stem which was correlated with the number of teeth on the lamina (LT); (2) the length of the petiole one leaf pair above and (3) one leaf pair below the leaf pair subtending the lowermost inflorescence, both correlated with the length of the petiole subtending the lowermost inflorescence (LP); (4) the length and (5) density of hairs on the upper side of the lamina next to the base of the lamina and (6) the length and (7) density of hairs on the lower side of the lamina next to the apex of the lamina, which were correlated with the length (LUBL) and density (LUBD) of hairs on the upper side of the lamina next to the apex of the lamina, respectively.

Of the remaining twelve quantitative characters (the qualitative character was treated separately) a matrix of pairwise Gower distances (S15 (Legendre and Legendre, 1998)) was calculated with R Package 3.0 (Legendre and Vaudor, 1991) and served as basis for a Principal Co-ordinate Analysis (PCoA) using NTSYS-PC 2.0 (modules DCENTER and EIGEN; (Rohlf, 1998)). To check for correspondence of the morphological characters with the first two principle co-ordinates, a test for association between paired samples using Spearman's rho was carried out. To test for significant morphological differentiation between the Southern Group and the remaining samples (see Results), t-tests were undertaken and subsequently boxplots produced for characters with significant differences. The latter analyses were conducted with R 2.7.2 (R Development Core Team, 2008).

3. Results

3.1. Flow cytometry,

Flow cytometry analyses yielded histograms with mean CVs of G1 peaks of the sample and internal standard of 9.1% and 4.2%, respectively. DNA ploidy levels (Suda et al., 2006) inferred from measured genome sizes revealed that DNA diploids (for simplicity in the following referred to as “diploids”) and DNA tetraploids (referred to as “tetraploids”) were present throughout most of the study area (Fig. 2). However, tetraploids were more frequent in the North of the distribution area, resulting in a significant association of cytotypes with latitude (Spearman's rho = 0.337, $p < 0.001$). In contrast, no altitudinal separation could be detected (rho = -0.029, $p = 0.756$).

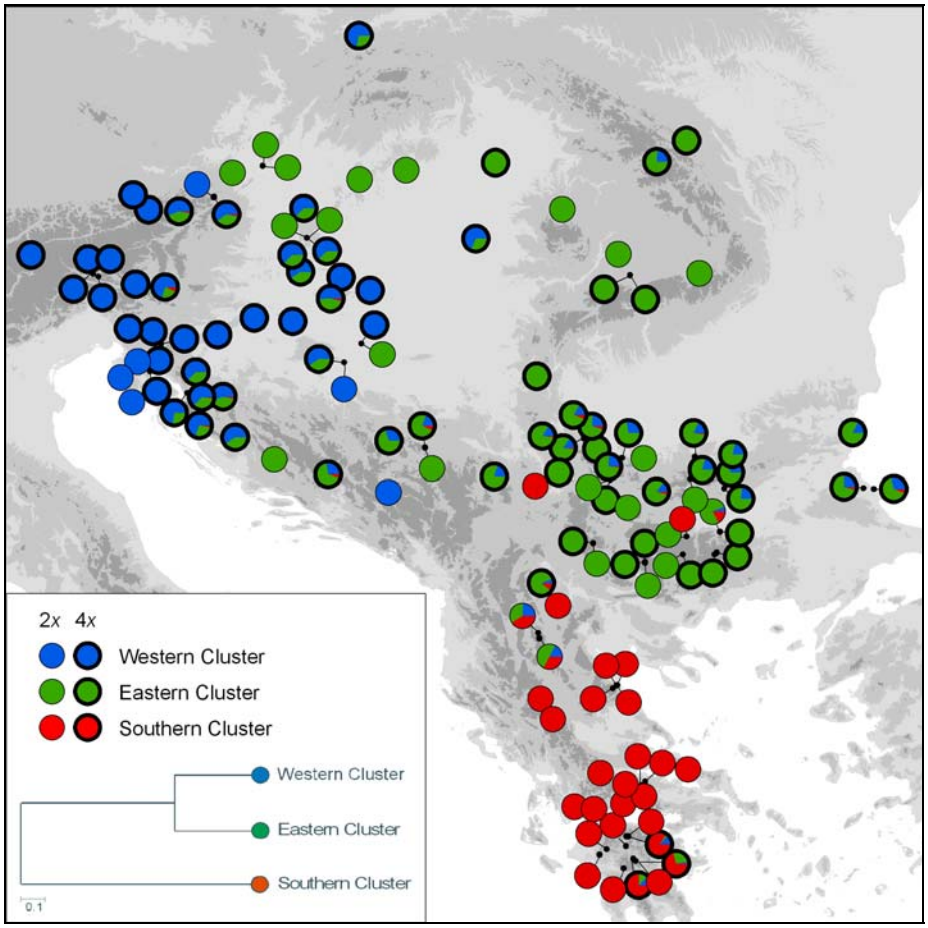

Fig. 2. Distribution of ploidy level (diploid, thin outline; tetraploid, thick outline) and of three genetic clusters derived from BAPS analysis of AFLP markers in 129 populations of the *Veronica chamaedrys* group from southeastern Europe. The insert shows a UPGMA tree of Kullback-Leibler distances among clusters. Population identifiers are given in Fig. 1.

3.2. AFLPs

We scored 468 AFLP fragments ranging from 101 to 498 base pairs. The error rate amounted to 1.6% and was thus well within the range mentioned by Bonin et al. (2004). Mixture analysis including only diploid individuals identified three clusters (data not shown). Subsequent admixture analysis assigned most individuals to a single cluster; only two individuals (from populations 58 and 59) were admixed and were excluded from the "training clusters". The subsequent admixture analysis including diploid as well as tetraploid individuals (Fig. 2) identified three clusters, hereinafter called *Southern*, *Western* and *Eastern Cluster*, each containing both diploid and tetraploid individuals. Allele distributions of the Western and the Eastern Cluster were more similar to each other than they were to the Southern Cluster (insert in

Fig. 2). Admixture was encountered among all clusters and mostly concerned tetraploid individuals, albeit not all tetraploids were admixed, while only three diploid individuals were admixed. The three clusters were geographically somewhat separated: The Western Cluster was most prominent in the northwest, the Eastern Cluster in the east and the Southern Cluster in the south, but especially Western and Eastern Clusters were widely geographically overlapping (Fig. 2). In the NeighbourNet (Fig. 3), two groups (*Southern Group*, *Western Group*) corresponding to two clusters resolved by the admixture analysis were separated along strongly weighted splits.

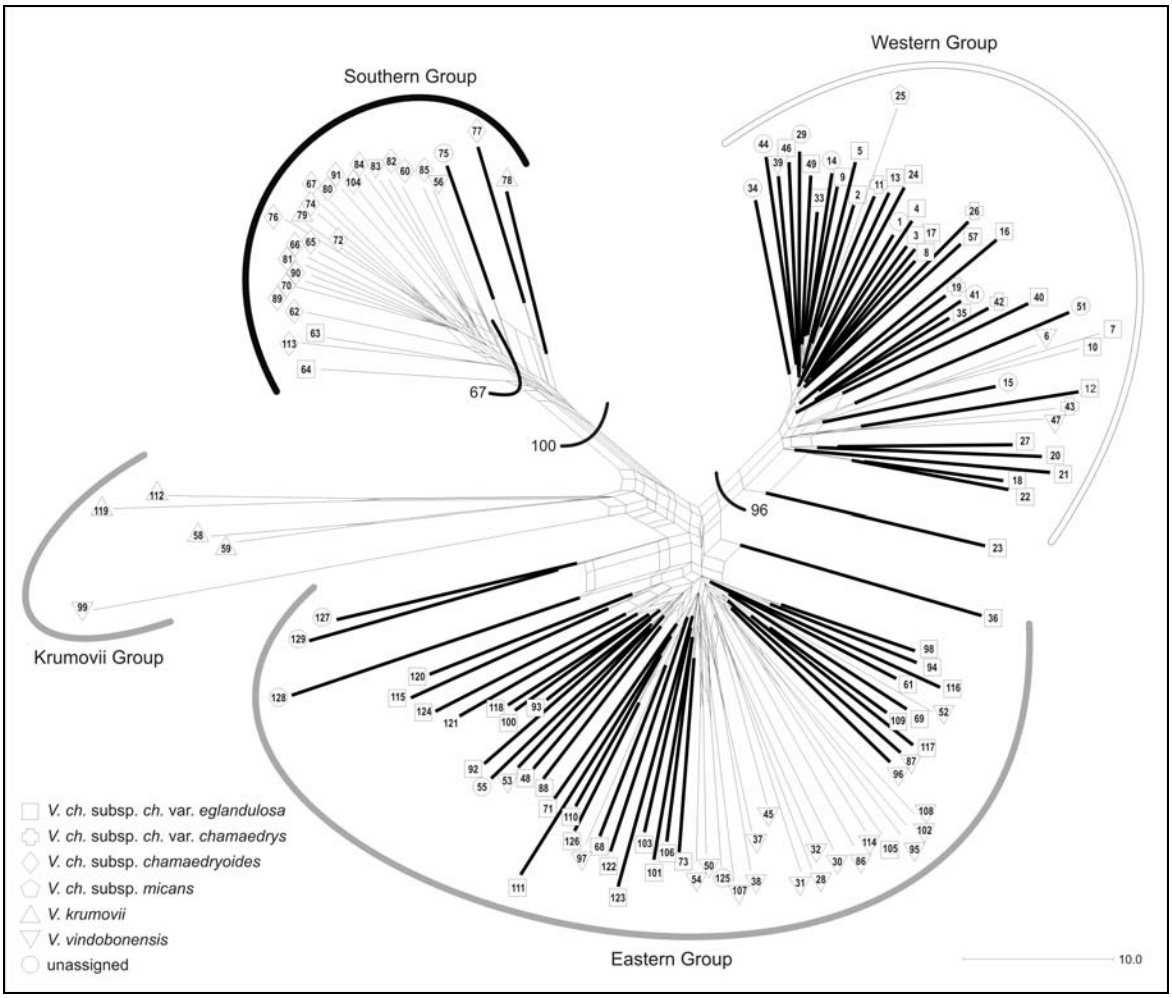

Fig. 3. NeighbourNet diagram of AFLP data constructed for 129 populations of the *Veronica chamaedrys* group from southeastern Europe. Splits with weight <0.5 have been omitted to aid legibility. Numbers along branches are bootstrap values above 50% derived from a Neighbour Joining analysis and are given for major branches only. Bold branches mark tetraploid individuals.

The remaining populations fell into the weakly differentiated *Eastern Group* and *Krumovii Group*, the latter being positioned between the Southern and the Eastern Group. All groups comprised more than one taxonomic entity, and *V. vindobonensis* was found in all four groups. In the Neighbour Joining analysis (not shown) both the Southern and the Western Group received high bootstrap support (100 BS and 96 BS, respectively). Neither the Krumovii nor the Eastern Group had bootstrap support > 50% in agreement with the lack of strongly weighted splits.

3.3. cpDNA sequencing

The *trnH-psbA* sequences were 295–304 bp, the *rps16 – trnK* sequences 701–709 bp and the *rpl32-trnL* sequences 843–867 bp long; the length of the concatenated alignment was 1978 bp. Concatenating the sequences yielded 79 haplotypes (Table 1). Phylogenetic analysis using BEAST (Fig. 4) revealed three major clades, from here on referred to as *Southern, Widespread* and *Eastern+Western Lineage*, and haplotype h71 found in a tetraploid individual. The oldest differentiation among the lineages (age given as median and its 95 % highest posterior density interval) occurred 0.81 (0.22–3.01) million years ago. The Southern and the Widespread Lineage (posterior probabilities of 0.82 and 0.97, respectively) diversified nearly simultaneously 0.40 (0.10–1.55) and 0.37 (0.10–1.36) million years ago, respectively. The Southern Lineage (h59–h70, h72–h79) was distributed on the southern Balkan Peninsula (Greece and northerly adjacent areas; Fig. 5). The Widespread Lineage (h39–h58) extended from the southeastern Alps (Austria, Slovenia) to the Bulgarian Stara Planina and was the sole lineage in the Carpathians (Romania). A deep phylogenetic split was evident in the Eastern+Western Lineage (posterior probability 0.84). The Eastern and the Western Lineages, which separated 0.47 (0.14–1.72) million years ago, started to diversify at 0.33 (0.09–1.20) and 0.29 (0.07–1.05) million years ago, respectively. The Western Lineage (h23–h38) extended from the southeastern Alps (Austria, Slovenia) to the northern Dinaric Mountains (Croatia, Bosnia and Herzegovina) and the eastern Pannonian Plain (Hungary), the Eastern Lineage (h1–h22) from western Serbia to Bulgaria. Both lineages geographically overlapped with the Widespread Lineage. Rapid population expansion for all lineages was inferred with the Bayesian Skyline Analysis (Fig. 4) to have occurred about 0.05 million years ago.

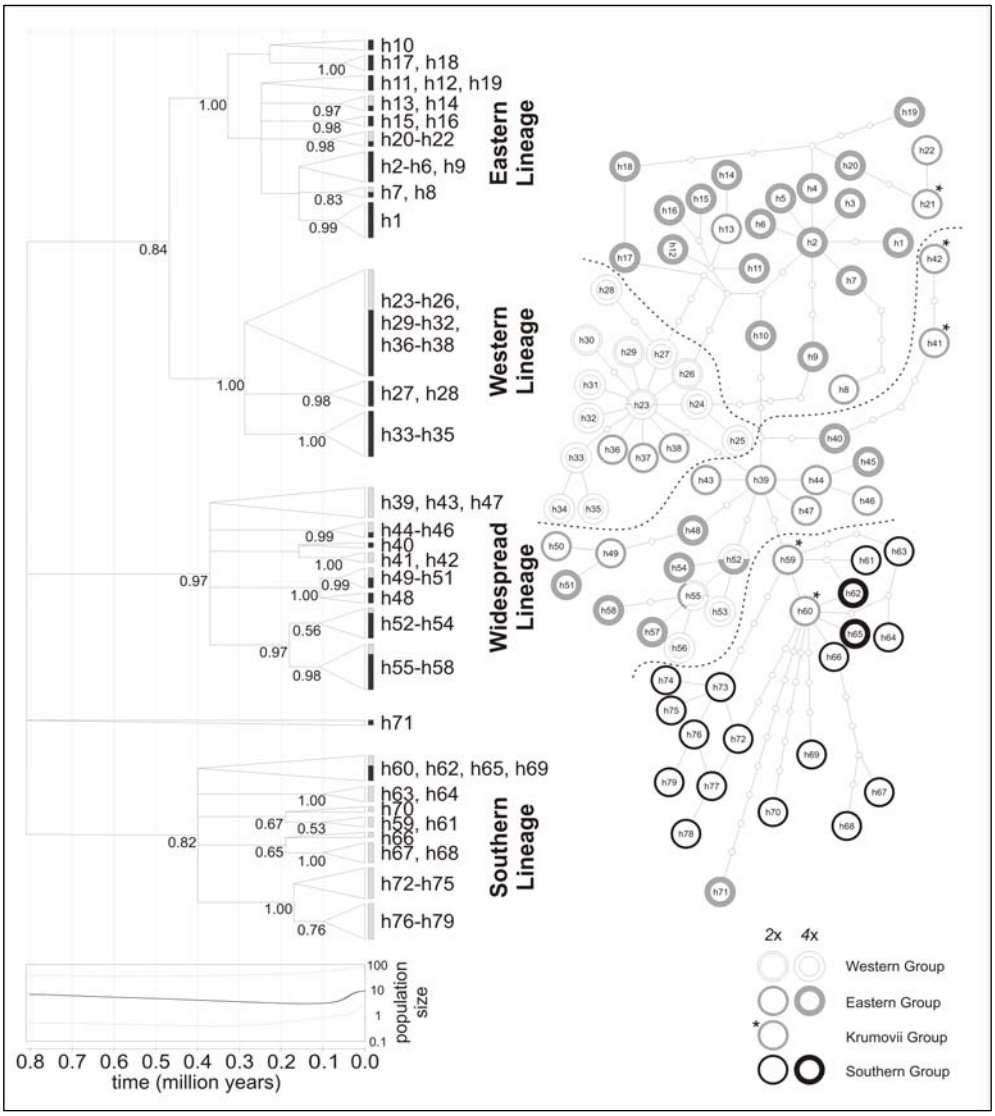

Fig. 4. Plastid DNA haplotype diversity encountered in 129 populations of the *Veronica chamaedrys* group from southeastern Europe. Left, simplified majority rule consensus tree from relaxed clock Bayesian analysis with the software BEAST. Node heights correspond to median ages (see text for details); nodes with age estimates younger than 0.04 million years are not shown and are thus not distinguishable from unresolved lineages. Numbers along branches are Bayesian posterior probabilities; identical haplotypes or unresolved polytomies are collapsed as triangles, their vertical extension being proportional to the number of individuals. The bar depicts the ploidy level of a lineage (light grey, diploid; dark grey, tetraploid). The insert at the bottom shows the Bayesian Skyline Plot (median and 95% high posterior density limits). Right, statistical parsimony network of plastid DNA haplotypes. Haplotypes that were not sampled are shown with small open circles. Color-coding corresponds to the three main AFLP groups presented in Fig. 3; individuals of the Krumovii Group are marked with an asterisk. Diploids and tetraploids have thin and thick outlines, respectively. The four main lineages identified by Bayesian analysis (at the left) are indicated.

In contrast to the clearly divergent Southern Lineage, differentiation among the Widespread, Eastern and Western Lineages identified by BEAST was only weakly reflected in the parsimony network (Fig. 4). Relatively few haplotypes were frequent and many were found in a single population only. The most frequent haplotypes were found in three separate regions (Fig. 5): (i) central to southern Greece (h67, h73, h76), (ii) Bulgaria and westerly adjacent areas (h1), and (iii) northern Croatia to eastern Austria and southern Poland (h23, h24, h27, h33 and h55); the only exception of this pattern were the haplotypes h39 and h52 from the Widespread Lineage which occurred in the West as well as in the East. Most haplotypes corresponding to the Western Lineage were closely related and maximally three mutational steps apart. Haplotype lineages were only partly congruent with AFLP-derived clusters (BAPS) or groups (NeighbourNet). The Southern Lineage formed a concise group in the network and comprised all individuals from the Southern Group as well as two haplotypes corresponding to the Krumovii Group (h59, h60) and haplotype h71, which belongs to the Eastern Group. With the exception of haplotypes h36–h38 sampled in populations pertaining to the Eastern Group, the Western Lineage comprised mainly haplotypes of the Western Group, albeit not all. The Eastern Lineage included exclusively haplotypes which occurred in the Eastern Group, whereas the Widespread Lineage contained mostly haplotypes of the Eastern Group plus four sampled in the Western Group (h52, h53, h55, h56). Haplotypes sampled in individuals from the Krumovii Group were spread over the network and were partly internal to those corresponding to the Southern Lineage and partly related to those of the Eastern as well as the Widespread Lineages (Fig. 4).

The mean number of pairwise differences (π) calculated for sets of haplotypes of the Western, Eastern, Widespread and Southern Lineages amounted to 2.13±1.21, 4.06±2.08, 4.85±2.45 and 6.34±3.09, respectively. The mismatch distributions showed a unimodal distribution only for the Eastern and Western Lineages, the distribution of the Widespread Lineage was close to unimodal, and that of the Southern Lineage was multimodal; only the North-Eastern distribution was significant ($P=0.02$).

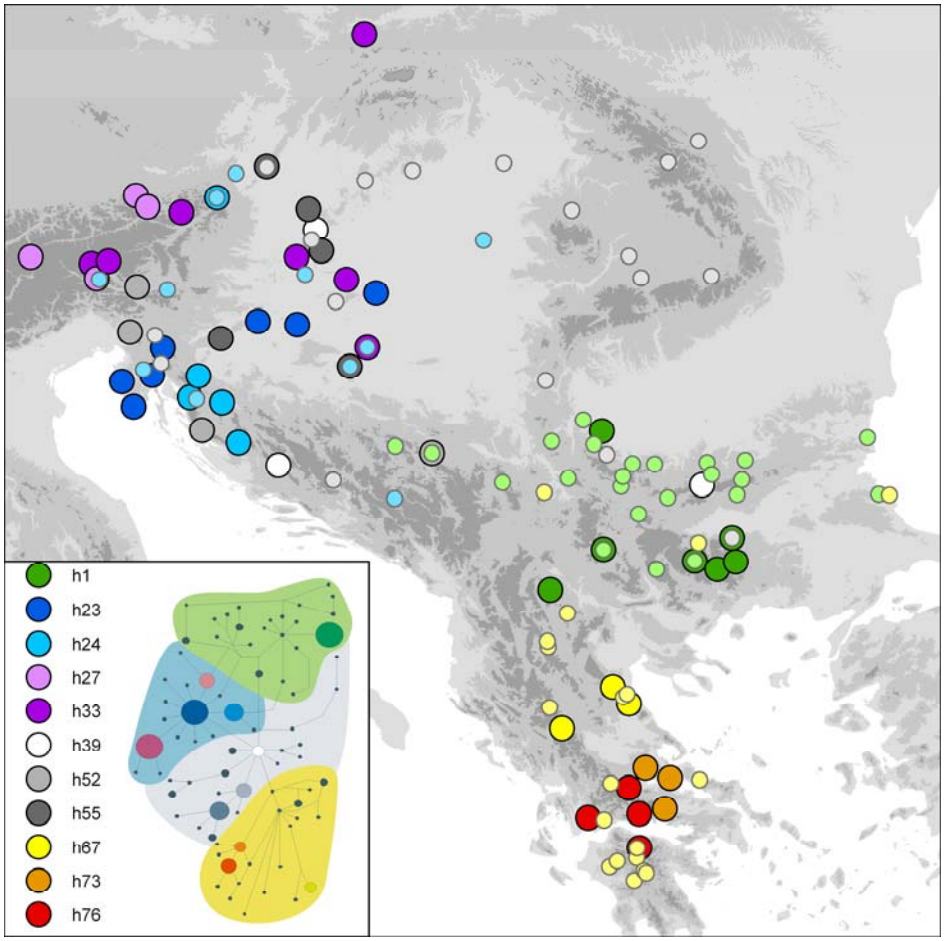

Fig. 5. Geographical distribution of plastid DNA haplotypes encountered in 129 populations of the *Veronica chamaedrys* group from southeastern Europe. In the insert, the most frequently sampled haplotypes (> 2 occurrences) are listed, and their position is indicated in the statistical parsimony network onto which the four main lineages identified by BEAST are overlaid (green, Eastern Lineage; blue, Western Lineage; grey, Widespread Lineage; yellow, Southern Lineage). The map shows the geographic distribution of the eleven most frequent haplotypes (big dots) as well as the rarer ones (small dots, colored as the overlay in the insert).

3.4. Morphometry

The PCoA of the morphometric data (Fig. 6) showed a weak segregation of di- and tetraploids along the first factor (16.8%; second factor: 10.3%), but failed to reflect the taxonomic grouping. There was, however, a weak clinal separation mainly along the first factor with *V. chamaedrys* subsp. *chamaedryoides* and subsp. *chamaedrys* var. *eglandulosa* being most distant and subsp. *chamaedrys* var.*chamaedrys* as well as *V. vindobonensis* and *V. krumovii* being intermediate. The first factor was positively correlated with different length characters (e.g. length of hairs on

different organs), the number of teeth on the lamina and the distance between the first tooth and the lamina base, but negatively correlated with the densities of hairs, the length-to-width ratio of the tooth at the broadest part of the lamina or the length of the petiole. The second factor showed positive correlation with the length-to-width ratio of the tooth at the broadest part of the lamina, but was negatively correlated with the density of hairs on different organs and the length-to-width ratio of the tooth at the broadest part of the lamina (Appendix 4). Differentiation along the first axis was also detected when the only qualitative character, i.e. presence of glandular hairs on the calyx, was plotted (plot not shown); importantly, all individuals of subsp. *chamaedryoides* had a glandular hairy calyx. When displaying the genetic groups obtained from the NeighbourNet of the AFLP data onto the morphometric plot, individuals of the Southern, Western and Krumovii Groups were separated but the Eastern Group blurred the separation (Fig. 6).

Significant differences (at $p = 0.001$) between the Southern Group and the remaining samples (excluding the Krumovii Group, for details see Discussion) were found for seven characters (details in Appendix 5, Appendix 6). Altogether, individuals of the Southern Group had denser but shorter hairs and the teeth at the margin of the lamina were longer and narrower than in the Western and the Eastern Group.

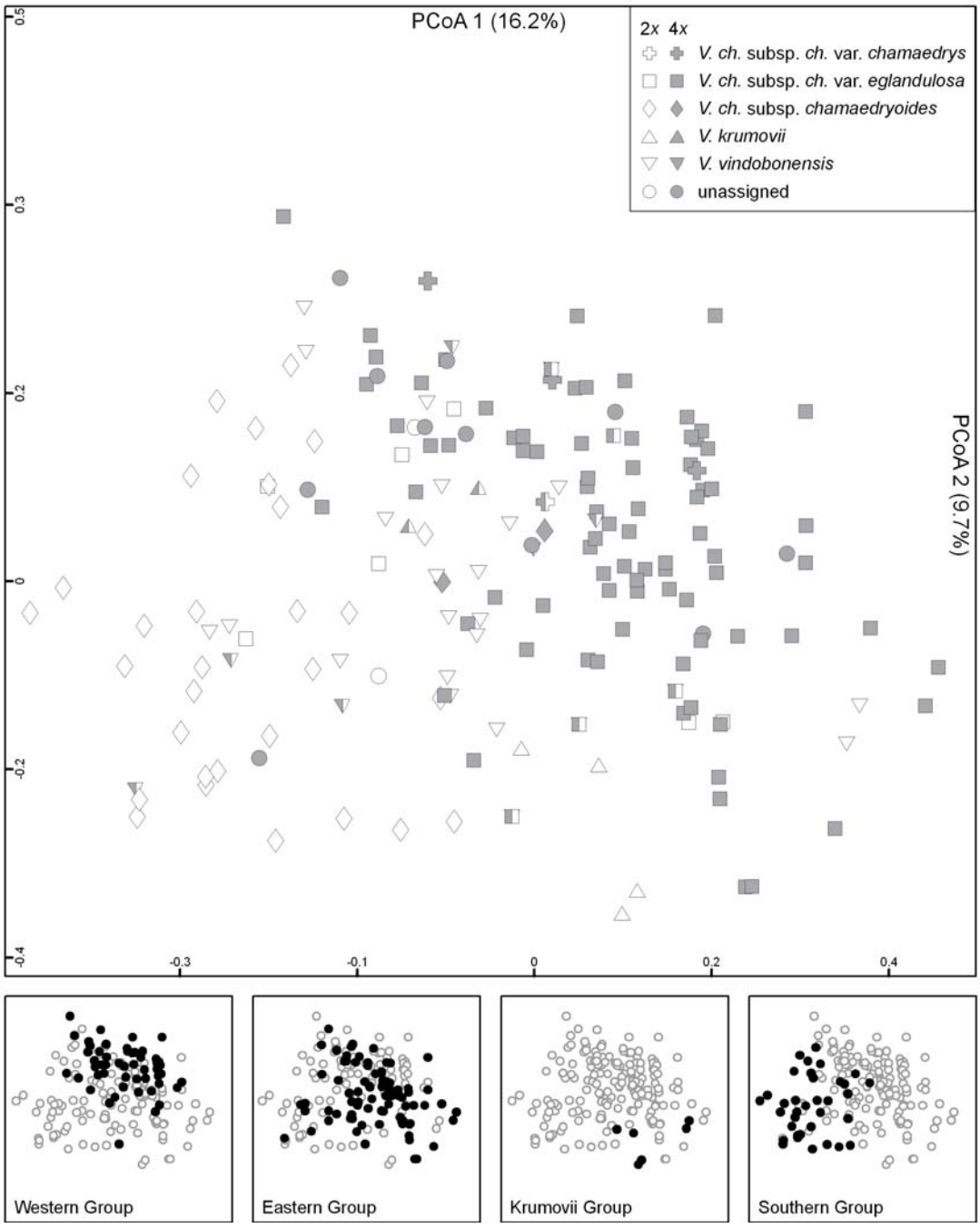

Fig. 6. Principal Co-ordinate Analysis of a matrix of pair-wise Gower distances based on 12 morphological characters scored for 187 individuals from 98 populations of the *Veronica chamaedrys* group from southeastern Europe. Symbols of the upper graph correspond to taxa as used in Fig. 1; diploid individuals are white, tetraploids are shaded in grey. Sampling sites with both cytotypes are marked both grey and white. The graphs at the bottom show the correspondence with the four AFLP groups as indicated in Fig. 3.

4. Discussion

4.1. Distribution of cytotypes within the Veronica chamaedrys *group in southeastern Europe*

Di- and tetraploid cytotypes within the *V. chamaedrys* group occurred all over southeastern Europe, as has been assumed previously (Mirek and Fischer, 1986). There was, however, a significant association with latitude in the study area as diploids were predominant in the south, whereas in the north both di- and tetraploids occurred (Fig. 2). Previous studies revealed similar patterns of cytotype distribution across Europe. Autotetraploids of *Rorippa amphibia* (Luttikhuizen et al., 2007), for example, occur in northern Europe, whereas diploids grow in Central and Western Europe. A similar pattern was also found in *Plantago media* in which diploids had small fragmented distributions in southern and southeastern Europe but tetraploids were more widespread and continuously distributed over the continent (Van Dijk and Bakx-Schotman, 1997).

The cytotypes of the *V. chamaedrys* group did neither show altitudinal segregation (Table 1), as observed in *Lotus corniculatus* s. l. (Gauthier et al., 1998) or *Taraxacum* sect. *Ruderalia*, (Calame and Felber, 2000), nor was there obvious habitat differentiation between the cytotypes, as has been shown for *Anthoxanthum alpinum* (Felber-Girard et al., 1996), *Claytonia virginica* (Lewis and Suda, 1976), *Dactylis glomerata* (Lumaret et al., 1987) or *Senecio carniolicus* (Hülber et al., 2009). Our results are in concert with studies on *Solidago altissima,* where autopolyploids co-exist with diploids in a broad zone of overlap (Halverson et al., 2008), or *Ranunculus adoneus* with no ecological differentiation between di- and tetraploids (Baack and Stanton, 2005).

Tetraploid individuals of the *V. chamaedrys* group were mainly of autopolyploid origin and evolved several times independently within each genetic group. This is supported by the unambiguous assignment of tetraploid individuals to clusters including also diploids (Fig. 2), the NeighbourNet diagram uniting di- and tetraploid individuals with heavily weighted splits (Fig. 3), as well as the co-occurrence of diploids and tetraploids in plastid lineages (Figs. 4, 5). Our results are thus adding to an ample body of evidence that multiple origins of polyploids are rather the rule than the exception (e.g. Doyle et al., 1990; Soltis et al., 2003; Albach, 2007). Tetraploids mainly evolved within various northern genetic lineages, and only a few originated within the southern refugium (Figs. 2–5). Although the exact causes remain elusive, we speculate that the more stable conditions on the southern Balkans (Tzedakis et al., 2002) may

have prevented the successful establishment of polyploids. More pronounced climatic oscillations on the northern Balkan Peninsula and adjacent areas in the north, in contrast, triggered massive range shifts of forest vegetation that also concerned the *V. chamaedrys* group and were probably a driving force allowing tetraploid establishment.

Whereas our data strongly support prevalence of autopolyploidy in the *V. chamaedrys* group in southeastern Europe, they are inconclusive with respect to the role of allopolyploidy. The admixed state of many tetraploid individuals in the Bayesian clustering analysis (Fig. 2) may indicate that they originated by crossings between divergent diploid lineages. Alternatively, these crosses may as well have occurred at the tetraploid level, in line with the close geographical proximity of non-admixed tetraploid populations. In any event it needs to be pointed out that the NeighbourNet, which is a method of choice for uncovering reticulate relationships (Huson and Bryant, 2006), only identified two tetraploid individuals (populations 23 and 36; Fig. 3) as being intermediate between the Western and Eastern Groups.

4.2. Spatio-temporal evolution of the Veronica chamaedrys *group*

Slowly mutating, maternally inherited (Zhang et al., 2003) plastid DNA sequence data (Figs. 4–6) and rapidly evolving, nearly entirely nuclear-derived (Bussell et al., 2005) AFLP marker (Figs. 2–3) do not yield congruent patterns of spatio-temporal evolution of the *V. chamaedrys* group, most probably because both marker systems trace differentiation processes taking place at different time horizons. Integrating information obtained from both marker systems, however, allows reconstructing a detailed scenario from the earliest diversification in the mid-Pleistocene over the relatively constant diversification during the last 0.4 my (Fig. 4) to the allopatric differentiation accompanying isolation in refugia during the Last Glacial Maximum and postglacial secondary contact leading to admixture of gene pools (Fig. 2).

The oldest traceable phylogeographic pattern in the *V. chamaedrys* group is the simultaneous differentiation of three plastid DNA lineages in the mid-Pleistocene (Fig. 4). A fourth lineage is constituted by haplotype h71 sampled in population 129 from the Bulgarian Black Sea coast. Since this sample is not differentiated in the AFLP data set (Figs. 2, 3) and our sampling from the southeastern-most Balkan Peninsula is very scarce, we here only point out the possibility of a divergent plastid lineage probably distributed along the southern coast of the Black Sea. Reconstructing the palaeo-distribution of the three main lineages is straightforward for only one of them. The Southern Lineage is restricted to the south of the Balkan Peninsula (Fig. 5) except

for two isolated occurrences in southern Serbia (population 63) and central Bulgaria (population 113). In contrast to the other lineages, the multimodal mismatch distribution indicates distributional stasis and lack of population expansion. The distinctness of the genetic entity from the southern Balkans including its two northern outliers is corroborated by AFLP data (Figs. 2, 3), and the nearly perfect congruence between the data sets with contrasting inheritance strongly suggests that hybridisation with other entities was at least rare. It remains to be tested whether the observed integrity has only historical reasons or if also ecological or crossing reasons are involved. The mountain ranges of northern Greece were glaciated (e.g. Hughes et al., 2007) and probably acted as strong barriers, contributing to the genetic isolation of the Southern Lineage/Group. Accordingly, the southernmost Balkan Peninsula is well-known as one of the centres of endemicity in Europe (Tan et al., 2001).

The distributions of the Widespread and Eastern+Western Lineages (Fig. 4) overlap throughout their entire distribution area from the Alps to the Rhodope Mountains and both lineages sometimes co-occur in immediate vicinity (e.g., populations 52, 53; Fig. 5). Our data do not allow discriminating if both lineages either co-occurred in sympatry (“ancient polymorphism”) after their split or if they regained secondary contact after an initial allopatric phase. The Carpathians, however, were obviously never reached by the Western+Eastern Lineage and were colonised by the Widespread Lineage in the course of the first diversification event at about 0.4 my (Fig. 4), as evidenced by the presence of micro-lineages that have their most internal haplotypes in the Carpathians (h44–h46, h48–h51; Figs. 4–6).

The Eastern+Western Lineage was disrupted into the vicariant Western and the Eastern Lineages at about 0.47 my (Fig. 4). The two entities occur from the Alps and the Western Hungarian Plains to the northern Dinaric Mountains and from the Stara Planina to the Rhodope Mountains, respectively (Fig. 5). Diversification within the two lineages started roughly simultaneously at about 0.29 and 0.33 my ago (Fig. 4). Longitudinal vicariance is also seen in the AFLP data (Fig. 2, 3). The Eastern Group is distributed from the Hungarian Plain and the Carpathians to the Stara Planina and the Rhodope Mountains. It comprises the entire Eastern Lineage and most haplotypes of the Widespread Lineage (Fig. 5). In contrast to the other main AFLP groups, the Eastern Group lacks strongly weighted splits and bootstrap support (Fig. 3). It is unclear if the lack of divergence is a consequence of the relatively recent contact of two formerly differentiated genetic entities corresponding to the Widespread and Eastern plastid Lineages.

The northwestern genetic entities encountered in the *V. chamaedrys* group, the Western Lineage and the Western Group, are roughly congruent and occur from the Alps and the Hungarian Plain to the Dinaric Mountains. The Western Lineage possesses a set of closely related haplotypes some of which were sampled frequently (Figs. 5, 6), and thus exhibits the lowest value of pairwise differences, amounting to only one third of that of the Southern Lineage. All this suggests either a comparatively young age or a stronger bottleneck within the contemporary distribution range. The Western AFLP group comprises mostly tetraploid individuals (Fig. 2); diploids were scattered throughout the range but were most frequent in Istria.

Allopatric differentiation during the Last Glacial Maximum presumably shaped the pattern seen in the AFLP data (Figs. 2, 3). It is mainly governed by three refugia in the southern, northwestern and eastern parts of southeastern Europe whose existence was already previously proposed for associated tree species based on macrofossil charcoal, pollen evidence and phylogeographical studies. (1) The southern Balkan Peninsula (Pindus mountain range and central Greece) provided buffered climatic oscillations (Tzedakis et al., 2002) and refugia of tree and shrub species such as beech and hornbeam were hypothesised (Grivet and Petit, 2003; Magri et al., 2006), overlapping with the Southern Cluster/Group. (2) On the eastern Balkan Peninsula (Eastwood, 2004) and the Carpathians (Willis and van Andel, 2004), refugia for beech, hornbeam and ash were proposed (Grivet and Petit, 2003; Heuertz et al., 2006; Magri et al., 2006) within the distribution area of the Eastern Cluster/Group. (3) Refugia for beech and ash (Heuertz et al., 2006; Magri et al., 2006) have been suggested on the north-western Balkan Peninsula and adjacent areas, namely Istria, the Pannonian Basin and the valley of River Danube in Austria (Willis and van Andel, 2004), roughly congruent with the extent of the Western Group/Cluster. Generally, the forest refugia were either separated by glaciated mountain massifs as the climatic snow line was about 1000 m lower than today (e.g. Bognar and Prugovečki, 1997; Reuther et al., 2007), or by *Artemisia*-steppes prevailing in the lowlands (Willis, 1994).

Triggered by most probably postglacial climatic amelioration, the *V. chamaedrys* group underwent massive range expansions as suggested by the Bayesian Skyline Plot (Fig. 4) for c. 0.05 mya. As substitution rates derived from phylogenetic studies with usually deeper time coverage (older than 1–2 million years) will often be gross underestimates of more recent substitution rates (Ho et al., 2005) the obtained age estimate will be biased towards older ages; thus the rapid expansion may have occurred after the last glacial maximum. Subsequently, as

suggested by the admixture analysis (Fig. 2) but not by the NeighbourNet diagram (Fig. 3), secondary contact between Western and Eastern Groups partially broke down previously existing genetic differentiation by hybridisation involving frequent independent polyploidisation (Fig. 2).

4.3. Taxonomic considerations

Our data show that current taxonomy does not reflect genetic lineages (Figs. 2–5). Furthermore, there is no tight association between taxa and ploidy level, as previously suggested, but diploids and polyploids are found in all recognized taxa as well as in all major genetic lineages (Fig. 3). Morphometric analysis does not recover current taxonomic entities, either (Fig. 6). Therefore, our data suggest changes to the currently used taxonomic framework of the *V. chamaedrys* group. The only entity that is congruently identified by different data types and thus merits taxonomic recognition is *V. ch.* subsp. *chamaedryoides* from the southern Balkan Peninsula. It is mainly (but not exclusively, see Fig. 3) diploid and its genetic distinctness was confirmed by both the AFLP and the cpDNA data sets (Figs. 3, 5, 6). Although only weakly separated in the PCoA plot of the morphometric data, subsp. *chamaedryoides* can be separated from the other taxa using only slightly overlapping characters (Appendix 5). Specifically, indumentum characters as well as the shape of the teeth can be used to distinguish subsp. *chamaedryoides* (Appendix 5, 6). The identifiability of this taxon is evidenced by the fact that about eighty percent of the voucher specimens have been correctly identified in the initial phase of our study.

The two widespread taxa *V. ch.* subsp. *chamaedrys* and *V. vindobonensis* could neither be differentiated by morphometry (Fig. 6) nor by molecular data (Figs. 2–6) and both comprise diploid and tetraploid cytotypes (Fig. 3). Additionally, in contrast to the northwestern distribution edge of *V. vindobonensis* in eastern Austria (Fischer, 2008), the vegetation of the sampling sites did not reveal obvious habitat differentiation between these entities and both entities were found from wet to dry habitats, in open forests or meadows (K. Bardy, personal observations). Although the AFLP data (Fig. 3) suggested two genetic entities, these can neither be paralleled with morphometric data nor are they congruent with a priori determinations of voucher specimens (Figs. 3, 7). Although we cannot entirely exclude the possibility that morphological differential characters might still be found, we favor to treat these two lineages as informal phylogeographical groups within *V. ch.* subsp. *chamaedrys*, which thus becomes a morphologically and cytologically variable taxon comprising diploids as well as mainly autotetraploid derivatives.

Although *V. orbelica* was described as diploid (Peev, 1972), we found only tetraploid individuals morphologically resembling this taxon. As continuous variation of characters connects *V. orbelica* (Peev, 1972) and *V. ch.* subsp. *chamaedrys* var. *eglandulosa* (Mirek and Fischer, 1986; see Appendix 1), and it is not genetically divergent, either, *V. orbelica* should be included in subsp. *chamaedrys* var. *eglandulosa*. Similarly, genetic indistinctness and the lack of morphometric differentiation of *V. ch.* subsp. *micans* suggest merging this taxon with subsp. *chamaedrys*.

Veronica krumovii morphologically resembles *V. ch.* subsp. *chamaedrys* (Fig. 6) but differs by glandular hairs of varying length scattered over the whole plant. According to the AFLP data (Fig. 3), the majority of populations appears to be intermediate between the Southern and the Eastern Group, and may actually be hybrids between subsp. *chamaedrys* and *chamaedryoides* as circumscribed here. Plants morphologically belonging to *V. krumovii* also occur disjunctly in southern Greece (Fig. 1), probably suggesting that the erratically occurring, but conspicuous diagnostic character is under simple genetic control and consequently of no taxonomic value.

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Appendix 1. Previously recognised taxa of the *Veronica chamaedrys* group in southeastern Europe (see Appendix 2 for discriminative characters).

V. vindobonensis

(M. [A.] Fischer) M. [A.] Fischer, in Österr. Bot. Z. 122: 287 (1974); basionym: *V. chamaedrys subsp. vindobonensis* M. [A.] Fischer, in Österr. Bot. Z. 118: 207 (1970); type: Austria, Austria Inferior (Niederösterreich), Weinviertel, prope pagum Markt Pirawarth haud procul ab oppido Mistelbach, leg. M. [A.] Fischer, 23.V.1969; herb. WU.

V. vindobonensis has been originally described at the rank of subspecies (Fischer, 1970) but later, on the basis of subsequent studies revealing a consistent complex of correlated characters, raised to specific rank (Mirek and Fischer, 1986). It is a mainly pannonian-pontic species ranging continuously from eastern Austria and southern Germany to climatically continental regions in northern Poland (Mirek and Fischer, 1986), to the Caucasus and to climatically pontic parts of easternmost Turkey (Manfred A. Fischer, unpublished), but also to the south, though rather scattered, on mountains of the Balkan peninsula (Mirek and Fischer, 1986). On the Balkan Peninsula, however, *V. vindobonensis* is in close contact with several other taxa of the *V. chamaedrys* group, some of them strongly approaching *V. vindobonensis*, thus blurring the species distinction.

V. krumovii

(Peev) Peev, in Flora Reipubl. Bulgaricae 10: 159 (1995); basionym: *V. chamaedrys* subsp. *krumovii* Peev, Compt. Rend. Acad. Bulg. Sci. 25: 6 (1972); type: Bulgaria, Stara planina (Haemus mons), in valle „Kuru dere“ prope Kăzănlak; herb. SOM.

V. krumovii was described by Peev (1972) from a small population in a valley on the mountain range of Stara planina (central Bulgaria), and is considered to be endemic to Bulgaria – “spread in Bulgaria in grassy and rocky habitats at forest edges from lowlands to mountain foothills up to 800 m a. s. l.“ (Peev, 1995). It differs from *V. chamaedrys* by a single, though conspicuous character: stem and leaves are covered with glandular hairs with varying length and density, a trait not present in any of the other taxa within *V. chamaedrys* group.

V. orbelica* and *V. chamaedrys

V. chamaedrys* subsp. *chamaedrys

V. chamaedrys* subsp. *chamaedrys* var. *chamaedrys

L., Sp. Pl. 13 (1753); type: Europe. (Calyx hairs glandular)

V. chamaedrys* subsp. *chamaedrys* var. *eglandulosa

M. A. Fischer, in Phyton (Austria) 26(1): 127 (1986); type: Austria, Styria media, in pago Kleinstübing dicto prope Peggau, leg. M. A. & G. Fischer, 12.V.1972; herb. WU. (Calyx hairs eglandular)

Syn.: ***V. orbelica***

(Peev) Peev, in Flora Reipubl. Bulgaricae 10: 160 (1995); basionym: *V. chamaedrys* subsp. *orbelica* Peev, Compt. Rend. Acad. Bulg. Sci. 25: 6 (1972); type: Bulgaria, mons Pirin septentrionalis, prope refugium alpinum Javorov; herb. SOM

V. orbelica, has been described from Pirin mountains (southwestern Bulgaria) as diploid *V. chamaedrys* subsp. *orbelica* (Peev, 1972) and later on raised to specific rank and considered endemic to the Pirin mountains (Peev, 1995), differing from *V. vindobonensis* only by eglandular calyx hairs. Samples of the *V. chamaedrys* group collected on the Balkan Peninsula in the present study did not allow to distinguish the type population of *„V. orbelica“* from other populations all over this area because leave shape varies from *vindobonensis*-like (deeply serrate to almost pinnatisect) to regularly serrate like in *V. chamaedrys* s. str. (as is shown in Table 38, Fig. 3 in Peev, 1995). Specimens with some resemblance to *V. vindobonensis* and other traits of *V. vindobonensis* – the two stem hair lines are dense without hairs in between – and with relatively short hairs on calyx and leave surface, could tentatively be determined as *„V. orbelica“* but most of the specimens throughout the Balkan Peninsula have longer hairs and do not evidently resemble typical *V. orbelica*. It was not possible to find any objective boundary between *„V. orbelica“* and *V. chamaedrys* with eglandular calyx indumentums as there exists a continuous variation range connecting populations of *V. orbelica* with eglandular *V. chamaedrys*, described as ***V. ch.* subsp. *chamaedrys* var. *eglandulosa*** in Mirek and Fischer (1986: 127), a tetraploid spreading from southern Austria to the Balkan Peninsula. In the present study *V. orbelica* was therefore included in *V. ch.* subsp. *chamaedrys* var. *eglandulosa.*

Typical ***V. chamaedrys* subsp. *chamaedrys* var. *chamaedrys*** (calyx hairs few, all glandular), is very rare on the Balkan Peninsula. *Veronica chamaedrys* in the sense of Peev (1995) evidently includes both var. *eglandulosa* and var. *chamaedrys*.

V. chamaedrys* subsp*. micans

M. [A.] Fischer, in Österr. Bot. Z. 121: 73 (1973); type: Austria, Austria Inferior (Niederösterreich), Praealpes Calcarei Septentr., in apertis piceeti altomontani supra vallem Lechnergraben montis Dürrenstein prope Lunz am See, leg. M. [A.] & G. Físcher, 4.VII.1971; herb. WU.

V. chamaedrys subsp*. micans* is a diploid taxon, differing from subsp*. chamaedrys* by leaf characters and short eglandular calyx hairs and is known from mountains of the North-eastern Calcareous Alps. In low regions of southern Carinthia and Styria diploid populations close to subsp. *micans* growing in nutrient-poor habitats (Fischer, 1973) are still not sufficiently investigated.

V. chamaedrys* subsp. *chamaedryoides

(Bory & Chaub.) M. A. Fischer, in Strid A. & Kit Tan: Montain Flora of Greece 2: 222 (1991); basionym: *V. chamaedryoides* Bory & Chaub., Expéd. Sci. Morée 3(2) Bot.: 15 (1832); type: Graecia: Peloponnisos: Les lieux montueux au bord des ombrages en allant à Phigalée, et en montant au Taygète.

V. chamaedrys subsp. *chamaedryoides* is diploid, endemic to Greece and morphologically distinguishable from the very variable subsp. *chamaedrys* by a few well correlated characters. It also resembles *V. vindobonensis*; both have glandular calyx hairs, a small and often whitish corolla und incised leave margin, but nevertheless differs by longer glandular calyx hairs, a stem indumentum less strictly confined to two lines, leaf and tooth shape, usually shorter pedicels, smaller corolla size and shorter style (Fischer, 1991; see also map in Kit Tan and Iatrou, 2001). Morphologically it seems less distinct from subsp. *chamaedrys* than *V. vindobonensis* from subsp. *chamaedrys* – as expressed by different taxonomic rank.

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Appendix 2. Discriminative characters used for determination of previously recognised taxa of the *Veronica chamaedrys* group in southeastern Europe (see Appendix 1 for the taxonomic circumscription of the taxa).

	V. chamaedrys subsp. *chamaedrys*	*V. cham.* subsp. *chamaedryoides*	*V. chamaedrys* subsp. *micans*	*V. „krumovii“*	*V. „orbelica“*	*V. vindobonensis*
hairs in the two opposite lines on the stem	(0.5)1.0–1.6(2.0) mm long, lines dense to loose, sometimes some hairs scattered between them	(0.4)0.5 –0.9(1.1) mm, lines sense to loose, often hairs between them	0.8–1.4 mm long, lines loose, often sparse hairs scattered between them	0.5–1.0 mm long glandular hairs scattered around the stem and additionally in two lines	upper stem scattered, lower stem in 2 opposite lines, 0.8–1.5 mm long	(0.7)1.0–1.3 (1.5) mm long, lines dense, no hairs between them
petiole length	(0.5)1.0–2.0(3.0) mm	1.0–2.5(4.0) mm	2.0–3.0 (4.0) mm	0.0–8.0 mm	not given	0.0–1.0 (1.5) mm
leave lamina: size and teeth	(17)20–35(45) × (12)15–25(30) mm, regularly serrate (not pinnatisect), teeth (4)5–9(13), wide, ovate	20–40 × 15–30 mm, usually deeply serrate, teeth 5–8, narrowly triangular to linear	20–35 × 12–20 mm, regularly serrate, teeth (6)9–11 (14), narrow, sublinear, especially in lower leaves	35–40 × 25–35 mm, irregularly dentate [serrate]	30–35 × 18–25 mm, irregularly incised-dentate [-serrate]	(9)11–22 (32) × (5)7–15 (24) mm, almost pinnatisect, teeth (5)6–8(11), narrow, almost linear
upper side of leave lamina: pubes-cence	± sparsely pubescent, hairs 0.3–1.0 mm, on tip of leave 0.2–0.5(0.7) mm long	loosely puberulent, hairs 0.2–0.4 mm, on tip of leave 0.1–0.2(0.3) mm long	± sparsely pubescent, hairs 0.1–0.6 mm, on tip of leave 0.1–0.3 mm long	pubescent with 0.25–0.30 mm long glandular hairs	pubescent with hairs 0.5–1 mm long	± densely puberulent only near edge, hairs 0.2–0.4 mm, on tip of leave 0.1–0.3 mm long
leaves	deep green, not shining	bright green, slightly shining?	± bright green, weakly but distinctly shining	upper side green, lower side purplish	green to bright green	dull yellowish green, not shining
fruiting pedicel	(4)5–8(10) mm long	2–3(4) mm long	4–8 mm long	3–8 mm long	4–7 mm long	3–5(8) mm long
calyx indument	loose to dense, hairs glandular or (in main investigation area) eglandular and (0.3)0.5 – 0.8(1.2) mm long	± loose, hairs glandular, (0.2)0.3–0.4(0.5) mm long	± loose, hairs eglandular, (0.3)0.4–0.7 mm long	hairs glandular, 0.35–0.40 mm long	hairs eglandular, 0.15–0.2 mm long	± dense, hairs glandular, 0.2–0.4(0.5) mm long
corolla	10–13(15) mm ∅, bright to dark blue	6–8 mm ∅, bright blue or often whitish with blue centre	9–12 mm ∅, ± bright blue	10–12 mm ∅, white or bright blue with darker veins	7–9 mm ∅, with scattered eglandular hairs, bright blue	9–12 mm ∅, bright to pale blue, margins often whitish
style length	4.5–5.0(5.5) mm	2.5–3.5(4) mm	4.0–5.0 mm	3.0–5.0 mm	3.8–5.5 mm	3–4.5(5.0) mm
ploidy level	4*x*	2*x*	2*x*	2*x* and 4*x*	2*x*	2*x*
habitat	meadows, forest edges, lowland up to mountains	grassland, forest edges (?)	mountain forest clearings, subalpine grasslands	dry, stony places at forest edges, up to 800 m a. s. l.	dry, calcareous places	dry grassland, lowland up to subalpine grassland
distribution	entire Europe	Greece	N. Calcareous Alps (only?)	Balkan Pensinsula (?)	endemic to Pirin mountains (SW Bul-garia)	from SE. C. to E. Europe to Caucasia

Appendix 3. Data matrix of morphological characters or ratios of characters employed in a morphometric analysis of 98 populations of the *Veronica chamaedrys* group from southeastern Europe. Length characters are in mm, density is per mm^2, qualitative characters were coded as in Table 2. Missing values are denoted "NA".

Population	KG	KHD	KHL	LLW	LP	LS	LT	LTB	LTLW	LUBD	LUBL	SRD	SRL
1	1	14	0.52	1.24	1.3	309	7	7.3	0.33	3	0.38	3	0.93
2	0	15	0.61	1.18	0.0	149	7	4.0	0.63	8	0.31	2	1.17
2	0	16	0.65	1.29	1.4	264	8	6.5	0.38	6	0.46	1	1.09
3	0	17	0.67	1.49	1.7	197	8	8.8	0.53	4	0.41	10	0.89
3	0	20	0.73	1.33	1.4	168	8	6.6	1.00	8	0.42	10	1.16
4	0	13	0.84	1.27	1.0	210	8	8.0	1.14	5	0.49	5	1.08
4	0	15	0.80	1.59	2.3	291	11	10.1	0.69	9	0.59	6	1.15
5	0	10	0.69	0.95	1.5	123	9	3.1	0.47	7	0.64	5	1.36
5	0	21	0.69	1.45	1.8	154	7	6.0	0.45	NA	NA	8	1.24
6	1	20	0.24	1.00	3.4	126	8	5.3	0.48	6	0.13	0	0.50
6	1	21	0.35	1.33	3.2	275	9	6.1	0.65	14	0.17	2	0.96
7	0	19	0.44	1.01	3.7	187	8	4.0	1.10	15	0.22	0	0.98
7	1	21	0.29	1.49	2.7	260	8	3.2	0.59	32	0.15	0	0.78
9	1	15	0.41	1.17	1.7	207	10	4.3	0.85	7	0.54	6	1.73
9	0	19	0.91	1.74	0.0	157	7	5.3	0.64	6	0.56	21	1.38
10	0	13	0.48	1.28	1.5	270	8	5.8	0.78	6	0.28	12	0.74
10	0	29	0.32	1.23	0.7	195	8	3.9	0.69	15	0.39	8	0.76
12	1	12	0.26	1.69	2.5	129	7	5.3	0.42	5	0.13	6	0.47
12	0	14	0.82	1.20	1.9	207	9	6.0	0.61	6	0.37	4	1.68
13	0	10	0.93	NA	4.0	204	7	10.3	0.41	4	0.30	10	1.41
13	0	13	0.70	1.54	3.8	202	10	6.0	0.77	5	0.27	9	1.08
16	0	12	0.85	1.05	2.2	516	9	4.9	0.96	10	0.36	3	1.56
16	0	15	0.60	1.41	0.7	277	6	5.9	0.49	4	0.47	6	1.24
17	0	24	0.70	1.79	4.7	145	11	7.2	0.63	6	0.43	2	1.20
17	1	25	0.39	1.58	0.0	184	11	4.1	0.73	3	0.31	13	1.04
18	0	19	0.56	1.07	NA	65	7	2.8	0.63	16	0.22	8	0.80
18	0	24	0.65	1.15	0.9	132	8	4.6	0.86	13	0.25	10	0.78
19	1	14	0.51	1.53	1.0	244	7	7.7	0.75	10	0.44	10	1.70
19	1	15	0.43	1.27	1.3	197	5	4.8	0.42	5	0.41	4	1.19
20	0	17	0.75	1.09	0.0	334	8	2.5	0.37	4	0.30	0	0.91
20	0	23	0.52	1.21	0.0	NA	6	4.7	0.56	NA	NA	0	1.29
21	0	21	0.48	1.36	NA	65	7	3.0	0.55	17	0.41	0	0.76
21	0	23	0.55	1.28	0.7	89	6	2.0	0.50	14	0.31	0	0.72
22	0	12	0.69	1.20	0.9	275	6	3.2	0.86	6	0.32	0	0.79
22	0	17	0.60	1.56	2.0	293	9	6.9	1.16	13	0.47	2	1.24
24	0	17	0.59	1.59	0.0	153	7	4.2	1.00	10	0.36	25	0.74
24	0	21	0.62	1.23	1.2	232	11	6.8	0.71	6	0.40	13	1.09
26	0	16	0.51	NA	NA	NA	NA	NA	NA	NA	NA	0	0.39
27	0	17	0.40	1.54	0.9	NA	7	5.8	0.51	13	0.21	0	0.58
27	0	20	0.28	1.38	0.0	223	5	4.4	0.88	13	0.19	0	0.61
28	1	25	0.27	1.69	1.5	351	6	6.6	0.40	9	0.34	0	1.22
28	1	32	0.40	1.48	0.0	190	6	5.5	0.80	15	0.29	0	1.11
29	0	16	0.55	1.47	0.0	167	8	3.8	0.79	6	0.35	10	1.01
29	0	17	0.53	1.23	1.6	NA	5	4.0	0.64	8	0.20	5	0.69
35	0	28	0.60	NA	NA	361	NA	NA	NA	13	0.37	2	1.02
37	1	26	0.30	1.14	1.2	170	7	6.5	1.72	19	0.40	0	1.20
37	1	33	0.33	1.47	1.2	198	7	6.2	0.73	15	0.38	0	1.17
38	1	13	0.35	1.22	2.0	199	8	4.8	0.62	3	0.37	5	0.85
38	1	23	0.32	1.22	1.6	203	9	5.3	1.13	8	0.46	0	1.07
39	1	13	0.35	1.22	2.0	199	8	4.8	0.62	3	0.37	5	0.85
39	1	23	0.32	1.22	1.6	203	9	5.3	1.13	8	0.46	0	1.07
40	0	16	0.59	1.61	NA	149	6	6.0	0.52	9	0.60	0	0.69
40	0	17	0.82	NA	1.3	241	9	6.4	0.67	13	0.45	0	1.04
42	1	NA	0.37	1.32	1.7	94	9	3.1	0.85	3	0.60	0	1.37
43	1	NA	0.37	1.32	1.7	94	9	3.1	0.85	3	0.60	0	1.37
44	0	14	0.70	0.91	NA	256	10	4.4	1.14	NA	NA	6	1.54
44	0	25	0.56	NA	2.2	263	NA	NA	NA	7	0.59	3	1.38
45	0	13	0.60	1.24	2.0	224	7	7.0	0.62	9	0.31	6	0.90
45	1	27	0.33	NA	0.0	291	NA	NA	NA	NA	NA	0	0.97
46	0	13	0.60	1.24	2.0	224	7	7.0	0.62	9	0.31	6	0.90

Population	KG	KHD	KHL	LLW	LP	LS	LT	LTB	LTLW	LUBD	LUBL	SRD	SRL
46	1	27	0.33	NA	0.0	291	NA	NA	NA	NA	NA	0	0.97
48	0	17	0.53	NA	0.0	143	8	4.5	0.44	6	0.27	0	0.52
48	0	20	0.49	1.43	1.0	175	8	6.6	0.67	3	0.37	0	1.20
50	1	23	0.30	1.21	1.9	159	8	5.5	1.16	16	0.20	0	1.11
50	1	33	0.31	1.19	1.2	273	9	4.4	0.89	17	0.28	0	1.29
52	1	37	0.21	1.24	0.0	173	4	2.2	1.19	9	0.15	0	0.75
52	1	49	0.20	1.24	0.0	173	4	1.6	1.64	29	0.15	0	0.78
53	1	37	0.21	1.24	0.0	173	4	2.2	1.19	9	0.15	0	0.75
53	1	49	0.20	1.24	0.0	173	4	1.6	1.64	29	0.15	0	0.78
54	1	26	0.33	2.36	1.8	177	6	10.5	0.93	NA	NA	0	1.07
54	1	35	0.32	0.95	1.5	132	8	4.2	1.36	NA	NA	0	1.12
55	1	21	0.41	1.24	0.0	110	9	6.3	0.84	13	0.46	0	0.78
55	1	40	0.23	0.91	0.0	210	8	4.6	1.14	21	0.15	0	0.80
57	0	24	0.25	1.31	1.3	113	8	3.4	0.54	10	0.31	0	1.11
57	0	26	0.54	NA	1.4	161	9	4.2	2.00	NA	NA	0	1.08
62	1	40	0.41	1.20	2.9	338	9	7.5	1.51	49	0.18	18	0.66
62	1	44	0.37	1.10	1.2	219	10	4.4	1.23	48	0.17	21	0.70
63	1	25	0.40	1.27	1.3	170	10	2.4	1.71	23	0.21	13	0.71
65	1	33	0.37	1.59	1.1	88	6	2.0	1.29	59	0.18	52	0.55
65	1	37	0.36	1.29	0.9	60	7	2.7	0.96	60	0.22	25	0.91
66	1	40	0.29	1.30	2.1	174	5	4.9	0.62	49	0.20	20	0.50
66	1	41	0.37	1.34	1.3	259	10	3.7	1.36	44	0.18	15	0.62
67	1	38	0.49	1.14	1.8	264	11	7.4	1.66	28	0.22	8	1.02
67	1	44	0.44	1.33	0.0	242	10	4.9	0.58	55	0.19	15	0.95
68	0	25	0.52	NA	NA	425	NA	NA	NA	13	0.22	0	1.17
69	0	17	0.73	NA	0.8	NA	7	5.9	1.05	11	0.48	9	0.88
69	0	21	0.74	1.14	1.1	NA	10	7.9	0.70	2	0.69	7	1.23
70	1	21	0.37	1.61	1.9	196	7	5.2	0.67	13	0.22	15	0.53
70	1	25	0.31	1.39	1.3	235	10	5.7	1.21	NA	NA	0	0.52
72	1	37	0.54	1.08	1.8	331	10	6.2	1.90	31	0.35	9	1.07
72	1	44	0.43	1.68	1.5	266	14	6.1	0.97	41	0.22	10	1.02
73	0	19	0.57	1.41	0.6	181	8	6.6	0.58	NA	NA	5	0.84
73	0	23	0.69	1.24	0.0	287	14	9.5	1.29	16	0.57	0	1.40
74	1	44	0.37	1.08	2.3	324	6	7.8	1.35	11	0.17	7	0.78
74	1	47	0.35	1.19	2.5	262	11	4.5	0.86	33	0.17	14	0.84
76	1	39	0.44	1.37	1.4	216	9	3.3	1.46	33	0.19	11	0.74
76	1	50	0.41	1.53	1.6	185	10	3.5	1.10	29	0.24	5	0.65
77	1	21	0.69	1.04	0.0	73	5	3.5	1.78	21	0.49	10	0.72
77	1	21	0.74	1.17	1.3	166	9	7.4	1.18	13	0.46	13	0.63
78	1	15	0.57	1.06	0.0	115	6	6.0	2.00	17	0.34	32	0.69
78	1	17	0.45	0.93	0.0	116	8	4.2	1.11	3	0.47	15	0.77
79	1	15	0.57	1.06	0.0	115	6	6.0	2.00	17	0.34	32	0.69
79	1	17	0.45	0.93	0.0	116	8	4.2	1.11	3	0.47	15	0.77
80	1	33	0.45	1.16	0.9	209	9	6.1	2.72	29	0.12	0	0.59
80	1	47	0.44	1.03	1.2	200	8	5.2	1.56	46	0.26	14	0.86
81	1	24	0.32	1.18	2.1	149	8	6.2	0.91	NA	NA	0	0.50
81	1	31	0.23	1.33	2.5	146	8	7.5	1.00	NA	NA	0	0.45
88	0	21	0.63	1.78	0.0	214	11	8.7	0.47	13	0.59	0	1.11
88	0	31	0.68	1.54	1.2	206	9	6.9	0.76	3	0.48	0	0.94
89	1	32	0.36	1.48	1.2	284	8	4.4	1.45	32	0.19	2	0.50
89	1	41	0.43	1.50	1.9	178	8	5.8	0.73	6	0.18	4	0.52
90	1	30	0.39	1.13	1.1	232	11	5.1	1.53	3	0.23	0	1.01
90	1	31	0.29	1.19	0.0	175	8	3.6	1.26	3	0.22	0	0.66
91	1	35	0.33	1.18	2.0	179	7	2.9	0.88	4	0.19	11	0.42
91	1	40	0.43	1.30	2.1	213	7	4.8	0.96	26	0.19	9	0.72
92	0	25	0.50	1.07	3.0	274	7	9.4	0.59	9	0.37	3	0.89
92	0	26	0.70	NA	NA	256	NA	NA	NA	9	0.75	0	1.69
93	0	28	0.67	1.19	1.0	228	7	5.0	0.76	10	0.81	0	1.55
93	0	37	0.62	1.28	0.0	258	10	8.9	0.74	12	0.46	0	1.21
94	0	25	0.53	1.32	0.0	204	7	7.2	0.83	3	0.38	2	0.94
94	0	27	0.55	1.24	1.1	277	8	8.7	0.54	13	0.48	6	0.93
95	1	21	0.48	1.01	0.0	197	7	4.3	0.58	9	0.43	0	1.08
95	1	40	0.22	1.19	0.0	190	5	4.8	0.63	NA	NA	0	1.02
96	1	21	0.48	1.01	0.0	197	7	4.3	0.58	9	0.43	0	1.08
96	1	40	0.22	1.19	0.0	190	5	4.8	0.63	NA	NA	0	1.02
97	0	23	0.23	1.94	1.3	155	6	4.4	0.21	6	0.17	0	0.69
97	1	25	0.27	1.55	1.5	129	6	7.4	0.35	6	0.22	0	1.01
98	0	20	0.59	1.44	0.0	174	8	4.6	0.79	5	0.48	2	0.89
98	0	27	0.49	1.26	0.0	264	9	6.9	0.78	12	0.46	0	0.72

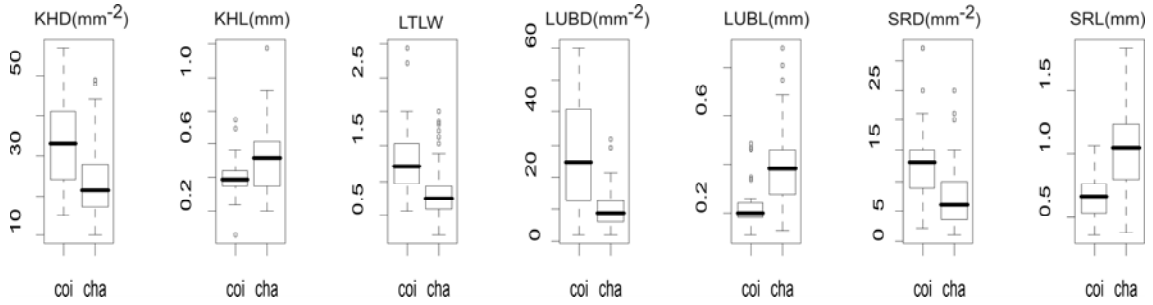
Population	KG	KHD	KHL	LLW	LP	LS	LT	LTB	LTLW	LUBD	LUBL	SRD	SRL
99	0	19	0.75	1.01	0.0	262	12	7.4	0.80	10	0.58	0	1.39
99	0	24	0.62	0.96	0.0	281	12	8.5	0.55	11	0.48	0	1.50
100	0	17	0.49	1.29	0.0	249	8	9.6	0.71	6	0.31	0	0.93
100	0	28	0.51	1.16	0.0	260	9	6.4	0.51	12	0.35	0	0.95
101	0	20	0.33	1.01	0.0	167	9	7.8	1.11	7	0.21	0	1.14
102	1	35	0.31	1.27	0.0	133	7	3.1	1.81	6	0.23	15	0.73
102	1	37	0.32	1.44	0.0	84	5	4.0	1.86	19	0.31	0	0.39
103	0	21	0.75	0.96	0.0	123	7	3.2	0.94	9	0.41	0	1.10
103	0	26	0.45	0.93	0.0	88	5	7.9	0.81	3	0.46	0	1.26
104	1	33	0.27	1.42	2.0	176	6	4.9	0.55	2	0.20	8	0.36
104	1	57	0.05	1.24	1.6	207	7	6.0	1.00	20	0.13	13	0.54
105	0	24	0.54	1.11	0.0	270	10	6.1	1.17	10	0.43	0	1.15
105	0	29	0.55	1.37	0.0	344	11	10.6	0.61	3	0.33	0	1.15
106	0	22	0.60	1.10	0.0	209	12	6.8	1.26	11	0.51	0	1.07
106	0	22	0.57	1.10	0.0	180	8	7.5	1.38	10	0.50	0	1.23
107	1	35	0.36	1.31	1.0	195	7	5.8	0.89	18	0.36	0	1.48
107	1	40	0.25	1.55	1.5	227	7	8.0	0.67	2	0.18	2	1.29
108	0	29	0.76	1.26	0.0	260	7	9.8	1.08	10	0.40	0	0.99
108	0	44	0.56	1.21	0.0	271	8	10.3	0.78	6	0.41	0	0.78
109	0	29	0.76	1.26	0.0	260	7	9.8	1.08	10	0.40	0	0.99
109	0	44	0.56	1.21	0.0	271	8	10.3	0.78	6	0.41	0	0.78
110	0	19	0.48	1.25	NA	338	7	6.8	0.22	9	0.25	0	0.79
110	0	26	1.18	1.17	1.9	500	8	6.8	0.83	NA	NA	1	1.02
111	0	16	0.62	1.33	1.0	255	10	6.6	0.94	11	0.41	0	1.25
111	0	17	0.53	NA	NA	183	NA	NA	NA	5	0.44	0	1.38
112	1	29	0.64	1.12	0.0	332	13	7.5	1.38	30	0.39	17	1.14
112	1	40	0.72	1.03	0.0	353	12	6.8	0.77	23	0.41	13	1.15
113	1	19	0.36	1.32	1.7	58	7	6.6	2.93	18	0.11	6	0.50
113	1	20	0.33	0.88	2.0	250	10	7.8	0.70	15	0.16	0	0.64
114	1	37	0.31	1.13	0.0	212	6	4.9	1.13	13	0.40	0	1.45
114	1	38	0.35	1.20	0.0	206	6	5.2	0.88	11	0.35	0	1.09
115	0	29	0.50	1.12	0.0	206	10	3.3	0.72	20	0.31	0	0.81
115	0	39	0.36	1.67	0.0	254	13	6.2	0.79	11	0.28	0	0.87
116	1	17	0.52	1.20	0.0	296	10	6.0	0.56	15	0.48	0	1.30
116	0	23	0.69	NA	0.0	399	NA	NA	NA	9	0.58	0	1.41
117	0	18	0.58	1.04	0.0	175	8	3.5	0.94	11	0.45	0	1.47
117	0	19	0.53	1.13	0.0	156	8	6.4	0.71	13	0.59	0	1.37
118	0	22	0.65	NA	0.0	NA	NA	NA	NA	11	0.44	0	1.32
118	0	25	0.50	NA	NA	292	NA	NA	NA	NA	NA	0	0.83
119	1	26	0.60	1.35	0.0	229	9	9.1	1.00	30	0.37	22	1.20
119	1	33	0.52	1.07	0.0	189	11	6.8	0.56	21	0.56	19	0.73
120	0	29	0.48	1.00	0.0	240	9	6.3	0.75	12	0.51	0	1.23
120	0	42	0.72	1.36	0.0	319	10	8.6	1.25	12	0.63	8	1.83
122	0	17	0.53	1.31	0.0	293	9	4.3	0.66	13	0.40	0	1.26
122	0	21	0.50	1.36	1.3	274	10	6.2	0.59	10	0.32	0	1.02
123	0	29	0.75	1.25	0.0	174	11	6.7	1.20	15	0.56	10	1.53
123	0	29	0.59	1.31	0.0	120	7	6.4	0.56	15	0.51	5	1.26
124	0	31	0.65	1.24	0.0	206	10	7.5	0.70	5	0.88	11	1.05
124	0	48	0.69	1.33	0.0	264	11	8.5	0.87	15	0.59	0	1.62
125	1	21	0.37	1.34	1.0	145	6	5.8	0.33	17	0.28	0	0.94
125	1	25	0.27	1.44	0.0	252	11	4.9	1.53	9	0.22	0	0.60
126	0	21	0.52	1.48	1.6	173	6	5.0	0.88	9	0.31	0	1.04
127	1	10	0.34	1.36	0.0	NA	7	8.2	0.56	10	0.32	20	0.67
127	1	21	0.34	1.35	NA	112	7	7.2	0.44	16	0.27	0	0.62
128	1	17	0.36	1.38	1.4	93	7	6.4	0.61	4	0.23	5	0.38
128	1	25	0.29	1.44	0.0	115	7	5.2	0.81	NA	NA	0	0.51
129	1	16	0.36	1.18	1.1	179	10	4.8	0.61	9	0.35	6	0.57
129	1	16	0.36	1.62	1.2	NA	8	9.2	0.70	5	0.27	11	0.60

Appendix 4. Correlation of morphological characters with the first two axes derived from a Principal Co-ordinate Analysis (Figure 5) of a matrix of Gower distances among 187 individuals from 98 populations of the *Veronica chamaedrys* group from southeastern Europe.

	Spearman´s rho	
Character	PCoA 1	PCoA 2
Length of hairs on the upper side of the lamina next to the apex of the lamina (LUBL)	0.83***	-0.05 n.s.
Length of hairs on the calyx lobes (KHL)	0.75***	0.02 n.s.
Length of hairs on the two opposite lines of hairs (SRL)	0.77***	-0.21**.
Vertical distance from first tooth to the base of the lamina (negative if lamina base is rounded to cuneate, positive if base is cordate) (LTB)	0.53***	-0.10 n.s.
Length of the style (LS)	0.38***	-0.43***
Number of teeth on one half of the lamina (LT)	0.35***	-0.41***
Density of the hairs between the two opposite lines of hairs on the stem (SRD)	-0.18*	0.07 n.s.
Density of hairs on the upper side of the lamina next to the apex of the lamina (LUBD)	-0.36***	-0.52***
Length/ width of lamina tooth at maximum width line (LTLW)	-0.25***	-0.55***
Density of hairs on the calyx lobes (KHD)	-0.38***	-0.75***
Length of the petiole (LP)	-0.24**	0.44***
Length/ maximum width of lamina (LLW)	-0.10 n.s.	0.27***

* significant at P = 0.05; ** significant at P = 0.01; *** significant at P = 0.001

Appendix 5. Morphological differentiation in the *Veronica chamaedrys* group from southeastern Europe. Boxplots (boxes indicate 25th and 75th percentile, whiskers extend to the 1.5-fold of the interquartile range) are shown for seven morphometric characters that exhibited significant differences (at p=0.001) between the Southern Group as defined by AFLPs (referred to as "coi" in the plot) and the remaining samples, excluding the Krumovii Group ("cha"). KHD, density of the hairs per mm^2 on the calyx lobes; KHL, length of the hairs on the calyx lobes in mm; LTLW, length to width ratio of lamina tooth at maximum width line; LUBD, density of the hairs per mm^2 on the upper side of the lamina next to the apex of the lamina; LUBL, length of the hairs in mm on the upper side of the lamina next to the apex of the lamina; SRD, density of hairs per mm^2 between the two opposite lines of hairs on the stem; SRL, length of hairs in mm on the two opposite lines of hairs.



Appendix 6. Mean and standard deviation (SD) of seven morphometric characters with significant differences on the 0.1% level (t-test). KHD, density of hairs per mm^2 on the calyx lobes; KHL, length of hairs on the calyx lobes in mm; LTLW, length to width ratio of lamina tooth at maximum width line; LUBD, density of the hairs per mm^2 on the upper side of the lamina next to the apex of the lamina; LUBL, length of hairs in mm on the upper side of the lamina next to the apex of the lamina; SRD, density of hairs per mm^2 between the two opposite lines of hairs on the stem; SRL, length of hairs in mm on the two opposite lines of hairs.

	Southern Group		**Western & Eastern Groups**	
character	mean	SD	mean	SD
KHD	33	11	24	9
KHL	0.40	0.12	0.51	0.18
LTLW	1.29	0.54	0.80	0.33
LUBD	26	18	10	5
LUBL	0.24	0.10	0.38	0.14
SRD	15	10	7	5
SRL	0.68	0.18	1.04	0.30

Extensive gene flow blurs species boundaries among *Veronica barrelieri, V. orchidea* and *V. spicata* (Plantaginaceae) in southeastern Europe

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(submitted to Taxon)

ABSTRACT

Little is known about the contribution of interspecific hybridization, a frequent phenomenon in plants, to the high plant diversity in southeastern Europe, one of the continent's diversity hot spots. A good system to study the relevance of hybridization for biodiversity in this region is *Veronica* subg. *Pseudolysimachion* sect. *Pseudolysimachion* (Plantaginaceae). Depending on the presumed frequency of hybridization, existing taxonomic concepts in this group range from distinguishing only morphological races without explicit taxonomic status to recognizing several species each with a series of intraspecific taxa. Using genetic (plastid sequences and AFLP fingerprints), ploidy level and morphometric data, three core groups, pertaining to the currently recognized species *V. barrelieri, V. orchidea,* and *V. spicata*, are congruently identified. All three species are, however, connected by numerous and gradual genotypic transitions and show rampant discrepancies between genetic and morphometric-taxonomic assignments. Complete homogenization of the three core groups is probably prevented by geographic isolation, ecological divergence and ploidy differences. Misinterpretation of these hybrid swarms as separate taxa and the mosaic distribution of different indumentum types have led to a gross overestimation of taxonomic diversity of sect. *Pseudolysimachion* in southeastern Europe. Taxonomically, this might be accommodated by reducing *V. barrelieri, V. orchidea* and *V. spicata* to subspecific rank and abandoning recognition at least of those subspecies and varieties included in our study.

KEYWORDS: AFLP, genome size, hybridization, morphometrics, plastid DNA, polyploidy

INTRODUCTION

Interspecific hybridization is frequent (Rieseberg, 1997; Seehausen, 2004) and involves at least one quarter of plant species (Mallet 2005). Hybridization can affect biodiversity in two counteracting ways. On the one hand, introgression may blur species boundaries in hybrid zones if isolating factors are lacking or cease to exist (Vilà & al., 2000; Suehs & al., 2004; Tovar-Sánchez & Oyama, 2004) and even lead to merging species that were formerly separated (Raudnitschka & al., 2007). On the other hand, hybridization may enhance speciation if hybrid lineages are maintained and stabilized and follow distinct evolutionary trajectories (Mallet, 2008). Mutually not exclusive mechanisms include spatial isolation or ecological selection (Buerkle & al., 2000) sometimes involving extreme hybrid phenotypes (”transgressive segregation”; Rieseberg & al., 2003) as well as allopolyploidization. The latter facilitates genome stabilization and may reduce gene flow via the triploid block (Köhler & al., 2010), although unidirectional gene flow from diploid parents to their polyploid hybrids may still be possible (Chapman & Abbott, 2010). Another effect concerns ploidy level dependent differences in the likelihood of introgression. For example, in the polyploid complex of *Achillea millefolium*, stronger introgression on the tetraploid as compared to the diploid level was suggested (Guo & al., 2005). The outcome of interspecific hybridization thus depends on various factors and may lead to reduced biodiversity by blurring species boundaries and eventually merging of species or to enhanced biodiversity via the formation of new species.

One of the major European biodiversity hotspots is southeastern Europe, in particular the Balkan Peninsula (Turrill, 1929; Horvat & al., 1974; Kryštufek & Reed, 2004). Although this high diversity may be the result of different processes, it is traditionally explained by the limited impact of Pleistocene glaciations, allowing the preservation of a high number of often endemic or disjunct, “relict” species (Turrill, 1929). Inflation of diversity might, however, be caused by misinterpretation of hybrids as separate taxa. In a different geographic setting, this is well exemplified by the Louisiana irises (reviewed in Arnold, 2006), where about eighty species have been described, which are in fact products of interspecific hybridization among three species forming natural hybrid zones when growing in sympatry. Essentially nothing is, however, known about the possible contribution of interspecific hybridization to plant diversity in southeastern Europe.

A good system to study the relevance of hybridization for southeastern European biodiversity is *Veronica* subg. *Pseudolysimachium* sect. *Pseudolysimachion*. This section has its centre of diversity in southeastern Europe (Trávniček, 1998), where it includes in addition to *V. barrelieri* H. Schott ex Roem. & Schult*, V. orchidea* Crantz, and *V. spicata* L. several geographically restricted entities originally described on specific rank, but currently treated as intraspecific taxa of these more widely distributed species (Albach & Fischer, 2003). A fourth species of this group, *V. incana*, reaches southeastern Europe only marginally. Two competing hypotheses have been put forward to explain the high diversity of this group. The ease of successful cross-pollination in particular between individuals of the same ploidy level (Graze, 1933, 1935) prompted Härle (1932) to suggest that hybridization played a major role in the formation of sect. *Pseudolysimachion* and that groups of morphological races ("Formenkreise") connected by transitional forms rather than species should be distinguished. In contrast, later authors (Fischer, 1974; Trávniček, 1998; Albach & Fischer, 2003; Trávniček & al., 2004) maintained several species differentiated from each other mainly by indumentum characters such as the presence of glandular hairs. They acknowledged the role of current and historical gene flow giving rise to interspecific hybrids and taxa with hybridogenic origins (Trávniček, 1998; Albach & Fischer, 2003), respectively. Both processes were, however, regarded insufficient to break down species barriers. A low incidence of hybridization would be in line with differentiation among species, which may be ecological, geographical or, as in case of the regularly co-occurring *V. orchidea* and *V. spicata* (K. Bardy, pers. obs.), cytological, *V. orchidea* being diploid and *V. spicata* being mostly tetraploid (Graze, 1933; Trávniček & al., 2004). These contradicting taxonomic hypotheses have, however, never been tested using genetic data.

Here, we use genetic (plastid sequences and AFLP fingerprints), ploidy level and morphometric data to assess the role of hybridization in affecting diversity of sect. *Pseudolysimachion* in southeastern Europe. Specifically, we address the following questions. (1) Do *V. barrelieri*, *V. orchidea* and *V. spicata* form discrete entities? (2) What is the origin of the geographically restricted taxa on the Balkan Peninsula? Are they relicts or are they of hybridogenic origin? (3) If they are of hybridogenic origin, what is the ploidy level of these taxa? Is there evidence for different levels of introgression on different ploidy levels, in particular stronger introgression on the tetraploid level? (4) How can the inferred patterns and relationships be best reflected in taxonomy?

MATERIAL AND METHODS

Study group. --- Apart from *V. incana* that reaches southeastern Europe only marginally, the study area harbours *V. barrelieri* with five subspecies, *V. orchidea* with one subspecies divided into five varieties, and *V. spicata* with four subspecies (Albach & Fischer, 2003), all of which are included here with the exceptions of *V. barrelieri* subsp. *andrasovszkyi* (Albania and Kosovo), *V. orchidea* subsp. *orchidea* var. *bulgarica* (northern and northeastern Bulgaria and southern Romania) and *V. spicata* subsp. *euxina* (northeastern Bulgaria and maybe adjacent Ukraine). *Veronica orchidea* differs from *V. spicata* and *V. barrelieri* mainly by its taller growth, flowers smelling like burned horn, longer, slightly twisted and recurved petals, the usually glandular hairy calyx, and the indumentum of the upper stem usually consisting of upwardly bent hairs (Albach & Fischer, 2003). *Veronica orchidea* is found in moderately dry to periodically wet grasslands and open oak forests (Fischer, 1974) from eastern Austria to the Black Sea coast and the foothills of the Great Caucasus in Georgia (reviewed in Albach & Fischer, 2003). In contrast to *V. orchidea* and *V. spicata, V. barrelieri* has hairs of a more robust structure occurring only at the rim of the sepals and not on their entire surface, and the petals are more obtuse than those of *V. spicata. Veronica barrelieri* occurs on sunny and dry rocky grassland, and in open oak forests on limestone (Fischer, 1974) and is distributed from the northern and eastern Adriatic coast and adjacent inland areas to Bulgaria and the northern Black Sea coast (reviewed in Härle, 1932). *Veronica spicata* – which is additionally characterized by thin hairs on the stem that are not bent upwards, both stem and lamina are densely pubescent – grows in dry grasslands with shallow soil (Fischer, 1974) and is distributed throughout most of Europe to Central Asia (reviewed in Trávniček & al., 2004).

Sampling. --- *Veronica barrelieri, V. orchidea* and *V. spicata* were sampled in 72 sites (in the following referred to as “populations”, Fig. 1, Appendix 1). Leaf material was collected and immediately stored in silica gel. Voucher specimens are deposited at the Institute of Botany, University of Vienna, Austria (WU, voucher numbers given in Appendix 1) unless stated otherwise. Voucher specimens were determined by a taxonomic expert of this group (M. A. F.) based on Albach & Fischer (2003).

Flow cytometry and DNA extraction. --- DNA ploidy levels (Suda & al., 2006) were estimated from five individuals per population, except for populations 40 and 68, where only

single individuals were found. Flow cytometry was conducted on a CyFlow ML (Partec GmbH, Münster, Germany) equipped with a green laser (Cobolt Samba 532 nm, Cobolt AB, Solna, Sweden) using silica gel dried material and *Pisum sativum* cultivar ´Kleine Rheinländerin` as internal standard. We used propidium iodide staining following the protocol of Baranyi & Greilhuber (1996).

Total genomic DNA was extracted from silica gel dried tissue (c. 10 mg) of two plants per population, where available; in populations with mixed cytotypes one individual per cytotype was analyzed. Extraction followed the CTAB-protocol of Doyle & Doyle (1987) with a few modifications: after precipitation with isopropanol and subsequent centrifugation, the DNA pellet was washed in 70% ethanol, dried at 37°C and re-suspended in TE-buffer. The quality of the extracted DNA was checked on 1% TAE-agarose gels.

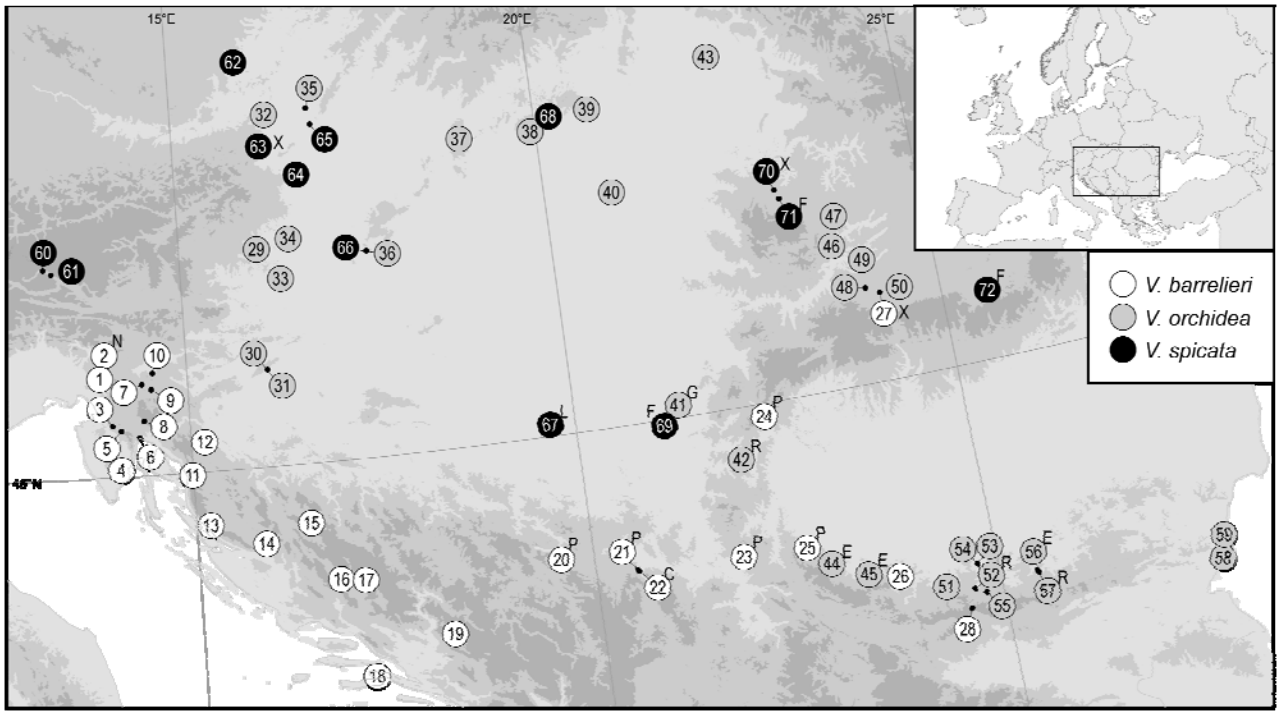

Fig. 1. Sampled populations of *Veronica barrelieri, V. orchidea and V. spicata* in southeastern Europe (details in Table 1). Colors reflect morphology-based determinations. Populations assigned to non-nominal taxa are indicated with superscript capital letters: C = subsp*. crassifolia*, N = subsp*. nitens*, P = subsp*. prodanii*; E = var*. eglandulosa*, G = var. *glandulopilosa*, R = "var*. rumelica*"; F = subsp*. fischeri*, L = subsp*. lanisepala*. Morphologically untypical populations of *V. spicata* and *V. barrelieri* are indicated with superscript X (see Table 1).

AFLPs. --- The AFLP procedure followed Vos & al. (1995) with the modifications described in Schönswetter & al. (2009). Initially, selective primers were screened using 23 primer combinations. The six final primer combinations for the selective PCR (fluorescent dye in brackets) were *Eco*RI (6-Fam)-ACA / *Mse*I-CAC, *Eco*RI (VIC)-AAG / *Mse*I-CAT, *Eco*RI (NED)-AGG / *Mse*I-CAA, *Eco*RI (6-Fam)-ATC / *Mse*I-CTA, *Eco*RI (VIC)-AAG / *Mse*I-CAA, and *Eco*RI (NED)-ACA / *Mse*I-CAT. 5 µl each of 6-FAM, NED and VIC labeled selective PCR products were combined and purified using Sephadex G-50 Superfine (GE Healthcare Bio-Sciences, Uppsala, Sweden) applied to a Multi Screen-HV plate (Millipore, Molsheim, France). 1.2µl of the elution product was mixed with 10µl formamide (Applied Biosystems, Foster City, CA, USA) and 0.1µl GeneScan 500 ROX (Applied Biosystems) and run on an ABI 3130x automated capillary sequencer. Twenty individuals were replicated to calculate the error rate allowing non-reproducible fragments to be excluded from the analysis. Raw AFLP data were aligned with the internal size standard using ABI Prism GENESCAN 3.7.1 (Applied Biosystems), and imported into GENOGRAPHER 1.6.0 (available at http://hordeum.oscs.montana.edu/genographer) for scoring. The error rate (Bonin & al., 2004) was calculated as the ratio of mismatches (scoring of 0 vs. 1) over matches (1 vs. 1) in AFLP profiles of replicated individuals.

The software STRUCTURE 2.2 with a Bayesian clustering approach developed for dominant markers (Pritchard & al., 2000; Falush & al., 2007) was used with an admixture model with uncorrelated allele frequencies and recessive alleles. Ten replicate runs for each K (number of groups) ranging from 1 to 10 were carried out at the Bioportal of the University of Oslo (http://www.bioportal.uio.no/), using a burn-in of 10^5 iterations followed by 10^6 additional MCMC iterations. Similarity among results of different runs for the same K was calculated according to Nordborg & al. (2005) using AFLPsum (Ehrich, 2006). We identified the optimal number of groups as the value of K where the increase in likelihood started to flatten out; the results of replicate runs were identical, and no empty groups were encountered. Replicate runs of the best K were merged with CLUMPP 1.1.1. (Jakobsson & Rosenberg, 2007). The relative 'cluster membership coefficients' of all individuals were then averaged for each population. A Principal Coordinate Analysis (PCoA) based on a matrix of Jaccard distances among individuals was calculated using NTSYS-pc 2.0 (modules SIMQUAL, DCENTER, and EIGEN (Rohlf, 1998)). A Neighbor-joining (NJ) analysis based on a matrix of Nei-Li distances (Nei & Li, 1979) was conducted and bootstrapped employing 2,000 pseudo-replicates with TREECON 1.3b (Van de Peer & De Wachter, 1997). Using the program SPLITSTREE4 ver. 4.6 (Hudson & Bryant, 2006), a NeighborNet was calculated based on the same distance matrix.

Plastid DNA. --- Eight regions of the plastid genome have been tested for variation. The two regions that yielded highest variability, the *trnH-psbA* spacer and the *rps16-trnK* spacer, were sequenced from the same individuals as used for the AFLP analysis using primers *trnH*-F and *psbA*-R (Tate & Simpson, 2003) and *rps16*x2F2 and *trnK*(UUU)x1 (Shaw & al., 2007), respectively. PCR conditions for both regions were 5 min at 95°C followed by 30 cycles of 1 min at 95°C, 1 min at 50°C and 3 min at 65°C, followed by 7 min at 65°C. Reaction volumes were 24 µl, comprising 8 µl REDTaq ReadyMix PCR Reaction Mix (Sigma-Aldrich, Vienna, Austria), 2 µl of each primer (10 µM; genXpress, Wiener Neudorf, Austria), 8 µl of H_2O, and 4 µl of 1:10 diluted template DNA of unknown concentration. The PCR products were cleaned with Exonuclease I and Calf Intestine Alkaline Phosphatase (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. All reactions were carried out on a GeneAmp 9700 thermocycler (Applied Biosystems). BigDye Terminator chemistry (Applied Biosystems) was used according to the manufacturer's instructions for cycle sequencing following electrophoresis on a 3130xl Genetic Analyzer capillary sequencer (Applied Biosystems). Sequences were edited with SEQMAN II v. 5.05 (DNAStar, Madison, WI, USA) and aligned using BIOEDIT 7.0.4.1 (Hall, 1999). All sequences were deposited in GenBank (Appendix 1).

Sequences from the two plastid regions were concatenated based on the assumption that the plastid forms a single linkage group. Phylogenetic analyses were conducted using the approach implemented in BEAST 1.4.8 (Drummond & Rambaut, 2007), as this allows taking into account the genealogical uncertainty due to the stochastic nature of the coalescence process. After inversions have been reverted in the concatenated plastid sequences, the best-fit substitution model was determined using the AIC as implemented in MODELTEST 3.6 (Posada & Crandall, 1998). As the set of models until the cumulative Akaike weight exceeded 0.95 included often non-nested models with at least 2 substitution rates, we finally used a GTR+Γ model subsuming the proportion of invariable sites in the gamma distribution and using Jeffrey´s priors for the substitution model parameters. Since a relaxed clock model was not feasible (lack of convergence and low effective sample sizes [ESS] for many parameters, likely due to over-parameterization), rate evolution was modeled in a strict clock framework. Due to the lack of external calibrations, we used a strong prior on the substitution rate, derived from previously published substitution rates for plastid regions (Smith & al., 2008; Yamane & al., 2003), and modeled it with a normal distribution with a mean and standard deviation of 4×10^{-3} and 2×10^{-3} substitutions per site and million years, respectively. We used the Bayesian skyline plot (Drummond & al., 2005) as the most general demographic model, as it also allows fluctuations in

population size to be detected. Using different group intervals (m=3, 5, 10) gave very similar results (absolute Bayes factors <0.7), and only those with m=3 are shown. Stationarity of the Markov chain, which was run for 3×10^7 generations with sampling every $1{,}000^{th}$ generation, was determined using TRACER 1.4. The first 10% of sampled generations was discarded as burn-in, after which all ESS values were greater than 430. A second run was conducted to confirm convergence of the Markov chain on the stationary distribution. All parameter estimates were based on these two runs combined (54,000 sampling points). A chronogram was constructed based on the majority rule consensus tree calculated using PAUP 4.0b10 (Swofford, 2001) with node heights being the median values of the age estimates.

A statistical parsimony haplotype network was constructed using TCS 1.21 (Clement & al., 2000). For this analysis, insertions/deletions longer than one base pair as well as inversions were coded as single step mutations, and sequence gaps were treated as a fifth character state. Mononucleotide repeats of varying length were excluded, since they are prone to homoplasy at larger geographic scales (Ingvarsson & al., 2003).

Morphometry. --- In 58 of the 72 sampled populations, one or two individuals with generative shoots were available, whose phenological stage allowed scoring of more than half of the morphometric characters. One qualitative and 17 quantitative characters, including one ratio, which are suggested as diagnostic characters in current taxonomic treatments (Albach & Fischer, 2003), were scored. Length characters were measured in mm, hair density in numbers per mm^2; measurements of leaf characters were undertaken on the leaf pair below the one subtending the inflorescence, the stem indumentum was scored on the internodium above this leaf pair. The qualitative character was measured once; quantitative characters other than leaf dimensions, which were measured for both leaves of the respective leaf pair, and shoot height were measured five times and averaged. Of the characters that were highly correlated ($p< 0.001$; Spearman´s rho for quantitative data, logistic regression for qualitative data; both calculated with R 2.10.1; data not shown) only one was included in the analysis. Finally, the following nine characters were excluded: the densities of hairs (1) on the calyx lobes, (2) on their margins and (3) on the upper leaf surface were correlated with the density of hairs on the stem (SHD); lengths of hairs (4) on the calyx lobes, (5) on their margins and (6) on the upper leaf surface were correlated with the length of hairs on the stem (SHL); the (7) length of the corolla tube and (8) the zygomorphy of the corolla (the single qualitative character) were correlated with the length of the corolla (CL); finally, (9) the length of the calyx tube was correlated with the

length of the entire calyx (KL). The remaining nine quantitative characters (Table 1; data matrix: Appendix 3) were used to construct a matrix of Gower similarity coefficients (`gowdis´, Laliberté & Shipley, 2010), which served as the basis of a Principal Coordinates Analysis (PCoA). To check for correspondence of the morphological characters with the first two factors, a test for association between paired samples using Spearman's rho was carried out. All analyses were conducted in R 2.10.1.

Table 1. Morphometric characters employed in an analysis of 58 populations of *Veronica barrelieri, V. orchidea* and *V. spicata* from southeastern Europe.

Abbreviation	Morphological character or ratio
CL	Length of the longest petal
CW	Width of corolla
KCG	Percentage of stalked glandular hairs on sepals
KCS	Percentage of sessile glandular hairs on sepals
KL	Length of the longest sepal
LLW	Length / width of lamina of the leaf pair below the one subtending the inflorescence
PH	Total plant height
SHD	Density of hairs on stem on the internodium below the inflorescence
SHL	Length of hairs on stem on the internodium below the inflorescence

RESULTS

Flow cytometry. --- Flow cytometric analyses yielded histograms with mean CVs of G1 peaks of the sample and internal standard of 11.4% and 5.2%, respectively. DNA ploidy levels inferred from measured genome sizes revealed that DNA diploids (for simplicity in the following referred to as “diploids”) and DNA tetraploids (referred to as “tetraploids”) were present throughout most of the study area (Fig. 2), but tetraploids were absent in the southwest. All populations except population 21 contained only a single cytotype. All three species comprised both cytotypes, yet with different proportions. In *V. barrelieri* and *V. orchidea* diploids predominated, whereas in *V. spicata* diploids were restricted to the two northwestern-most populations (Fig. 2).

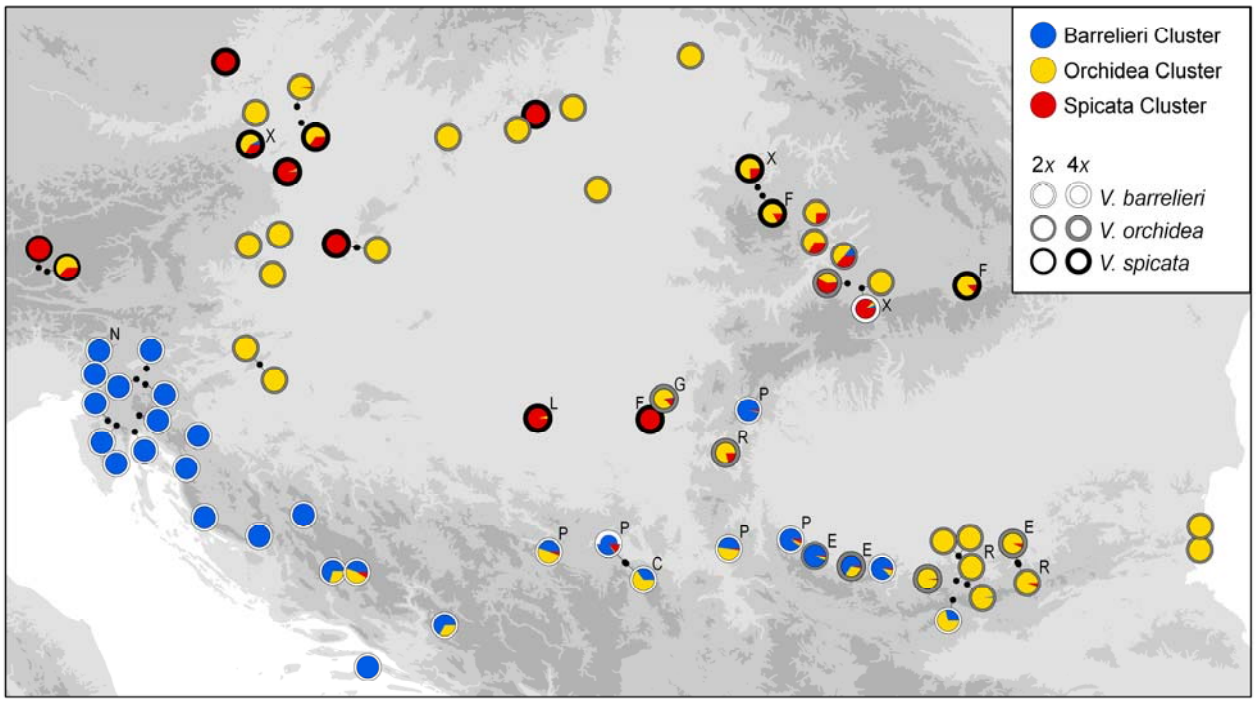

Fig. 2. Distribution of ploidy levels and of the genetic clusters derived from STRUCTURE analysis of AFLP data of *Veronica barrelieri, V. orchidea* and *V. spicata*. Outline colors and thickness reflect morphology-based determinations and ploidy level, respectively; indication of non-nominal intraspecific taxa and of morphologically untypical populations as in Fig. 1.

AFLPs. --- We scored 661 AFLP fragments ranging from 101 to 499 base pairs. The error rate amounted to 1.2% and was thus well within the range deemed acceptable by Bonin et al. (2004). Model-based clustering with STRUCTURE (presented on a geographical basis in Fig. 2) resulted in three clusters, termed in the following Barrelieri Cluster, Orchidea Cluster, and Spicata Cluster. The Barrelieri Cluster mostly occurred in the southwest of the study area. The Orchidea and Spicata Clusters were geographically overlapping to a large extent, the Orchidea Cluster being more frequent towards the southeast. 30% of the investigated individuals were admixed, when admixture was arbitrarily defined as a second or third cluster contributing at least 10% to an individual's gene pool. Most admixed populations possessed significant fractions of the Orchidea Cluster. The non-admixed populations perfectly agreed with the morphologically determined taxa; for instance, all non-admixed populations of the Barrelieri Cluster were from *V. barrelieri*. The reverse was, however, not true: only 66% of plants identified as *V. barrelieri* fell into the Barrelieri Cluster, 72% *V. orchidea* into the Orchidea Cluster, and 52% *V. spicata* into the Spicata Cluster. Admixed populations occurred in all intraspecific taxa except *V. barrelieri* ssp. *nitens* and *V. spicata* subsp. *lanisepala*, but dominated only in the non-nominal taxa (Fig. 3). All three clusters contained diploid and tetraploid individuals. Tetraploids amounted to 88% when the Spicata Cluster was the predominant cluster, but only 24% and 6% of predominating Orchidea and Barrelieri Clusters, respectively, were composed of tetraploids.

The PCoA (Fig. 3) separated the Barrelieri Cluster from the Orchidea and Spicata Clusters along the first factor and the Orchidea Cluster from the Spicata Cluster along the second factor. Whereas non-admixed individuals were well separated along the two axes, admixed individuals occupied intermediate positions. While the nominal taxa *V. barrelieri* subsp. *barrelieri*, *V. orchidea* (subsp. *orchidea*) var. *orchidea,* and *Veronica spicata* subsp. *spicata* overlapped only slightly, the other intraspecific taxa were either found at intermediate positions or completely overlapping with the nominal taxa.

The NeighbourNet analysis indicates a weakly supported separation of *V. barrelieri* (except pop. 27) from *V. orchidea* and *V. spicata* (bootstrap support 65; Appendix 2). Two smaller groups (pops. 16, 17, 19 and pops. 20, 22, 23, 28, respectively) comprise populations with significant admixture between the Barrelieri and the Orchidea Cluster (Fig. 2) and occupy an intermediate position between *V. barrelieri* and *V. orchidea* plus *V. spicata* (Appendix 2). Populations in a taxonomically heterogeneous third group (pops. 21, 25, 27 of *V. barrelieri* and pops. 44, 45 of *V. orchidea*) belong to the Barrelieri Cluster, only population 45 showing

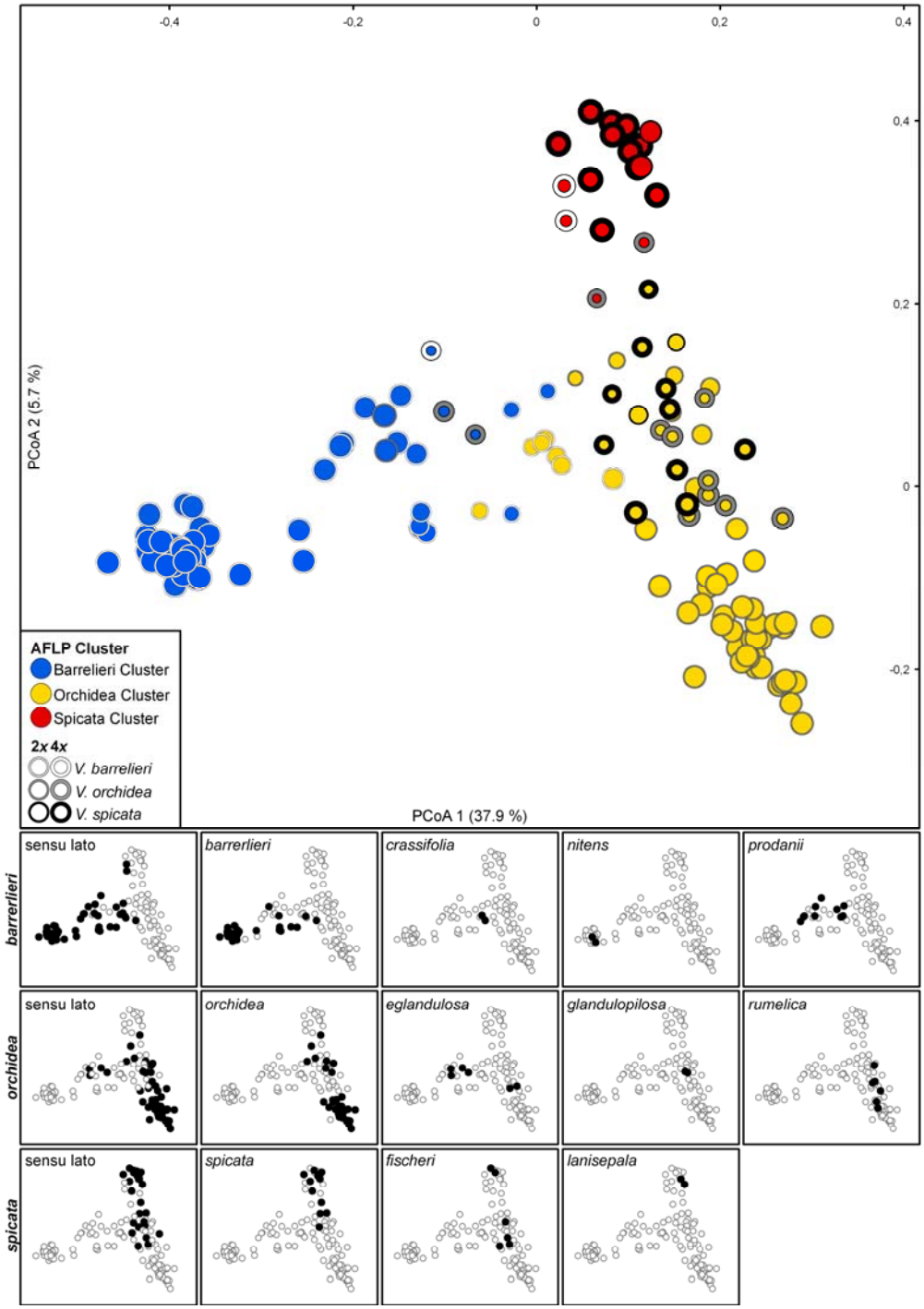

Fig. 3. Principal Coordinates Analysis of a matrix of Jaccard distances based on AFLP data of *Veronica barrelieri, V. orchidea,* and *V. spicata*. Dot colors correspond to three genetic clusters derived from STRUCTURE analysis of AFLP markers (see Fig. 2); dot sizes indicate the proportion of the predominant AFLP cluster (i.e., smaller dots indicate higher admixture). Outline colors and thickness reflect morphology-based determinations and ploidy level, respectively. The small panels highlight the position of the species (left column) and their intraspecific taxa (remaining columns) within the scatter plot.

considerable admixture with the Orchidea Cluster. There is no discernible separation between *V. orchidea* and *V. spicata*.

Plastid DNA sequences. --- The *trnH-psbA* sequences were 267--269 bp and the *rps16-trnK* sequences 780--782 bp long. Concatenating the sequences yielded 39 haplotypes (Appendix 1; Fig. 4). Phylogenetic analysis using BEAST (Fig. 4A) revealed numerous small clades, consisting of one to few haplotypes, with poorly resolved and often insufficiently supported relationships. The only exceptions are a clade consisting of haplotypes from diploid and a single tetraploid *V. orchidea* plus three diploid individuals of *V. barrelieri* from the geographical contact area of *V. orchidea* and *V. barrelieri* (100% posterior probability; Group A) and another clade containing haplotypes found in diploid *V. barrelieri* plus diploid and tetraploid *V. spicata* (85% posterior probability; Group B). The remaining 15 haplotypes are found in tetraploids of all three species and several diploid individuals of *V. barrelieri* and *V. orchidea*. The diversification of the entire group (age given as median and its 95 % highest posterior density interval) occurred 0.59 (0.11--2.28) million years ago (mya), those of Groups A and B at 0.17 (0.03--0.73) mya and 0.22 (0.04--0.88) mya, respectively.

Groups A and B identified by BEAST (Fig. 4A) occupied opposite ends in the parsimony network and were connected by haplotypes of the paraphyletic remainder from the tree-based analysis (Fig. 4B). Both the AFLP Clusters and the two taxa *V. barrelieri* and *V. orchidea* showed good congruence with distinct regions of the haplotype network, whereas *V. spicata* was found in two separate areas of the network (Figs. 4C and D). Non-admixed populations identified from AFLP data had nearly mutually exclusive sets of haplotypes (data not shown), even if these did not form coherent lineages. The most frequent haplotypes belonged nearly exclusively to the Orchidea (haplotypes h32, h34, h35) or the Barrelieri Cluster (h1) with the exception of h22 distributed roughly equally between the Orchidea and the Spicata Clusters (Fig. 4C). Nominal and non-nominal intraspecific taxa in *V. orchidea* and *V. spicata* shared several haplotypes, whereas in *V. barrelieri* most haplotypes where characteristic for a single intraspecific taxon (Fig. 4D).

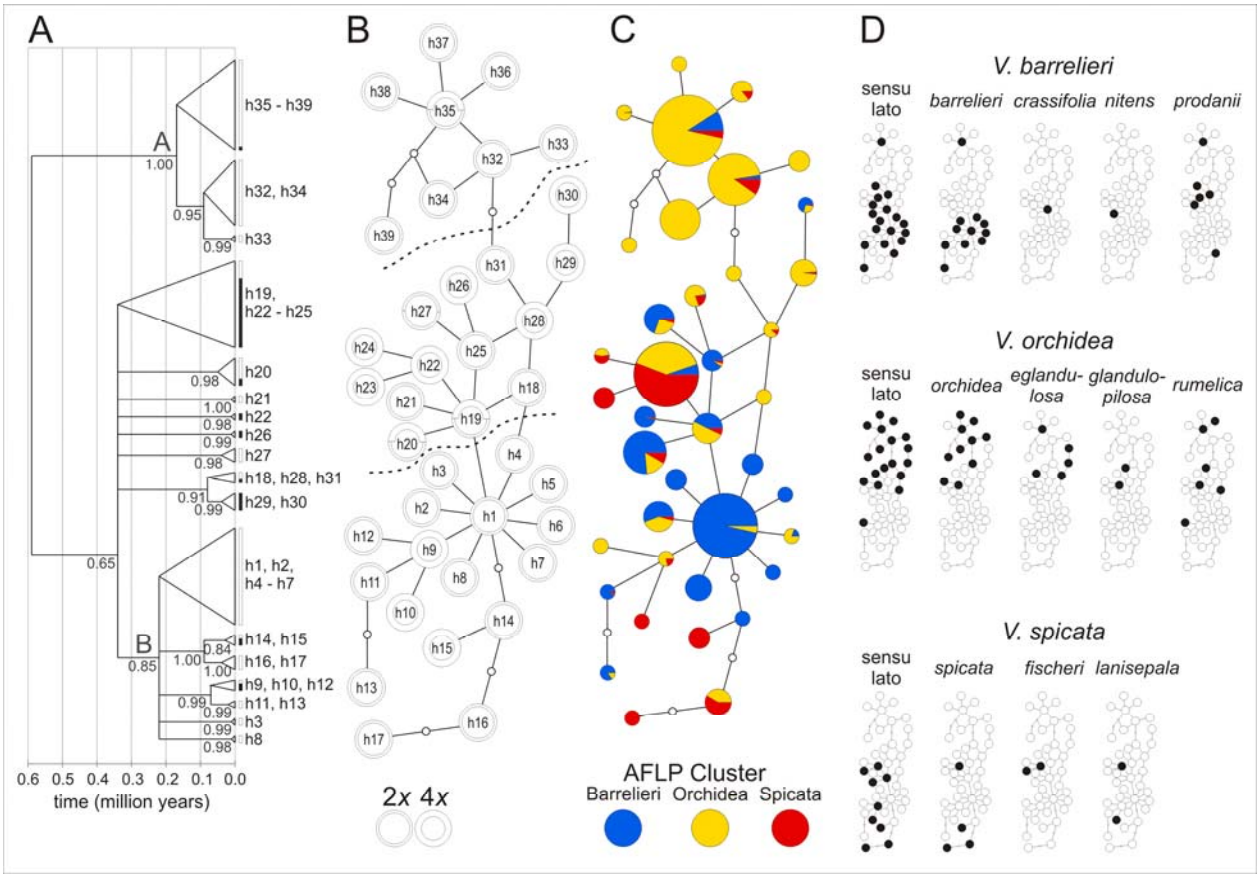

Fig. 4. Plastid DNA haplotypes of *Veronica barrelieri, V. orchidea* and *V. spicata*. (A) Majority rule consensus tree from the BEAST analysis; node heights correspond to median ages. Numbers **along branches are Bayesian posterior probabilities above 0.60; polytomies are collapsed as triangles, their vertical extension being proportional to the number of individuals. The bar depicts the ploidy level of a lineage (white, diploid; black, tetraploid). (B) Statistical parsimony network of the 39 haplotypes (numbered from h1 to h39, haplotypes not sampled shown as small open circles); outline thickness corresponds to ploidy level. Dashed lines indicate borders between Group A, Group B and the paraphyletic remainder as illustrated in (A). (C) Statistical parsimony network as in (B), showing the mean proportions of the three AFLP clusters derived from STRUCTURE analyses in the individual(s) possessing the respective haplotype; the size of the circle corresponds to the frequency of the haplotype (square-root transformed to aid legibility). (D) Positions of the three species (left column) and their intraspecific taxa (remaining columns) in the haplotype network.**

Morphometry. --- The PCoA of the morphometric data set (Fig. 5) showed only partial segregation of the three taxa. *Veronica barrelieri* was separated from *V. orchidea* along the first PCoA factor. In accordance, the majority of individuals of the Orchidea Cluster was, together with the diploid and some tetraploid individuals of the Spicata Cluster, separated from a group constituted by the Barrelieri Cluster and some, mostly tetraploid, individuals of the Orchidea and Spicata Clusters. The first factor was positively correlated with the proportions of stalked and sessile glandular hairs on the calyx, corolla length and the density of hairs on the stem, but negatively correlated with the length of hairs on the stem and the calyx length (Appendix 4). Along the second factor some *V. spicata* samples were separated from *V. orchidea*. This factor was positively correlated with plant height and calyx and corolla lengths, but negatively correlated with the proportion of glandular hairs and the density of hairs on the stem (Appendix 4). The intraspecific taxa of *V. barrelieri* overlapped with each other. Whereas all samples of *V. orchidea* var. *orchidea* except the tetraploid population 48 were clearly separated from the other species, the other intraspecific taxa of *V. orchidea* overlapped with *V. barrelieri*. Samples of *V. spicata* subsp. *spicata* formed a compact group close to *V. orchidea* and were separated along the first axis from the other intraspecific taxa, which instead were spread across the group mainly containing *V. barrelieri*. There was no association between genetic admixture, as identified from STRUCTURE analysis of AFLP data, and morphological intermediacy, as inferred from morphometric data.

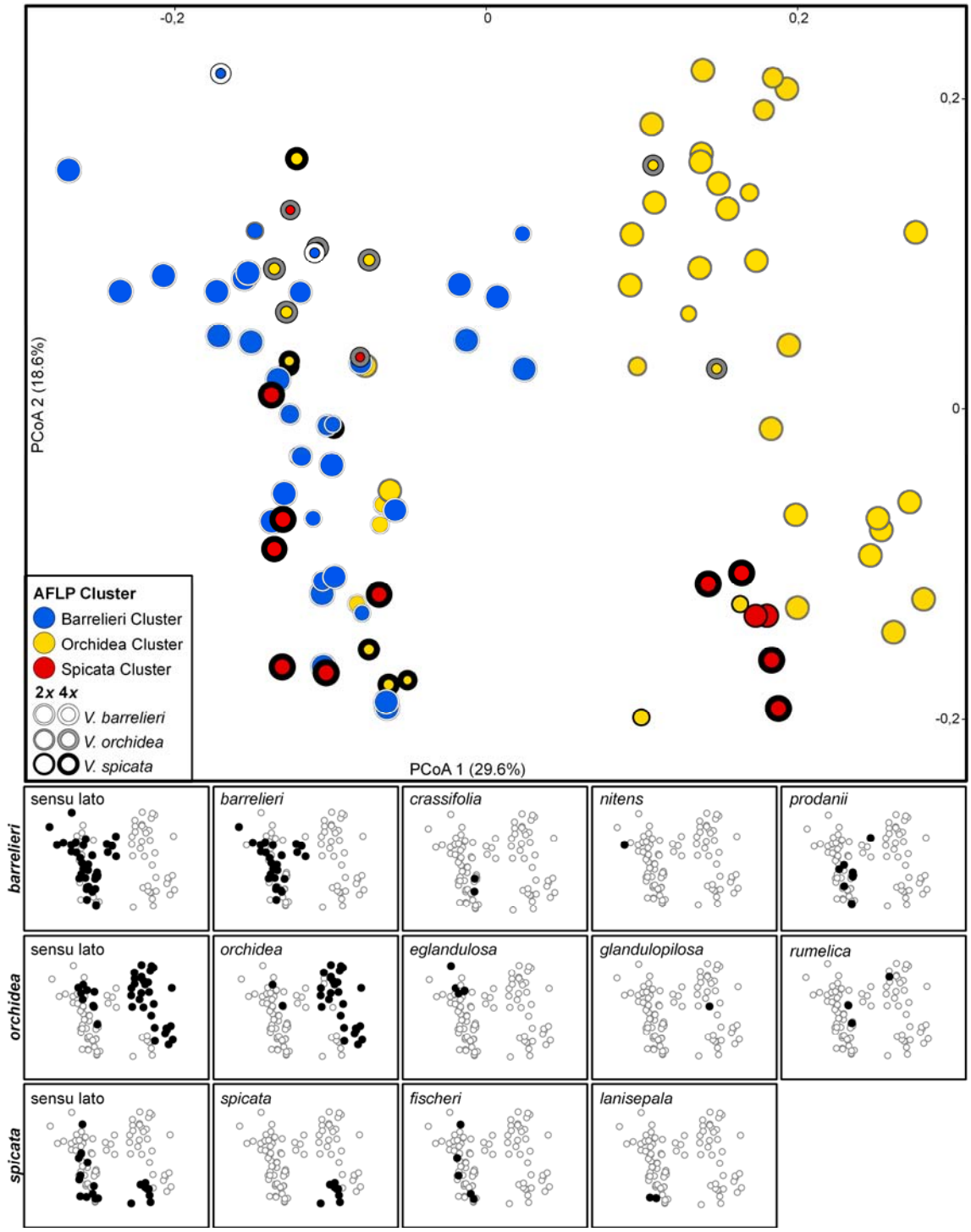

Fig. 5. Principal Coordinates Analysis of a matrix of Gower similarity coefficients based on nine morphological characters of *Veronica barrelieri, V. orchidea* and *V. spicata*. Dot colors correspond to three genetic clusters derived from STRUCTURE analysis of AFLP markers (see Fig. 2); dot sizes indicate the proportion of the predominant AFLP cluster (i.e., smaller dots indicate higher admixture). Outline colors and thickness reflect morphology-based determinations and ploidy level, respectively. The small panels highlight the position of the species (left column) and their intraspecific taxa (remaining columns) within the scatter plot.

DISCUSSION

Hybridization has been long recognised as an important force in the evolution of *Veronica* subg. *Pseudolysimachium*, but its consequences for taxon integrity have been interpreted differently. Based on extensive cross-compatibility, Härle (1932) stressed that in this group hybridization prevented clear-cut recognition of species due to the ample presence of transitional forms. In contrast, later authors (Fischer, 1974; Trávniček, 1998; Albach & Fischer, 2003; Trávniček & al., 2004) did acknowledge the occurrence of current and historical gene flow, but regarded both processes insufficient to break down species barriers. Genetic data from nuclear and plastid genomes as well as morphometric data clearly support Härle's (1932) interpretation. Although three "core" groups, pertaining to *V. barrelieri*, *V. orchidea*, and *V. spicata*, are congruently identified (Figs. 2--5), these are connected by numerous and gradual genotypic transitions (Figs. 2--3, Appendix 2) and are burdened by discrepancies between genetic and morphometric as well as taxonomic assignments (Fig. 5).

Genetic intergrading is best illustrated by the AFLP data, where admixture among gene pools increases with growing distance from the core groups (Fig. 3). Further support for widespread gene flow comes from instances of chloroplast capture. These concern population 52, which was determined as *V. orchidea* and falls into the Orchidea Cluster (Fig. 2), but possesses haplotype h12 that is closely related to and derived from haplotypes typical for *V. barrelieri* (Fig. 4BC), and population 28, which was assigned to *V. barrelieri* and is admixed between the Barrelieri and the Orchidea Cluster (Fig. 2), but possesses haplotype h35 characteristic for and frequent in *V. orchidea* (Fig. 4BC). Chloroplast capture might also be responsible for the occurrence of two divergent haplotype groups in *V. spicata*. Whereas the first group (haplotypes h10, h15--h17) is likely *spicata*-specific (it includes the only two diploid populations [pops. 60 and 61] included in our study), the second group (haplotypes h19, h20, h22, h23) might be connected to *V. incana*, since the haplotype found in the single analysed individual of *V. incana* (from southern Russia) was only four mutational steps from haplotypes h19 and h22 (K. Bardy, unpubl.). Further data of this species, which is widely distributed in eastern European and Asian steppes, but only marginally reaches our study area (Trávniček, 1998) will be necessary to test this hypothesis. Morphological intermediates have been identified before, but mainly within species, such as among the varieties of *V. orchidea* subsp. *orchidea* (Albach & Fischer, 2003: their Fig. 2) or among subspecies of *V. barrelieri* (Albach & Fischer, 2003: p. 417). Additionally, mosaic combinations of indumentum characters led to the suggestion that *V. spicata* subspp. *fischeri* and *lanisepala* as well as *V. orchidea* subsp.

transcaucasica (outside of our study area) might be due to introgression from *V. incana* (Trávniček, 1998; Albach & Fischer, 2003). A similar scenario (introgression from *V. orchidea* subsp. *transcaucasica* or from *V. spicata* subsp. *lanisepala* into *V. orchidea* subsp. *orchidea*) has been suggested to explain the considerable indumentum variability of *V. orchidea* subsp. *orchidea* in Bulgaria and Romania (Albach & Fischer, 2003). In combination with genetic data, it becomes now clear that gene flow among taxa is rampant, but may have remained undetected due to morphological resemblance to a parental taxon (Fig. 5), as has been observed in other hybrid swarms (Lihova, 2007).

Whereas gene flow obviously blurs the boundaries among *V. barrelieri*, *V. orchidea* and *V. spicata* (Figs. 2--4), a combination of geographical isolation and ploidy differentiation appears to be responsible for the presence of three genetic core groups (Fig. 3) and to prevent their total homogenization, rendering them at least to some extent morphologically recognisable (Fig. 5). The predominantly diploid *V. barrelieri* and *V. orchidea* are geographically separated; the former mainly occurs in the Dinaric Mountains on the western Balkan Peninsula, whereas the latter is mostly found in and around the Hungarian Plain and in Bulgaria (Fig. 1). In the contact area (Bosnia and Herzegovina, Serbia and western Bulgaria), populations are admixed (Fig. 2, Appendix 2) in accordance with previously suggested within-ploidy cross compatibility (Härle, 1932). The geographical separation of *V. barrelieri* and *V. orchidea*, which corresponds with strong geographic structure in the genetic data (Fig. 2, 4A--C), might reflect location of different refugia enforced by Pleistocene climate fluctuations (Médail & Diadema, 2009) in line with the inferred diversification ages (Fig. 4A), but a phylogeographic approach with a denser sampling also outside our study area would be necessary to test this hypothesis. In contrast, possible gene flow between (mainly) tetraploid *V. spicata* and (mainly) diploid *V. orchidea*, which in the north of our study area (Austria, Hungary and Romania) can occur in sympatry (sometimes within a few dozens of meters; K. Bardy, pers. obs.), is reduced due to cytological differences, as previously suggested by Härle (1932). In agreement with the hypothesis that inter-cytotype gene flow, if occurring, should be unidirectional from diploids into tetraploids (Chapman & Abbott, 2010), intermixing of gene pools was more frequent in tetraploid individuals than in diploids (Fig. 2).

The majority of the non-nominal intraspecific taxa, originally often described as separate species (reviewed in Albach & Fischer, 2003), occur in areas of geographical contact among *V. barrelieri*, *V. spicata*, and *V. orchidea* (Fig. 2). Among others, the high incidence of genetic

admixture (Fig. 2) and the often genetically intermediate position (Fig. 3) clearly support their hybrid origins. Taxa within sect. *Pseudolysimachion*, whose hybridogenic origin has been deduced entirely from morphology, are known from outside our study area, including *V. semiglabrata* V. M. Ostapko (Ukraine) involving *V. incana* s. l. and *V. barrelieri* s. l. (Ostapko, 1994) and *V. spicata* subspp. *maeoticum* Klokov (Crimea eastwards) and *klokovii* Tzvelev (Caucasus), both involving *V. spicata* s. l. and *V. barrelieri* s. l. (Trávniček, 1998). Given sufficient representation in our sampling, our data provide no evidence that any intraspecific taxa were characterised by specific plastid lineages (Fig. 4), exclusive AFLP clusters (Fig. 3) or coherent morphology (Fig. 5). Therefore, these entities do not represent independent, established lineages, but rather hybrid swarms. Gene flow does not necessarily lead to a morphological intermediate or to novel combinations of morphological characters allowing distinct entities to be recognised, as is evident from the occurrence of genetically admixed individuals also among the nominal intraspecific taxa (Figs. 3, 5). Conversely, not all of the non-nominal intraspecific taxa are of hybridogenic origin; *V. barrelieri* subsp*. nitens* and *V. spicata* subsp*. lanisepala* show no traces of hybridization and genetically overlap with the nominal subspecies (Fig. 3). Their taxonomic recognition is, however, based on single characters, such as organ-specific presence or absence of hairs, which may be under simple genetic control as is the case in *Arabidopsis lyrata* (Kivimäki & al., 2007) and consequently of little taxonomic value. We cannot, however, exclude the alternative hypothesis that *V. spicata* subsp*. lanisepala* originated from hybridization between diploid *V. incana* and diploid *V. spicata* (Trávniček, 1998). Further data including also diploid cytotypes of *V. spicata* subsp. *lanisepala* (Fischer, 1974; we found only tetraploid cytotypes: Appendix 2) and *V. incana* will be necessary to test this hypothesis. Altogether, the existence of hybrid swarms led systematists to overestimate the diversity in sect. *Pseudolysimachion*, as was also the case in the Lousiana irises, where numerous hybrids have been described as separate taxa (reviewed in Arnold, 2006).

There was no clear association between ploidy level and the status of an intraspecific taxon as hybridogenic or not. Whereas some are either di- or tetraploid, such as the diploid *V. barrelieri* subsp. *crassifolia* or the tetraploid *V. spicata* subsp*. fischeri*, others comprise both cytotypes, such as *V. orchidea* var. *eglandulosa* and “var. *rumelica*” (Fig. 2). Generally, we observed a correspondence of elevated ploidy level and admixture, but it was only significant when admixed individuals were defined as having a proportion of the dominant Cluster < 90 % (Fisher´s exact test, P = 0.001), whereas with more stringent requirements for admixture the differences were no longer significant (proportion of the dominant Cluster < 80 %: P = 0.069; <

75 %: P = 0.104). Most tetraploids show high levels of admixture, and only very few possess a single gene pool (Fig. 2). The low number of non-admixed tetraploid populations suggests that tetraploids mostly originated from allopolyploidisation involving distinct diploid lineages rather than mixture after hybridization of autotetraploids. In contrast to other polyploids, which despite their allopolyploid origin possess distinct gene pools separated from the parental gene pools (Dixon & al., 2009), polyploids in sect. *Pseudolysimachion* are not characterised by own gene pools, supporting that polyploid speciation plays no role in the diversification of sect. *Pseudolysimachion*.

Taxonomic considerations

In the most widely applied taxonomic concept (Fischer, 1974; Trávniček, 1998; Albach & Fischer, 2003), sect. *Pseudolysimachion* is separated into several species, including *V. barrelieri*, *V. orchidea*, and *V. spicata*. The high morphological diversity was accounted for by distinguishing intraspecific taxa, usually defined by their indumentum. Härle (1932) found that under experimental conditions species, especially if on the same ploidy level, can easily hybridize. Therefore, he suggested considering those rather as groups of morphological races ("Formenkreise") than as species. Here, we have shown that gene flow among species has been rampant also in the wild, which is likely responsible for the complex morphological patterns in this group and the only weak congruence between genetic and morphological entities (Fig. 5). This might be accommodated by treating *V. barrelieri*, *V. orchidea* and *V. spicata* on the subspecific rank (subspecies of *V. spicata*, which is the oldest available name). The same might be necessary for *V. incana*, once genetic data are available. Furthermore, the subspecies of *V. barrelieri* and of *V. spicata* as well as the varieties of *V. orchidea* ssp. *orchidea* that have been included in our study do not merit taxonomic recognition.

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Appendix 1. Population number, intraspecific taxon, collecting locality, collector and voucher number, coordinates, DNA ploidy level, cpDNA haplotype and GenBank accession numbers of 72 sampled populations of *Veronica barrelieri* H. Schott ex Roem. & Schult*, V. orchidea* Crantz and *V. spicata* L. from southeastern Europe.

Population	Intra-specific taxon [1)]	Locality	Coordinates (E, N)	Ploidy	Haplotype	GenBank accession no. [2)]
barrelieri						
1	barr	SLO: Mt. Čaven (Frajman & Schönswetter 11132)	13°51′16′′, 45°55′28′′	2*x*	h1	### ###
2	nits	SLO: Idrija (Frajman & Schönswetter 11735)	13°54′38′′, 46°07′02′′	2*x*	h20	### ###
3	--	SLO: Mt. Kavčič (Surina, no voucher)	13°59′19′′, 45°28′16′′	2*x*	h20	### ###
4	barr	HR: Raša (Bardy pseu4)	14°03′57′′, 45°03′53′′	2*x*	h1, h4	### ###
5	barr	HR: Lupoglav (Bardy pseu5)	14°06′06′′, 45°25′12′′	2*x*	h1	### ###
6	barr	HR: Rijeka (Bardy pseu6)	14°20′11′′, 45°21′04′′	2*x*	h1, h20	### ###
7	barr	SLO: Cerknica (Frajman 12021)	14°24′00′′, 45°50′25′′	2*x*	h1, h3	### ###
8	--	HR: Gomance valley (Modrić & Surina NHMR 425) [c)]	14°24′37′′, 45°29′58′′	2*x*	h4	### ###
9	barr	SLO: Bloke (Frajman 12025)	14°31′01′′, 45°47′18′′	2*x*	h8	### ###
10	barr	SLO: Draga pri Igu (Frajman & Schönswetter 11790)	14°32′46′′, 45°56′15′′	2*x*	h1	### ###
11	barr	HR: Vratnik pass (Bardy pseu11)	14°59′09′′, 44°58′43′′	2*x*	h1	### ###
12	barr	HR: Ogulin (Bardy pseu12)	15°10′19′′, 45°16′34′′	2*x*	h3, h20	### ###
13	barr	HR: Baške Oštarije (Frajman & Schönswetter 12064)	15°10′40′′, 44°30′20′′	2*x*	h5, h7	### ###
14	barr	HR: Gračac (Bardy pseu14)	15°52′08′′, 44°17′48′′	2*x*	h1	### ###
15	--	BIH: Klekovača (Frajman & Schönswetter, no voucher)	16°27′60′′, 44°27′16′′	2*x*	h1, h19	### ###
16	barr	BIH: Livanjsko polje (Modrić & Surina pseu16)	16°48′03′′, 43°54′35′′	2*x*	h1	### ###
17	barr	BIH: Begovac (Surina pseu17)	17°02′52′′, 43°53′02′′	2*x*	h2	### ###
18	barr	HR: Mt. Sveti Ilija (Frajman & Schönswetter pseu18)	17°06′25′′, 42°59′37′′	2*x*	h1	### ###
19	barr	BIH: Mt. Veliki Velež (Surina pseu19)	18°08′17′′, 43°18′20′′	2*x*	h2	### ###

Population	Intra-specific taxon [1)]	Locality	Coordinates (E, N)	Ploidy	Haplotype	GenBank accession no. [2)]
20	prod	SRB: Kremna (Bardy pseu20)	19°34′40′′, 43°51′43′′	2*x*	h27	### ###
21	prod	SRB: Kraljevo (Frajman & Schönswetter 11719)	20°31′53′′, 43°40′03′′	2*x*, 4*x*	h14, h22	### ###
22	crass	SRB: Kraljevo (Frajman & Schönswetter 11718)	20°32′41′′, 43°39′17′′	2*x*	h19	### ###
23	prod	SRB: Sokobanja (Bardy pseu23)	21°52′26′′, 43°38′19′′	2*x*	h35	### ###
24	prod	RO: Băile Herculane (Modrić & Surina pseu24)	22°25′41′′, 44°53′02′′	2*x*	h21	### ###
25	prod	BG: Belogradčik (Bardy pseu25)	22°40′35′′, 43°37′25′′	2*x*	h25	### ###
26	barr	BG: Vraca (Bardy pseu26)	23°45′39′′, 43°12′31′′	2*x*	h11, h13	### ###
27	-- [a)]	RO: Sibiu (Bardy pseu27)	24°12′24′′, 45°48′36′′	4*x*	h22	### ###
28	barr	BG: Trojan pass (Bardy pseu28)	24°33′57′′, 42°47′28′′	2*x*	h6, h35	### ###
orchidea						
29	orch	A: Fürstenfeld (Bardy pseu29)	16°03′06′′, 47°01′11′′	2*x*	h32, h35	### ###
30	--	HR: Zagreb (Hsuševar, no voucher)	16°03′55′′, 45°53′49′′	2*x*	h34	### ###
31	orch	HR: Zagreb (Bardy pseu31)	16°03′55′′, 45°53′49′′	2*x*	h34	### ###
32	orch	A: Wien (Tod FT020907)	16°18′21′′, 48°15′20′′	2*x*	h35	### ###
33	orch	SLO: Pordašinci (Bardy pseu33)	16°20′28′′, 46°43′42′′	2*x*	h32	### ###
34	orch	H: Körmend (Bardy pseu34)	16°29′18′′, 47°05′36′′	2*x*	h32, h34	### ###
35	orch	A: Marchegg (Bardy pseu35)	16°53′46′′, 48°16′46′′	2*x*	h32	### ###
36	orch	H: Hegyesd (Bardy pseu36)	17°31′49′′, 46°54′12′′	2*x*	h35, h36	### ###
37	orch	H: Nagymaros (Bardy pseu37)	18°57′05′′, 47°50′24′′	2*x*	h32, h35	### ###
38	orch	H: Gyöngyös (Bardy pseu38)	19°56′24′′, 47°49′28′′	2*x*	h31, h32	### ###
39	orch	H: Miskolc (Bardy pseu39)	20°44′23′′, 47°57′10′′	2*x*	h37, h39	### ###
40	--	H: Dévavanya (Bardy, no voucher)	20°54′29′′, 47°09′29′′	2*x*	h35	### ###
41	glan	SRB: Vrašac (Bardy pseu41)	21°21′15′′, 45°07′05′′	4*x*	h22, h26	### ###

Population	Intra-specific taxon [1)]	Locality	Coordinates (E, N)	Ploidy	Haplotype	GenBank accession no. [2)]
42	ruml	SRB: Boljetin (Bardy pseu42)	22°02′28′′, 44°31′33′′	4*x*	h22, h26	### ###
43	orch	UKR: Mukatschewo (Greimler pseu43)	22°29′04′′, 48°15′20′′	2*x*	h34	### ###
44	egld	BG: Montana (Bardy pseu44)	22°56′50′′, 43°26′15′′	2*x*	h27	### ###
45	egld	BG: Vraca (Bardy pseu45)	23°22′41′′, 43°16′57′′	4*x*	h30, h35	### ###
46	orch	RO: Buru (Bardy pseu46)	23°41′53′′, 46°19′09′′	2*x*	h32, h36	### ###
47	orch	RO: Ploscos (Bardy pseu47)	23°48′03′′, 46°35′25′′	2*x*	h35	### ###
48	orch	RO: Sibiliu (Bardy pseu48)	24°01′57′′, 45°52′48′′	4*x*	h22, h24	### ###
49	orch	RO: Valea Lunga (Bardy pseu49)	24°03′27′′, 46°08′31′′	2*x*	h32	### ###
50	orch	RO: Sibiu (Bardy pseu50)	24°12′24′′, 45°48′36′′	2*x*	h33	### ###
51	--	BG: Bulgarski Izvor (Bardy pseu51)	24°18′09′′, 43°01′59′′	4*x*	h29	### ###
52	ruml	BG: Dălbok Dol (Bardy pseu52)	24°39′22′′, 42°57′49′′	2*x*	h12, h18	### ###
53	orch	BG: Levtski (Bardy pseu53)	24°44′56′′, 43°10′49′′	2*x*	h35	### ###
54	orch	BG: Levtski (Bardy pseu54)	24°44′56′′, 43°10′49′′	2*x*	h35	### ###
55	--	BG: Orešak (Bardy pseu55)	24°47′01′′, 42°54′34′′	2*x*	h35	### ###
56	egld	BG: Veliko Tărnova (Bardy pseu56)	25°27′52′′, 43°00′25′′	4*x*	h28, h29	### ###
57	ruml	BG: Veliko Tărnova (Bardy pseu57)	25°28′15′′, 42°59′21′′	2*x*	h35	### ###
58	--	BG: Varna (Albach, no voucher)	27°45′11′′, 42°44′49′′	2*x*	h35, h38	### ###
59	orch	BG: Goritsa (Albach 2007_22)	27°49′13′′, 42°55′01′′	2*x*	h32, h35	### ###
spicata						
60	spic	A: Söbriach (Bardy pseu60)	13°09′34′′, 46°56′18′′	2*x*	h16, h17	### ###
61	spic	A: Obervellach (Bardy pseu61)	13°16′12′′, 46°53′51′′	2*x*	h16	### ###
62	spic	A: Retz (Bardy-Durchhalter pseu62)	15°56′38′′, 48°45′45′′	4*x*	h22	### ###
63	-- [b)]	A: Gainfarn (Bardy pseu63)	16°11′44′′, 47°58′00′′	4*x*	h20	### ###

Population	Intra-specific taxon [1)]	Locality	Coordinates (E, N)	Ploidy	Haplotype	GenBank accession no. [2)]
64	spic	H: Balf (Bardy pseu64)	16°40´37´´, 47°40´37´´	4*x*	h22	### ###
65	spic	A: Mt. Hundsheimer Berg (Bardy pseu65)	16°56´03´´, 48°07´26´´	4*x*	h22	### ###
66	spic	H: Hegyesd (Bardy pseu66)	17°31´21´´, 46°54´17´´	4*x*	h15	### ###
67	lani	SRB: Sremska Mitrovica (Frajman & Schönswetter 11726)	19°40´05´´, 45°06´36´´	4*x*	h10, h22	### ###
68	--	H: Sirok (Bardy pseu68)	20°11´48´´, 47°56´08´´	4*x*	h22	### ###
69	fisc	SRB: Šušara (Bardy pseu69)	21°07´55´´, 44°56´52´´	4*x*	h23	### ###
70	-- [b)]	RO: Fildu de Jos (Bardy pseu70)	23°04´55´´, 46°55´31´´	4*x*	h19; h9	### ###
71	fisc	RO: Izvorului (Bardy pseu71)	23°07´30´´, 46°50´19´´	4*x*	h22	### ###
72	fisc	RO: Braşov (Bardy pseu72)	25°35´34´´, 45°38´02´´	4*x*	h22	### ###

[1)] Populations that could not be assigned to an intraspecific taxon are indicated with "--"; *barr* = *V. barrelieri* subsp*. barrelieri, crass* = *V. barrelieri* "subsp*. crassifolia*" (sensu Albach & Fischer, 2003)*, nits* = *V. barrelieri* subsp*. nitens* (Host) Albach*, prod* = *V. barrelieri* subsp*. prodanii* (Degen) Albach*, orch* = *V. orchidea* subsp. *orchidea* var*. orchidea, egld* = *V. orchidea* subsp. *o.* var*. eglandulosa* (Peev & M. A. Fisch.) Albach*, glan* = *V. orchidea* subsp. *o.* var. *glandulopilosa* (Peev & M. A. Fisch.) Albach*, ruml* = *V. orchidea* subsp. *o.* "var*. rumelica*" (sensu Albach & Fischer, 2003)*, spic* = *V. spicata* subsp*. spicata, fisc* = *V. spicata* subsp*. fischeri* (Trávn.) Albach*, lani* = *V. spicata* subsp*. lanisepala* (Trávn.) Albach.

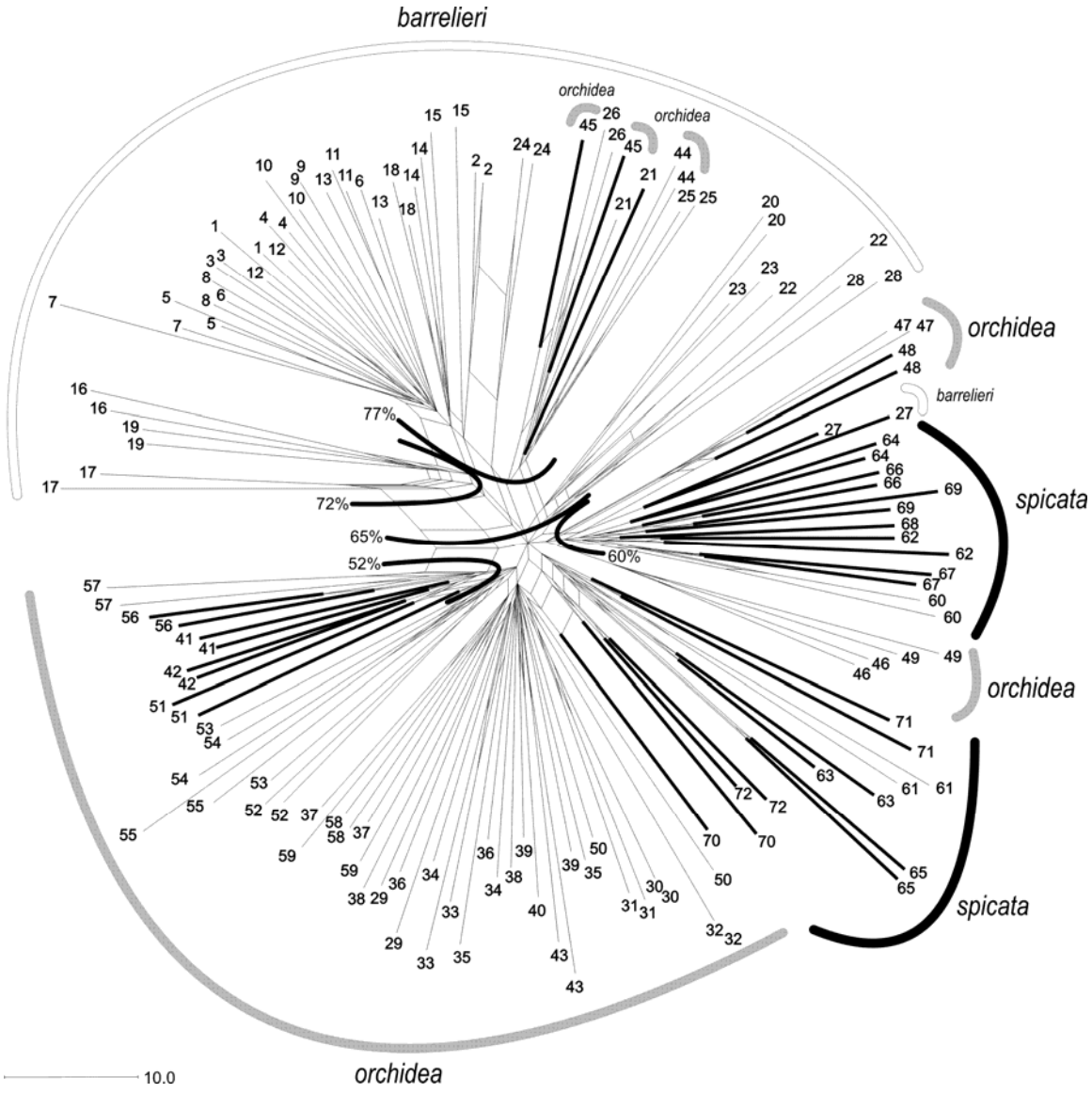
[2)] The first number refers to the trnH-psbA spacer sequence and the second to the rps16-trnK spacer.

[a)] Untypical *V. barrelieri*, in some characters close to *V. orchidea* or *V. spicata.*

[b)] Untypical *V. spicata* (indumentum characters are aberrant).

[c)] voucher specimen deposited at the Natural History Museum Rijeka (NHMR)

Appendix 2. NeighborNet diagram based on a Nei-Li distance matrix of AFLP data constructed for 142 individuals from 72 populations of *Veronica barrelieri, V. orchidea and V. spicata* from southeastern Europe. Splits with weight <0.5 have been omitted to aid legibility. Numbers along branches are bootstrap values above 50% derived from a Neighbor Joining analysis of the same distance matrix and are given for major branches only. Bold branches mark tetraploid individuals.



Appendix 3. Matrix of morphometric characters (see Table 1) employed in an analysis of 58 populations of *Veronica barrelieri, V. orchidea* and *V. spicata* from southeastern Europe; length characters are in mm, density is per mm^2.

Population	PH	LLW	KL	CL	CW	SHL	SHD	KCS	KCG
2	215	5.66	2.67	6.02	2.17	NA	NA	0.00	0.00
4	371	3.50	2.62	4.62	2.50	0.29	28.13	0.00	0.00
4	NA	NA	2.35	5.75	2.25	0.34	31.25	0.40	0.00
5	324	3.25	NA	NA	NA	0.39	12.50	0.00	0.00
5	292	4.47	NA	NA	NA	0.27	68.75	NA	NA
6	404	2.28	NA	NA	NA	0.41	50.00	NA	NA
6	412	2.91	NA	NA	NA	0.33	59.38	NA	NA
7	318	4.11	3.32	4.30	2.55	0.30	25.00	0.40	0.00
7	NA	NA	1.63	4.80	2.55	0.30	65.63	0.60	0.00
9	NA	NA	1.95	3.98	2.37	0.19	34.38	0.00	0.00
9	NA	NA	2.23	3.97	2.60	0.22	56.25	0.60	0.00
10	373	4.98	2.38	4.58	2.45	0.25	21.88	0.60	0.00
11	255	2.51	3.13	4.02	1.87	0.32	28.13	0.00	0.00
11	324	3.26	NA	NA	NA	0.39	46.88	NA	NA
12	295	2.41	2.55	5.12	2.35	0.37	12.50	1.00	0.00
12	216	1.88	2.17	4.25	1.90	0.34	15.63	1.00	0.00
14	309	8.36	2.72	5.55	1.83	0.24	28.13	0.00	0.00
14	335	3.88	2.57	3.90	1.63	0.38	28.13	0.20	0.00
16	264	3.61	2.28	4.68	1.82	0.24	25.00	0.40	0.00
16	238	7.18	2.32	4.08	1.37	0.23	37.50	0.40	0.00
17	239	4.18	2.20	4.48	2.80	0.38	78.13	0.60	0.00
17	229	3.66	2.38	5.02	2.47	0.31	62.50	1.00	0.00
19	220	4.54	2.68	4.47	2.25	0.33	43.75	0.40	0.00
19	208	3.36	2.22	4.47	2.50	0.35	50.00	0.80	0.00
20	454	2.43	2.75	5.20	2.68	0.09	168.75	0.00	0.60
20	515	3.39	1.65	3.65	1.55	0.07	56.25	0.40	0.00
21	267	3.71	2.05	6.18	1.82	0.27	131.25	0.60	0.00
21	462	3.08	2.65	5.45	1.53	0.24	90.63	0.80	0.00
22	387	3.47	1.95	4.73	2.43	0.08	106.25	0.60	0.00
22	225	3.32	2.08	4.52	2.20	0.09	81.25	0.80	0.00
23	391	2.28	2.35	4.88	2.92	0.10	325.00	0.40	0.00
24	285	3.56	NA	NA	NA	0.10	112.50	1.00	0.00
24	299	4.12	NA	NA	NA	0.09	118.75	1.00	0.00
25	385	2.74	2.03	4.67	1.90	0.16	153.13	0.60	0.00
25	237	1.94	2.22	5.32	2.40	0.12	321.88	0.60	0.00
26	334	2.32	2.62	5.20	1.55	0.24	84.38	0.00	0.00
26	428	2.19	3.22	6.45	2.17	0.23	50.00	0.60	0.00
27	310	6.84	2.08	5.07	2.32	0.61	118.75	0.60	0.00
27	201	5.27	2.53	6.07	2.12	0.35	84.38	1.00	0.00
28	397	2.97	2.77	6.05	1.90	0.33	59.38	1.00	0.00
28	225	2.95	2.73	5.15	1.97	0.39	90.63	1.00	0.00
29	NA	NA	2.63	9.57	1.88	0.15	84.38	0.20	1.00
29	NA	NA	2.02	8.35	1.92	0.26	93.75	0.60	1.00

Population	PH	LLW	KL	CL	CW	SHL	SHD	KCS	KCG
31	409	5.93	3.25	9.55	2.53	0.19	75.00	0.00	1.00
32	414	4.03	NA	NA	NA	0.26	115.63	1.00	1.00
32	372	4.69	NA	NA	NA	0.28	115.63	1.00	1.00
33	NA	NA	4.27	9.67	1.87	0.12	56.25	0.60	1.00
34	210	2.86	2.00	7.98	1.68	0.15	100.00	1.00	1.00
34	262	9.61	2.12	6.25	2.27	0.09	184.38	1.00	1.00
35	NA	NA	2.12	5.48	1.47	0.15	100.00	0.60	1.00
35	NA	NA	2.10	6.20	1.73	0.14	218.75	1.00	1.00
36	364	2.74	NA	NA	NA	0.21	84.38	1.00	1.00
36	283	2.75	NA	NA	NA	0.19	168.75	1.00	1.00
37	300	3.45	2.18	6.75	2.07	0.35	118.75	0.00	1.00
37	323	5.21	2.03	5.52	1.98	0.34	175.00	0.00	1.00
38	521	3.50	NA	NA	NA	0.25	112.50	1.00	1.00
39	373	4.39	NA	NA	NA	0.23	106.25	0.40	1.00
41	697	4.13	2.12	6.10	1.50	0.38	137.50	0.60	1.00
42	471	3.87	3.17	5.68	1.42	0.31	137.50	0.00	1.00
43	740	4.27	2.87	6.60	2.37	0.20	128.13	0.20	1.00
43	758	3.70	2.57	7.78	2.63	0.20	84.38	0.40	1.00
44	NA	NA	2.28	6.88	2.05	0.24	153.13	0.00	0.00
45	467	2.76	2.98	8.43	3.68	NA	81.25	0.00	0.00
45	779	4.31	2.22	7.83	2.38	0.38	65.63	0.40	0.00
46	619	4.85	2.77	10.20	2.03	0.31	153.13	0.40	1.00
46	NA	NA	2.55	8.18	2.58	0.17	125.00	0.60	0.60
47	NA	NA	2.28	9.45	2.95	0.15	34.38	0.20	1.00
47	NA	NA	2.55	8.02	1.27	0.21	90.63	0.20	1.00
48	NA	NA	2.75	7.48	2.23	0.20	93.75	0.20	0.00
48	283	5.10	2.12	6.57	0.93	0.16	75.00	0.20	0.00
49	263	2.44	2.22	7.98	2.03	0.27	121.88	0.40	1.00
50	462	4.14	2.33	7.73	2.28	0.14	90.63	0.40	1.00
51	487	3.59	2.65	8.53	2.00	0.22	46.88	0.40	0.00
51	509	3.97	2.80	6.87	1.95	0.63	162.50	0.40	0.00
52	279	3.25	2.43	6.73	2.45	0.23	81.25	0.40	0.00
52	440	5.47	2.52	6.60	3.30	0.27	125.00	0.80	0.00
53	431	3.54	2.12	7.38	1.28	0.12	71.88	0.00	1.00
54	466	3.09	3.17	6.45	2.22	0.20	81.25	0.00	1.00
54	486	3.98	2.97	8.02	3.28	0.15	93.75	0.40	1.00
55	295	3.65	2.57	6.87	2.20	0.15	90.63	0.00	1.00
56	493	4.72	2.60	4.63	0.88	0.26	75.00	0.00	0.00
56	471	2.58	2.90	5.55	1.17	0.21	118.75	0.00	0.00
60	204	5.28	2.05	4.88	1.27	0.14	118.75	1.00	1.00
60	264	5.41	1.98	5.15	1.12	0.14	143.75	1.00	1.00
61	187	3.84	2.00	4.77	2.07	0.10	115.63	1.00	1.00
61	140	3.97	1.73	3.50	1.33	0.13	168.75	1.00	0.60
63	230	4.45	2.42	4.87	1.68	0.31	115.63	0.00	0.00
63	139	5.70	1.75	5.30	1.77	0.18	84.38	1.00	0.00
64	195	5.38	2.10	5.22	2.00	0.28	103.13	1.00	1.00
64	285	4.19	2.17	5.50	2.63	0.13	153.13	1.00	1.00
66	166	6.26	1.65	5.20	2.50	0.12	171.88	1.00	1.00
66	159	6.85	1.70	4.10	2.18	0.12	221.88	1.00	1.00

Population	PH	LLW	KL	CL	CW	SHL	SHD	KCS	KCG
67	246	6.05	2.35	5.83	3.25	0.60	59.38	1.00	0.00
67	484	7.81	NA	NA	NA	0.31	121.88	1.00	0.00
69	505	5.11	2.68	4.75	2.83	0.44	184.38	0.40	0.00
69	391	6.58	2.67	4.77	2.95	0.36	256.25	0.60	0.00
70	187	8.25	1.87	5.75	1.30	0.17	71.88	0.00	0.00
70	194	7.13	2.37	5.55	1.87	0.24	68.75	0.40	0.00
71	403	11.01	3.05	7.50	2.28	0.14	90.63	0.00	0.00
72	163	4.94	2.18	6.23	2.05	0.21	343.75	0.80	0.00
72	280	4.82	1.92	5.83	1.93	0.33	203.13	1.00	0.00

Appendix 3. Correlation of morphological characters with the first two axes derived from a Principal Coordinates Analysis (Fig. 5) of morphometric data from *Veronica barrelieri, V. orchidea* and *V. spicata* from southeastern Europe; significance of Spearman's rho is indicated: n.s., non-significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

	Spearman's rho	
Character	PCoA 1	PCoA 2
Percentage of stalked glandular hairs on sepals (KCG)	0.86***	0.16 n.s.
Length of the longest petal (CL)	0.42***	0.54***
Percentage of sessile glandular hairs on sepals (KCS)	0.42***	-0.88***
Density of hairs on stem on the internodium below the inflorescence (SHD)	0.40***	-0.27**
Length of hairs on stem on the internodium below the inflorescence (SHL)	-0.43***	0.04 n.s.
Length of the longest sepal (KL)	-0.24*	0.58***
Width of corolla (CW)	-0.17 n.s.	0.03 n.s.
Total plant height (PH)	0.02 n.s.	0.61***
Length / width of lamina of the leaf pair below the one subtending the inflorescence (LLW)	-0.02 n.s.	-0.16 n.s.

Conclusions

Southeastern Europe, and the Balkan Peninsula in particular, harbor an elevated level of species diversity (Kryštufek & Reed, 2004) and are therefore well-suited to study patterns and processes contributing to the richness we observe today. The Pleistocene had a strong impact on taxa of the Balkan Peninsula, climate fluctuations led to range expansions and contractions (e.g. Magri & al., 2006; Médail & Diadema, 2009). As each taxon responded independently to climate oscillations (Taberlet & al., 1998), closely related species groups occurring in different habitats – such as the alpine species *Veronica saturejoides*, *V. thessalica* and *V. erinoides*, the woodland species group of *V. chamaedrys,* and the grassland species *V. barrelieri, V. orchidea* and *V. spicata* – are an ideal study system to elucidate these processes.

The alpine taxa *Veronica saturejoides*, *V. erinoides* and *V. thessalica*, which are all endemic to the Balkan Peninsula, occur on different mountain ranges and do not overlap geographically. Plant material of *V. saturejoides* subsp. *munellensis,* one of three subspecies, was not available for analysis. For the other taxa, which are all diploid, incongruence between nuclear (DNA sequences and AFLPs) and plastid data sets could be detected, especially concerning the phylogenetic position of *V. saturejoides* subsp*. saturejoides.* Nuclear sequences and AFLPs revealed monophyly for the two subspecies of *V. saturejoides,* subsp. *saturejoides* and subsp. *kellereri*, with *V. erinoides* as sister to them and *V. thessalica* closely related, but with an unresolved position. Plastid data, on the other hand, detected a monophyletic clade of *V. thessalica* and *V. saturejoides* subsp. *saturejoides,* closely related to them but with unresolved positions were *V. saturejoides* subsp. *kellereri* and *V. erinoides.* The most likely scenario involves introgression from *V. thessalica* into *V. saturejoides* subsp. *saturejoides,* thus leading to chloroplast capture. The position of the inflorescences, a character earlier considered of high taxonomic value in traditional *Veronica* systematic, seems to be of low phylogenetic significance.

Within populations of *V. saturejoides* subsp. *saturejoides* genetic homogeneity of plastid data was detected. The other investigated taxa on the other hand, which occur in the south of our study area – in Albania, Macedonia, Bulgaria and Greece – showed diverse haplotypes even within populations. Higher diversity in the south might indicate the origin of the group in this region. Range expansions therefrom and subsequent speciation probably led to the formation of the present taxa. Pleistocenic restriction of alpine animals to the southern Dinaric Mountains has previously been shown (Sotiropoulos & al., 2007) and this region was also inferred as a

speciation hotspot in *Edraianthus* (Stefanović & al., 2008). Our data thus indicate that *V. thessalica* and *V. erinoides* should be kept as separate species but treated within the same subgenus, in contrast to previous assumptions. The subspecific rank of *V. saturejoides* subsp. *kellereri* could be confirmed.

Within the woodland species group *V. chamaedrys* diploids were predominant in the south, whereas in the north both di- and tetraploids occurred. More pronounced climatic oscillations on the northern Balkan Peninsula triggering massive range shifts of forest vegetation were probably a driving force allowing tetraploid establishment within the *V. chamaedrys* group, whereas more stable conditions on the southern Balkans (Tzedakis & al., 2002) may have prevented it in this region. Both plastid and AFLP data revealed several genetic lineages with distribution centres in the southern, northwestern and eastern parts of southeastern Europe, overlapping with areas of previously proposed forest refugia of associated tree species (Eastwood, 2004; Grivet & Petit, 2003; Heuertz & al., 2006; Willis & van Andel, 2004). As sequence data and AFLPs likely trace different time horizons, gross geographic congruence of the two data sets suggest that refugia at different time scales were located within the same region. The distinctness of the genetic entity from the southern Balkans strongly suggests that hybridisation with other entities was at least rare, which might be due to glaciated mountain ranges of northern Greece (e.g. Hughes & al., 2007) acting as strong barriers. The northern lineage was subsequently disrupted into vicariant lineages in the west and east, a third lineage probably existed in the northeast. These lineages differentiated in phases of isolation in different refugia but came into secondary contact and intermixed in climatically more benign phases when forests became more widespread again. Finally, the *V. chamaedrys* group underwent massive postglacial range expansions. As genetic as well as morphometric analysis do not recover the currently recognized taxonomic entities, we suggest changes to the currently used taxonomic framework of the *V. chamaedrys* group. The only entity that merits taxonomic recognition is *V. ch.* subsp. *chamaedryoides* from the southern Balkan Peninsula. All other taxa should be included in *V. ch.* subsp. *chamaedrys.*

Hybridization between the grassland species *V. barrelieri*, *V. orchidea*, and *V. spicata* has been recognised as an important force in the evolution of the species group already by Härle (1932), who based his conclusions on extensive crossing experiments. Hybridization was, however, not regarded to be sufficient to break down species barriers by later authors (Albach & Fischer, 2003; Fischer, 1974; Trávniček, 1998; Trávniček & al., 2004). Our genetic data from nuclear and plastid genomes as well as morphometric data clearly support Härle's (1932)

interpretation. Although three “core” groups, pertaining to *Veronica barrelieri*, *V. orchidea*, and *V. spicata*, are congruently identified, these are connected by numerous and gradual transitions, supplemented by discrepancies between genetic and morphometric-taxonomic assignments. Genetic intergrading is best illustrated by the AFLP data with admixture among gene pools and is supported by occurrence of chloroplast capture from *V. barrelieri* into *V. orchidea* and *vice versa.* This might also be the case in *V. spicata*, where two divergent haplotype groups occur. Whereas the first group is likely *V. spicata* specific, the second one might be connected to *V. incana.* Morphological intermediates have been identified before, but mainly within species (e.g. Albach & Fischer, 2003). Additionally, mosaic combinations of indumentum characters led to suggestions of introgression among several species or subspecies (e.g. Albach & Fischer, 2003; Trávniček, 1998). Our genetic data indicate, that gene flow among taxa is rampant, but may have remained undetected due to morphological resemblance to a parental taxon, as has been observed in other hybrid swarms (Lihova, 2007). Whereas gene flow obviously blurs the boundaries among *V. barrelieri*, *V. orchidea* and *V. spicata*, a combination of geographical isolation and ploidy differentiation appears to be responsible for the presence of the three genetic core groups and to prevent total homogenization. The predominantly diploid *V. barrelieri* and *V. orchidea* are geographically separated, whereas the geographically overlapping *V. orchidea* and *V. spicata* inhibit different ploidy levels – *V. orchidea* is mainly diploid, *V. spicata* mainly tetraploid. Additionally to the nominal taxa, several non-nominal intraspecific taxa have been described. The majority of these taxa occur in areas of geographical contact among *Veronica barrelieri*, *V. orchidea*, and *V. spicata*. The high incidence of genetic admixture and the often genetically intermediate position clearly support a hybrid origin of most of these non-nominal intraspecific taxa. There was no clear association between ploidy level and the status of an intraspecific taxon as hybridogenic or not. Generally, the low number of non-admixed tetraploid populations suggests that tetraploids mostly originated from allopolyploidisation involving distinct diploid lineages rather than mixture after hybridization of autotetraploids.

As gene flow among species is rampant, which is likely responsible for the complex morphological patterns in this group, we suggest treating *V. barrelieri, V. orchidea*, and *V. spicata* as subspecies. Furthermore, subspecies or varieties included in our study do not merit taxonomic recognition.

All three investigated groups – *V. saturejoides*, *V. thessalica* and *V. erinoides*, the *V. chamaedrys* group, and *V. barrelieri, V. orchidea* and *V. spicata* – show the importance of gene flow and polyploidization in the diversification of taxa in southeastern Europe. They differentiated

allopatrically, but intermixed at least partially when they came into secondary contact. Patterns of diversification on a broader geographical range, however, remain to be tested. Ecological niche modelling of taxa distribution during the Pleistocene will further elucidate their evolutionary history.

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Willis, K.J., & van Andel, T.H. 2004. Trees or no trees? The environments ofcentral and eastern Europe during the Last Glaciation. *Quat. Sci. Rev.* 23:2369–2387.

Curriculum vitae

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Personal data

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Education

since04/ 2006	PhD-thesis *Disentangling the evolutionary history of* Veronica *(Plantaginaceae) in southeastern Europe*, supervised by D. C. Albach, M. A. Fischer & P. Schönswetter at the Department of Biogeography and the Department of Systematic and Evolutionary Botany, University of Vienna
18.10.2005	Master-equivalent (*Magistra rerum naturarum*), graduation with distinction
12/ 2003 – 10/ 2005	Diploma thesis *Establishment of snowbed plant species of the North-Eastern Calcareous Alps*, supervised by G. Grabherr, Department of Conservation Biology, Vegetation and Landscape Ecology, University of Vienna
10/ 1998 – 10/ 2005	Studies of Biology (Botany) with major subject Vegetation Ecology, University of Vienna
01/ 2003 – 09/ 2003	Erasmus student at the University of Bergen and at The University Centre in Svalbard (UNIS), Norway
1990 – 1998	High school (Humanistisches Gymnasium), AKG, Vienna

Working experience

since12/ 2008	Lab work for the project *Landscape-scale dynamics of alpine plants*, Department of Conservation Biology, Vegetation and Landscape Ecology, University of Vienna
01 – 03/ 2006	Supporting financial control and administrative activities, EU-project *IntraBioDiv*, Department of Biogeography, University of Vienna
06/ 2005 – 04/ 2006	Habitat mapping, *Biotopkartierung Oberösterreich*, Arbeitsgemeinschaft Vegetationsökologie und Landschaftsplanung (AVL), Vienna
08 – 09/ 2004	Plant mapping, *Schrankogel Campaign 2004*, Gloria network, Department of Conservation Biology, Vegetation and Landscape Ecology, University of Vienna
08 – 09/ 2001	Practical at the Institute for Plant Cultivation and Cultural Landscape, Agricultural Research and Education Centre, Gumpenstein, Austria

Teaching experience

SS* 2009	Tutor for the course *DNA Marker und Chromosomen in Pflanzen-systematik und Evolutionsforschung*, Department of Biogeographie/ Department of Systematic and Evolutionary Botany, University of Vienna
SS* 2009 & SS* 2008	Tutor for the course *Übungen Systematische Botanik,* Institute of Botany, University of Natural Resources and Applied Life Sciences, Vienna
SS* 2006	Tutor for the project practical *Vegetation and landscape ecology*, Department of Conservation Biology, Vegetation and Landscape Ecology, University of Vienna
since 1997	Environmental and creative educator - Freiraum, Vienna (2000 – 2006) - Grüne Schule, Botanical Garden, University of Vienna (since 2004) - OeAV, Klagenfurt (08/ 2002; 07/ 2005) - Umweltspürnasenclub, Vienna (2000 – 2002) - WWF-Seewinkelhof, Vienna (06/ 2004) Taking care of children and juveniles during excursions and project weeks; planning excursions and workshops; preparing exhibitions.

(*SS = summer term)

Further education

14.-17.03/ 2009	*Biogeographie-Workshop* organized by JuSys, with Isabel Sanmartin (Real Jardín Botánico de Madrid) & Richard Ree (University of Chicago), Institut für Spezielle Botanik und Botanischer Garten, Mainz, Germany
31.01.-09.02/ 2008	*Phylogeographical Analysis* – Workshop by Dorothee Ehrich (Universitetet i Tromsø) & Andreas Tribsch (Universität Salzburg), Fakultät für Naturwissenschaften, University of Salzburg
26.-28.11/ 2007	*Plant Ecological Genetics*, Workshop by Brian C. Husband (University of Guelph, USA), Prague, Czech Republic
14.-15.01/ 2006	*Biodiversität und Footprint* – Seminars for project in schools, WWF, Vienna
05.-08.12/ 2005	*Understanding competition for space*, Workshop by Beáta Oborny, (Eötvös Loránd Tudományegytem, Budapest), Prague, Czech Republic
29.-31.10/ 2004	*Klima in Bewegung* – Seminar for a school project, WWF, Vienna
20.-21.05/ 2003	*Stølskulturlandskapet* (Cultural landscape of summer farms), Workshop for the EU-project *Transhumance*, Høgskulen i Sogn og Fjordane, Sogndal, Norway
12.07-05.08/ 2000	Norwegian language course at the University of Bergen, Norway
2000 – 2005	Pedagogic training courses, *Trainer for social competence*, Freiraum, Vienna

Additional qualifications

English (fluent, written and spoken)

Norwegian (good knowledge)

Good knowledge in MS Office, Adobe Photoshop, CorelDraw, SigmaPlot, R, Arc-GIS, programs for analyses of molecular data (Splitstree, TCS, STRUCTURE, BAPS, BEAST etc), SAP-application, Html, etc.

Voluntary work (1996 – 2007)

- Atlantis – *Working guest* on a farm, Norway (1998)
- Caritas – Helping on a farm, Eastern Tyrol (1997)
- Entwicklungshilfeklub, Vienna (1996)
- OeAV – *Umweltbaustelle (*Environmental construction sites), Northern and Eastern Tyrol (1997 & 1999)
- Service Civil International (SCI) – *Workcamp*, Slovakia (1999)
- WWF, Vienna (1997 – 2007)

Publications

Publications – Articles

Bardy, K. E., D. C. Albach, G. M. Schneeweiss, M. A. Fischer, P. Schönswetter (accepted, pending revision): Disentangling phylogeography, polyploid evolution and taxonomy of a woodland herb (*Veronica chamaedrys* group, Plantaginaceae) in southeastern Europe — Molecular Phylogenetics and Evolution.

Rebernig, C. A., G. M. Schneeweiss, **K. Bardy**, P. Schönswetter, J. Luis Villaseñor, T. F. Stuessy, H. Weiss-Schneeweiss (accepted, pending revision): Multiple Pleistocene refugia and Holocene range expansion of an abundant southwestern American desert species — Molecular Ecology.

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Articles in review

Bardy, K. E., P. Schönswetter, G. M. Schneeweiss, M. A. Fischer & D. C. Albach (in review): Extensive gene flow blurs species boundaries among Veronica barrelieri, V. orchidea and V. spicata (Plantaginaceae) in southeastern Europe — Taxon.

Hülber, K., S. Dullinger & **K. Bardy** (in review): Effects of snowmelt timing and competition on the performance of alpine snowbed plants — Perspectives in Plant Ecology, Evolution and Systematics.

Talks

5th Balkan Botanical Congress (Belgrade, Serbia). 7.-11. September 2009. **Bardy,K.,** P. Schönswetter, M. A. Fischer & D. C. Albach: *Multiple origins of tetraploid* Veronica chamaedrys *on the Balkan Peninsula*.

Symposium *Flora in Vegetacija Slovenije 2008* (Ljubljana, Slovenia). 17.-20. Oktober 2008. **Bardy**, **K.,** P. Schönswetter, D. C. Albach: *Phylogeography on the Balkan Peninsula – two examples from* Veronica *(Plantaginaceae).*

13. Österreichisches Botanikertreffen (Salzburg, Austria). 11.-13.September 2008. **Bardy**, **K.,** D.C. Albach, P. Schönswetter, & M.A. Fischer: *Phylogeography on the Balkan Peninsula – two examples from* Veronica *(Plantaginaceae).*

Xth Symposium of the International Organization of Plant Biosystematists (Štrbske Pleso, Slovakia). 2.-4. Juli 2008. **Bardy**, **K.,** P. Schönswetter, D. C. Albach & Manfred A. Fischer: *Phylogeography on the Balkan Peninsula – examples from* Veronica *(Plantaginaceae)*.

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Wissenschaftstag 2005 des Vienna Ecology Centres (Vienna, Austria). 31.März 2005. **Bardy, K.**, Durisin, N, Dullinger, S, Hülber, K. & G. Grabherr: *Germination and establishment of snowbed plant species of the North-Eastern Calcareous Alps.*

Poster

XIII.Optima Congress (Antalya, Turkey), 22.-26.March 2010. “Multiple origins of tetraploid *Veronica chamaedrys* on the Balkan Peninsula”. **Bardy, K.**, P. Schönswetter, M.A. Fischer & D.C. Albach

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BSS Symposium *History, Evolution and Future of Arctic and Alpine Flora* (St. Andrews, Scotland, UK). 25.-27.Juni 2007. **Bardy**, **K.,** D.C. Albach, P. Schönswetter, & M.A. Fischer: *Speciation on the Balkan Peninsula – examples from* Veronica *(Plantaginacae).*

9. Jahrestagung der Gesellschaft für Biologische Systematik (Vienna, Austria). 20.-23.Februar 2007. **Bardy**, **K.,** D.C. Albach, P. Schönswetter, & M.A. Fischer: *Speciation on the Balkan Peninsula – examples from* Veronica *(Veronicaceae).*

IV Balkan Botanical Congress (Sophia, Bulgaria). 20.-26.Juni 2006. Albach, D. C., **K. Bardy**, M. von Sternburg, P. Schönswetter & M. A. Fischer: *Phylogeography of* Veronica *on the Balkan Peninsula - an ongoing project.*