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# „Multigene approach on the Phylogeny of Scaphopoda (Mollusca)“

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# Abstract

Although scaphopods have a worldwide marine distribution, they are still little studied. Above all, the evolutionary relationships of the approximately 570 species are still unclear today. In particular, the monophyly of several genus- und family-level taxa is uncertain. Previous studies based on morphological or genetic data suffer from a lack of informative characters and/or low taxon sampling. The present study increases the number of taxa and genetic markers (*CO1*, *16S* rRNA, *18S* rRNA and *28S* rRNA), with a focus on Dentaliida, allowing also to test the monophyly of some of the represented genera and families.

The phylogenetic trees include 23 genera from ten of the fourteen currently described families of Scaphopoda, allowing a more comprehensive analysis with improved resolution of the evolutionary relationships of scaphopods. There is support for a clade of the smooth-shelled taxa Anulidentaliidae, Gadilinidae, Laevidentaliidae, and Rhabdidae, being the sister group to the mostly ribbed Dentaliidae and Calliodentaliidae. The trees also show the dentaliid genera *Antalis* and *Fissidentalium* para- or polyphyletic. Similarly, the monophyly of most genera in the Gadilida is not supported, although the data is limited. The present study emphasizes that genus und family assignment based on shell characters alone is prone to error due to parallelism and convergence and needs to be supplemented by DNA data.

# Zusammenfassung

Obwohl Scaphopoden weltweit im Meer verbreitet sind, sind sie noch wenig erforscht. Vor allem die evolutionären Beziehungen der rund 570 Arten sind heute noch unklar. Insbesondere die Monophylie mehrerer Taxa auf Gattungs- und Familienebene ist unsicher. Frühere Studien, die sich auf morphologische oder genetische Daten stützen, leiden unter einem Mangel an informativen Merkmalen und/oder einer geringen Anzahl von Taxa. In der vorliegenden Arbeit wurde die Anzahl der Taxa und der genetischen Marker (*CO1*, *16S* rRNA, *18S* rRNA und *28S* rRNA) erhöht, wobei der Schwerpunkt auf den Dentaliida liegt, sodass auch die Monophylie einiger der vertretenen Gattungen und Familien geprüft werden kann.

Die phylogenetischen Bäume umfassen 23 Gattungen aus zehn der vierzehn derzeit beschriebenen Familien der Scaphopoda, was eine umfassendere Analyse mit verbesserter Auflösung der evolutionären Beziehungen der Scaphopoden ermöglicht. Es gibt Unterstützung für eine Klade der glattschaligen Taxa Anulidentaliidae, Gadilinidae, Laevidentaliidae und Rhabdidae, die die Schwestergruppe der meist gerippten Dentaliidae und Calliodentaliidae bilden. Die Bäume zeigen auch, dass die Dentaliidae-Gattungen *Antalis* und *Fissidentalium* para- oder polyphyletisch sind. Auch die Monophylie der meisten Gattungen bei den Gadilida wird nicht bestätigt, obgleich die Daten dazu begrenzt sind. Die vorliegende Arbeit unterstreicht, dass die Zuordnung von Gattungen und Familien allein anhand von Schalenmerkmalen aufgrund von Parallelität und Konvergenz fehleranfällig ist und durch DNA-Daten ergänzt werden muss.

# 1. Introduction

## 1.1. Scaphopoda

Scaphopoda is a phylogenetically little studied taxon of infaunal marine molluscs which live burrowing in sediment from intertidal to abyssal. They are characterised by their conical, tube-shaped shell and captacula, which are tentacle-like, cerebrally innervated head appendages (Shimek, 1988; Lamprell & Healy, 1998; Reynolds & Steiner, 2008). Their shell has a slight to strong curvature with the convex side being ventral. The larger aperture of the shell is anterior and the smaller one posterior (Reynolds & Steiner, 2008). Using their foot protruding from the anterior opening of the shell, scaphopods actively burrow and create a feeding cavity in which they feed mostly on foraminifera (e.g. Poon, 1987; Shimek, 1990; Langer et al., 1995; Glover et al., 2003; Gudmundsson et al., 2003; Dantas et al., 2017). The head is weakly differentiated and houses a large stereoglossate radula in the buccal cavity. Scaphopoda lack gills, ctenidia, osphradia, a differentiated heart, and hypobranchial glands (Lamprell & Healy, 1998; Reynolds, 2002; Reynolds & Steiner, 2008). The taxonomic description of species of the Scaphopoda is mostly based on shell morphology and only for a few species on radula morphology (Scarabino, 1995; Lamprell & Healy, 1998). In two recent comprehensive catalogues, 517 extant species of Scaphopoda were listed and reviewed (Steiner & Kabat, 2001; 2004). Since then, around 50 species were described (e.g. Scarabino & Scarabino, 2010; 2011; Sahlmann, 2012; Martínez-Ortí & Cádiz, 2012; Sahlmann et al., 2016; Souza et al., 2020), adding up to around 570 recent species.

## 1.2. The position of Scaphopoda within Mollusca

There has been a long debate about the phylogenetic relationships of the Scaphopoda within the phylum Mollusca since the late 19th century. Based on morphological characters two opposing concepts were proposed (for summary: Steiner & Dreyer, 2003). The Helcionellid concept places fossil Helcionellida (Monoplacophora) as stem group to both Scaphopoda + Cephalopoda with Gastropoda as their sister group (Waller, 1998). Opposed to that is the Diasoma-Cyrtosoma or Loboconcha-Visceroconcha concept, which places Scaphopoda as sister group to Bivalvia (Runnegar & Pojeta, 1974; Salvini-Plawen, 1990). Problematic is the fossil record, since assignment of many specimens previously thought to belong to the Scaphopoda seem to be unreliable and are presumably fossils of Cephalopoda (for summary: Reynolds & Steiner, 2008). Currently, the oldest accepted fossil belonging to the Dentaliidae is from the Carboniferous (Yochelson, 1999), and the oldest described gadilid fossil is from the Paleogene (Emerson, 1962). This results in a  gap of at least 126 million years to the origin of most other Mollusca which are thought to be from the Cambrian (Wanninger & Wollesen, 2019; Kocot et al., 2020). More recent studies based on molecular genetic data suggest that Scaphopoda are more closely related to Gastropoda and Bivalvia. Either as sister group to (Bivalvia + Gastropoda) (Kocot et al., 2011) or closer related to Gastropoda as ((Gastropoda + Scaphopoda) + Bivalvia) with Cephalopoda as sister group to all other Conchifera (Smith et al., 2011; Kocot et al., 2020).

## 1.3. Phylogeny of Scaphopoda

The phylogenetic relationships within the Scaphopoda are poorly resolved. Independently, Starobogatov (1974) and Palmer (1974) proposed a new classification, separating the order Gadilida Starobogatov, 1974 from the Dentaliida Da Costa, 1798 based on a series of morphological characters (Reynolds & Steiner, 2008). Based on a cladistic analysis of characters of the pedal musculature, which was performed with a broad sample of scaphopod taxa, Gadilida were further subdivided into the suborders Entalimorpha and Gadilimorpha (Steiner, 1992a; 1992b). Since then a number of studies concerning the phylogeny of Scaphopoda, mostly based on a broad set of morphological characters were published (Steiner, 1996; 1998; 1999; Reynolds, 1997; Reynolds & Okusu, 1999). All these phylogenetic studies, except that of Reynolds (1997), clearly support the separation of Dentaliida and Gadilida. However, these studies struggle to reveal clear phylogenetic relationships on family and genus level. For instance, a strict consensus tree in the study by Steiner (1998, Fig. 4), based on a sample of 40 scaphopod OTUs (operational taxonomic units) and a morphological character matrix with 25 characters coded on species level, shows low resolution, pointing out the lack of informative characters. A previous study by Reynolds (1997) comes to a similar result. Reynolds and Okusu (1999) tried to increase the resolution by raising the taxon sampling and coding their character matrice on family level based on 34 morphological characters. However, the authors themselves point out that it is questionable whether those families are actually monophyletic (Reynolds & Okusu, 1999). The difficulty in assessing the monophyly of families and genera of Scaphopoda is primarily because species are classified almost exclusively on the basis of shell morphology (Scarabino, 1995; Lamprell & Healy, 1998). Although classification is well applicable to some species groups, possible convergences may be overlooked due to the low complexity of shell sculpture (Reynolds & Steiner, 2008).

Lamprell and Healy (1998, p. 10) describes the exclusive use of shell characters to assign species to genera "”workable” in the absence of comparative anatomical information”. For example, they chose to not recognise the genus *Antalis* H. Adams & A. Adams, 1854 since there is no clear differentiation to the genus *Dentalium* Linnaeus*,* 1758 based on shell characteristics (Lamprell and Healy, 1998, p. 173). Therefore, the question arises why there are not more informative morphological characteristics. The examination of some smaller morphological characters can be problematic since it requires a very good to excellent condition of examined specimens (Steiner, 1998). Some characters can only be found by using an approach with multiple different microscopic methods (Reynolds & Okusu, 1999). Also, there are several characters whose homology is questionable and it could be that they are a result of convergent evolution (Steiner, 1998). Other characters are simply not well enough examined in regard of their taxonomic and phylogenetic value (Reynolds, 1997; Lamprell & Healy, 1998; Steiner, 1998; Reynolds & Okusu, 1999). Not being able to assign the species correctly to genera or families itself then questions the coding of morphological characters above species level. So there is a need for more data collection, improvement of information on the phylogeny of Scaphopoda, and following the establishment of new morphological characters (Reynolds, 1997; Lamprell & Healy, 1998; Steiner, 1998; Reynolds & Okusu, 1999; Reynolds & Steiner, 2008).

Two studies using DNA sequence data were published. Steiner and Reynolds (2003) is based on the *cytochrome c oxidase subunit 1* (*CO1*) gene, whereas Steiner and Dreyer (2003, Fig. 3) is based on the *18S* rRNA gene. Like the studies based on morphological characters, both analyses show strong support for Dentaliida and Gadilida. However, the analysis based on the *CO1* gene does not support the monophyly of Gadilimorpha and shows the genus *Antalis* and the family Dentaliidae Children, 1834 paraphyletic (Steiner & Reynolds, 2003). In contrast, the study based on the *18S* rRNA gene supports the monophyly of Gadilimorpha and Entalimorpha, but also shows the taxa *Antalis* and Dentaliidae paraphyletic (Steiner & Dreyer, 2003). The *18S* rRNA tree reveals distinct branch length differences in Dentaliida and Gadilida. Steiner and Dreyer (2003) also point out that the genetic distances in the *18S* rRNA gene within families (especially Dentaliidae) are surprisingly small and yield little signal. However, both studies are limited by the small taxon sample. Collecting live specimens for morphological or molecular analyses of a broad range of taxa often requires research vessels, as the diversity peak of scaphopods is below 100 m depth (Steiner & Kabat, 2004).

### 1.4. Aim of the study

Therefore, this study especially aims to expand the molecular phylogenetic taxon sample of Scaphopoda by using museum material with a focus on dentaliid species. A combination of mitochondrial and nuclear markers is used to gain more insight into their phylogenetic signals in Scaphopoda. This increased taxon sample will allow to reconstruct the scaphopod phylogeny more accurately and to test the monophyly of the represented family- and genus-level taxa.

## 2. Material and Methods

### 2.1. Sampling and morphological identification

In total 35 individual scaphopods were processed, from which 19 specimens were obtained from the Muséum national d'histoire naturelle (Paris, Île-de-France, France). Additionally, 7 specimens were collected in the Sea of Japan, 8 specimens in the North Atlantic Sea off Roscoff (Finistère, France), and 1 specimen in the Mediterranean Sea off Rovinj (Istria, Croatia) (Appendix 1). Specimens were stored in ethanol (96%) and identified by their shells (Boissevain, 1906; Scarabino, 1995; Lamprell & Healy, 1998; Scarabino, 2008; Sahlmann et al., 2016). A portion of the foot or the whole soft body was sampled for DNA extraction depending on the size of the animal.

### 2.2. DNA extraction

For the DNA extraction of most samples the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) was used, following the manufacturers protocol. However, as a modification the DNA was eluted in 50µl instead of 200µl to gain a higher concentrated DNA solution. Very small samples were extracted using the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) following the manufactures protocol.

### 2.3. Amplification & primer design

For this study, the following genetic markers were examined: *18S* and *28S* rRNA, *internal transcribed spacer* (*ITS*, consisting of *ITS1*, *5,8S* rRNA and *ITS2*), *16S* rRNA, and *cytochrome c oxidase subunit 1* (*CO1*). Primers from the literature were chosen for the *CO1* gene, *18S* rRNA gene, *28S* rRNA gene, and *16S* rRNA gene based on the best fitting for Scaphopoda (Table 1). The forward primer for *16S* rRNA and the primer on the 3' end of *28S* rRNA were modified. Additionally, 8 new primers for the *28S* rRNA gene and 4 new primers for the *ITS* region were designed using AmplifX 1.7.0 (Jullien, 2019) (Table 1). Due to possible fragmentation of the DNA from the museum material, the *18S* and *28S* rRNA gene fragments each were divided into 3 and the *ITS* into 2 overlapping fragments. Since there were differences between Dentaliida and Gadilida in the *28S* rRNA gene, it was necessary to design some taxon specific primers (Table 1). PCRs were done using the Biozym Red HS Taq Master Mix (Biozym, Hessisch Oldendorf, Germany) and using a Biometra TProfessional Standard Gradient Thermocycler (Biometra, Göttingen, Germany). When testing the new primers, it became apparent that it was problematic to obtain the expected fragments of the *ITS* region, as they were much longer than expected.  Therefore, only a few test sequences were obtained using a cloning approach. As cloning procedure, the pGEM®-T Easy Vector System (Promega, Madison, Wisconsin, USA) was used following the manufacturers protocol. Vector positive colonies were chosen randomly. Lysis and extraction of plasmids were done by adding nuclease free water and a denaturation step (95°C for 5 min) in a PCR cycler. Amplification of vectors was done using PCR conditions (Table 1). Every PCR product was checked by agarose gel electrophoresis for correct length and quality. Gels were made with LE Agarose (1,3 %; Biozym, Hessisch Oldendorf, Germany), dissolved in either TRIS-Borat-EDTA or TRIS-Acetat-EDTA buffer and 4,4 µl SYBR Gold nucleic acid gel stain (Invitrogen, Paisley, United Kingdom) per 100 ml buffer. 3 µl PCR product and 1,2 µl DNA-ladder Quantitas Fast DNA Marker (Biozym, Hessisch Oldendorf, Germany) were used. Gel electrophoresis runs were done with 100V for 40 min. Although all primers work well, some sequences are incomplete as a result of degraded template DNA.

**Table 1:** List of primers and their sequences (top) and annealing temperatures for the primer pairs (bottom).

| **Fragment** | **Name of Primer** | **Sequence 5'-3'** | **Source** | **Note** |
|---|---|---|---|---|
| *CO1* | LCO1490 | GGTCAACAAATCATAAAGATA | Folmer *et al.*, 1994 | |
| | HCO2198 | TAAACTTCAGGGTGACCAAAA | Folmer *et al.*, 1994 | |
| *16S* rRNA | 16S_sch_fwd_sca | CGCAGTATCCTGACTGTGC | Jaksch *et al.*, 2016 | modified |
| | 16S_sch_rev | CGCCGGTCTGAACTCAGATC | Jaksch *et al.*, 2016 | |
| *18S* rRNA | 18A1 | CCTACCTGGTTGATCCTGCCAG | Steiner and Dreyer, 2003 | |
| | 18S3F | GTTCGATTCCGGAGAGGGA | Stöger *et al.*, 2013 | |
| | 600r | CCGAGATCCAACTACGAGCT | Steiner and Dreyer, 2003 | |
| | NS5 | AACTTAAAGGAATTGACGGAAG | White *et al.*, 1990 | |
| | 1400r | GCATCACAGACCTGTTATTGCTC | Steiner and Dreyer, 2003 | |
| | 1800r | ATGATCCTTCCGCAGGTTCACC | Steiner and Dreyer, 2003 | |
| *28S* rRNA | 28SF1_Sca | CCTCAGATCGGGCGAGAC | This Study | |
| | 28SF2_ScaD | CTCCATCTAAGGCTAAATACG | This Study | |
| | 28SF2_ScaG | CTCCATCTAAGGCTAAATACT | This Study | |
| | 28SR1_Sca | CGGTTTCACGTACTCTTG | This Study | |
| | 28SF3_Sca | CCACCCGACCCGTCTTG | This Study | |
| | 28SR2_ScaD | GTACGCTCTCGCTCCGC | This Study | |
| | 28SR2_ScaD2 | GTACGCTCTTGCTCCGC | This Study | |
| | 28SR2_ScaG | GCTCGCTCTTGCTCGGC | This Study | |
| | 28nn_sca | CAGCTACTAGATGGTTCG | Passamaneck *et al.*, 2004 | modified |
| ITS | ITS1_ScaF | GCGGTTTACTGAGGGACAA | This Study | |
| | ITS2_ScaF | GCGGTGGATCACTCGGCTCGT | This Study | |
| | ITS1_ScaR | CGATGTTCAATGTGTCCTGC | This Study | |
| | ITS2_ScaR | GCCTGACACCCGCTCTGGATG | This Study | |

| **Primer Combinations and PCR-Conditions** | | | | |
|---|---|---|---|---|
| Fragment | Forward Primer | Reverse Primer | Size | Primer Annealing |
| CO1 | LCO1490 | HCO2198 | 668-671 bp | 0:45 min 49°C |
| 16S | 16S_sch_fwd_sca | 16S_sch_rev | 361-414 bp | 0:45 min 60°C |
| 18S | 18A1 | 1800r | 1805-2151 bp | 0:45 min 57°C |
| 18S1 | 18A1 | 600r | 629-709 bp | 0:45 min 55°C |
| 18S2 | 18S3F | 1400r | 1041-1269 bp | 0:45 min 55-60°C |
| 18S3 | NS5 | 1800r | 634-785 bp | 0:45 min 55-56°C |
| 28S | 28SF1_Sca | 28nn_sca | 1170-1246 bp | 0:45 min 60°C |
| 28S1 | 28SF1_Sca | 28SR1_Sca | 379-387 bp | 0:45 min 55°C |
| 28S2 | 28SF2_ScaD/28SF2_ScaG | 28SR2_ScaD1 or D2/28SR2_ScaG | 701-778 bp | 0:45 min 55°C |
| 28S3 | 28SF3_Sca | 28nn_sca | 324-359 bp | 0:45 min 55°C |
| ITS1 | ITS1_ScaF | ITS1_ScaR | 832-1020 bp | 0:45 min 60°C |
| ITS2 | ITS2_ScaF | ITS2_ScaR | 674-824 bp | 0:45 min 60°C |
| **Cloning PCR Condition** | | | | |
| Fragment | Forward Primer | Reverse Primer | Size | Primer Annealing |
| Vector | M13F | M13R | -/- | 0:45 min 55°C |

## 2.4. Sequencing and alignment

Purification of PCR products was done enzymatically using the A'SAP PCR clean-up Kit (ArcticZymes, Tromsø, Norway). The purified PCR products were sequenced using PCR primers at Microsynth Austria (Vienna, Austria) and Eurofins Genomics Germany (Ebersberg, Germany). Chromatograms were checked using Finch TV 1.4.0 (Geospiza Inc.). Sequences were edited using GeneDoc 2.7.0 (Nicholas & Nicholas, 1997) and MEGA X 10.0.5 (Kumar et al., 2018). For the final alignments additional 65 sequences published in the NCBI Genbank (National Center for Biotechnology Information), 7 published in the BOLD database (Barcode of Life Data System) and 41 unpublished sequences from previous work by Gerhard Steiner were added to the sampling. Newly generated sequences and unpublished sequences were uploaded to the BOLD database (Table 2).

**Table 2:** Systematic list of specimens/OTUs and NCBI accession numbers / BOLD IDs used in the present study. Sequences generated in the present study and unpublished sequences are in bold.

| **Order Dentaliida** | | ***CO1*** | ***16S* rRNA** | ***18S* rRNA** | ***28S* rRNA** |
|---|---|---|---|---|---|
| Anulidentaliidae | | | | | |
| | Anulidentalium bambusa 1 | **DPSP023-22** | **DPSP023-22** | - | - |
| Calliodentaliidae | | | | | |
| | Calliodentalium crocinum 1 | **DPSP006-22** | **DPSP006-22** | **DPSP006-22** | **DPSP006-22** |
| | Calliodentalium crocinum 2 | - | **DPSP034-22** | **DPSP034-22** | - |
| Dentaliidae | | | | | |
| | Antalis antillaris 1 | AY260813.1 | - | - | - |
| | Antalis antillaris 2 | AY260814.1 | - | - | - |
| | Antalis antillaris 3 | FPMAR064-08 | - | - | - |
| | Antalis dentalis 1 | AY260815.1 | - | - | - |
| | Antalis entalis 1 | DQ280016.1 | DQ280027.1 | DQ279936 | - |
| | Antalis entalis 2 | KR084703.1 | - | - | - |
| | Antalis entalis 3 | KR084941.1 | - | - | - |
| | Antalis entalis 4 | AY260816.1 | - | - | - |
| | Antalis entalis 5 | AY260817.1 | - | - | - |
| | Antalis entalis 6 | AY260818.1 | - | - | - |
| | Antalis entalis 7 | KR084424.1 | - | - | - |
| | Antalis entalis 8 | - | - | AY145363 | AY145388.1 |
| | Antalis entalis 9 | - | - | KJ193772.1 | - |
| | Antalis inaequicostata 1 | DQ280015.1 | DQ280026.1 | DQ279935 | - |
| | Antalis inaequicostata 2 | AY260819.1 | - | - | - |
| | Antalis inaequicostata 3 | DQ093531.1 | - | DQ093444 | - |
| | Antalis inaequicostata 4 | - | - | AJ389660.1 | - |
| | Antalis perinvoluta 1 | AY260820.1 | - | AJ389663.1 | - |
| | Antalis pilsbryi 1 | AY260821.1 | - | - | - |

| | | | | | |
|---|---|---|---|---|---|
| | Antalis pilsbryi 2 | AF120639.1 | - | AF120522.1 | - |
| | Antalis sp. 1 | AY260822.1 | - | - | - |
| | Antalis vulgaris 1 | **DPSP012-22** | **DPSP012-22** | **DPSP012-22** | **DPSP012-22** |
| | Antalis vulgaris 2 | **DPSP013-22** | **DPSP013-22** | **DPSP013-22** | **DPSP013-22** |
| | Antalis vulgaris 3 | **DPSP014-22** | - | - | - |
| | Antalis vulgaris 4 | **DPSP015-22** | - | - | - |
| | Antalis vulgaris 5 | **DPSP016-22** | - | - | - |
| | Antalis vulgaris 6 | **DPSP017-22** | - | - | - |
| | Antalis vulgaris 7 | **DPSP024-22** | **DPSP024-22** | **DPSP024-22** | **DPSP024-22** |
| | Antalis weinkauffi 1 | - | **DPSP030-22** | - | - |
| | Compressidentalium clathratum 1 | - | **DPSP035-22** | - | - |
| | Compressidentalium clathratum 2 | - | **DPSP036-22** | - | - |
| | Compressidentalium hungerfordi 1 | - | - | **DPSP037-22** | - |
| | Compressidentalium sibogae 1 | **DPSP004-22** | **DPSP004-22** | **DPSP004-22** | **DPSP004-22** |
| | Compressidentalium sibogae 2 | **DPSP005-22** | **DPSP005-22** | **DPSP005-22** | **DPSP005-22** |
| | Compressidentalium sp. 1 | - | **DPSP038-22** | - | - |
| | Dentaliidae sp. 1 | - | - | - | **DPSP018-22** |
| | Dentalium aprinum 1 | - | **DPSP011-22** | **DPSP011-22** | **DPSP011-22** |
| | Dentalium austini 1 | - | **DPSP039-22** | AF490594.1 | - |
| | Dentalium elephantinum 1 | **DPSP008-22** | **DPSP008-22** | **DPSP008-22** | **DPSP008-22** |
| | Dentalium elephantinum 2 | **DPSP010-22** | **DPSP010-22** | **DPSP010-22** | **DPSP010-22** |
| | Dentalium majorinum 1 | AY260823.1 | - | - | - |
| | Dentalium majorinum 2 | BASKL221-09 | - | - | - |
| | Dentalium majorinum 3 | BASKL270-09 | - | - | - |
| | Dentalium octangulatum 1 | - | - | AY145372 | AY145403.1 |
| | Fissidentalium candidum 1 | AY260824.1 | - | - | - |
| | Fissidentalium candidum 2 | - | **DPSP044-22** | - | - |
| | Fissidentalium candidum 3 | - | **DPSP045-22** | - | - |
| | Fissidentalium candidum 4 | - | - | AF490595.1 | - |
| | Fissidentalium capillosum 1 | - | **DPSP046-22** | - | - |
| | Fissidentalium capillosum 2 | - | - | AF490596.1 | - |
| | Fissidentalium magnificum 1 | **DPSP003-22** | **DPSP003-22** | **DPSP003-22** | **DPSP003-22** |
| | Fissidentalium magnificum 2 | - | **DPSP047-22** | - | - |
| | Fissidentalium profundorum 1 | - | **DPSP048-22** | - | - |
| | Fissidentalium sp. 1 | MF157511.1 | MF157461.1 | MF157489.1 | - |
| | Graptacme acicula 1 | - | - | **DPSP055-22** | - |
| | Graptacme eborea 1 | AY260825.1 | - | - | - |
| | Graptacme eborea 2 | NC_006162.1 | NC_006162.1 | - | - |
| | Paradentalium healyi 1 | - | **DPSP019-22** | **-** | **DPSP019-22** |
| Fustiariidae | | | | | |
| | Fustiaria rubescens 1 | - | - | AF490597 | - |
| Gadilinidae | | | | | |
| | Episiphon candelatum 1 | - | **DPSP022-22** | - | **DPSP022-22** |
| | Episiphon virgula 1 | **DPSP001-22** | **DPSP001-22** | **DPSP001-22** | **DPSP001-22** |
| | Episiphon virgula 2 | **DPSP027-22** | - | - | - |

| | | | | | |
|---|---|---|---|---|---|
| | Episiphon yamakawai 1 | AB084110.1 | - | - | - |
| | Gadilina insolita 1 | **DPSP021-22** | **DPSP021-22** | - | - |
| | Gadilina insolita 2 | - | - | **DPSP053-22** | - |
| | Gadilina insolita 3 | - | - | **DPSP054-22** | - |
| Laevidentaliidae | | | | | |
| | Laevidentalium eburneum 1 | **DPSP007-22** | **DPSP007-22** | **DPSP007-22** | **DPSP007-22** |
| | Laevidentalium lubricatum 1 | - | **DPSP057-22** | - | - |
| Rhabdidae | | | | | |
| | Rhabdus rectius 1 | AF120640.1 | - | AF120523 | - |
| | Rhabdus rectius 2 | AY260826.1 | - | - | - |
| | Rhabdus rectius 3 | AY260827.1 | - | - | - |
| | Rhabdus rectius 4 | KF643483.1 | - | - | - |
| | Rhabdus rectius 5 | CMBIA342-11 | - | - | - |
| | Rhabdus rectius 6 | - | AY377619.1 | - | - |
| | Rhabdus toyamaense 1 | **DPSP025-22** | **DPSP025-22** | **DPSP025-22** | **DPSP025-22** |
| | Rhabdus toyamaense 2 | **DPSP028-22** | - | - | - |
| | Rhabdus toyamaense 3 | **DPSP029-22** | **DPSP029-22** | **DPSP029-22** | **DPSP029-22** |
| | Rhabdus toyamaense 4 | - | **DPSP061-22** | **DPSP061-22** | - |

| **Order Gadilida** | | ***CO1*** | ***16S* rRNA** | ***18S* rRNA** | ***28S* rRNA** |
|---|---|---|---|---|---|
| Entalinidae | | | | | |
| | Entalina mirifica 1 | - | **DPSP040-22** | - | - |
| | Entalina mirifica 2 | - | **DPSP041-22** | - | - |
| | Entalina tetragona 1 | AY260828.1 | **DPSP042-22** | AF490598.1 | - |
| | Entalina tetragona 2 | - | **DPSP043-22** | - | - |
| | Entalinopsis habutae 1 | **DPSP026-22** | **DPSP026-22** | **DPSP026-22** | **DPSP026-22** |
| | Heteroschismoides subterfissus 1 | - | **DPSP056-22** | AF490599.1 | - |
| | Rhomboxiphus tricarinatus 1 | - | **DPSP062-22** | - | - |
| Gadilidae | | | | | |
| | Cadulus jeffreysi 1 | - | **DPSP031-22** | **DPSP031-22** | - |
| | Cadulus sp. 1 | - | - | AF490604 | - |
| | Cadulus sp. 2 | - | - | AF490605 | - |
| | Cadulus subfusiformis 1 | - | **DPSP032-22** | **DPSP032-22** | - |
| | Cadulus subfusiformis 2 | - | **DPSP033-22** | - | - |
| | Cadulus subfusiformis 3 | - | - | AF490603.1 | - |
| | Gadila aberrans 1 | AY260829.1 | - | - | - |
| | Gadila aberrans 2 | AY260830.1 | - | - | - |
| | Gadila cf. cobbi 3 | - | - | **DPSP049-22** | - |
| | Gadila cobbi 1 | **DPSP009-22** | **DPSP009-22** | **DPSP009-22** | **DPSP009-22** |
| | Gadila cobbi 2 | - | **DPSP050-22** | - | - |
| | Gadila sagamiensis 1 | - | **DPSP051-22** | **DPSP051-22** | - |
| | Gadila sp. 1 | MF157518.1 | MF157463.1 | MF157493.1 | - |
| | Gadila virginalis 1 | - | **DPSP052-22** | **DPSP052-22** | - |
| | Polyschides carolinensis 1 | AY260831.1 | - | - | - |
| | Polyschides olivi 1 | - | **DPSP058-22** | AF490602.1 | - |

| | | | | | |
|---|---|---|---|---|---|
| | Polyschides quadrifissatus 1 | CMBIA340-11 | - | - | - |
| | Polyschides quadrifissatus 2 | CMBIA341-11 | - | - | - |
| | Polyschides quadrifissatus 3 | CMBIA392-11 | - | - | - |
| | Siphonodentalium japonicum 1 | **DPSP002-22** | **DPSP002-22** | **DPSP002-22** | **DPSP002-22** |
| | Siphonodentalium lobatum 1 | AY342055.1 | AY342055.1 | AF490601.1 | - |
| | Siphonodentalium magnum 1 | - | **DPSP063-22** | - | - |
| | Striocadulus sagei 1 | **DPSP020-22** | **DPSP020-22** | **DPSP020-22** | **DPSP020-22** |
| Pulsellidae | | | | | |
| | Pulsellum affine 1 | - | **DPSP059-22** | - | - |
| | Pulsellum affine 2 | - | - | AF490600.1 | - |
| | Pulsellum lofotense 1 | - | **DPSP060-22** | **DPSP060-22** | - |
| | Pulsellum salishorum 1 | AY260832.1 | - | - | - |
| | Pulsellum salishorum 2 | AY260833.1 | - | - | - |

Alignments were made with MAFFT version 7.452 (Katoh et al., 2019). For *CO1* and *16S* rRNA sequences the L-INS-i strategy and for *18S* and *28S* rRNA sequences the E-INS-i strategy were used. There is no overlap between the sequences from primers *28SF2_ScaD*/*28SF2_ScaG* and *28SR1_Sca* in *Compressidentalium sibogae* 1, Dentaliidae sp. 1, and *Fissidentalium magnificum* 1, resulting in a gap of 15-16 bp in the alignment. The work on the *ITS* region sequences was aborted because of the difficulties in amplification and sequencing resulting from length heterogeneities and suspected allelic heterogeneities. The nucleotide composition for every alignment was calculated with MEGA X 10.0.5.

## 2.5. Tree reconstruction

Phylogenetic analyses were conducted for single gene matrices of *CO1*, *16S* rRNA, *18S* rRNA, and *28S* rRNA data and for concatenated gene matrices. One dataset incorporates taxa in which all genetic markers are present (4gTree), one dataset incorporates taxa in which at least three genetic markers are present (3gTree), and one dataset incorporates taxa in which at least two genetic markers are present (2gTree) (Table 3).

**Table 3:** List of datasets and their OTU composition used for tree searches, depending on data availability. Gene(s) refer(s) to the *CO1* gene, *16S* rRNA gene, *18S* rRNA gene, and *28S* rRNA gene.

| **Name of Tree** | **Description of Dataset** | **Alignment length** |
|---|---|---|
| 4gTree | Only OTUs with data for all four genes available | 4451 bp |
| 3gTree | Only OTUs with data of at least 3 of 4 genes available | 4456 bp |
| 2gTree | Only OTUs with data of at least 2 of 4 genes available | 4586 bp |
| *CO1* | All OTUs with *CO1* data available | 668 bp |
| *16S* | All OTUs with *16S* data available | 442 bp |
| *18S* | All OTUs with *18S* data available | 2134 bp |
| *28S* | All OTUs with *28S* data available | 1363 bp |

*Entalinopsis habutae* was eliminated from the tree searches as a long-branch taxon. Concatenated alignments were divided into six partitions: one each for *16S* rRNA, *18S* rRNA and *28S* rRNA data, and the three codon positions of *CO1*. Evolutionary models for each partition were optimised using jModelTest 2.1.10 (Guindon et al., 2010; Darriba et al., 2012) with standard settings and were chosen based on the Akaike information criterion (AIC) as well as with ModelFinder (Kalyaanamoorthy et al., 2017) within the IQ-Tree 2.1.1 software by using the -m TEST command. Estimations of phylogeny were done using Bayesian Inference and Maximum Likelihood approaches. Bayesian Inference calculations were done using MrBayes 3.2.6 (Ronquist et al., 2012) with 2x4 Markov Chain Monte Carlo iterations of $1x10^7$ generations, sampling every 200th generation and a 10% burnin (which was set after examining the stationarity of log-likelihood values). Maximum Likelihood searches were done using IQ-Tree 2.1.1 with edge-linked partition models (Chernomor et al., 2016; Minh et al., 2020). Branch support was tested with 1000 standard bootstrap replicates with the IQ-Tree software. The evolutionary models used for the reconstruction of all trees as well as the results for the ln likelihoods (Maximum Likelihood analyses) and the arithmetic and harmonic means (Bayesian Inference analyses) are given in Appendix 2. Masking the ribosomal gene alignments with various settings of Gblocks v0.91b (Castresana, 2000) led to no gain in overall node support. Therefore, unmasked ribosomal alignments were used for phylogenetic analyses. Final trees were visualised using ITol Interactive tree of life v5.6.3 (Letunic & Bork, 2021), edited using Inkscape v1.0.2-2 (https://www.inkscape.org) and exported using GIMP v2.10.24 (https://www.gimp.org/).

# 3. Results

## 3.1. The analysis of used primers and obtained sequences

The amplification of the DNA templates resulted in the following sequence lengths: a length of 667-670 bp for *CO1* with *Entalinopsis habutae* having an additional codon; a length of 361-414 bp for *16S* rRNA; a length of 1805-2151 bp for *18S* rRNA with *Entalinopsis habutae* being 171 bp longer than every other OTU; and a length of 1170-1246 for *28S* rRNA. Amplification of the full *ITS* region sequences was only possible for 2 specimens: *Episiphon virgula* 1 with a sequence length for *ITS1* of 832 bp and for *ITS2* of 674 bp and *Antalis vulgaris* 1 with a sequence length for *ITS1* of 1020 bp and for *ITS2* of 824 bp. The nucleotide composition in the four markers varies considerably (Table 4).

**Table 4:** Nucleotide composition in percent of the individual genetic markers. *CO1* 1P, *CO1* 2P, and *CO1* 3P refer to the different codon positions. A+T and C+G is the sum of the corresponding nucleotides.

| Alignment | A (%) | T (%) | C (%) | G (%) | A+T (%) | C+G (%) |
|---|---|---|---|---|---|---|
| *CO1* | 26,2 | 40,1 | 15,9 | 17,8 | 66,3 | 33,7 |
| *CO1* 1P | 26,7 | 29,2 | 16,6 | 27,6 | 55,9 | 44,1 |
| *CO1* 2P | 14,5 | 44,2 | 23,6 | 17,7 | 58,7 | 41,3 |
| *CO1* 3P | 37,3 | 46,9 | 7,6 | 8,1 | 84,3 | 15,7 |
| *16S* | 36,6 | 35,2 | 12,1 | 16,1 | 71,8 | 28,2 |
| *18S* | 23,7 | 22,9 | 24,4 | 29,0 | 46,6 | 53,4 |
| *28S* | 20,4 | 17,6 | 28,7 | 33,3 | 38,0 | 62,0 |

The alignment of the 4gTree has a length of 4451 bp, the alignment of the 3gTree has a length of 4456 bp, and the alignment of the 2gTree has a length of 4586 bp. The alignments of the single gene trees have the following lengths: *CO1* has 668 bp, *16S* has 442 bp, *18S* has 2134 bp, and *28S* has 1363 bp (Table 3). The following fragments could not be amplified: *Gadila cobbi* 1, *Gadilina insolita* 2, *Gadilina insolita* 3 and *Gadila sagamiensis* 1 are missing the 18S3 fragment. *Gadila cf. cobbi* 3 is missing the 18S2 and the 18S3 fragment. *Paradentalium healyi* 1 and *Episiphon candelatum* 1 are missing the 28S2 fragment. *Calliodentalium crocinum* 1 is missing a small part of the 28S2 fragment. *Laevidentalium eburneum* 1 is missing the 28S2 fragment of the *18S* rRNA gene and the 28S2 fragment of the *28S* rRNA gene (Table 1).

## 3.2. Phylogenetic analyses

### 3.2.1. Order and suborder level taxa

The overall result of the six trees obtained in this study can be seen in Table 5 as well as in the separate trees (Figs. 1-6). Dentaliida and Gadilida are monophyletic in all trees with high support. The Entalimorpha are present and highly supported in the 2gTree and the *18S* tree (Figs. 2, 5). In the *16S* tree they are also monophyletic, but they are positioned within the Gadilimorpha (Fig. 4). There is not enough data to assess the monophyly or position of the Entalimorpha in the 4gTree and 3gTree as well as in the *CO1* and *28S* trees (Figs. 1, 3, 6). The Gadilimorpha are represented in both the 3gTree and 2gTree and in the *18S* tree, but the node support for the taxon is poor in all three trees (Figs. 1-2, 6). Since *Entalina tetragona* 1 is located within the Gadilimorpha in the *CO1* tree and all Entalimorpha are clustered within the Gadilimorpha in the *16S* tree there is no clear result of monophyly for the Gadilimorpha in either of the two trees (Figs. 3-4). There are not enough taxa to assess the monophyly or position of the Gadilimorpha in the 4gTree and in the *28S* tree. The representatives of Anulidentaliidae, Fustiariidae, Gadilinidae, Laevidentaliidae and Rhabdidae are the sister group to the remaining Dentaliida in all trees from the concatenated datasets (Figs. 1-2). As the species of these families have smooth shells, this clade is here referred to as the 'Smooth Shell Clade'. This taxon is robustly supported in the 4gTree, 3gTree, and 2gTree and moderately supported in the *28S* tree (Figs. 1-2, 6). The species of the 'Smooth Shell Clade' also appear in the *CO1* tree as a monophyletic group but display an unresolved polytomy together with the rest of the Dentaliida. They are also only poorly supported with *Fissidentalium candidum* 1 as the closest basal branch (Fig. 3). In the trees based on *16S* and *18S* gene data the species comprising the 'Smooth Shell Clade' are not monophyletic, but they are all positioned at the base of the Dentaliida in a paraphyletic (*16S*) or unresolved (*18S*) relationship (Figs. 4-5).

**Table 5:** Node support by Bayesian Posterior Probability (PP) and ML bootstrap percentage (ML BP) for various scaphopod taxa and unnamed clades (in systematic and alphabetic order) in each dataset. Colour code: Green - 100-90, Yellow - 89-70, Orange - 69-50, Red - <50, White – no data. A cell with "n.a." indicates insufficient or inapplicable data. Abbreviations: ADFC = *Antalis*, *Dentalium*, *Fissidentalium* and *Compressidentalium* Clade; *Antalis* Clade 1 = *A. inaequicostata* and *A. vulgaris*; Smooth Shell Clade = Anulidentaliidae, Fustiariidae, Gadilinidae, Laevidentaliidae, and Rhabdidae.

| | **PP** | | | | | | | **ML BP** | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 4gTree | 3gTree | 2gTree | *CO1* | *16S* | *18S* | *28S* | 4gTree | 3gTree | 2gTree | *CO1* | *16S* | *18S* | *28S* |
| Dentaliida | 🟩 | 🟩 | 🟩 | 🟩 | 🟩 | 🟩 | 🟩 | 🟩 | 🟩 | 🟩 | 🟩 | 🟩 | 🟩 | 🟩 |
| Gadilida | 🟩 | 🟩 | 🟩 | 🟩 | 🟩 | 🟩 | 🟩 | 🟩 | 🟩 | 🟩 | 🟩 | 🟩 | 🟩 | 🟩 |
| Entalimorpha | | n.a. | 🟩 | n.a. | 🟩 | 🟩 | | | n.a. | 🟩 | n.a. | 🟩 | 🟩 | |
| Gadilimorpha | n.a. | 🟧 | 🟨 | 🟥 | 🟥 | 🟨 | n.a. | n.a. | 🟧 | 🟧 | 🟥 | 🟥 | 🟥 | n.a. |
| Dentaliidae | 🟩 | 🟩 | 🟩 | 🟥 | 🟥 | 🟩 | 🟩 | 🟩 | 🟩 | 🟨 | 🟥 | 🟥 | 🟧 | 🟩 |
| Gadilidae | 🟩 | 🟧 | 🟥 | 🟥 | 🟥 | 🟥 | 🟩 | 🟩 | 🟧 | 🟥 | 🟥 | 🟥 | 🟥 | 🟩 |
| Gadilinidae | n.a. | n.a. | 🟥 | 🟥 | 🟥 | 🟥 | 🟥 | n.a. | n.a. | 🟥 | 🟥 | 🟥 | 🟥 | 🟥 |
| Laevidentaliidae | n.a. | n.a. | n.a. | n.a. | 🟥 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | 🟥 | n.a. | n.a. |
| Pulsellidae | | | n.a. | 🟩 | 🟥 | 🟥 | | | | n.a. | 🟩 | 🟥 | 🟥 | |
| Rhabdidae | n.a. | n.a. | n.a. | 🟥 | 🟥 | n.a. | n.a. | n.a. | n.a. | n.a. | 🟥 | 🟥 | n.a. | n.a. |
| *Antalis* | n.a. | 🟥 | 🟥 | 🟥 | 🟥 | 🟥 | 🟥 | n.a. | 🟥 | 🟥 | 🟥 | 🟥 | 🟥 | 🟥 |
| *Cadulus* | | | 🟥 | | 🟥 | 🟥 | | | | 🟥 | | 🟥 | 🟥 | |
| *Compressidentalium* | n.a. | n.a. | n.a. | n.a. | 🟥 | 🟥 | n.a. | n.a. | n.a. | n.a. | n.a. | 🟥 | 🟥 | n.a. |
| *Dentalium* | n.a. | 🟩 | 🟥 | 🟥 | 🟥 | 🟥 | 🟩 | n.a. | 🟩 | 🟥 | 🟥 | 🟥 | 🟥 | 🟨 |
| *Entalina* | | n.a. | n.a. | n.a. | 🟩 | n.a. | | | n.a. | n.a. | n.a. | 🟨 | n.a. | |
| *Episiphon* | n.a. | n.a. | 🟥 | n.a. | 🟥 | n.a. | 🟥 | n.a. | n.a. | 🟥 | n.a. | 🟥 | n.a. | 🟥 |
| *Fissidentalium* | n.a. | 🟥 | 🟥 | 🟥 | 🟥 | 🟥 | n.a. | n.a. | 🟥 | 🟥 | 🟥 | 🟥 | 🟥 | n.a. |
| *Gadila* | n.a. | 🟥 | 🟥 | 🟥 | 🟥 | 🟥 | n.a. | n.a. | 🟥 | 🟥 | 🟥 | 🟥 | 🟥 | n.a. |
| *Gadilina* | | | n.a. | n.a. | n.a. | 🟥 | | | | n.a. | n.a. | n.a. | 🟥 | |
| *Laevidentalium* | n.a. | n.a. | n.a. | n.a. | 🟥 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | 🟥 | n.a. | n.a. |
| *Polyschides* | | | n.a. | 🟩 | n.a. | n.a. | | | | n.a. | 🟨 | n.a. | n.a. | |
| *Pulsellum* | | | n.a. | n.a. | 🟥 | 🟥 | | | | n.a. | n.a. | 🟥 | 🟥 | |
| *Rhabdus* | n.a. | n.a. | n.a. | 🟥 | 🟥 | n.a. | n.a. | n.a. | n.a. | n.a. | 🟥 | 🟥 | n.a. | n.a. |
| *Siphonodentalium* | n.a. | 🟥 | 🟥 | 🟥 | 🟥 | 🟥 | n.a. | n.a. | 🟥 | 🟥 | 🟥 | 🟥 | 🟥 | n.a. |
| ADFC Clade | | 🟩 | 🟨 | 🟧 | 🟥 | 🟩 | 🟥 | | 🟨 | 🟥 | 🟥 | 🟥 | 🟥 | 🟥 |
| *Antalis* clade 1 | | 🟩 | 🟩 | 🟩 | 🟩 | 🟩 | | | 🟩 | 🟩 | 🟩 | 🟩 | 🟩 | |
| *Calliodentalium* + Dentaliidae | 🟨 | 🟨 | 🟩 | 🟥 | 🟥 | 🟩 | 🟨 | 🟧 | 🟧 | 🟨 | 🟥 | 🟥 | 🟧 | 🟧 |
| Smooth Shell Clade | 🟩 | 🟩 | 🟩 | 🟧 | 🟥 | 🟥 | 🟧 | 🟩 | 🟩 | 🟩 | 🟥 | 🟥 | 🟥 | 🟧 |

### 3.2.2. Family, genus, and species level taxa

The Dentaliidae is the family-level taxon with the highest number of represented species in the data. The monophyly of this taxon is supported by the 4gTree, 3gTree, 2gTree, and the trees based on *18S* and *28S* gene data with in overall high support (Figs. 1-2, 5-6). In the *16S* tree, the two specimens of *Calliodentalium crocinum* (Calliodentaliidae) are placed within the Dentaliidae rendering them paraphyletic (Fig. 4). In the *CO1* tree the Dentaliida are resolved as polytomy which splits the Dentaliidae in multiple clades (Fig. 3).

Two robust subclades of Dentaliidae are represented in most trees in the present study. The first one includes *Antalis vulgaris* and *Antalis inaequicostata* which are sister taxa in all trees (except for the 4gTree which lacks *A. inaequicostata*) of the present study and represent one part of the genus *Antalis* (*Antalis* clade 1). This clade is always highly supported (Figs 1-6). Another major clade (ADFC clade) which is also present in most trees represents a group consisting of closely related members of the genus *Antalis* (*A. entalis* and *A. pilsbryi*), all species of *Fissidentalium* (*F. capillosum*, *F. candidum*, *F. magnificum*, *F. profundorum*, and one unidentified species) and *Compressidentalium* (*C. clathratum*, *C. hungerfordi*, *C. sibogae*, and one unidentified species) with most species of *Dentalium* either as a sister group (*D. aprinum*, *D. elephantinum*, and *D. octangulatum*) or as part of the group (*D. majorinum*). However, the node is only moderately supported by posterior probabilities and not by bootstrap values (Figs. 1-6). Within the ADFC clade there is no clear picture of the phylogenetic relationships of the members of the genera *Antalis*, *Fissidentalium* and *Compressidentalium*. In the 3gTree and 2gTree *A. entalis* and *A. pilsbryi* are the basal branch or sister taxon to the genera *Fissidentalium* + *Compressidentalium* (Figs. 1-3). On the other hand, there is a close relationship between *A. entalis* and *C. clathratum* in the *16S* tree while there is almost no distinction between the three genera in the trees based on *18S* and *28S* gene data (Figs. 4-6). This is again well supported by posterior probability and only moderately by bootstrap values. The genera *Fissidentalium* and *Compressidentalium* cannot be separated when two or more species of both genera are present in the tree (Figs. 1-4). *Antalis weinkauffi* and *Paradentalium healyi* may also be closely related to this group of taxa (Figs. 4, 6), but the data is not clear (Fig. 2). There is also third part of the genus *Antalis* which can be distinguished (*Antalis* clade 2). It is comprised of the two species *A. perinvoluta* and *A. antillaris* as well as another *A. pilsbryi* sequence. However, the position of this clade is unstable (Figs. 2-3, 5). Also unclear is the position of *Dentalium austini* and the members of the genus *Graptacme*.

*Calliodentalium crocinum*, the only member of the Calliodentaliidae, is the sister taxon to the Dentaliidae (Figs. 1-2, 4-6), but there is only moderate support for this in all shown trees (Table 5). The 'Smooth Shell Clade' taxa, *i.e.*, the Gadilinidae (*Episiphon candelatum*, *E. virgula, E. yamakawai* and *Gadilina insolita*), Rhabdidae (*Rhabdus rectius* and *R. toyamaense*), and Laevidentaliidae (*Laevidentalium eburneum* and *L. lubricatum*), appear not monophyletic when represented by more than one species (Figs. 2-6). *Episiphon virgula* is in all presented trees with high support the sister clade to *Laevidentalium eburneum* (Figs. 1-6). *Rhabdus toyamaense* is closely related to *E. candelatum* (Figs. 2, 4, 6) with *Gadilina insolita* positioned as next closest relative (Figs. 2-3, 5) which is also highly supported. There is few data for the Anulidentaliidae and Fustiariidae, but *Anulidentalium bambusa* is closely related to *R. rectius* (Figs. 3-4). The OTU *R. rectius* 1 (NCBI Acc.No. AF120640.1) is unrelated to all other *R. rectius* sequences (Figs. 2-3, 5).

There is not a single genus in the Gadilimorpha that appears monophyletic in this dataset (Figs. 2-6). The members of the genera *Cadulus*, *Gadila*, and *Siphonodentalium* are entirely mixed (Figs. 2-5). The genus *Polyschides* is shown to be monophyletic in the *CO1* tree (Fig. 3), but there is no data for *Polyschides olivi*, which, based on other genetic markers, is positioned in a different clade (Figs. 2, 3, 5). The members of the Pulsellidae are positioned within the Gadilidae, except in the *CO1* tree (Fig. 3). Within the Gadilimorpha, there are some species that show a stable phylogenetic relationship with high node supports. The first are *Striocadulus sagei* and *Gadila sagamiensis* which, when both present, are always sister species (Figs. 2, 4, 5). These two species show a close phylogenetic relationship with *Gadila virginalis* in the 2gTree and the *16S* tree (Figs. 2, 4). The second group of species consists of *Gadila cobbi*, *Polyschides olivi*, and *Siphonodentalium lobatum* with the first two being closer related except in the *18S* tree as this tree does not resolve the group (Figs. 1-5). The position and phylogenetic relationships of the other members of Gadilimorpha are unclear since they are different in every tree. There is also little data on the genera of the Entalinidae. In the *CO1* tree, the genus *Entalina* is more closely related to *Heteroschismoides* than to *Rhomboxiphus* (Fig. 3).

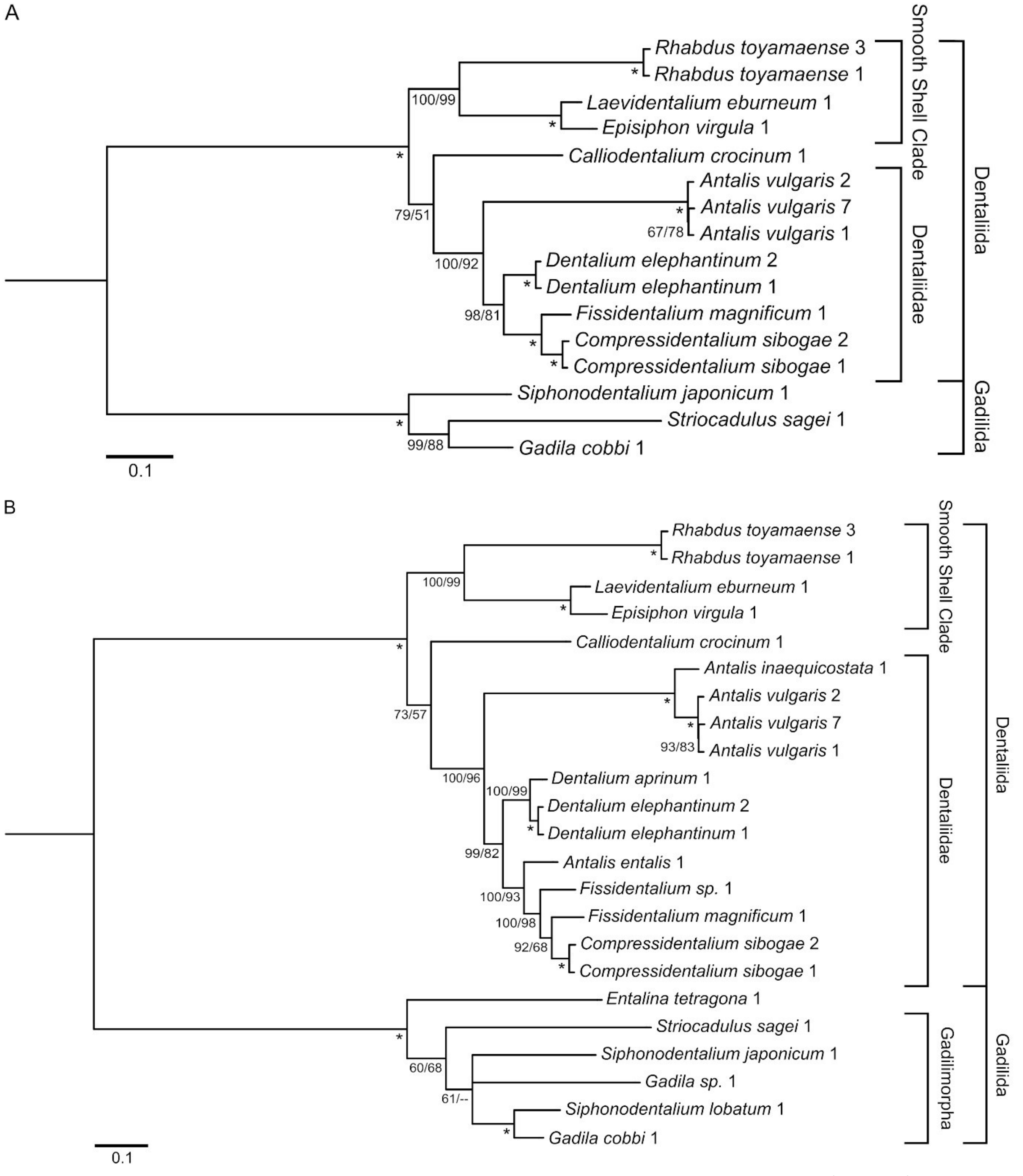

**Figure 1:** Bayesian Inference tree of scaphopod species based on all four markers (4gTree), *CO1*, *16S* rRNA, *18S* rRNA, and *28S* rRNA (A), and based on at least three markers (3gTree) (B). Branch support (≥ 50) from Posterior probabilities (left) and bootstrap values (right, in %). A star (*) indicates full support (100/100).

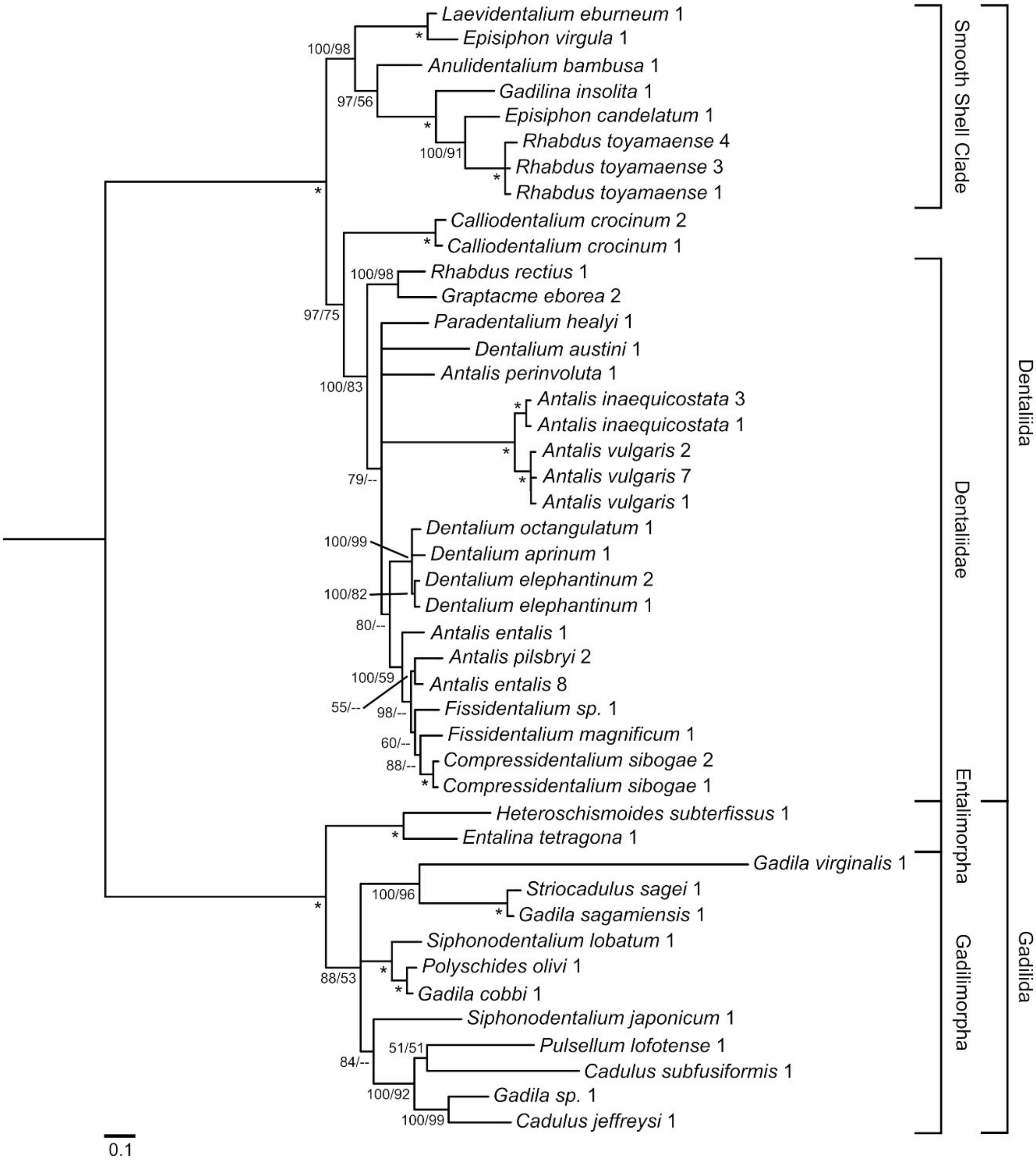

**Figure 2:** Bayesian Inference tree of scaphopod species based on at least 2 of 4 markers (2gTree). Branch support (≥ 50) from Posterior probabilities (left) and bootstrap values (right, in %). A star (*) indicates full support (100/100).

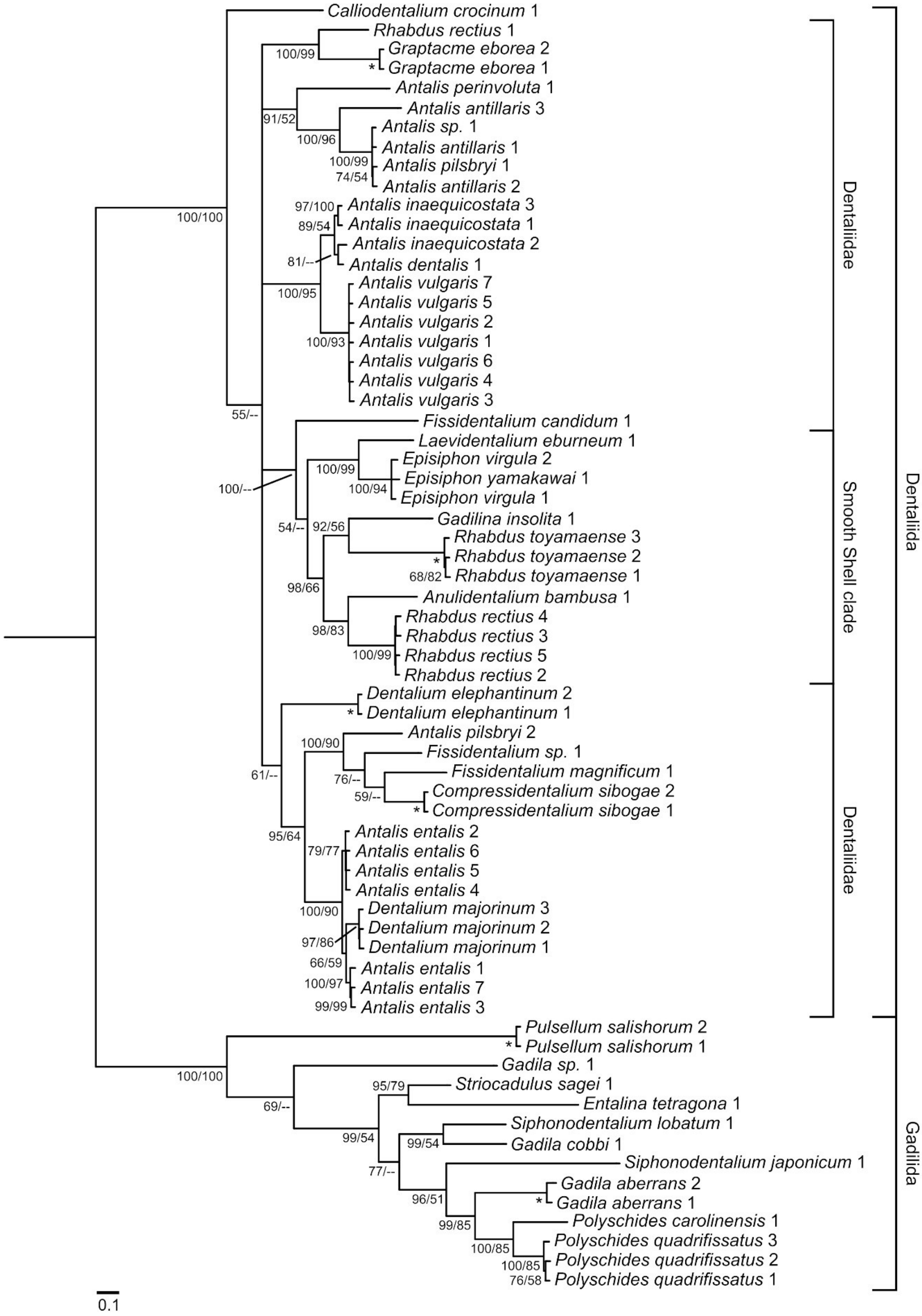

**Figure 3:** Bayesian Inference tree of scaphopod species based on the *CO1* marker. Branch support (≥ 50) from Posterior probabilities (left) and bootstrap values (right, in %). A star (*) indicates full support (100/100).

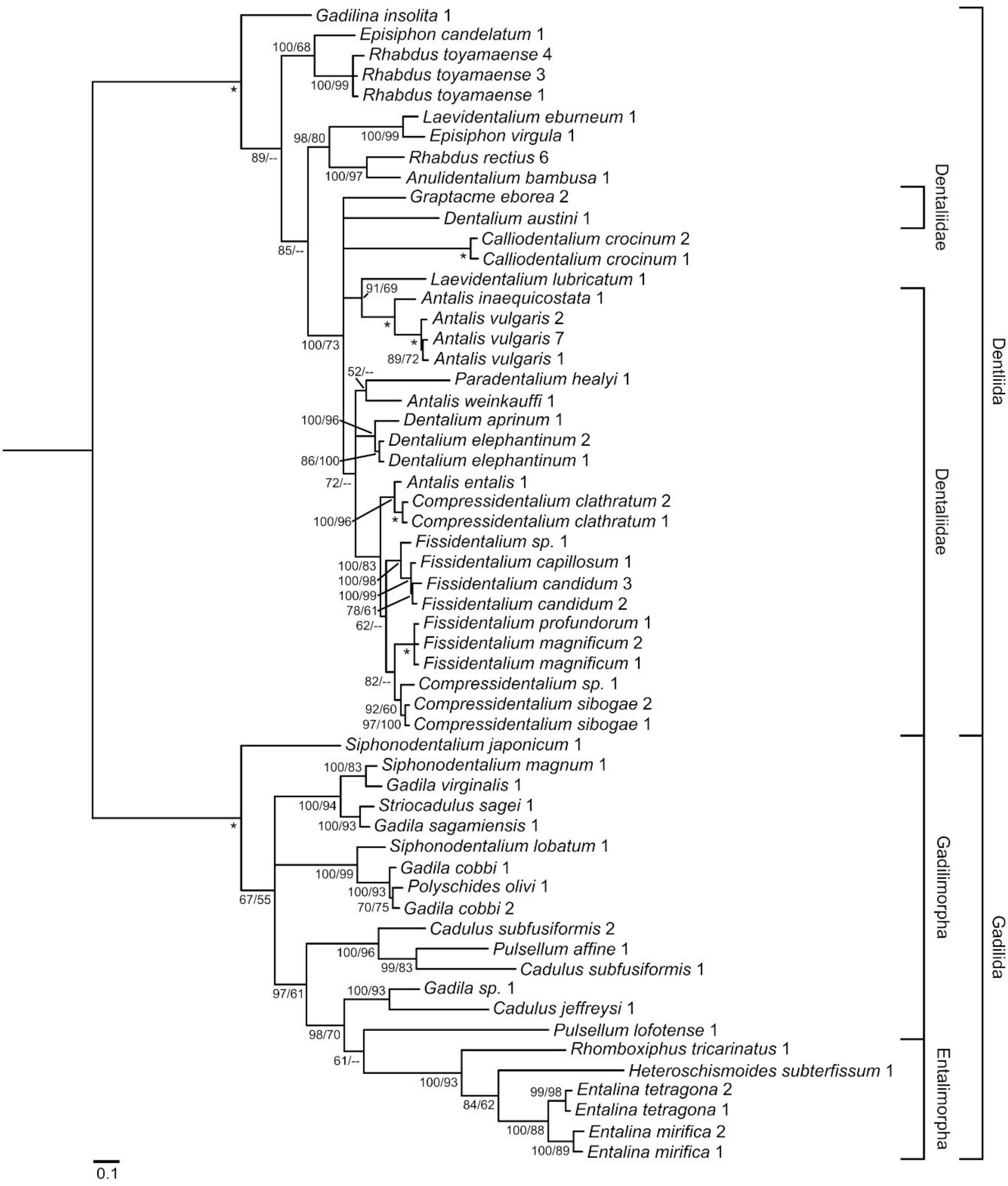

**Figure 4:** Bayesian Inference tree of scaphopod species based on the *16S* rRNA marker. Branch support (≥ 50) from Posterior probabilities (left) and bootstrap values (right, in %). A star (*) indicates full support (100/100).

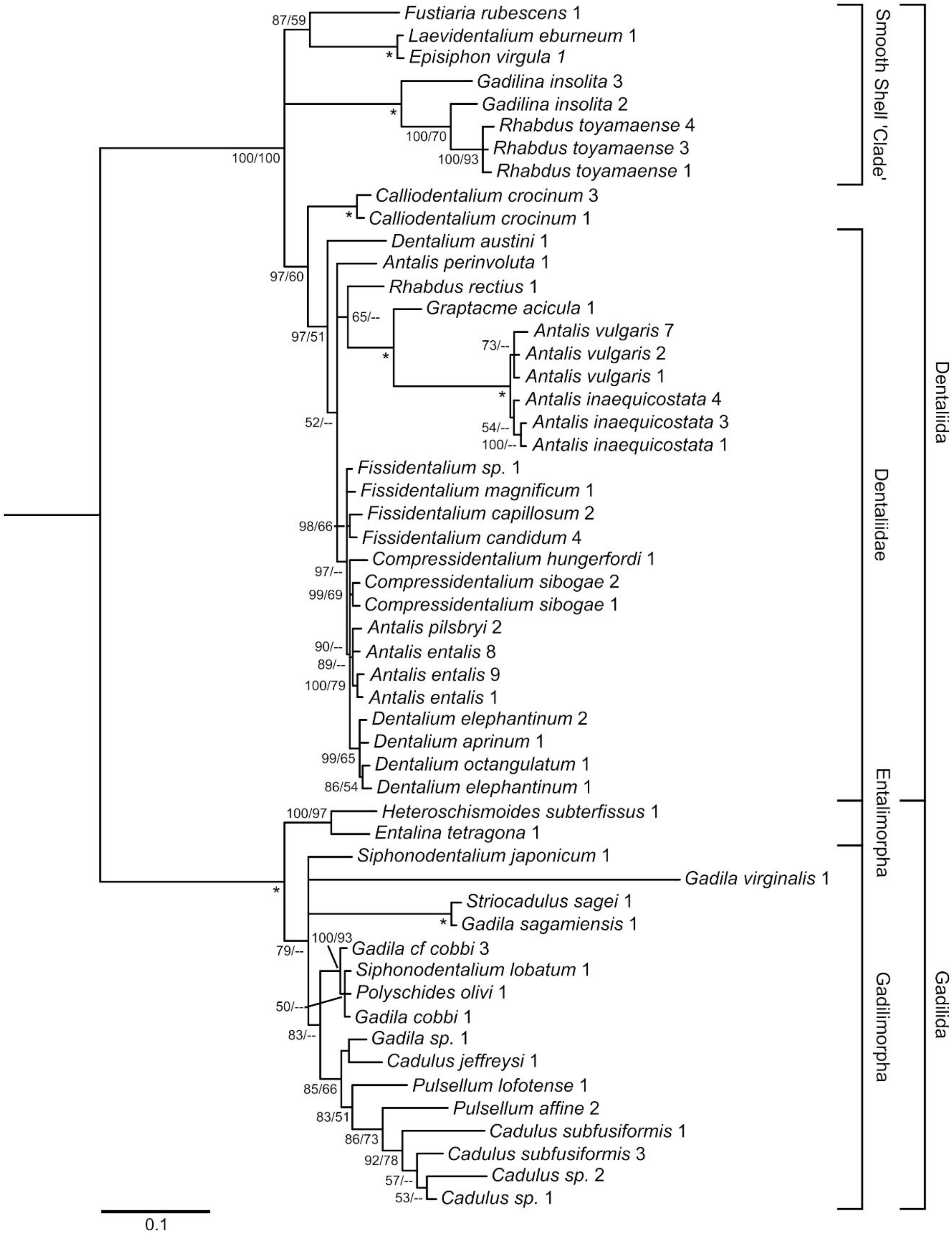

**Figure 5:** Bayesian Inference tree of scaphopod species based on the *18S* rRNA marker. Branch support (≥ 50) from Posterior probabilities (left) and bootstrap values (right, in %). A star (*) indicates full support (100/100).

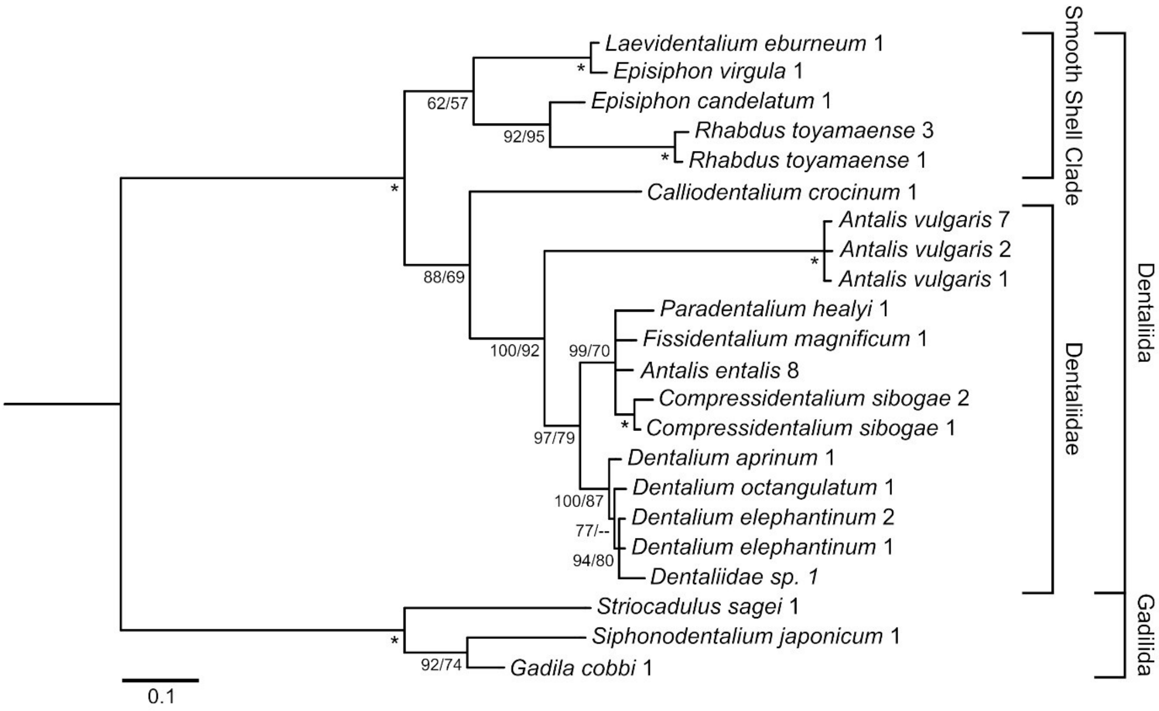

**Figure 6:** Bayesian Inference tree of scaphopod species based on the 2*8S* rRNA marker. Branch support (≥ 50) from Posterior probabilities (left) and bootstrap values (right, in %). A star (*) indicates full support (100/100).

# 4. Discussion

## 4.1. Primers and phylogenetic signal

The present study expands the sequence dataset for Scaphopoda by almost doubling the previously available genetic data. The adaptation of primers to scaphopod-specific sequences increased the efficiency and reduced amplification of non-target organisms such as fungi. However, the best primers cannot compensate severe DNA fragmentation that leads to the gaps in some of the sequences and failure to amplify some markers. Unfortunately, obtaining *ITS* sequences for most of the individuals was not possible, although they were successfully used for other mollusc groups (e.g. Nakano et al., 2009; Psonis et al., 2015; Pfeiffer et al., 2018). Even though the *ITS* primers have a 100% fit to the flanking sequences, they produced amplicons of different lengths making sequencing impossible.

Compared to previous studies (Steiner & Reynolds, 2003; Steiner & Dreyer, 2003), the now increased dataset sheds more light on the phylogenetic relationships of scaphopod species. Based on the data of the obtained trees, the phylogenetic signal to investigate the phylogeny of scaphopods effectively seems to need at least 3 of the genetic markers used in the present study. The 4gTree is fully resolved and shows full support in all but one node (Fig. 1, Table 5). Naturally, the 4gTree also has the fewest taxa, which at the same time reduces the potential conflict in the dataset. The 3gTree has in overall also almost perfect resolution and support values (Table 5). In addition to the mentioned node in the 4gTree there is also a poor supported section within the Gadilida (Fig. 1). Interestingly, it seems that the poorly supported node that separates *Calliodentalium crocinum* 1 from the Dentaliidae could be based on the short missing section of its *28S* rRNA gene marker since there is a similar picture in the *28S* tree (Fig. 6). The poorly resolved and supported section within the Gadilida in the 3gTree may be based in a similar way on the missing *28S* rRNA gene markers of *Gadila sp.* 1 and *Siphonodentalium lobatum* 1 (Table 2). However, there is also comparable low resolution at the same phylogenetic region in the trees based on *16S* and *18S* gene data, which could indicate that the signal in this region is generally low (Figs. 4-5). The 2gTree based on at least 2 available genetic markers has generally high support values but, depending on which sequences are available, fails to resolve some nodes (Fig. 2, Table 5). This is primarily visible in parts of the Dentaliidae involving mostly OTUs which have only two markers available (*Antalis perinvoluta* 1, *Dentalium austini* 1, and *Paradentalium healyi* 1 as well as A. *entalis* 8 and *A. pilsbryi* 2; Table 2). The same is true for the weakly supported part of the Gadilida section of the 2gTree where *Cadulus subfusiformis* 1 and *Pulsellum lofotense* 1 both have only two markers available (Fig. 2, Table 2). It is striking that almost all these mentioned OTUs (the exception is *P. healyi*) have the *18S* rRNA gene marker available as one of the two used markers (Table 2), which may explain the low signal additionally to the missing markers. The *18S* tree has in overall the poorest resolution and support values compared to the other trees obtained, especially within the Dentaliidae (Fig. 5). However, it is known that the *18S* rRNA has strong phylogenetic signals for old speciations but performs poorly among closely related species (Steiner & Müller, 1996). This was tested several times in molluscs with single-gene approaches (e.g. Steiner & Müller, 1996; Winnepenninckx et al., 1996; Steiner & Dreyer, 2003; Vonnemann et al., 2005). However, there are other OTUs in the tree with only two markers but well supported branches. Since all other single gene trees have the same or a very similar overall topology as the 2gTree, it could be that an accumulation of missing genetic markers and thus missing 'linkage' between the OTUs in these areas of the 2gTree causes the local aggregation of poor support values (Figs. 2-6, Table 2). For the OTUs mentioned, more data would be needed to see if this problem can be solved or if there is simply not enough signal in these areas of the 2gTree using this specific taxon set. There is no surprise that the *CO1* tree does not resolve the deep nodes of the Dentaliida since this marker is mostly used for species discrimination. Hence it is much more interesting that the deep nodes of the Gadilida are rather well supported, and the branches are much longer than those of the Dentaliida (Fig. 3). A similar picture concerning the branch lengths can be seen in the trees based on *16S* and *18S* genetic data (Figs. 4-5). In accordance with Steiner & Dreyer's (2003, p. 354) assessment that the Dentaliida have "a low substitution rate and/or a recent series of cladogenetic events" this would, in contrast, indicate a high rate of substitution and/or an older series of cladogenetic events in the Gadilida. This would be especially surprising as there is currently a gap in the fossil history between Dentaliidae and Gadilida, with the Gadilida being much younger, as mentioned earlier (Wanninger & Wollesen, 2019). The signal of the distal nodes of the *CO1* tree is very high, and the corresponding nodes have almost full support, encouraging further use in barcoding approaches for Scaphopoda (Fig. 3). The *16S* tree is characterised by a mixture of low signal in some parts (within the Dentaliidae and at the base of the Gadilida) and high signal on other parts of the basal nodes (Fig. 4). The reason for this could be the high number of Indels and the high overall variability of this marker which may lead to a higher signal-to-noise ratio compared to the other markers. However, it is still possible to discriminate most clades which are also present in the other trees although the branch lengths within the Dentaliidae are very short (Fig. 4). The strong phylogenetic signal of the *28S* rRNA marker decreases slightly towards the deep nodes (Fig. 6). However, only few *28S* rRNA genetic marker sequences are available, so this statement will have to be revised as more data becomes available. In the present *28S* tree, there is only one polytomy of four dentaliids, which only incorporates one incomplete sequence (*P. healyi* 1), so missing data is probably not the reason for the poor resolution. Since masking the hypervariable region of the alignment had no influence on the support values, the most plausible explanation is an overall lack of signal for these speciations in the *28S* rRNA sequences.

### 4.2. Phylogenetic relationships

As in many previous phylogenetic studies based on morphological and molecular data there is a good supported separation of Dentaliida and Gadilida visible in all trees of the present study (Steiner, 1992a; 1998; Reynolds & Okusu, 1999; Steiner & Reynolds, 2003; Reynolds & Steiner, 2008). For the monophyly of Entalimorpha and Gadilimorpha, on the other hand, there are contradictory results, as only little data is available to confirm the monophyly. It is only supported by the 2gTree and the *18S* tree (Figs. 2, 5). These two trees show the Entalinidae as a basal branch of the Gadilida, as the previous tree based on *18S* data by Steiner and Dreyer (2003) and the trees based on morphological characters do (Steiner, 1992a; Reynolds, 1997; Reynolds & Okusu, 1999). However, the *16S* tree shows the opposite placing the monophyletic Entalimorpha as most distal branch within the Gadilimorpha (Fig. 4). Additionally, *Entalina tetragona* 1 is in the middle of the Gadilimorpha in the *CO1* tree (Fig. 3) as it is in the previous published tree based on *CO1* gene data by Steiner & Reynolds (2003). The Gadilida need a sound revision once more data for Gadilimorpha and especially Entalimorpha becomes available. The results on the newly found 'Smooth Shell Clade' within the Dentaliida are much clearer. Most trees show a distinct monophyletic clade including all available members (except *L. lubricatum*) of the Anulidentaliidae, Gadilinidae, Laevidentaliidae, and Rhabdidae (Figs. 1-3, 6) or show a basal position of these taxa at the base of the Dentaliida (Figs. 4, 5). It may be that Fustiariidae are also part of this clade but there is so far only a *18S* rRNA gene sequence available which places *Fustiaria rubescens* 1 with moderate support closer to members of the 'Smooth Shell Clade' (Fig. 5). Previous studies based on morphological data place the Fustiariidae as sister clade to the Dentaliidae (Reynolds & Okusu, 1999) or as a closely related clade (Steiner, 1992a; 1999). The monophyly of the family-level taxa and genera (with more than one OTU) within the 'Smooth Shell Clade' is not supported by any tree in the present study. This is also reported for the Gadilinidae and the genus *Episiphon* by previous morphological studies (Steiner, 1998; 1999; Reynolds & Okusu, 1999). However, until now this is not backed by molecular data. The phylogenetic relationships between the taxa of the 'Smooth Shell Clade' presented here were also never reported before and the possible para- or polyphyly of all members at the family level of this clade limits comparison to previous studies due to their small taxon samples. Earlier studies report a closer relationship of Laevidentaliidae and Rhabdidae (Steiner, 1992a; 1998; 1999) which is not visible in the trees of the present study. However, Lamprell & Healy (1998) also state that the Laevidentaliidae may be not monophyletic and need revision. Therefore, a closer phylogenetic relationship to some parts of the Rhabdidae cannot be excluded since there are only two representatives of the Laevidentaliidae in this study. The study by Steiner (1999, Fig. 9B) also reports a possible close relationship between a representative species of Anulidentaliidae (*Epirhabdoides ivanovi*) and *Rhabdus rectius* (which is present in this study, Figs. 3-4). Since there is only one other species (*Anulidentalium bambusa*) from this group in the present study, it would have to be assumed that the Anulidentaliidae are monophyletic. However, this is not certain given the data on the other genera and families of the 'Smooth Shell Clade’. The close phylogenetic relationship of Anulidentaliidae, Gadilinidae, Laevidentaliidae, and Rhabdidae (and perhaps Fustiariidae) resembles the *Plagioglypta*/*Fustiaria* lineage postulated already by Emerson (1962). In this context, it would be interesting to have genetic data for the genera *Plagioglypta* and *Omniglypta,* which were since placed in the Dentaliidae and the family Omniglyptidae, respectively (Steiner & Kabat, 2001). The Omniglyptidae could be a member of the ‘Smooth Shell Clade’ as a previous study by Steiner (1998) and general morphological data indicates (Lamprell & Healy, 1998). The Calliodentaliidae (here only represented by *Calliodentalium crocinum*) seem to be closer related to the Dentaliidae based on the trees of the present study, but only with moderate support (Table 5). In older phylogenetic studies, the Calliodentaliidae are closer related to the Rhabdidae (Reynolds & Okusu, 1999) or to the Rhabdidae and the Laevidentaliidae (Steiner, 1998; 1999). The species *Calliodentalium crocinum* was also long considered to be a member of the Laevidentaliidae (Lamprell & Healy, 1998). On the other hand, the new data on the Calliodentaliidae supports the decision by Scarabino (1995) to erect the Calliodentaliidae as a family-level taxon. The Dentaliidae are monophyletic based on the data obtained in the present study. However, the genera within the Dentaliidae that are present in this study are not. There are only two genera (*Graptacme* and *Paradentalium*) that have not enough data to check for monophyly. The result that most if not all other genera (here represented by *Antalis*, *Compressidentalium*, *Dentalium*, and *Fissidentalium)* of the Dentaliidae are not monophyletic is supported by concerns by other authors that there is not enough morphological data (especially shell and radula morphology since mostly used) to actually differentiate the genera (Lamprell & Healy, 1998; Steiner & Dreyer, 2003; Reynolds & Steiner, 2008; Scarabino, 2008). The data of the present study indicates that there are at least two clades (*Antalis* clade 1 and ADFC clade) involving members of the genus *Antalis* (Figs. 1-6). Based on the *CO1* gene data there is probably at least a third additional clade (*Antalis* clade 2). This confirms previous reports about the  paraphyly of the genus (Steiner & Dreyer, 2003; Reynolds & Steiner, 2008) and, as previously mentioned, there are also authors that already do not recognise *Antalis* as a valid genus (Lamprell & Healy, 1998). However, the new data also indicates the para- or polyphyly of *Dentalium*, *Fissidentalium*, and *Compressidentalium* (Figs. 1-6). The use of the genus *Compressidentalium* was also rejected by previous authors due to missing data and convincing morphological evidence for a separation of the concerning species from *Fissidentalium* (Lamprell & Healy, 1998; Sahlmann et al., 2016) in contrast to the acceptance of the genus by Scarabino (1995). Additionally, there are comments by Lamprell & Healy (1998) that *Fissidentalium* may be two separate groups of species based on radula features. The here presented data shows that these genera are indeed in need of a complete revision. Since they appear especially closely related and intertwined it may need even more data of more species and a clear focus on this clade to get better insight. There is a good support for *Graptacme* as member of Dentaliidae as this was also questioned before (Lamprell & Healy, 1998), but there is no clear signal for the position of the genus. It is possible that *Graptacme eborea* is basal within the Dentaliidae, as already indicated by Steiner and Reynolds (2003) based on *CO1* gene data, but *Graptacme acicula* is more closely related to *A. vulgaris* and *A. inaequicostata*. It needs more data for the markers used and further species of the genus, since it seems there is barely enough signal in the 2gTree, the *CO1* tree, and the *16S* tree (Figs. 2-4). A final remark on the Dentaliida: One species, *Laevidentalium lubricatum*, which is at the base of the *Antalis* clade 1 in the *16S* tree, shows the difficulty of species assignment of Dentaliid species (Fig. 4). This species is originally described as *Dentalium lubricatum*, then placed within the genus *Laevidentalium* (summary: Steiner & Kabat, 2004) and finally placed in the genus *Antalis* (as synonym of *Antalis tibanum*) by Scarabino (1995). The latter movement was criticised by Lamprell & Healy (1998) due to the problems concerning the genera as mentioned above. However, it seems that this is supported by the new data, although there is no information on which genus is most suitable. The data of the present study also shows that there are problems with species identification of Dentaliida. There are two *A. pilsbryi* sequences in completely different locations of the Dentaliidae (ADFC clade and Antalis clade 2). There is *A. dentalis* which is genetically almost indistinguishable from and probably is more likely *A. inaequicostata* and there is also *Rhabdus rectius* 1, which is positioned in the Dentaliidae whereas every other OTU of *R. rectius* in the *CO1* tree is within the ‘Smooth Shell Clade’ (Fig. 3). There is a high probability that this single OTU (*R. rectius* 1) is a misidentification. There are also examples that are not so clear. In the *CO1* tree, there is *Fissidentalium candidum* 1 which is more closely related to the members of the 'Smooth Shell Clade’, while new *16S* gene data positions this species in the ADFC clade (Figs. 3-4). In the *CO1* tree, there is also *Antalis antillaris* 3 which might be something else compared to the other *Antalis antillaris* since there is quite some genetic distance (Fig. 3).

Less data is available for the Gadilida, but based on the available data, the relationships within the Gadilida are characterised by an implied para- or polyphyly of all available genera. In all here presented trees, only the genus *Entalina* appears to be a monophyly with no data indicating otherwise (Fig. 4). The placement of species of the Pulsellidae within the Gadilidae is already reported based on *18S* gene data (Steiner & Dreyer, 2003). This previous result is supported by all genetic markers except the *CO1* gene marker (Figs. 3-4, 6). The phylogenetic relationships between a larger number of species and genera of the Gadilidae were so far only studied once based on morphological data (Steiner, 1998). The species-level coded tree presented in that study displays the Gadilidae as a complete polytomy with the Pulsellidae as part of an earlier branch together with members of the Wemersoniellidae. Since there is a complete lack of molecular data for the Wemersoniellidae as well as most genera of the Gadilidae it is impossible to deduce any significant or clear phylogenetic relationships within the Gadilidae. As already mentioned for the Entalimorpha, more data is needed to establish a sound phylogeny of the Gadilida in general.

In summary, this study, based on molecular data from a broader taxon set, provides important information on the phylogeny of Scaphopoda. The previous phylogenetic studies based on morphological data are limited by their morphological character sets (Steiner, 1992a; 1996; 1998; 1999; Reynolds, 1997; Reynolds & Okusu, 1999) as are the previous two studies on molecular data limited by their taxon sample (Steiner & Reynolds, 2003; Steiner & Dreyer, 2003). The new data indicates para- or polyphyly for almost all genera for which molecular data is now available for more than one species. This also indicates that the assignment of most species almost solely on shell characteristics and sometimes radula features is not sufficient. Further anatomical data and newly found morphological characters are probably needed to facilitate species assignment. Since it is difficult to obtain fresh samples of Scaphopoda it might also help to resort more to morphometrics, as there are now some promising results concerning congeneric species differentiation (Shimek, 1989; Steiner & Linse, 2000; Absalão et al., 2005; Scarabino & Caetano, 2008; Caetano et al., 2010; Sahlmann et al., 2016; Souza & Caetano, 2020). However, more molecular data for additional species in combination with a multigene approach is needed to further research the overall molecular phylogeny of Scaphopoda.

# 5. Appendix

**Appendix 1:** List with data for the scaphopod specimens processed for the present study.

| **Specimen ID** | **Original-Description** | **Expedition** | **Country** | **Locality** | **Coordinates** | **Depth (m)** | **Date** | **Collected by** |
|---|---|---|---|---|---|---|---|---|
| *Episiphon virgula* 1 | GS MI5 | Japan 2018 | Japan | Sea of Japan | N 35° 45.020 / E 135° 19.994 | 91.6 - 89.7 | 19.09.2018 | Gerhard Steiner |
| *Siphonodentalium japonicum* 1 | GS MI19 | Japan 2018 | Japan | Sea of Japan | N 35° 45.020 / E 135° 19.994 | 91.6 - 89.7 | 19.09.2018 | Gerhard Steiner |
| *Fissidentalium magnificum* 1 | MNHN IM 2013 62881 | Biopapua | Papua New Guinea | Bismarck Sea | S 05° 20.000 / E 145° 51.000 | 720 - 676 | 05.10.2010 | Samadi & Corbari |
| *Compressidentalium sibogae* 1 | MNHN IM 2013 62889 | NanHai 2014 | | South China Sea | N 10° 25.000 / E 114° 46.000 | 1076 - 464 | 07.01.2014 | Chen Wei-jen |
| *Compressidentalium sibogae* 2 | MNHN IM 2013 62888 | NanHai 2014 | | South China Sea | N 10° 25.000 / E 114° 46.000 | 1076 - 464 | 07.01.2014 | Chen Wei-jen |
| *Calliodentalium crocinum* 1 | MNHN IM 2009 4655 | Aurora 2007 | Philippines | Philippine Sea | N 15° 27.000 / E 121° 36.000 | 556 | 31.05.2007 | MNHN-NUS-NFRDI |
| *Laevidentalium eburneum* 1 | MNHN IM 2009 4625 | Panglao 2005 | Philippines | Bohol Sea | N 09° 29.000 / E 123° 44.000 | 318 - 271 | 23.05.2005 | MNHN-NUS-NFRDI |
| *Dentalium elephantinum* 1 | MNHN IM 2013 54039 | Kavieng 2014 | Papua New Guinea | Kavieng Lagoon | S 02° 37.700 / E 150° 33.000 | 16 - 3 | 16.06.2014 | |
| *Gadila cobbi* 1 | MNHN IM 2013 10049 | Papua Niugini | Papua New Guinea | Bismarck Sea | S 05° 07.000 / E 145° 53.000 | 980 | 02.12.2012 | |
| *Dentalium elephantinum* 2 | MNHN IM 2013 54507 | Kavieng 2014 | Papua New Guinea | Kavieng Lagoon | S 02° 34.500 / E 150° 33.600 | 20 - 15 | 18.06.2014 | |
| *Dentalium aprinum* 1 | MNHN IM 2013 47513 | Kavieng 2014 | Papua New Guinea | Kavieng Lagoon | S 02° 41.200 / E 150° 41.200 | 12 - 3 | 04.06.2014 | |
| *Antalis vulgaris* 1 | EZ RO3 | Roscoff 2019 | France | English Channel | N 48° 42.879 / W 03° 54.170 | | 07.2019 | Elisabeth Zieger § |
| *Antalis vulgaris* 2 | EZ RO4 | Roscoff 2019 | France | English Channel | N 48° 42.879 / W 03° 54.170 | | 07.2019 | Elisabeth Zieger § |
| *Antalis vulgaris* 3 | EZ RO5 | Roscoff 2019 | France | English Channel | N 48° 42.879 / W 03° 54.170 | | 07.2019 | Elisabeth Zieger § |
| *Antalis vulgaris* 4 | EZ RO6 | Roscoff 2019 | France | English Channel | N 48° 42.879 / W 03° 54.170 | | 07.2019 | Elisabeth Zieger § |
| *Antalis vulgaris* 5 | EZ RO7 | Roscoff 2019 | France | English Channel | N 48° 42.879 / W 03° 54.170 | | 07.2019 | Elisabeth Zieger § |
| *Antalis vulgaris* 6 | EZ RO8 | Roscoff 2019 | France | English Channel | N 48° 42.879 / W 03° 54.170 | | 07.2019 | Elisabeth Zieger § |
| Dentaliidae sp. 1 | MNHN IM 2013 62883C | Biopapua | Papua New Guinea | Bismarck Sea | S 04° 40.000 / E 151° 33.000 | 866 - 760 | 23.09.2010 | Samadi & Corbari |
| *Paradentalium healyi* 1 | MNHN IM 2013 62878 | Biopapua | Papua New Guinea | Solomon Sea | S 05° 35.000 / E 151° 35.000 | 680 - 470 | 16.10.2010 | Samadi & Corbari |
| *Striocadulus sagei* 1 | MNHN IM 2013 62882 | Biopapua | Papua New Guinea | Bismarck Sea | S 03° 48.000 / E 144° 39.000 | 729 - 720 | 01.10.2010 | Samadi & Corbari |
| *Gadilina insolita* 1 | MNHN IM 2013 62883B | Biopapua | Papua New Guinea | Bismarck Sea | S 04° 40.000 / E 151° 33.000 | 866 - 760 | 23.09.2010 | Samadi & Corbari |
| *Episiphon candelatum* 1 | MNHN IM 2009 4636 | Taiwan 2001 | Taiwan | NE Coast | S 24° 48.000 / E 122° 08.000 | 331 - 326 | 19.05.2001 | Bouchet, Richer de Forges-IRD & Chan |
| *Anulidentalium bambusa* 1 | MNHN IM 2013 62887 | Aurora 2007 | Philippines | Philippine Sea | N 15° 20.000 / E 121° 34.000 | 339 - 327 | 31.05.2007 | MNHN-NUS-NFRDI |
| *Antalis vulgaris* 7 | GS Rov1 | Rovinj 2019 | Croatia | Ronvinj | | 5 | 07.2019 | Gerhard Steiner |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Rhabdus toyamaense* 1 | GS MI38 | Japan 2018 | Japan | Sea of Japan | N 35° 45.020 / E 135° 19.994 | 91.6-89.7 | 19.09.2018 | Gerhard Steiner |
| *Entalinopsis habutae* 1 | GS MI36 | Japan 2018 | Japan | Sea of Japan | N 35° 45.020 / E 135° 19.994 | 91.6-89.7 | 19.09.2018 | Gerhard Steiner |
| *Episiphon virgula* 3 | GS 74 | Japan 2019 | Japan | Sea of Japan | N 35° 45.020 / E 135° 19.994 | 91.6-89.7 | 09.2019 | Gerhard Steiner |
| *Rhabdus toyamaense* 2 | GS 75 | Japan 2019 | Japan | Sea of Japan | N 35° 45.020 / E 135° 19.994 | 91.6-89.7 | 09.2019 | Gerhard Steiner |
| *Rhabdus toyamaense* 3 | GS 76 | Japan 2019 | Japan | Sea of Japan | N 35° 45.020 / E 135° 19.994 | 91.6-89.7 | 09.2019 | Gerhard Steiner |

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**Appendix 2:** List of evolutionary models used for tree reconstruction in IQ-Tree and MrBayes. For the maximum likelihood analyses, the ln likelihood and for the Bayesian Inference analyses, the arithmetic and harmonic mean are given. P1, P2, and P3 refer to the different codon positions of *CO1*.

| | **4gTree** | **3gTree** | **2gTree** | ***CO1*** | ***16S*** | ***18S*** | ***28S*** |
|---|---|---|---|---|---|---|---|
| **Models IQ-Tree** | P1: TIM2+F+G4 | P1: TIM2+F+I+G4 | P1: TIM2+F+I+G4 | P1: TN+F+I+G4 | GTR+F+I+G4 | TN+F+I+G4 | TN+F+G4 |
| | P2: TVM+F+I | P2: TVM+F+G4 | P2: TVM+F+G4 | P2: GTR+F+G4 | | | |
| | P3: HKY+F+G4 | P3: K3Pu+F+G4 | P3: HKY+F+G4 | P3: HKY+F+G4 | | | |
| | 16S: TIM2+F+G4 | 16S: TIM2+F+G4 | 16S: TIM2+F+I+G4 | | | | |
| | 18S: TNe+G4 | 18S: TNe+I+G4 | 18S: TIM3+F+I+G4 | | | | |
| | 28S: TN+F+G4 | 28S: TN+F+G4 | 28S: TN+F+G4 | | | | |
| **ln likelihood** | -21848.535 | -25179.616 | -31832.763 | -9996.535 | -7429.197 | -10337.498 | -6741.097 |
| **Models MrBayes** | P1: nst = 6<br>rates = propinv | P1: nst = 6<br>rates = propinv | P1: nst = 6<br>rates = invgamma | P1: nst = 6<br>rates = invgamma | nst = 6<br>rates = invgamma | nst = 6<br>rates = invgamma | nst = 6<br>rates = gamma |
| | P2: nst = 6<br>rates = invgamma | P2: nst = 6<br>rates = propinv | P2: nst = 6<br>rates = propinv | P2: nst = 6<br>rates = gamma | | | |
| | P3: nst = 2<br>rates = invgamma | P3: nst = 6<br>rates = gamma | P3: nst = 2<br>rates = gamma | P3: nst = 2<br>rates = gamma | | | |
| | 16S: nst = 6 rates = invgamma | 16S: nst = 6<br>rates = gamma | 16S: nst = 6<br>rates = invgamma | | | | |
| | 18S: nst = 6 rates = invgamma | 18S: nst = 6<br>rates = invgamma | 18S: nst = 6<br>rates = invgamma | | | | |
| | 28S: nst=6 rates = gamma | 28S: nst = 6<br>rates = gamma | 28S: nst = 6<br>rates = invgamma | | | | |
| **Arithmetic mean (Total)** | -18975.08 | -22292.27 | -28965.12 | -9995.70 | -7469.74 | -10374.06 | -6757.28 |
| **Harmonic mean (Total)** | -19011.06 | -22333.69 | -29018.35 | -10071.85 | -7524.91 | -10428.65 | -6790.91 |

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