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Phylogeny of Serpulidae (Annelida, Polychaeta) Inferred from Morphology and DNA Sequences, with a New Classification

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Abstract: Serpulidae Rafinesque, 1815 is a speciose group of polychaetes that all inhabit calcareous tubes. The family was traditionally subdivided into Serpulinae, Filograninae, and Spirorbinae. Recent phylogenetic analyses have suggested that both Filograninae and Serpulinae are paraphyletic, though with limited sampling. Here we report the first phylogenetic analysis of Serpulidae based on comprehensive sampling of genera (though excluding most spirorbin genera). We include a much-needed revision of serpulid taxonomy based on a phylogenetic hypothesis derived from both morphological and molecular data. We analysed 18S, 28S, histone H3 ribosomal nuclear DNA and cytochrome b (cytb) mitochondrial sequences, combined with morphological data. The proposed new classification includes the re-formulated Serpulinae (with tribes Serpulini and Ficopomatini), Spirorbinae, and Filograninae, with apomorphies highlighted for major taxa.

Keywords: Annelida; Serpulidae; phylogeny; Bayesian analysis; maximum likelihood

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1. Introduction

Serpulidae Rafinesque, 1815 is a clade of polychaetes permanently living in calcareous tubes. Recent reviews have assessed Serpulidae as having 506–576 accepted species [1]. A comprehensive review of Sabellida by Capa et al. [2] lists 69 genera of Serpulidae, which includes 48 genera with 374 extant species of Serpulinae *sensu lato* and 23 genera with 188 extant species of Spirorbinae. Over half of the nominal serpulin species belong to four genera: *Hydroides* (105), *Spirobranchus* (42), *Serpula* (26), and *Spiraserpula* (18). Twenty genera are monotypic, some of them being rare and/or found in the deep sea only (e.g., *Bathyditrupa*, *Chitinopomoides*, *Microprotula*, *Vitreotubus*, *Zibrovermilia*), known only from type material (*Tanturia*) or, at the extreme, known only from a poorly preserved holotype (*Paumotella*). The recent revisionary studies within Serpulidae include morphology-based studies of *Pseudochitinopoma* by Kupriyanova et al. [3] and *Spirodiscus* by Kupriyanova and Ippolitov [4] as well as integrative phylogenetic studies of *Hydroides* by Sun et al. [5] and *Laminatubus* by Rouse and Kupriyanova [6]. There are also several other relevant sources on the biodiversity and taxonomy of Serpulidae [7–25].

Serpulidae is placed within the large annelid clade Sabellida that was initially named by Dales [26] for just Sabellidae and Serpulidae. Sabellida became commonly used in major taxonomic works and though the composition of the group has varied (e.g., Fauchald [27], Pettibone [28], Rouse et al. [1]), Serpulidae and Sabellidae have always been maintained as part of this taxon though views on the membership and relationships within have otherwise varied. Smith [29] argued that Sabellidae was paraphyletic with Serpulidae, being the sister group to Sabellinae. This arrangement was also found by Rousset et al. [30], though with limited taxon sampling. Subsequently, further sampling using nuclear and mitochondrial DNA data also showed sabellid paraphyly and a sister-group relationship

between Serpulidae and Fabriciinae (Kupriyanova and Rouse [31]). As a result, Fabriciinae was raised to the family rank, Fabriciidae. Most recently, Tilic et al. [32] found Serpulidae to be closer to Sabellidae than to Fabriciidae, based on phylogenomic data.

Earlier hypotheses about relationships within Serpulidae are reviewed in Kupriyanova et al. [33]. Briefly, Serpulidae was initially divided into the subfamilies Serpulinae Rafinesque, 1815 [34] and Spirorbinae Chamberlin, 1919 [35] until Rioja [36] established the subfamily Filograninae in 1923 and later Ficopomatinae was established by Pillai [37] in 1960. The most drastic proposed revision of Serpulidae by Uchida [38] proposed the creation of 11 subfamilies and numerous new genera based on small (partly presumed) differences in chaetal structure. The phylogenetic trees of Uchida [38] were not based on formal datasets with repeatable analyses and, as a result, his classification has hardly been used by other authors.

Spirorbinae was elevated to Spirorbidae by Pillai [39] in 1970. However, Fitzhugh [40] and then Smith [29] suggested that Spirorbidae were more closely related to Serpulinae than to Filograninae and concluded that maintaining the family Spirorbidae was not justified. An analysis of relationships among spirorbin genera by Macdonald [41] also showed that Spirorbinae were more closely related to Serpulinae than to Filograninae. All analyses of molecular data have demonstrated that Spirorbinae should be treated as a sub-taxon of Serpulidae (Kupriyanova et al. [33]; Lehrke et al. [42]; Kupriyanova and Rouse [31]; Kupriyanova et al. [43]; Kupriyanova and Nishi [44]), which was adopted by Rzhavsky et al. [45].

Ficopomatinae was proposed [37,46] with the diagnosis: "stout teeth in collar chaetae, wingless opercular peduncle, vesicular opercula and geniculate abdominal chaetae". The subfamily was revised by ten Hove and Weerdenburg [47] in 1978 and, as a result, four brackish-water monotypic genera were placed into *Ficopomatus*. More recently, *Ficopomatus talehsapensis* Pillai, 2008 [48] and *Ficopomatus shenzhensis* Li et al., 2012 [49] were added to the genus, while Styan et al. [50] demonstrated that *Ficopomatus enigmaticus* (Fauvel, 1923) [51] in Australia (supposedly its native range) consists of three genetic groups with overlapping ranges, one of which is morphologically distinct from the other two. *Marifugia cavatica* Absolon and Hrabě, 1930 [52], the only freshwater serpulid, has been shown to be a part of a monophyletic *Ficopomatus-Marifugia* clade [43,48], which is treated as Ficopomatinae by some authors [48,49,53].

Both Filograninae and Serpulinae have been problematic from the phylogenetic point of view. When Filograninae was proposed by Rioja [36], he stated that "presence of pinnules on the opercular peduncle . . . . indicates that the species included in this subfamily are very primitive, . . . , corroborated by a hardly developed operculum". Rioja included *Filograna* and *Salmacina* that have fin-and-blade special collar chaetae, as well as *Apomatus* and *Protula* with simple collar chaetae. He also tentatively included *Josephella marenzelleri* Caullery and Mesnil, 1896 [54] in Filograninae. Rioja also included *Spirodiscus grimaldii* (Fauvel, 1909) [55] in the subfamily because of the pinnulated opercular peduncle, even though this species has a well-developed chitinized operculum. Finally, Rioja [36] also mentioned *Protis* as a possible member of the group, with collar chaetae like those of *Filograna* and *Salmacina* and without an operculum. In her catalogue, Hartman [56] classified *Apomatus*, *Protula*, and *Spirodiscus* as Serpulinae. Fauchald [27] included *Filogranula*, *Filograna*, *Salmacina*, *Salmacinopsis*, and *Spirodiscus* in Filograninae, but listed *Apomatus*, *Protis*, and *Protula* as Serpulinae. Neither Hartman [56] nor Fauchald [27] gave any reasoning behind such arrangements. ten Hove [57] was the first to suggest that Filograninae may be paraphyletic and a morphology-based cladistic morphological analysis by Kupriyanova [58] also recovered Filograninae as a grade.

Later molecular phylogenetic studies showed Filograninae to potentially be polyphyletic (Kupriyanova et al. [33]) or at least paraphyletic (Lehrke et al. [42]). Kupriyanova et al. [33] assessed phylogenetic relationships within Serpulidae using both molecular and morphological characters while Lehrke et al. [42] conducted a similar analysis using 18S ribosomal DNA data and fewer terminals. Both studies found Serpulinae to be paraphyletic. Kupriyanova et al. [33] refrained from revising serpulid classification but suggested that Serpulidae needed further comprehensive phylogenetic analyses.

Here the phylogenetic relationships within Serpulidae are revisited. We added available and newly obtained molecular sequence data to create a total evidence phylogeny of serpulids based on combined molecular and morphological datasets.

2. Materials and Methods

2.1. Taxa Used in This Study and Morphological Matrix

This study is based on all genera currently included in Serpulidae according to Capa et al. [2] but excluding most Spirorbinae. At least one representative from each genus was used in the analysis. The selection of taxa was based on the availability of fresh material for combined analyses of morphological and molecular data. The type species of a genus could not always be used to score the characters, as material was not available and/or the original description was inadequate. In total, molecular data were available for 93 ingroup terminals from 35 genera (Table 1). Phylogenetic positions of poorly known genera *Bathyditrupa*, *Chitinopomoides*, *Microprotula*, *Neomicrorbis*, *Omphalopomopsis*, *Paumotella*, *Spirodiscus*, *Tanturia*, *Vitreotubus*, and *Zibrovermilia* were inferred from morphological data only. Both previously published and new sequences were used. Whether the currently accepted as valid serpulid genera are monophyletic remains unknown, even questionable (e.g., ten Hove and Kupriyanova [59]: 66, 71, 83, 102 on *Neovermilia*, *Paraprotis*, *Protula*, and *Vermiliopsis*, respectively, Kupriyanova and Rouse [31]) and the monophyly of each non-monotypic genus with only a single representative included here needs to be assessed in more restricted analyses.

Table 1. Terminals with vouchers and GenBank accession numbers. FMNH–Field Museum of Natural History, Chicago, IL, USA; SIO–Scripps Institution of Oceanography, San Diego, CA, USA; NHMW–Museum of Natural History of Vienna (=Wien), Austria; USNM–United States National Museum, Washington, DC, USA.

Species	Vouchers	28S	18S	Histone H3	Cytochrome b
Apomatus globifer	ZMA V.Pol. 5250	EU195362	EU195378	OQ397982	OQ427448
Apomatus sp.	FMNH 5201	OQ389662	OQ379428	OQ397983	OQ427449
Apomatus voightae	FMNH 6217	OQ389663	GU441856	-	OQ427450
Bathyditrupa hovei	ZMA V.Pol 5325	-	-	-	-
Bathyvermilia eliasoni	FMNH 6189	-	GU441857	-	-
Chitinopoma serrula	SAM E3524	EU195350	DQ317112	OQ397984	-
Chitinopomoides wilsoni	ZMA V.Pol. 3166	-	-	-	-
Crucigera inconstans	SAM E3525	EU184071	DQ317113	-	EU190464
Crucigera tricornis	SAM E3587	EU184067	EU184056	-	EU190474
Crucigera zygophora	SAM E3503	DQ242577	DQ242543	EF192929	EU190470
Dasynema chrysogyrus	AM W.45087	OQ397664	OQ379429	-	-
Ditrupa arietina	SAM E3527	EU195351	DQ317114	EF192933	-
Ficopomatus enigmaticus	SAM E3356	EU195373	DQ317115	OQ427487	OQ427451
Ficopomatus macrodon	SAM E3618	EU167535	EU167532	OQ412612	KP863778
Ficopomatus miamiensis	SAM E3617	EU167534	EU167531	OQ397989	KP863779
Filograna implexa	SAM E3528	EU195347	DQ317116	-	OQ427452
Filogranella elatensis	SAM E3661	EU195370	EU195385	-	-
Filogranula stellata	SAM E3606	EU195358	EU195374	OQ397985	-
Floriprotis sabiuraensis	SAM E3659	EU195371	EU195386	-	OQ427453
Floriprotis sabiuraensis	SAM E7192	OQ389665	OQ379430	-	OQ427454
Galeolaria caespitosa	SAM E3529	OQ389666	OQ379431	OQ412631	EU184054
Galeolaria hystrix	SAM E3526	EU256550	DQ314839	OQ397988	EU200441
Helicosiphon biscoeensis	SIO-BIC A4000	OQ392408	OQ379432	OQ412613	-
Hyalopomatus mironovi	SAM E3728	OQ651975	GU063862	MT468421	MT468442
Hydroides elegans	SAM E3616	EU195369	EU195384	OQ412614	OQ427455
Hydroides ezoensis	SAM E3584	EU184077	EU184062	-	OQ427456
Hydroides nikae	SAM E3530	EU184072	DQ317117	-	EU190466
Hydroides minax	SAM E3597	EU184074	EU184063	-	EU190475

Table 1. *Cont.*

Species	Vouchers	28S	18S	Histone H3	Cytochrome b
Hydroides pseudouncinata	ZMA V.Pol. 5240	EU184075	DQ140403	-	EU190467
Hydroides sanctaecrucis	SAM E3625	EU184076	EU184061	-	-
Hydroides trivesiculosa	SAM E3601	EU184073	EU184060	OQ397992	EU190476
Hydroides tuberculata	SAM E3596	OQ389667	EU184059	-	EU190473
Janita fimbriata	AM W.42388	OQ389668	OQ379433	-	OQ427457
Josephella marenzelleri	SAM E3620	EU195359	EU195375	-	OQ427458
Laminatubus alvini	SAM E3531	EU195355	DQ317118	OQ412616	OQ427459
Marifugia cavatica	SAM E3612	EU167533	EU167530	OQ397990	OQ427460
Metavermilia acanthophora	SAM E3533	EU195352	DQ317119	-	OQ427461
Microprotula ovicellata	ZMA V.Pol. 4046	-	-	-	-
Neomicrorbis azoricus	ZMA V.Pol. 3905	-	-	-	-
Neovermilia globula	SAM E3586	EU195363	EU195379	-	-
Spirodiscus grimaldii	ZMA V.Pol. 3906	-	-	-	-
Omphalopomopsis langerhansii	NHMWAN14552.2054	-	-	-	-
Paraprotis dendrova	SAM E3591	EU195361	EU195377	-	-
Paraprotis pulchra	SAM E3665	OQ389669	OQ379434	OQ412629	OQ427462
Paumotella takemoana	USNM 19432	-	-	-	-
Placostegus sp.	SAM E3589	OQ397665	OQ379435	OQ412628	-
Placostegus tridentatus	SAM E3585	EU195364	OQ379436	OQ412622	-
Pomatostegus actinoceras	AM W.42378	OQ389670	OQ379437	-	-
Pomatostegus stellatus	SAM E3607	EU195367	EU195382	-	-
Protis hydrothermica	SAM E3541	EU195356	DQ317122	-	-
Protis sp.	SAM E3727	OQ389671	OQ379438	-	OQ427463
Protula bispiralis	SAM E3657	OQ389672	OQ379439	OQ412609	OQ427464
Protula tubularia	SAM E3542	EU195349	DQ317123	EF192934	OQ427465
Pseudochitinopoma occidentalis	SAM E3501	DQ242575	DQ242542	OQ412626	OQ427466
Pseudochitinopoma pavimentata	SAM E3660	OQ397666	OQ379440	OQ412627	OQ427467
Pseudovermilia occidentalis	SAM E3613	EU195368	EU195383	-	OQ427468
Pyrgopolon ctenactis	SIO-BIC A25451	OQ389673	OQ379441	OQ412625	-
Rhodopsis pusilla	SAM E3621	EU195360	EU195376	OQ397987	OQ427469
Salmacina sp.	SAM E3499	EU256545	DQ317126	-	OQ427470
Semivermilia annehoggettae	SAM E3628	OQ389674	OQ379442	-	OQ427471
Semivermilia elliptica	SAM E3664	EU195372	EU195387	OQ397986	OQ427472
Semivermilia lylevaili	SAM E3629	OQ397667	OQ389601	-	-
Serpula columbiana	SAM E3505	DQ242576	DQ317127	-	EU190469
Serpula concharum	ZMA V.Pol. 5245	EU184066	DQ140408	-	EU190468
Serpula jukesii	SAM E3536	EU184069	DQ317129	-	EU190465
Serpula narconensis	SIO-BIC A3469	OQ389676	OQ379443	OQ397991	-
Serpula uschakovi	SAM E3593	EU184078	EU184065	-	EU190477
Serpula vermicularis	SAM E3537	EU184070	DQ317128	-	EU190479
Serpula vittata	SAM E3594	EU184079	EU184064	-	EU190471
Serpula watsoni	SAM E3595	EU184068	EU184057	-	EU190472
Spiraserpula iugoconvexa	AM W.42093	OQ389680	OQ379444	-	OQ427473
Spirobranchus akitsushima	ZMA V.Pol. 3201	EU195365	EU195380	-	OQ427474
Spirobranchus corniculatus	ZMA V.Pol. 5247	OQ389677	OQ379446	-	OQ427475
Spirobranchus corniculatus	SAM E3608	EU195366	EU195381	-	OQ427476
Spirobranchus coronatus	SAM E3609	OQ389678	OQ379445	OQ412624	OQ427477
Spirobranchus kraussii	AM W.49977	OQ397668	MK308673	OQ412619	MK308658
Spirobranchus lima	SAM E3538	EU256547	DQ317130	EF192930	OQ427478
Spirobranchus richardsmithi	SAM E3610	OQ389679	OQ379447	-	OQ427479
Spirobranchus taeniatus	SAM E3532	EU195353	DQ317120	OQ412618	OQ427480
Spirobranchus triqueter	SAM E3534	EU195348	DQ317121	EF192932	OQ427481
Tanturia zibrowii	ZMA V.Pol. 4668	-	-	-	-
Turbocavus secretus	USNM 251863	-	OQ379448	OQ412611	OQ427483
Vermiliopsis infundibulum	ZMA V.Pol. 5248	OQ389681	DQ140411	-	OQ427484
Vermiliopsis labiata	SAM E3543	EU256549	DQ317131	-	OQ427485
Vermiliopsis pygidialis	SAM E3544	EU256546	DQ317132	-	-

Table 1. *Cont.*

Species	Vouchers	28S	18S	Histone H3	Cytochrome b
Vermiliopsis striaticeps	SAM E3545	EU256548	DQ317133	EF192931	OQ427486
Vitreotubus digeronimoi	ZMA V.Pol.3907	-	-	-	-
Zibrovermilia zibrowii	AM W.46387	-	-	-	-
Protolaeospira tricostalis	SAM E3487	DQ242606	DQ318587	EF192936	-
Romanchella quadricostalis	SAM E3491	DQ242608	DQ242559	EF192935	-
Spirorbis tridentatus	SAM E3477	DQ242602	DQ242573	OQ412623	-
Manayunkia athalassia	SAM E3518	DQ209245	EF116202	EF192917	-
Schizobranchia insignis	GenBank	AY732225	AY732222	-	-

The genera not included in the analysis are *Membranopsis*, synonymised with *Protula* by ten Hove and Kupriyanova [59], *Pomatoleios* and *Pomatoceros* synonymized with *Spirobranchus* by Pillai [15], as well as monotypic genera *Kimberleya* and *Pseudoprotula* that have not been formally synonymised yet, but in our opinion most likely should be attributed to *Protula*. Given that the monophyly of Spirorbinae is undisputed, its position within Serpulidae has been determined in previous studies [33,42,58] and phylogenetic relationships among Spirorbinae have been assessed [41,60], only four spirorbins were included in this study.

The morphology of Serpulidae *sensu lato* was reviewed and illustrated by ten Hove and Kupriyanova [59] with special reference to the features that can provide characters for a phylogenetic analysis. The morphology of all species used in the analysis was examined by us and details of the structure of their chaetae and uncini were elucidated with the help of SEM. For complex features where the whole feature (such as the operculum) could be absent, absence–presence was treated as a separate character, whereas different states of the compound character were coded as subsidiary characters. Terminals coded as absent for the more general characters were coded as 'inapplicable' with a "-" [61] for the subsidiary characters, which is treated as ? by PAUP * version 40b10 [62]. Unknown character states were coded with a "?". The characters for the morphological matrix are listed in Appendix A, the description of characters is available in Supplementary File S1, morphological matrix in nexus format as Supplementary File S2, and combined matrix of morphological and molecular data in nexus format as Supplementary File S3.

2.2. DNA Extraction, Amplification, and Sequencing

Specimens for molecular work were preserved in ethanol and stored at −20 °C. Voucher specimens were preserved in 10% formalin and transferred to 70% ethanol after rinsing in water. The vouchers were deposited in South Australian Museum (SAM), Naturalis Biodiversity Centre (including the former Zoological Museum of University of Amsterdam, ZMA), and Australian Museum (AM), unless indicated otherwise (see Table 1).

Molecular work was conducted at the University of Adelaide Evolutionary Biology Unit (EBU), molecular laboratory of Japanese Agency for Marine Science and Technology, Yukosuka, Japan (JAMSTEC), and Australian Center for Wildlife Genomics at the Australian Museum (ACWG AM).

Total DNA was extracted using Qiagen DNeasy Kit or using a Bioline Isolate II genomic DNA kit following the manufacturer's protocols. Stock DNA was diluted 1:10 or 1:100 with deionized water to produce optimal template strength DNA for Polymerase Chain Reactions (PCR).

Partial or near complete 18S rRNA gene (18S) sequences were amplified in two overlapping fragments, one of approximately 1100 bp with the primers TimA (AMCTGGTTGATCCTGCCA G) and 1100R2 (CGGTATCTGATCGTCTTCGA) [63]; the other of approximately 1300 bp using 18s2F (GTTGCT GCAGTTAAA) and 18s2R (ACCTTGTTAGCTGTTTTACTTCCTC) [33]. An approximately 900 bp fragment of 28S rRNA gene (28S) was amplified using primers LSUD1F (ACCCGCTGAATTTAAGCATA) and D3ar (ACGAACGATTTGCACGTCAG) [64]. In some cases, a shorter D1 fragment (approximately 350 bp) was amplified using primers ACCCSCTGAAYTTAAGCAT and AACTCTCTCMTTCARAGTTC [65].

A part of the mitochondrial Cytochrome b (Cytb) gene (approximately 350 bp) was amplified with the primer pair Cytb424F (GGWTAYGTWYTWCCWTGRGGWCARAT) and cobr825 (AARTAYCAYTCYGGYTTRATRTG) [66]. An approximately 350 bp fragment of Histone H3 (H3) gene was amplified with the primers (1) ATGGCTCGTACCAAGCAGACVGC and ATATCCTTRGGC ATRATRGTGAC or (2) ATGGCTCGTACCAAGCAGAC and ATRTCCTTGGGCATGATTGTTAC [65].

The PCR products were separated by gel electrophoresis in 1.5% agarose gel and visualized under UV after staining with ethidium bromide or staining with gel red (Biotium TM, San Francisco, CA, USA).

At AM, successful PCR products were sent to Macrogen TM, South Korea for purification and standard Sanger sequencing. The successful PCR products were purified with UltraClean PCR Clean-up DNA purification kit by MoBio Laboratories following the manufacturer's protocol (EBU) or with Gel and PCR Clean-up DNA purification kit (Promega) following the manufacturer's protocol (JAMSTEC). At EBU and JAMSTEC, PCR products were sequenced in both directions using Big Dye Ver. 3 chemistry with the same primers as in PCR.

At EBU, sequenced products were purified using the magnetic method with CleanSeq kit by Agencourt Biosciences Corporation, whereas the Performa® DTR Gel Filtration Cartridge kit (EdgeBio) was used at JAMSTEC. Products of the sequencing reactions were read on an automated capillary sequencer ABI 3130 Genetic Analyzer (Applied Biosystems) at the Institute of Medical and Veterinary Sciences (IMVS) in Adelaide or at JAMSTEC molecular laboratory.

Sequences were edited using SeqEd ver. 1.0.3 (Applied Biosystems Inc.) or Geneious. BLAST searches [67] confirmed the correct gene regions had been amplified and the new sequences were submitted to GenBank (Table 1).

2.3. Phylogenetic Analyses

Analyses were performed on three datasets: Molecular data only, Morphology data only, and a combined Morphology and Molecular dataset. Morphology was coded for representatives of all available genera, but molecular data was not available for *Bathyditrupa*, *Chitinopomoides*, *Omphalopomopsis*, *Microprotula*, *Neomicrorbis*, *Paumotella*, *Spirodiscus*, *Vitreotubus*, *Tanturia*, and *Zibrovermilia*. *Manayunkia athalassia* (Fabriciidae) and *Schizobranchia insignis* (Sabellidae) were chosen as the most appropriate outgroups based on previous phylogenomic results [32].

2.3.1. Morphology Only Dataset

The 75-character morphology dataset was analysed using PAUP* v.4.0a166 [62]. Characters were initially treated as unordered. Owing to their being 93 terminals and many 'inapplicable' character scores, the dataset could not be run with simple parameters to explore tree space properly. Therefore, a parsimony ratchet approach [68] was used with PRAP v. 2.0 [69] in association with PAUP*. Ten runs with 200 ratchet iterations were performed and the resulting shortest trees, after filtering for duplicates, were then used for further searching with the command "hsearch start = current swap = TBR steepest = no multrees = yes" to find all the shortest trees. A strict consensus tree was generated from the resulting trees.

2.3.2. Molecular Dataset

The gene partitions were aligned using Muscle (H3, CytB) [70] or MAFFT (18S, 28S) [71] and concatenated using Sequence Matrix [72]) or RAxML-NG [73,74], resulting in an alignment of 4483 base pairs. This data set was partitioned by gene and appropriate models selected by ModelTest-NG [75] under the Bayesian information criterion (BIC) before maximum likelihood (ML) analysis with RaxML-NG. Models used were GTR + I + Γ (18S, 28S, Cytb) or TIM2 + I + Γ (Histone H3). Fifty random addition searches were performed as well as node support assessment via 'thorough' bootstrapping (with 1000 pseudorepli-

cates). Bayesian inference (BI) analysis of the concatenated data, partitioned by gene, was conducted using Mr. Bayes v.3.2.7a [76]. All partitions were run using GTR + I + Γ. Default priors in MrBayes were used and data partitions were unlinked for parameter estimations. Two iterations of four Markov chain Monte Carlo (MCMC) chains were run for 50 million generations, sampling every thousand generations. A majority rule consensus tree was made from the trees remaining after discarding 25% of trees as burn-in, after checking with Tracer 1.7.1 [77].

2.3.3. Molecular + Morphology Dataset

The molecular dataset was treated the same as in the molecular-only analyses above. These partitions were concatenated with the morphology dataset. In RaXML-NG, the morphology partition was run with the MULTIx MK model (with x as 6 for the maximum six-character states), while in MrBayes, the morphology dataset was analysed under the Mkv model. Both these models are derived from Lewis' [78] likelihood model for discrete morphology data. Several morphological characters were traced on the BI tree topology from the molecular + morphology dataset in Mesquite [79] using likelihood ancestral state reconstruction, with the Mk1 probability model.

3. Results

3.1. Morphology Only Dataset

The parsimony ratchet analysis and subsequent heuristic search of the morphology data matrix resulted in 1,192,317 shortest trees of 352 steps. Seventy of the seventy-five characters used were parsimony informative. The consistency index was 0.27 and the retention index 0.78. The strict consensus of these trees resulted in a major polytomy (Figure 1). The only partially resolved larger clades included *Apomatus-Protula*, *Crucigera-Serpula-Hydroides-Floriprotis*, *Galeolaria-Pyrgopolon-Spirobranchus*, *Ditrupa-Ficopomatus-Hyalopomatus-Placostegus-Marifugia*, and *Spirodiscus-Bathyditrupa*. Spirorbin taxa were recovered as a clade with *Helicosiphon* as sister group to *Spirorbis*-(*Protolaeospira-Romanchella*). Apart from Spirorbinae, no major clades were recovered and the relationships within Serpulidae were largely unresolved. Genera with multiple terminals such as *Apomatus*, *Protula*, *Crucigera*, *Ficopomatus*, *Galeolaria*, *Pomatostegus*, and *Semivermilia* were recovered as clades. Notably other important genera such as *Hydroides*, *Protis*, *Serpula*, *Spirobranchus*, and *Vermiliopsis* were not recovered as monophyletic.

3.2. Molecular Only Dataset

The ML and BI analyses of the molecular-only data set resulted in identical tree topologies (Figure 2). The analyses inferred three well-supported major clades within Serpulidae. One included those taxa typically attributed to the subfamily Serpulinae and was further split into two well-supported sister clades. One included a monophyletic well-supported *Hydroides* clade along with representatives *Serpula*, *Crucigera*, *Spiraserpula*, and *Floriprotis*. We refer to this as Serpulini. The other major serpulin clade is referred to here as Ficopomatini. Notably, *Neovermilia globula* was the sister group to all other Ficopomatini, which had a relatively long branch and formed a highly supported clade. Within Ficopomatini, *Spirobranchus* formed a well-supported clade that was the sister group to *Pyrgopolon*. *Laminatubus alvini* was recovered as sister to the *Spirobranchus–Pyrgopolon* clade and similarly *Ditrupa* was sister to a *Pseudochitinopoma* clade, both with high support. Other highly supported groups include monophyletic *Placostegus* and *Galeolaria* clades. *Marifugia cavatica* was nested within *Ficopomatus* forming a well-supported clade that was sister to *Galeolaria*, though with low support. The phylogenetic positions of *Hyalopomatus mironovi* and relationships among the *Placostegus* and *Pseudochitinopoma-Ditrupa* clades can be regarded as relatively uncertain owing to low support.

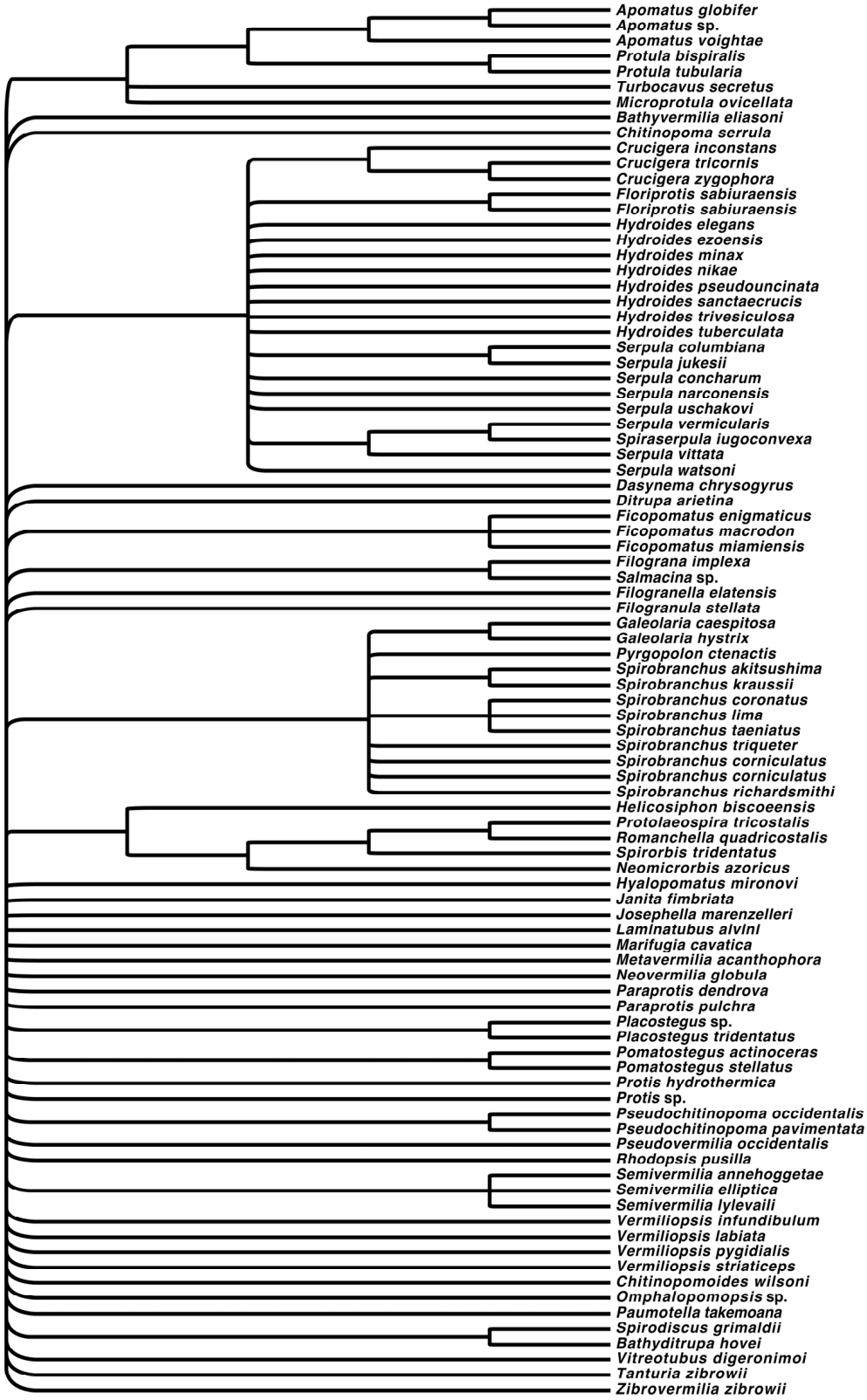

Figure 1. Strict consensus of 1,192,317 shortest trees resulted from the maximum parsimony analysis of 75-character morphology dataset. Outgroups are excluded.

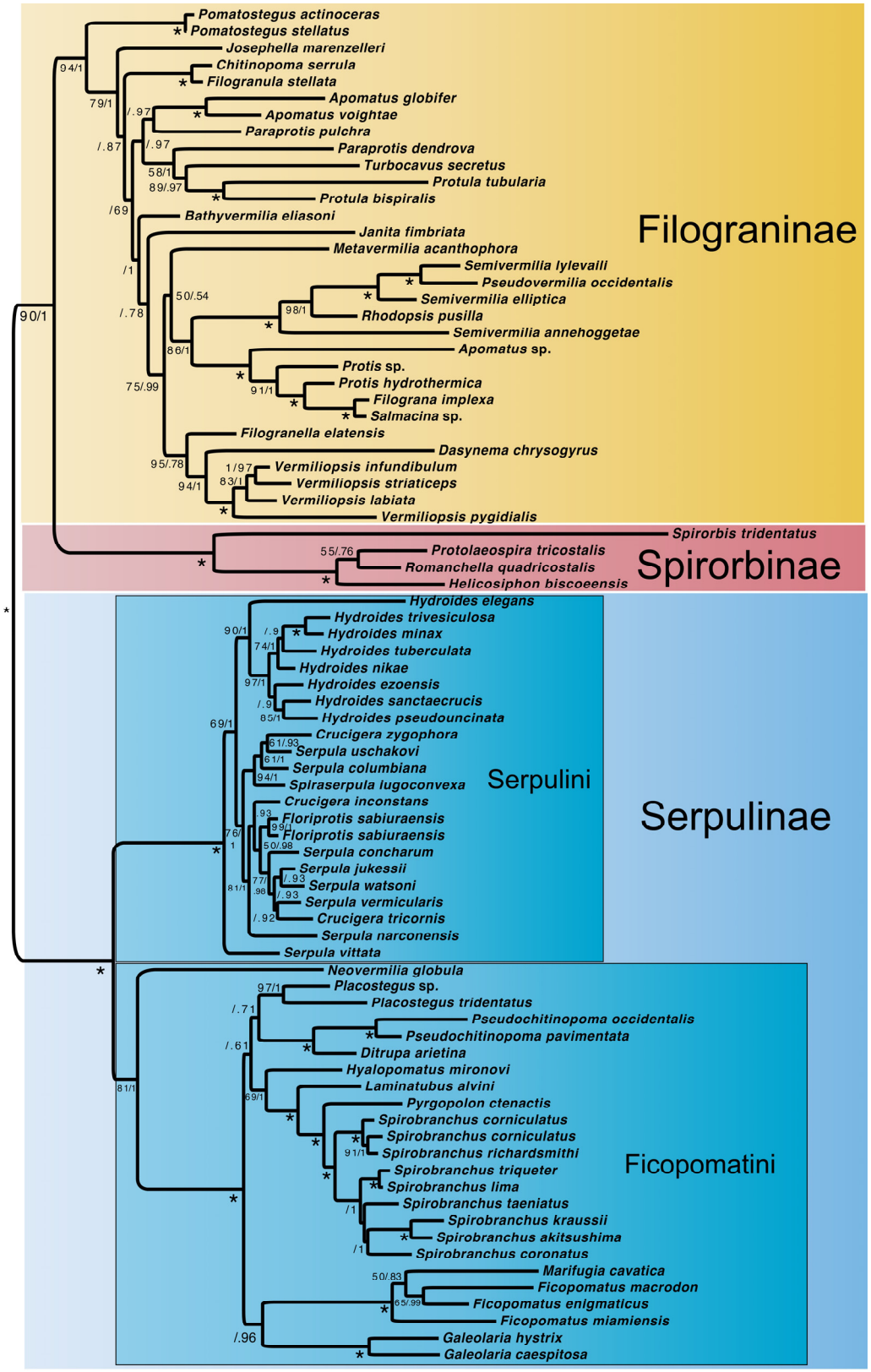

Figure 2. Maximum likelihood (RaXML) best tree based on the molecular data set. Outgroups are excluded. The BI analysis topology was congruent. ML bootstrap values are shown followed by posterior probabilities for the BI analysis. Nodes with bootstrap values of 100 and posterior probabilities of 1.0 are indicated with *. Nodes with bootstrap values < 50% and posterior probabilities < 0.5 are blank.

The second well-supported major clade included many terminals that had been attributed to Filograninae (*Apomatus*, *Filograna*, *Filogranula*, *Josephella*, *Protis*, *Protula*, *Rhodopsis*, *Salmacina*), as well as several traditionally attributed to Serpulinae (*Bathyvermilia*, *Chitinopoma*, *Dasynema*, *Metavermilia*, *Pomatostegus*, *Pseudovermilia*, *Semivermilia*, *Vermiliopsis*) and others not explicitly attributed to a subfamily before (*Janita*, *Filogranella*, *Neovermilia*, *Turbocavus*, and *Paraprotis*). We apply the name Filograninae to this clade. Together, this assemblage constituted the sister group to a highly supported Spirorbinae clade, which included *Spirorbis* as the sister group to a *Romanchella-Protolaeospira-Helicosiphon* clade. Other minor clades with good support were: *Pomatostegus*, *Chitinopoma-Filogranula*, *Protula*, *Apomatus*, *Filograna-Salmacina*, *Vermiliopsis*, *Dasynema-Vermiliopsis*, and *Filogranella-Dasynema-Vermiliopsis*. The *Filograna-Salmacina* clade was nested within *Protis*, forming a well-supported clade. *Semivermilia* and *Pseudovermilia* were not recovered as monophyletic, but instead formed a well-supported mixed clade along with *Rhodopsis*. The two *Paraprotis* terminals did not form a clade and the three *Apomatus* terminals appeared in two places within Filograninae. The positions of *Josephella*, *Bathyvermilia*, *Janita*, and *Metavermilia* within Filograninae were poorly supported.

3.3. Molecular + Morphology Dataset

The BI and ML analyses of the combined morphological and molecular data set resulted in slightly different topologies, with the BI result shown here and congruent nodes for the ML analysis are indicated (Figure 3). Overall support values were markedly lower than seen in the molecular-only analyses but allowing for the additional taxa, the relationships were nearly identical to those shown in Figure 2. Most terminals for which DNA sequence data were not available (*Bathyditrupa*, *Chitinopomoides*, *Microprotula*, *Neomicrorbis*, *Omphalopomopsis*, *Paumotella*, *Spirodiscus*, *Tanturia*, *Vitreotubus*, and *Zibrovermilia*) were recovered within Filograninae and in this clade some differences between the ML and BI analyses were observed. Notably, while the BI placed *Omphalopomopsis* as sister to all other Filograninae terminals except for *Pomatostegus* (Figure 3), in the ML result (not shown) *Omphalopomopsis* was recovered as sister to the Filograninae + Spirorbinae clade. Furthermore, in the ML analysis *Chitinopomoides* was recovered outside the clade containing *Rhodopsis*, *Semivermilia-Pseudovermilia*, and (*Bathyditrupa-Spirodiscus*) *Zibrovermilia* clade, while in the BI analysis *Chitinopomoides* formed sister to the (*Semivermilia-Pseudovermilia*)-*Rhodopsis* clade, but support for both placements was low. The deep-sea (abyssal) terminals *Spirodiscus* and *Bathyditrupa* formed a highly supported clade and were recovered with low support as sister to bathyal *Zibrovermilia*. *Paumotella* was recovered as sister taxon to *Dasynema* with this clade being the sister group to *Vermiliopsis*. *Tanturia* was found in a poorly supported clade with *Josephella. Microprotula* and *Turbocavus* formed a clade and were the sister group to *Protula*. A questionable spirorbin *Neomicrorbis* was recovered within Spirorbinae as the sister taxon to *Helicosiphon* with low support. Within Serpulinae, the topology was the same as in the molecular-only analyses with the difference being the placement of *Vitreotubus* (morphology only data) recovered in a clade with *Laminatubus* with a reasonable support.

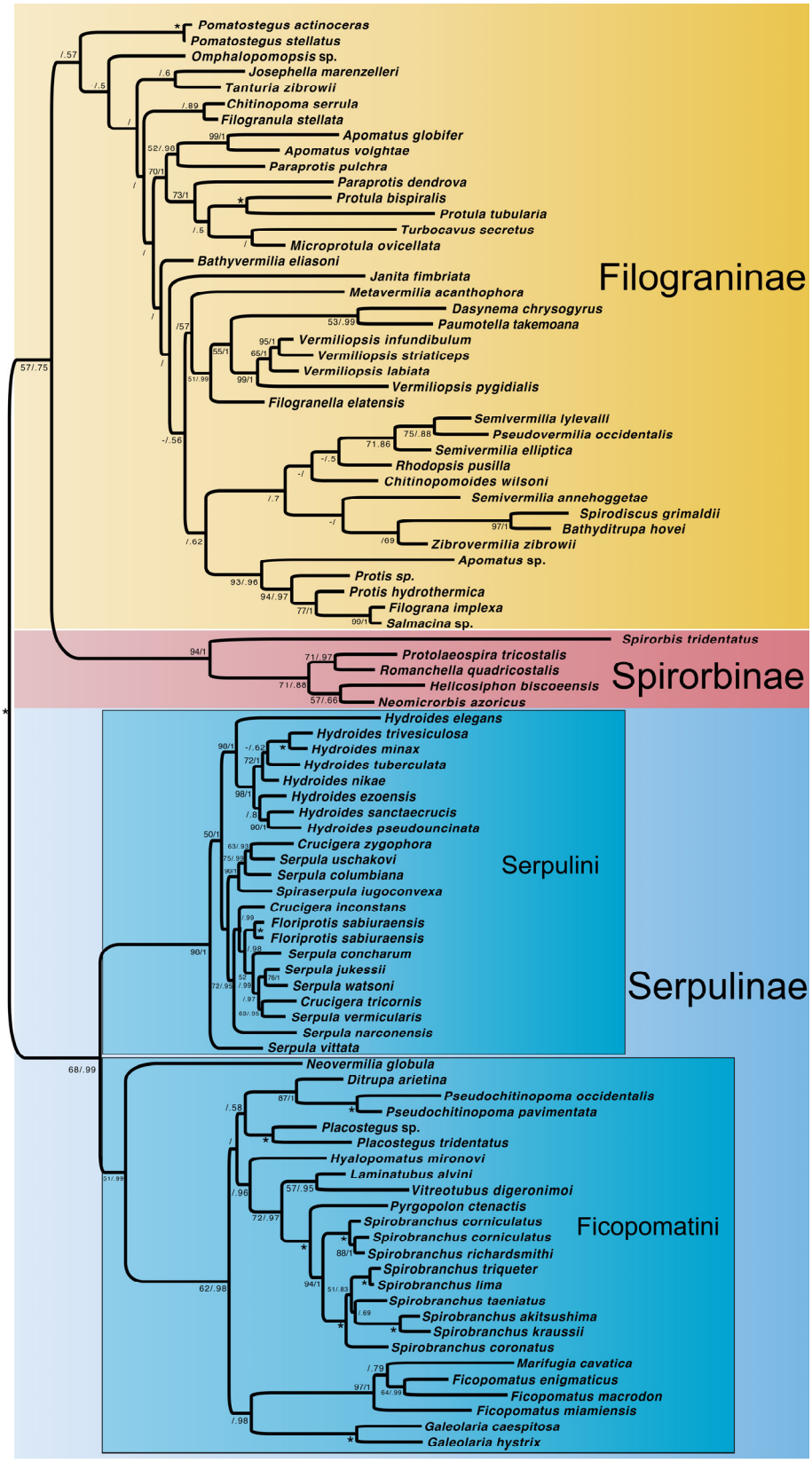

Figure 3. Bayesian majority rule consensus phylogram of the combined molecular and morphological data set. The ML analysis of the same dataset had some incongruities. Bootstrap values of 100 and posterior probabilities 1.0 are indicated with *. Nodes with bootstrap values < 50% and posterior probabilities < 0.5 are blank. Nodes that were not found in the ML analysis are indicated by -.

3.4. Transformations

Monophyly of Serpulidae was unequivocally supported by synapomorphies such as the presence of a calcareous tube (char 8), the presence of the operculum (char 22, Figure 4), though with subsequent reversals (see below), and the presence of the thoracic membranes (char 53, Figure 5A).

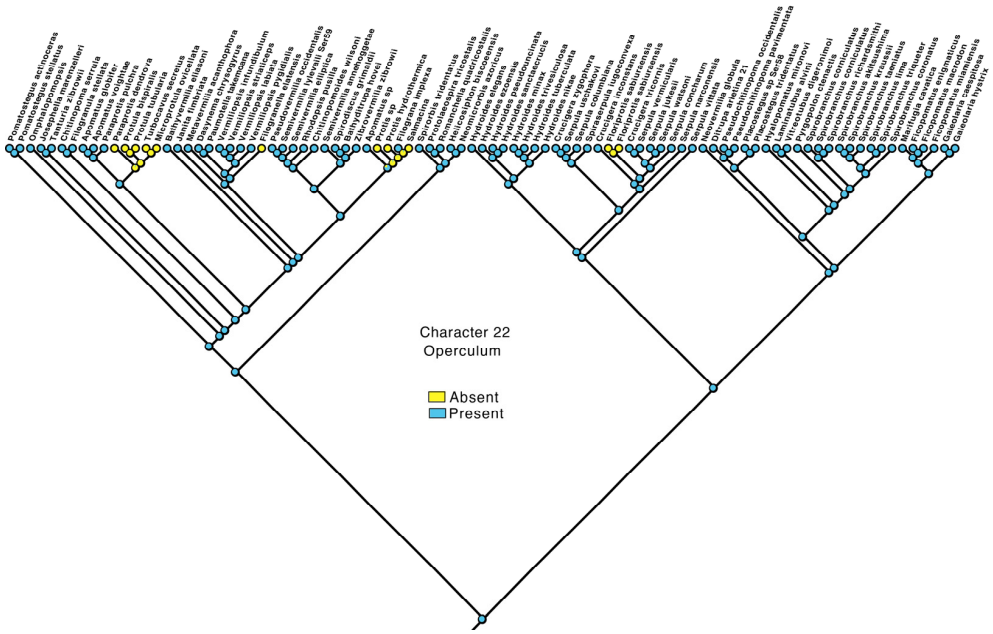

Figure 4. Ancestral state reconstruction, using Mesquite under a likelihood model, of the character 22 from the morphology matrix, Operculum, traced on the Bayesian majority rule consensus phylogram of the combined molecular and morphological data set. The plesiomorphic state for Serpulidae can be inferred as operculum present with four subsequent losses and one reappearance.

Serpulinae was not supported by any morphological apomorphies. Filograninae-Spirorbinae was supported by the presence of *Apomatus* chaetae (char 56, Figure 5A). Filograninae was supported by the presence of abdominal flat geniculate chaetae (char 75). Body asymmetry due to tube coiling (char 16) appeared in Spirorbinae and in *Spiraserpula*. Spirorbinae was also characterised by incomplete chaetal inversion (char 2), distinguished from a more synapomorphy for Sabellida which is characterised by complete chaetal inversion. Serpulini was supported by several apomorphies such as *Serpula*-type operculum (char 32), bayonet collar chaetae (char 52), the presence of pseudoperculum (char 23), and abdominal flat trumpet shaped chaetae (char 70, Figure 5B). Within Serpulini, the presence of opercular verticil (a crown of chitinous spines, char 33) was an apomorphy for *Hydroides*. The presence of abdominal true trumpet chaetae (char 71, Figure 5C) was an apomorphy for Ficopomatini, while *Ficopomatus* was supported by distinct collar chaetae with stout teeth (char 50, *Ficopomatus*-type chaetae). Other apomorphies with ficopomatin groups were collar tonguelets (char 48) found in *Placostegus* and *Spirobranchus-Pyrgopolon* and peduncular wings (char 41) of *Spirobranchus*.

Transformations for the 75 morphological characters can be traced on the BI topology of the combined morphological and molecular data set (Supplemental File S3). Here some of them are highlighted. Figure 4 shows the transformation for the binary character based on the operculum. Under a likelihood transformation, the plesiomorphic state for Serpulidae can be inferred as operculum present with four subsequent losses and one reappearance.

Figure 5 shows the transformation of four chaetal characters. Figure 5A shows the transformation for the distinctive *Apomatus* chaetae and that their presence is an apomorphy for the Filograninae plus Spirorbinae clade with four losses. Figure 5B shows the transformation for abdominal flat trumpet-shaped chaetae and that their presence is an apomorphy for the Serpulini. Figure 5C shows the transformation for abdominal true

trumpet-shaped chaetae and that their presence is an apomorphy for the Ficopomatini. Figure 5D shows the transformation for the flat geniculate chaetae and that their presence is an apomorphy for the Filograninae plus Spirorbinae clade with two losses.

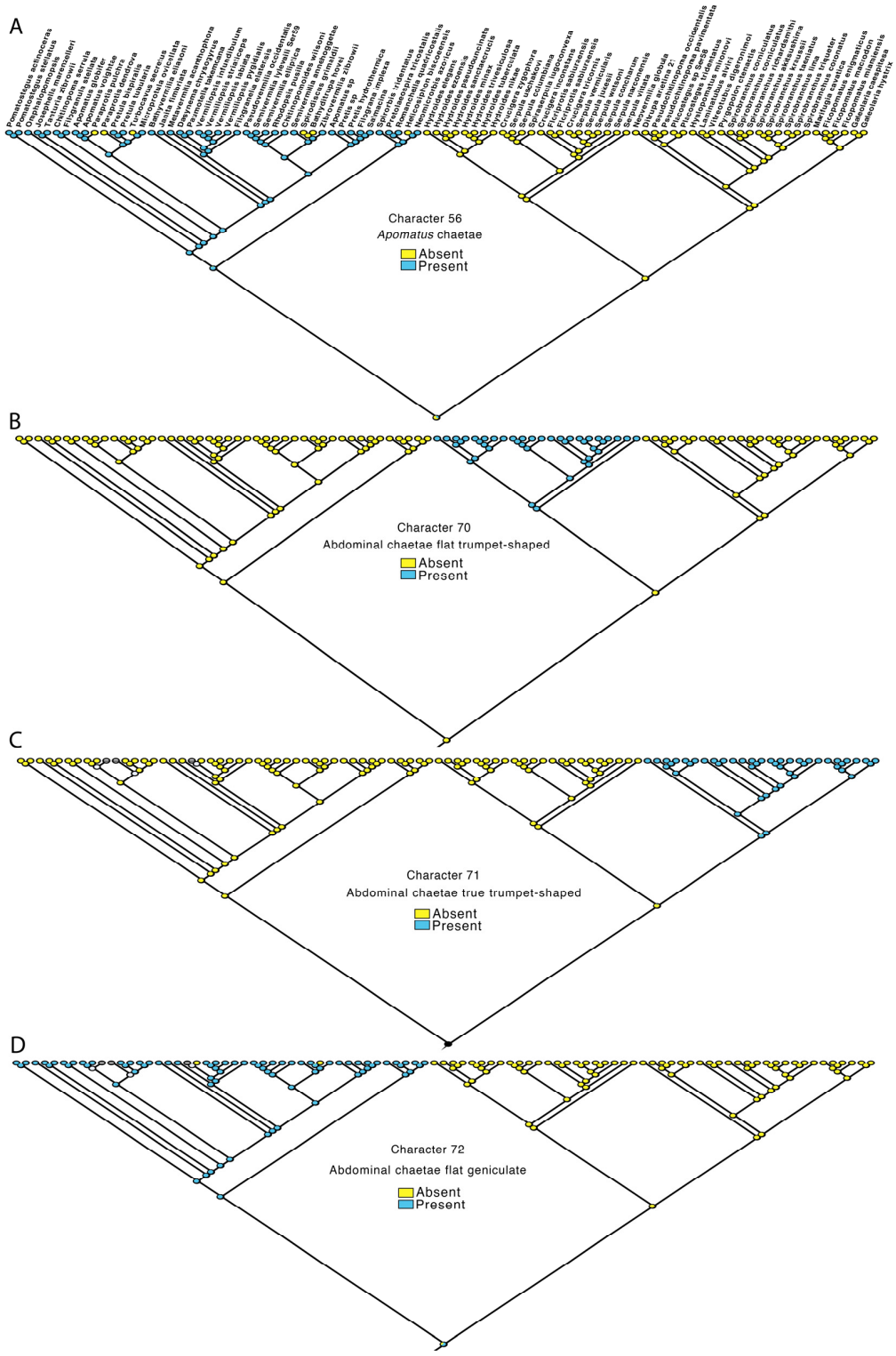

Figure 5. Ancestral state reconstruction, using Mesquite under a likelihood model, of four of the chaetal character from the morphology matrix traced on the Bayesian majority rule consensus phylogram of the combined molecular and morphological data set. (**A**)—Character 56 *Apomatus* chaetae, (**B**)—Character 70 (abdominal chaetae flat trumpet-shaped), (**C**)—Character 71 (abdominal chaetae true trumpet-shaped, (**D**)—Character 72 (abdominal chaetae flat geniculate).

4. Discussion

This study presents the first analysis of phylogenetic relationships within Serpulidae *sensu lato* based on comprehensive taxonomic sampling of the genera and combined molecular and morphological data. The results of this study are generally consistent with those of earlier molecular studies [33,42–44] based on a more restricted taxonomic sampling and DNA sequence data.

The molecular and combined data analyses of Kupriyanova et al. [33] in 2006 provided the first well-supported phylogenetic tree topologies conflicting those obtained from earlier morphology-only analyses where Spirorbinae was recovered as the sister group to Serpulinae [58]. As in this study, in [33] Spirorbinae was recovered as the sister group to a clade composed of both 'filogranin' and 'serpulin' taxa, thus demonstrating that the traditionally formulated subfamilies Serpulinae and Filograninae were not monophyletic. The authors [33] called for a major revision of serpulid taxonomy but refrained from doing so suggesting that further taxon sampling and molecular sequencing were required. The results based on comprehensive sampling here further confirm non-monophyly of both traditional serpulid subfamilies Filograninae and Serpulinae and allow us to propose a new classification within Serpulidae.

The traditional taxonomy of serpulids relied largely on the absence (=many Filograninae) or presence (=all Serpulinae) of an operculum. When present, the structural details were used to delineate genera, such as being simple and membranous or reinforced with chitinous and/or structures such as endplates or spines of varying complexity. The operculum-bearing radiole could be unmodified and pinnulate, or modified into a smooth thickened peduncle (reviewed in [59]). No morphological synapomorphies have previously been proposed to support the traditional Filograninae. Moreover, in 1984 ten Hove [57] had noted that Filograninae was erected on the basis of apparently plesiomorphic (or possibly paedomorphic) features of pinnules on the opercular peduncle.

While all species characterized by unmodified pinnulated operculum-bearing radioles (or thickened pinnulated peduncle in *Spirodiscus* and *Bathyditrupa*) belong to Filograninae, many taxa in the filogranin clade have smooth peduncles and some even have complex chitinized opercula, notable examples being *Pomatostegus*, *Metavermilia*, or *Vermiliopsis*. While some filogranins are non-operculate (e.g., *Protula*, *Turbocavus*, *Filogranella*, *Salmacina*, some *Protis* spp.), some serpulins, such as *Floriprotis sabiuraensis*, *Spirobranchus nigranucha*, and some *Hyalopomatus* and *Spiraserpula* spp. also lack opercula, secondarily according to Figure 4. Furthermore, whereas opercular calcification is a common feature of serpulins in *Galeolaria* and especially in the *Pyrgopolon-Spirobranchus* clade, some *Bathyvermilia* spp. and *Vermiliopsis labiata* have calcified opercular endplates. The observed incongruence of molecular and morphological results in [33] led to the suggestion that the morphological characters traditionally used in serpulid taxonomy, especially opercular structures, may be misleading.

The results of this study strongly support the subdivision of Serpulidae into two major clades, and thus, we suggest that these groups should retain ranks of two previously erected subfamilies, Serpulinae and Filograninae, as well as maintaining the subfamily Spirorbinae, with the type genus *Spirorbis*. However, here we propose the formulation of the subfamilies based on chaetal rather than opercular characters. The suggested characters are the presence/absence of thoracic *Apomatus* chaetae and the structure of abdominal chaetae.

The presence of thoracic *Apomatus* chaetae (sometimes also termed sickle-shaped chaetae) (Figure 6G,H) was recovered as a synapomorphy for the first time and supported Filograninae plus Spirorbinae (Figure 5A). Another synapomorphy supporting subfamilies was the structure of the abdominal chaetae (Figure 5B–D, Figure 6E,F, Figures 7F and 8E). In Filograninae, these chaetae are always some variant of flat geniculate type (sickle-shaped, flat triangular, flat narrow geniculate, retro-geniculate *sensu* ten Hove and Kupriyanova [59], Figure 6E,F). The notable exceptions are capillary abdominal chaetae of *Bathyditrupa* (although the closely related *Spirodiscus* shows typical for filogranin chaetae) and acicular chaetae of *Paumotella*. This newly formulated subfamily contains very morphologically variable taxa (Figure 6A–D) ranging from non-operculate taxa (e.g., *Salmacina*, *Protula*, *Filogranella*, *Turbocavus*) to taxa

with simple membranous opercula lacking any re-enforcements (*Apomatus*, *Filograna*, *Paraprotis*) and those with distinct chitinous opercular re-enforcements (e.g., *Vermiliopsis*, *Dasynema*, *Semivermilia*, *Pseudovermilia*, and especially *Pomatostegus*).

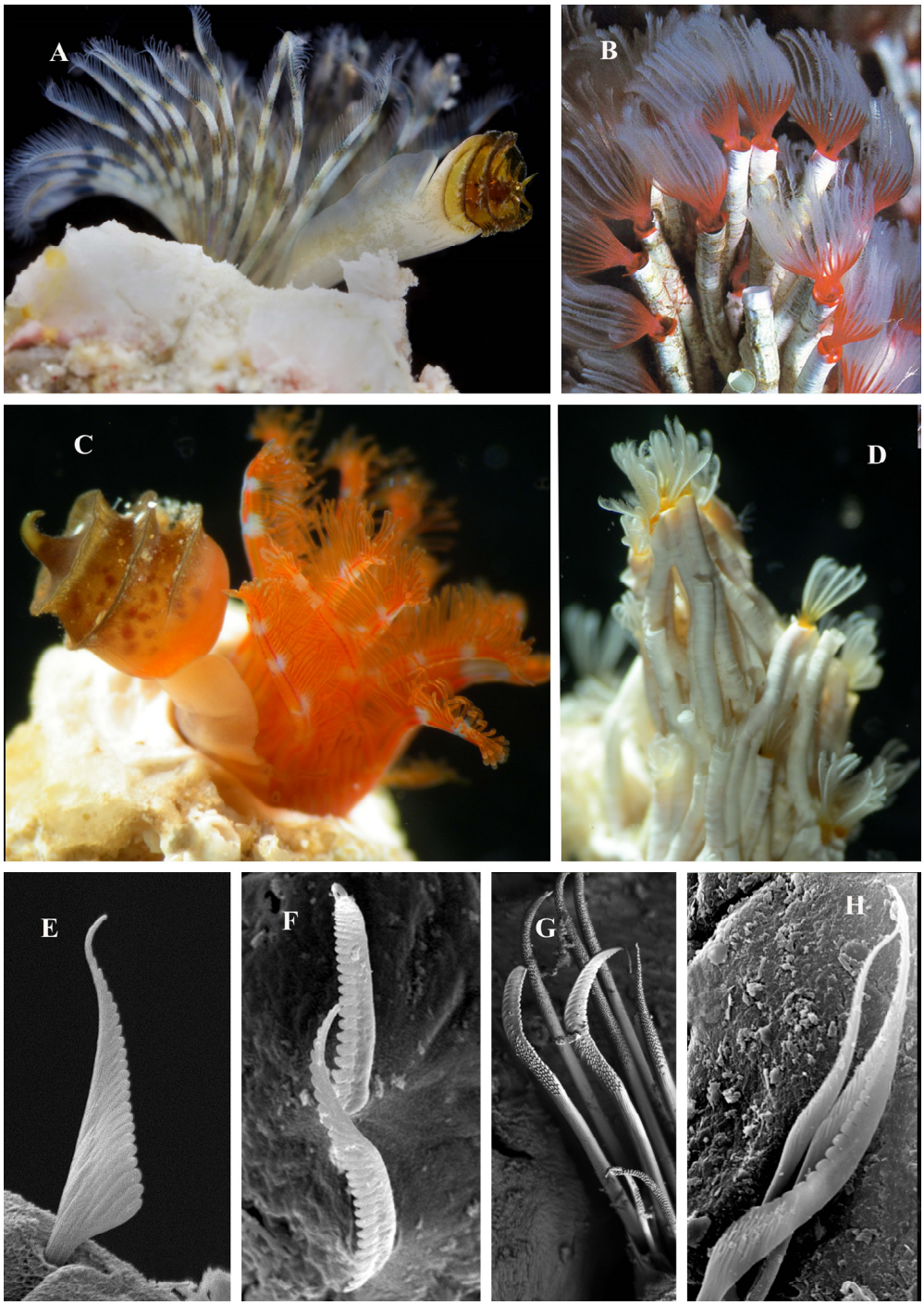

Figure 6. Representatives of Filograninae and their chaetal characters. (**A**)—*Pomatostegus actinoceras* (photo A. Semenov); (**B**)—*Filogranella* sp. (photo E. Nishi); (**C**)—*Metavermilia acanthophora* (photo G. Rouse); (**D**)—*Salmacina* sp. (photo G. Rouse); (**E**)—SEM of abdominal flat geniculate chaetae in *Chitinopoma*, (**F**)—SEM of abdominal flat geniculate chaetae of *Metavermilia*; (**G**)—SEM of thoracic *Apomatus* chaetae of *Filograna*; (**H**)—SEM of thoracic *Apomatus* chaetae of *Filogranula* ((**E**–**H**) photos S. Lindsay).

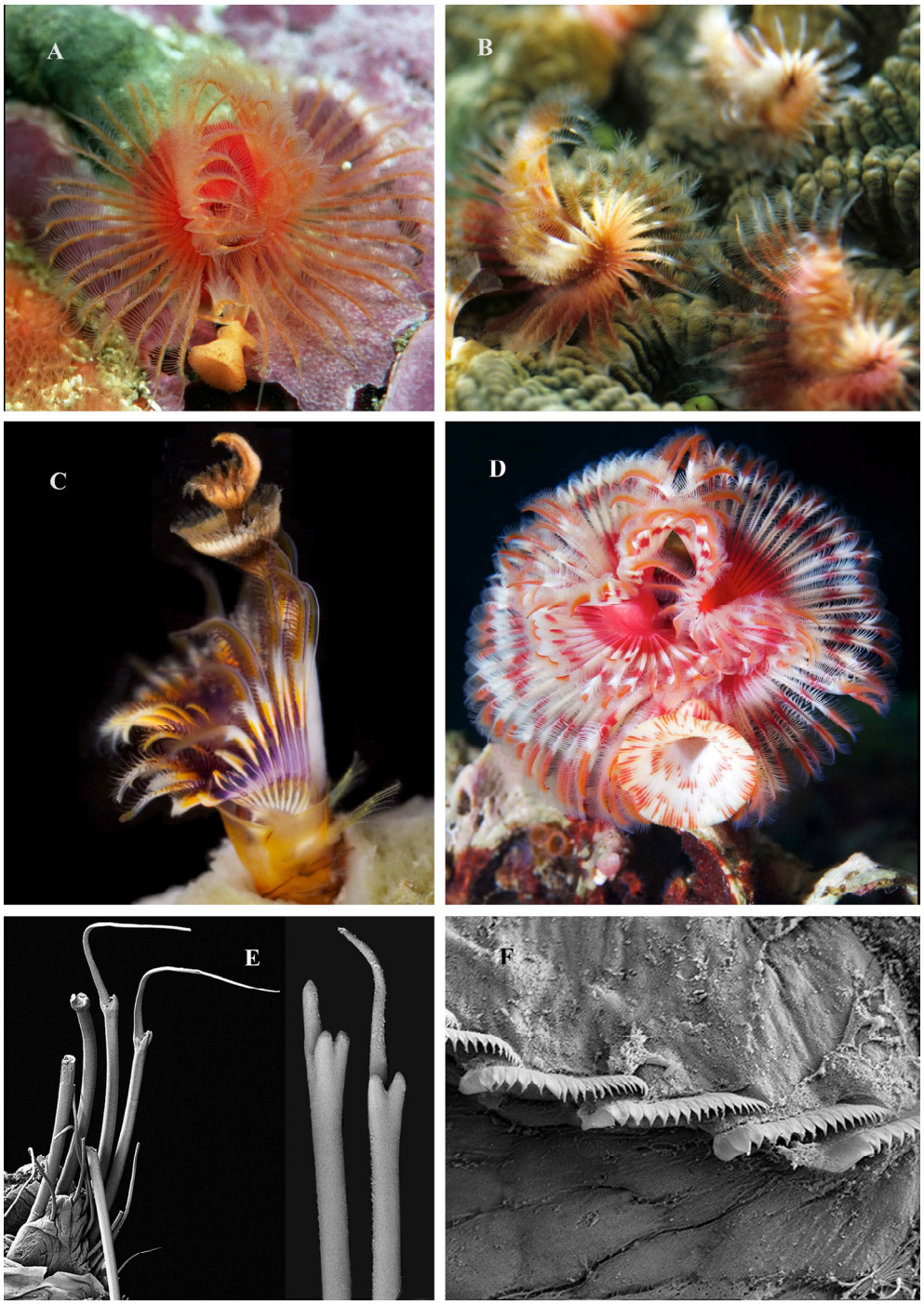

Figure 7. Representatives of Serpulini and their chaetal characters. (**A**)—*Crucigera zygophora* (photo K. Sanamyan); (**B**)—*Floriprotis sabiuraensis* (photo K. Nomura); (**C**)—*Hydroides lirs* (photo A. Semenov); (**D**)—*Serpula columbiana* (photo A. Semenov); (**E**)—SEM of collar chaetae of *Hydroides*; (**F**)—SEM of flat trumpet chaetae of *Hydroides* ((**E**,**F**) photos S. Lindsay).

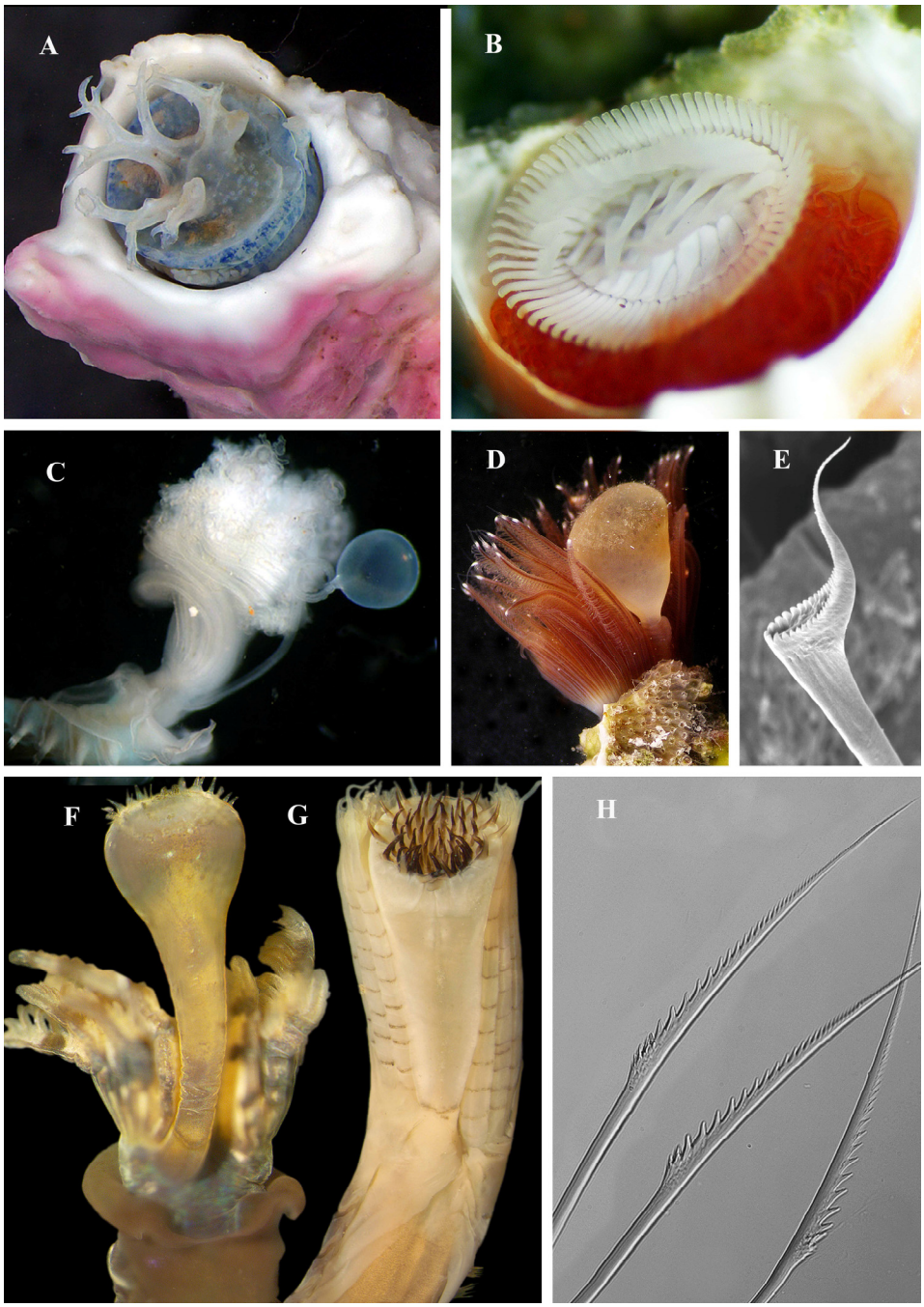

Figure 8. Representatives of Ficopomatini and their chaetal characters. (**A**)—*Spirobranchus tetraceros* (photo W. Zhang); (**B**)—*Galeolaria hystrix*; (**C**)—*Hyalopomatus* sp.; (**D**)—*Neovermilia globula* ((**B**–**D**) photo G. Rouse); (**E**)—SEM of true trumpet-shaped abdominal chaeta of *Spirobranchus*; (**F**)—*Ficopomatus* cf. *uschakovi*, (**G**)—*F. enigmaticus*; (**H**)—collar chaetae of *Ficopomatus* sp. ((**E**,**F**) photos E. Wong).

In Serpulinae, for which we recovered no apomorphy, thoracic *Apomatus* chaetae are invariably absent and two very distinct types of abdominal chaetae, flat trumpet-shaped and true trumpet-shaped, are found (Figure 5B,C and Figure 7F). The original "trumpet-shaped chaetae" received this name because, when examined under a compound microscope, they looked widened into what in profile resembles a chalice or trumpet edged with apparently two rows of thin elongated teeth. However, examination with SEM showed [59] that these chaetae are not hollow as the name might suggest, but rather flat, with a single row of marginal acute teeth. This is in contrast with true trumpet-shaped

abdominal chaetae, which when examined with SEM proved to be distally hollow, with two parallel rows of sharp denticles, extending into a long lateral spine [59]. These had been incorrectly lumped together with the completely different flat geniculate abdominal chaetae of filogranins. We suggest that the flat trumpet-shaped and true trumpet-shaped chaetae are synapomorphies supporting two main groups within Serpulinae, the tribes Serpulini and Ficopomatini, respectively.

Serpulini, including *Crucigera*, *Floriprotis*, *Hydroides*, and *Spiraserpula*, with type genus *Serpula*, is well supported by morphological apomorphies (Figure 7A–D, Supplemental File S3). In addition to the flat trumpet-shaped chaetae, Serpulini synapomorphies include the presence of funnel-shaped (Figure 7A,C,D) *Serpula*-type opercula (additionally topped with a chitinous verticil of chitinous spines in *Hydroides* (Figure 7C) and provided with basal opercular bosses (Figure 7A) in *Crucigera*), pseudoperculum on a shortened peduncle, and distinct bayonet-shaped special collar chaetae (Figure 7E). Based on morphology alone, the genera *Serpula*, *Crucigera*, and *Hydroides* formed the earliest monophyletic group recognized within the family [57]. *Hydroides* was recently revised, its monophyly confirmed by DNA data and the phylogenetic relationships within the genus have been accessed (Sun et al. [5]), but it was not recovered as a monophyletic group on morphology alone in this study. On the contrary, *Crucigera* and *Serpula* were not recovered as monophyletic in this study, a result already demonstrated previously [80], so the clade including terminals of *Crucigera*, *Serpula*, and *Spiraserpula* was a not unexpected outcome of this study. Interestingly, the fact that the usually non-operculate coral-associated *Floriprotis* (Figure 7B) also belongs to this group has not been explicitly proposed before, even though the taxon shows the chaetal pattern identical to that found in *Serpula* and *Hydroides*. Whether *Spiraserpula* is monophyletic needs to be tested in further analyses.

Ficopomatini, as proposed here, includes serpulins with true trumpet-shaped chaetae (Figure 8E), but no other synapomorphy supported the clade. Originally, Ficopomatinae was proposed by Pillai [37] for four monotypic brackish water serpulid genera (*Neopomatus*, *Ficopomatus*, *Mercierella*, and *Sphaeropomatus*), which were later synonymised with *Ficopomatus* [47]. The freshwater monotypic genus *Marifugia* was subsequently added to Ficopomatinae by Pillai, the author of the original subfamily [48]. Here we maintain *Ficopomatus* (supported by distinct collar chaetae (Figure 8H) with stout teeth, an apomorphy for the clade) and *Marifugia* (collar chaetae absent, no clear apomorphies proposed), even though in our analysis *Marifugia* was recovered as nested within *Ficopomatus*. Furthermore, we lower the previously erected subfamilial name Ficopomatinae to the tribe Ficopomatini and broaden the composition of the new tribe to include all genera from *Neovermilia* (positioned as sister to all other members of the tribe) to *Galeolaria* (Figures 2, 3 and 8) with the type genus *Ficopomatus*. This new tribe contains morphologically variable taxa with distinct significant opercular calcification (*Galeolaria*, *Spirobranchus*, and especially *Pyrgopolon*), with chitinous opercular endplates (*Placostegus*, *Pseudochitinopoma*, *Ditrupa*, *Neovermilia*, *Laminatubus*) and even with soft vesicular opercula without any re-enforcement (*Hyalopomatus*).

The phylogenetic position of poorly known and/or deep-sea taxa *Bathyditrupa*, *Chitinopomoides*, *Microprotula*, *Neomicrorbis*, *Spirodiscus*, *Omphalopomopsis*, *Paumotella*, *Tanturia* (all filogranins), as well as *Vitreotubus* and *Zibrovermilia* (Ficopomatini) inferred from morphological data only in this study needs to be confirmed with DNA sequence data when the molecular grade material becomes available. Furthermore, to obtain a robust phylogeny with optimal support, further analyses of serpulid phylogenetic relationships should be based on transcriptome data as performed for Sabellidae [32] or mitogenomes.

5. Conclusions

Morphological characters traditionally used in serpulid taxonomy, especially opercular and peduncular structures, appear to be poor indicators of phylogenetic relationships within the family. Based on results of comprehensive phylogenetic analyses of combined molecular and morphological data, we propose a new classification of Serpulidae that includes re-formulated subfamilies Serpulinae (with tribes Serpulini and Ficopomatini),

Spirorbinae, and Filograninae supported by chaetal characters (presence of thoracic *Apomatus* chaetae and the structure of abdominal chaetae).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15030398/s1, File S1: Description of morphological characters. File S2: Matrix of morphological characters used in this study in nexus format. File S3: Combined molecular and morphology dataset and BI tree used for Figure 3 and show transformations of morphological characters (Figures 4 and 5). References [81–85] are cited in the supplementary materials (File S1).

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Matrix of combined A/P and multistate morphological characters. Unknown is coded with "?"

1. Body symmetry: symmetrical—0, asymmetrical—1.
2. Chaetal inversion: complete—0, incomplete—1.
3. Radiolar lobes: fused—0, separate—1.
4. Inter-radiolar membrane: absent—0, present —1.
5. Radiolar eyespots: absent—0, present—1.
6. Arrangements of radioles: in semi-circles—0, pectinately—1, in spiral—2.
7. Radiolar stylodes: present—0, absent—1.
8. Tube material: mucous—0, calcareous—1.
9. Tube keels: absent—0, present—1.
10. Tube (semi)circular in cross-section: no—0, yes—1.
11. Tube triangular in cross-section: no—0, yes—1.

12. Tube trapezoid in cross-section: no—0, yes—1.
13. Tube quadrangular in cross-section: no—0, yes—1.
14. Granular overlay: absent—0, present—1.
15. Tube wall transparency: completely opaque—0, with outer hyaline and inner opaque layer—1, completely hyaline—2.
16. Tube coiling: straight or irregular—1, spirally coiled—2.
17. Colonies due to asexual budding: absent—0, present—1.
18. Adult tube attachment: attached—0, unattached—1.
19. Internal tube structures: absent—0, present—1.
20. Tabulae: absent—0, present—1.
21. Colour of opaque tubes: white opaque—0, coloured—2.
22. Operculum: absent—0, present—1.
23. Pseudoperculum: absent—0, present—1.
24. Pseudoperculum: borne on pinnulated radiole—0, borne on smooth short radiole—1.
25. Opercular reinforcement: absent—0, present—1.
26. Chitinous reinforcement: absent—0, present—1.
27. Calcareous reinforcement: absent—0, present—1.
28. Thickened cuticle: absent—0, present—1.
29. Chitinous opercular reinforcement: without spines—0, with spines—1.
30. Chitinous endplate: flat opercular plate or concave—0, elongated opercular cap—1, multi-tiered structure—2.
31. Basal processes below operculum: absent—0, present—1.
32. *Serpula*-type operculum: absent—0, present—1.
33. Verticil on *Serpula*-type operculum: absent—0; present—1
34. Type of calcareous opercular reinforcement: operculum infested with calcareous flakes—1, calcareous deposits forming distal plate—2, entirely calcified operculum—3.
35. Calcareous opercular spines: absent—0, non-movable—1, movable—2.
36. Calcareous opercular talon: absent—0, short, embedded in opercular ampulla—1, long, continues into opercular peduncle—2.
37. Opercular constriction: operculum gradually merges into peduncle without constriction—0, operculum separated from the peduncle by a constriction—1.
38. Ontogeny of operculum: indirect—0, direct—1.
39. The operculum-bearing radiole is not different from all other radioles—0, operculum-bearing radiole is modified into a thickened peduncle—1.
40. Peduncle smooth, without pinnules—0, peduncle with pinnules—1.
41. Distal peduncular wings: absent—0, present—1.
42. Proximal peduncular wings: absent—0, present—1.
43. Insertion of the opercular peduncle: as second dorsal radiole—0, as the first radiole—1, at the base of radiolar crown, median insertion covering several opercular radioles—2.
44. Peduncle cross-section: circular—0, triangular—1, flattened—2.
45. Peduncle width: as wide as normal radioles—0, wider than normal radioles—1, much wider than normal radioles—2.
46. Peduncle surface texture: smooth—0, wrinkled—1.
47. Collar: unlobed—0, trilobed—1.
48. Collar tonguelets: absent—0, present—1.
49. Chaetae on the collar segment (collar chaetae): absent—0, present—1.
50. Special collar chaetae: absent—0, with basal modification—1, with distal modification—2.
51. Special fin-and-blade collar chaetae: absent—0, present—1
52. Special bayonet collar chaetae: absent—0, present—1.
53. Special *Spirobranchus* collar chaetae: absent—0, present—1.
54. Thoracic membranes: absent—0, present—1.
55. Thoracic membranes end: short, second segment—0, mid-thorax—1, end of thorax—2, form apron—3.
56. Thoracic *Apomatus* chaetae: absent—0, present—1.

57. Thoracic uncini rasp-shaped: absent—0, present—1.
58. Thoracic uncini saw-to-rasp: absent—0, present—1.
59. Thoracic uncini saw-shaped: absent—0, present—1.
60. Anterior tooth of thoracic uncini pointed: absent—0, present—1.
61. Anterior tooth of thoracic uncini blunt elongated, with rows of teeth implanted over almost entire length of peg (*Protula* type): absent—0, present—1.
62. Anterior tooth of thoracic uncini blunt rounded: absent—0, present—1.
63. Anterior tooth of thoracic uncini blunt flattened, often gouged underneath: absent—0, present—1.
64. Number of teeth in thoracic uncini in profile: < 8–0; 8–19–1; > 20–2.
65. Number of uncinigerous thoracic segments: seven—0, six—1, five—2, four—3, three—4.
66. Variable number of thoracic uncinigerous chaetigers: no—0, yes—1.
67. Ventral arrangement of thoracic uncini: parallel, not forming triangular depression—0, converging posteriorly forming triangular depression—1, fused—2.
68. Achaetous region in the beginning of abdomen: absent—0, present—1.
69. Abdominal chaetae capillary: no—0, yes—1.
70. Abdominal chaetae flat trumpet-shaped: no—0, yes—1.
71. Abdominal chaetae true trumpet shaped: no—0, yes—1.
72. Abdominal chaetae flat geniculate: no—0, yes—1.
73. Abdominal chaetae acicular: no—0, yes—1.
74. Posterior glandular pad: absent—0, present—1.
75. Long capillary chaetae in posterior abdominal segments: absent—0, present—1.

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