Syst. Biol. 57(3):388–405, 2008

ISSN: 1063-5157 print / 1076-836X online
DOI: 10.1080/10635150802161088

Phylogeny and Evolution of Glass Sponges (Porifera, Hexactinellida)

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Abstract.—Reconstructing the phylogeny of sponges (Porifera) is one of the remaining challenges to resolve the metazoan Tree of Life and is a prerequisite for understanding early animal evolution. Molecular phylogenetic analyses for two of the three extant classes of the phylum, Demospongiae and Calcarea, are largely incongruent with traditional classifications, most likely because of a paucity of informative morphological characters and high levels of homoplasy. For the third class, Hexactinellida (glass sponges)—predominantly deep-sea inhabitants with unusual morphology and biology—we present the first molecular phylogeny, along with a cladistic analysis of morphological characters. We collected 18S, 28S, and mitochondrial 16S ribosomal DNA sequences of 34 glass sponge species from 27 genera, 9 families, and 3 orders and conducted partitioned Bayesian analyses using RNA secondary structure-specific substitution models (paired-sites models) for stem regions. Bayes factor comparisons of different paired-sites models against each other and conventional (independent-sites) models revealed a significantly better fit of the former but, contrary to previous predictions, the least parameter-rich of the tested paired-sites models provided the best fit to our data. In contrast to Demospongiae and Calcarea, our rDNA phylogeny agrees well with the traditional classification and a previously proposed phylogenetic system, which we ascribe to a more informative morphology in Hexactinellida. We find high support for a close relationship of glass sponges and Demospongiae *sensu stricto*, though the latter may be paraphyletic with respect to Hexactinellida. Homoscleromorpha appears to be the sister group of Calcarea. Contrary to most previous findings from rDNA, we recover Porifera as monophyletic, although support for this clade is low under paired-sites models. [Bayes factors; data partitioning; Hexactinellida; metazoan phylogeny; model comparison; Porifera; ribosomal DNA; RNA secondary structure.]

As the earliest branching phylum in the Metazoa (e.g., Halanych, 2004), sponges (Porifera) are crucial for understanding animal origins and evolution. Relationships among the major sponge groups ("classes"), and issues of mono- versus paraphyly of Porifera, have been intensely debated (e.g., Ax, 1996; Medina et al., 2001), but a consensus view has yet to emerge. Relationships within Calcarea and Demospongiae have recently been investigated with molecular phylogenetic approaches (e.g., Manuel et al., 2003; Borchiellini et al., 2004; Nichols, 2005; Dohrmann et al., 2006). However, the third, and arguably most remarkable class (see Leys et al., 2007), Hexactinellida (glass sponges), is largely restricted to deep, cold-water habitats. As a result, there have been no comprehensive phylogenetic studies of extant hexactinellid taxa or molecular phylogenetic analyses of intraclass relationships published to date.

Hexactinellids are exclusively marine, siliceous sponges that live in all the world's oceans, primarily in deeper waters. They are important components of sessile benthic communities and can constitute a major fraction of their biomass (e.g., Rice et al., 1990; Barthel and Tendal, 1994; Krautter et al., 2001). Their fossil record dates back to the late Neoproterozoic (Steiner et al., 1993; Gehling and Rigby, 1996; Brasier et al., 1997), and no major modifications of the body plan have occurred since then (Mehl, 1992, 1996). During the Jurassic, glass sponges contributed to the formation of massive reefs along the coasts of the Tethys Sea (e.g., Leinfelder et al., 1994), modern analogues of which have recently been discovered off the coasts of British Columbia, Canada (Conway et al., 1991, 2001; Krautter et al., 2001). In the late Cretaceous they reached their peak of diversity, which gradually declined after this period, presumably due to restrictions of their Mesozoic shelf habitats (Mehl, 1992) and/or changes in ocean chemistry (Maldonado et al., 1999). Currently, approximately 500 extant species, representing roughly 7% of total sponge diversity, are recognized. Given the substantial collections in need of taxonomic treatment (Reiswig, 2002a) and, most importantly, the enormous regions of the deep sea still unknown to science, the actual richness of Hexactinellida is probably much greater than is currently recognized (e.g., Janussen et al., 2004). Glass sponges have recently come to the attention of materials scientists because of the optical and structural properties of their skeletal elements (spicules or scleres; e.g., Sundar et al., 2003; Aizenberg et al., 2005). Their capability of impulse conduction in the absence of a nervous system makes them attractive models for studying the early evolution of conduction systems in the Metazoa (Leys and Mackie, 1997; Leys et al., 2007).

The unique, largely syncytial, organization of their soft tissues (e.g., Mackie and Singla, 1983; Reiswig and Mehl, 1991; Leys et al., 2006) and a triaxonic spicule symmetry (see Leys et al., 2007: chapter 6) are putative synapomorphies of such distinction that the monophyly of Hexactinellida is not doubted (Mehl, 1992). Nevertheless, the hypothesis of hexactinellid monophyly has never been tested with a molecular analysis and there is little certainty about relationships within the group. The currently used classification system (Hooper and van Soest, 2002:1201–1509), based primarily on spicule morphology and skeletal architecture, does not rest on

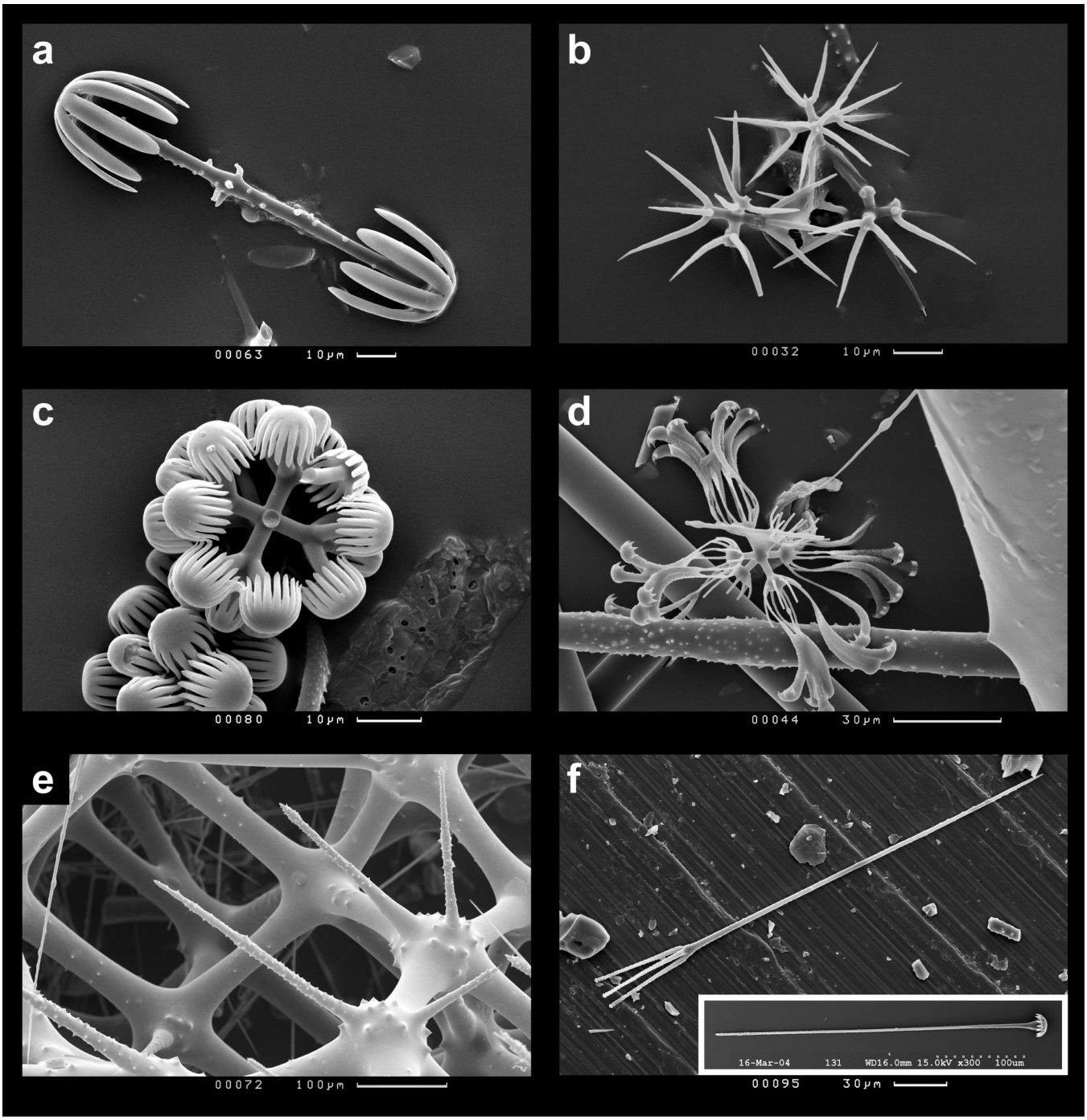


FIGURE 1. Hexactinellid skeletal features. Scanning electron micrographs (SEM). (a) Amphidisc of *Hyalonema* sp. (HBOI 23-VIII-93–4-001). (b to d) Hexasters. (b) Oxyhexasters of *Heterochone calyx* (HBOI 20-X-95–3-001). (c) Discohexaster of *Rhabdopectella tintinnus* (HBOI 4-X-88–2-014). (d) Floricome of *Euplectella* sp. (HBOI 12-XI-86–1-054). (e) Section of dictyonal framework of *Hexactinella carolinensis* (HBOI 25-V-92–2-010). (f) Sceptrules. Main picture: sceptrule (in the form of a scopule) from *Heterochone* sp. (SMF 10523). Inset: sceptrule (in the form of a clavule) from *Farrea* sp. (courtesy of H. M. Reiswig).

principles of phylogenetic systematics (Hennig, 1966). There has been only one attempt to erect a phylogenetic system of glass sponges (doctoral dissertation of D.J.; published as Mehl, 1992), and this study was based mainly on fossil taxa.

Division of Hexactinellida into the subclasses (Reiswig, 2002a) or sister groups (Mehl, 1992) Amphidiscophora and Hexasterophora (Schulze, 1886) is now generally accepted due to their very distinct types of microscleres ("small spicules"), namely amphidiscs and

hexasters, respectively (Figs. 1a to 1d). A phylogenetic system of Amphidiscophora has never been proposed (but see Tabachnick and Menshenina, 1999), as this group has a relatively poor fossil record and was thus not treated in detail by Mehl (1992). Hexasterophora is classically divided into taxa with a rigid skeleton composed of fused hexactine megascleres (the dictyonal framework; Fig. 1e; see Leys et al., 2007: chapter 6) and those lacking such structures (Lyssacinosida; lyssacine skeletal organization). However, dictyonal frameworks are also absent in Amphidiscophora, implying that the lyssacine skeletal organization is plesiomorphic (Mehl, 1992). In the current classification (see Hooper and van Soest, 2002), the dictyonal taxa are divided into several orders according to structural differences of their frameworks. In contrast, Mehl (1992) interpreted sceptrules (Fig. 1f) as an autapomorphy of a new taxon Sceptrulophora and treated sceptrule-lacking dictyonal taxa—except Lychniscosida—as *incertae sedis* within a tentatively maintained "Hexactinosa." With respect to relationships between families and genera, and monophyly of these groups, Mehl (1992) proposed a number of (often weakly supported) hypotheses, but many extant taxa were not investigated. In the classical system, information about interordinal, interfamiliar, and intergeneric relationships is sparse, although some of the more species-rich families are further divided into subfamilies. Clearly, investigation of molecular data and a rigorous cladistic analysis of morphological characters are needed to further elucidate the phylogeny of glass sponges.

Materials and Methods

Specimens and Laboratory Procedures

Specimens were provided to us by colleagues (see Acknowledgments) or collected by D. J. during the ANDEEP III expedition (Brandt et al., 2007; Janussen and Tendal, 2007) and in Sagami Bay, Japan. Voucher numbers, sequence accession numbers, collection regions, and current taxonomic status of the species used in this study are summarized in Table 1.

Genomic DNA was prepared for polymerase chain reaction (PCR) from ethanol-preserved or freeze-dried tissue using 5% to 20% Chelex (Sigma-Aldrich; Singer-Sam et al., 1989). Alternatively, DNA was extracted by phenol/chloroform extraction following ethanol precipitation or by using the DNeasy tissue kit (QIAGEN) according to manufacturer's instructions. For *Aphrocallistes vastus* and *Acanthascus dawsoni*, cDNA produced with a ProtoScript First Strand cDNA Synthesis kit (New England Biolabs) was used for amplification (RNA was extracted with QIAGEN's RNeasy plant tissue kit).

Amplification primers for full-length 18S (~2 kb), partial 28S (~1.2 kb), and partial 16S (~0.5 kb) ribosomal DNA (rDNA) are given in Table 2. Reaction mixes were either as described in Dohrmann et al. (2006), or Promega's GoTaq was used (25-μL reaction mixes contained 5 μL 5 $\times$ GoTaq Flexi Buffer, 4 μL 25 mM $MgCl_2$, 0.5 μL dNTPs [10 mM each], 0.125 μL GoTaq DNA polymerase, 1 μL of each primer [10 μM], and 1 to 8 μL template DNA). Most 18S and 28S amplicons were obtained by seminested PCR with hexactinellid-specific internal primers (Table 2). Thermocycling was carried out using mostly "touchdown" protocols modified from West and Powers (1993) with annealing temperatures ranging from 65°C to 45°C, depending on melting temperature of primers. Bands of expected size were cut out from agarose gels and purified following Boyle and Lew (1995). Both strands of PCR products were sequenced directly with BigDye Terminator 3.1 chemistry and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems), or PCR products were cloned with the TOPO Cloning Kit for Sequencing (Invitrogen) prior to sequencing. Additional sequencing primers for 18S are given in Dohrmann et al. (2006: supplementary online material); for 28S we used primers F1586 and R1630 from Medina et al. (2001) in addition to PCR primers. Tracefiles were inspected, assembled, and edited with CodonCode Aligner (www.codoncode.com) and hexactinellid origin verified with BLAST searches (Altschul et al., 1990) in GenBank. Polymorphic or ambiguous sites were recoded according to the ambiguity code of the International Union of Pure and Applied Chemistry (IUPAC). 18S and 28S sequences of *Acanthascus dawsoni* and 18S sequences of *Farrea occa* and *Oopsacas minuta* were downloaded from GenBank (Table 1); for the latter two no tissue or DNA for amplification of 28S and 16S was available to us. Despite substantial efforts, we were not able to obtain 18S sequences for some species, and in some cases only parts of the 18S and 28S partitions, respectively, could be amplified (see Table 1 for distribution of missing data).

Choice of Outgroups

Outgroup sequences were downloaded from GenBank (Table 3). To achieve a reasonable trade-off between representativeness of outgroup taxa and ease of alignment, and because our prime interests were relationships within Hexactinellida, we included only a few members of the other key sponge groups and restricted sampling of nonsponge animals to Cnidaria and Placozoa. For rooting purposes, *Monosiga brevicollis* (Choanoflagellata) was also included. The apparent high evolutionary rate of the 16S fragment did not allow reliable alignment of outgroups to the hexactinellid sequences, so this partition was analyzed only with ingroup sequences. For outgroups used in morphological analyses, see Morphology.

Alignments

Sequences were initially aligned with ClustalX 1.81 (Thompson et al., 1997) and alignments refined by eye in Se-Al (Rambaut, 1996) and SeaView (Galtier et al., 1996) to match conserved secondary structures of the

TABLE 1. Specimen and sequence information for species used in this study. BX, Biosys-Hermes 2005 field number (see www.nioz.nl/public/dmg/rpt/crs/64pe238.pdf); HBOI, Harbor Branch Oceanographic Institution (Florida); HMR, collection of Henry M. Reiswig (Victoria, Canada); QM, Queensland Museum (Brisbane, Australia); SMF; Senckenberg Museum (Frankfurt am Main, Germany); ZMA, Zoölogisch Museum Amsterdam (The Netherlands); *, previously published sequences.

Subclass	Order	Family	Subfamily	Genus (subgenus)	Species	Voucher no.	Collection region	Accession no. 18S	28S	16S
Amphidiscophora	Amphidiscosida[a]	Hyalonematidae		*Hyalonema*	sp. 1	HBOI 23-VIII-93-4-001	Jamaica		AM886383[h]	AM886321
					sp. 2	HBOI 21-V-93-1-006	Bahamas		AM886382[h]	AM886322
		Pheronematidae		*Pheronema*	sp.	HBOI 10-VI-91-1-001	Canary Isl.		AM886381[h]	AM886323
				Semperella	*schulzei*	QM G318562	Norfolk Ridge, New Caledonia	AM886410	AM886372	AM886324
				Sericolophus	*hawaiicus*	HMR 02-04-16-08	Hawaii	AM886409	AM886380	AM886325
Hexasterophora	Hexactinosida	Aphrocallistidae		*Aphrocallistes*	*vastus*		British Columbia	AM886406	AM886377	AM886328
				Heterochone	*calyx*	HBOI 20-X-95-3-001	Galapagos Isl.	AM886405	AM886379	AM886326
					sp.	SMF 10523	Sagami Bay, Japan	AM886404	AM886378	AM886327
		Dactylocalycidae		*Iphiteon*	*panicea*	HBOI 30-X-94-1-006	Turks and Caicos Isl.	AM886403	AM886374	AM886331
		Farreidae		*Farrea*	*occa*			AF159623*		
		Tretodictyidae		*Tretodictyum*	*tubulosum*[b]	BX63/5	Rockall Bank, Ireland	AM886407	AM886375	AM886329
				Hexactinella	*carolinensis*[c]	HBOI 25-V-92-2-010	South Carolina	AM886408[f]	AM886376	AM886330
	Lyssacinosida	Euplectellidae	Euplectellinae	*Euplectella*	sp. 1	HBOI 19-XI-86-1-001	Galapagos Isl.	AM886397	AM886368	AM886335
					sp. 2	HBOI 12-XI-86-1-054	Galapagos Isl.	AM886398	AM886367	AM886336
				Acoelocalyx	*brucei*	SMF 10530	Weddell Sea, Antarctica	AM886401	AM886370	AM886333
				Malacosaccus	*coatsi*	SMF 10521	Weddell Sea, Antarctica	AM886400	AM886369	AM886334
			Bolosominae	*Rhabdopectella*	*tintinnus*[c]	HBOI 4-X-88-2-014	Bahamas	AM886402	AM886371	AM886332
			Corbitellinae	*Walteria*	*leuckarti*	SMF 10522	Sagami Bay, Japan	AM886399	AM886373	AM886337
		Leucopsacidae		*Leucopsacus*	sp.	BX12/6	Rockall Bank, Ireland	AM886396	AM886365	AM886338
				Oopsacas	*minuta*			AF207844*		
		Rossellidae	Rossellinae	*Rossella*	*nodastrella*	ZMA POR18484	Rockall Bank, Ireland	AM886386	AM886354	AM886344
					nuda	SMF 10531	Weddell Sea, Antarctica	AM886384	AM886355	AM886343
					racovitzae	SMF 10532	Weddell Sea, Antarctica	AM886385	AM886356	AM886342
				Acanthascus (Rhabdocalyptus)	*dawsoni*		British Columbia	AF100949*	AY026379*	AM886340
				Aulosaccus	cf. *mitsukurii*	HBOI 20-XI-86-3-006	Galapagos Isl.	AM886387[g]	AM886353	AM886346
				Bathydorus	*spinosus*	SMF 10526	Weddell Sea, Antarctica	AM886390	AM886358	AM886341
				Caulophacella	*tenuis*	SMF 10533	Weddell Sea, Antarctica	AM886392	AM886363	AM886351
				Caulophacus (Caulophacus)	*arcticus*	SMF 10520	Arctic Ocean	AM886395	AM886360	AM886350
				Caulophacus (Caulodiscus)	*valdiviae*	SMF 10528	Weddell Sea, Antarctica	AM886394	AM886362	AM886348
				Caulophacus (Oxydiscus)	*weddelli*	SMF 10527	Weddell Sea, Antarctica	AM886393	AM886361	AM886349
				Crateromorpha (Crateromorpha)	*meyeri*	SMF 10525	Sagami Bay, Japan	AM886389	AM886359	AM886347
				n. gen.	n. sp.[d]	QM G316480	Norfolk Ridge, New Caledonia	AM886388	AM886357	AM886345
			Lanuginellinae	*Lophocalyx*	n. sp.[e]	SMF 10524	Weddell Sea, Antarctica	AM886391	AM886364	AM886352
		incertae sedis		*Clathrochone*	*clathroclada*	QM G318604	Norfolk Ridge, New Caledonia		AM886366[h]	AM886339

[a] Amphidiscosida is the only extant order of subclass Amphidiscophora (Reiswig, 2002b).
[b] Only reports from Japan and the China Sea are considered valid representatives of *T. tubulosum* by Reiswig (2002c), so this specimen might belong to a new species.
[c] *H. carolinensis* is a recently described species (Reiswig et al., 2008), whereas *R. tintinnus* will be redescribed and transferred from Corbitellinae (Tabachnick, 2002c) to Bolosominae (Reiswig et al., in progress).
[d] H. M. Reiswig, description in progress.
[e] D. Janussen and H. M. Reiswig, description in progress.
[f] Only 5′ half could be amplified.
[g] The two amplified fragments did not overlap, leaving a gap of ~250 bp.
[h] Only 3′ half could be amplified.

TABLE 2. PCR primers.

Gene	Primer name	Sequence (5′-3′)	Reference
18S	18S-AF	CTGGTTGATCCTGCCAG	Collins (2002)
	18S-BR	CTGCAGGTTCACCTAC	Collins (2002)
	Hexa18Sint1	CGTAAGGTGCCAACGAC	This study
	Hexa18Sint2a	GGAATAGGACTCTGCGC	This study
	Hexa18Sint2b	GGAATAGGACTCTGTGT	This study
	Hexa18Sint3	TTCAAACCAACAAAATAGRCACAGA	This study
	Hexa18Sint4	GTCGTTGGCACCTTAC	This study
28S	NL4F	GACCCGAAAGATGGTGAACTA	Nichols (2005)
	NL4R	ACCTTGGAGACCTGATGCG	Nichols (2005)
	Hexa28Sint1	TCTGCACCCTTACTAACCAGGCTTTCG	This study
	Hexa28Sint2	ACGTGAACAGCAYTTGGACGTGGGTG	This study
16S	16S1fw	TCGACTGTTTACCAAAAACATAGC	Watkins and Beckenbach (1999)
	16SH_mod	YRTAATTCAACATCGAGGTC	Oliver Voigt (pers. comm.)

encoded RNA molecules. We used secondary structure predictions of *Acanthascus dawsoni* (Hexactinellida) for 18S (Voigt et al., 2008) and *Mycale fibrexilis* (Demospongiae *s. str.*) for 28S (Wuyts et al., 2004, with modifications; O. Voigt, personal communication) as reference structures. A consensus structure of the 16S fragment could not be reliably predicted, so we aligned and analyzed these sequences without structural information. With the exception of a few positions where primary homology could be determined according to secondary structure, regions that could not be unambiguously aligned were excluded; in cases where ambiguity was present only between ingroup and outgroup sequences, the outgroups were recoded as missing data in these regions to maximize phylogenetic signal for the ingroup. Insertions composed entirely of uninformative sites were also excluded. For the final analyses (see below), the three alignments were concatenated into a supermatrix (51 taxa, 3.435 kb). The total amount of missing data was 18.75% (see also Table 1, Table 3). Sequences of *Dysidea* sp. (18S) and *Dysidea etheria* (28S), and of *Hydra littoralis* (18S) and *Hydra circumcincta* (28S), respectively, were concatenated to form chimeric sequences. Full alignments including information on secondary structure and excluded sites are available at www.systematicbiology.org. The supermatrix is deposited in TreeBASE (www.treebase.org).

Phylogenetic Analysis of Molecular Data

Single-gene analyses and selection of conventional substitution models.—Initial phylogenetic analyses of each gene were performed with the parallel version of MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003; Altekar et al., 2004). Comparison between unpartitioned and partitioned analyses using the Bayes factor (Kass and Raftery, 1995) revealed a significantly better fit of partitioned models to our data (results not shown; see also Nylander et al., 2004; Brandley et al., 2005; Brown and Lemmon, 2007). Substitution models for each

TABLE 3. Outgroup sequences. Demospongiae = Demospongiae *sensu stricto* (Borchiellini et al., 2004).

Species	Taxonomy	18S Accession	28S Accession
Monosiga brevicollis	Choanoflagellata	AF100940	AY026374
Montastraea franksi	Cnidaria: Anthozoa	AY026382	AY026375
Antipathes galapagensis	Cnidaria: Anthozoa	AF100943	AY026365
Hydra circumcincta	Cnidaria: Hydrozoa		AY026371
Hydra littoralis	Cnidaria: Hydrozoa	AF358082	
Atolla vanhoeffeni	Cnidaria: Scyphozoa	AF100942	AY026368
Trichoplax sp.	Placozoa	AY652581	AY652583
Leucascus sp.	Calcarea: Calcinea	AM180954	AM180981
Soleneiscus stolonifer	Calcarea: Calcinea	AM180955	AM180983
Grantiopsis sp.	Calcarea: Calcaronea	AM180977	AM181008
Plectroninia neocaledoniense	Calcarea: Calcaronea	AM180979	AM181011
Oscarella tuberculata	Homoscleromorpha	AY348883	
Plakortis simplex	Homoscleromorpha	AY348884	
Dysidea etheria	Demospongiae: Dictyoceratida		AY561954
Dysidea sp.	Demospongiae: Dictyoceratida	AY734449	
Aplysina fistularis	Demospongiae: Verongida	AJ621545	AY561864
Spongilla lacustris	Demospongiae: Haplosclerida	AF121112	AY561873
Mycale fibrexilis	Demospongiae: Poecilosclerida	AF100946	AY026376
Suberites ficus	Demospongiae: Hadromerida	AF100947	AY026381

partition—18S helices (stems), 18S single-stranded regions (loops), 28S stems, 28S loops, and 16S—were chosen on the basis of the Akaike information criterion (AIC; Akaike, 1974) implemented in ModelTest 3.7 (Posada and Crandall, 1998; likelihood scores were obtained with PAUP* 4.0b10 [Swofford, 2003]). The AIC favored the GTR model (Lanave et al., 1984) for 18S loops and 16S, the TrN93 model (Tamura and Nei, 1993) for 18S stems and 28S loops, and the TVM (transversion) model for 28S stems. Because the TVM and the TrN93 models are not implemented in MrBayes, we chose the HKY85 model (Hasegawa et al., 1985) for 28S stems, because it was favored by the hierarchical likelihood-ratio test (hLRT), and the GTR model for 18S stems and 28S loops. Among-site rate variation (ASRV) was modeled with a discrete gamma distribution with four rate categories (+G; Gu et al., 1995) for 16S, 18S and 28S, plus a proportion of invariable sites (+I; Gu et al., 1995) for 18S and 28S, as favored by the AIC. All model parameters except tree topology and branch lengths were estimated independently for 18S and 28S stems and loops, respectively. The rate prior was set to "variable" to account for among-partition rate variation (APRV; see Marshall et al., 2006). All other priors were left at their default values. For each analysis, two simultaneous independent runs with eight chains each (one cold and seven heated chains; heating parameter = 0.025) were employed. Chains were run until the standard deviation of split frequencies dropped below a threshold of 0.005, sampling every 100 generations. Convergence of parameters and likelihood values was examined with Tracer 1.4 (Rambaut and Drummond, 2007). The first 10% of samples was discarded as burn-in, and the remaining trees were summarized in 50% majority-rule consensus trees with MrBayes.

Combined analyses and comparison of paired-sites models.—Paired nucleotides in RNA helices do not evolve independently of each other in order to maintain secondary structure (Wheeler and Honeycutt, 1988; Dixon and Hillis, 1993; Stephan, 1996). Therefore, it is advisable to use substitution models that take this fact into account (paired-sites models; reviewed in Savill et al., 2001) when analyzing RNA genes. Paired-sites models have been shown to provide a significantly better fit to real data sets and yield trees that are in better agreement with other evidence than standard models that make the simplifying assumption of independent evolution of all sites (e.g., Schöniger and von Haeseler, 1999; Hudelot et al., 2003; Telford et al., 2005; Dohrmann et al., 2006; Erpenbeck et al., 2007).

We analyzed our supermatrix with the Bayesian Markov chain Monte Carlo (MCMC) program mcmcphase from the PHASE 2.0 software package (www.bioinf.manchester.ac.uk/resources/phase/). We tested the performance of four different paired-sites models in comparison to each other and to the conventional (independent-sites) models selected as described above, by means of the Bayes factor (Kass and Raftery, 1995): RNA6A, RNA6B, RNA7A, and RNA7D (see Savill et al., 2001; Gowri-Shankar and Jow, 2006). RNA6A and RNA6B are six-state models that treat mismatches (base pairings other than AU, UA, CG, GC, GU, and UG) as ambiguities, whereas RNA7A and RNA7D are seven-state models where all possible types of mismatches are combined into a single state, MM. RNA6A and RNA7A are the most general time-reversible models of the respective category (six-state or seven-state), and RNA6B and RNA7D are "biologically plausible restriction[s]" of these models (Gowri-Shankar and Jow, 2006:45). Due to computational limitations, we did not test 16-state models, which differentiate between all the possible types of mismatches, as well as the remaining six-state and seven-state models implemented in PHASE (see Gowri-Shankar and Jow, 2006). For each model, we included a proportion of invariable sites and a gamma (4) distribution to account for ASRV (see above); all parameter values except topology and branch lengths were estimated independently for 18S and 28S stems. Substitution models for 18S and 28S loops, and 16S as well as 18S and 28S stems in the analyses with independent-site models, were chosen as described in Single-Gene Analyses and Selection of Conventional Substitution Models, but for 28S loops (and 18S stems in the independent-sites analyses), the originally favored TrN93 model was used, because it is implemented in PHASE. Nucleotide sites in the 28S partition with binding partners lying outside the range of the alignment were also treated as loops. We used mostly default priors, but for branch lengths we followed Yang and Rannala (2005) and implemented an exponential (10) prior.

We ran four chains with different random starting seeds per analysis to check whether the same stationary distribution was sampled independent of initial parameter values. Chains were allowed to burn in for 1.2 million generations, followed by 12 million generations during which tree topologies, branch lengths, and model parameters were sampled every 100th generation. A final analysis with the paired-sites model favored by the Bayes factor (see Results) was run for 22 million generations, sampling after 2 million generations. Because the 18S sequence of *F. occa* used here (from West and Powers, 1993) apparently contains many sequencing errors or might even represent a pseudogene (Voigt et al., 2008), it could have had adverse effects on the rest of the tree. We therefore repeated the final analysis of the supermatrix without this sequence, and compared the results.

Output files from the PHASE analyses were formatted for further use with Perl scripts written by Matt Yoder (available from http://hymenoptera.tamu.edu/rna/download.php). Convergence of parameters and likelihood values was examined with Tracer 1.4 (Rambaut and Drummond, 2007). Consensus phylograms were produced with the program mcmcsummarize from the PHASE package, and clade posterior probabilities (PPs) were obtained by summarizing the sampled trees in extended 50% majority-rule consensus trees (i.e., clades with <50% PP were kept if they were compatible with the rest of the topology) using PAUP* 4.0b10 (Swofford, 2003). Bayes factors were calculated

from the sampled likelihood values using the method of Newton and Raftery (1994; with modifications proposed by Suchard et al., 2001) as implemented in Tracer 1.4 (default settings).

To obtain an alternative measure of clade support, we also performed a partitioned maximum likelihood (ML) analysis on the supermatrix using RAxML-VI-HPC 2.2.3 (Stamatakis, 2006a). We analyzed 1000 bootstrap replicates (Felsenstein, 1985) under the GTRCAT approximation (Stamatakis, 2006b) with the default number of categories (c = 25) and random starting trees. Model parameters were estimated independently for the five partitions of the supermatrix. Note that (to our knowledge) bootstrapping under paired-sites models is currently not implemented in any available software, so comparability of bootstrap proportions (BPs) to PPs obtained from the analyses described above is limited.

Morphology

For the purposes of this study, we only investigated those hexactinellid genera that are present in the molecular data set (Table 1) and restricted ourselves to those characters that we considered most informative for subclass- to genus-level taxonomy of Hexactinellida. Genera were a priori assumed to be monophyletic. We also included Demospongiae *s. str.* (Borchiellini et al., 2004), Homoscleromorpha, Calcarea, Eumetazoa, and Choanoflagellata as outgroups, although very few characters shared with hexactinellids are applicable across these taxa. Note that Choanoflagellata was included for rooting purposes only; it is coded as "0" or "—" for all characters. Character state information was retrieved from Hooper and van Soest (2002), Janussen et al. (2004), Menshenina et al. (2007), and through personal communication with H.M. Reiswig. Data were assembled with Nexus Data Editor (Page, 2001). We produced two alternative matrices following different approaches to character coding: (1) characters were coded as present for a terminal taxon even if they are absent in some of its members, assuming that the character was present in its ancestor and subsequently lost in some species (Table 4); (2) characters were coded as polymorphic for a given terminal when its members exhibited more than one state ("0/1"; Supplementary Table 1, available at www.systematicbiology.org). Only parsimony-informative characters were coded, yielding matrices of 46 characters for 32 terminal taxa. Detailed comments on the characters are given in Appendix (available online at www.systematicbiology.org).

Phylogenetic analysis was performed with PAUP* 4.0b10 (Swofford, 2003) under maximum parsimony (MP), employing a heuristic search with 20 random taxon addition replicates followed by tree-bisection-reconnection (TBR) branch swapping, saving all shortest trees (multrees = yes). All characters were treated as unweighted and unordered. The most parsimonious trees (MPTs) were summarized in strict consensus trees. Clade frequencies in the sets of MPTs were obtained by constructing 50% majority-rule consensus trees in PAUP*. Clade support was assessed by bootstrapping (500 bootstrap replicates with 20 random addition replicates each). Additionally, decay indices (Bremer, 1994) were calculated using PRAP 1.0 (Müller, 2004) in combination with PAUP*. Data matrices and strict consensus trees are available at TreeBASE.

RESULTS

Molecular Phylogenetic Analyses

Single-gene analyses.—Comparison of the single-gene trees (available at www.systematicbiology.org) revealed some topological conflict, but most differences were not statistically significant (PPs < 0.90). Overall, trees from the shorter 28S and 16S alignments were less resolved than the 18S tree and trees from the combined analyses. Therefore, sampling error is the most plausible explanation for most of the conflicting nodes. In a few cases, however, there were more striking differences, which will be discussed below.

Model comparison.—Tree topologies and PPs obtained from the four independent chains under each modeling scheme did not markedly differ; therefore, we randomly chose output from one of the chains to compute Bayes factors. Results from the Bayes factor analyses are summarized in Table 5. These data clearly show that the paired-sites models outperform traditional models that assume independent evolution of all sites. Among the paired-sites models we tested, the most general time-reversible models (RNA6A, RNA7A) of each category (six-state and seven-state) performed poorer than the less parameter-rich restrictions RNA6B and RNA7D. Also, many parameters of RNA6A and RNA7A had not converged to stable values after 13.2 million generations, as indicated by effective sample sizes (ESS) < 100 (see Drummond et al., 2007: chapter 5), and the simpler six-state models performed significantly better than the more complex seven-state models in terms of sampled likelihood values. Thus, although the assumption of independent evolution of stem sites is clearly violated, overparameterization of paired-sites models seems to be a problem with our dataset.

Overall, tree topologies and PPs obtained with the different modeling schemes were very similar. However, the phylogenetic position of *Iphiteon panicea* (Hexasterophora: Dactylocalycidae) was reconstructed differently under six-state models as compared to seven-state models or independent-sites models, and PPs for two outgroup nodes significantly differed between trees derived with paired-sites and independent-sites models, respectively (see below).

In the final 22 million generation PHASE run using RNA6B for stem regions, likelihood values and all model parameters showed ESS > 200, as recommended by Drummond et al. (2007: chapter 5). The consensus tree from this analysis, together with ML bootstrap proportions, is shown in Figure 2 (also available in TreeBASE). Exclusion of the putatively erroneous *F. occa* sequence (see Materials and Methods) resulted in the same

TABLE 4. Morphological character matrix with characters coded as present (1) even if they are absent in some members of a genus. —, inapplicable. See Appendix (available online at www.systematicbiology.org) for comments on characters. An alternative coding scheme that allows for polymorphisms (0/1) within terminal taxa is given in Supplementary Table 1 at www.systematicbiology.org.

	Characters																																													
Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
Hyalonema	0	1	1	0	1	1	1	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	1	1	0	1	0
Pheronema	0	1	1	0	1	1	1	1	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	1	0	1	0
Semperella	0	1	1	0	1	1	1	1	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	1	0	1	0
Sericolophus	0	1	1	0	1	1	1	1	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	1	0	1	0
Aphrocallistes	0	1	1	0	1	1	1	1	1	0	0	0	1	0	1	1	1	2	0	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	1	0	0
Heterochone	0	1	1	0	1	1	1	1	1	0	0	0	1	0	1	1	1	2	0	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1
Tretodictyum	0	1	1	0	1	1	1	1	1	0	0	0	1	0	1	1	1	1	0	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0
Hexactinella	0	1	1	0	1	1	1	1	1	0	0	0	1	0	1	1	1	1	0	1	1	1	1	1	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	1	0
Farrea	0	1	1	0	1	1	1	1	1	0	0	0	1	0	1	1	2	3	0	1	1	1	0	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0
Iphiteon	0	1	1	0	1	1	1	1	1	0	0	0	1	0	1	1	3	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0
Rhabdopectella	0	1	1	0	1	1	1	1	1	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	1	1	1	1	0	0	1	0
Walteria	0	1	1	0	1	1	1	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0
Euplectella	0	1	1	0	1	1	1	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	1	0	1	0	1	1	1	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0
Acoelocalyx	0	1	1	0	1	1	1	1	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1
Malacosaccus	0	1	1	0	1	1	1	1	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1
Rossella	0	1	1	0	1	1	1	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	1	0	1	1	0	0	0	1	0	0	1	0	0	1	1	0	0	0	0	0	0	1
Rossellinae n. gen.	0	1	1	0	1	1	1	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	1
Acanthascus	0	1	1	0	1	1	1	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	1	1
Aulosaccus	0	1	1	0	1	1	1	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	0	1
Bathydorus	0	1	1	0	1	1	1	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	0	1
Caulophacus	0	1	1	0	1	1	1	1	1	0	0	1	1	0	1	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	1	0	0	1	1	0	1	1	0	0	1	1	0	1	1
Caulophacella	0	1	1	0	1	1	1	1	1	0	0	1	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	1	1	0	1	0
Crateromorpha	0	1	1	0	1	1	1	1	1	0	0	1	1	0	1	0	0	0	0	0	0	0	0	1	1	0	1	1	0	1	0	0	0	1	1	0	1	1	1	0	0	0	0	1	1	0
Lophocalyx	0	1	1	0	1	1	1	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	1	1	1	1	1	0	0	1	1	0	1	1
Leucopsacus	0	1	1	0	1	1	1	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1
Oopsacas	0	1	1	0	1	1	1	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1
Clathrochone	0	1	1	0	1	1	1	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1
Demospongiae	0	1	1	1	1	1	0	0	—	0	0	0	0	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Homoscleromorpha	1	1	1	1	1	1	0	0	—	0	0	0	0	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Calcarea	0	1	1	1	0	0	0	0	—	0	0	0	0	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Eumetazoa	1	0	0	0	0	0	0	0	—	0	0	0	0	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Choanoflagellata	0	0	0	0	0	0	0	0	—	0	0	0	0	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

TABLE 5. Harmonic means (HM) calculated from post–burn-in log-likelihoods of PHASE analyses using different models for stem regions (see text) and associated Bayes factors (2lnBF). See Materials and Methods for models applied to loop regions. 2lnBF > 10 indicates "very strong" evidence against the null hypothesis (Kass and Raftery, 1995:777); for example, RNA7A (paired-sites) has 2lnBF = 5855.112 against TrN/HKY (independent-sites) and thus provides a significantly better fit to the underlying data. RNA6B outperforms all other models in this comparison. SE, standard error. HM, SE, and lnBF were calculated in Tracer 1.4 (Rambaut and Drummond, 2007) under default settings (see also Newton and Raftery, 1994; Suchard et al., 2001).

Model (+I+G)	HM	SE	RNA6B	RNA6A	RNA7D	RNA7A	TrN/HKY
RNA6B	−17,681.249	0.114	—	92.952	2942.248	2993.736	8848.848
RNA6A	−17,727.725	0.134	−92.952	—	2849.296	2900.784	8755.896
RNA7D	−19,152.373	0.126	−2942.248	−2849.296	—	51.488	5906.600
RNA7A	−19,178.117	0.146	−2993.736	−2900.784	−51.488	—	5855.112
TrN/HKY	−22,105.673	0.125	−8848.848	−8755.896	−5906.600	−5855.112	—

topology, but PPs for two ingroup nodes significantly differed (see below).

Molecular Phylogeny of Hexactinellida and Relationships among Poriferans

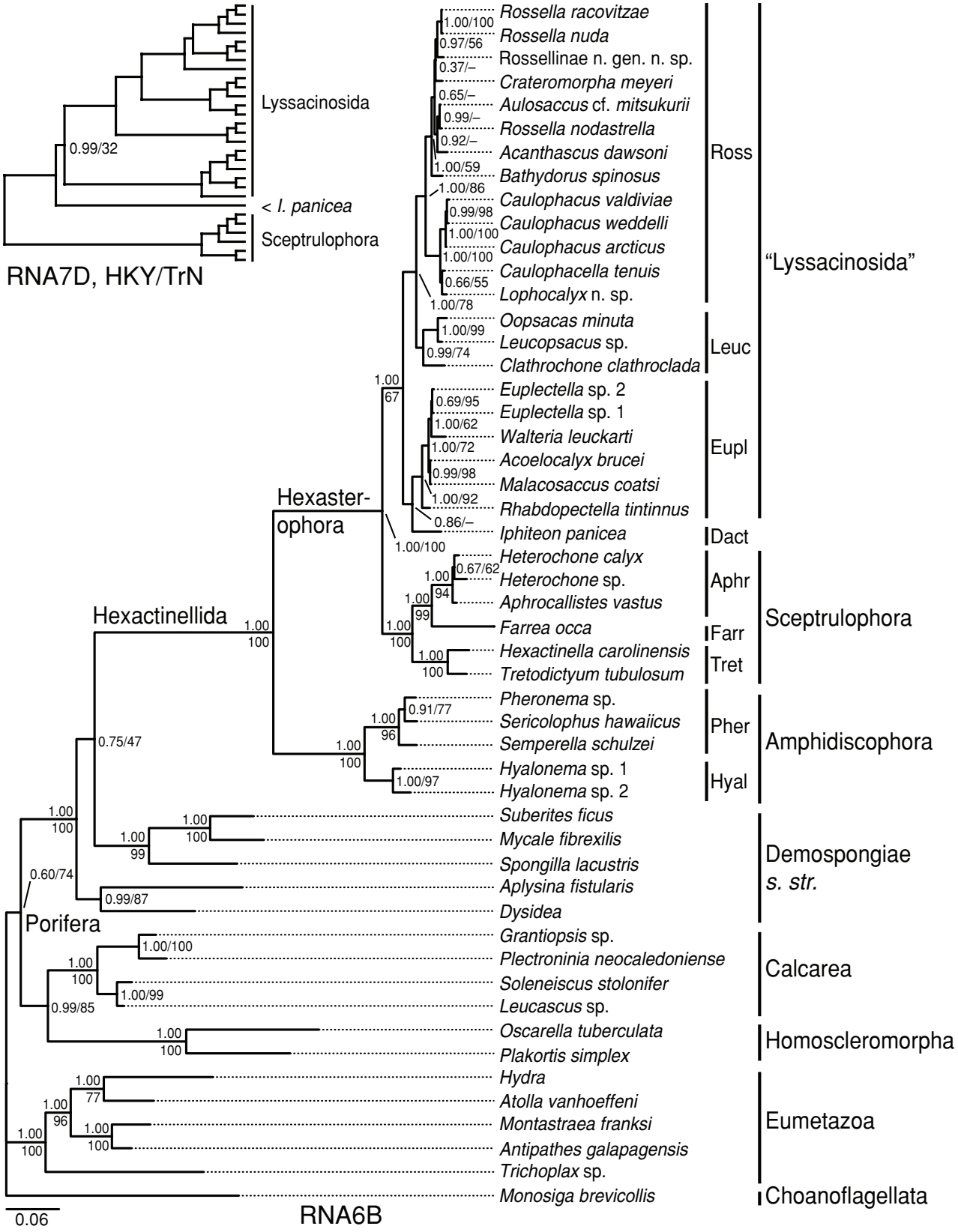
Hexactinellida is clearly monophyletic and separated by a very long branch from the rest of the tree. It forms a highly supported clade with Demospongiae *sensu stricto* (Borchiellini et al., 2004). However, the latter is not monophyletic, as a clade comprising *Mycale fibrexilis*, *Suberites ficus*, and *Spongilla lacustris* appears to be more closely related to hexactinellids than to the remaining two demosponges (*s. str.*), *Aplysina fistularis* and *Dysidea*. However, this result receives only 0.75 PP and 47% BP. In the analysis with independent-sites models applied to stem regions, support for this hypothesis was 0.63 (not shown). Homoscleromorpha is the sister group of Calcarea, and Porifera is monophyletic, albeit with only 0.60 PP and 74% BP. Application of independent-sites models to stem regions resulted in 0.94 PP for poriferan monophyly (not shown), indicating high sensitivity to model choice for this part of the tree. Monophyly of Cnidaria and Eumetazoa (*Trichoplax* + Cnidaria) is highly supported.

Both Amphidiscophora and Hexasterophora are recovered with full support. Within Amphidiscophora, the three pheronematid genera form the sister group to a clade consisting of the two *Hyalonema* species (Hyalonematidae). *Pheronema* sp. is more closely related to *Sericolophus hawaiicus* than it is to *Semperella schulzei*. In the hexasterophoran subtree, all sceptrule-bearing taxa (Sceptrulophora) group together with high support. Both *Aphrocallistes* and *Heterochone* and *Tretodictyum* and *Hexactinella* form clades, consistent with monophyly of Aphrocallistidae and Tretodictyidae. Monophyly of *Heterochone* (Aphrocallistidae) is only weakly supported (0.67), but support was significantly higher (0.83) when *Farrea occa* (Farreidae) was excluded from the analysis (not shown). *Farrea occa* is most closely related to Aphrocallistidae, which is at odds with the division of Sceptrulophora into Clavularia (here: *F. occa*) and Scopularia (here: Aphrocallistidae, Tretodictyidae), introduced by Schulze (1886).

The only sceptrule-lacking dictyonal species investigated here, *Iphiteon panicea* (Dactylocalycidae), appears nested within Lyssacinosida rather than forming a clade with the remaining dictyonal taxa, rendering Hexactinosida diphyletic and Lyssacinosida paraphyletic. However, under seven-state paired-sites models or independent-sites models applied to stem regions, *I. panicea* was sister to Lyssacinosida, monophyly of which was supported by 0.99 PP, but only 32% BP (Fig. 2, inset). In the 28S tree, *I. panicea* was highly supported as sister to Sceptrulophora. However, under paired-sites models, *I. panicea* grouped with Lyssacinosida (not shown), suggesting that the result from the MrBayes analysis was due to model-misspecification and re-emphasizes the importance of paired-sites models for 28S rDNA studies (see Erpenbeck et al., 2007).

All three families of Lyssacinosida are monophyletic given the current taxon sampling: Euplectellidae, Rossellidae, and Leucopsacidae. *Iphiteon panicea* is sister to Euplectellidae under RNA6B, but support for this hypothesis is moderate (0.86 PP) and was very low (0.59) when *F. occa* was excluded from the analysis (not shown). In the 28S tree, monophyly of Euplectellidae was not supported, but as in the case of *I. panicea* (see above), this changed when paired-sites models were applied (not shown). *Clathrochone clathroclada* (Lyssacinosida *incertae sedis*; Tabachnick, 2002a) groups with *Oopsacas*/*Leucopsacus*, indicating that it also belongs to

FIGURE 2. Phylogeny of Hexactinellida inferred from combined 18S, 28S, and 16S rDNA. Substitution models used were RNA6B+I+G for 18S and 28S stems, GTR+I+G for 18S loops, TrN+I+G for 28S loops, and GTR+G for 16S (see text for details of analysis). Bayesian consensus tree (extended 50% majority-rule). Posterior probabilities (PP) given above or left and maximum likelihood bootstrap proportions (BP; %) below or right (—, clade not present in ML tree). Inset: phylogenetic placement of *I. panicea* when seven-state or independent-sites models (see text) were applied to 18S and 28S stems. Support values: PP under RNA7D/ML BP. Aphr, Aphrocallistidae; Dact, Dactylocalycidae; Eupl, Euplectellidae; Farr, Farreidae; Hyal, Hyalonematidae; Leuc; Leucopsacidae (*sensu novo*; see text); Pher, Pheronematidae; Ross, Rossellidae; *s. str.*, *sensu stricto*; Tret, Tretodictyidae. Scale bar, number of expected substitutions per site. Alignment of taxon labels is for readability purposes only.



Leucopsacidae. This "Leucopsacidae *sensu novo*" is the sister group of Rossellidae.

Among Euplectellidae, the sole included representative of subfamily Bolosominae (*Rhabdopectella tintinnus*; see footnote in Table 1) is sister to the remaining euplectellids. *Acoelocalyx brucei* and *Malacosaccus coatsi* (Euplectellinae) are closely related. *Walteria leuckarti* (Corbitellinae) groups with *Euplectella* to the exclusion of the other Euplectellinae, rendering this subfamily paraphyletic. However, the 16S tree displayed monophyletic Euplectellinae (0.99 PP), with *W. leuckarti* and *R. tintinnus* as successive sister groups (not shown), indicating conflicting phylogenetic signal between nuclear and mitochondrial genomes for this region of the tree. Monophyly of *Euplectella* is only weakly supported by the Bayesian analysis, but has high bootstrap support. Among Rossellidae, subfamily Rossellinae appears paraphyletic: *Lophocalyx* n. sp., the only investigated representative of the second subfamily, Lanuginellinae, is part of a fully supported clade otherwise comprising *Caulophacus* spp. and *Caulophacella tenuis*. *Lophocalyx* n. sp. and *C. tenuis* appear to have a close relationship, but this hypothesis is poorly supported. The three *Caulophacus* species unambiguously group together, with the two Antarctic species (*C. valdiviae* and *C. weddelli*) forming a clade to the exclusion of *C. arcticus*. The remaining rossellids fall into one clade, with *Bathydorus spinosus* branching off first in the Bayesian tree. The two Antarctic *Rossella* species, *R. nuda* and *R. racovitzae*, are very closely related, whereas the N. Atlantic *R. nodastrella* clusters with *Aulosaccus* cf. *mitsukurii*, rendering *Rossella* nonmonophyletic. In the ML tree (not shown), the positions of *R. nodastrella* and *A.* cf. *mitsukurii* were not resolved at the 50% bootstrap consensus level; the same holds true for *Acanthascus dawsoni*, *Crateromorpha meyeri*, and *B. spinosus*. In the Bayesian tree (Fig. 2), *A. dawsoni* is most closely related to *A.* cf. *mitsukurii*/*R. nodastrella*, and the position of *C. meyeri* is not resolved at the 50% majority-rule consensus level. Rossellinae n. gen. n. sp. is sister to *R. nuda*/*R. racovitzae*.

Phylogenetic Analysis of Morphological Data

The heuristic MP search on the character matrix without polymorphisms (Table 4) found 2208 MPTs 105 steps long; the strict consensus tree is shown in Fig. 3a. Hexactinellida, Silicea (Demospongiae + Hexactinellida), and Porifera are monophyletic, but relationships between Demospongiae *s. str.*, Homoscleromorpha, and Hexactinellida are not resolved. Resolution within Hexactinellida is poor: along with three two-species clades from the lyssacine hexasterophorans (*Walteria* + *Euplectella*, *Acoelocalyx* + *Malacosaccus*, and *Rossella* + Rossellinae n. gen.), only Amphidiscophora, Pheronematidae, Hexactinosida (*Iphiteon* + Sceptrulophora), Sceptrulophora, and Scopularia are recovered (note that Hexactinosida and Scopularia are not found in the rDNA tree [Fig. 2]). However, Hexasterophora, Euplectellidae, Rossellidae, and some other clades compatible with the rDNA tree (Fig. 2) were found in high to moderate proportions in the set of MPTs (Table 6).

TABLE 6. Frequencies of important clades in the sets of MPTs inferred from the morphological data sets. Freq, frequency (%); poly = no, coding scheme without polymorphisms allowed (Table 4); poly = yes, coding scheme with polymorphisms allowed (Supplementary Table 1).

Taxon/clade	Freq (poly = no)	Freq (poly = yes)
Amphidiscophora	100	72
Hexasterophora	91	—
Euplectellidae	97	100
Rossellidae	91	—
Aphrocallistidae	67	100
Tretodictyidae	67	—
(*Lophocalyx* (*Caulophacus*, *Caulophacella*))	91	—
Clathrochone + Rossellidae	91	—

The alternative character matrix with polymorphisms allowed (Supplementary Table 1) yielded 839 MPTs 92 steps long. The strict consensus tree (Fig. 3b) is similar to that from the other coding scheme (Fig. 3a) but appears slightly better resolved. Silicea and Amphidiscophora are not recovered at the strict consensus level (but see Table 6), whereas Aphrocallistidae and Euplectellidae are found in all MPTs (note that the position of *Rhabdopectella* differs from that in Fig. 2). There is also some additional resolution within Rossellidae. However, in 72% of MPTs, Amphidiscophora formed a clade with a paraphyletic Rossellidae, Hexactinosida, and the two leucopsacids to the exclusion of Euplectellidae; i.e., in contrast to the other coding scheme (see Table 6), Hexasterophora and Rossellidae are not recovered at the 50% majority-rule consensus level. The same holds true for Tretodictyidae, as 53% of MPTs contained a *Tretodictyum* + Aphrocallistidae clade to the exclusion of *Hexactinella*.

DISCUSSION

Model Comparisons

In agreement with previous results (e.g., Telford et al., 2005; Dohrmann et al., 2006; Erpenbeck et al., 2007), we find paired-sites models to show a significantly better fit to rDNA alignments than conventional models; i.e., the assumption of site independence is clearly violated. A surprising result of our model comparison was that the least complex paired-sites model (RNA6B) performed best with our data set and that the most general time-reversible models performed poorer than their less parameter-rich restrictions (although the difference in log-likelihood was much greater between six-state and seven-state models than between the two models of each category; see Table 5). These results are contrary to the findings of Savill et al. (2001), who found a significantly better fit of the most general time-reversible models of each category. Although they admit that their results are specific to their particular data set, Savill et al. (2001:409) predicted that they could be extrapolated to other real sequences. At least for our data, this is not

the case: too many rate parameters lead to lower log-likelihood values. However, model comparison in an ML framework, as in Savill et al. (2001; see also Telford et al., 2005; Biffin et al., 2007), could have principally produced different results (V. Gowri-Shankar, personal communication). Although the fact that many parameters of RNA6A and RNA7A had not converged to stable values could have biased the likelihood scores, this fact alone indicates that these models are overparameterized with respect to our data and the priors used (V. Gowri-Shankar, personal communication). The better performance of six-state models found here requires further investigation: they only differ from seven-state models in that they treat mismatches as ambiguities; it is unclear if this ignorance of information actually leads to more accurate trees. This is of direct importance for the present study because under six-state models the phylogenetic position of one key taxon, *Iphiteon panicea*, is reconstructed differently than under the other models tested (Fig. 2).

Morphological Analyses

From a theoretical point of view, it seems more objective to code characters that can be absent or present in members of a terminal taxon as polymorphic instead of coding the assumed or reconstructed ground pattern (e.g., Jenner, 2001). In the present study, using a coding scheme that allows for polymorphisms resulted in less and shorter MPTs and a slightly better resolved strict consensus tree (Fig. 3). However, taxonomically well-defined groups such as Hexasterophora or Tretodictyidae were not recovered at the 50% majority-rule consensus level (Table 6), in favor of more doubtful groupings (e.g., *Caulophacella* + Amphidiscophora; not shown). In contrast, when coding assumed ground patterns, Hexasterophora and most of its families (except

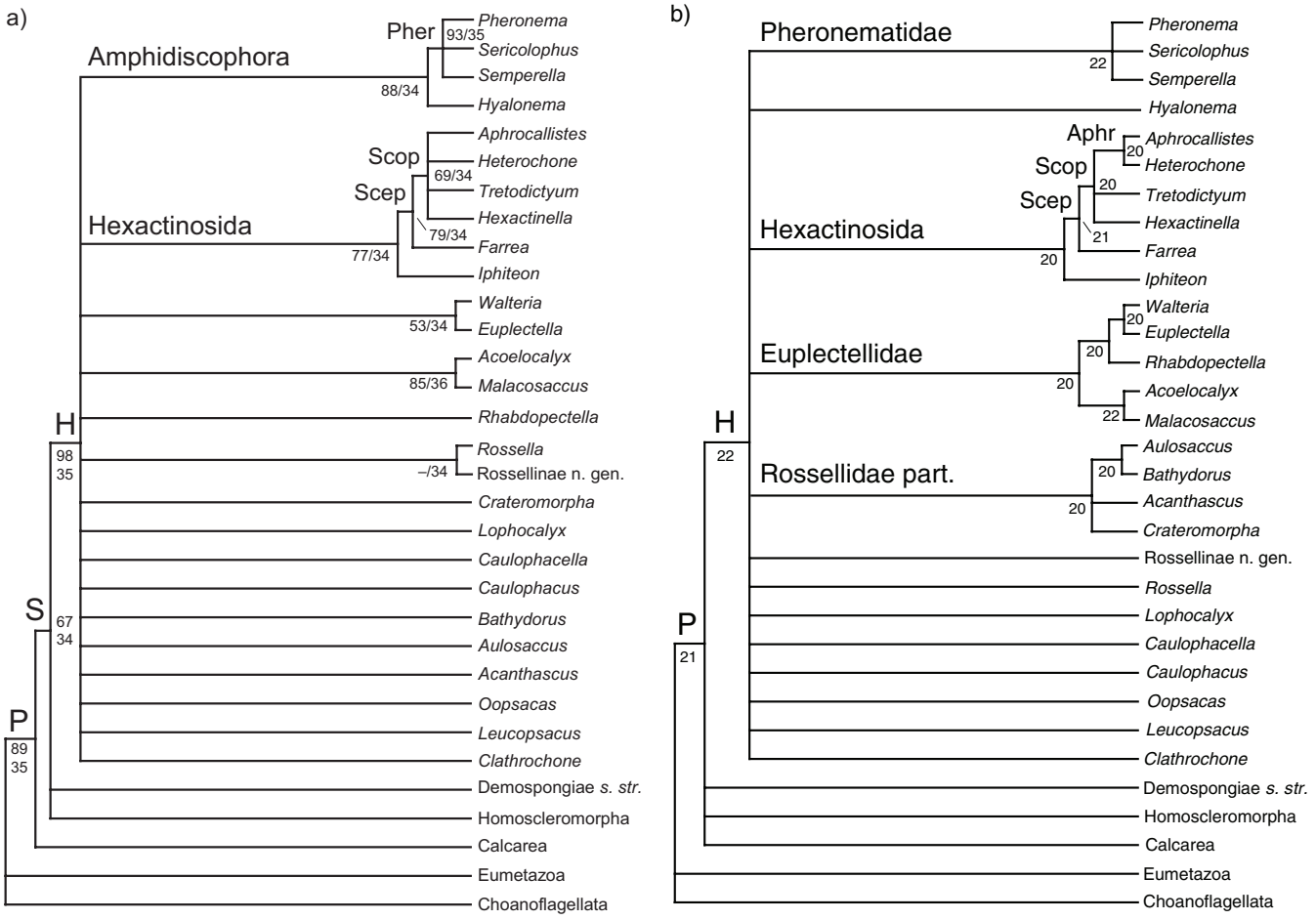


FIGURE 3. Phylogeny of Hexactinellida inferred from morphological data. Maximum parsimony strict consensus trees. (a) Tree inferred from the character matrix given in Table 4 (no polymorphisms allowed within terminal taxa). Length = 72, consistency index (CI) = 0.69; retention index (RI) = 0.90; rescaled consistency index (RC) = 0.63. Numbers at branches are bootstrap proportions (top or left; –, BP < 50%) and decay indices (bottom or right). (b) Tree inferred from the character matrix given in Supplementary Table 1 (polymorphisms allowed within terminal taxa). Length = 73, CI = 0.66; RI = 0.88; RC = 0.58. Numbers at branches are decay indices. BPs are not given because the heuristic search on the bootstrap replicates was not able to finish within reasonable time. Aphr, Aphrocallistidae; H, Hexactinellida; P, Porifera; Pher, Pheronematidae; S, Silicea; Scep, Sceptrulophora; Scop, Scopularia; *s. str.*, *sensu stricto*.

Leucopsacidae)—as well as some unnamed clades compatible with the rDNA phylogeny—are found in high or moderate proportions in the set of MPTs (Table 6). We suspect that allowing for polymorphisms introduced a substantial amount of noise that masked the true phylogenetic signal in our dataset. However, the poor resolution of the strict consensus trees indicates that most clades are supported by only a few characters, or character states that independently evolved elsewhere on the tree, which is in agreement with the notion that it is usually a *combination* of characters that makes a good taxon definition in Hexactinellida (e.g., Amphidiscophora: hypodermal pentactins *and amphidiscs*; Rossellidae: hypodermal pentactins *and hexasters*).

Relationships of the Major Sponge Groups

Previous rDNA analyses (e.g., Collins, 1998; Medina et al., 2001; Manuel et al., 2003) found a close relationship of hexactinellids to demosponges, although only one or two hexactinellid sequences were used in these studies. By substantially increasing hexactinellid taxon sampling, we were able to confirm this relationship, which is also supported by the shared cytoplasmic production of siliceous spicules along a protein filament (Böger, 1988; Ax, 1996), and the shared presence of "demospongic acids" (Thiel et al., 2002; Blumenberg, 2003; see character 5 in online Appendix). A Demospongiae + Hexactinellida clade (Silicea) was also recovered from our morphological analysis (Fig. 3a), although allowing for polymorphisms made this result ambiguous (Fig. 3b, Table 6), apparently because some demosponge groups do not produce spicules at all (see character 6 in online Appendix).

However, Homoscleromorpha, traditionally assigned to Demospongiae, poses a substantial challenge for the Silicea hypothesis. Homoscleromorphs are the only sponges known to possess basement membranes and therefore allegedly true epithelia (e.g., Boury-Esnault et al., 2003; Ereskovsky and Tokina, 2007), leading to the hypothesis that they might be more closely related to Eumetazoa than to other sponges (Boury-Esnault et al., 2003; Sperling et al., 2007). Although we included this character in our morphological analysis, a Homoscleromorpha/Eumetazoa clade was not found (Fig. 3). Using 18S rDNA, Borchiellini et al. (2004) found Homoscleromorpha to group outside the remaining demosponges, but could not resolve their exact relationship to other metazoan taxa. Sperling et al. (2007) have analyzed a set of seven nuclear genes for a small sampling of sponges and conclude that Homoscleromorpha is indeed the sister group to Eumetazoa and that Calcarea represents the sister group to these two taxa. In this study, we found Homoscleromorpha to be the sister group of Calcarea (see also Nichols, 2005; Erpenbeck et al., 2007). Calcarean affinities of homoscleromorphs had been suggested earlier on the grounds of larval similarities (van Soest, 1984; Grothe, 1989), although this interpretation has been questioned (Boury-Esnault et al., 2003). A grouping of Homoscleromorpha with Calcarea would imply that the production of siliceous spicules along a protein filament and the synthesis of demospongic acids either evolved at least twice, or that calcareans have secondarily lost these features; both scenarios are difficult to imagine.

A surprising result of our study was that monophyly of Demospongiae *s. str.* was not recovered (see also Cavalier-Smith and Chao, 2003; Nichols, 2005). According to our rDNA analysis, Demospongiae *s. str.* is paraphyletic with respect to Hexactinellida, although with low support. These results highlight the need to continue investigating relationships between the major groups of siliceous sponges. It should be noted that Demospongiae *s. str.* is not well supported by any morphological character (Borchiellini et al., 2004).

From a morphological perspective, monophyly of Porifera seems unambiguous (e.g., Ax, 1996; Reitner and Mehl, 1996; Nielsen, 2003). The fact that our morphological analysis supports sponge monophyly (Fig. 3) therefore comes as no surprise. In contrast, most molecular phylogenetic studies found sponges to be paraphyletic, usually with Calcarea being more closely related to Eumetazoa (e.g., Collins, 1998; Borchiellini et al., 2001; Medina et al., 2001; Peterson and Butterfield, 2005). Although support for such a scenario is generally low, it apparently has become generally accepted that sponges are paraphyletic (see, for example, Pennisi, 2003; Halanych, 2004). In the present study, we did not explicitly address the question of sponge monophyly, and our taxon sampling might be inadequate to answer it. Nevertheless, the fact that we found a monophyletic Porifera—albeit with low support under paired-sites models—indicates that rDNA does harbor phylogenetic signal for sponge monophyly. Therefore, paraphyly of Porifera should not be taken as accepted or as generally indicated by rDNA data.

Phylogeny of Hexactinellida

This paper presents the first reconstruction of glass sponge phylogeny from molecular data, as well as the first computerized cladistic analysis of hexactinellid morphology. These analyses confirm the monophyly of Hexactinellida and its primary subgroups, Amphidiscophora and Hexasterophora. The very long branches leading to these three clades in the rDNA tree (Fig. 2) suggest that hexactinellids have undergone an accelerated rate of molecular evolution, which accords well with their highly derived morphology (see Leys et al., 2007). However, the selection pressures responsible for these phenomena remain enigmatic. Molecular dating techniques, involving paleontological and geological data, should help to better understand the environmental conditions under which glass sponges originated. Hexasterophora and Amphidiscophora must have already diverged from each other in the Early Paleozoic, as indicated by isolated hexasters and amphidiscs from Ordovician and Silurian strata, respectively (Mostler, 1986; Mostler and Mehl, 1990).

In order to clarify the discussion of our inferred phylogeny of Hexactinellida and its implications for the

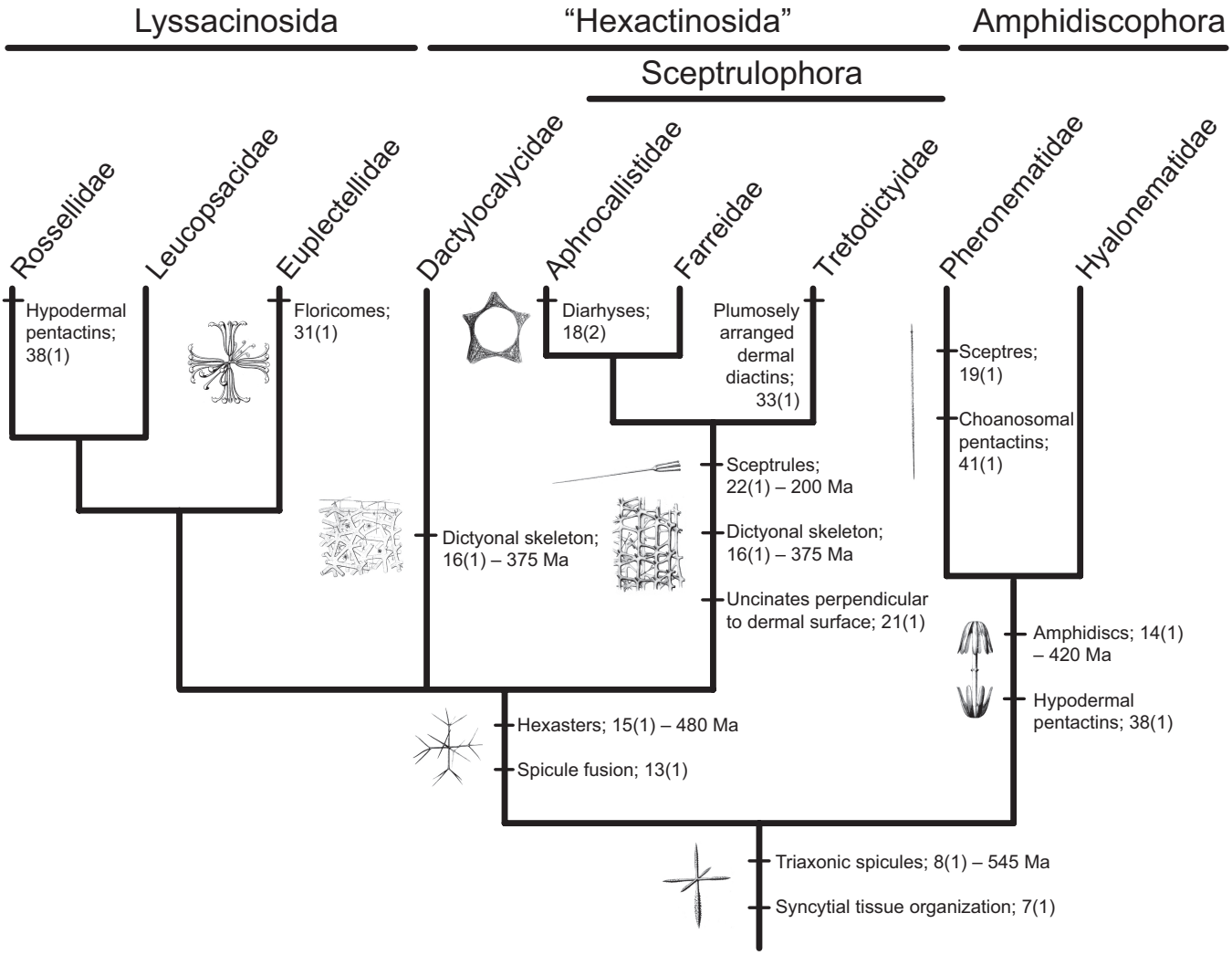


FIGURE 4. Morphological apomorphies of selected hexactinellid taxa and first appearances of characters in the fossil record (where known). The topology is a simplified version of the rDNA phylogeny (Fig. 2). Phylogenetic position of Dactylocalycidae is drawn as unresolved because it differed depending on the substitution model applied to stem regions (see text). Characters (character states) indicated at branches (see online Appendix for more detailed descriptions). Ma, million years ago. Dates from Steiner et al. (1993) and Brasier et al. (1997; triaxonic spicules); Mostler (1986; amphidiscs and hexasters); Rigby et al. (1981) and Mehl (1996; dictyonal skeletons); Donofrio (1991; sceptrules). Icons from Schulze (1887, 1899, 1902, 1904) and Ijima (1926).

evolution of morphological characters, we present a summary tree (Fig. 4), with important apomorphies and first occurrences of spicule types in the fossil record indicated.

Amphidiscophora.—As far as the current taxon sampling is concerned (three of six genera), monophyly of Pheronematidae is highly supported by both our molecular and morphological analyses (see Fig. 4 for apomorphies). Because the current morphology-based classification is essentially uninformative about internal relationships of this family (see, however, Tabachnick and Menshenina, 1999), our study provides a first glimpse into how the pheronematid genera might be related. Our molecular results also show a clade consisting of the two investigated *Hyalonema* species, which is at least consistent with monophyly of Hyalonematidae, but the family is diverse and contains four other genera and many subgenera. More extensive sampling of amphidiscophoran diversity is necessary to obtain a broader picture of this taxon's evolution.

Sceptrulophora and Hexactinosida.—Mehl's (1992) Sceptrulophora hypothesis is strongly corroborated by our results. Our molecular analyses further indicate a sister group relationship of the sceptrule-lacking dictyonal species *Iphiteon panicea* (Dactylocalycidae) to the lyssacinosidan family Euplectellidae, or possibly to Lyssacinosida as a whole (Fig. 2), thereby rejecting monophyly of Hexactinosida. However, Hexactinosida is monophyletic in our morphology-based trees, with *Iphiteon* sister to Sceptrulophora (Fig. 3). Thus, although our morphological analysis supports a single origin of dictyonal frameworks, the rDNA analyses suggest that these structures evolved at least twice. Finding evidence for convergence is not surprising, however, because structural

organization of dictyonal frameworks varies substantially across taxa (Leys et al., 2007) and their homology is a tentative hypothesis (Mehl, 1992). The occurrence of hexactinellids with dictyonal frameworks in the Late Devonian (e.g., Rigby et al., 1981; Rigby, 1986; Mehl, 1996), their absence in Late Paleozoic strata, and their reappearance in the Mid-Triassic may also point towards convergent evolution.

The internal relationships of Sceptrulophora found here are in good agreement with the classical system; Aphrocallistidae and Tretodictyidae are morphologically well-defined taxa (Mehl, 1992; Reiswig, 2002c, 2002d; see Fig. 4). However, although Aphrocallistidae consists solely of *Aphrocallistes* and *Heterochone*, we only sampled two out of eight tretodictyid genera, and these are probably very closely related (see Mehl, 1992; Reiswig, 2002c); therefore, a thorough test of monophyly of this group awaits broader taxon sampling.

A division of Sceptrulophora into Scopularia and Clavularia according to the type of sceptrules (see Fig. 1f) (Schulze, 1886; Mehl, 1992) is supported by our morphological analysis (Fig. 3), but the molecular results strongly suggest paraphyly of Scopularia (Fig. 2). Given that scopules are widespread among dictyonal hexactinellids and clavules are restricted to Farreidae, scopularian paraphyly seems plausible. However, a broader taxon sampling is required to further investigate this issue.

Lyssacinosida.—Monophyly of Lyssacinosida is only supported under seven-state paired-sites models or independent-sites models (Fig. 2, inset). Application of six-state models, which provide a significantly better fit to our data (Table 5), changes the position of *I. panicea* from sister group of Lyssacinosida to sister group of Euplectellidae, rendering Lyssacinosida paraphyletic (Fig. 2). Also, ML bootstrap support for Lyssacinosida is low (Fig. 2, inset). These results are consistent with the observation that no morphological synapomorphies are known for Lyssacinosida (Mehl, 1992). The order is simply defined by a lack of dictyonal frameworks (Reiswig, 2002e), which easily explains its absence from our morphology-based trees (Fig. 3). On the other hand, we are not aware of potential synapomorphies of *I. panicea* and Euplectellidae or Lyssacinosida. Given its sensitivity to model choice, the exact phylogenetic placement of *I. panicea* (or Dactylocalycidae) requires further research.

Monophyly of the three lyssacinosidan families is corroborated by our study, at least with molecular data (the morphological results are more ambiguous; see above). We were also able to resolve the position of *Clathrochone clathroclada*, formerly *incertae sedis* (Tabachnick, 2002a). The species apparently belongs to Leucopsacidae (see Tabachnick, 2002b), which consists of only 16 described species in three genera and appears as sister to Rossellidae in our rDNA phylogeny (Fig. 2). Rossellidae and Euplectellidae are the most diverse hexasterophoran families, with 23 and 27 described genera, respectively (Leys et al., 2007:117). Although monophyly of these families therefore should be further tested with molecular sampling of additional taxa, we feel confident in our results because of the high support from the rDNA analysis (Fig. 2) and the relatively high morphological homogeneity within these two groups (see Hooper and van Soest, 2002).

Due to the scarcity of taxonomic hypotheses regarding internal relationships of Rossellidae and Euplectellidae (see Tabachnick, 2002c, 2002d), we discuss only a few key points here. First, the subfamily division of both taxa gains no support from our study: within Rossellidae, subfamily Rossellinae appears paraphyletic because the only investigated species of the second subfamily (Lanuginellinae), *Lophocalyx* n. sp., is nested within it as a close relative of *Caulophacus* spp. and *Caulophacella*. This result also gains some support from the morphological analysis (see Table 6) and is not surprising given the weak definition of Rossellinae (see Tabachnick, 2002d). It should also be noted that the suspected close relationship of *Caulophacus* and *Caulophacella* to *Crateromorpha* (Tabachnick, 2002d; Menshenina et al., 2007) is not supported by our study. Similarly, the euplectellid subfamily Euplectellinae appears paraphyletic due to the position of *Walteria* (the sole included member of Corbitellinae) as sister to *Euplectella* (Euplectellinae; Figs. 2, 3; see also position of *Rhabdopectella* [Bolosominae] in Fig. 3b). However, euplectellid subfamiles are not clearly delineated (see Tabachnick, 2002d), and a *Walteria*/*Euplectella* clade is at least consistent with Mehl's (1992) proposed phylogeny. The close relationship of *Acoelocalyx* and *Malacosaccus* found in both molecular (Fig. 2) and morphological (Fig. 3) analyses is supported by the presence of a long peduncle composed of anchorate basalia supplemented by tauactins (characters 10 and 11; see Tabachnick, 2002c), providing a nice example of congruence between molecular and morphological data sets.

A final point worth mentioning is the nonmonophyly of *Rossella* (see Fig. 2). *Rossella nodastrella* is the only N Atlantic member of the genus (see van Soest et al., 2007) and does not group with the two Antarctic species in our rDNA tree, suggesting a potential error in the generic assignment of *R. nodastrella*. Although the Bayesian analyses under both paired-sites (Fig. 2) and independent-sites (not shown) models yielded the same well-resolved and supported relationships among these taxa, it should be noted that these relationships are not well supported by the ML bootstrap analysis. Better resolution in the Bayesian topologies may result from an artifact caused by the difficulty of currently implemented priors to correctly handle very short branch lengths (Lewis et al., 2005; Steel and Matsen, 2007; Yang, 2007). Thus, results associated with this part of the topology should be interpreted with some caution.

CONCLUSION

Of the three poriferan classes, Hexactinellida is by far the least well studied. For the first time, this study investigates the group's phylogenetic relationships using molecular and morphological data, thus filling another gap in our knowledge of the metazoan Tree of

Life. In contrast to Demospongiae and Calcarea, where substantial conflicts between morphology-based systems and molecular phylogenies have been revealed (e.g., Borchiellini et al., 2004; Dohrmann et al., 2006), we found our rDNA phylogeny to be largely consistent with the currently used Linnaean system, the phylogeny proposed by Mehl (1992), and the results of our morphological analyses. The reason for this is probably very simple: hexactinellids are generally characterized by a rich suite of morphological characters, especially an "amazing array of spicules of various shapes and sizes" (Leys et al., 2007:59), providing taxonomists and phylogeneticists with sufficient information to delineate natural groups. Nevertheless, monophyly of Hexactinosida, Lyssacinosida, and Scopularia is not supported by our rDNA analyses, and the current subfamily classification of Rossellidae and Euplectellidae seems to be at odds with both our molecular and morphological results. These findings can readily be explained by the poor taxonomic definitions of the respective groups. Incorporation of additional taxa and characters in future studies will further enhance our knowledge of glass sponge phylogeny and evolution and thereby serve as an indispensable basis for understanding all aspects of the biology of these remarkable animals.

ACKNOWLEDGMENTS

Shirley Pomponi and Volker Thiel, John Hooper, Rob van Soest, Sally Leys, Henry Reiswig, and Carsten Eckert are thanked for providing specimens. Henry Reiswig and Konstantin Tabachnick helped with identification of specimens and gave advice on morphology. Oliver Voigt and Dirk Erpenbeck provided useful discussions on laboratory procedures. Oliver Voigt is also thanked for help with RNA secondary structure and for providing 16S primers and 16S sequences of *Aphrocallistes vastus* and *Acanthascus dawsoni*. Daniel Jackson kindly provided cDNA of *A. vastus* and *A. dawsoni*. Vivek Gowri-Shankar, Kai Müller, and Alexandros Stamatakis provided comments on and help with their software. We gratefully acknowledge the Gesellschaft für wissenschaftliche Datenverarbeitung Göttingen (GWDG; www.gwdg.de) and Burkhard Morgenstern (Department of Bioinformatics, University of Göttingen) for access to Linux clusters. Henry Reiswig provided the picture of a clavule (Fig. 1f, inset). Comments by Frank Anderson, Jack Sullivan, Henry Reiswig, and an anonymous reviewer helped to improve this manuscript. M.D. would like to thank the staff and students of the Workshop on Molecular Evolution 2006 for inspiring and fruitful discussions, and the Boehringer Ingelheim Fonds for funding his stay in Woods Hole. M.D. also gratefully acknowledges a Mini-PEET award from the Society of Systematic Biologists. This work was funded by grants of the Deutsche Forschungsgemeinschaft (DFG) to G.W. (project Wo896/5–1, 2) and D.J. (project JA1063/11–2). We acknowledge funding through the DFG Priority Program SPP1174 "Deep Metazoan Phylogeny".

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First submitted 13 August 2007; reviews returned 2 October 2007; final acceptance 11 February 2008
Associate Editor: Frank Anderson