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Speculation with spiculation?—Three independent gene fragments and biochemical characters versus morphology in demosponge higher classification

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Abstract

Demosponge higher-level systematics is currently a subject of major changes due to the simplicity and paucity of complex morphological characters. Still, sponge classification is primarily based on morphological features. The systematics of the demosponge order Agelasida has been exceptionally problematic in the past. Here, we present the first molecular phylogenetic analysis based on three partially independent genes in demosponges in combination with a comprehensive search for biochemical synapomorphies to indicate their phylogenetic relationships. We show how sponges with fundamentally different skeletons can be in fact closely related and discuss examples of the misleading nature of morphological systematics in sponges.

Keywords: Porifera; Demospongiae; Morphology; Chemotaxonomy; Molecular systematics; Agelasida

1. Introduction

1.1. Pitfalls of demosponge systematics

Lévi (1957) dubbed the Porifera as the last major group of Metazoa in which the orders were still not clearly defined. Difficulties in demosponge systematics are clearly due to the simple 'bauplan' of this taxon resulting in a shortage of characters required for a robust phylogenetic reconstruction. Sponges bear only a few different cell types of which the sclerocytes produce the characteristic spicules, i.e., siliceous structures, which are often needle-shaped. These spicules often form a distinct skeleton, but occasionally they are loosely distributed throughout the sponge body without identifiable order or they are lacking entirely. Size, type, shape, combination of spicules, and their skeletal arrangements are the fundamentals of current sponge systematics. Although morphogenesis of those spicules has been studied (see Dendy, 1921; Jones, 1997; Uriz et al., 2003), the evolution of all spicule features is not fully understood, which prevents their unambiguous phylogenetic interpretation. Various alternative morphological characters such as shape, surface, texture or color can be variable with microhabitat conditions or season (e.g., Barthel, 1991; Jones, 1984; Schönberg and Barthel, 1997) or are observable only in situ because sponges might shrink and loose their coloration during preservation in ethanol. The use of cytological features in sponge systematics has also been examined (Boury-Esnault et al., 1994), but these are prone to preparation artifacts, and their phylogenetic information content is not fully analyzed and might not be sufficient for higher sponge taxonomy.

1.2. The enigmatic relationship between the demosponge orders Halichondrida and Agelasida

The two demosponge orders Halichondrida and Agelasida are considered to be only distantly related because their skeletal features differ dramatically.

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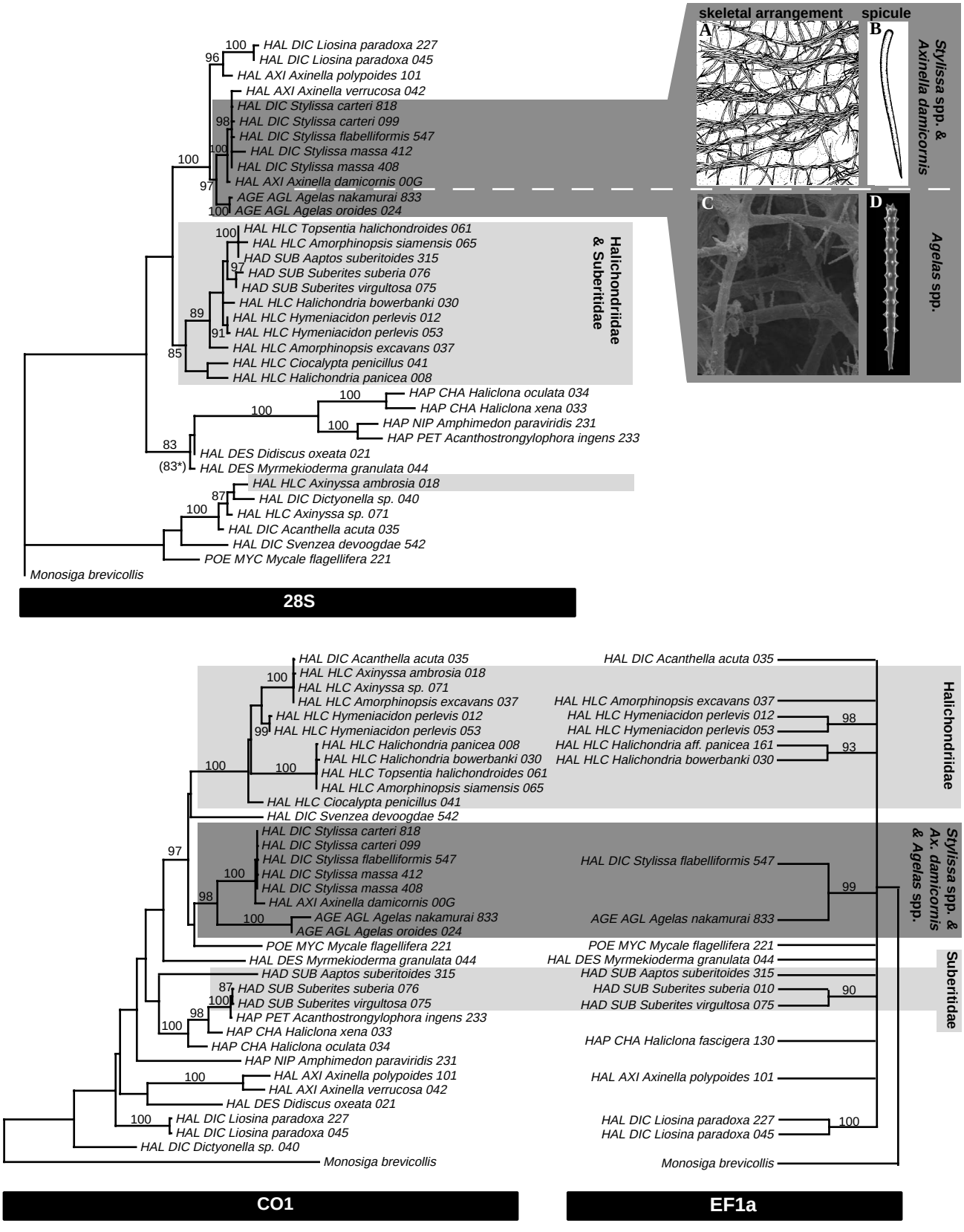
Agelasida currently comprises two families: Agelasidae Verrill 1907 and Astroscleridae Lister 1900 (Van Soest and Hooper, 2002). The family Agelasidae comprises only one genus: *Agelas*, which is also the nominal genus of the order. Its skeleton is a reticulation of spongin fibers, which are cored (usually main fibres only) and heavily echinated by verticillately spined spicules (Fig. 1, inset C). These verticillately spined spicules (Fig. 1, inset D) are the only type



of spicules found in Agelasida and are unique for Porifera, which makes their use for classification difficult. The Astroscleridae have a fundamentally different skeleton: a basal calcareous skeleton made of sclerodermites in aragonite (Vacelet, 2002). Nevertheless, the presence of the characteristic verticillately spined spicules in most genera in combination with molecular 28S sequence data (Alvarez et al., 2000; Chombard et al., 1997; Nichols, 2005) and biochemical evidence (review in Wörheide, 1998) clearly showed a sister-group relationship of the Astroscleridae *s.s.* to Agelasidae and justified their combination in a taxon "Agelasida" despite their fundamentally different skeleton types.

Such a spongin skeleton in combination with echinating spicules, verticilliate spined styles or a coralline basal skeleton, as present in Agelasida, is unknown throughout the demosponge order Halichondrida. The skeleton of the halichondrid genus *Axinella*, the nominal genus of the family Axinellidae, is differentiated in an axial and extra-axial part, both based on spicules and not on spongin fibres (Fig. 1, inset A). The spicules (styles) of *Axinella* are smooth and never echinating (Fig. 1, inset B). A similar skeleton and spiculation is present in the genus *Stylissa* of the halichondrid family Dictyonellidae. Several *Stylissa* species such as *S. massa* and *S. carteri* were previously regarded as *Axinella* because of their vaguely reticulate skeleton of styles, lacking a distinct surface specialization. A relationship with Agelasida was hardly ever considered from the morphological point of view.

Biochemical and molecular rDNA data gave rise to new phylogenetic hypotheses. Biochemical data for sponges have increased immensely in the recent literature (Van Soest and Braekman, 1999), after discovery of sponge-produced bioactive and pharmaceutically valuable molecules (Bergmann and Freeney, 1950). Presence and absence of particular biochemical compounds or pathways were used as synapomorphic characters in sponge systematics to augment the poorness of morphological characters. On the basis of such biochemical data, Bergquist (1978) concluded that Agelasidae have closer affinities to Axinellidae. Nevertheless, in subsequent classifications Agelasidae remained placed distant from axinellids and even obtained order status (Hartman, 1980). Later, Braekman et al. (1992) noted an exclusive occurrence of pyrrole-2-imidazole derivates in both Agelasida and axinellid species and provided additional evidence for a potential relationship between these two groups. In the same year, Lafay et al. (1992) published one of the first molecular phylogenies on sponges. Their 28SrDNA fragment analysis also favored a close relationship between *Agelas oroides* and *Axinella damicornis* species and was supported by the 28SrDNA data from Alvarez et al. (2000) and others.

1.3. Pitfalls of molecular and biochemical systematics in sponges

These analyses of Lafay et al. (1992) and Alvarez et al. (2000) were, as almost entirely all molecular phylogenies of higher sponge taxa, based on fragments of the nuclear rDNA gene family (18S and 28SrDNA) such as phylogenetic studies on "Sclerosponges" (Chombard et al., 1997), Hadromerida (Chombard and Boury-Esnault, 1999), "Tetractinellida" (Chombard et al., 1998), "Lithistida" (Kelly-Borges and Pomponi, 1994; McInerney et al., 1999), Hadromerida (Kelly-Borges et al., 1991; McCormack and Kelly, 2002), Haplosclerida (McCormack et al., 2002), Demospongiae (Borchiellini et al., 2004), and Calcarea (Manuel et al., 2003) (see also Borchiellini et al., 2000 for an overview). Many of these studies resulted in gene tree topologies that raised more questions than they answered or were highly incongruent with the morphological expectations (e.g., McCormack et al., 2002). Chombard and Boury-Esnault (1999) reconstructed a partial rDNA tree, which mixed taxa of the Halichondriidae (order Halichondrida) with the Suberitidae (order Hadromerida). The resulting proposed order "Suberitina" to date lacks support from other character sets and has not been accepted so far (Van Soest and Hooper, 2002).

Erpenbeck et al. (2004) showed that significant differences in 28SrRNA secondary structure and evolutionary rates exist. They might bias phylogenetic signal and phylogenetic reconstruction, which clearly suggests additional gene trees based on other, independent genes are necessary. (See also Wörheide et al. (2004) on intragenomic variation in sponge ITS and Vollmer and Palumbi (2004) on Anthozoa.)

Similarly, the biochemical compounds are not problem-free either. Both Bergquist (1978) and Van Soest and Braekman (1999) drew attention to the importance of a more precise identification, which often suffers from lack of experienced identifiers. Further pitfalls with chemosystematics are difficulties with homologization of pathways and a strong emphasis on *new* compounds in the chemical

Fig. 1. Phylogenetic trees of the three fragments analyzed. The numbers on the branches are bayesian inference posterior probabilities >80%. Grey shaded fields highlight taxon groups discussed in the text. Insets: (A) Schematic drawing of a plumo-reticulated skeleton with its reticulated skeleton of smooth styles (B). (A and B with friendly permission from B. Alvarez.) (C) Cross-section of the skeleton of *Agelas clathrodes* with the spongin fibres and echinating verticillately spined spicules (SEM). (D) Verticillately spined spicule of *Agelas dispar* (SEM). Tree above: 28SrDNA maximum likelihood tree constructed under the TrN + I + G model with bayesian support values under the GTR + I + G model. The asterisk indicates a sister-group relationship for the Desmoxyidae *Didiscus oxeata* and *Myrmekioderma granulata* as present in the 28S Bayesian inference reconstruction, which is in this part different from the ML-tree. Below left: ML tree under the TVM + I + G model with bayesian support values under the GTR + I + G model. Below right: EF1α bayesian inference tree under the SYM + G + I model (80% consensus). *Orders*: AGE, Agelasida; HAD, Hadromerida; HAL, Halichondrida; HAP, Haplosclerida; POE, Poecilosclerida. *Families*: AGL, Agelasidae; AXI, Axinellidae; CHA, Chalinidae; DIC, Dictyonellidae; DES, Desmoxyidae; HLC, Halichondriidae; MYC, Mycalidae; NIP, Niphatidae; PET, Petrosiidae; SUB, Suberitidae.

literature. In contrast with this practice, only reports of *similar* compounds in different sponges could be used as potential synapomorphies.

1.4. The approach

Single gene family approaches frequently suffer from systematic error and can be influenced by taxon-specific peculiarities (Erpenbeck et al., 2004), which prevents them from displaying the species tree. Therefore, only the congruence of several independent gene trees will provide us with a sufficient degree of certainty about their correctness. Phylogenies with two independent genes (e.g., Schröder et al., 2003; Nichols et al., 2005; with partial 28S and CO1) are still scarce as mitochondrial markers have only relatively recently been introduced in sponge systematics (Wörheide et al., 2000). However, the recent sequencing of the first complete sponge mitochondrial genome (Lavrov et al., 2005) will provide a wealth of new opportunities. Here, we report on the first three independent gene approach in demosponge systematics to unravel the systematic position of the Agelasida and to verify their close relationship to halichondrid taxa. We employed the D3–D5 region of the cytoplasmatic ribosomal long subunit gene (LSU, 28S), a mitochondrial gene fragment (cytochrome oxidase subunit 1, CO1) and a nuclear protein (elongation factor 1-α, EF1α). In addition, we investigate potential support and coherence of the proposed taxon "Suberitina," which is to date only defined by partial 28SrDNA sequences.

2. Materials and methods

2.1. Taxon set, DNA extraction, and PCR setup

The list of specimens studied with their sample locations is given in Table 1. Samples were either freshly collected by SCUBA diving or taken from collection material of the Zoological Museum Amsterdam (ZMA), where all vouchers for this investigation are kept. Total DNA was extracted from the choanosome to reduce the chance of amplifying non-sponge DNA. For the extraction, we used standard protocols (see Sambrook et al., 1989) or a commercial DNA extraction kit (Quiamp DNA Mini Kit, Quiagen). The PCRs volume was 50 µl and contained 3 mM $MgCl_2$, 1 U SuperTaq Polymerase (Promega) with reaction buffer (Promega), 2 mM dNTPs (Gibco), 0.03 mg BSA (Sigma), 10 ng DNA template and 4 pmol of each primer (Misof et al., 2000). The SuperTaq/H_2O mix was added 1 min after starting the initial denaturation.

2.2. Amplification of 28S fragments

PCR primers for the 28S fragment were taken from McCormack and Kelly (2002) (primers: RD3A, GACC CGTCTTGAAACACGA and RD5B2, ACACACTCCT TAGCGGA; temperature regime, 94 °C 3 min during which the SuperTaq/H_2O mix was added, 35× (94 °C 30 s; 50 °C 20 s; 72 °C 60 s), 72 °C 10 min).

2.3. Amplification of CO1 fragments

DNA fragments of EF1α and CO1 were amplified in a two-step nested PCR. A first PCR product of CO1 was obtained with universal PCR primers under a temperature regime as used in previous studies (Erpenbeck et al., 2002). 1 µl of the PCR product was taken as DNA template in a consecutive step in order to specifically re-amplify the sponge fragments. For the second step, the following specific primers were designed: CO1porF1, 5′-CCN CAN TTN KCN GMN AAA AAA CA-3′ and CO1porR1, 5′-AAN TGN TGN GGR AAR AAN G-3′ and used under a temperature regime of 3 min 94 °C, followed by 35 cycles of (30 s 94 °C, 30 s 45 °C, 1 min 72 °C) and a final elongation time of 10 min 72 °C.

2.4. Amplification of EF1α fragments

The amplification of EF1α fragments follows Erpenbeck et al. (2005). Fragments were either amplified in a single step using the universal forward primers EF-599 (ATC TCC GGA TGG CAC GGY GAC AA, B. Normack) or EF1α-5F (AAR AAR RTN GGN TAY AAY CC) with the reverse primer EF-923 (ACG TTC TTC ACG TTG AAR CCA, B. Normark) under the following temperature regime: 94 °C 3 min, 40× (94 °C 30 s, 50 °C 30 s, and 72 °C 1 min), 72 °C 20 min or under a "touch-down-PCR" (94 °C 3 min, 20× (94 °C 30 s, 54 °C with 0.5 °C less each cycle, 72 °C 1 min) followed by 25× (94 °C 30 s, 50 °C 30 s, and 72 °C 1 min), 72 °C 20 min).

Alternatively, DNA was amplified in a nested approach. In the first step, a primary amplification was performed with the primers EF-F140 (GGN CAR ACN MGN GAR CA) and EF-R370c (ATR TGN GMN GTG TGR CAR TC, Erpenbeck et al. (2005); 94 °C 3 min, 40× (94 °C 30 s, 53 °C 30 s, and 72 °C 1 min), 72 °C 20 min). Second step: 1 µl of the PCR product was re-amplified using the internal primers EF-F200 (TGG WTG GMA NGG NGA YAA YAT G) and EF-R350 (ATC TNN CCA GGR TGR TTG A) under 55 °C annealing temperature, no BSA and only half concentration of $MgCl_2$.

2.5. PCR product processing, cloning, and sequencing

All PCR products were excised from a 2% TAE gel and extracted with glassmilk in a 65 °C water bath, washed three times with 80% ethanol, dried and re-eluted in 10 µl H_2O followed by ligation in a pGEM T-easy vector (Promega) and cloned in *Escherichia coli* according to the manufacturer's protocol. Plasmid DNA of at least three colonies was extracted using the alkaline lysis method (Sambrook et al., 1989) and cycle sequenced (Amersham) with labelled M13 primers. Both strands of the template

Table 1
Samples used in this analysis with voucher number, sampling locations, and the GenBank accession numbers for the amplified fragments

#	Species	POR[a]	Location	28S Accession No.	CO1 Accession No.	EF1α Accession No.
Order Agelasida						
833	*Agelas nakamurai*	17662	Sulawesi	AY618704	AY625654	AY623842
024	*Agelas oroides*	14435	Blanes/E	AY319311	AF437296	
Order Hadromerida						
315	*Aaptos suberitoides*	17498	Sulawesi	AY319308	AY625653	AY623837
010	*Suberites suberia*	14124	Roscoff/F			AY623867
076	*Suberites suberia*	9726	North Sea	AY319309	AF437295	
075	*Suberites virgultosa*	12969	North Sea	AY618725	AY625674	AY623868
Order Halichondrida						
035	*Acanthella acuta*	14589	Spain	AY319322	AY625652	AY623841
037	*Amorphinopsis excavans*	14627	Oman	AY319313	AF437297	AY623843
065	*Amorphinopsis siamensis*	17672	Gulf of Siam	AY618705	AY625655	
00G	*Axinella damicornis*	14097	Roscoff/F	AY319314	AF437304	
036	*Axinella polypoides*	14093	Roscoff/F	AY618728	AY625656	AY623844
042	*Axinella verrucosa*	14590	Blanes/E	AY319312	AY625657	
018	*Axinyssa ambrosia*	14311	Curacao	AY618707	AY625658	
071	*Axinyssa* sp.	14501	Sulawesi	AY618709	AY625659	
041	*Ciocalypta penicillus*	14111	Roscoff/F	AY618710	AF437302	
040	*Dictyonella* sp.	14614	Oman	AY319325	AY625660	
021	*Didiscus oxeata*	14326	Curacao	AY319320	AF437298	
030	*Halichondria bowerbanki*	17683	Netherlands	AY646836	AF437299	AY623845
008	*Halichondria panicea*	14125	Roscoff/F	AY319315	AF437294	
161	*Halichondria cf. panicea*	17691	Alaska			AY623846
012	*Hymeniacidon perlevis*	14140	Roscoff/F	AY319317	AF437301	AY623853
053	*Hymeniacidon perlevis*	17676	Netherlands	AY618715	AY625662	AY623854
045	*Liosina paradoxa*	14499	Sulawesi	AY618716	AF437303	AY623858
227	*Liosina paradoxa*	17499	Sulawesi	AY319318	AY625663	AY623859
044	*Myrmekioderma granulata*	14530	Sulawesi	AY319319	AY625665	AY623862
818	*Stylissa carteri*	17666	Sulawesi	AY618718	AY625669	
099	*Stylissa carteri*	17667	Sulawesi	AY618719	AY625670	
547	*Stylissa flabelliformis*	17668	Sulawesi	AY618720	AY625671	AY623866
408	*Stylissa massa*	17669	Sulawesi	AY618722	AY625673	
412	*Stylissa massa*	17670	Sulawesi	AY618721	AY625672	
542	*Svenzea devoogdae*	17671	Sulawesi	AY618726	AY625675	
061	*Topsentia halichondroides*	8450	Sri Lanka	AY618727	AY625676	
Order Haplosclerida						
233	*Acanthostrongylophora ingens*	17500	Sulawesi	AY319326	AY625667	
231	*Amphimedon paraviridis*	17685	Sulawesi	AF441350[b]	AY625666	
130	*Haliclona fascigera*	17700	Sulawesi			AY623849
034	*Haliclona oculata*	17501	Netherlands	AF441330[b]	AY625661	
033	*Haliclona xena*	17504	Netherlands	AY319327	AF437300	
Order Poecilosclerida						
221	*Mycale flagellifera*	17503	Sulawesi	AY319321	AY625664	AY623860
Outgroup (*Choanoflagellida*)						
	Monosiga brevicollis	taken from GenBank		AY026374	AF538053	AY026073

[a] Porifera collection of the Zoological Museum Amsterdam.
[b] Sequenced by us, but identical with those previously published by McCormack et al. (2002), whose accession number is given instead.

were sequenced on a LiCor automated sequencer. Because of the enhanced chance of amplifying symbionts in sponge systematics, several specimens were double-checked by re-sequencing of either the PCR products or the cloned plasmids by direct sequencing with an automated (ABI) sequencer using the BigDye Terminator v1.1 according to the manufacturer's protocol. Positions in contradiction between two sequences were coded with question marks. The CO1 and EF1α-exon alignment were unambiguous. The 28S sequence alignment was performed with secondary structure information given in Erpenbeck et al. (2004). All alignments were performed by eye.

2.6. Validation of the taxonomic origin

Sponges contain numerous microbial symbionts or otherwise ingested DNA templates. To prevent the incorporation of non-sponge sequences in our data set we

verified the poriferan origin of all sequences with phenetic BLAST searches (Altschul et al., 1990) and cladistic tree-reconstructions as described in Erpenbeck et al. (2002).

2.7. Phylogenetic reconstructions

Only nucleotide data has been used for the reconstructions, while the resulting phylogenies were compared with reconstructions on amino acid data for potential incongruence. Sequences of *Monosiga brevicollis* (Choanoflagellida) were used as uniform outgroup sequence for all analyses. We performed phylogenetic reconstructions under maximum-likelihood and Bayesian inference criteria on the nucleotide data sets. PAUP*4.10b (Swofford, 2002) was used for the heuristic maximum likelihood analyses under the relatively best-fitting model. Bayesian inference reconstructions were obtained with MrBayes 3.04b (Huelsenbeck and Ronquist, 2001) under 1,000,000 generations and four Metropolis-coupled Markov chains. The relatively best-fitting models were estimated with the likelihood-ratio test as implemented in Modeltest 3.06 (Posada and Crandall, 1998) or MrModeltest 1.1 (Nylander, 2002). To compare the congruent gene trees statistically, we tested them with the partition homogeneity test (Farris et al., 1994) as implemented in PAUP*4.10b.

2.8. Collection of biochemical data

Biochemical data were obtained from the literature as available from MarinLit (Blunt and Munro, 2003), a compilation of marine biochemical literature of the last 60 years until present. Until spring 2004 MarinLit comprised 15,485 records, which were subsequently screened for Halichondrida and potential synapomorphic markers with the orders Agelasida or Hadromerida. Pre-compiled records were taken from the surveys of Van Soest and Braekman (1999) and from Erpenbeck (2004). Compounds of assumed symbiotic or otherwise non-sponge origin were disregarded because genealogical relationships cannot be inferred from this unambiguously. Every potential marker for halichondrid–agelasid or halichondrid–suberitid relationship was re-checked against the database for presence among other demosponge taxa.

3. Results

Ambiguously alignable positions such as the hypervariable loop positions in 28S (Erpenbeck et al., 2004) and the EF1α-introns (Erpenbeck et al., 2005) were disregarded for all analyses. The resulting data sets comprised 36 taxa (see Table 1) with 560 characters (142 parsimony informative, ts/tv ratio = 1.22) for the 28S fragment, 36 taxa with 459 nucleotides (172 parsimony informative, ts/tv ratio = 2.81) for CO1, and 18 taxa with 302 nucleotides (129 parsimony informative, ts/tv ratio = 1.22) for EF1α. The resulting phylogenies are displayed in Fig. 1. As Bayesian posterior probabilities tend to have higher values than bootstrap probabilities (Huelsenbeck et al., 2002) we restricted branch support to a posterior probability of 80 or higher. The number of sponge sequences obtained for the EF1α was considerably lower and resulted in a smaller tree than for the 28S or CO1 fragment. To verify the suitability of *Monosiga* as outgroup taxon (see Lavrov et al., 2005), we used in different approaches calcareous sponges (*Leucosolenia*) and cnidarians (*Sarcophyton*, *Anemonia*, and *Eugymanthea*) as outgroup taxa and observed no differences in the supported outcome (Erpenbeck, 2004).

The partition homogeneity test revealed significant differences ($p < 0.01$) between the resulting 28S and CO1 topologies. The usage of (a) the second codon positions only and (b) first + second codon positions in CO1 had no significant impact on the outcome.

All three trees equivocally favor a close relationship of *Agelas* with *Stylissa*, respectively, the *Stylissa*/*Axinella damicornis* species complex in CO1 and 28S (posterior probabilities: 28S, 97; CO1, 98; EF1α, 99). The 28S tree places the agelasid taxa with *Stylissa* spp., *Axinella damicornis*, *A. verrucosa*, and close to *A. polypoides*, while the positions of the latter two is more basal in CO1 and inconclusive in EF1α. Nevertheless, our data displays strong evidence for a close relationship between the halichondrids *Axinella damicornis* and particularly *Stylissa* to Agelasidae, as all data sets are equivocal on their positions.

Suberitidae and Halichondriidae are clustered together in the 28S tree whereas they appear distant in the CO1 tree. The EF1α tree is inconclusive on this question (Fig. 1).

Furthermore, there is evidence for a coherence of the halichondriid taxa disregarding the uncertain relationship to the Suberitidae and *Axinyssa* in the 28S tree and *Acanthella* in the CO1 tree. The Dictyonellidae, however, are scattered all over the topology in both trees. We could not establish whether the families Desmoxyidae and Axinellidae were monophyletic because the number of taxa in our data set was not representative.

3.1. Potential biochemical markers uniting Halichondrida with Agelasida

The survey of MarinLit yielded the following potential biochemical markers and features shared by both agelasid and halichondrid taxa (Table 2):

(a) Pyrroloaminopropylimidazoles in Agelasida, Axinellidae, and Dictyonellidae: They are utilized by sponges as defense against predators and have already previously been discussed as combining markers for these taxa (Braekman et al., 1992; Van Soest and Braekman, 1999; and others). They still can be regarded as synapomorphic marker for these groups.
(b) Triple (C2,3,6)—sulfation of the steroid nucleus in Halichondrida and the astrosclerid *Ceratoporella*: Sulfation of the steroids, however, is widespread in

the demosponges, especially sulfation of the nucleus. The fact that some Halichondrida and the astrosclerid *Ceratoporella* sp. share compounds with the relatively rare triple sulfation at C 2,3,6, might discriminate these groups from the other sponges, but one must assume convergent evolution to regard this feature as potential halichondrid/agelasid synapomorphy as a similar structure is found in petrosiid sponges (e.g., Aoki et al., 2002). It remains not entirely certain, if such a triple sulfation is a solid synapomorphic character as the same enzymes might be involved, which (mono- or di-)sulfate the nuclei in other taxa.

(c) Galactosylceramides, which are a unique class of the membrane glycosphingolipids and shared by *Agelas* and *Axinella* spp. (Costantino et al., 1995, 1999). However, most of these glycosphingolipids appear to be derived from bacteria (Wicke et al., 2000), which might diminish their suitability as synapomorphies.

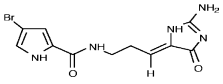
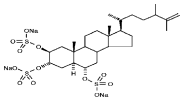
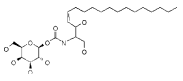
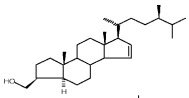
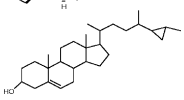
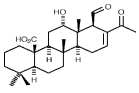
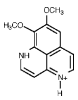
There were only relatively few records for biochemical components in Suberitidae and in particular for suberitid components shared with Halichondrida (tab. 2). None of the biochemical records for Hadromerida proved to be a potential synapomorphic marker with Halichondrida towards a taxon "Suberitina," or other potential hadromerid–halichondrid relationships:

(a) In several halichondrid taxa the A-ring of certain steroids is modified from a C6- to a C5-ring forming a 3B-hydroxymethyl-A-nor-steroid (see also Bergquist, 1978). This feature is also reported from a *Homaxinella* species (Eggersdorfer et al., 1982). The genus *Homaxinella* is currently assigned to the hadromerid family Suberitidae. However, other members of that genus such as the investigated *Homaxinella trachys* are probably axinellids (Erpenbeck, 2004; see also Barnathan et al., 2004), which revokes the current compound as Suberitidae/Halichondriidae marker.

(b) Cyclosterols with a cyclopropane ring at C25 and C27 are assumed to be rare (Umeyama et al., 2000) and have been recorded from several halichondrid genera (e.g., McKee et al., 1993) and also from the hadromerid genus *Spheciospongia* (Clionaidae, Catalan et al., 1982). However, we found neither chemotaxonomic value for the pure presence of a cyclopropane ring at C25 and C27 nor for its specific positions at the sterol side chain as it occurs frequently in species of other orders (Verongida and Haplosclerida).

(c) Sesterterpenes form only a small fraction of the biologically active terpene composition in Halichondrida and are also reported form the suberitid *Suberites* (Shin et al., 1995). However, they have been extracted from many other Dictyoceratida (Tasdemir et al., 2000), Dendroceratida (Cardellina et al., 1991),

Table 2
Potential biochemical compounds combining Halichondrida with Agelasida or Hadromerida

Compound	Family	Genus (e.g.)	Author (e.g.)
Pyrroloaminopropyl-imidazoles 	**Agelasidae**	***Agelas***	Iwagawa et al. (1998)
	Astroscleridae	***Astrosclera***	Williams and Faulkner (1996)
	Astroscleridae	***Goreauiella***	Rinehart (1989)
	Axinellidae	***Axinella***	Cimino et al. (1982)
	Dictyonellidae	***Stylissa***	Mattia et al. (1982)
(C 2,3,6) sulfation of the steroid nucleus 	Halichondriidae	*Halichondria*	Makarieva et al. (1995)
	Astroscleridae	*Ceratoporella*	Yang et al. (2003)
Galactosylceramides 	**Agelasidae**	***Agelas***	Costantino et al. (1995, 1999)
	Axinellidae	***Axinella***	Costantino et al. (1995, 1999)
3B-hydroxymethyl-A-or-steroids 	Axinellidae	*Axinella*	Aknin et al. (1996)
	Dictyonellidae	*Stylissa*[a]	Bohlin et al. (1980)
	(Suberitidae)[b]	*Homaxinella*[b]	Eggersdorfer et al. (1982)
Cyclosterols with cyclopropane rings at C25 and C27 	Halichondriidae	*Topsentia*	McKee et al. (1993)
	Dictyonellidae	*Stylissa*	Kelecom et al. (1979)
	Axinellidae	*Axinella*	Crist and Djerassi (1983)
	Clionaidae	*Spheciospongia*	Catalan et al. (1982)
Sesterterpenes 	Halichondriidae	*Halichondria*	Nakagawa et al. (1987)
	Suberitidae	*Suberites*	Shin et al. (1995)
Aaptamines 	Halichondriidae	*Hymeniacidon*	Pettit et al. (2004)
	Suberitidae	*Suberites*	Bergquist et al. (1991)

Their suitability is discussed in the text. Compounds, which have not been reported from any other demosponge order, are highlighted in bold.

[a] As *Stylotella agminata*.

[b] This *Homaxinella* (*trachys*) is probably an Axinellidae, see text.

Verongida (Jimenez et al., 1991), and Poecilosclerida (Yamada and Yamagishi, 1993), which revokes their suitability as combining character.

(d) Aaptamine-type alkaloids are a suggested marker for Hadromerida (Bergquist et al., 1991), respectively, the family Suberitidae (Van Soest and Braekman, 1999) and also reported for the halichondrid *Hymeniacidon* (Pettit et al., 2004). However, they have recently been isolated from the Haplosclerida *Xestospongia* (Calcul et al., 2003), and are known from the Dictyoceratida *Luffariella* (Park et al., 1995). They cannot be considered as a combining character for Suberitidae and halichondrids.

4. Discussion

4.1. The need for independent markers in demosponge molecular phylogeny

Congruent results from independent data sets provide strong support for any phylogenetic hypothesis. We could show, that three molecular and a biochemical data sets display congruencies (e.g., the phylogenetic affinities of the Agelasida within the demosponges) and provide strong support for morphology-incompatible classifications. However, in our approach, the CO1 gene tree is in major parts different from the 28SrDNA tree. 28SrDNA reconstructions of demosponge phylogenies repeatedly lead to topologies that are largely inconsistent with morphological expectation and classification (e.g., McCormack et al., 2002; McCormack and Kelly, 2002). Although this is no objective criterion to prefer one gene tree above another in the case of contradiction (Nichols, 2005; this study), the comparison of trees based on independent genes (e.g., nuclear rDNA against mtDNA and/or against a nuclear protein) appears highly necessary.

Moore (1995) discusses mitochondrial gene trees as more likely to be congruent with the species tree than nuclear gene trees, but they are also more strongly affected by historical events such as founder effects or bottlenecks than nuclear genes (Arnaud-Haond et al., 2003).

On the other hand, the diploblast mitochondrion appears in many aspects different from its bilaterian counterpart. Lavrov et al. (2005) revealed in the first published complete poriferan mt-DNA sequences extra genes in sponges (such as atp9, which is present in mtDNA of fungi, plants, and protists). Furthermore, they found relatively large intergenic regions and bacterial-like rRNA and tRNA features.

There are further peculiarities of diploblast mtDNA compared to bilaterian mtDNA. First, certain cnidarians bear a linear mtDNA structure instead of the typical circular strands (Bridge et al., 1992). Second, mtDNA of Anthozoa (and probably of other diploblasts as well) encodes only 1 to 2 tRNA genes instead of 22 as usual in Metazoa (Wolstenholme, 1992), which requires import of tRNAs and causes different evolutionary constraints. Furthermore, the evolutionary rate of mtDNA is found to be up to 10–20 times lower in diploblasts (Shearer et al., 2002), which may be caused by a special mtDNA mismatch repair system as found in corals (Pont-Kingdon et al., 1995). This lower evolutionary rate in diploblasts might be suitable to resolve older clades or deeper phylogenies than in Bilateria.

Shearer et al. (2002) describe cases for diploblasts in which the mitochondrial gene clearly failed to reconstruct species trees: genetic variation among species also can be disproportionate to the taxonomic classification with a divergence rate too variable among clades. Romano and Palumbi (1996, 1997) report six identical mitochondrial 16S strands from taxa of three different scleractinian families, which Shearer et al. (2002) interpret as additional reason not to overestimate the phylogenetic inference of mitochondrial gene trees.

Consequently, the need of multiple *independent* gene trees in (sponge) phylogenetics is highly evident, regarding the specific peculiarities of every gene fragment recruited for phylogenetic reconstructions. A combination out of mtDNA, nuclear rDNA and a suitable nuclear protein, as shown in our example, appears desirable.

4.2. Multiple, independent support for agelasidlhalichondrid relationships

A close relationship between Agelasidae and halichondrid taxa, especially to the *Stylissa*/*Axinella damicornis* species complex, is indicated equivocally by all three gene fragments and biochemical compounds. Our data confirms the analyses of Lafay et al. (1992) and Alvarez et al. (2000) with a different ribosomal fragment and provides support from two other partially independent genes, a mitochondrial (CO1) and a nuclear (EF1α) protein and the chemosystematic analyses of Bergquist (1985), Braekman et al. (1992), and Costantino et al. (1996). A suborder Agelasina may be employed alongside other halichondrid subtaxa, which will have to be distinguished in future studies (Erpenbeck, 2004; Van Soest and Hooper, 2002). Although this hypothesis does not match the morphological expectations (which clearly would be desirable and would provide overwhelming support), we believe that independent molecular and biochemical data outperform morphological characters in phylogenetic reconstructions.

Indeed, morphological synapomorphies between the agelasids and halichondrids are not immediately obvious. A close relationship is morphologically masked by the accumulation of autapomorphies in Agelasidae. They differ essentially in form and function of the styles (verticillately spined and echinating in Agelasidae, smooth and plumoreticulate in *Axinella* and *Stylissa*), which justified the placement in different orders in previous systems (e.g., Lévi, 1973). Numerous other taxa share the style types of *Stylissa* and *Axinella*, which therefore imply their plesiomorphic character state. Therefore, an autapomorphic nature of the verticillately spined echinating styles has to be assumed in

this group. Echinating spicules are undoubtedly important for sorting relationships at the species level, but are of questionable significance for higher levels of classification (see also Hooper, 1990).

There are other fundamental differences in skeletal architecture masking close phylogenetic relationships in sponges: The order Agelasida contains the family Astroscleridae, whose members possess a calcareous basal skeleton, and have been earlier placed in a different class of sponges (''sclerosponges''), prior to the availability of morphological (Van Soest, 1984a; Vacelet, 1985), biochemical, and molecular data (e.g., Alvarez et al., 2000; Chombard et al., 1997). With our current data we can assess even more, independent lines of evidence for a close relationship of morphologically different demosponge taxa. Consequently, we cannot ignore the fact that sponge morphological systematics is frequently misleading and masks true evolutionary histories. This frequently misleading nature might have been due to a strong evolutionary pressure on the morphology of spicules, which appear to be unchanged since their earliest fossil records. Due to such potential constraints, morphologically similar spicules at different positions in the skeleton (i.e., with different functions) do not necessarily have to be homologous and require a more detailed functional distinction of characters to improve their homologization (Fromont and Bergquist, 1990).

4.3. Further phylogenetic implications supported by multiple data sets

Our independent gene tree approach also provides additional insight in the phylogenetic classification of the Halichondrida, which should be briefly taken into account:

We have not found further indication for the coherence of a taxon ''Suberitina,'' i.e., the predicted relationship between the families Halichondriidae (order Halichondrida) and Suberitidae (order Hadromerida), as suggested by Chombard and Boury-Esnault (1999). While their (and our) partial 28SrDNA trees clearly favor the close relationship between halichondriid and suberitid demosponges, our CO1, EF1α and biochemical data provide no extra evidence to merge these two families.

The halichondrid family Dictyonellidae Van Soest, Diaz and Pomponi, is not supported as a monophyletic group. Its genera are positioned in various parts of the tree indicating that the family Dictyonellidae is polyphyletic. The taxon Dictyonellidae is based on mainly negative characters (such as the lack of an ectosomal skeleton) and apparently an assemblage of genera related to different higher taxa, sharing the absence of their taxonomic relevant characters.

There is phylogenetic signal, suggesting coherence of a taxon Halichondriidae comprising of (at least) the genera *Halichondria*, *Hymeniacidon*, *Ciocalypta*, and *Topsentia* and *Amorphinopsis*. Their close relationship is supported by the CO1 and 28SrDNA data, while their relationship to other taxa with contradicting position in the gene trees such as *Axinyssa* or *Acanthella* has to be verified with additional data.

Despite their placement in two different families, *Stylissa* and *Axinella* might be closely related. *Stylissa massa* and *S. carteri*, both included in our data set together with *S. flabelliformis*, the type species of the genus *Stylissa*, were previously regarded as species of the genus *Axinella* and both genera share the occurrence of 3B-hydroxymethyl-A-nor-steroids (Erpenbeck, 2004).

4.4. Implications for sponge morphological systematics

The ancient origin of demosponges in combination with their simple morphology causes peculiar problems (homoplasies) in developing a morphological demosponge phylogeny. Therefore, molecular markers are used to examine the polarity of character evolution (e.g., Borchiellini et al., 2004; Manuel et al., 2003). Evolutionary constraints appear to have kept the sponge bauplan basically unchanged since the late Cambrian (Reitner and Wörheide, 2002) and inhibit the recognition of any additional morphological features. Based on these constraints, spicule elements were therefore either modified, and/or obtained different functions in different parts of the skeleton. Examples of convergent microsclere evolution are the ''didiscorhabds'' of *Didiscus* (order Halichondrida, Fig. 2A) and the convergently re-developed ''chessman-spicules'' of *Latrunculia* (order Poecilosclerida *i.s.*, Fig. 2B), of which clearly could be shown that they originate *analogously* from a different spicule type (Hiemstra and Van Soest, 1991).

While theories of microsclere evolution are abundant, homology assessments appear more difficult for principal styles (megascleres). It remains unclear if the ''tylote'' (Fig. 2C) spicules, as found in *Hymeniacidon* (Fig. 2D), are homologous to the ''tylostyles'' of Suberitidae (Figs. 2C and E), which could therefore be a potential synapomorphic character for the proposed taxon Suberitina. Both types of spicules appear morphologically different but could be either autapomorphic modifications, or convergent development of characters. The same problem is given with the ''mycalostyles'' of *Mycale* spp. (Fig. 2F). Mycalostyles are spicules that become narrower before the rounded end, resulting in a tylostyle-like appearance. They could be derived from common ''styles'' (Fig. 1B), tylostyles (Fig. 2C) or none, or both.

Our present data clearly show how morphological systematics, based on our understanding of morphology, is not sufficient for a rigorous cladistic reconstruction of a phylogenetic tree of all clades and all taxonomic levels *simultaneously*. Our molecular CO1 and 28S data sets support morphological expectations that the Halichondriidae genera *Amorphinopsis*, *Ciocalypta*, *Halichondria*, *Hymeniacidon*, and *Topsentia* are in fact closely related. To date these relationships cannot be technically supported by exhaustive cladistic analyses of the morphological characters because the possession of distinct spicules (''oxeas''

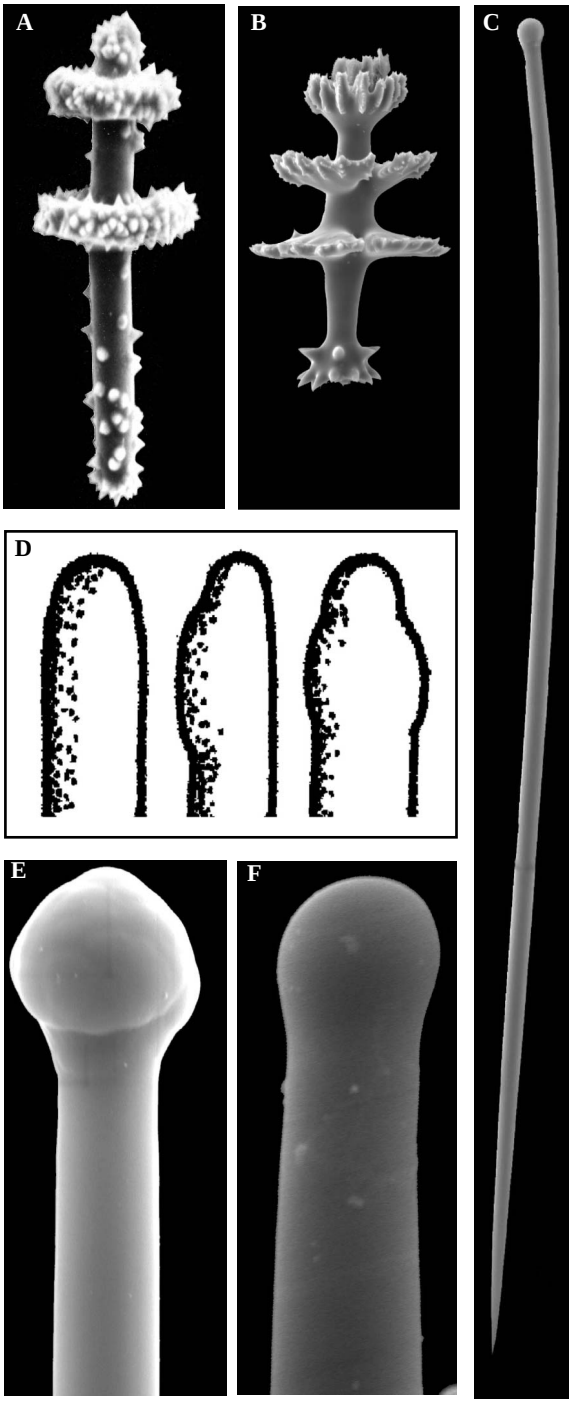


Fig. 2. (A) Didiscorhabd from *Didiscus aceratus*. (B) Discorhabd from *Latrunculia brevis*. (C) Tylostyle of *Suberites domuncula*. (D) Apices of styles of *Hymeniacidon perlevis*. (E) Apex of tylostyle of *Suberites domuncula*. (F) Apex of "Mycalostyle" from *Mycale diversisigmata*.

or "styles") separates the two genera *Halichondria* and *Hymeniacidon* as two different genera, but in the same family the same features can be used to distinguish two species of a genus (*Ciocalypta penicilus* and *C. tyleri*). Since both types of spicules are found in the same species in the genus *Amorphinopsis*, the character "spicule type" looses its power to resolve phylogenetic relationships in this family.

Skeletal arrangements are likewise differently suitable as phylogenetic characters among taxa. For example, the genus *Higginsia* (Halichondrida: Desmoxyidae) possesses in its different species a very large range of choanosomal skeletal arrangements, which would be in other groups of sponges distinctive for even higher taxa.

In conclusion, if morphological systematics shall remain valuable for demosponge systematics, we have to do a better job judging taxon-wise which characters may be recruited. General character value estimations (e.g., Hajdu and Van Soest, 1996) for characters based on a large range of demosponge taxa are therefore misleading. An a priori comparison with (congruent) molecular phylogenies could provide the taxonomist insight in the distribution of the particular character among higher demosponge taxa and reveal potential homoplasies and "good" apomorphic characters.

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