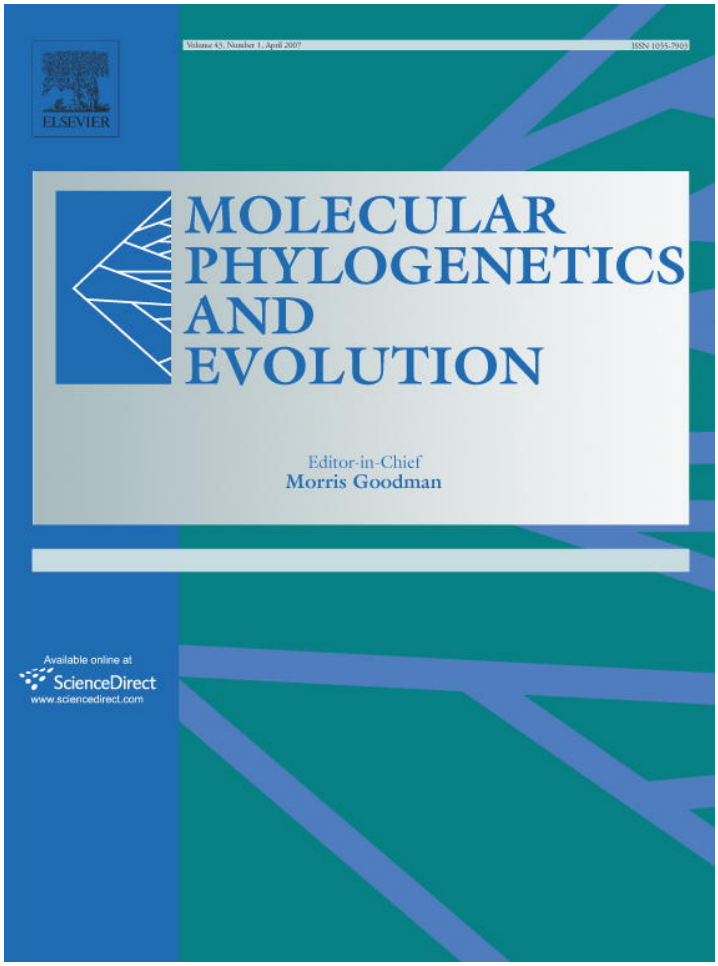

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Short communication

Reassessment of the classification of the Order Haplosclerida (Class Demospongiae, Phylum Porifera) using 18S rRNA gene sequence data

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1. Introduction

Classification and phylogeny reconstruction in the Porifera is difficult at all levels. The low number and potential plasticity of morphological characters have meant a precise taxonomical classification of this phylum continues to be problematical. Sponge spicule morphology and spongin fibre architecture have long been the main focus of sponge taxonomy. However, the difficulty in defining the range of a character has caused problems in employing these features to reconstruct robust phylogenies. Non-traditional characters such as the secondary metabolites present, molecular sequences and cytological and embryological characteristics, have been employed in more recent studies to address this problem (e.g. Boury-Esnault et al., 1994; van Soest and Braekman, 1999; McCormack et al., 2002; Borchiellini et al., 2004; Addis and Peterson, 2005; Erpenbeck et al., 2006) often contradicting many previously established classifications, which adds to the difficulty in determining the relationships between poriferan taxa.

Class Demospongiae is the largest and most variable sponge class, comprising about 85% of living species of which the Order Haplosclerida is a member. This order contains a large number of shallow-water marine sponges and all freshwater sponges, comprising the highest biodiversity of sponges in terms of habitat and species (van Soest and Hooper, 2002a). The Haplosclerida, as a group, have been well-described morphologically (e.g. van Soest, 1980; van Soest and Hooper, 2002a) but some higher-level definitions appear to be largely groupings of convenience, containing a large number of ancient and diverse sponges and the existing classification may not represent true evolutionary relationships. The major difficulty is that there is a very limited range of spicule types present in sponges of this order, megascleres include oxea or strongyloxeas and microscleres, if present, include sigmas, smooth toxas, microxeas or microstrongyles (van Soest and Hooper, 2002a), and despite the considerable range of architectures of the spicule and spongin skeleton, classification is difficult. In the most recent classification of the Haplosclerida, (van Soest and Hooper, 2002a) three suborders are recognised, Suborder Haplosclerina (families Callyspongiidae, Chalinidae and Niphatidae), Suborder Petrosina (families Calcifibrospongiidae, Petrosiidae and Phloeodictyidae), and Suborder Spongillina (families Spongillidae, Malawispongiidae, Metaniidae, Metschnikowiidae, Palaeospongillidae, Potamolepiidae and Lubomirskiidae). The latter suborder contains all the freshwater sponges while the other two contain only marine sponges.

Although the two marine suborders are closely related morphologically they are controversial higher taxa (van Soest and Hooper, 2002a). Originally, two groups of marine

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haplosclerids were divided into chalinid and renierid sponges based on the presence or absence of spongin in the skeleton, respectively (Schmidt, 1870). Topsent (1928) amalgamated these into one group but the families within continued to be recognised as chalinids and renierids. This continued until Bergquist (1980) proposed two orders (Haplosclerida and Nepheliospongida) based on reproductive and biochemical strategies even though they more or less covered the chalinid vs. renierid lines. In the current classification these two orders are joined into one Order Haplosclerida because of their shared unique chemistry (van Soest and Braekman, 1999) and similar spicule size and form (van Soest and Hooper, 2002a) but they now occupy the position of suborders. de Weerdt (1989), following a character analysis of the marine families had previously proposed this, but the morphological synapomorphies supporting these as suborders are vague and elusive, many of them being shared by sponges in other groups (van Soest and Hooper, 2002b,c).

The Suborder Spongillina contains exclusively freshwater sponges and it appears to be somewhat distantly related to the suborders Haplosclerina and Petrosina. There are seven families in this suborder and of the six extant families, three are geographically widespread (Metaniidae, Potamolepiae and Spongillidae) and three are endemic. The family Lubomirskiidae is found only in Lake Baikal in Siberia, Malawispongiidae are found in ancient lakes in the Middle East, African Rift Valley, Eastern Europe and Sulawesi while Metschnikowiidae sponges are found only in the Caspian Sea (Manconi and Pronzato, 2002). Gemmules, gemmoscleres, the arrangement of the skeletal network and microscleres are the main morphological characters used in freshwater classification (Manconi and Pronzato, 2002) but only four families produce gemmules thus the systematics of the remaining species may be problematical.

Recent molecular studies are not congruent with the currently accepted morphological classification. McCormack et al. (2002) employed the D3 region of 28S rRNA gene to study the relationships between 20 Haplosclerid taxa and the order, families and genera were found to be polyphyletic. No firm conclusions could be drawn from this study, as only one gene region was employed and this region is very variable in haplosclerid taxa. However, Borchiellini et al. (2004) and Nichols (2005) also found the order not to be monophyletic while the latter had support for the formation of a single monophyletic group containing the marine suborders. In this study, we examine the classification as hypothesised by Hooper and van Soest (2002) and test the monophyly of the Order Haplosclerida, the three suborders and five of the six families designated to suborders Haplosclerina and Petrosina, using 18S rRNA sequences from 32 haplosclerid sponges.

2. Materials and methods

The species included in this study were acquired on loan from the Zoological Museum Amsterdam and also from the National Institute of Water and Atmospheric Research in Auckland, New Zealand (details are listed in Table 1). Genomic DNA was extracted from samples held in 6 M guanidinium chloride solution by standard phenol–chloroform–isoamyl extractions followed by ethanol precipitation.

2.1. PCR amplification and sequencing

Initial attempts to amplify the complete 18S rDNA were not very successful probably due to the degraded nature of some of the DNA samples. Subsequently, the 18S rDNA gene was amplified and sequenced in three overlapping fragments (approx. 600, 900bp and 600 bp) after designing three pairs of haplosclerid-specific primers from a Haliclona sp. 18S rDNA sequence using the PrimerSelect program from the Lasergene package (DNASTAR Inc). All PCR and sequencing primers are described in Table 2. The 18S rRNA gene fragments were amplified in 50 μl reactions, which comprised 5 μl 10× PCR Buffer (Promega), 10 mM dNTPs (Promega), 1 μl DMSO (Finnzymes), 2 μM primers and 1 U *Taq* polymerase (Promega). The temperature regime was an initial denaturation of 94 °C for 5 min followed by 30 cycles of 1 min at 94 °C, 30 s at annealing temperature (between 38 and 48 °C depending on specimen) and 1 min at 72 °C. A final extension step of 5 min at 72 °C finished the regime. PCR products were gel purified on a 1% Seakem agarose gel (BioWhittaker Molecular Applications) and automatically sequenced in both directions. Sequences were assembled into contigs using the SeqMan II software from the Lasergene package (DNASTAR Inc.) and edited by eye in MacClade 4.0 (Maddison and Maddison, 1998). The fully edited consensus sequence was entered into a BLAST algorithm (www.ncbi.nlm.nih.gov) search to check for possible contamination.

2.2. Phylogenetic analysis

Sequences were retrieved from a representative of each of the three putative haplosclerid suborders (one freshwater sponge family and from five of the six other haplosclerid families). 18S rRNA sequence data from 98 sponge species were downloaded from GenBank, including all those that were complete or almost complete. These included eight calcarean, five homoscleromorph, two hexactinellid and four cnidarian sequences to explore suitable outgroups. The homoscleromorph and hexactinellid sequences were subsequently excluded due to their unstable position, as were a number of demosponge sequences due to being too short, identical to other sequences or where the identification of the specimen was known to be incorrect. Details of all sequences included in the final analyses are also listed in Table 1. The final alignment had a total of 86 sequences, of which 32 were haplosclerids, and was a total of 2044 bp in length. A conservative alignment strategy was employed where all positions that could not be reliably aligned were excluded

Table 1
List of specimens used in this study including geographical origin, GenBank Accession and voucher numbers and author

Classification	Origin	Accession/voucher Nos.	Author
Phylum Porifera			
Class Demospongiae			
Order Haplosclerida			
Suborder Haplosclerina			
Family Callyspongiidae			
Callyspongia (*Euplacella*) sp.	PNG	DQ927314/NIWAKD1668	This study
Callyspongia sp.	Oman	DQ927310/POR14635	This study
Siphonochalina sp.	Oman	DQ927311/POR14630	This study
Family Chaliniidae			
Chalinula hooperi	Indonesia	DQ927319/POR17651	This study
Haliclona (*Reniera*) *cinerea*	France	DQ927306/POR14138	This study
Haliclona (*Haliclona*) *oculata*	France	DQ927307/POR14116	This study
Haliclona (*Haliclona*) *oculata*	GenBank	AY734450	Direct submission
Haliclona (*Reniera*) *fascigera*	Micronesia	DQ927315/NIWAKD150	This study
Haliclona (*Halichoclona*) sp.	Bahamas	DQ927309/NIWAKD550	This study
Haliclona sp. A	GenBank	AJ703889	Direct submission
Haliclona sp. B	GenBank	AY73444	Direct submission
Haliclona amphioxa	GenBank	AJ703887	Direct submission
Haliclona (*Reniera*) *mediterranea*	GenBank	AY348879	Borchiellini et al. (2004)
Family Niphatidae			
Cribochalina vasculum	Bahamas	DQ927308/NIWAKD538	This study
Dasychalina fragilis	Indonesia	DQ927316/POR14455	This study
Niphates sp.	Micronesia	DQ927312/NIWAKD148	This study
Suborder Petrosina			
Family Petrosiidae			
Acanthostrongylophora ingens	Indonesia	DQ927318/POR17500	This study
Petrosia sp. A	PNG	DQ927321/NIWAKD1020	This study
Petrosia sp. B	PNG	DQ927320/NIWAKD1068	This study
Xestospongia muta	GenBank	AY621510	Direct submission
Family Phloeodictyidae			
Aka mucosa	Bahamas	DQ927322/NIWAKD1068	This study
Calyx sp.	PNG	DQ927313/NIWAKD1132	This study
Oceanapia sp.	Bahamas	DQ927317/NIWAKD586	This study
Suborder Spongillina			
Family Lubomirskiidae			
Baikalospongia bacillifera	GenBank	DQ176775	Addis and Peterson (2005)
Family Metaniidae			
Corvomeyenia sp.	GenBank	DQ176774	Addis and Peterson (2005)
Family Spongillidae			
Nudospongilla sp.	Tanzania	DQ927323/POR16913	This study
Ephydatia fluviatilis	GenBank	AY578146.1	Direct submission
Ephydatia muelleri	GenBank	AF121110	Direct submission
Eunapius fragilis	GenBank	AF121111	Direct submission
Spongillina lacustris	GenBank	AF121112	Direct submission
Trochospongillina horrida	GenBank	AY609320	Direct submission
Trochospongilla pennsylvanicas	GenBank	DQ087507	Direct submission
Order Dictyoceratida			
Dysidea avara	GenBank	AF456326.1	Direct submission
Dysidea sp.	GenBank	AY734449	Direct submission
Ircinia felix	GenBank	AY734448	Direct submission
Sponia officinalis	GenBank	AY348888.1	Borchiellini et al. (2004)
Order Halichondrida			
Axinella corrugata	GenBank	AY737637	Direct submission
Axinella damicornis	GenBank	AY348887.3	Borchiellini et al. (2004)
Axinella polypoides	GenBank	APU43190	Direct submission
Dictyonella incisa	GenBank	AY348880	Borchiellini et al. (2004)
Halichondria melanodocia	GenBank	AY737639	Direct submission
Pseudoaxinella lunaecharta	GenBank	AY734442	Direct submission
Ptilocaulis gracilis	GenBank	AY737638	Direct submission
Scopalina ruetzleri	GenBank	AJ621546	Direct submission
Spongosorites genitrix	GenBank	AY348885.1	Borchiellini et al. (2004)

Table 1 (*continued*)

Classification	Origin	Accession/voucher Nos.	Author
Order Poecilosclerida			
Crella elegans	GenBank	AY348882	Borchiellini et al. (2004)
Iotrochota birotulata	GenBank	AY737641	Direct submission
Mycale fibrexilis	GenBank	AF100946	Collins (1998)
Mycale sp.	GenBank	AY737643	Direct submission
Phorbas tenacior	GenBank	AY348881	Borchiellini et al. (2004)
Tedania ignis	GenBank	AY737642	Direct submission
Order Verongida			
Aiolochroia crassa	GenBank	AY591805.1	Schmitt et al. (2005)
Aplysina aerophoba	GenBank	AY591799.1	Schmitt et al. (2005)
Aplysina archeri	GenBank	AY591801.1	Schmitt et al. (2005)
Aplysina cavernicola A	GenBank	AY348875.1	Borchiellini et al. (2004)
Aplysina cavernicola B	GenBank	AY591800.1	Schmitt et al. (2005)
Aplysina lacunosa 'hard'	GenBank	AY591802.1	Schmitt et al. (2005)
Aplysina lacunosa 'soft'	GenBank	AY591803.1	Schmitt et al. (2005)
Hexadella pruvoti	GenBank	AY348877.1	Borchiellini et al. (2004)
Verongula gigantea	GenBank	AY591804.1	Schmitt et al. (2005)
Order Agelasida			
Agelas clathrodes	GenBank	AY769087	Direct submission
Agelas conifera	GenBank	AY734443	Direct submission
Agelas dipar	GenBank	AY737640	Direct submission
Agelas oroides	GenBank	AY348886.2	Borchiellini et al. (2004)
Order Astrophorida			
Geodia cydonium	GenBank	AY348878.1	Borchiellini et al. (2004)
Geodia neptuni	GenBank	AY737635	Direct submission
Order Hadromerida			
Chondrosia reniformis	GenBank	AY348876.1	Borchiellini et al. (2004)
Spheciospongia vesparium	GenBank	AY734440	Direct submission
Suberites domuncula	GenBank	AJ620112	Direct submission
Suberites ficus	GenBank	AF100947	Collins (1998)
Tethya actinia	GenBank	AY878079	Lavrov et al. (2005)
Order Spirophorida			
Cinachyrella sp.	GenBank	AY734439	Direct submission
Tetilla japonica	GenBank	D15067.1	Direct submission
Order Lithistida			
Corallistes sp.	GenBank	AY737636	Direct submission
Phylum Porifera			
Class Calcarea			
Anamixilla sp.	GenBank	AF182192	Direct submission
Baeria nivea	GenBank	AF182191	Borchiellini et al. (2001)
Clathrina cerebrum	GenBank	U42452	Direct submission
Grantiopsis sp.	GenBank	AF182193	Direct submission
Leucetta sp.	GenBank	AY737644	Direct submission
Leucosolenia sp.	GenBank	AF100945	Collins (1998)
Petrobiona massiliana	GenBank	AF260902	Direct submission
Sycon calcaravis	GenBank	D15066.1	Direct submission
Phylum Cnidaria			
Antipathes galapagenesis	GenBank	AF100943	Collins (1998)
Hydra circumcincta	GenBank	AF358080	Medina et al. (2001)
Montastraea franksi	GenBank	AY026382	Medina et al. (2001)
Nectopyramis sp.	GenBank	AF358068	Medina et al. (2001)

The prefix NIWAKD corresponds to specimens that are part of Michelle Kelly's Porifera collection held at NIWA, Auckland, NZ and the prefix POR refers to specimens received from the Porifera collection at the Zoological Museum of Amsterdam. PNG, Papua New Guinea.

from further analyses (i.e. 0–36, 109–116, 158–163, 172–184, 216–259, 289–292, 671–762, 795–80, 838–853, 898–907, 944–1001, 1234–1243, 1353–1357, 1553–1628, 1653–1665, 1754–1759 and 1954–1993). The final alignment contained 1595 characters. The sequences obtained during this study are available in GenBank (Accession Nos. DQ927306–DQ927323) and alignments are available from the author upon request.

Table 2
PCR and sequencing primers

Primer name	Sequence 5′–3′	Primer use
18S rRNA forward primers		
1F18S	AAC CTG GTT GAT CCT GCC AGT	PCR and sequencing
210F18S	TTA GAT CCA AAA CCA ATG	Sequencing
400F18S	CCT GAG AAA CGG CTA CCA CA	PCR and sequencing
560F18S	GAG GAA CAA TTG GAG GGC	Sequencing
830F18S	TTC GGG ACG TTT ACT TTG	Sequencing
1200F18S	TAA TTT GAC TCA ACA CGG G	PCR and Sequencing
18S rRNA reverse primers		
360R18S	GGG CAG AAA CTT GAA TGA AC	Sequencing
600R18S	CGA GCT TTT TAA CTG CAA	PCR and Sequencing
1350R18S	CGG GAC TAG TTA GCA GGT TAA	PCR and Sequencing
1800R18S	GTT CAC CTA CYG AAA CCT TGT T	PCR and Sequencing

An appropriate model of evolution (TrN + I + G model with base frequencies A = 0.24, C = 0.21, G = 0.30, T = 0.25; R(A–G) = 0.272, R(C–T) = 5.7, other rates = 1; I = 0.42; G = 0.57) was chosen by the implementation of the hierarchical likelihood ratio test (Huelsenbeck and Crandall, 1997) in MODELTEST3.06 (Posada and Crandall, 1998). The reconstruction of phylogenetic hypotheses employing maximum parsimony (MP) and bootstrap resampling (1000 replicates) were carried out using PAUP* 4.0b10 (Swofford, 1998), while maxim likelihood (ML) analysis and bootstrapping (1000 replicates) was carried out using DPRml, a parallelised version of ML, (Keane et al., 2005) employing 40 desktop processors. Tree searches were carried out using a heuristic search strategy, where a total of ten random addition sequence replicates were performed, with the tree-bisection-reconnection (TBR) branch swapping option in effect. Tests for saturation were carried out using DAMBE (Xia and Xie, 2001) and no saturation was detected in the dataset. Topological constraints were enforced upon the 18S rRNA conservative dataset, and probability scores of alternative topologies were estimated using the Shimodaira–Hasegawa test (Shimodaira and Hasegawa, 1999). These constraints were consistent with the hypotheses of the current classification that the order and each of the suborders, families and genera were all monophyletic.

3. Results

Two trees were reconstructed (under ML and MP) that differed only between taxa in the unresolved parts of the topologies and the ML topology is presented in Fig. 1. This topology indicated that both the Class Calcarea and Class Demospongiae were monophyletic when the four Cnidarian sequences were employed as outgroups. Within this Demospongiae clade the freshwater haplosclerids were removed from the clade containing the marine haplosclerids rendering the Order Haplosclerida polyphyletic. The freshwater haplosclerids (Suborder Spongillina) formed a single clade that was a sister to a large clade containing representatives of seven other demosponge orders. Resolution within the freshwater sponge clade was poor but *Corvomeyenia* sp. was basal followed by the pairing of two species of *Trochospongilla*. While *Eunapius fragilis* and *Nudospongilla* sp. grouped together so too did *Ephydatia fluviatilis*, *E. muelleri* and *Baikalospongia bacillifera*. The halichondrid sequence *Scopalina ruetzleri* was positioned alone and basal to the freshwater clade but with high bootstrap support (Fig. 1). The sister clade to the freshwater sponges (and *Scopalina*) was not highly supported and contained sequences from the Agelasida, Astrophorida, Hadromerida, Halichondrida, Lithistida, Poecilosclerida and Spirophorida. Each of the orders Agelasida, Poecilosclerida and Spirophorida were monophyletic, while neither the orders Hadromerida nor Halichondrida formed discrete groups. There was only one representative of the lithistids included and this sequence grouped with *Geodia cydonium* and *Geodia neptuni.* All nine representatives of the Order Verongida and a sequence from *Chondrosia reniformis* (AY348876) formed a highly supported clade, 100BP, (ML and MP) towards the base of the demosponges as did the four Dictyoceratid sequences.

The clade that included all of the marine haplosclerids was very highly supported (99/100BP; ML/MP) and contained two smaller clades. One clade contained two sequences from *Petrosia* and one from *Acanthostrongylophora* (all Suborder Petrosina), in addition to sequences from *Niphates* sp. and *Chalinula hooperi* (both Suborder Haplosclerina) and was supported by 100BP (ML and MP). Resolution within this clade was poor. The other clade (supported by 99/100BP; ML/MP) contained 14 members of the Suborder Haplosclerina and three members of the Suborder Petrosina (*Oceanapia* sp, *Calyx* sp. and *Xestospongia muta*). The two *Callyspongia* and eight (of the nine) *Haliclona* sequences grouped in a supported clade with *Siphonochalina* sp. and *Calyx* sp. (Fig. 1). Both *Haliclona oculata* sequences grouped together with 93BP (ML), and were found basal to a small clade containing *Haliclona* sp. A, *Haliclona cinerea, Haliclona fascigera, Siphonochalina* sp. and *Calyx* sp. The relationships of *Cribochalina vasculum*, *Dasychalina fragilis*, *Oceanapia* sp. and *X. muta* were undetermined within this clade. *Aka mucosa* was positioned alone between the two clades mentioned above and none of the marine families represented formed distinct clades.

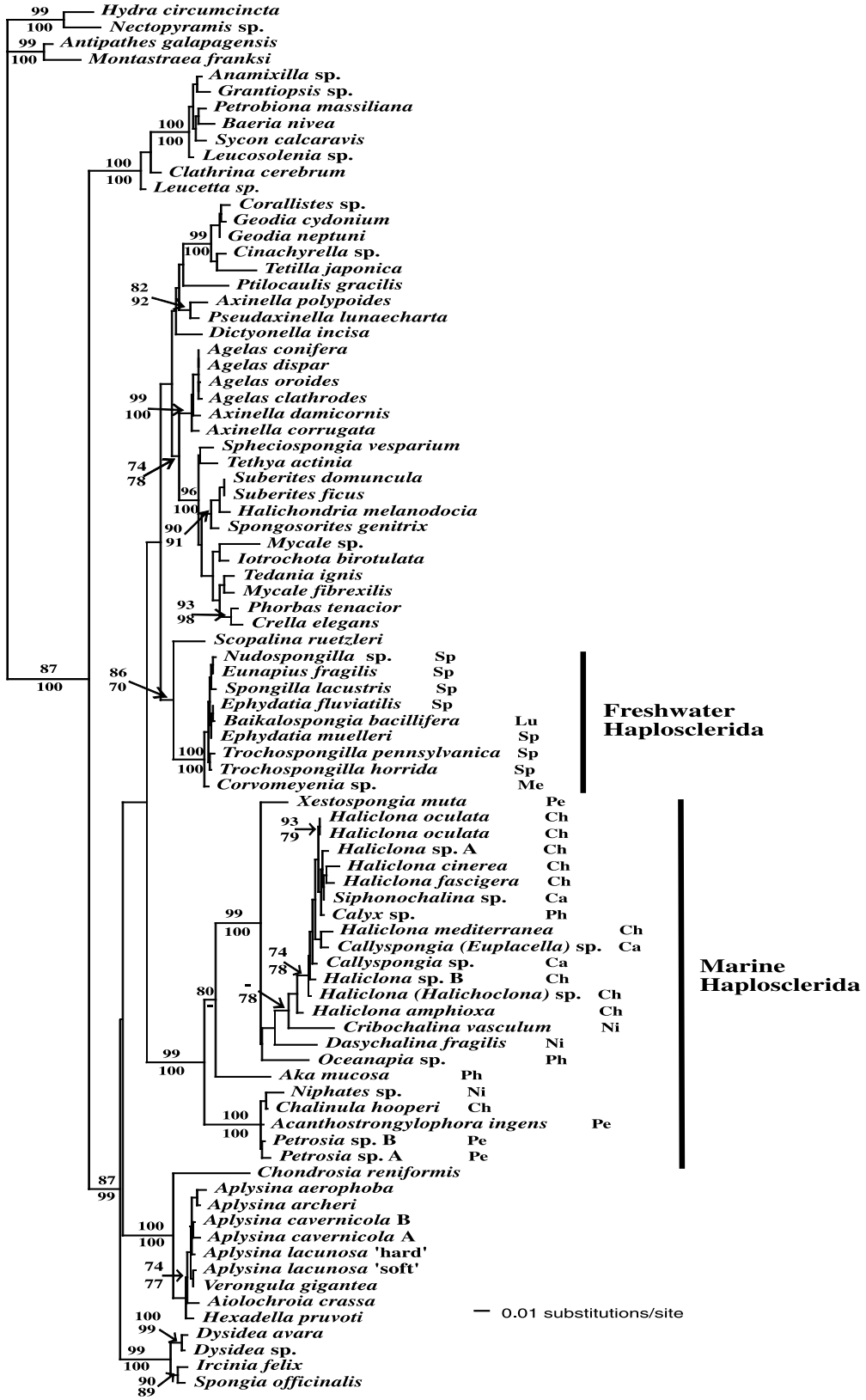

Fig. 1. ML phylogeny of 18S rRNA gene sequences. Numbers on branches correspond to bootstrap values, above the branches, ML and below the branches, MP. Only values above 70BP are shown, a '–' symbol indicates the value was less than 70BP for one of the two analyses. Some BP values of >70 BP were not included due to clarity (only those not relevant to this manuscript). Families: Ca, Callyspongiidae; Ch, Chalinidae; Ni, Niphatidae; Pe, Petrosiidae; Ph, Phloeodictyidae; Lu, Lubomirskiidae; Me, Metaniidae; Sp, Spongillidae.

Constraint analysis indicated that trees containing a monophyletic order, each suborder, or families Chalinidae, Niphatidae and Phloeodictyidae were significantly worse than the best tree. Although the families Callyspongiidae and Petrosiidae, and the genera *Callyspongia* and *Haliclona* were all polyphyletic in the molecular phylogeny, forcing each of these to be monophyletic was not significantly worse than the best tree. Branch lengths at that level in the tree are very short.

Many of the 18S rRNA gene sequences isolated from the marine haplosclerids contained large insertion sequences that were not present in any other sponge taxa. All the sequences containing insertions fell into a single clade (the larger of the haplosclerid clades, containing *Haliclona* and *Callyspongia* etc.) and were not present in the *Aka*, *Niphates*, *Chalinula*, *Acanthostrongylophora* or *Petrosia* sequences examined. These insertion sequences were not included in the analysis presented above. However, analysis of the data when such areas were included did not alter the evolutionary relationships postulated in Fig. 1.

4. Discussion

Order Haplosclerida contains a high diversity of sponge taxa. Taxonomy within the order is controversial as far as recognition, interpretation and definition of internal relationships is concerned. This work forms part of a larger project attempting to build a robust phylogeny of the order employing a number of gene regions and reviewing morphological criteria. The 18S rRNA gene data was employed to elucidate relationships at higher levels in the order, i.e. within and between families and suborders in an attempt to corroborate other data (e.g. McCormack et al., 2002). This study implies strong support for an alternative taxonomic scheme in relation to the order, all three suborders and many if not all of the families investigated and supports previous hypothesis drawn from molecular data.

Previous studies (McCormack et al., 2002; Borchiellini et al., 2004; Nichols, 2005) found the Order Haplosclerida polyphyletic. The former did not include the freshwater sponges in their analysis and the polyphyly is thought to be due to the presence of contaminating sequences. However, Borchiellini et al. (2004) showed that the freshwater Haplosclerida appeared as a sister-group to a clade that did not include the marine haplosclerids but contained members from seven other Demosponge orders including the Agelasida, Hadromerida, Halichondrida and Poecilosclerida, a result reflected in our study. In Nichols (2005) the freshwater haplosclerid LSU sequence from *Spongilla lacustris* grouped with the halichondrid *Scopalina ruetzleri* (a relationship also found in this analysis) and the hadromerid *Timea lowchoyi*. These molecular data strongly suggest that the freshwater sponges should be removed from this order. The relationships between the freshwater sponges were similar to those shown in Addis and Peterson (2005) in that the *Corvomeyenia* sp. sequence was the most basal followed by the *Trochospongilla* sequences. The addition of a *Nudospongilla* sequence as part of this work was found to alter the relationship between *Spongilla lacustris* and *Eunapius fragilis* from that shown in Addis and Peterson (2005). The *Nudospongilla* sequence grouped with the *E. fragilis* sequence. However branch lengths are very short at this level in the phylogeny and the addition of further freshwater sponge sequences will likely further alter the relationships within the freshwater sponge clade.

Sequences from members of the suborders Haplosclerina and Petrosina were interspersed in two separate clades that together formed one large marine haplosclerid group that was highly supported by bootstrapping. This pattern supports that which was produced by McCormack et al. (2002), who employed partial 28S rDNA sequences to reconstruct a haplosclerid phylogeny. Furthermore, in both studies *Haliclona* and *Callyspongia* sequences fell in one clade, while *Niphates* and *Acanthostrongylophora* sequences amongst others, fell in another. Between these two clades an *Aka* sequence was positioned on its own. This same pattern occurred in the two different studies even though two different gene regions, and different species of each genus, were employed. At the family level none of the families grouped together in individual clades. In Systema Porifera (Desqueyroux-Faúndez and Valentine, 2002) the history of the family Phloeodictyidae is described as being a "relative mess". Thus, it may not be surprising that two representatives of this family (*Oceanapia* sp. and *Calyx* sp.) were positioned with the members of the genera *Callyspongia*, *Haliclona* and *Siphonochalina* and the third representative from this family, *A. mucosa*, was positioned basal to this clade. Also, in the constraint analysis, it was significantly worse to force a monophyletic Phloeodictyidae. Three members of the family Petrosiidae grouped together with a Niphatidae and a Chalinidae and although the two *Petrosia* species did group together the family was not monophyletic as would be expected.

Despite the low-resolution at this level within the 18S rDNA gene tree, the pattern of relationships casts some doubt on the monophyly of the genus *Haliclona* (>70BP places *H. amphioxa* outside a clade containing *Haliclona* and *Callyspongia* sequences amongst others). McCormack et al. (2002) indicated that both *Callyspongia* and *Haliclona* might be polyphyletic. de Weerdt (2002) reorganized the hierarchies of the family Chalinidae reducing the number of genera. Molecular evidence may suggest that more diversity is present in the family than is currently allowed for in the classification of the order. The 18S rRNA gene is not suitable to accurately determine relationships between closely related species and additional molecular data (e.g. mitochondrial genes) is necessary.

The haplosclerid species that had insertions in the SSU in this study (e.g. members of the genera *Callyspongia* and *Haliclona* and not Niphatidae and *Acanthostrongylophora*) were similar to those that showed insertions in their LSU in Erpenbeck et al. (2004a) perhaps highlighting further the similar evolutionary patterns in the two ribosomal genes. Recent work suggested that the Haplosclerida LSU D3

structure has a higher substitution rate and displays greater variability to that of other demosponges (Erpenbeck et al., 2004a). In addition, the putative secondary structure of the LSU D3 region in haplosclerids displays major differences to those of other demosponges (Erpenbeck et al., 2004a) due to the insertion sequences, a pattern similar to that found in this work. Insertions in the SSU have been reported in various organisms including protists (Busse and Preisfeld, 2002) and platyhelminthes (Picón et al., 1996; Liu et al., 1997). The latter study demonstrated that the region with the insertion (which was 210 nt in length) still formed a stable single helix despite the sequence divergence. Thus, it may be possible that the insertion sequences found in the haplosclerid sponges do not substantially affect the secondary structure but the functional implications of these insertions are still unclear. However, the presence of these insertion sequences, and their size differences and nucleotide complement in some haplosclerid taxa may be strong synapomorphies supporting an alternative taxonomic scheme.

In conclusion, the SSU data presented here support the monophyly of two novel clades of marine haplosclerids that do not correspond to the existing taxonomic classification. The monophyly of the suborder Spongillina is supported but its inclusion in the Order Haplosclerida must now be questioned considering its close relationship with other demosponge orders.

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