J. Mar. Biol. Ass. U.K. (2007), **87**, 1577–1584
Printed in the United Kingdom

doi: 10.1017/S0025315407058341

Mitochondrial Cytochrome oxidase 1 phylogeny supports alternative taxonomic scheme for the marine Haplosclerida

Jean Raleigh*, Niamh E. Redmond*, Emma Delahan[†], Seamus Torpey[‡], Rob W.M. van Soest[ʃ], Michelle Kelly[§] and Grace P. McCormack*[¶].

*Molecular Evolution and Systematics Laboratory, Department of Zoology, Martin Ryan Institute, National University of Ireland, Galway, Ireland. [†]Department of Biology, National University of Ireland Maynooth, Maynooth, Co. Kildare, Ireland. [‡]Department of Information Technology, National University of Ireland, Galway, Ireland. [ʃ]Zoological Museum, University of Amsterdam, PO Box 94766, 1090 GT Amsterdam, The Netherlands. [§]National Centre for Aquatic Biodiversity and Biosecurity, National Institute of Water and Atmospheric Research, 269 Khyber Pass Rd., New Market, Auckland, New Zealand. [¶]Corresponding author, e-mail: grace.mccormack@nuigalway.ie

Recent molecular studies have shown that the sponge order Haploslcerida is polyphyletic as the freshwater sponges appear to be more closely related to other demosponges than they are to the marine haplosclerids. Within the marine haplosclerid clade relationships viewed via 18S and 28S rRNA gene phylogenies suggest that the suborders and many families and genera are also polyphyletic. However, both of these genes are on the same locus and do not evolve completely independently. We have analysed mitochondrial Cytochrome oxidase 1 gene fragments from 44 species of marine Haplosclerida and show conclusively that the classification of this group needs complete revision. Molecular data show a very complicated phylogeny supporting very few morphological hypotheses and little geographical pattern. However, the molecular data contain a great deal of phylogenetic signal at many taxonomic levels and support phylogenies drawn from the other genes.

INTRODUCTION

Sponges (Phylum Porifera), the earliest branching metazoans, have a simple body plan, are sessile and exclusively aquatic. Despite their simplicity the Porifera are very common, with approximately 7000 species currently described and an estimated additional 8000 undescribed species occupying the full range of depths in both fresh water and marine environments (Hooper & van Soest, 2002). The phylogenetic classification of the Porifera still remains an area of much debate and is in constant flux. To date, the main method of sponge classification has been the use of skeletal and gross morphological characters. However, the fact that not only is there a paucity of morphological characters available but that some can be highly plastic makes their use in classification problematical. Haplosclerid sponges, possibly the most successful order being the most diverse in terms of habitat and species number, pose a particular problem in this regard (van Soest & Hooper, 2002a; Erpenbeck et al., 2007). Recent molecular studies have suggested that the phylogenetic classification of the entire order needs to be re-evaluated (McCormack et al., 2002; Redmond et al., 2007).

Originally thought to comprise three suborders (Haplosclerina Topsent, 1928, Petrosina Boury-Esnault & Van Beveren, 1982 and Spongillina Manconi & Pronzato, 2002) molecular evidence suggests that the order Haplosclerida Topsent, 1928 is polyphyletic. Employing 18S rDNA and 28S rDNA three separate studies found that the freshwater sponges (suborder Spongillina) appeared as a sister clade to a group comprising members of the orders Hadromerida Topsent, 1894, Halichondrida Vosmaer, 1887 and Astrophorida Sollas, 1887 amongst others, rather than with the other haplosclerid suborders (Borchellini et al., 2004; Nichols, 2005; Redmond et al., 2007). According to Hooper & van Soest (2002) the two marine haplosclerid suborders, i.e. Haplosclerina and Petrosina, are recognizable and definable on the basis of skeletal architecture in addition to having different reproductive strategies (members of the Haplosclerina are viviparous while those in the suborder Petrosina are oviparous). However, both show many similarities in spicule form and size and also share a unique chemistry (van Soest & Braekman, 1999). McCormack et al. (2002) employing phylogenetic analysis of the D3–D5 region of the 28S rDNA initially suggested that these suborders and some of the families and genera were not monophyletic. Subsequently, two studies of relationships within the Demospongiae shed additional light on the marine Haplosclerida. Although only three haplosclerid 28S rRNA gene sequences were included in their work, Borchiellini et al. (2004) indicated that *Haliclona fulva* Topsent, 1893 grouped with *Petrosia ficiformis* Poiret, 1789 in preference to its con-gener *H. mucosa* Griessinger, 1971 although the relationships at this level in the tree were not supported. In the 28S rDNA phylogeny shown in Nichols (2005) high support was shown for a single marine haplosclerid clade containing 12 sequences. Within this clade were two groups each containing members of both marine suborders. As the

https://doi.org/10.1017/S0025315407058341 Published online by Cambridge University Press

Table 1. *Specimens examined, with voucher number, geographical origin and GenBank accession numbers (those in bold were produced for this work).*

Sample No.	Species	Geographical origin	Voucher no.	Accession no.
Order Haplosclerida				
Family Callyspongiidae de Laubenfels, 1936				
1	*Callyspongia plicifera* Lamarck, 1818	Curaçao	POR 14276	**EF655732**
3	*C. fallax* Duchassaing & Michelotti, 1864	Curaçao	POR 14314	**EF655712**
21	*C. pseudoreticulata* Desqueyroux-Faúndez, 1984	Indonesia	POR 14552	**EF655748**
32	*Callyspongia* sp.	Oman	POR 14597	**EF655718**
33	*Callyspongia* sp.	Oman	POR 14623	**EF655717**
34	*Callyspongia* sp.	Oman	POR 14635	**EF655706**
36	*Siphonochalina* sp.	Oman	POR 14630	**EF655725**
38	*Callyspongia* sp.	Oman	POR 14599	**EF655726**
39	*Callyspongia* sp.	Oman	POR14628	**EF655742**
42	*Callyspongia* aff. *carens*	USA	POR 16410	**EF655741**
93	*C. fallax*	USA	MKB219	**EF655716**
118	*C. rosa*	Papua New Guinea	MKB1031	**EF655729**
126	*Callyspongia* sp.	Papua New Guinea	MKB1618	**EF655735**
132	*Callyspongia* (*Euplacella*) sp.	Papua New Guinea	MKB1668	**EF655734**
133	*C. ramosa* Gray, 1843	New Zealand	MKB3091	**EF655714**
134	*C. ramosa*	New Zealand	MKB3092	**EF655707**
135	*C. (Euplacella) latituba* Dendy, 1924	New Zealand	MKB3093	**EF655715**
205	*C. siphonella* Lendenfeld, 1887	Egypt	POR16627	**EF655720**
211	*C. multiformis* Pulitzer-Finali, 1986	Caribbean	POR14294	**EF655749**
Family Chaliniidae				
5	*Haliclona cinerea* Grant, 1886	France	POR 14139	**EF655730**
6	*H. oculata* Pallas, 1766	France	POR 14116	**EF655743**
8	*H. cinerea*	France	POR 14138	**EF655738**
28	*H. vanderlandi* de Weerdt & van Soest, 2001	Indonesia	POR 14497	**EF655739**
56	*H. rudis* Topsent, 1901		POR 13247	**EF655711**
59	*H. toxius* Topsent,1897		POR 14642	**EF655727**
69	*Chalinula hooperi* Bakus & Nishiyama, 2000		POR 17651	**EF655724**
92	*H. fascigera* Hentschel, 1912	Micronesia	MKB150	**EF655733**
102	*Haliclona (Halichoclona)* sp.	Bahamas	MKB550	**EF655708**
124	*H. koremella* de Laubenfels, 1954	Papua New Guinea	MKB1097	**EF655719**
167	*H. vansoesti* de Weerdt, Kluiver & Gomez, 1999	Curaçao	POR14240	**EF655747**
	Haliclona xena de Weerdt, 1986	Netherlands	POR 17504	AF437300
	H. oculata	Netherlands	POR 17501	AY625661
Family Niphatidae				
10	*Dasychalina fragilis* Ridley & Dendy, 1886	Indonesia	POR 14455	**EF655740**
13	*Niphates* sp.	Indonesia	POR 14462	**EF655721**
97	*Amphimedon compressa* Duchassaing & Michelotti, 1864	Bahamas	MKB519	**EF655737**
114	*Cribochalina* sp.	Papua New Guinea	MKB1023	**EF655728**
115	*Gelliodes fibulata* Carter, 1881	Papua New Guinea	MKB1026	**EF655731**
	Amphimedon paraviridis Fromont, 1993	Sulawesi	POR 17685	AY625666
Family Petrosiidae				
16	*Petrosia hoeksemai* de Voogd & van Soest, 2002	Indonesia	POR 14474	**EF655722**
25	*P. hoeksemai*	Indonesia	POR 14517	**EF655723**
85	Petrosiidae sp.	New Zealand	KAH9907/48	**EF655710**
104	*Petrosia* sp.	Bahamas	MKB506	**EF655744**
111	*Petrosia* sp.	Fiji	MKB983	**EF655709**
112	*Petrosia* sp.	Papua New Guinea	MKB1020	**EF655746**
	Acanthostrongylophora ingens Thiele, 1899	Sulawesi	POR 17500	AY625667
Family Phloeodictyidae				
30	*Oceanapia* sp.	Indonesia	POR 14507	**EF655736**
109	*Oceanapia* sp.	Bahamas	MKB586	**EF655745**
423	Haplosclerina sp.		UCMPWC1082	**EF655713**
Order Hadromerida				
	Suberites suberia Montagu, 1818	North Sea	POR 9726	AF437295
	Suberites virgultosa Johnston, 1842	North Sea	POR 12969	AY625674

https://doi.org/10.1017/S0025315407058341 Published online by Cambridge University Press

Table 1. *(Continued.)*

Outgroup				
Order Halichondrida				
	Acanthella acuta Schmidt, 1862	Spain	POR 14589	AY625652
	Axinyssa ambrosia de Laubenfels, 1954	Curaçao	POR 14311	AY625658
	Axinyssa sp.	Sulawesi	POR 14501	AY625659
	Amorphinopsis siamensis Topsent, 1925	Gulf of Siam	POR 17672	AY625655
	Ciocalypta penicillus Bowerbank, 1862	France	POR 14111	AF437302
	Halichondria bowerbanki Burton, 1930	Netherlands	POR 17683	AF437299
	Halichondria panicea Pallas, 1766	France	POR 14125	AF437294
	Hymeniacidon perlevis Montagu, 1818	Netherlands	POR 17676	AY625662
	Topsentia halichondroides Dendy, 1905	Sri Lanka	POR 8450	AY625676
	Topsentia ophiraphidites de Laubenfels, 1934	?	?	AY62577

Nichols (2005) study did not focus on the Haplosclerida, unfortunately many of the haplosclerid specimens were not identified below suborder level. Furthermore, on the 28S rDNA phylogeny in Erpenbeck et al. (2006) an *Amphimedon paraviridis* Fromont, 1993 sequence (suborder Haplosclerina; family Niphatidae van Soest, 1980) clustered with an *Acanthostrongylophora ingens* Thiele, 1889 sequence (suborder Petrosina; family Petrosiidae van Soest, 1980) rather than with other Haplosclerina sequences from the family Chalinidae Gray, 1867 (two *Haliclona* sequences). This pattern was fully supported by Redmond et al. (2007) who employed the 18S rRNA gene to elucidate haplosclerid relationships at the family level and above. In that study the order, suborders and families Phloeodictyidae Carter, 1882, Niphatidae and Chalinidae did not form monophyletic groups. Therefore, it is clear that ribosomal DNA supports an alternative phylogeny within the order Haplosclerida.

It could be argued that both genes so far employed for the purposes of haplosclerid phylogeny are both on the same locus, evolve in a similar manner and therefore might not be independent markers. Thus it is important to examine the phylogeny by employing a totally independent marker. Fragments of the mitochondrial Cytochrome oxidase subunit 1 gene (CO1) have been extensively and successfully used to infer lower level phylogenies in higher metazoans, but this marker has only recently been employed in sponge systematics (e.g. Erpenbeck et al., 2002, 2006; Schroeder et al., 2003; Addis & Peterson 2005; Nichols, 2005). Being one of the loci of choice for population genetics studies this locus has been shown to evolve more slowly in sponges and thus may be informative at the species to family level (Duran et al., 2003; Wörheide et al., 2005). Erpenbeck et al. (2006) employed CO1 sequences to reconstruct a phylogeny of the Demospongiae, which was then compared to that reconstructed using 28S rDNA data and with morphological hypotheses. Their CO1 phylogeny also indicated problems with the current view of haplosclerid relationships. Despite the low numbers of haplosclerid sequences examined the family Chalinidae was not monophyletic and neither were the marine haplosclerid suborders.

In this paper we have employed the CO1 region to reconstruct a phylogeny of the Haplosclerida focusing on lower level relationships, testing the monophyly of genera and families. We show that the phylogeny reconstructed using mitochondrial data fully supports those produced using 18S and 28S rDNA data and also suggests that this locus contains signal to help elucidate relationships relatively deep in the phylogeny in addition to more recent relationships.

MATERIALS AND METHODS

Material

Samples were acquired on loan from the Zoological Museum Amsterdam (ZMA) and also from the National Institute of Water and Atmospheric Research in Auckland, New Zealand (NIWAKD). A list of the specimens included in the present study along with their authority, details of their geographical origin and their voucher numbers is given in Table 1. Specimens from the Netherlands (those with a POR voucher number) were primarily preserved in ethanol for varying lengths of time, many from the late 1990s. Those from NZ (vouchers with an MKB number) were preserved in 6M guanidinium chloride (GnCl) for approximately one year only. Specimens in ethanol were air-dried, chopped into very fine pieces in 8M GnCl buffer to which proteinase K was added at 20 mg/ml.

DNA extraction, PCR and sequencing

Total DNA was extracted from samples held in 8M/6M guanidinium chloride solution (Sigma) by standard phenol-chloroform-isoamyl extractions followed by ethanol precipitation. The PCR amplification of the 3' end of the CO1 gene (also known as the Erpenbeck fragment) were initially amplified using C1-J2165 (5'-GAAGTTTATATTTTAATTTTACCCC(AG T)GG-3') as forward primer and C-Npor 2760 (5'-TCTAGGTAATCCAGCTAAACC-3') as reverse primer, both universal metazoan primers (Erpenbeck et al., 2002). Haplosclerid specific primers were subsequently designed from resulting sequences. For the majority of specimens CO1F4 (5'-TGATTTTTTGGTCACCCTGAAGTTTA-3') and CO1R4 (5'-GCTACCTAGCGATATTCTGA-3') were successful, for some specimens one or other of the following two primer sets were employed, CO1F1

https://doi.org/10.1017/S0025315407058341 Published online by Cambridge University Press

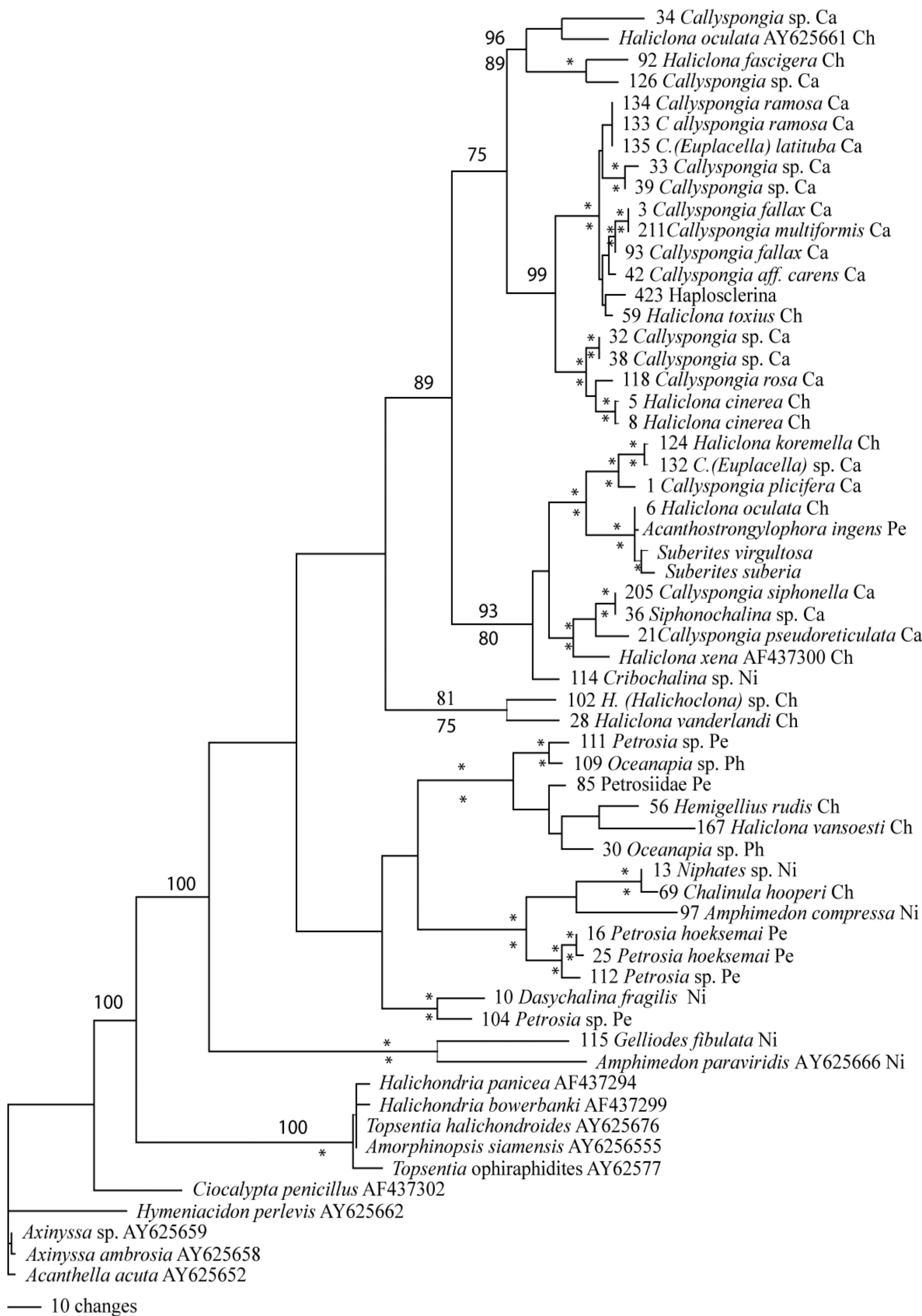

Figure 1. Maximum likelihood phylogeny of marine haplosclerid taxa reconstructed from mt CO1 gene data with halichondrid taxa employed as outgroups. The numbers on the branches represent bootstrap values. For clarity we did not attempt to show all bootstrap values on the tree. Instead where space is limited asterisks are provided where bootstrap values were over 75%. The number preceding the species name is the laboratory number, which is also listed in Table 1 and will accompany the sequence in GenBank. The letters after the species name refer to the family (Ca, Callyspongiidae; Ch, Chaliniidae; Pe, Petrosiidae; Ph, Phloeodictyidae; Ni, Niphatidae).

(5'-AATTTTACCGGGGTTGG-3') with CO1R1 (5'-GCATTTTTTAGGTTTAGCTGG-3') and the forward primer CO1F2 (5'-CCGGGGTTT(GT)ATG(AG)T-3') with CO1R2 (5'-AAC(CT)TTTTTT(CT)CC(GT)CAGCA-3'). The PCR amplification was carried out on a Techne™ Thermocycler in 50 µl reactions, which comprised 5 µl 10× PCR buffer (Promega), 10 mM dNTPs (Promega), 2 mM $MgCl_2$, 2 µM primers, 1 unit of Taq Polymerase (Promega)

https://doi.org/10.1017/S0025315407058341 Published online by Cambridge University Press

and 2–4 µl of DNA template. A step-up protocol (after Folmer et al., 1994) was employed which involved an initial denaturation of 94°C for 10 mins followed by 5 cycles of 94°C for 1 min, 45°C for 1.5 min and 72°C for 1.5 min; this was followed by 35 cycles of 93°C for 1 min, 50°C for 1.5 min and 72°C for 1.5 min. The PCR products were gel purified on a 1% Seakem agarose gel (BioWhittaker Molecular Applications). Purified DNA was automatically sequenced in both directions, assembled into contigs using the SeqMan II software from the Lasergene package (DNASTAR Inc.) and edited by eye in MacClade v. 4.0 (Maddison & Maddison, 1998). To ensure all sequences generated were of poriferan origin the fully edited consensus sequence was then entered into a BLAST algorithm (www.ncbi.nlm.nih.gov). Resulting sequences have been placed in GenBank (Accession numbers EFF655706–EFF655749) and will also be deposited in the sponge barcoding website (http://www.spongebarcoding.org).

Phylogenetic analysis

Forty-four sequences (556 bp in length) were retrieved from a representative from five of the six marine haplosclerid families of both suborders (Petrosina and Haplosclerina). Initially all available additional poriferan sequences (39) of the same region of the CO1 gene were downloaded from GenBank and aligned to those retrieved from this study in an attempt to identify suitable outgroup sequences. Two substitution models (TVM+I+G and GTR+I+G) were identified as being optimal for the CO1 data by the implementation of the hierarchical likelihood ratio test and the Akaike information criterion respectively in MODELTEST 3.7 (Posada & Crandall, 1998). Phylogenetic reconstructions with bootstrap resampling (1000 replicates), were performed under both models using maximum likelihood as implemented in DPRml (Keane et al., 2005) and under neighbour-joining as implemented in PAUP* 4.0b10 (Sinauer Assoc. Inc.). No single clade emerged from this analysis as being the closest sister group to the marine haplosclerids. A monophyletic group of halichondrid sequences were employed as outgroups to the marine haplosclerid clade due to their stability under analyses and the majority of the remaining poriferan sequences excluded from analysis (two hadromerid sequences remained as discussed below). Model selection and phylogenetic reconstruction was repeated for the smaller alignment (60 sequences) as described above. Genetic distances were estimated using PAUP* 4.0b10 under the TVM+I+G substitution model.

RESULTS

Phylogenetic reconstructions under maximum likelihood analysis with both substitution models were identical and that reconstructed employing the TVM+I+G model is shown in Figure 1. Bootstrap support was strong deep within the tree (supporting the monophyly of the marine haplosclerids when halichondrid sequences were employed as outgroups) and was also strong toward the tips of the tree, supporting small groups of sequences. Support was weaker towards the centre of the tree where some larger groups of sequences were not supported. Sequences from the marine haplosclerids were separated into a number of smaller groups, none of which conformed to the suborders Haplosclerina or Petrosina. One clade with 89 BP (bootstrap proportions) into which the majority of the sequences fell (32 taxa) was dominated by taxa of the suborder Haplosclerina but also contained a sequence from *Acanthostrongylophora ingens*. This large clade was subdivided into a number of smaller subclades that were also highly supported but contained a mixture of species from different genera. Additional sequences from other members of this suborder (e.g. species of *Niphates*, *Haliclona*, *Chalinula*, *Amphimedon* etc.) fell outside this clade.

The majority of representatives of the suborder Petrosina (families Petrosiidae and Phloeodictyidae) fell into an unsupported clade, which consisted of two highly-supported subclades. Members of the families Niphatidae and Chalinidae (suborder Haplosclerina) also grouped within one of the subclades. Basal to this large group *Dasychalina fragilis* (Niphatidae) and *Petrosia* sp. (Petrosiidae) grouped together with high bootstrap support. *Gelliodes fibulata* and *Amphimedon paraviridis* (Niphatidae) grouped together with 89 BP at the base of the rest of the marine Haplosclerids, while the exact position of two *Haliclona* sequences (no. 102 and no. 28) were undetermined.

As indicated above many of the haploslcerid families (i.e. Niphatidae, Phloeodictyidae and Petrosiidae), were shown on this phylogeny to be polyphyletic. Furthermore, because *Haliclona* sequences were dispersed among *Callyspongia* sequences, family Callyspongiidae was also polyphyletic (represented by many *Callyspongia* sequences as well as a *Siphonochalina* sequence). Family Chalinidae was also polyphyletic with some *Haliclona* sequences and a *Chalinula* sequence grouping with *Petrosia* sequences well away from the majority of other *Haliclona* sequences. The phylogeny in Figure 1 also indicated serious problems with the current classification at the genus level. *Haliclona* sequences were dispersed throughout the entire haplosclerid phylogeny. Sequences from *Callyspongia* fell into three separate groupings each supported by >80 BP and each contained at least one *Haliclona* sequence and one group also contained two sequences from the family Niphatidae. It is also of interest to look at how similar or dissimilar some sequences actually are from one another. While the genetic distance between all marine haplosclerid sequences ranged from zero to 0.6 substitutions per site (mean 0.2, standard deviation 0.1), the genetic distances between sequences from a single genus, i.e. *Haliclona* were relatively large ranging from zero to 0.3 (mean 0.15, standard deviation 0.1).

In almost all instances where two sequences from different voucher specimens of the same species were generated, i.e. *Callyspongia ramosa* (no. 133, no. 134), *Haliclona cinerea* (no. 5, no. 8) and *Petrosia hoeksemai* (no. 16, no. 25) these grouped together on the tree with high bootstrap support and for the first two species the sequences were identical. In the case of *C. fallax* (no. 3, no. 93) although the two sequences did not group together they were positioned very close to each other. One *C. fallax* sequence was identical to a sequence from *C. multiformis*, which is considered to be a synonym of *C. fallax*. Other identical sequences included the *C. ramosa* sequences and a *C.* (*Euplacella*) *latituba* sequence, a *Siphonochalina* sp. sequence with a *C. siphonella* sequence and two specimens of a 'purple-grey' *Callyspongia* species. However, two sequences

https://doi.org/10.1017/S0025315407058341 Published online by Cambridge University Press

from the species *Haliclona oculata*, one generated by the current study (no. 6) and one (AY625661) downloaded from GenBank, were positioned far apart from each other on the tree. The *H. oculata* from the present study clustered in a highly supported (100 BP) group with a sequence from *Acanthostrongylophora ingens* (Petrosiidae) and two *Suberites* sequences (*S. suberia*, *S. virgultosa*) from the order Hadromerida, which were also downloaded from GenBank. Furthermore, the genetic distance between the *H. oculata* sequence and the *A. ingens* sequences was very similar to that between the two *P. hoeksemai* and two *H. cinerea* sequences at <0.005 substitutions per site per year. In contrast the genetic distance between the two *H. oculata* sequences was 0.15, which is equal to the mean genetic distance between all *Haliclona* sequences.

DISCUSSION

The current classification of the Order Haplosclerida, comprises three suborders and 13 families. Recent investigations, which employed molecular data suggested strongly that the order should be limited to two suborders both of which are marine (e.g. Borchellini et al., 2004; Nichols, 2005; Redmond et al., 2007). The affinities of the freshwater sponges have not yet being elucidated. While almost certainly not belonging to the order Haplosclerida they form a sister group to a large clade of sponges from many other orders and so far do not show affinity for one over the other (Borchiellini et al., 2004; Nichols, 2005; Redmond et al., 2007). Indeed the exact relationships of the marine haplosclerids are also undetermined. In a search for a suitable outgroup for this analysis all CO1 sequences available in GenBank were included and none showed a particularly close relationship to the marine haplosclerida. Many other sponge orders have been shown not to be monophyletic, e.g. Halichondrida, Axinellida and Lithistida (Kelly-Borges & Pomponi, 1994; Alvarez et al., 2000; Erpenbeck et al., 2002). Furthermore, it is also now generally accepted that the Demosponge subclasses Tetractinomorpha and Ceractinomorpha should no longer be used due to several phylogenetic studies that suggest neither subclass is monophyletic (e.g. Lafay et al., 1992; Borchiellini et al., 2001, 2004). Thus the structure of higher-level relationships within the Demospongiae is still elusive.

Molecular systematics is gaining popularity in the sponge research community to help address questions in poriferan classification. So far results have been mixed with phylogenetic reconstructions from molecular data sometimes being far removed from those expected according to morphological characters (e.g. this study). Without doubt additional molecular data are highly desirable; from more genes to corroborate the phylogenies reconstructed from a single gene region and from more taxa to fill in gaps that have newly emerged. Perhaps the most common loci of choice to date for sponge molecular systematics have been the 18S and the 28S rRNA genes. While the mt CO1 gene was originally employed for close relationships at the population level and above (e.g. Avise, 2000) recent research has shown that this gene evolves more slowly in sponges than in other animal groups (Duran et al., 2003; Wörheide et al., 2005). Thus this locus is informative at genus and family levels. Here we contribute 44 mt CO1 gene sequences from a single sponge order, more than doubling the number of sequences of that region available in GenBank. The data shown confirm our previous work with both the 18S and 28S rRNA genes and show that problems in haplosclerid systematics are very serious with re-evaluation necessary at all taxonomic levels within the order.

According to Desqueyroux-Faúndez & Valentine (2002) the secondary ectosomal reticulation described by de Laubenfels (1936) should be sufficient to separate the Callyspongiidae from the Chalinidae, a view that was also shared by Bergquist & Warne (1980). Wiedenmayer (1977) found this character to be unsuitable as a diagnostic trait though he subsequently accepted the family Callyspongiidae in 1989 (Wiedenmayer, 1989). In Figure 1 neither the family Callyspongiidae nor the genus *Callyspongia* were monophyletic. While all the members of the family and genus were in the same clade, this clade also contained sequences from a number of *Haliclona* species in addition to a sequence of *Acanthostrongylophora ingens* (Petrosina: Petrosiidae) and a *Cribochalina* sp. (family Niphatidae). Figure 1 shows a number of small highly supported clades containing one or two *Callyspongia* sequences clustered tightly to a *Haliclona* sequence. The pattern shown in Figure 1 also confirms the polyphyly of the family Chalinidae and the genus *Haliclona*. The picture for this group is more complicated than for Callyspongiidae because its members were spread right across the haplosclerid phylogeny, grouping with sequences from *Petrosia* and *Oceanapia* as well as with *Callyspongia* sequences. Chalinid sponges are common, present in all seas and at all depths. In the most recent classification of the Chalinidae by de Weerdt (2002) the family is described as having a 'long and complicated' history with many attempts being made to classify them. Gray (1867) and Lendenfeld (1887) suggested that the group contained highly unrelated sponges. De Laubenfels (1932) created three subfamilies comprising the Gelliinae for sponges with microscleres, the Renierinae for sponges with skeletons reinforced with spicules and the Chalininae for sponges with skeletons reinforced with spongin. Van Soest (1980) suggested that many of the characters used previously may not be homologous and used the structure of the choanosomal skeleton as a synapomorphy for the family. De Weerdt in 1986 abandoned most of the nominal genera so far attributed to the family, recognizing only eight 'species groups' and, by 2002, of the 27 genera that had been described for the Chalinidae only four were considered valid with *Haliclona* thought to contain six subgenera (de Weerdt, 2002). Molecular data strongly suggests that the structure of the skeleton is not homologous in chalinid sponges and that this family and the genus *Haliclona* contains a large number of unrelated species.

The tree in Figure 1 contained some single sequence representatives of particular haplosclerid genera. In some cases the species shown may not be a 'typical' member of that genus (e.g. *Chalinula*, *Dasychalina fragilis*, *Cribochalina* sp.) While every attempt was made to obtain suitable data from 'good' representatives, this was not possible in many cases due to the lack of availability of a suitable specimen or the age of the preserved material leading to degraded DNA. As

https://doi.org/10.1017/S0025315407058341 Published online by Cambridge University Press

a consequence some sequences were placed alone on the tree or in a perhaps unusual position and their affinities remain uncertain. The addition of further taxa in the future will likely alter their positions. However, they do suggest a greater diversity within the marine Haplosclerida than might have originally been expected and suggest that further sampling of this order is necessary to gain additional insights in to its taxonomic structure.

The phylogeny shown in Figure 1 also highlights a number of very puzzling relationships. Nestled amongst the Haplosclerida in a highly supported clade with *H. oculata* and *Acanthostronglyophora ingens*, were two sequences from members of the order Hadromerida (*Suberites suberia*, *S. virgultosa*). This pattern of relationships was also found on the phylogeny from Erpenbeck et al. (2006). There are two regions of the CO1 gene that have been utilized in sponge systematics. The fragment presented here is known as the Erpenbeck fragment after Erpenbeck (2002), the other is known as the Folmer fragment after Folmer (1994). Two different datasets are available from GenBank as it can be difficult to sequence the entire CO1 from sponges, and different researchers have selected different regions. We are attempting to sequence both fragments from the haplosclerid sponges and have an as yet incomplete dataset for the Folmer fragment. However, analysis of our Folmer fragment dataset shows *Suberites* sequences (kindly provided by Isobel Heim) to fall outside of the haplosclerid clade. Furthermore, after the phylogeny shown in Figure 1 was produced, an Erpenbeck fragment sequence was generated from *S. carnosus* DNA (DNA kindly provided by Isobel Heim) and was found to cluster outside the haposclerid clade in a neighbour-joining tree reconstructed from all available sequences (data not shown). It is likely that the position of *Suberites* suggested by Figure 1 is spurious and due to something unusual about the *Suberites* sequences which we downloaded from GenBank, perhaps contamination.

Another potential problem is suggested by the fact that a *Haliclona oculata* sequence generated during this work (no. 6 on Figure 1) did not cluster with the *H. oculata* sequence from the study of Erpenbeck et al. (2006). In fact, the genetic distance between these sequences was 0.15 substitutions/site, which is the same as the mean genetic distance between all *Haliclona* sequences and only 0.05 substitutions/site less than the genetic distance between all haplosclerid sequences. This may suggest a problem with one of the *H. oculata* sequences or it might suggest that multiple species are present within the *H. oculata* taxon. The specimen from which the GenBank sequence was produced was collected in the south-western Netherlands and is being held in the ZMA (POR17501). While of delicate form it is certainly a recognizable form of *H. oculata*. The *H. oculata* specimen sequenced during this study (POR 14116) and collected in France also falls within normal *H. oculata* variation. It lacks a surface reticulation, a character found in *H. oculata*, but shares features in common with a related species, *H. simulans*. To further investigate this issue another *H. oculata* specimen, collected in the Netherlands (kindly provided by Marieke Koopmans) was sequenced and was identical to the one from this study. This scenario has also been found with *H. cinerea*, where of three specimens sequenced to date for the CO1 Folmer fragment and the ITS region, two were found to be identical and one was found to be very different (Redmond et al., unpublished data). The same pattern is also being suggested with other common *Haliclona* species.

The phylogeny generated from the CO1 data mirrors closely those produced using 18S and 28S rDNA, despite the difference in substitution rates observed between the genes and lends confidence to the molecular data. Ten of the specimens employed in this analysis also had their 18S rRNA gene completely sequenced (Redmond et al., 2007). Their positions in both trees are very similar despite the presence of additional sequences in each case. The *Chalinula* sp. and *Petrosia* sp. sequences clustered together away from the *Callyspongia* and *Haliclona* sequences, while the *Dasychalina fragilis* sequence was in between the two groups. While the same *Niphates* specimen did not produce both CO1 and 18S rRNA gene sequences there was a *Niphates* representative in each analysis and in each case it fell in to the clade shared by *Petrosia* and *Chalinula*. Although sequences from *Haliclona* and *Callyspongia* were intermixed on the 18S rRNA phylogeny from Redmond et al. (2007) their relationships were poorly resolved. The CO1 data further elucidate the relationships between haplosclerid taxa at this level in the tree (i.e. within and between genera) showing clearly, and with high support, the need for an alternative taxonomic regime for the marine Haplosclerida.

One feature, common in systematics projects, which may create additional problems in attempting to reconstruct large molecular phylogenies is that of regional faunas. Many systematic studies are focused on a particular geographical region and when the fauna of different regions are combined for a large study such as this some genera that may appear similar may instead belong to regional clades that have evolved in isolation from a basal ancestor. However, there does not appear to be any consistent geographical pattern for the clustering apparent in Figure 1. While the sequence from *H. fascigera* collected in Micronesia clustered with high support with a *Callyspongia* sp. collected in Papua New Guinea, and sequences from *H. koremella* and *C.* (*Euplacella*) sp. both collected in Papua New Guinea clustered together, two sequences from *H. cinerea* specimens collected in France clustered in a clade with a number of *Callyspongia* sequences from specimens collected in Oman. A new strategy needs to be adopted in order to reconstruct a robust phylogeny for the marine Haplosclerida that will reconcile molecular and other evidence. Attempts to focus on a single genus, e.g. *Haliclona* have met with problems due to the diversity evident within genera (this study; Redmond et al., unpublished data), and focusing on a single regional fauna may only perpetuate the problems highlighted here. Perhaps by starting with a clade that is highly supported by molecular evidence and attempting to find synapomorphies from other approaches we can build a database of well-described species from around the world and begin to unravel the true evolutionary path taken by these important sponges.

This work was funded by the Irish Research Council for Science and Technology (IRCSET). The authors would like to thank Isabel Heim, Marieke Koopmans and Scott Nichols for specimens and/or DNA. All experiments were carried out in Ireland and complied with current Irish law.

https://doi.org/10.1017/S0025315407058341 Published online by Cambridge University Press

REFERENCES

Addis, J.S. & Peterson, K.J., 2005. Phylogenetic relationships of freshwater sponges (Porifera, Spongillina) inferred from analyses of 18S rDNA, COI mtDNA and ITS2 rDNA sequences. *Zoologica Scripta*, **34**, 549–557.

Alvarez, B., Crisp, M.D., Driver, F., Hooper, J.N.A & Soest, R.W.M. van, 2000. Phylogenetic relationships of the family Axinellidae (Porifera: Demospongiae) using morphological and molecular data. *Zoologica Scripta*, **29**, 169–198.

Avise, J., 2000. *Phylogeography: the history and formation of species*. Cambridge MA: Harvard University Press.

Borchiellini, C., Chombard, C., Manuel, M., Alivon, E., Vacelet, J. & Boury-Esnault, N., 2004. Molecular phylogeny of Demospongiae: implications for classification and scenarios of character evolution. *Molecular Phylogenetics and Evolution*, **32**, 823–837.

Borchiellini, C., Manuel, M., Alivon, E., Boury-Esnault, N., Vacelet, J. & Le Parco, Y., 2001. Sponge paraphyly and the origin of Metazoa. *Journal of Evolutionary Biology,* **122**, 187–202.

Desqueyroux-Faúndez, R. & Valentine C., 2002. Family Callyspongiidae de Laubenfels, 1936. In *Systema Porifera* (ed. J.N.A. Hooper and R.W.M. van Soest), pp. 835–851. New York: Kluwer Academic/Plenum Publishers.

Duran, S., Pascual, M. & Turon, X., 2003. Low levels of genetic variation in mtDNA sequences over the western Mediterranean and Atlantic range of the sponge *Crambe crambe* (Poecilosclerida). *Marine Biology*, **144**, 31–35.

Erpenbeck, D., Breeuwer, J.A., Parra-Velandia, F.J. & Soest, R.W.M. van, 2006. Speculation with spiculation?—three independent gene fragments and biochemical characters versus morphology in demosponge higher classification. *Molecular Phylogenetics and Evolution*, **38**, 293–305.

Erpenbeck, D., Breeuwer, J.A.J., Velde, H.C. van der & Soest, R.W.M. van, 2002. Unravelling host and symbiont phylogenies of halichondrid sponges (Demospongiae, Porifera) using a mitochondrial marker. *Marine Biology*, **141**, 377–386.

Erpenbeck, D.,Voigt, O., Adamski, M., Adamska, M., Hooper, J.N., Wörheide, G. & Degnan, B.M., 2007. Mitochondrial diversity of early-branching metazoa is revealed by the complete mt genome of a haplosclerid demosponge. *Molecular Biology and Evolution*, **24**, 19–22.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, **3**, 294–299.

Gray, J.E., 1867. Notes on the arrangement of sponges, with the description of some new genera. *Proceedings of the Zoological Society of London*, 492–558.

Hooper, J.N.A., Soest, R.W.M. van & Derenne, F., 2002. Phylum Porifera Grant, 1836. In *Systema Porifera* (ed. J.N.A. Hooper and R.W.M. van Soest), pp. 9–13. New York: Kluwer Academic/ Plenum Publishers.

Keane, T.M., Naughton, T.J., Travers, S.A.A., McInerney, J.O. & McCormack, G.P., 2005. DPRml: distributed phylogeny reconstruction by maximum likelihood. *Bioinformatics*, **21**, 969–974.

Kelly-Borges, M. & Pomponi, S.A., 1994. Phylogeny and classification of lithistid sponges (Porifera: Demospongiae): a preliminary assessment using ribosomal DNA sequence comparisons. *Molecular Marine Biology and Biotechnology*, **3**, 87–103.

Lafay, B., Boury-Esnault, N., Vacelet, J. & Christen, R., 1992. An analysis of partial 28S ribosomal RNA sequences suggests early radiations of sponges. *Biosystems*, **28**, 139–151.

Laubenfels, M.W. de, 1932. The marine and freshwater sponges of California. *Proceedings of the United States National Museum*, **81**(2927), 1–140.

Laubenfels, M.W. de, 1936. A discussion of the sponge fauna of the dry Tortugas in particular and the West Indies in general, with material for a revision of the families and orders of the Porifera. *Carnegie Institute of Washington (Tortugas Laboratory paper no. 467)*, **30**, 1–225.

Lendenfeld, R. von, 1887. Die Chalineen des australischen Gebietes. *Zoologische Jahrbucher*, **2**, 723–828.

Maddison, W.P. & Maddison, D.R., 1998. *MacClade, Version 4.03*. Sunderland, MA: Sinauer Associates.

McCormack, G.P., Erpenbeck, D. & Soest, R.W.M. van, 2002. Major discrepancy between phylogenetic hypotheses based on molecular and morphological criteria within the Order Haplosclerida (Phylum Porifera: Class Demospongiae). *Journal of Zoological Systematics and Evolutionary Research*, **40**, 237–240.

Nichols, S.A., 2005. An evaluation of support for order level monophyly and interrelationships within the class Demospongiae using partial data from the large subunit rDNA and cytochrome oxidase subunit I. *Molecular Phylogenetics and Evolution*, **34**, 81–96.

Posada, D. & Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics*, **14**, 817–818.

Redmond, N.E., Soest, R.W.M. van, Kelly, M., Raleigh, J., Travers, S.A.A. & McCormack, G.P., 2007. Reassessment of the classification of the Order Haplosclerida (Class Demospongiae, Phylum Porifera) using 18S rRNA gene sequence data. *Molecular Phylogenetics and Evolution*, **43**, 344–352.

Schroeder, H.C., Efremova, S.M., Itskovitch, V.B., Masuda, Y., Krasko, A., Mueller, I.M. & Mueller, W.E.G., 2003. Molecular phylogeny of freshwater sponges in Lake Baikal. *Journal of Zoological Systematics and Evolutionary Research*, **41**, 80–86.

Soest, R.W.M. van, 1980. Marine sponges from Curaçao and other Caribbean localities. Part II. Haplosclerida. *Studies of the Fauna of Curaçao and other Caribbean Islands*, **62**, 1–173.

Soest, R.W.M. van & Braekman, J.C., 1999. Chemosystematics of Porifera: a review. *Memoires of the Queensland Museum*, **44**, 569–589.

Soest, R.W.M. van & Hooper, J.N.A., 2002a. Order Haplosclerida Topsent 1928. In *Systema Porifera* (ed. J.N.A. Hooper and R.W.M. van Soest), pp. 831–832. New York; Kluwer Academic/Plenum Publishers.

Soest, R.W.M. van & Hooper, J.N.A., 2002b. Suborder Haplosclerina Topsent 1928. In *Systema Porifera* (ed. J.N.A. Hooper and R.W.M. van Soest), pp. 833–834. New York: Kluwer Academic/Plenum Publishers.

Soest, R.W.M. van & Hooper, J.N.A., 2002c. Suborder Petrosina Topsent 1928. In *Systema Porifera* (ed. J.N.A. Hooper and R.W.M. van Soest), pp. 891–892. New York: Kluwer Academic/Plenum Publishers.

Weerdt, W.H. de, 1986. A systematic revision of the north-eastern Atlantic shallow-water Haplosclerida (Porifera, Demospongiae), part II: Chalinidae. *Beaufortia*, **36**, 81–165.

Weerdt, W.H. de, 1989. Phylogeny and vicariance biogeography of North Atlantic Chalinidae (Haplosclerida: Demospongiae). *Beaufortia*, **39**, 55–88.

Weerdt, W.H. de, 2002. Family Chalinidae Gray, 1867. In *Systema Porifera* (ed. J.N.A. Hooper and R.W.M. van Soest), pp. 852–873. New York: Kluwer Academic/Plenum Publishers.

Wiedenmayer, F., 1977. Shallow-water sponges of the western Bahamas. *Experientia Supplementum*, **28**, 1–287.

Wiedenmayer, F., 1989. Demospongiae (Porifera). The stratigraphic record, ecology and global distribution of intermediate and higher taxa. *Schweizerische Palaontologische Abhandlungen*, **116**, 1–147.

Wörheide, G., Sole-cava, A.M. & Hooper, J.N.A., 2005. Biodiversity, molecular ecology and phylogeography of marine sponges; patterns, implications and outlooks. *Integrative and Comparative Biology*, **45**, 377–385.

Submitted 30 April 2007. Accepted 18 June 2007.

https://doi.org/10.1017/S0025315407058341 Published online by Cambridge University Press