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Phylogeny and systematics of deep-sea sea pens (Anthozoa: Octocorallia: Pennatulacea)

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ABSTRACT

Molecular methods have been used for the first time to determine the phylogeny of families, genera and species within the Pennatulacea (sea pens). Variation in *ND2* and *mtMutS* mitochondrial protein-coding genes proved adequate to resolve phylogenetic relationships among pennatulacean families. The gene *mtMutS* is more variable than *ND2* and differentiates all genera, and many pennatulacean species. A molecular phylogeny based on a Bayesian analysis reveals that suborder Sessiliflorae is paraphyletic and Subselliflorae is polyphyletic. Many families of pennatulaceans do not represent monophyletic groups including Umbellulidae, Pteroeididae, and Kophobelemnidae. The high frequency of morphological homoplasy in pennatulaceans has led to many misinterpretations in the systematics of the group. The traditional classification scheme for pennatulaceans requires revision.

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1. Introduction

Octocorals (Cnidaria: Anthozoa) are ecologically diverse and important members of a variety of marine communities, from the warm shallow-water tropics to the cold depths of the deep sea where they are often abundant megafaunal filter feeders (Tyler, 2003). Indeed, most families of octocorals have representatives living in the deep sea, and some reach their highest diversity at depths >200 m (Watling et al., 2011). In contrast to other major groups of cnidarians, for which there is a long and rich history of phylogenetic study (for example Veron et al., 1996; Collins et al., 2006), our knowledge of the evolutionary relationships within the octocorals is poor and under-studied (Bayer, 1981). Endeavours to improve our understanding have been impeded by a scarcity of useful taxonomic characters, a high frequency of homoplasy (parallelisms, convergences, and reversals), and high degrees of intraspecific variability (Williams, 1992). In the past, systematic work has focused mainly on alpha-taxonomy (Kölliker, 1880; Hickson, 1916; Bayer, 1955; Williams, 1992, 1997), yet the difficulty in polarising taxonomic characters for phylogenetic reconstructions has been exacerbated by the near absence of octocorals in the fossil record (Bayer, 1956).

1.1. Pennatulacea systematics

Within the Octocorallia, the sea pens (Pennatulacea) can be readily distinguished based on morphology (Bayer, 1956, 1973). The pennatulaceans are the most advanced of octocorals in terms of their colonial complexity, functional specialisation of polyps, and colonial integration (Hickson, 1909; Bayer, 1956, 1973). Uniquely, mature colonies develop from a single large primary polyp that produces secondary polyps by lateral budding of its body wall (Williams, 2011). Also exclusive to the pennatulaceans, is the character of a muscular peduncle, which anchors the colony by peristaltic contractions into soft substrata such as sand, mud, or abyssal ooze (Williams, 2011). Some species have a peduncle that is expanded to form a sucker-like structure and can attach to rocky substrata (Williams and Alderslade, 2011).

1.2. Historical classification

Kükenthal and Broch (1911) and Kükenthal (1915) developed a higher classification scheme of two suborders (and six sections) within the pennatulaceans: the Sessiliflorae for the taxa with polyps emanating directly from the rachis and the Subselliflorae for the taxa with polyps located on polyp leaves or raised ridges. Very few authors have attempted to deal with the subject of phylogeny and the origins of the pennatulaceans, the majority of which predate current cladistic methods (Kölliker, 1870, 1880; Koch, 1878;

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Marshall, 1887; Kükenthal and Broch, 1911; Kükenthal, 1915; Hickson, 1916). Williams (1992) initiated modern phylogenetic studies of the group based on a cladistic analysis of morphological characters for nine of the fifteen pennatulacean families.

1.3. Origins of pennatulaceans

There is still much speculation with regards to the origins of pennatulaceans. Many believe that the Ediacaran and Burgess Shale frond-like fauna are fossilised pennatulacean-like octocorals (Bergström, 1991). However, 'similarities' i.e. the lateral branches of the frond-like fossils and the polyp leaves of many pennatulaceans appear to be non-homologous and not even functionally convergent (Williams, 1997; Antcliffe and Braiser, 2007). Instead, Williams (1997) proposed that pennatulaceans evolved from a soft coral ancestor similar to the alcyoniid genus *Anthomastus*. Molecular evidence suggests that *Anthomastus* may be more closely related to the pennatulaceans than other soft corals, but these data do not support a sister-group relationship (Berntson et al., 1999, 2001; McFadden et al., 2006). Instead, there is strong evidence to support the calcaxonian sea fan family Ellisellidae as the sister group to the Pennatulacea (Bayer, 1955; McFadden et al., 2006). This would make the Calcaxonia a paraphyletic assemblage (Williams, 2011) and requires the re-evaluation of the classification of the Pennatulacea as an order.

Distributional and phylogenetic data based on morphology suggest that pennatulaceans first differentiated in tropical shallow-waters and subsequently dispersed to and diversified in temperate and polar regions, and to all ocean depths, as well as the shallow-water tropics (Williams, 1997).

1.4. Modern genetic work

The advent of molecular approaches has considerably improved our understanding of the evolutionary relationships among anthozoans (France et al., 1996; Song and Won, 1997; Bridge et al., 1995; Brugler and France, 2007; Medina et al., 2006; Rogers, 2009). Octocorallia has been shown to form a monophyletic group, sister to all other anthozoan orders (Berntson et al., 1999, 2001; McFadden et al., 2006; Daly et al., 2007; Rogers, 2009).

To date, studies have addressed a variety of questions at different systematic levels, generally with well-established octocorallian taxa. They range from analyses carried out at species and genus levels (McFadden, 1999; Song and Lee, 2000; McFadden and Hutchinson, 2004) to boarder studies of genera within families of selected sections of octocorals (France and Hoover, 2001, 2002; Sánchez et al., 2003; Pante et al., 2012). Very little research has focused on the pennatulaceans. Berntson et al. (2001) examined the phylogenetics of Octocorallia based on nuclear *18S* rDNA sequences. Unexpectedly, Pennatulacea was found to be polyphyletic because of the inclusion of the pennatulacean *Umbellula* sp. in a clade with the alcyoniids, *Anthomastus* and *Corallium*. This result, however, was not supported by mitochondrial data (*ND2* and *mtMutS*), which recovered the pennatulaceans as a monophyletic order (McFadden et al., 2006).

To understand sub-ordinal relationships within the octocorals, mitochondrial molecular markers were developed for the octocoral-specific gene, *mtMutS* (France and Hoover, 2001; McFadden et al., 2004). It is now recognised that all octocorals exhibit the mitochondrial protein-coding gene, *mtMutS*, a homologue of the bacterial DNA mismatch repair gene, *mutS*, which is not known to occur in any other cnidarians or metazoans (Pont-Kingdon et al., 1995; Culligan et al., 2000). The *mtMutS* gene is believed to evolve two times faster than either *ND3* or *ND4L* (France and Hoover, 2001), making it potentially informative for family- and genus-level phylogenetic analyses. Mitochondrial sequence data using a combination of *ND2* and *mtMutS* genes have unveiled better-resolved phylogenies within the octocorals (Sánchez et al., 2003; McFadden et al., 2006).

To date, there are no phylogenetic or systematic studies among or within any families of Pennatulacea based on molecular data (Daly et al., 2007). The recent collections of pennatulaceans for molecular analysis, representing a suite of taxa of wide geographic and bathymetric scope, have enabled a reassessment of the systematics and phylogenetic relationships among 11 of the 15 pennatulacean families (classification based on Williams, 1995a). This study offers the first genetic analysis of Pennatulacea and addresses the following questions:

1. Is the current classification scheme of Pennatulacea supported by molecular systematics?
2. Is there molecular evidence to support the higher classification scheme (the suborders Sessiliflorae and Subselliflorae) of Kükenthal and Broch (1911) and Kükenthal (1915)?
3. Are the two mitochondrial protein-coding genes, *ND2* and *mtMutS*, useful for addressing phylogenetic questions within Pennatulacea?

2. Materials and methods

2.1. Specimens

A total of 132 frozen and ethanol-preserved pennatulacean specimens were collected during research cruises and from other sources. Samples included representatives from all oceans (Atlantic, Arctic, Indian, Pacific and Southern), ranging in depth from 12 m to 4229 m (Table 1). See Appendix A for a full list of cruises and sources.

Pacific material was identified to generic level by J.A. Sanchez. All other individuals were identified to at least the generic level by E. Dolan using the taxonomic treatments of Bayer et al. (1983), Broch (1913, 1957, 1958, 1961), Danielssen and Koren (1884), Grasshoff (1972, 1981), Gray (1860, 1870), Hickson (1916, 1937), Jungersen (1904), Kölliker (1880), Kükenthal (1915), Kükenthal and Broch (1911), Lindahl (1874), Marshall (1887), Pasternak (1962, 1964, 1975, 1993) and Williams (1990, 1995a,b). Type specimens housed at the Natural History Museum (London, UK) were studied and photographed for reference. Morphological characters were determined by examination under a stereo microscope and sclerites were examined by means of compound microscope and/or imaged with scanning electron microscopy (Dolan, 2008). Species of *Umbellula* were identified as part of a comprehensive revision of the group (Dolan, 2008). Voucher material is deposited in the Natural History Museum (London, UK) or catalogued at NIWA (Table 1). Depth range was coded as 'shallow' for taxa found between 0–200 m and 'deep' for below 200 m, by searching the literature and examining the depths of available specimens.

2.2. DNA extraction

From 103 of 132 pennatulacean specimens, total genomic DNA was extracted from 15 to 25 mg of polyp tissue using Qiagen DNeasy extraction kits according the manufacturer's instructions. DNA from 53 of these was used for the final analysis in this study (see below). Pennatulacean tissue tended to yield large quantities of DNA as detected on the NanoDrop ND-1000 spectrophotometer (Labtech International), and often had to be significantly diluted to obtain optimum concentrations of 2 ng μl^{-1}.

2.3. Primers, amplification and sequencing

Six different genes were examined for their suitability for sequence analysis of pennatulaceans: part of the mitochondrial enzyme-complex gene, succinate dehydrogenase (*SDH*); the

Table 1
Pennatulid specimens analysed in this study.

Taxon	Date	Depth	Location	Catalogue #	*ND2*	*mtMutS*
Anthoptilidae						
Anthoptilum murrayi			**Tasman Sea, AUS**		**DQ302938**	**–**
Anthoptilum sp.1	2006	–	Subantarctic	2010.18[a]	KF313805	KF313832
Anthoptilum sp.2	2005	1714	Sumatra, Indian Ocean	NOCS	KF313806	–
Funiculinidae						
Funiculina armata	2007	350	NE Atlantic	2010.11[a]	KF313807	KF313833
Funiculina quadrangularis	2006	173	NE Atlantic	NOCS	–	KF313834
Halipteridae						
Halipteris finmarchica			**Tasman Sea, AUS**		**DQ302941**	**DQ302868**
Halipteris finmarchica	2007	555	New Zealand, W Pacific	28801[b]	KF313808	KF313835
Kophobelemnidae						
Kophobelemnon macrospinosum			**Tasman Sea, AUS**		**DQ302937**	**DQ302865**
Kophobelemnon pauciflorum	2005	4189	Crozet, S Atlantic	2010.21[a]	KF313809	KF313836
Kophobelemnon sp.1	2007	70	Koster Channel, NE Atlantic	2010.13[a]	KF313810	KF313837
Kophobelemnon sp.2	2006	2456	Monterey, E Pacific	2010.10[a]	KF313811	KF313838
Kophobelemnon sp.3	2007	1812	New Zealand, W Pacific	28827[b]	KF313812	KF313839
Sclerobelemnon theseus			**Colombia**		**DQ311678**	**DQ311679**
Pennatulidae						
Pennatula aculeata	2006	2456	Monterey, E Pacific	2010.15[a]	KF313813	KF313840
Pennatula phosphorea	2006	55	Millport, NE Atlantic	NOCS	KF313814	KF313841
Pennatula murrayi	2006	3208	Monterey, E Pacific	2010.9[a]	KF313815	KF313842
***Pennatula* sp.**			**Tasman Sea, AUS**		**DQ302943**	**DQ302870**
Protoptilidae						
Distichoptilum gracile			**Tasman Sea, AUS**		**DQ302939**	**DQ302866**
Distichoptilum gracile	2007	1211	New Zealand, W Pacific	28813[b]	KF313816	KF313843
Distichoptilum gracile	2006	2456	Monterey, E Pacific	NOCS	KF313817	–
Protoptilum sp.	2006	3208	Monterey, E Pacific	2010.20[a]	KF313818	KF313844
Pteroeidae						
Gyrophyllum sp.	2006	1580	NE Atlantic	NOCS	–	KF313845
Gyrophyllum sp.	2007	997	New Zealand, W Pacific	28779[b]	KF313819	KF313846
***Pteroeides* sp.**			**Tasman Sea, AUS**		**DQ302944**	**DQ302871**
Renillidae						
Renilla muelleri			**GOM, Florida, USA**		**DQ297451**	**DQ297432**
Scleroptilidae						
Scleroptilum grandiflorum	2007	2190	Mid-Atlantic Ridge	2010.14[a]	KF313820	KF313847
Umbellulidae						
Umbellula carpenteri	2005	4189	Crozet, S Atlantic	NOCS	KF313821	KF313848
Umbellula encrinus	2001	1400	Arctic Ocean	2010.8[a]	KF313822	KF313849
Umbellula huxleyi	2006	1512	NE Atlantic	2010.17[a]	KF313823	KF313850
Umbellula magniflora	2007	840	Marguerite Bay, Antarctica	2010.22[a]	KF313824	KF313851
Umbellula monocephalus	2005	4229	Indian ocean	2010.16[a]	KF313825	KF313852
Umbellula thomsoni 1	2005	4189	Crozet, S Atlantic	2010.19[a]	KF313826	KF313853
Umbellula thomsoni 2	2007	3476	Cascais Canyon, NE Atlantic	NOCS	KF313827	KF313854
Umbellula sp.1	2007	4040	Whittard Canyon, NE Atlantic	2009.8[a]	KF313828	KF313855
Umbellula sp.2	2007	4189	Crozet, S Atlantic	2009.6[a]	KF313829	KF313856
Virgulariidae						
Virgularia mirabilis	2007	36.5	Sweden, NE Atlantic	2010.7[a]	KF313830	KF313857
Virgularia mirabilis	2006	12	Portland, UK, NE Atlantic	2010.23[a]	KF313831	KF313858
Outgroup: Ellisellidae						
Ctenocella barbadensis			**Unknown**		**AY534736**	**AY533651**
***Verrucella* sp.**			**Unknown**		**DQ302936**	**DQ302864**

Entries in boldface were obtained from GenBank (McFadden et al., 2006). NOCS, National Oceanography Centre, Southampton (UK).
[a] Natural History Museum (London, UK) catalogue number.
[b] NIWA (New Zealand) catalogue number.

non-coding region of the mitochondrial genome between *COI* and *COII* (*COI–COII* intergenic spacer); the large subunit of the mitochondrial ribosomal DNA gene (*16S rDNA*); the small subunit of the nuclear ribosomal DNA gene (*18S rDNA*); and two mitochondrial protein-coding genes, NADH-dehydrogenase subunit 2 (*ND2*) and *mtMutS*, a homologue of the bacterial DNA mismatch repair gene, *mutS*. Only *ND2* and *mtMutS* proved to be suitable for phylogenetic analysis of the Pennatulacea.

The primers used to amplify NADH-dehydrogenase subunit 2 (*ND2*) were:

16647F (5′-ACACAGCTCGGTTTCTATCTACCA-3′) and ND21418R (5′-ACATCGGGAGCCCACATA-3′) (McFadden et al., 2004).For *mtMutS*, the primers used were:

ND42599F (5′-GCCATTATGGTTAACTATTAC-3′) (France and Hoover, 2002) and Mut-3458R (5′-TSGAGCAAAAGCCACTCC-3′) (Sánchez et al., 2003).

The PCR solutions contained (in 50 μl volumes): 5 μl of 10× PCR buffer, 2 μl of 3 mM $MgCl_2$, 2 μl of 0.2 mM dNTP, 5 μl of "Q-solution", 0.5 μl of *Taq* Polymerase (all reagents from Qiagen), 2 μl of each 10 pmol μl^{-1} primer and 5 μl of 2 ng μl^{-1} DNA template.

Amplification was carried out over 35 cycles of 90 s at 94 °C, 90 s at 58 °C, 60 s at 72 °C, followed by a 5 min extension at 72 °C.

DNA was purified using QIAquick Gel Extraction kits (Qiagen) following the manufacturer's instructions. Clean PCR products were sent to Macrogen Ltd., South Korea, for sequencing.

2.4. Sequence analysis of ND2 and mtMutS

Both strands of corresponding sequences were aligned inBioEdit using ClustalW (Thompson et al., 1994) with default alignment parameters, and then corrected by eye to produce a consensus sequence. Raw sequence traces were consulted to verify base calls. A BLAST (Basic Local Alignment Search Tool) search was performed in GenBank (Benson et al., 2006, http://www.ncbi.nlm.nih.gov/) and the matching homologous pennatulacean sequences (an additional 8 sequences for *ND2* and 7 for *mtMutS*, of Table 1) were retained for subsequent alignment to complement the analysis of sequences generated in this study. Two members of the closely-related calcaxonian family Ellisellidae, *Ctenocella barbadensis* and *Verrucella* sp., were chosen as outgroups (McFadden et al., 2006).

Sequences were analysed both separately and in combination. For each data set, sequences were aligned in MEGA4 (Tamura et al., 2007) with ClustalW (Thompson et al., 1994) using the default alignment settings, and trimmed to the shortest sequence.

Models of sequence evolution were selected for each alignment using Modeltest 3.7 (Posada and Crandall, 1998) using the Akaike Information Criterion. A Bayesian analysis was performed using the program MrBayes Version 3.1.2 (Huelsenbeck and Ronquist, 2001) using 10 million generations and 4 chains. The burn-in was assessed by examining the model output using Tracer v1.4 (http://beast.bio.ed.ac.uk/). The first 1 million generations were discarded, subsequent generations were used to construct consensus trees and determine clade support using the "sumt" command in MrBayes. Maximum-likelihood, maximum parsimony and neighbour-joining analyses were performed using PAUP* version 4.0b10 (Swofford, 1993). Maximum likelihood analyses were run using a heuristic search with TBR branch-swapping, for 100 bootstrap replicates with the model parameters chosen by Modeltest 3.7. For maximum parsimony, a heuristic search with TBR branch-swapping was used, for 1000 bootstraps with a maximum of 1000 trees saved per replicate. Neighbour-joining (distance method) was conducted for 1000 bootstrap replicates.

Further analyses were performed on the combined data set, constraining the monophyly of each family and genus in turn and another constraining the monophyly of all families. An SH test (Shimodaira and Hasegawa, 1999) was performed on the most likely tree from each analysis compared with the most likely tree from the unconstrained analysis using PAUP* version 4.0b10 (Swofford, 1993). Morphological and depth characters were mapped onto the combined-data consensus phylogeny using the maximum parsimony character optimisation in Mesquite v2.5 (Maddison and Maddison, 2008).

3. Results

3.1. Sequences

Amplifications were often impeded by DNA deterioration in many older specimens as well as those stored in <90% EtOH. Amplifications from 53 individuals were of sufficient quality to generate sequences for analysis (45 *ND2* and 47 *mtMutS*). The combined analysis, which included five additional pairs of each gene acquired from GenBank, corresponded to 29 distinct haplotypes of 14 pennatulacean genera and 11 families. Wherever possible, at least two representatives of each species were sequenced. In nearly all cases, sequences were identical between individuals of the same species, however, where two sequences differed; both sequences were included in the phylogenetic analysis (viz. *U. thomsoni*). Our sequences of *Distichoptilum gracile*, *Halipteris finmarchica* and *Kophobelemnon macrospinosum* were identical to those previously deposited in GenBank (McFadden et al., 2006). The sequences obtained here for *K. macrospinosum* were of poor quality, and accordingly, the GenBank sequences of this taxon were used in the analyses.

The *ND2* gene fragment was found to be less variable than *mtMutS*. *ND2* sequences of *Pennatula aculeata* and *P. phosphorea* were invariant between individuals, whereas the corresponding *mtMutS* sequences revealed differences in haplotype. This was also the case for *Umbellula thomsoni*: *mtMutS* showed interspecific variation, whereas *ND2* sequences were invariant among this species.

3.2. Alignments

ND2 fragments generated in this study ranged from 684 to 717 base pairs (bp) in length. The alignment of all *ND2* sequences revealed 3 insertions/deletions (indels). Differences in length were mainly attributable to a large indel near the 3′ end of the fragment, with insertions of 24 bp in *Anthoptilum* spp. and *Virgularia mirabilis* sequences. This variable region was removed in the final analysis, with longer new sequences trimmed to match the shorter ones obtained from GenBank. The final alignment was 465 base pairs. Five of 9 *Umbellula* spp. displayed a deletion of 3 bp, in alignment position 125–127, shared by species without sclerites in the polyp/rachis tissue. *Halipteris finmarchica* possessed a unique deletion of 6 bp.

MtMutS fragments generated in this study ranged from 733 to 776 bp. The sequence for *Anthoptilum* sp.1 contained a section of sequence 32 bp in length that would not align adequately with the other sequences (position 165–196); this resulted in 15 bp of insertion unique to *Anthoptilum* sp.1. Nearby this section, there were two other indels shared by multiple taxa (8 bp at position 202–207 and 9 bp at position 184–193). Again, *Umbellula* species contained indels corresponding to those species with and without sclerites in the polyp/rachis tissue.

The combined-data alignment was 1196 bp (*ND2* = 465 bp and *mtMutS* = 731 bp). Of these, 865 bp were invariant, and 197 of 331 variable sites were parsimony-informative.

3.3. Trees

Bayesian, maximum parsimony, maximum-likelihood, and neighbour-joining analyses all recovered very similar topologies for the combined dataset of *ND2* and *mtMutS* partial sequences. Phylogenetic analyses of each gene separately produced trees similar in topology to the combined analyses but generally had lower posterior probability (PP) support values and were less resolved relative to the combined analysis. *ND2* proved to be the less informative of the two genes. Single-gene analyses however, allowed the examination of the phylogenetic positions of some taxa for which it was possible to sequence only one of the two genes. For simplicity, we present the combined Bayesian analysis only (Fig. 1).

All pennatulaceans of this study formed a well-supported monophyletic group with respect to the chosen outgroups. *Anthoptilum* sp.1 (F. Anthoptilidae) was positioned as a sister taxon to all other pennatulaceans: this is probably because of the large and unique insertion for this taxon mentioned above. The *ND2* analysis included two other *Anthoptilum* species (*Anthoptilum* sp.2 and *A. murrayi*), which were positioned as sister taxa to each other, but their relationship with *Anthoptilum* sp.1 was unresolved. The rest of the pennatulaceans can be separated into four distinct and well-supported clades (PP = 1).

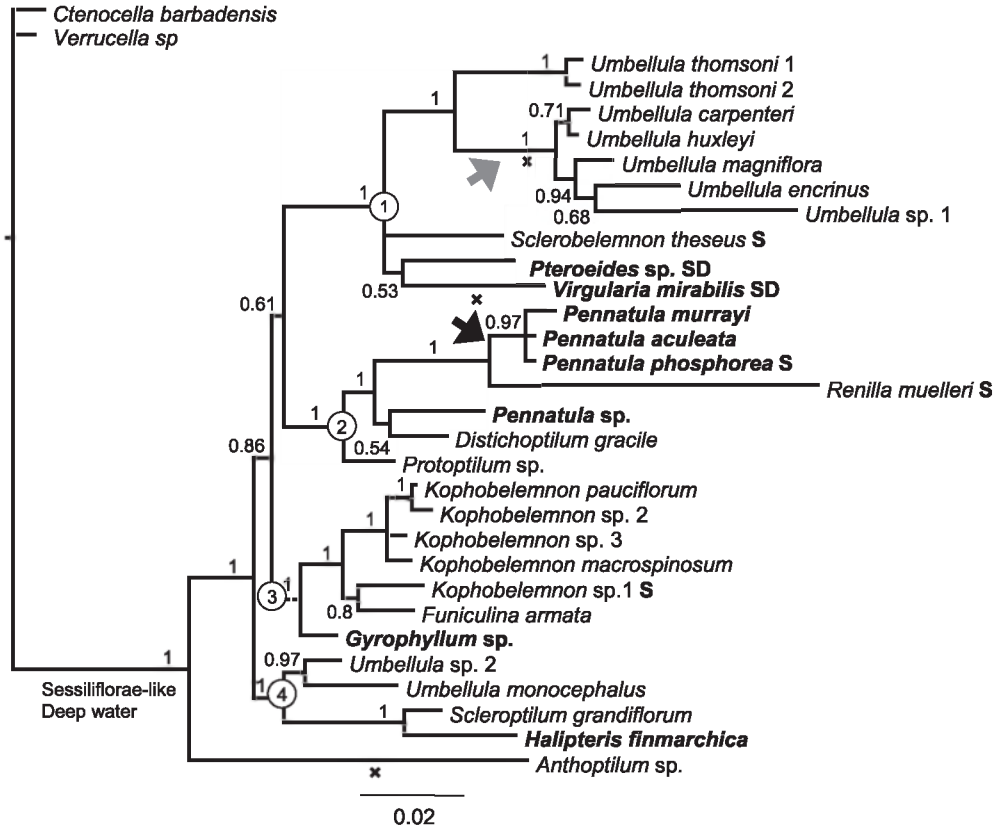


Fig. 1. Phylogenetic relationships among 11 families in Pennatulacea for the combined analysis of *ND2* and *mtMutS*. Consensus tree from Bayesian analysis, scale bar indicates number of nucleotide changes, numbers on nodes indicate posterior probabilities. Circled numbers indicate the clades discussed in the text. Taxa are Sessiliflorae unless indicated in boldface (**Subselliflorae**); **x** (on branches) indicates sclerites absent from polyps and rachis. All taxa are deep water (>200 m) unless indicated with **S** (shallow water < 200 m) or **SD** (shallow and deep water) following taxon names. Ancestral reconstruction reveals Sessiliflorae-like, deep-water taxa at every internal node except that indicated with a black arrow, which signifies a change in character state to Subselliflorae-like taxa. The grey arrow indicates sclerite loss at an internal node.

3.3.1. Clade 1

All but two members of the monogeneric family Umbellulidae (*Umbellula* spp.) are in this clade. The hypothesis that Umbellulidae forms a monophyletic group is rejected by the SH test ($P = 0.005$, see Fig. 2). However, the *Umbellula* species within clade 1 do form a monophyletic group and can be sub-divided into clades according to the presence or absence of sclerites. Although the relationships of *Pteroeides* (F. Pteroeididae), *Virgularia* (F. Virgulariidae) and *Sclerobelemnon* (F. Kophobelemnidae) within this clade were unresolved, clade membership was highly supported in all phylogenetic analyses. No trees grouped *Sclerobelemnon* with the other members of the family Kophobelemnidae. Constraining the monophyly of Kophobelemnidae produces significantly worse likelihoods (Fig. 2).

3.3.2. Clade 2

Pennatula species do not form a monophyletic group. It is possible that individuals assigned to the genus *Pennatula* may have been misidentified, although we note that *Pennatula* sp. was identified and sequenced for McFadden et al. (2006) and the authors of this

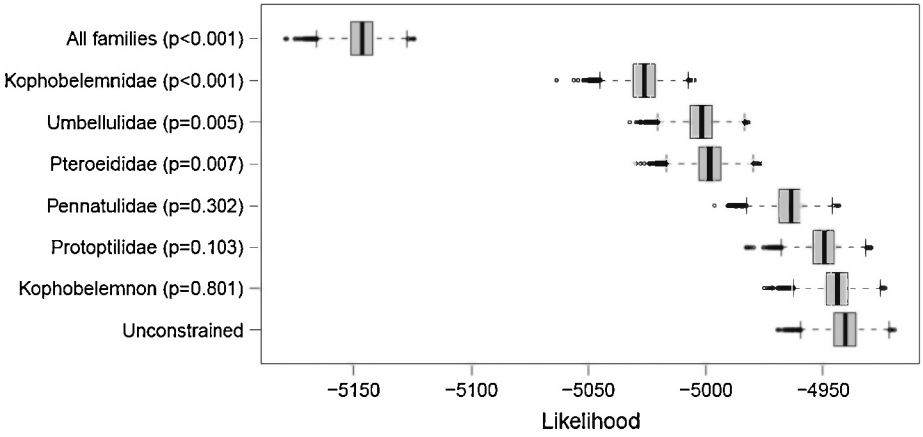


Fig. 2. Box plot of likelihoods from Bayesian analyses constraining the monophyly of indicated groups. *p*-Values are from an SH test comparing the best tree from each analysis with that of the unconstrained analysis.

paper include several scientists with expertise in octocorals taxonomy (particularly Alderslade). Our own material was carefully identified on the basis of the current morphological classification scheme. A more likely explanation is that the morphological criteria that are used to identify members of the genus *Pennatula* poorly resolve their phylogenetic relationships compared to analysis based on DNA sequence data. In the latter case, a redefinition of the genus *Pennatula* may be warranted. *Renilla muelleri* (Renillidae) is strongly supported (PP = 1) as sister to *P. murrayi*, *P. aculeata* and *P. phosphorea*, whilst the other *Pennatula* species is very weakly grouped with *Distichoptilum gracile*. Constraining the monophyly of *Pennatula* produced trees with significantly worse likelihood values. The family Protoptilidae contains two genera, *Protoptilum* and *Distichoptilum*. The two representatives of the Protoptilidae (*Protoptilum* sp. and *D. gracile*) are not resolved as sister species in the consensus phylogeny, but we cannot formally reject the monophyly of Protoptilidae ($P > 0.1$).

3.3.3. Clade 3

All *Kophobelemnon* spp. and the monogeneric F. Funiculinidae (*Funiculina armata*) form a well-supported group within this clade in all analyses. This is further supported on the basis of analyses of *mtMutS* alone, where *Funiculina quadrangularis* is grouped with *Funiculina armata* and *Kophobelemnon* sp.1 (PP = 0.8). All but one sample of the genus *Kophobelemnon* form a well-supported monophyletic group (PP = 1), however the monophyly of genus *Kophobelemnon* cannot be rejected (Fig. 2). The family Pteroeididae is represented by *Gyrophyllum* and *Pteroeides*. *Gyrophyllum* is sister to *Kophobelemnon* + *Funiculina*, whilst *Pteroeides* is firmly placed within clade 1. The monophyly of Pteroeididae is rejected ($P < 0.01$).

3.3.4. Clade 4

This clade includes the two other *Umbellula* species, which form a sister group (PP = 0.97). The topology of all trees suggests a close relationship between *Halipteris finmarchica* Sars 1851 (F. Halipteridae, Williams, 1995a) and *Scleroptilum grandiflorum* Kölliker, 1880 (F. Scleroptilidae, Jungersen, 1904).

4. Discussion

The recent collections of pennatulaceans represent a suite of taxa of wide geographic and bathymetric scope and have enabled a reassessment of the systematics and phylogenetic relationships of 11 of the 15 families. Phylogenetic analysis produced well-supported phylogenetic relationships for representative deep-sea and shallow-water pennatulaceans. In this study, the *ND2* region was found to be more conserved than *mtMutS*, supporting the finding that *mtMutS* is better suited to lower level phylogenetic analysis than other mitochondrial genes (France and Hoover, 2001; van der Ham et al., 2009).

A summary of the classification (Williams, 1995a) alongside the results of this analysis is found in Table 2, demonstrating the high level of incongruence between the traditional classification based on morphology and the molecular phylogeny. Our consensus phylogeny fails to recover monophyly for any genus with more than a single representative (*Umbellula*, *Pennatula* and *Kophobelemnon*). Additionally, the topology of the consensus phylogenetic tree suggests that the five families Pteroeididae Umbellulidae, Kophobelemnidae, Pennatulidae and Protoptilidae are not monophyletic, but the monophyly of Pennatulidae and Protoptilidae could not be formally rejected by an SH test. Similar findings have been demonstrated for other octocorals. Polyphyly has recently been shown in chrysogorgiid octocorals, suggesting a call for re-assessment of the diagnostic characters used to differentiate the Chrysogorgiidae (Pante et al., 2012).

Table 2
A summary of the classification used in this analysis.

Suborder	Family	Genus	Clade
Sessiliflorae*	Anthoptilidae	*Anthoptilum*	0
	Funiculinidae	*Funiculina*	3
	Kophobelemnidae*	*Kophobelemnon**	3
		Sclerobelemnon	4
	Protoptilidae*	*Distichoptilum*	2
		Protoptilum	2
	Renillidae	*Renilla*	2
	Scleroptilidae	*Scleroptilum*	1
	Umbellulidae	*Umbellula**	1 and 4
Subselliflorae*	Halipteridae	*Halipteris*	4
	Pennatulidae*	*Pennatula**	2
	Pteroeididae*	*Gyrophyllum*	3
		Pteroeides	1
	Virgulariidae	*Virgularia*	1

Clade placement follows the tree in Fig. 2. Taxa marked with an asterisk are not supported as monophyletic by this analysis.

4.1. Umbellulidae

Umbellula spp. are morphologically distinct from all other pennatulaceans in that species of this genus have exceptionally large and localised polyps, situated at the most distal portion of the rachis. These traits are considered highly specialised and adapted towards macrophagy/carnivory, which has also occurred in several groups of sessile organisms as an adaptation to life in the deep sea where availability of particulate organic matter is limited, making suspension feeding energetically unfavourable (Gage and Tyler, 1991).

Umbellula are an exclusively deep-sea and cosmopolitan taxon, representing one of the more diverse groups (in terms of number of recognised species), thus supporting the hypothesis that some taxa radiated and diversified in the deep sea.

Our analysis suggests that species currently classified as *Umbellula* represent two different lineages that have undergone evolutionary convergence, indicating that localisation of feeding polyps is a homoplastic trait. Sclerite loss, as seen in one clade of *Umbellula* species, can be considered apomorphic within this clade, as these species evolved from a common ancestor, within a single lineage, in which sclerites were most likely present.

Two of the most important traits for the identification of *Umbellula* species are the cross-section shape of the axis and the presence/absence of sclerites. Those *Umbellula* species grouped in Clade 4 possess both a round axis and sclerites, whereas the *Umbellula* species grouped in Clade 1 do not exhibit this combination of characteristics. The molecular evidence that distinguishes these two groups of *Umbellula* species is therefore consistent with morphological differences between them. A more detailed study of the genus *Umbellula* is currently in preparation examining both molecular and morphological data to further resolve the systematics of this group of ecologically important species.

4.2. Pennatulidae

Pennatula are defined by the characters of tubular autozooids with spiculiferous calyces and eight terminal teeth; autozooids emanate from lateral leaves. Our phylogeny does not support the unique evolutionary development of these traits. However, it should be noted that the single *Pennatula* species breaking the monophyly of *Pennatula* was not identified to the specific level by McFadden et al. (2006).

Renillidae (the sea pansy) was considered a close relation to Veretillidae and Echinoptilidae based on morphology (Pérez and Ocampo, 2001). Our very limited data available for *Renilla* suggests a sister relationship with some members of Pennatulidae. However, taking into account the branch length leading to *Renilla*, long-branch attraction cannot be rejected, particularly given the lack of representatives of Veretillidae and Echinoptilidae.

4.3. Pteroeididae

Historically, there has been much discussion concerning how to classify the members of the families Pennatulidae and Pteroeididae. Kölliker (1869) originally unified the subfamilies 'Pennatulinae' and 'Pteroeidinae' into one family and then subsequently elevated the status of the subfamilies to separate families (Kölliker, 1880). Studer (1901) placed the new genus *Gyrophyllum* in the family Pteroeididae, but Kükenthal and Broch (1911) disputed this stating the presence of three-flanged sclerites are characteristic of the family Pennatulidae. Kükenthal (1915) made a distinction between Pennatulidae and Pteroeididae based on sclerite morphology. More recently, Williams (1995b) suggested that *Gyrophyllum* represents a morphological intermediate between the two families and proposed that Pennatulidae and Pteroeididae represent a single monophyletic taxon. On this basis, only one family was recognised, the Pennatulidae, comprising the six genera *Gyrophyllum*, *Pennatula*, *Ptilosarcus*, *Sarcoptilus*, *Crassophyllum*, and *Pteroeides*. Genetically, however, these do not form a natural group, and instead *Gyrophyllum* and *Pteroeides* (Pteroeididae), and *Pennatula* (Pennatulidae) are separated into three different clades. Further work incorporating more taxa is required to make any firm conclusions on the reclassification of these three genera. The two representatives of Pteroeididae (*Gyrophyllum* and *Pteroeides*) do not form a sister group.

4.4. Kophobelemnidae

Although the genetic data do not support the monophyly of *Kophobelemnon*, we cannot reject the hypothesis. There is a single taxon outlier, and it is noted that it could not be identified to the specific level. The case for the family is different. *Sclerobelemnon* is shown to be very distant from *Kophobelemnon*. Misidentification of *Sclerobelemnon*, for which the GenBank sequence was obtained, cannot be ruled out but we view this as unlikely given the specimen was identified by McFadden et al. (2006), who included, as previously stated, taxonomic experts for octocorals. It is more likely that the definition of genera and possibly the family require revision as suggested by the molecular evidence presented here.

4.5. Protoptilidae

The monophyly of Protoptilidae is not recovered in our phylogeny, but we cannot formally reject monophyly for this family. Previously, *Protoptilum* and *Funiculina* were considered members of closely related families (Kükenthal and Broch, 1911; Williams, 1997), but the molecular evidence strongly suggests that *Protoptilum* and *Distichoptilum* share a common ancestry with *Pennatula*.

4.6. Origins of pennatulaceans

The morphological characters examined in this study demonstrate multiple origins (Fig. 1), for example, the loss of sclerites occurred at least three times in the evolutionary history of this group. The plastic nature of such characters suggests that traditional classifications based on morphology may be unreliable. Loss of sclerites has been reported in other Octocorallia such as *Phenganax parrini* (Alderslade and McFadden, 2011) and *Acanthoaxis wirtzi* (van Ofwegen and McFadden, 2009). This will be examined in *Umbellula* in more detail in a future publication.

Kükenthal and Broch (1911) and Kükenthal (1915) developed a morphology-based higher classification scheme for the Pennatulacea, consisting of two sub groups (termed suborders in their work). The subgroup Subselliflorae comprised those pennatulaceans with a feather-like appearance where polyps are positioned on leaves or raised ridges. In contrast, Sessiliflorae lack leaves/ridges and instead polyps emanate directly from the rachis. Recent identification and comparison of characters of many pennatulaceans has shown that this higher classification scheme is problematic and largely inadequate (Williams, 1992, 1995a, 1997). Williams (1995a) suggested that Sessiliflorae should be considered paraphyletic, which is supported by our findings. The ancestral reconstruction suggests that the ancestral pennatulacean was Sessiliflorae-like, but the characters associated with this group have been lost on at least four separate occasions.

Williams (1995a) considered the Subselliflorae as monophyletic based on synapomorphy of polyp leaves. This analysis clearly separates the Subselliflorae into evolutionarily distinct lineages with independent histories.

The parsimony reconstruction of the depth character suggests that deep-water groups may have given rise to shallow-water lineages of pennatulaceans. However, the lack of veretillids in the present analyses, and the bias towards deep-sea taxa, prevent firm conclusions being drawn, but it does seem likely that multiple invasions of shallow-water ecosystems from the deep sea have occurred. This is in agreement with other studies. Lindner et al. (2008) found that stylasterid corals initially radiated in the deep sea but subsequently invaded shallow-waters multiple times, and more recently Pante et al., (2012) hypothesised that the shallowest members of *Chrysogorgia* (octocoral family Chrysogorgiidae) have evolved from deep-water ancestors.

Finally, we point out that this phylogenetic study is limited to two mitochondrial genes. Because of the lack of recombination in the mitochondrial genome these genes effectively share the same history (Ballard and Whitlock, 2004). This not only represents a small sample of the genome of the pennatulaceans investigated but because they are mitochondrial genes they are more sensitive to processes such as introgression or selective sweeps and mitochondrial genealogy may not be representative of a larger selection of genes or the evolutionary history of the taxa as a whole (reviewed in Ballard and Whitlock (2004)). A more comprehensive sampling of the genome to infer phylogeny, in other words the sequencing of nuclear genes, is desirable and must be part of future analyses of pennatulacean phylogeny. We are, however, encouraged by the apparent resolution offered by *ND2* and *mtMutS* and this study offers a step change in our understanding of the evolution and taxonomy of these ecologically important animals.

5. Conclusions

The genes *mtMutS* and *ND2* proved useful in differentiating between all genera, and many (if not all) pennatulacean species. The high frequency of morphological homoplasy in pennatulaceans, possibly driven by natural selection associated with the environmental characteristics of the deep sea, has led to many misinterpretations in the systematics of the group. Further work incorporating more taxa is necessary to provide better clarity on the phylogeny and systematics of the group. However, the traditional classification scheme for pennatulaceans requires revision.

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Appendix A. A summary of the origins of specimens gathered for this study

Samples were collected during the following research cruises: a suite of frozen and ethanol-preserved material obtained from the Benthic CROZET RRS Discovery cruise (D300) (National Oceanography Centre, Southampton); ethanol-preserved specimens collected aboard the Western Flyer with the ROV Tiburon (Monterey Bay Research Institute) off Monterey; several ethanol-preserved specimens provided by Edward McCormack (Marine Institute, Galway) from the NE Atlantic; five ethanol-preserved specimens from Marguerite Bay, Antarctica, collected aboard RRS James Clark Ross during JCR166 with the ROV Isis (National Oceanography Centre, Southampton/Scott Polar Research Institute); three ethanol-preserved specimens obtained from the NE Atlantic during HERMES cruises aboard RRS James Cook (JC10 and JC11) with the ROV Isis (National Oceanography Centre, Southampton); an array of specimens preserved in ethanol collected during the Oceans 2020 voyages, courtesy of National Institute of Water and Atmospheric Research (New Zealand); and a further two specimens were obtained from the Indian Ocean off Sumatra by Paul Tyler on board RV The Performer.

Additional material was obtained from a variety of sources: samples collected by means of SCUBA diving from Portland Harbour (Dorset, UK) and preserved in ethanol; ethanol-preserved tissue of an Arctic specimen acquired by Peter Lamont of the Scottish Association of Marine Science; two specimens donated by Hans G. Hansson, Tjärnö Marine Biological Laboratory, University of Gothenburg, taken from the Koster Channel, Sweden; eleven ethanol-preserved specimens from the Subantarctic courtesy of Rhian Waller (Woods Hole Oceanographic Institution); material obtained from the collections housed at the California Academy of Sciences, courtesy of Gary Williams; and further specimens were obtained through the Millport Marine Station, Scotland.

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