Comprehensive phylogenetic reconstruction of relationships in *Octocorallia (Cnidaria: Anthozoa)* from the Atlantic ocean using *mtMutS* and *nad2* genes tree reconstructions

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Abstract

Despite being an abundant group of significant ecological importance the phylogenetic relationships of the Octocorallia remain poorly understood and very much understudied. We used 1132 bp of two mitochondrial protein-coding genes, *nad2* and *mtMutS* (previously referred to as *msh1*), to construct a phylogeny for 161 octocoral specimens from the Atlantic, including both *Isididae* and non-*Isididae* species. We found that four clades were supported using a concatenated alignment. Two of these (A and B) were in general agreement with the of Holaxonia—Alcyoniina and Anthomastus—Corallium clades identified by previous work. The third and fourth clades represent a split of the Calcarellinae—Pennatulacea clade resulting in a clade containing the Pennatulacea and a small number of Isididae specimens and a second clade containing the remaining Calcarellinae. When individual genes were considered *nad2* largely agreed with previous work with *MtMutS* also producing a fourth clade corresponding to a split of *Isididae* species from the Calcarellinae—Pennatulacea clade. It is expected these difference are a consequence of the inclusion of Isididae species that have undergone a gene inversion in the *mtMutS* gene causing their separation in the *MtMutS* only tree. The fourth clade in the concatenated tree is also suspected to be a result of this gene inversion, as there were very few Isididae species included in previous work tree and thus this separation would not be clearly resolved. A larger phylogeny including both *Isididae* and non-*Isididae* species is required to further resolve these clades.

1 Introduction

The Octocorallia (*Cnidaria: Anthozoa*) are cosmopolitan in the marine environment, occurring in marine habitats ranging from the Arctic to the Antarctic and from intertidal to abyssal depths. An estimated 65 % of Octocorallia occur in cold water environments. (Sánchez and Lasker, 2003; Roberts et al., 2009; Morris et al., 2012). Octocorals can grow individually or as coral gardens and are important structural components of many marine habitats (McFadden et al., 2010). The evolutionary history of octocorals remains poorly understood as a result of problematic taxonomic distinctions (Bayer, 1981). The current taxonomic system in use was proposed by Bayer (1981), identifying three Octocorallia orders: Pennatulacea (sea pens), Helioporacea (blue corals), and Alcyonacea (soft corals gorgonians and stoloniferous forms) (see review by McFadden et al., 2010). However, morphological identification has difficulties; very few deep-sea coral species have taxonomic descriptions published and many which are published lack informative illustrations, or any illustrations at all (Sánchez, 2007). Features that have traditionally been used to identify the vast number of species include: growth form, axis composition, shape and arrangement of sclerites (McFadden et al., 2010). However, with high levels of phenotypic plasticity and homoplasy hindering attempts at understanding evolutionary relationships (Williams, 1992; Roberts et al., 2009), there has been a steady increase in the use of molecular taxonomic approaches (France et al., 1996; Berntson et al., 2001; Sánchez and Lasker, 2003; Sánchez et al., 2003; McFadden et al., 2004, 2006b, 2010, 2011; Wirshing et al., 2005; France, 2007). Despite the now more widespread use of genetic tools the phylogenetic relationships within Octocorallia still remain challenging, with taxonomic revisions occurring frequently (McFadden et al., 2006a, 2010).

Species- and population-level phylogenetics of Octocorallia lags behind most other invertebrate groups (McFadden et al., 2010). Early phylogenetic work, based on 18S and 28S rDNA markers supported a monophyly within the Octocorallia and Hexacorallia (McFadden et al., 2010). The *mtMutS* gene (previously referred to as *msh1*), which codes for a DNA mitochondrial mismatch repair protein homologous to the bacterial *mutS*, was later discovered to be a synapomorphic character in octocorals (McFadden et al., 2010; Bilewitch and Degnan, 2011). The *mtMutS* gene has been found in all octocoral families, but is yet to be found in any other metazoans (Culligan et al., 2000). Evolutionary rates in Octocorallia are often 50 to100 times slower than in the mitochondrial genomes of most other eukaryotic organisms (Shearer et al., 2002; Hellberg, 2006; McFadden et al., 2011). *mtMutS* has been shown in some cases to reach
twice the amount variation found in other protein-coding regions in the octocoral mitochondrial genome (France and Hoover, 2001; Wirshing et al., 2005; Van der Ham et al., 2009; McFadden et al., 2010), making it a suitable marker for determining phylogenetic relationships among Octocorallia (e.g. McFadden et al., 2006; Herrera et al., 2012). The nad2 gene (NADH dehydrogenase subunit 2) also codes for a mitochondrial protein involved in the cellular respiration (Nakamaru-Ogiso et al., 2010), and has also been widely used in phylogenetic analyses of groups of octocorals (Shearer et al., 2002; Wirshing et al., 2003; Sánchez and Lasker, 2003; McFadden et al., 2006b). nad2 and mtMutS genes have both been used in the most comprehensive phylogenetic reconstructions of Octocorallia by McFadden et al. (2006b), Wirshing et al. (2003) and Sánchez and Lasker (2003).

nad2 and mtMutS genes have both been used in the most comprehensive phylogenetic reconstructions of Octocorallia by McFadden et al. (2006b), Wirshing et al. (2003) and Sánchez and Lasker (2003). The most complete phylogenetic analysis of Octocorallia to date was carried out by McFadden et al. (2006b) using partial sequences of nad2 and mtMutS for 115 genera encompassing 46 families. Their results indicated that there were two large distinct clades, one including Holaxonia, some Scleraxonia and Stolonifera, and the other including Pennatulacea, Heliopora and Calcaxonia. Additionally, there was a third smaller unresolved clade that included families from Alcyoniidae and Corallidae. These results mainly agreed with previous studies by France et al. (1996), Berntson et al. (2001) and Sánchez et al. (2003). All studies differed slightly in the topographical form of which clades occurred, nevertheless there was a general agreement throughout. However, none of these studies showed consistency of the phylogenetic hypothesis with the traditional ordinal divisions placed within the Octocorallia (Bayer, 1981; McFadden et al., 2006b).

There has never been an in depth study of Octocorallia from the Atlantic, with previous studies focusing on specimens predominantly from the Pacific and tropical origin. In this study, we have created a phylogeny for Octocorallia from the Atlantic using partial nad2 and mtMutS genes using newly collected specimens and publically available sequences, with the inclusion of both Isididae and non-Isididae octocoral species and an increased number of deep-sea specimens. This has produced a larger phylogeny than has previously been published to specifically address the following questions: (a) Do the alignments of individual genes produce different phylogenetic predictions from those of a concatenated alignment tree? (b) Does the phylogeny prediction from a concatenated alignment trees relate closely to current taxonomic groupings? (c) How do the trees produced relate to previous phylogenetic predictions? (c) and (d) Is there evidence of bathymetric distribution within the predicted clades in the Octocorallia?

2 Materials and methods

A total 161 octocoral specimens were included in this study of which 87 were new. These were collected from a range of sources and depths (Supplement Table 1) in the North Atlantic and Caribbean basins. All existing sequences were retrieved from the GenBank nucleotide database. The majority of the new specimens were collected using the remotely operated vehicle (ROV) Isis during R.R.S. James Cook cruises JC24 along the Mid-Atlantic Ridge (MAR) at 45°N 27°W, and JC36 within the Whittard Canyon 48°15′N, 10°30′W. Other specimens were obtained from the R.V. Celtic Explorer cruise CE10014 within the Rockall Trough and the Belgica Mound in the North Atlantic using the ROV Holland. Additional specimens were obtained from the Bahamas and Antarctic, more still were recovered from the MAR obtained from R.R.S. James Cook cruise JC48 and from the Azores collected using Lula 500 submersible and as by catch of fisheries. New samples were also obtained from the collections of the National Museum of Natural History (NHNMM) of the Smithsonian Institution (SI) (Washington DC, USA) and the National Institute of Water and Atmospheric Research (NIWA, Wellington, New Zealand) (Fig. 1).

Specimens were identified using species descriptions and keys where possible to the highest possible level, the small amount of tissue made this difficult in some places. Guidance from Watling at the University of Hawaii, was sought on difficult specimens
Phylogenetic analyses were performed on three data sets: (1) concatenated *mtMutS-nad2* and (2) *mtMutS* only (3) *nad2* only. The most appropriate models of sequence evolution were selected using the Akaike Information Criterion in MrModeltest 2.3 (Nylander, 2004). Bayesian phylogenetic analysis was conducted using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) as implemented in the CIPRES Science Gateway portal (Miller et al., 2010), each data set was run using four chains with two parallel runs set at a temperature of range of 0.02–0.3 until the average standard deviation between the split frequencies was less than 0.01 corresponding to 150–200 million generations dependent on the tree. Other convergence diagnostics were examined as in Herrera et al. (2010). Maximum parsimony models were run using PAUP* 4.0b (Swofford, 2002) with 1000 bootstrap replicates. Maximum-likelihood analysis was performed using GARLI 2.0 as implemented in the CIPRES Science Gateway portal (Miller et al., 2010), and 1000 bootstrap replicates were run in RAxML 7.3.2 (Stamatakis et al., 2008). Resulting trees were visualized using FigTree v3.1 rooted with *Erythrophodium sp.* as suggested by McFadden et al. (2006b). Once rooted, tree topologies within a data set were compared and a consensus tree created. Finally, levels of species identification and intra and inter-specific variation were examined following calculations of pairwise genetic distances (uncorrected $p$) on PAUP* 4.0b (Swofford, 2002) for the least conserved gene *mtMutS* dataset, as suggested by McFadden, et al. (2011).

3 Results

A total of 1132 nucleotide bases were aligned to generate the concatenated phylogenetic prediction. This consisted of 709 base pairs including indels from *mtMutS* with fragments ranging from 159 to 191 amino acids in length (477–573 nucleotides). 572 characters were parsimony-informative. The *nad2* portion contained 366 parsimony-informative characters out of a total of 423 base pairs including indels. Of these, 154 (36.41 %) sites were without polymorphism with the alignments ranging from 134 to 138 amino acids in length (402–414 nucleotides). Despite the presence of indels, nucleotide
sequences of both genes and the combined data set maintained the correct reading frame indicating a continuation in the coding function of the genes. Within the concatenated alignment 447 (39.49 %) sites were invariant and 647 sites were parsimony informative. The majority of sequence length variation in alignments can be attributed to large indels present within 

Bayesian, maximum parsimony (MP) and maximum-likelihood (ML) analyses all recovered very similar topologies within the different analyses allowing consensus trees to be produced. A monophyletic origin of all Octocorallia species was observed on the concatenated tree, this was also observed in the individual trees (Supplement Figs. 1 and 2). The first of these clades (A) (Fig. 2) which had high branch posterior probability support (> 0.9) and high bootstrap support (> 90 %) contained all specimens from the Parargorgiidae and Coralliidae. There was a grouping of the shallow species (50–500 m) indicated by the colour-coded names (pink colour) separated from those that are found in deeper water. Clade B (Fig. 2) was the largest of the clades and contained all specimens belonging to the sub-order Holaxonia as well as some Alcyoniinae; again this was a well-supported clade (> 0.9, > 90 %). Very few of the new sequences generated in this study occur within this clade. Acanthogorgia spp. appear at the top of the clade but were not very well resolved, with the shallower species being separated from the deeper specimens. Sample Ma33 representing a new undescribed species (as concurred with Watling, University of Hawaii) occurred within this clade and was grouped with samples belonging to the family Alcyoniinae. Eunicea species appear well clustered; however, there were a few changes in the position of the given species between tree topologies as highlighted in Fig. 2. The majority of this clade occurred at a depth of 0–50 m, with deeper samples tending to group together at the top of the clade. Individual branches were not always well supported. The third clade (C) was less well supported with a posterior probability of 0.87 and a bootstrap support of 61.5 %. This clade was made up almost entirely of sequences newly obtained in this study and belonging to the families Isididae and Chrysogorgiidae. Species of the family Isididae appear to be monophyletic. Whilst there is generally a good grouping between genera, there were some discrepancies where sub-clades contain more than one genus. This indicates taxonomic and molecular discrepancies and is supported by the fact that although sub-clades were well supported the branches were not. Radicipes spp. appear well grouped with all samples occurring in one well supported sub-clade. The fourth and final clade (D) is a small well supported clade occurring at the base of the tree (< 0.9 and 90 %). This group contained all specimens of Pennatulaceae included in the analysis. This also contained two samples of the family Isididae, a branch that is also well supported. When just the nad2 sequences were considered three clades were present (Supplement Fig. 1). These corresponded to Clade A, B and C within the concatenated tree with Clade D becoming subsumed within Clade C. Pennatulacea, falling in Clade C, were strongly grouped with some Acanella species, away from the main sub-clades. Individual branches within the clade C differed from the concatenated tree with reduced resolution in Isididae, resulting in an increase in the number of branches but lower branch support. Isididae species appear to be polyphyletic. Isididae genera were spread throughout the clades rather than grouped together indicating poor resolution at the genus level. Radicipes spp. were clustered in a well-supported monophyletic sub-clade, other Chrysogorgiidae species were also well grouped within well supported branches, indicating good resolution at genus level within the Chrysogorgiidae. Clade A and B are fairly consistent between the different analyses.

When the single gene tree for mtMutS was considered (Supplement Fig. 2), clades A and B from the concatenated tree remain intact with slight rearrangement of branches. These clades had high posterior probability (> 90 %; Supplement Fig. 2). However, there was a significant rearrangement of clades C and D. Clade C was split into two clades with all Isididae samples separating from the Primnoiidae and Chrysogorgiidae. Clade D became nested with the Isididae clade. These new clades were not very well supported with the separation only having a posterior probability
of 0.5. Despite this the separation of the *Isididae-Pennatulacea* and *Primnoidae-Chrysogorgiidae* from clade B is highly supported.

Pairwise genetic distances of uncorrected *P* for *mtMutS* gene showed an intraspecific variation ranging from 0 to 1.57%, whereas the congeneric uncorrected genetic distances varied from 0 to 13.73% (Fig. 3). Moreover, an overlap of sequence divergences on both family and subclass levels was detected, with values ranging from 0 to 18.11% and 0 to 24.16% respectively (Fig. 3).

4 Discussion

All tree topologies between different analyses were similar and generally well supported, indicating they are robust. However there are differences between the concatenated tree and the individual gene trees. Although all topologies agree on the occurrence of a monophyletic origin within the order *Pennatulacea* the placement of this differs between them. Within the *nad2* only tree *Pennatulacea* group in clade C to produce a *Calcaxonia–Pennatulacea* clade. However in the concatenated tree a fourth clade, not present within the *nad2* tree, represents a separation of the *Pennatulacea* and some *Isididae* species from the *nad2* only *Calcaxonia–Pennatulacea* clade, with the majority of the *Isididae* species remaining within the Calcaxonia clade (Fig. 2 and Supplement Fig. 1). The *mtMutS* tree (Supplement Fig. 2) also produces 4 clades in which the *Pennatulacea* are once again separated from the *Calcaxonia–Pennatulacea* clade, in this case they are grouped with the *Isididae* to produce two new clades – viz: *Isididae–Pennatulacea* and *Primnoidae–Chrysogorgiidae*.

This change in the clade assignment between the different trees could be a result of the inclusion of a large number of *Isididae* species within the study, which as previously stated have an inverted gene order with the *mtMutS* region, expected to have occurred in one event. This results in these species being unable to be amplified by the standard Octocoral *mtMutS* primers and must be amplified with Keratoisidinae specific primers (Brugler and France, 2008). This inversion leads to difficulties in aligning samples from a range of Octocoral species, resulting in the inclusion of a large number of indels within the alignment. This difficulty maybe a reason why there are very few studies that have dealt with both *Isididae* and non- *Isididae* species using the *mtMutS* gene; with a tendency to deal with the *Isididae* separately from other octocoral families (McFadden et al., 2006b; France, 2007). This inversion and the occurrence of a number of indels within the Keratoisidinae results in a predicted monophyletic origin within this sub-family on *mtMutS* only gene. The *nad2* gene within the Octocorals did not show this variation between the *Isididae* and non- *Isididae* species and was well conserved across species. This lead to the production a polyphyletic origin of *Isididae* species within both the concatenated tree and the *nad2* only tree. The increase in variance would indicate that *mtMutS* is a more informative gene for barcoding than *nad2*, with the *nad2* gene order appearing to be conserved (Beaton et al., 1998).

The concatenated tree has a higher resolution than either of the single gene trees as a result of higher number of informative sites which lead to an increased number of branches with a high level of support. This would indicate that most *Isididae* do have a polyphyletic origin. This would be in agreement with Duenas and Sánchez (2009) who believed that the high levels of lability within the *Isididae* indicate that they have evolved independently multiple times. McFadden et al. (2010) also noted that there has been increased evidence of polyphyletic origins in *Isididae* as well as other coral families from unpublished data.

Sub-clades do not tend to represent the ordinal family separations based on morphological segregations. This is in agreement with Wirshing et al. (2005) who found that although all phylogenetic reconstructions produced similar topologies, none of them agreed with familial arrangements hypothesized by Bayer (1981). Some sub-clades are more highly resolved than others; the *Chrysogorgiidae*, especially *Radicipes* species, grouped well in all treatments within this study. *Acanthogorgia* species were found to be polyphyletic occurring in more than one sub-clade within Clade B. This is in agreement with McFadden et al. (2006b) who showed two distinct lineages of *Chrysogorgiidae*. *Isididae* species are well grouped within all phylogenetic reconstructions.
produced. However, at genus level resolution becomes less defined with some subclades containing more than one genus i.e. Clade B (Fig. 2) where Acanthogorgia and Paramuricea occur in the same sub-clade; this is in agreement with France (2007) who found not only different genera occurring throughout sub-clades but also different branching type, indicating again that morphological identifications do not always agree with molecular ones.

When the pairwise genetic distances were calculated it was shown that intra-specific variation ranged from 0 to 1.57 %, with congeneric distances reaching up to 13.73 % and subclass levels of up to 24.16 %. The overlap of divergences on both family and subclass levels detected (Fig. 3) would indicate that despite mtMutS having approximately twice the level of variation of other protein coding genes in the mitochondrial genome (France, 2007; McFadden et al., 2010), it does not have a high enough resolution to separate out individual species. As a consequence of this, phylogenetic analyses are more likely to identify family differences than allow the identification of new species. However, intra-species differences rarely exceed 0.5 % and thus it has been argued that anything above that should be regarded a different species (McFadden et al., 2011). This is in agreement with Van der Ham et al. (2009) and McFadden et al. (2010, 2011) and is illustrated once again by the unclear separation of species within branches on the taxonomic tree (Fig. 2). This indicates that mtMutS is not suitable as a single gene for a barcoding. However, it must also be noted that it is quite common for genetic and taxonomic identification to disagree with one another and thus it is important that more joint work is completed to allow a more comprehensive picture of the phylogenetic and taxonomic relationships to be attained. (Van der Ham et al., 2009; McFadden et al., 2010, 2011).

Both the concatenated tree and the single gene nad2 tree clades are largely congruent with those produced by McFadden et al. (2006b) who found that there were two main clades of Octocorals; clade 1 (Holaxonia–Alcyoninna) and clade 2 (Calcaxonia–Pennatulacea). This outcome is also in agreement with Sánchez et al. (2003) who, using 16S and 18S markers, found two distinct clades – one containing Calcaxonia and Scleraxonia and the other Alcyoniina, Holaxonia and again Scleraxonia. McFadden et al. (2006b) clade 1 also closely corresponds to Berntson et al. (2001) clade C using the 16S and 18S genes; these were both the equivalent of the Clade B found within this study. McFadden’s clade 2 (Calcaxonia–Pennatulacea) corresponded with Clade C within this study for the separate trees. However, McFadden et al. (2006b) did not agree on the presence of a fourth clade within a concatenated tree and also considered these to be incorporated within the large clade C. This difference could be a result of increased sample numbers within this study, with 161 sequences in comparison to the 115 used by McFadden et al. (2006b), as well as the inclusion of a large number of Isididae as previously discussed.

McFadden et al. (2006b) also found a third small clade (Anthomastus–Corallium) corresponding to Clade A in the present study. In this study Paragorgidae species were also included in this clade. This finding is in agreement with Herrera et al. (2010) who found that there was strong support for a monophyly of Coralliidae species and Paragorgia species. No species of Paragorgidae were included in Sánchez and Lasker (2003), Wirshing et al. (2005) or McFadden et al. (2006b) so they cannot be compared with this finding.

From the trees it is possible to see a large grouping of the shallow water species within clade B of all treatments (Fig. 2, Supplement Figs. 1 and 2). Within clade A there was a grouping of the 50–500 m species at the bottom of the clade with the deeper species being grouped at the top of the clade. However, in clade C where there was a larger range of depths there are no clear groupings. This would indicate that the shallow water species tend to group together away from the deeper specimens, but no fine scale patterns are discernible in the deep-specimens. This would be in general agreement with Watling et al. (2011) who noted that within the phylogeny created by McFadden et al. (2006b) there were ten deep-sea species which all grouped together in a single clade, away from shallower specimens. These data support the contention that deep-sea corals have under gone in-situ radiation (Watling et al., 2011). However, when considering individuals from the same deep sea species Herrera et al. (2012)
found that depth did not appear to have a large scale impact on the structuring of a population, which would agree with the finding that there is no small scale pattern observed in the deeper specimens. Pante et al. (2012) indicate that there is a change in the Chrysogorgiidae species present deeper than 200 m than in shallower waters, but also do not describe any small scale grouping at depth. This has also been observed in the deep-sea prosobranch bivalve Deminucula atacellana, where populations above and below 2500 m are genetically distinct, resulting in high level diversification within the deep-sea (Chase et al., 1998). This would all indicate that there is a difference in the genetic makeup of shallow water species versus deep-water species but no finer scale detail is discernible.

5 Summary

This study has shown that to allow accurate Octocorallia taxonomic prediction to be made it is essential to use highly resolving markers. This was achieved by concatenating both the nad2 and mtMutS markers, resulting in a predicted fourth clade which has not been observed in previous studies. It is suspected this is a result of the increased number of species used and the inclusion of both Isididane and non-Isididae species. Overlap in the pairwise genetic divergences of both family and subclass level indicate that despite being the best marker for phylogenetic relationships at present mtMutS by itself does not offer high enough resolution to distinguish species-level differences, leading to a degree of disagreement between taxonomic and phylogenetic relationships. All trees indicated a general separation of shallow water species from deep-water species within the same clade, but no fine scale patterns with increased depth were identified, even from the concatenated prediction. The present approaches presented herein do allow a better evolutionary understanding of the Octocorallia; nonetheless, there is a need for further work to identify a superior marker for greater resolution, potentially including consideration of micro-satellite markers. Furthermore, it is clear that there is a continued need to revisit morphological taxonomic separations.

Supplementary material related to this article is available online at: http://www.biogeosciences-discuss.net/9/16977/2012/bgdi-9-16977-2012-supplement.zip.

Acknowledgements. We would like to thank Catherine McFadden for helpful insights and providing data to allow the completion of this manuscript and Scott France for provision of data and primer advice. We would also like to thank those involved in the procurement of the samples used within the study, Claudia Alt (University of Southampton), Dan Jones (NERC), and Kirsty Kemp (Zoological Society of London). In part, funding for this analysis was provided by the European Community’s Seventh Framework Programme (FP7/2007–2013) under the HERMIONE project, grant agreement no. 226354 and NERC studentship award NE/F009097/1.

References


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**Fig. 1.** Collection locations of the new Octocoral samples used within this study. Missing samples are the result of no geo-referencing data being available.
**Fig. 2.** Concatenated phylogenetic tree using mtMutS and NAD2 genes in Atlantic octocorals. *** indicates branches with > 90% Bayesian support and high MP support. Those branches with two species named indicate differences between Bayesian and MP tree topologies. Colours indicate depth of specimen collection.

**Fig. 3.** Uncorrected genetic distances $P$ for mtMutS of Octocorallia found in the Atlantic.