Molecular Phylogenetics and Evolution 64 (2012) 56–65

Contents lists available at SciVerse ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

The Melithaeidae (Cnidaria: Octocorallia) of the Ryukyu Archipelago: Molecular and morphological examinations

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ARTICLE INFO

Article history:
Received 8 August 2011
Revised 7 March 2012
Accepted 8 March 2012
Available online 21 March 2012

Keywords:
Melithaeidae
Octocoral
Systematics

ABSTRACT

The family Melithaeidae (Octocorallia: Alcyonacea) is distributed in the West Pacific, Indian Ocean and the Red Sea. They are most abundant in warmer waters but can also be found in temperate waters. At present six genera are assigned to this family (*Melithaea*, *Mopsella*, *Clathraria*, *Acabaria*, *Wrightella* and *Asperaxis*), however overlapping characteristics make this group's taxonomic identification difficult and their relationships unclear. There are only a few reports from the Ryukyu Archipelago in southern Japan of melithaeids and most other octocorals, despite the islands being an area of high octocoral diversity. To help resolve the taxonomic confusion in this family, samples from various Ryukyu Archipelago locations were collected and DNA sequences of nuclear 28S ribosomal DNA and mitochondrial cytochrome oxidase I (COI) were obtained. Additionally, SEM micrographs of the sclerites of specimens were taken to further confirm the molecular results. Three strongly supported clades were recovered from the COI and 28S rDNA analyses, corresponding to *Melithaea, Acabaria*, and *Mopsella*, and in most cases clades were clearly related with the sclerite shape reported for each genus. These results show clearly that molecular differences are present between the three genera, and also demonstrates the strong need of other molecular markers for resolving intra-generic phylogenies. Our results provide baseline data for future studies of this octocoral family, not only on taxonomy, but also with regards to their distribution in the Ryukyu Islands.

1. Introduction

Melithaeids (Melithaeidae: Octocorallia: Anthozoa: Cnidaria) are suspension feeding octocorals, and usually found on overhangs or in cracks and on reef walls where strong currents are present. They are generally found at depths from 1.5 m to 35 m, but have been found at depths greater than 200 m. Their colony size is variable, from a few centimeters to large fans that exceed 1 m in height. Melithaeids have been exploited as jewelry and decoration, and for this reason their populations are in decline (Muzik, personal communication). As components of coral reef ecosystems they are found in association with crinoids, copepods, seaworms and other organisms (Kumagai and Aoki, 2003), but comparatively little is known about their ecology, with for instance only a few studies describing their association with copepods (e.g., Kumagai, 2008).

Species in the family Melithaeidae are characterized by the presence of swollen nodes followed by rigid internodes (Fig. 1a and b), and having axes composed of cigar-shaped sclerites. Their colony shape, color and growth forms are unique to this family, but these characteristics are not diagnostic for generic or species level identification within Melithaeidae (Fabricius and Alderslade, 2001). Melithaeids are widely distributed in marine environments, and in Japan they are found not only in subtropical Okinawa, but also on the Pacific coasts of Kyushu, Shikoku and Honshu, and this group is not limited to tropical/subtropical waters (Fabricius and Alderslade, 2001). Few reports are available on the melithaeids of the Ryukyu Archipelago, as is the case for most octocorals (but see Imahara, 1991; Iwase, 1999). In 1909, Kükenthal described six *Melithaea* species and six *Acabaria* species from mainland Japanese waters, and since then melithaeid studies have continued to focus on the Japanese main islands, with some regions such as Sagami Bay (Iwase and Matsumoto, 2006; Matsumoto et al., 2007) having been widely investigated.

Melithaeid species descriptions include morphological characteristics such as colony size, colony color, polyp and calyx color,

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http://dx.doi.org/10.1016/j.ympev.2012.03.005

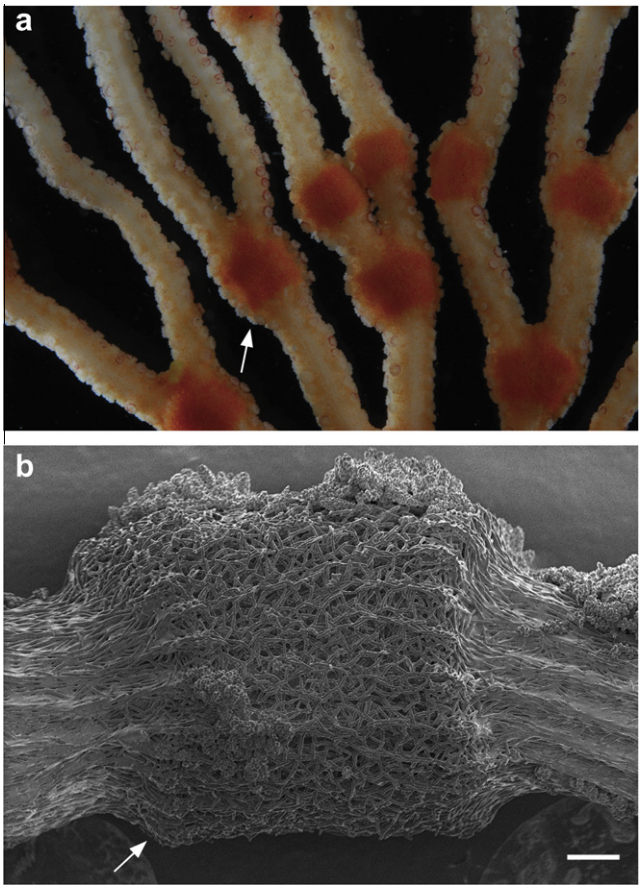

Fig. 1. Melithaeidae nodes. (a) A close up of a melithaeid (specimen 251ca; 22°80′S–113°69′E) from Ningaloo Reef, Australia. Specimen's axis with orange nodes followed by rigid white internodes. (b) Electronic micrograph of *Acabaria* (specimen 75ca) showing node sclerites. Scale bar = 0.2 mm. Arrow indicates the node.

type(s) of branching, presence or absence of anastomoses (connections between parts of the branches), degree of projection of calyces from the axes, calyx location in the colony branch (on three sides or on all the sides of the axis), and size of the internode (axis nodes are followed by rigid internodes) (van Ofwegen, 1987; van Ofwegen et al., 2000). However, these characters are variable and to properly describe or identify genera within this family sclerite descriptions are considered necessary. Thus, for melithaeid morphological identification, as for most other families in Alcyonacea, sclerite characteristics are utilized. Octocoral sclerites are made of calcite and are found in the coenenchyme of the colony where they provide support and protection. Their shapes, sizes and arrangement in the colony are the main diagnostic characteristics utilized in octocoral systematics, and have been used to establish the currently described six genera of melithaeids (van Ofwegen, 1987; Fabricius and Alderslade, 2001). The predominant sclerite forms within each melithaeid genus are described as follows: *Acabaria* Gray, 1859 rods and spindles, *Melithaea* Edwards and Haime, 1857 capstans, double-discs, and small clubs, *Mopsella* Gray, 1857 leafy spheroids and foliate capstans, *Clathraria* Gray, 1857 modified capstans, foliate spheroids, capstans and spindles, *Wrightela* Gray, 1870 foliate capstans and foliate spheroids and *Asperaxis* Alderslade, 2006 (1 sp.) rods and spindles with complex tubercles (Fabricius and Alderslade, 2001; Alderslade, 2006).

New melithaeid species have been found to have intermediate states and types of sclerites, with characters in between those originally used as diagnostic characteristics for the different genera, making new species' descriptions difficult (Alderslade, 2006). Most Melithaeidae species have many different types of sclerites that are found in all melithaeid genera. For example the genus *Clathraria* does not have a clear and predominant type of sclerite (van Ofwegen, 1987). Due to these complex and often conflicting relationships it is said that subordinal groups in Alcyonacea may represent grades of colony architecture rather than true clades (Fabricius and Alderslade, 2001).

The recent development of molecular techniques have allowed new examinations of octocoral systematics, and morphological variation has been compared to genetic variability, helping to resolve several taxonomic problems (van Ofwegen and Groenenberg, 2007; Vargas et al., 2010). In Melithaeidae, despite 105 described species only 13 sequences are currently found in GenBank (accessed June 2, 2011), demonstrating the lack of molecular studies for this group. At present, there have been no molecular studies focusing specifically on Melithaeidae, which means their molecular relationships with other octocoral groups, as well as their intrafamilal relationships, remain unknown.

Thus, there is a clear need to re-examine relationships within the Melithaeidae using not only morphological data but also molecular techniques. Although octocoral descriptions still are often based on morphological examinations only (e.g., López-González et al., 2002; Dautova, 2007) an increasing number use molecular data as well (van Ofwegen and McFadden, 2010; Alderslade and McFadden, 2007, 2011). Nevertheless, the use of molecular tools has thus far been limited to only a few relatively conservative molecular markers such as: mitochondrial cytochrome oxidase subunit I {COI} (but see Herrera et al., 2010 for a large set of markers), the mismatch repair homolog {msh1} (Wirshing et al., 2005; McFadden et al., 2006; Vargas et al., 2010), and the more quickly evolving internal transcribed spacer 2 of nuclear ribosomal DNA (ITS2) (Aguilar and Sánchez, 2007; Dueñas and Sánchez, 2009), which can have intragenomic variation (Sánchez and Dorado, 2008). While COI has been extensively used in bilaterians (Hebert et al., 2003), for anthozoans it has been found that COI divergence in this group is relatively low, with average interspecific distances of 1.42% (uncorrected mean), compared to 12.25% in Hydrozoa and 11.3% in congeneric Bilateria (Huang et al., 2008). Thus, although COI's utility as a species-level marker for anthozoans may be in question, its utility for phylogeny at the genus level and higher is unquestioned. Furthermore, different molecular markers seem to work well for different octocoral groups, and their variation rates can be different among different octocoral groups (McFadden et al., 2010), making octocoral systematics still difficult to comprehensively address (Berntson et al., 2001) using molecular methods.

The relatively unexplored melithaeid fauna of the Ryukyu Archipelago provide an ideal opportunity to examine morphological and molecular data in this understudied group of octocorals. We utilized the mitochondrial COI and nuclear 28S ribosomal DNA, and compare these genetic data to acquired morphological (sclerite) data in order to explore the relationships within the Melitheaidae specimens found throughout the Ryukyu Archipelago.

2. Materials and methods

2.1. Sampling

One hundred and six melithaeids and five additional octocorals (utilized as outgroups) (Supplementary material Table 1) were collected from October 2008 to May 2010 from 25 different locations in the Ryukyu Archipelago (see Fig. 2) by means of SCUBA diving with additional samples acquired from the Okinawa Churaumi Aquarium collection. The 111 samples are currently curated at

the Molecular Invertebrate Systematic and Ecology Laboratory (MISE) at the University of the Ryukyus, Okinawa, Japan, and will be deposited in appropriate institutions within Japan and abroad once species descriptions are complete (currently underway). Most samples were collected from depths of 1.5–35 m around Okinawa Island. Up to 15 cm samples of each colony were collected with scissors or by hand, and *in situ* pictures (with an Olympus μtough 8000) were taken with a scale to record colony size. Specimens were labeled, preserved in 90% ethanol, and brought back to MISE.

2.2. DNA extraction, amplification, and sequencing

DNA was extracted from 106 melithaeid specimens and five octocorals utilized as outgroups using the DNeasy Tissue and Blood Kit (Qiagen, Tokyo) and following the manufacturer's protocol. Before the addition of proteinase K the sample was homogenized using a 1.5 ml-sized pestle. Subsequently the DNA was diluted to 1/20 of original concentration.

PCR was performed for mitochondrial cytochrome oxidase subunit I (COI) using the primers COII-8068F (5′-CCATAACAGGACTAGCAGCATC-3′; McFadden et al., 2004) and COIOCTr the reverse complement of COIOCTf, (5′-ATCATAGCATAGACCATACC-3′; France and Hoover, 2002) and a COI fragment of 775–784 bp was amplified. Reactions were carried out in 20 μl, with 10 μl of ReadyMix, 7 μl of pure water, 1 μl of each primer, and 1 μl of template. PCR conditions for COI were: a denaturation step of 95 °C for 5 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 40 °C, and 1.5 min at 72 °C, followed by 7 min at 72 °C.

Nuclear 28S ribosomal DNA (28S rDNA) was amplified using the primers 528soctoF (5′-AGTAATGGCGAATGAAGAGGGAACA-3′) and 2D28octoR (5′-ATRGAGCCGTATGGYCGTCA-3′) designed from a 28S rDNA alignment of sequences from other octocorals, and a fragment ranging in length between 577–579 bp in length was amplified. The molecular marker for the msh1 (mismatch repair homolog) gene was also used in this study, but the fragment did not consistently amplify for all specimens. PCR conditions for 28S rDNA were: a denaturation first step of 94 °C for 5 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 50 °C, and 1.5 min at 72 °C, followed by 7 min at 72 °C.

All PCR products were then purified by adding 0.30 μl Exonuclease I, 0.15 μl of Shrimp Alkaline Phosphatase (SAP) and 2.55 μl of TE buffer (3.00 μl in total) to each PCR product, and run at 37 °C for 20 min, followed by 83 °C for 30 min. Sequencing was done on an ABI machine. Consensus sequences (sequencing from both directions) were done assembling the two complementary DNA chromatograms in BIOEDIT (Hall, 1999).

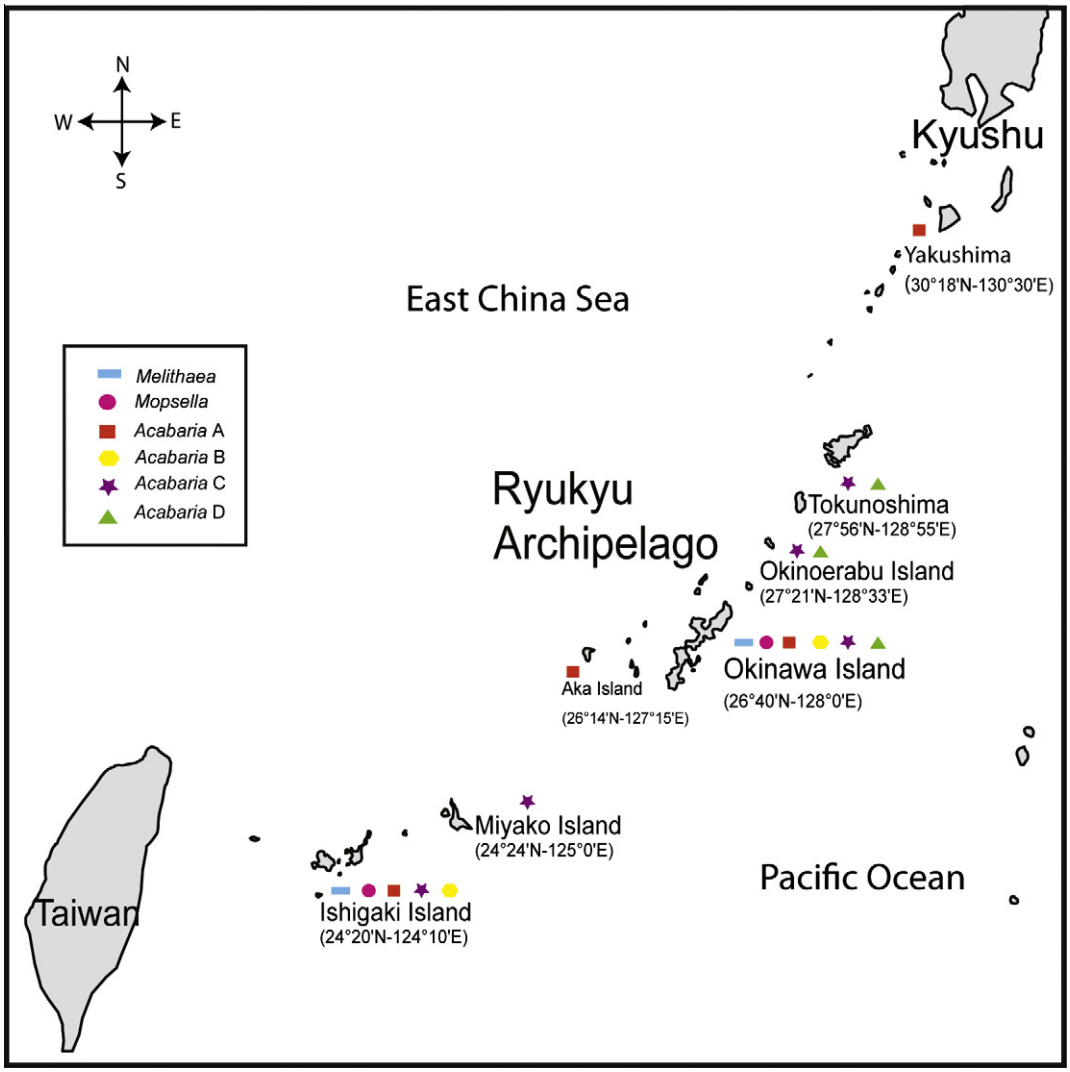

Fig. 2. Map of the Ryukyu Archipelago indicating the major islands where samples were collected. Colors represent each clade found at each respective location.

2.3. Sequences and phylogenetic analyses

All sequences were aligned using MAFFT default settings (Katoh and Toh, 2008) and corrected manually in BIOEDIT (Hall, 1999), and subsequently alignments of 783 bp for COI and 583 bp for 28S rDNA were obtained. The COI and 28S rDNA alignments were concatenated to obtain a 1364 bp alignment (designated "COI-28S combined"). Using the three final alignments (COI, 28S rDNA, combined) phylogenetic analyses were conducted under the following settings: (1) COI: GTR model of DNA evolution, number of substitution rates = 6, proportion of invariables sites = 0.00, and gamma shape parameter = 0.26; (2) 28S rDNA: TN93 model of DNA evolution and number of substitution rates = 6, proportion of invariables sites = 0.00, and gamma shape parameter = 0.5125; (3) Combined: GTR model of DNA evolution, number of substitution rates = 6, proportion of invariables sites = 0.612.

Models were selected according to MrModeltest (Nylander, 2004). The alignments were submitted with the above parameters and 1000 bootstrap replicates settings to PhyML 3.0 for maximum likelihood (ML) analyses (Guindon and Gascuel, 2003).

Bayesian inference (BI) was run on MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) for 10 million generations with topologies saved each 1000 generations, under the following best-fit models selected by AIC in MrModeltest (Nylander, 2004): (1) COI: GTR + G, statefreqpr = dirichlet(1,1,1,1), nst = 6, rates = gamma; (2) 28S rDNA: GTR + I, statefreqpr = dirichlet(1,1,1,1), nst = 6, rates = propinv; (3) Combined: using the previous models for each partition (COI: 1-783; 28S: 784-1364). Estimates of average evolutionary divergence over sequence pairs within groups and between groups (*Melithaea, Mopsella, Acabaria* A, B, C, and D) were performed in MEGA 4.0.2 (Tamura et al., 2007).

2.4. Morphological analyses

Tissue was taken from three different parts of each specimen (n = 67 specimens): the cortex (outer coenenchyme), calyx (anthostele), and anthocodia (polyp head). Sodium hypochlorite (household bleach) was used to remove coenenchyme tissue and examine the sclerites under a scanning microscope (Keyence VE-8800, 200× magnifications for the sclerite examination and 100× magnification for examining colony surface) at the Okinawa Churaumi Aquarium (Motobu, Okinawa, Japan). Generic identification was done by comparison with the sclerite plates from Fabricius and Alderslade (2001). Measurements from the micrographs of the sclerites, calyx heights and widths (upper part of the anthostele) were performed utilizing the computer software VE Series (Version 1.1, Keyence, Osaka, Japan). ANOVA and Tukey's post hoc test were done for the three major clades (*Melithea*, *Acabaria*, and *Mopsella*) in Statistical Analyses for Mac (Version 1.5b, Esumi, Tokyo).

3. Results

3.1. Molecular analyses

100 sequences of 28S rDNA (two *Euplexaura* spp., two *Villogorgia* spp. and a *Astrogorgia* sp. were included as outgroups), and 76 sequences of COI (*Villogorgia* sp. included as outgroup) from 111 specimens were obtained; all novel sequences were deposited in GenBank (accession numbers JQ323180–JQ323353). COI sequences were unambiguously aligned because they contain only eight single indels (due to outgroups); the 28S rDNA alignment had seven indels over the entire alignment. The PhyML tree had likelihood values of −2349.43768 for COI and −2204.51355for 28S rDNA.

The phylogenetic topologies for COI and 28S rDNA are shown in Figs. 3 and 4, with ML bootstrap (ML=) and Bayesian posterior probability (Bayes=) support. In both cases, Melithaeidae formed a completely supported monophyly (ML = 100%, Bayes = 1.00). In general, the major clades within Melithaeidae were well supported but relationships between them had low support, which has also been seen in other octocoral studies (e.g., McFadden et al., 2011).

In both CO1 and 28S rDNA phylogenies (Figs. 3 and 4), the genus *Acabaria* was divided into subclades (designated A–D). Subclade *Acabaria* D was a monophyletic clade in the COI phylogram but very divergent in the 28S rDNA phylogeny. The genus *Mopsella* was placed as the earliest diverging of all Melithaeidae clades in both phylogenies.

The *Melithaea* clade in the COI tree showed no divergence (Fig. 3), while in the 28S rDNA tree there was more distance between the samples. These results are clearly shown in distance analyses with *Melithaea* as a divergent clade (Table 1, p-distance = 0.0154) in the 28S rDNA tree, whereas in the COI tree *Melithaea* had no divergence (Table 1, p-distance = 0.00). Furthermore, in examining the distance between the clades in the 28S rDNA phylogeny, *Meltihaea* is the most genetically distinct (Table 2). The combined Bayesian analyses topology (see Supplementary material) was similar to 28S rDNA results, with *Acabaria* D not in a supported node like the rest of the *Acabaria* clades. Sequence 14–5 was located as a sister branch with *Acabaria* B (59ca and 63ca), which differs from its location with *Acabaria* A in COI results, suggesting mito-nuclear discordance. Combined analyses of COI and 28S rDNA placed 14–5 in the *Acabaria* B clade, as the sequences from this specimen share more base changes with *Acabaria* B in the 28S rDNA alignment than base changes from *Acabaria* A in the COI alignment. However, the combined analyses did not further resolve the low support of *Acabaria* D in the Melithaeidae phylogeny (Supplementary Fig. 1).

3.2. Morphological analyses

In situ pictures of the melitheid colonies (see Fig. 4) were used to identify some of the general gross morphological characteristics mentioned below. Sclerite shape allowed the clear identification of three genera: *Melithaea* (Fig. 5), *Mopsella* (Fig. 6) *Acabaria* (Fig. 7). The genus *Acabaria* was divided in four subclades: *Acabaria* A (Fig. 7), *Acabaria* B (Supplementary Fig. 2) *Acabaria* C (Supplementary Fig. 3) and *Acabaria* D (Supplementary Fig. 4). However, some specimens were difficult to identify since they did not have a predominant sclerite type. Sclerites from the cortex, calyx and anthocodia had clear morphological differences and sclerite arrangement in expanded polyps could be observed (Fig 5–7). The genus *Wrightella* was not found in the examined specimens and the genus *Clathraria* could not be conclusively identified. Overall, morphological features of each clade complemented the phylogenetic analyses (Table 3). *Melithaea* had larger colonies and a main branch thicker than observed in the rest of the melithaeids. Specimens in this genus had the smallest sclerite size when compared to the remaining clades (Fig. 5).

Mopsella included only three colonies (specimen 23ca, 53ca and 68ca), and two were collected at the same diving site (Marugu; Ishigaki Island), while sample 23ca was collected at Sesoko Station front reef (Okinawa, Japan). Two specimens (53ca and 68ca) were tentatively identified as *Mopsella retifera* (Lamarck, 1816) by the presence of small clubs in their coenenchyme, which look like flower buds (Fig. 6).

3.2.1. Acabaria subclades

Acabaria subclades had different morphological characteristics.

Acabaria A was the most common clade found in the samples; it had spindle type sclerites (Fig. 7) as described for *Acabaria*, and all colonies had anastomoses.

Acabaria B colonies had the smallest average size (2 cm) and the largest sclerite average size. Colonies varied in color with yellow

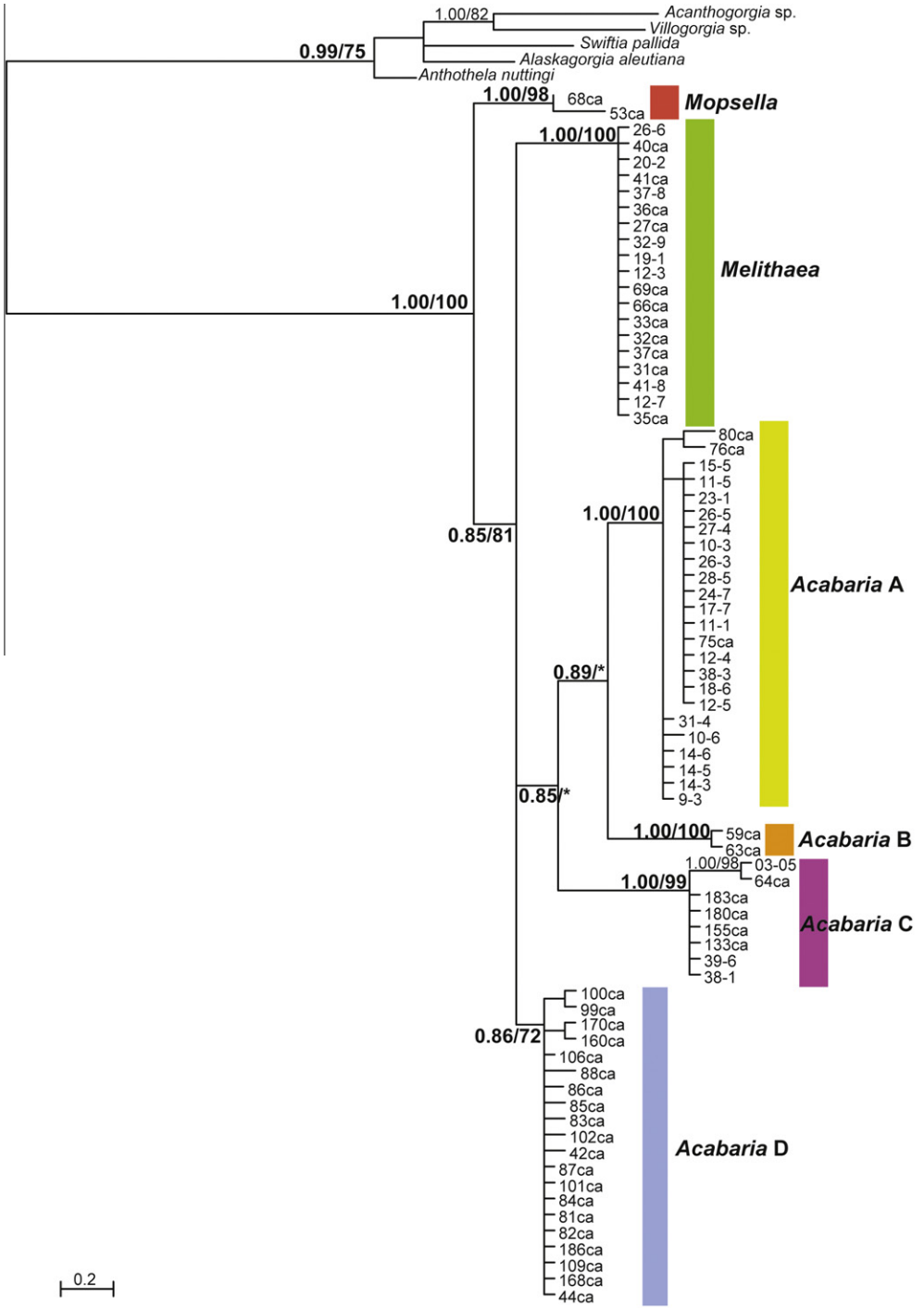

Fig. 3. Phylogram based on Bayesian inference analyses of the partial mitochondrial cytochrome oxidase subunit I (COI) gene from 75 Melithaeidae specimens. Numbers on branches show Bayesian posterior probability and ML bootstrap support, respectively. Hyphen (–) indicates low support (<50%, 0.50) from the respective method; asterisk (*) indicates branch difference from the MrBayes phylogram. *Acanthogorgia* sp. (FJ264912), *Villogorgia* sp. (49ca), *Swiftia pallida* (FJ264905), *Alaskagorgia aleutiana* (FJ264907), and *Anthothela nuttingi* (FJ264908) were included as outgroups.

the most predominant, and colonies in this clade generally did not have anastomoses.

Acabaria C colonies were small (8 cm max. size) and similar to *Acabaria* B. *Acabaria* C colonies were of variable color (pink, orange, red or white), and no anastomoses were seen in most of the samples. The predominant types of sclerites were unilaterally foliate spheroids and foliate capstans (Supplementary Fig. 3), which were initially classified as *Mopsella*, but the subsequent confirmation of the absence of dominant small clubs resulted in these specimens being identified as *Acabaria*; however, the presence of only one club (see Supplementary plate 3a) means clubs may have been inadvertently overlooked during micrograph shooting. Their calyx width was largest compared to the other clades.

Acabaria D colonies were predominantly red, and most colonies did not have anastomoses. Calyx width was the highest of all melithaeids at 0.93 mm (specimen 81ca).

Statistical analyses from the Tukey's Post-hoc test showed significant difference between the sclerite average sizes of the major clades (all comparisons $p < 0.01$).

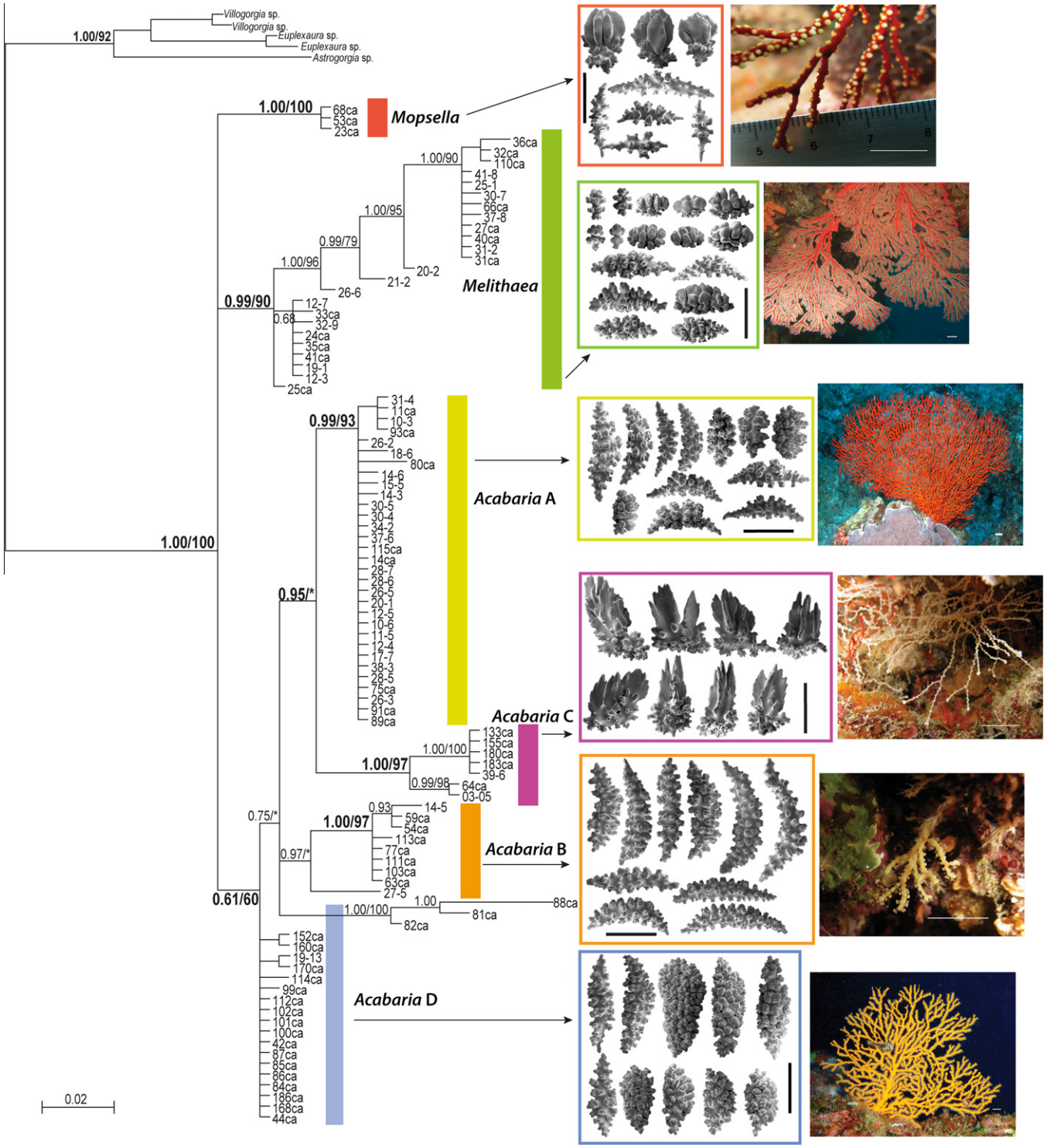

Fig. 4. Phylogram based on Bayesian inference analyses of the 28S ribosomal DNA gene from 95 Melithaeidae specimens. Numbers on branches show Bayesian posterior probability and ML bootstrap support, respectively. Hyphen (–) indicates low support (<50%, 0.50) from the respective method; asterisk (*) indicates branch difference from the MrBayes phylogram. Bar in the Melithaea clade indicates support for the same monophyletic group in MrBayes. Arrows point to sclerites of the coenenchyme/*in situ* picture of each clade: *Mopsella* (68ca/53ca); *Melithaea* (25ca/37ca); *Acabaria* A (75ca/11–5); *Acabaria* C (180ca/64ca); *Acabaria* B (77ca/77ca); *Acabaria* D (168ca/186ca). Scale in the sclerites plates 0.10 mm and approx. 10 mm for *in situ* pictures. Two *Villogorgia* spp., two *Euplexaura* spp., and *Astrogorgia* sp. were included as outgroups.

4. Discussion

4.1. General consensus of morphological and molecular results

The bayesian phylogenetic trees of COI and 28S rDNA both supported the distinction of three major clades inside the Melithaeidae family (Fig. 3 and 4). As no previous molecular data has been available for Melithaeidae, this is the first time Melithaeidae morphology has been correlated with molecular data, and this is expected to be of great help as a first step to clarify this family's taxonomic problems.

The phylogeny that presented higher resolution was the nuclear 28S rDNA marker that had greater congeneric divergence, in particular in the *Melithaea* clade and specimens (81ca, 82ca and

Table 1
Genetic distances (*p*-distance) and their standard deviation for two molecular markers (COI, 28S rDNA) within clades found in the Melithaeidae phylogeny. S.E. = standard error.

Clade	COI	S.E.	28S	S.E.
Melithaea	0	0	0.0154	0.0027
Acabaria	0.0136	0.0024	0.0168	0.0035
Mopsella	0.0052	0.0024	0	0

88ca) within *Acabaria* D clade. However, the *Acabaria* A and *Acabaria* C clades were the same in both the COI and 28S rDNA phylograms (Figs. 3 and 4). The changes in the *Melithaea* genus topologies between the COI and 28S rDNA trees could imply a faster evolution in this genus' nuclear genome compared with evolution in the other genera.

Mitonuclear discordance has been previously observed in some other octocoral subfamilies (e.g., Keratoisidinae, Dueñas and Sánchez, 2009), and is said to be evidence of hybridization in this group; our results here showed no major mitonuclear discordance in Melithaeidae except for sample 14–5 which was placed in different *Acabaria* clades in the COI and 28S rDNA phylogenies. Although the topologies had some differences the major clades were conserved in both COI and 28S rDNA trees (Fig. 3 and 4), and thus our results do not suggest evidence of hybridization between these genera.

Moreover, the increase in the length of the alignment when using the "combined" COI and 28S rDNA data sets did not resolve the uncertainties observed in the COI and/or 28S rDNA trees. The phylogram topology was similar to the 28s rDNA tree and did not result in higher support for the *Acabaria* clade (phylogram not shown see Supplementary Fig. 1).

4.2. Relations among clades

4.2.1. Mopsella

Mopsella was placed as basal in both phylogenies (Fig. 3 and 4). Specimen 53ca and 68ca's sclerites (see Fig. 6) were classified as belonging to the genus *Mopsella*, with sclerite characteristics belonging to those of *Mopsella retifera* Lamarck, 1816. As mentioned in the description of *M. retifera*: "*M. retifera* easily recognized by the clubs of the coenenchyme of the nodes and internodes, which look like flower-buds" (van Ofwegen, 2000), and specimens 53ca and 68ca (Fig. 3 and 4) had this same type of sclerites. *Mopsella* was closer to *Melithaea* in both 28S and COI distance analyses (see Table 2), which did not correlate with morphological characteristics that showed that specimens from *Acabaria* C were similar to *Mopsella* due to the presence of unilaterally foliate spheroids (Supplementary Fig. 3).

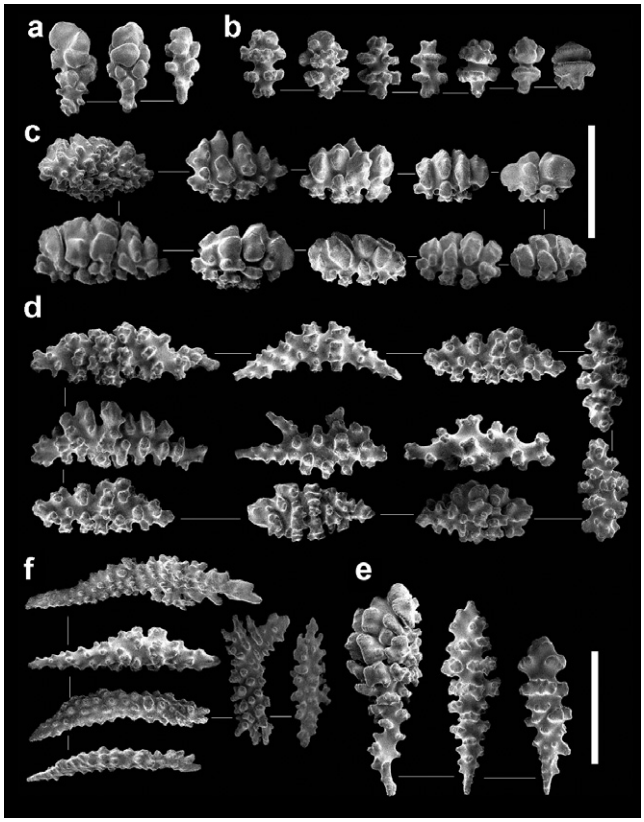

Fig. 5. *Melithea* sp., sclerites of specimen 25ca; a–d, coenenchyme of nodes and internodes; a, clubs; b, capstans; c, unilaterally spinose spindles; d, spindles; e, clubs of calyces; f, anthocodial sclerites. Scale = 0.10 mm.

4.2.2. Melithaea

The genus *Melithaea* had very distinct sclerites (capstans and double disk; Fig. 5) with the shortest sclerites among genera; moreover, their colony size was larger (up to 1.5 m) from the rest of melitheids. As the *Melithaea* clade was well supported in both phylogenies (Fig. 3 and 4) and their sequences had clear, particular traits, identification of *Melithaea* specimens was relatively easy.

4.2.3. Acabaria

In general specimens in *Acabaria* clades were predominated by spindles in the coenenchyme. Their colony size was very variable but no colonies were found to be as tall as *Melithaea*. *Acabaria* was the most abundant genus and thus its molecular results showed the most variability. However, there is a need for more detailed

Table 2
Genetic distances (*p*-distance) and their standard deviation to the right corner, for each molecular marker. Distance are between the clades found in the Melithaeidae phylogeny.

COI	*Melithaea*	*Mopsella*	*Acabaria* A	*Acabaria* B	*Acabaria* C	*Acabaria* D
Melithaea (*n* = 19)		*0.0055*	*0.0056*	*0.0051*	*0.0059*	*0.0042*
Mopsella (*n* = 2)	0.0264		*0.0056*	*0.0049*	*0.0057*	*0.0057*
Acabaria A (*n* = 24)	0.0249	0.0289		*0.0047*	*0.0055*	*0.0043*
Acabaria B (*n* = 2)	0.0196	0.0318	0.0182		*0.006*	*0.0049*
Acabaria C (*n* = 8)	0.028	0.0297	0.0161	0.0331		*0.0048*
Acabaria D (*n* = 20)	0.0151	0.0177	0.0161	0.0215	0.0223	
28S						
Melithaea (*n* = 24)		*0.0084*	*0.0074*	*0.008*	*0.0085*	*0.0064*
Mopsella (*n* = 3)	0.0414		*0.0076*	*0.0087*	*0.0087*	*0.0067*
Acabaria A(*n* = 31)	0.04	0.035		*0.0062*	*0.0063*	*0.0043*
Acabaria B (*n* = 7)	0.0449	0.0397	0.0255		*0.008*	*0.0049*
Acabaria C (*n* = 7)	0.0481	0.0434	0.0283	0.0421		*0.0067*
Acabaria D (*n* = 21)	0.0352	0.0298	0.0175	0.0194	0.0336	

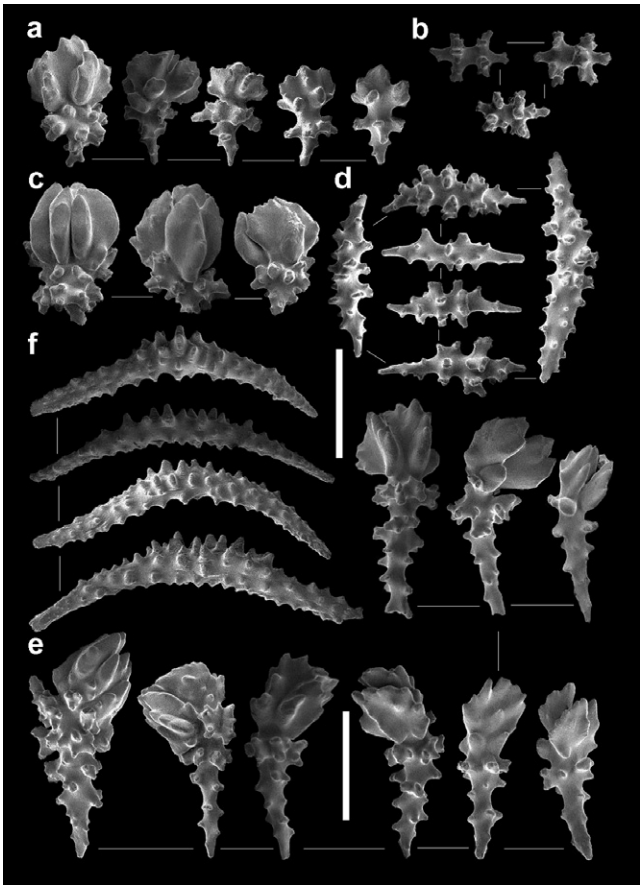

Fig. 6. *Mopsella* sp., sclerites of specimen 68ca; a–d, coenenchyme of nodes and internodes; a, clubs; b, capstans; c, unilaterally foliate spheroids; d, spindles; e, clubs of calyces; f, anthocodial sclerites. Scale = 0.10 mm.

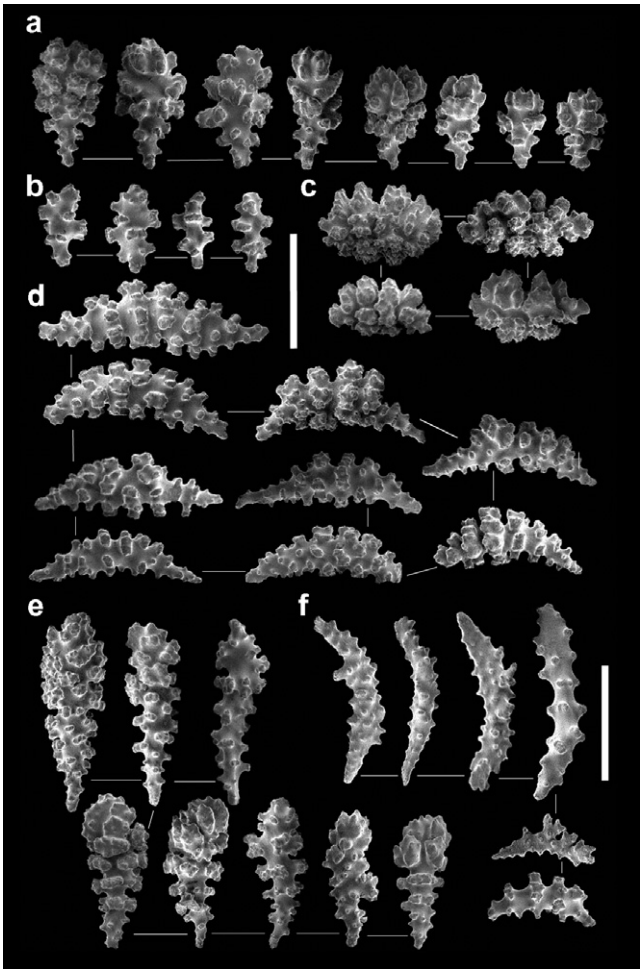

Fig. 7. *Acabaria* sp. A., sclerites of specimen 75ca; a–d, coenenchyme of nodes and internodes; a, clubs; b, capstans; c, unilaterally spinose spindles; d, spindles; e, clubs of calyces; f, anthocodial sclerites. Scale = 0.10 mm.

morphological studies to identify specific characteristics for each of the four observed clades from the molecular results.

4.2.3.1. Acabaria A and Acabaria D. *Acabaria* A and D were the *Acabaria* clades with most specimen numbers (31 and 21, respectively for 28S rDNA), and examined specimens for both clades had spindle type of sclerite. Even though there was a difference in sclerite size between *Acabaria* A (0.097 ± 0.0107 mm) and *Acabaria* D (0.124 ± 0.015 mm, $p = 0.0029$), there were no other clear morphological characteristics that could easily distinguish these two *Acabaria* clades from one another (Fig. 6, Supplementary Fig. 4). Specimens 81ca, 82ca and 88ca were divergent from the rest of the *Acabaria D* specimens in the 28S rDNA phylogeny (Fig. 4), and this genetic divergence could be indicative of species differentiation within this clade, which are in a monophyly in the COI phylogeny with no genetic variability (Fig. 3). The presence of subclades that seemingly belong to the genus *Acabaria* shows that this genus has more variability than *Melithaea* and *Mopsella*. In the distance analyses, *Acabaria A* and *Acabaria* D had the lowest genetic distance between them compared to the other melithaeid clades (0.016 ± 0.0042 = COI, 0.019 ± 0.0046 = 28S; see Table 2). These values are comparable to the minimum genetic distances among congeneric morphospecies found in other octocoral COI (mean = 1.2%; (McFadden et al., 2011). Thus, these results favor the hypotheses that these two clades are part of the same genus.

4.2.3.2. Acabaria B. In the COI phylogenetic tree *Acabaria* B (59ca and 63ca) grouped consistently with *Acabaria* (Bayes = 0.89), and also with *Melithaea* (but no support) in the ML phylogeny (ML < 50%, Fig. 3); however, their sclerites were not the predominant type that were found in *Melithaea* (capstans, double-disc, and small clubs) (Fig. 5). The two specimens' sequences had a 4 bp region that was otherwise exclusive to the *Melithaea* clade, but the remaining base pair changes had more characteristics of *Acabaria* sequences. It may be complex evolutionary event(s) occurred in the past that enabled the mitochondrial COI sequences of *Acabaria* B to have a unique region normally found in the COI sequences of *Melithaea*. Additionally, in the 28S rDNA phylogeny, specimens 59ca and 63ca were located outside the *Melithaea* clade with other specimens not present in the COI tree (Fig. 3, *Acabaria* B). Four specimens (59ca, 63ca, 77ca, and 90ca) had large spindles (0.195 ± 0.04 mm long, Supplementary Fig. 2) and were identified as *Acabaria* species; their sclerites were similar to sclerites in *Acabaria robusta* Shann, 1912 (in van Ofwegen et al., 2000). The distance results showed that *Acabaria* B was closer to *Acabaria* A (0.0182 ± 0.0043) in the COI tree and to *Acabaria* D (0.0202 ± 0.0047, Table 2) in the 28S rDNA tree, which also correlates with sclerite descriptions.

4.2.3.3. Acabaria C. *Acabaria* C and *Acabaria* A placed as sister groups in all the phylogenies; moreover, the closest clade to *Acabaria* C in the 28S rDNA distance analyses was *Acabaria* A (0.0277 ± 0.0063), and in COI analyses was *Acabaria* D (0.022 ± 0.0046) followed by *Acabaria* A (0.027 ± 0.005) (Table 2). Morphological examinations of *Acabaria* C (unilaterally foliate

Table 3
Morphological examinations of the Melithaeidae clades.

	Number of specimens	*Melithaea* 26	*Mopsella* 3	*Acabaria* A 37	*Acabaria* B 8	*Acabaria* C 8	*Acabaria* D 24
Cortex sclerites	Type[a]	Double disks and disc-spindles (n = 16) Fig. 5	Small clubs (like flower-buds) (n = 2) Fig. 6	Spindles and leaf-spindles (n = 25) Fig. 7	Spindles with bump-like processes (n = 6) Spl. Fig. 2	Unilaterally foliate spheroids and foliate capstans (n = 8) Spl. Fig. 3	Spindles and unilaterally foliate spindles (n = 10)Spl. Fig. 3
	Aver. Size[b](mm)	0.072 ± 0.009 (n = 159)	0.099 ± 0.013 (n = 27)	0.097 ± 0.010(n = 127)	0.182 ± 0.041 (n = 18)	0.151 ± 0.015 (n = 43)	0.124 ± 0.015 (n = 64)
Colony description	Color	Red (n = 24) cream (n = 1) yellow (n = 1)	Red (n = 3)	Red (n = 21) orange (n = 14) yellow (n = 2)	Yellow (n = 4) red (n = 2) pink (n = 1) white (n = 1)	Pink (n = 3)white (n = 2) orange (n = 1) red (n = 1) yellow (n = 1)	Red (n = 14) yellow (n = 4) orange (n = 2) white (n = 2)
	Size range (cm)	5–150	4–60	3–20	1.8–6	3–8	3–20
	Presence of anastomoses[c]	n = 14	n = 0	n = 37	n = 1	n = 2	n = 5
	Calyx width (mm)	0.256–0.594	0.901	0.279–0.599	0.421	0.586–0.821	0.66–0.93
	Calyx high (mm)	0.172–0.376	0.236	0.108–0.262	0.211–0.248	0.268–0.481	0.418
	Depth range (m)	3–35.5	6–10.4	1.5–28.3	3–22.9	6.4–27.4	2.8–26.7

[a] n = number of specimens analyzed.
[b] n = number of sclerites measured.
[c] n = number of colonies with anastomoses.

spheroids dominant in the coenenchyme Supplementary Fig. 3) originally suggested these specimens belonged to *Mopsella*. However, with molecular results, they were clearly part of the *Acabaria* clade (Fig. 3 and 4), by the absence of small clubs, and with the help of Dr. van Ofwegen (personal communication) we classified these specimens as *Acabaria* with special sclerite shape.

4.3. Intrageneric variation in Melithaeidae

Species boundaries in organisms such as octocorals are difficult to determine, especially when species descriptions are based solely on morphological data (Wolstenholme et al., 2003). As mentioned before, in this study no species identifications could be made based on the performed morphological characteristics alone.

4.3.1. COI

As expected from the COI genetic analyses, intrageneric genetic variability was relatively low and ranged from 0 to 0.0136 (p-distance) within observed Melithaeidae clades (see Table 1). Previous studies based on octocorals found that the minimum genetic distances among congeneric species pairs ranged from 0% to 4.75% (mean = 1.2%) (McFadden et al., 2011). Moreover, in the same study it was mentioned that for specimens that differed by <1% in COI sequences it was not possible to conclude whether or not species boundaries were present without additional biological, morphological or molecular data (McFadden et al., 2011). As seen in Table 1 p-distance values within *Melithaea* and *Mopsella* were <1%, and thus further studies are needed to clarify the specific status of the specimens inside each clade.

4.3.2. 28S rDNA

28S rDNA showed more divergence than COI, ranging from 0 to 0.0168 (p-distance, Table 1); however there is currently no clear information on congeneric divergence rates in 28S rDNA in octocorals. There are some studies that have used 28S rDNA for higher-level examinations such as the systematic relationships for the entire class Anthozoa (Chen et al., 1995) and the order Scleractinia utilizing a small fragment of 28S rDNA (Romano and Cairns, 2000). As mentioned in the results, there is a clear difference between the *Melithaea* clade in the COI tree with no divergence observed and in the 28S rDNA tree that had a high p-distance value, which shows the importance of using more than one marker to examine molecular phylogenies. *Melithaea* 28S rDNA divergence will allow further morphological analyses to use this genetic information in selecting appropriate specimens for further in-depth examinations, and in searching for congeneric morphospecies, taking this research to a more detailed level of identification and even allowing the application of methods such as sequence-based species delimitation (Pons et al., 2006).

4.4. Morphological overview

Melithaeidae morphology has troubled taxonomists for long time (Alderslade, 2006), making this family one of the many octocoral groups in need of revision. Species have been described by their morphological characteristics since Linneaus and recent re-examinations of some species have resulted in different generic placements for some species (van Ofwegen, 1987, p. 35). Intrageneric species are difficult to identify, as species differences in this family are currently based on morphological characteristics such as sclerite variation (cortex, calyx and anthocodia differences, Fig. 5–7), and colony shape (Fig. 4). Colony morphology seems to be very variable within the same genus, and aside from *Melithaea* with large colony sizes, no other colonial morphological characteristics (e.g., presence or absence of anastamoses) could be found to clearly identify each clade (see Table 3). Moreover, identification to genus level is not always obvious (Fabricius and Alderslade, 2001) since some specimens do not have the predominant type of sclerite specific to a genus, and thus appear to be placed between two genera. As mentioned in our morphological analyses we were able to identify three clear genera according to their predominant sclerite type (*Melithaea, Acabaria* and *Mopsella*, Fig. 5–7), but other genera were apparently absent (*Wrightella, Clathraria* and *Asperaxis*).

5. Conclusions

Genetic variability needs to be interpreted with morphological results, due to the lack of previous studies that have examined combined molecular and morphological results in this group. It is important to keep in mind that in reappraising Melithaeidae systematics both morphological and molecular analyses are needed. In particular, as this study is the first molecular examination of Melithaeidae, sclerite morphology data were very important for phylogram interpretation.

Despite the citation from Fabricius and Alderslade's (2001) book: "It seems likely that all five of the nominal genera will be found to represent variation of a single genus...", from our molecular and sclerite data there is a clear difference between at least

three Melithaeidae genera, validating the morphological-based (sclerite-based) taxonomy of this family. The implications of the phylogenetic analyses reported here, as the first molecular data available for this family, are the relatively clear relationships between molecular and sclerite analyses for both COI and 28S rDNA molecular markers. Finally, we hope these results will serve as baseline data for this octocoral group, and will be used in various studies in the future, not only for taxonomy, but also hopefully for biodiversity, ecological and conservation research.

Acknowledgments

We thank Leen van Ofwegen at the NCB Naturalis museum for his guidance on the specimen's sclerites identification. C.A. was supported by the Japanese Government (Monbukagakusho: MEXT) Scholarship in the Faculty of Science at the University of the Ryukyus. C.A. thanks the students of the Molecular Invertebrate Systematics and Ecology (MISE) Laboratory at the University of the Ryukyus for help with sampling, the Okinawa Churaumi Aquarium for the use of the electron microscope, and Aokawa Diving Service in Okinawa. J.D.R. was supported in part by the Rising Star Program and the International Research Hub Project for Climate Change and Coral Reef/Island Dynamics at the University of the Ryukyus, and a Japan Society for the Promotion of Science "Wakate B" (#21770089) grant-in-aid. Two anonymous reviewers greatly improved an earlier version of the manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.03.005.

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