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Phylogenetic relationships within Aglaopheniidae (Cnidaria, Hydrozoa) reveal unexpected generic diversity

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Morphology can be misleading in the representation of phylogenetic relationships, especially in simple organisms like cnidarians and particularly in hydrozoans. These suspension feeders are widely distributed in many marine ecosystems, and the family Aglaopheniidae Marktanner-Turneretscher, 1890 is among the most diverse and visible, especially on tropical coral reefs. The taxonomy of this family is based on morphological characters with emphasis on reproductive structures for the identification of genera. This study is the most comprehensive molecular phylogeny of the Aglaopheniidae to date, including six genera and 38 species, of which 13 were investigated for the first time and sampled on tropical coral reefs throughout the Indo-Pacific region. For newly sampled individuals, we sequenced the 16S rRNA, the nuclear locus comprising the complete ITS1-5.8S rRNA gene-ITS2 and the first intron of the calmodulin nuclear gene. Phylogenetic analyses of the data revealed and confirmed a general polyphyly, or doubtful monophyly, of all sampled genera in tropical regions based on both the mitochondrial and nuclear markers. Our results revealed that several morphological characters used today are unsuited to resolve phylogenetic relationships between species and genera, as well as the high phyletic diversity within this family. Future revision of the classification of this family will require extensive geographic sampling and the use of an integrative approach.

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Introduction

Prerequisites for conservation planning are inventories of species, which are often recognised and described using morphological clues (The House of Lords 2003; The Royal Society 2003; Mace 2004). However, taxa identification using morphological criteria may be challenging, especially in simple organisms that present few discriminant synapomorphies and high phenotypic plasticity, such as cnidarians [see Daly *et al.* (2007) for a review]. Moreover, molecular phylogenies have shown that a morphological approach is not always representative of the diversity and

phylogenetic relationships between species, genera, families and even higher levels in Cnidaria (e.g. Bridge *et al.* 1992; Chen *et al.* 1995; Berntson 1999; Daly *et al.* 2003; Benzoni *et al.* 2010; Budd *et al.* 2010; Brugler *et al.* 2013; Arrigoni *et al.* 2014; Kitano *et al.* 2014). This general problem in Cnidaria also pertains to Hydrozoa, (e.g. Bridge *et al.* 1992; Schuchert 1993; Bouillon & Boero 2000; Marques & Collins 2004; Leclère *et al.* 2009; Peña Cantero *et al.* 2009; Cartwright & Nawrocki 2010; Lindner *et al.* 2011), highlighting the need for a systematic revision of this cnidarian class.

The Aglaopheniidae (Marktanner-Turneretscher, 1890) hydrozoan family is one of the largest, comprising eight genera and over 250 described species (Bouillon *et al.* 2006). Genera are defined by reproductive apparatus (gonosome) morphology in addition to the general morphology of the rest of the colony (trophosome) (Allman 1883; Nutting 1900; Millard 1975; Calder 1997; Bouillon *et al.* 2006). However, gonosome morphology and the life cycle of hydrozoan species were recently shown to present several cases of plesiomorphy and convergent evolution (Leclère *et al.* 2007, 2009). Therefore, its use as criteria for identifying Aglaopheniidae genera is now arguable. A recently published molecular phylogeny of this family (Moura *et al.* 2012), based on 25 putative species belonging to six genera, partially supported the monophyly of some genera but rejected the existing classification based on gonosome morphology. The study by Moura *et al.* (2012) included a large number of species; however, most samples were collected from temperate regions in the Atlantic Ocean and Mediterranean Sea, implying that a part of the phyletic diversity within Aglaopheniidae might have been missed. Nutting (1900) already noted that the Aglaopheniidae diversity (family Statoplea according to the author) was particularly high in the tropical Atlantic Ocean (West Indies). Since then, an important volume of literature has confirmed the diversity of Aglaopheniidae in tropical seas, especially on coral reefs (see Vervoort 1967; Calder 1997; Schuchert 2003; Gravier-Bonnet & Bourmaud 2006a,b, 2012; Di Camillo *et al.* 2008). Consequently, sampling the tropical Indo-Pacific region represents a valuable enrichment of the data set, improving the assessment of the phyletic diversity of this family.

The 16S mitochondrial rRNA marker is widely used in cnidarian phylogenetic and phylogeographic studies (e.g. Bridge *et al.* 1995; Romano & Palumbi 1997; Collins *et al.* 2005; Peña Cantero *et al.* 2009; Cartwright & Nawrocki 2010; Addamo *et al.* 2012; Miglietta & Cunningham 2012; Hibino *et al.* 2014) and has shown a certain consistency with nuclear markers (Miranda *et al.* 2010; Schuchert 2014). However, using a single mitochondrial marker is not appropriate to study ancient phylogenetic relationships as it may present incomplete lineage sorting and introgression. Additional markers, each having a unique phylogenetic history and mutation rate, should be used to improve the resolution and the reliability of phylogenetic reconstructions. The internal transcribed spacers of the nuclear rDNA (ITS: including the entire ITS1, 5.8S gene and ITS2) present interesting characteristics for reconstructing phylogenetic relationships: biparental inheritance, easy amplification and universality (Barco *et al.* 2010; Imazu *et al.* 2014; Kitano

et al. 2014). However, due to its high variability, ITS is more appropriate for the study of interspecific phylogenetic relationships (see Schuchert 2014). For higher taxonomic ranks, the calmodulin, a ubiquitously conserved gene, has proved to be a reliable marker (Baba *et al.* 1984) used for reconstructing cnidarian phylogenies (Lindner *et al.* 2008).

This study aimed to improve knowledge on Aglaopheniidae phylogeny, primarily by enlarging the geographic coverage of included taxa, focussing sampling efforts on tropical coral reefs in the south-west Indian Ocean and the south-west Pacific – two marine biodiversity hotspots (Roberts *et al.* 2002) – as well as in the central Pacific. Using a multilocus approach, we provide a new insight into the phylogenetic relationships within this family and discuss current taxonomy and its validity.

Material and methods

Sampling

Samples were collected manually between 2007 and 2014 from shallow coral reef habitats (0–40 m depth) at six localities in the south-west Indian Ocean (SWIO, 15 sites per locality), one locality in the south-west Pacific (New Caledonia, 18 sites) and one in the central Pacific (Moorea, French Polynesia, three sites) (Fig. 1, Table S1). Each sampling site was prospected for Aglaopheniidae species using SCUBA or by snorkelling. To facilitate taxonomic identification, larger colonies (i.e. 3–40 cm high) were preferentially collected with visible gonosomes. Parts of colonies, referred to as individuals, were collected several metres apart to reduce the probability of sampling the same colony (genet). Samples were identified based on morphological characters by CAFB, NG-B and BP, using taxonomic literature (see references listed in Gravier-Bonnet & Bourmaud 2006a,b, 2012). For species and genera lists the ‘Hydrozoa Handbook’ (Bouillon *et al.* 2006) was used. A minimum of three individuals per species were collected and sequenced. Specimens were fixed and preserved in 90% ethanol for DNA extraction. Whenever possible, a voucher of each sequenced specimen was also preserved in a 3% formalin solution in sea water. Samples are currently stored in the laboratory at the University of Reunion Island and are available on request.

Sequencing

Prior to DNA extraction, all gonophores were removed from the colonies. DNA was extracted from one or two hydrocauli per colony using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol. Extraction quality was assessed on a 0.8% agarose gel stained with GelRed Nucleic Acid Stain,



Fig. 1 Sampling locations in the south-west Indian Ocean and west Pacific and central Pacific included in this study. EUR, Europa Island; GEY, Geyser bank; GLO, Glorieuses Islands; JUA, Juan de Nova Island; MAY, Mayotte; NCA, New Caledonia; RUN, Reunion Island; MOO, Moorea.

10 000 in DMSO (Gentaur, Kampenhout, Belgium). Each PCR was conducted in a total volume of 30 μ L: 15 μ L (0.625 U) of AmpliTaq mix (Applied Biosystems, Waltham, MA, USA), 0.5 μ L of each primer (10 μ M) and 4 μ L of template DNA (final concentration: 1.6 ng/ μ L) and completed with 10 μ L of ultra-pure water. We amplified and sequenced a fragment of the 16S rRNA (16S) mitochondrial gene with primers SHA (5'-ACG GAA TGA ACT CAA ATC ATG T-3') and SHB (5'-TCG ACT GTT TAC CAA AAA CAT A-3') (Cunningham & Buss 1993) and two nuclear regions: (i) a fragment of the calmodulin (CAM) gene with primers CAMF1 (5'-GAT CAA YTR CAN GAR GAA CAA ATT GC-3') and CAMR1 (5'-CCA TCN CCA TCR ATA TCA GC-3') (Lindner *et al.* 2008), and (ii) the complete internal transcribed spacer 1, 5.8S rDNA and internal transcribed spacer two region of the ribosomal DNA (ITS) with partial 18S and 28S ribosomal genes flanking the two ITS, respectively, upstream and downstream with primers ITSF (5'-CAC CGC CCG TCG CTA C TA CCG ATT GAA TGG-3') and ITSr (5'-CGC TTC ACT CGC CGT TAC TAG GGG AAT CC-3') (Martínez *et al.* 2010). The PCR conditions for 16S and CAM markers were as follows: (5 min at 95 $^{\circ}$ C), (30 s at 94 $^{\circ}$ C; 30 s at 46 $^{\circ}$ C; 1 min at 72 $^{\circ}$ C) \times 5, (30 s at 94 $^{\circ}$ C; 30 s at 51 $^{\circ}$ C; 1 min at 72 $^{\circ}$ C) \times 30, and (5 min at 72 $^{\circ}$ C). The PCR conditions for the ITS marker were as follows: (5 min at 95 $^{\circ}$ C), (30 s at 94 $^{\circ}$ C; 30 s at 55 $^{\circ}$ C; 1 min at 72 $^{\circ}$ C) \times 35 and (5 min at 72 $^{\circ}$ C). PCR products were visualised on a 1% agarose gel stained with GelRed Nucleic Acid Stain, 10 000 in DMSO (Gentaur, Kampenhout, Belgium). PCR products were sequenced in both directions by Genoscope (CEA Evry, France) and by Genoscreen (Lille, France) on capillary sequencer ABI3730XL.

Tree reconstruction

Sequences were checked and edited using Geneious 6.0 (<http://www.geneious.com>; Kearse *et al.* 2012) and deposited on GenBank (Table S2). Additional mitochondrial sequences previously published were retrieved from GenBank (Table S2). Sequences were aligned using Muscle algorithm (Geneious plug-in; Edgar 2004) and trimmed to the shortest sequence. To assess the general position of newly sampled species within the Aglaopheniidae phylogeny, 16S marker was analysed first, separately from the others. After checking for incongruences, phylogenetic relationships among the coral reef Aglaopheniidae species were analysed using the concatenated data set (i.e. mitochondrial and nuclear markers). We used Jmodeltest (stand-alone version; Darriba *et al.* 2012) to determine the best probabilistic model of sequence evolution based on AIC (16S: GTR+I+G; CAM: TPM2uf+I+G; ITS: TIM1+I+G). We performed maximum likelihood (ML) using PhyML (Geneious plug-in; Guindon *et al.* 2010) and Bayesian inferences (BI) analyses using MrBayes 3.2 (three independent runs of: 100.10⁶ generations, eight chains, temperature to 0.2, 10% burnin length, sampling every 1.10⁵ generations (Geneious plug-in; Ronquist *et al.* 2012). Nodes can be considered robust if their posterior probability (PP) is equal or higher to 0.95 when considering Bayesian reconstruction and when their bootstrap (BS) values are superior to 75% considering ML reconstruction (Erixon *et al.* 2003). These definitions were used thereafter. We used the same outgroups as Moura *et al.* (2012) for the 16S tree reconstruction, that is *Schizotricha turqueti* (Billard, 1906) and *Schizotricha nana* Peña Cantero *et al.* 1996. As no sequences of ITS and calmodulin were available for these species, sequences from a Campanulariidae species [*Chytia gracilis* (Fraser, 1938)] were used for

tree reconstructions, for the nuclear markers and for the concatenated data set.

Results and discussion

Our research represents the most comprehensive phylogenetic study of the family Aglaopheniidae to date, in terms of the number of taxa, genes included and geographic coverage, comprising sites from the Atlantic, south-west Indian Ocean and the south-west to central Pacific. For the 16S, a total of 238 sequences were analysed, comprising six genera and 38 species (Table S2); after alignment, 511 sites were retained of which 274 (53.6%) were parsimony-informative (i.e. at least two different bases for a given site, each represented at least twice). The calmodulin gene data set included 143 sequences of 330 sites, with 95 parsimony-informative sites (28.8%). For ITS, 143 sequences of 937 sites were obtained, with 432 parsimony-informative sites (46.1%). The results of BI and ML were almost identical, but BI analyses provided generally stronger nodal supports than ML. Phylogenies of the mitochondrial and nuclear markers (16S, calmodulin, ITS; Figs S1–S3, respectively) yielded similar topologies for the coral reef species studied, except for the position of *Macrorhynchia* sp. n. (Figs S1–S3). This species, primarily distinguished using morphological characters, formed a robust clade with other *Macrorhynchia* (Kirchenpauer, 1872) species when considering the nuclear markers separately (Figs S2 and S3), but not when considering only the mitochondrial marker (Fig. 2; Fig. S1). The marker 16S has been shown useful for barcoding hydrozoan species (Leclère *et al.* 2007, 2009; Moura *et al.* 2008, 2011, 2012; Zheng *et al.* 2014). However, several studies revealed its possible higher mutation rate in Aglaopheniidae compared to other hydrozoan families (Leclère *et al.* 2007; Moura *et al.* 2008; Peña Cantero *et al.* 2009). This characteristic might impede the reconstruction of intergeneric phylogenetic relationships in this family; to account for this possibility, we also used both slow (calmodulin) and fast (ITS) evolving nuclear markers (for tropical species only). The comparison of the different phylogenetic trees reconstructed using 16S, calmodulin or ITS (Figs S1–S3, respectively) revealed that none gave a good representation of ancient relationships within this family and that all genera are polyphyletic (except *Macrorhynchia* when using nuclear markers) (Figs S1–S3). Thus the high polyphyly of Aglaopheniidae genera might not be a simple artefact from the mitochondrial marker but highlight of the fact that we do not know to what extent evolutionary divergence is reflected in Aglaopheniidae morphology. Considering these results, and as the 16S marker might in addition not be representative of phylogenetic

relationships due to its maternal inheritance (Hurst & Jiggins 2005), we analysed the three markers concomitantly by concatenating them for the newly sequenced species.

Groups of taxa in Aglaopheniidae

Nodes discussed below (mitochondrial marker and/or concatenated data set) are supported by both Bayesian [posterior probability (PP)] and maximum likelihood [bootstrap (BS)] reconstructions and are considered robust (i.e. PP > 0.95 and BS > 75).

The phylogeny based on 16S, including all available sequences and newly sequenced species, is presented in Fig. 2. We identified three divergent and strongly supported clades within the family Aglaopheniidae: first, the genus *Streptocaulus* (Allman, 1883) at the base of the phylogeny (Fig. 2: A; PP = 1, BS = 100); second, the genus *Gymnangium* (Hincks, 1874) excluding *G. eximium* (Allman, 1874) and *G. gracilicaule* (Jäderholm, 1903) (Fig. 2: B; PP = 1, BS = 100); and third, the genera *Aglaophenia* Lamouroux, 1812; *Cladocarpus* (Allman, 1874), *Lytocarpia* (Kirchenpauer, 1872) and *Macrorhynchia*, and the two species *G. eximium* and *G. gracilicaule* (Fig. 2: C–N; PP = 0.98, BS = 79). The newly obtained sequences improved the resolution of the tree, and the third major clade (C–N) comprised three subclades: (a) the genus *Cladocarpus* (Fig. 2: C; PP = 1, BS = 98), (b) the genus *Macrorhynchia* and the species *G. eximium*, *G. gracilicaule* and *Lytocarpia nigra* (Nutting, 1905) gathered in a partially supported clade (Fig. 2: F–H; PP = 0.99, BS < 75), and (c) several *Lytocarpia* and *Aglaophenia* species (Fig. 2: D, E, I–N). The use of nuclear markers confirmed the major clade composed of subclades F to H (Fig. 3; PP = 0.99, BS = 97) and the monophyly of *L. brevirostris* (Fig. 3: J; PP = 1, BS = 100).

In the light of these new phylogenies, the genus *Gymnangium* is clearly polyphyletic, either when the 16S is analysed alone (Fig. 2: B and H), in agreement with previous studies (Moura *et al.* 2008, 2012; Leclère *et al.* 2009), or when the nuclear markers are included (Fig. 3: B and H). Likewise, the genera *Aglaophenia* and *Lytocarpia* are polyphyletic groups for both mitochondrial and nuclear markers, results congruent with those of Moura *et al.* (2012). In contrast, the *Macrorhynchia* species are closely related when excluding *Macrorhynchia* sp. n. based on the 16S (Fig. 2: G), but nuclear markers reveal the monophyletic status of this genus, including *Macrorhynchia* sp. n. (Fig. 3: G). Finally, the species of the genera *Cladocarpus* (Fig. 2: C) and *Streptocaulus* (Fig. 2: A), inhabiting deep waters, were not sampled in the present study: their monophyletic status can therefore neither be confirmed nor discussed.

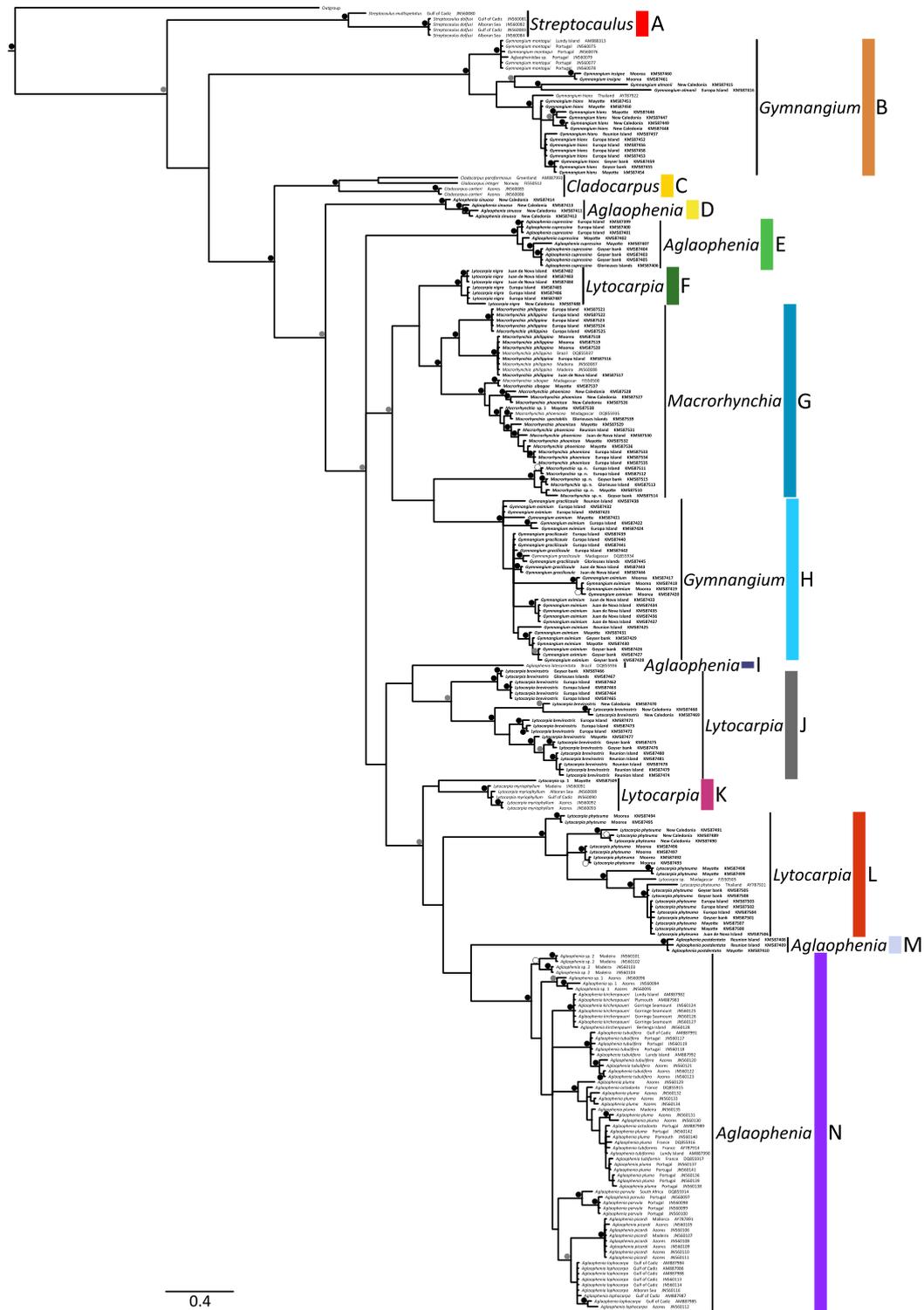


Fig. 2 Molecular phylogeny of Aglaopheniidae species generated by PhyML from 16S gene sequences. Black thick vertical lines indicate positions of genera. Support values of nodes are indicated: black dots indicate PP superior to 0.95 and ML bootstrap values superior to 75%; grey dots indicate PP superior to 0.95 and bootstrap values of maximum likelihood inferior to 75%; white dots indicate PP inferior to 0.95 and bootstrap values of maximum likelihood superior to 75%. Nodes with PP inferior to 0.95 and bootstrap values of maximum likelihood inferior to 75% are not indicated. Distances are expressed in number of substitution per site. Uppercase letters and colour codes delineate lineages referred to Table 1 and congruent with Fig. 3.

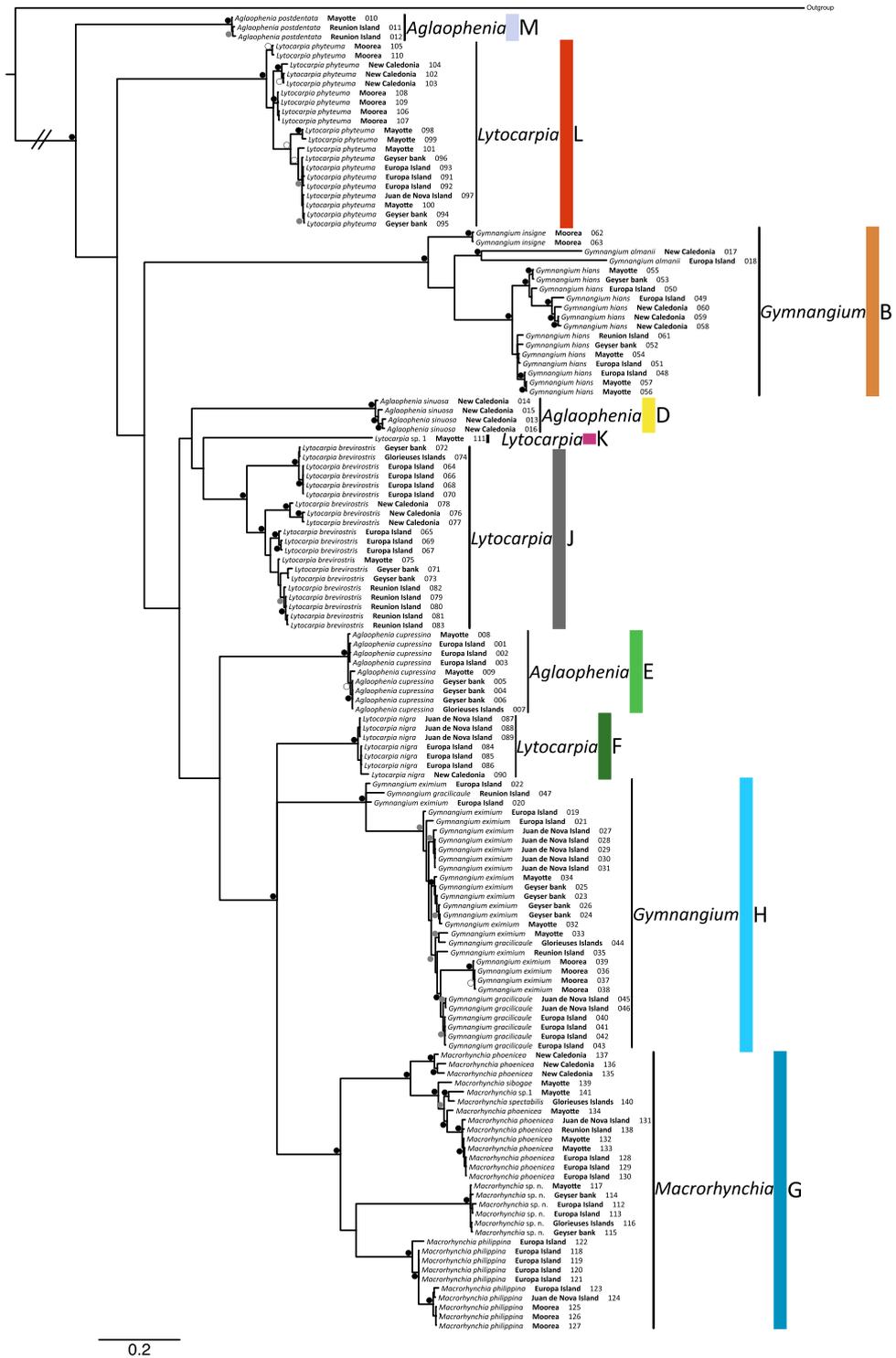


Fig. 3 Molecular phylogeny of 18 tropical Aglaopheniidae species generated by PhyML from 16S, ITS and calmodulin sequences. Black thick vertical lines indicate positions of genera. Support values of nodes are indicated: black dots indicate PP superior to 0.95 and bootstrap values of maximum likelihood superior to 75%; grey dots indicate PP superior to 0.95 and bootstrap values of maximum likelihood inferior to 75%; white dots indicate PP inferior to 0.95 and bootstrap values of maximum likelihood superior to 75%. Nodes with PP inferior to 0.95 and bootstrap values of maximum likelihood inferior to 75% are not indicated. Distances are expressed in number of substitution per site. Uppercase letters and colour codes delineate lineages referred to Table 1 and congruent with Fig. 2.

Phyletic richness of Aglaopheniidae genera

Moura *et al.* (2012) found the genus *Aglaophenia* to be polyphyletic due to the absence of grouping of *A. latecarinata* (Allman, 1877) with other *Aglaophenia* species. The inclusion of three newly sampled species of this genus (*A. cupressina* Lamouroux, 1816; *A. postdentata* Billard, 1913 and *A. sinuosa* Bale, 1888) supports this result. *Aglaophenia cupressina* (E) and *A. sinuosa* (D) are part of a large clade that includes the genera *Lytocarpia*, *Aglaophenia* and *Macrorhynchia* and two *Gymnangium* species, but are divergent among each other and separated from other *Aglaophenia* (Figs 2 and 3). With 75 valid species, the genus *Aglaophenia* is the most diverse of the family (Bouillon *et al.* 2006). Our results validate its polyphyletic status and suggest that it is composed of several divergent monophyletic taxa: based on our data, the genus *Aglaophenia* is composed by at least five divergent lineages (Figs 2 and 3: D, E, I, M, N; Table 1). These results question the validity of this genus and the reliability of the morphological characters used to define it, that is gonothecae protected by accessory structures, called corbulae, lacking hydrothecae at the base of the ribs (Millard 1975; Schuchert 2003; Bouillon *et al.* 2006). From its creation by Lamouroux (1812), the genus *Aglaophenia* has presented a very complex history, as summarised by Calder (1997). Today, with a more comprehensive sampling as a prerequisite, the genus *Aglaophenia* should attract attention for a taxonomic revision, which might lead to the denomination of new clades in the Aglaopheniidae family. Interestingly, *Aglaophenia*

species from the temperate Atlantic Ocean and Mediterranean Sea (Fig. 2: N) form a robust clade, which contains the type species (Table 1, *A. pluma* (Linnaeus, 1758)). This clade N is composed of 10 species with low interspecific genetic divergences (Moura *et al.* 2012) and strong morphologic similarities (P. Schuchert, pers. com.).

The addition of new species of *Lytocarpia* to the phylogeny also highlights the polyphyly of this taxon, consistent with the results of Moura *et al.* (2012). The species *Lytocarpia brevis* (Busk, 1852) (J), *L. myriophyllum* (Linnaeus, 1758) (K) and *L. phyteuma* (Kirchenpauer, 1876) (L) do not form a clade but seem related to *Aglaophenia* species from the Atlantic and the Mediterranean Sea (Fig. 2: N) and *A. postdentata* (Fig. 2: M), whereas *L. nigra* (F) is related to *Macrorhynchia* (G), *Gymnangium eximium* and *G. gracilicaule* (H). These results question the validity of the diagnostic character for distinguishing the genera *Lytocarpia* and *Aglaophenia*, that is the presence/absence of an hydrothecae at the basis of each corbula rib, respectively (Millard 1975). As previously shown by Moura *et al.* (2012), our results also indicate that the general classification of the Aglaopheniidae based on the morphological characters of the gonosome is not concordant with the phylogeny of the family. Furthermore, species of *Lytocarpia* belong to two of the major subclades of Aglaopheniidae (Fig. 2: F–H, I–N), indicating that the general diagnostic character of the genus might represent several cases of homoplasy. Indeed, the genus is composed of four divergent lineages, questioning again its general validity (Table 1). However, with only five of the 36 currently recognised species included in this study (Bouillon *et al.* 2006) and even if the type species is included (Table 1), our sampling of this genus remains limited and a taxonomically more inclusive analysis is needed before considering revising this genus.

Moura *et al.* (2012) could not conclude on the monophyly of *Macrorhynchia* as our results based on 16S (Fig. 2: G). Our results cautiously suggest the monophyletic status of this genus (Fig. 3), keeping in mind the scarce taxon sampling and that the type species is not included (Table 1). Furthermore, the phylogenetic relationships between sampled species are different considering the mitochondrial marker or the concatenated data set. Based on the 16S, *Macrorhynchia* sp. n. does not seem to be closely related to other *Macrorhynchia*, while *M. phoenicea* (Busk, 1852), *M. spectabilis* (Allman, 1883) and *M. sibogae* (Billard, 1913) form robust clades (Fig. 2: G). The addition of nuclear markers (i) supports the monophyly of the genus, (ii) supports the synonymy of *M. sibogae* and *M. phoenicea* suggested by Di Camillo *et al.* (2009), (iii) calls into question the validity of *M. spectabilis* (Fig. 3: G) and (iv) highlights the relatedness of *Macrorhynchia* sp. n. to

Table 1 Grouping at genus level of the 38 species analysed based on molecular data in this study

Clade	Genera	Species
A	<i>Streptocaulus</i> *	<i>Streptocaulus multiseptatus</i> , <i>S. dolfusi</i>
B	<i>Gymnangium</i>	<i>G. montagui</i> , <i>G. allmani</i> , <i>G. hians</i> , <i>G. insigne</i>
C	<i>Cladocarpus</i> *	<i>Cladocarpus cartieri</i> , <i>C. integer</i> , <i>C. paraformosus</i>
D	<i>Aglaophenia</i> ?	<i>Aglaophenia sinuosa</i>
E	<i>Aglaophenia</i> ?	<i>Aglaophenia cupressina</i>
F	<i>Lytocarpia</i> ?	<i>Lytocarpia nigra</i>
G	<i>Macrorhynchia</i> *	<i>Macrorhynchia phoenicea</i> , <i>M. philippina</i> , <i>M. sibogae</i> , <i>M. spectabilis</i> , <i>M</i> sp. n.
H	<i>Gymnangium</i> ?	<i>Gymnangium eximium</i> , <i>G. gracilicaule</i>
I	<i>Aglaophenia</i> ?	<i>Aglaophenia latecarinata</i>
J	<i>Lytocarpia</i> ?	<i>Lytocarpia brevis</i>
K	<i>Lytocarpia</i>	<i>Lytocarpia myriophyllum</i> , <i>L.</i> sp. 1
L	<i>Lytocarpia</i> ?	<i>Lytocarpia phyteuma</i>
M	<i>Aglaophenia</i> ?	<i>Aglaophenia postdentata</i>
N	<i>Aglaophenia</i>	<i>Aglaophenia pluma</i> , <i>A. kirchenpaueri</i> , <i>A. lophocarpa</i> , <i>A. octodonta</i> , <i>A. parvula</i> , <i>A. picardi</i> , <i>A.</i> sp. 1, <i>A.</i> sp. 2, <i>A. tubiformis</i> , <i>A. tubulifera</i>

Type species are in bold. Asterisks indicate clades where the type species of the corresponding genus was not sequenced; question marks indicate that the type species of the genus is not in the corresponding clade.

other *Macrorhynchia* species (Fig. 3; PP = 1, BS = 100). *Macrorhynchia philippina* (Kirchenpauer, 1872), on the other hand, forms a robust clade whatever the molecular marker considered (Figs 2 and 3; Figs S1–S3). Thus, we concur that the genus *Macrorhynchia* is represented in our phylogeny by at least three species (*M. philippina*, *M. phoenicea* and *Macrorhynchia* sp. n.) with unclear phylogenetic relationships.

The taxon *Gymnangium*, defined by the absence of protective structures on the gonothecae, is divided into two clades according to the 16S: one basal to the Aglaopheniidae, composed of *G. bians* (Busk, 1852), *G. montagui* (Billard, 1912) [type species], *G. allmani* (Marktanner-Turneretscher, 1890) and *G. insigne* (Allman, 1876) (Fig. 2: B) and another composed of *G. eximium* and *G. gracilicaule* related to *L. nigra* and *Macrorhynchia* spp (Fig. 2: H). Once more, our results highlight that the morphological characters of the gonosome are not synapomorphic in this taxon: the genus *Gymnangium* is composed of two divergent lineages (Fig. 2, Table 1). These results confirm those from previous studies (Leclère *et al.* 2007; Moura *et al.* 2012). Thus, this study poses a straightforward question to taxonomists: What common characters explain the phylogenetic relatedness of *Macrorhynchia* species to *L. nigra* and the clade composed of *G. eximium* and *G. gracilicaule*, considering that the morphological characters of the gonosome are not valid? It should be noted that approximately one century ago, Stechow (1921) proposed to split *Gymnangium* into three genera (*Gymnangium*, *Haliaria* and *Halicetta*), based on the trophosome characters (i.e. the whole animal except the temporary reproductive structures). One of our lineages (Figs 2 and 3: H; *G. gracilicaule* and *G. eximium*) enters in the genus *Halicetta* Stechow, 1921, while the second (Figs 2 and 3: B) is still in *Gymnangium sensu stricto*. Thus, the classification based on the trophosome seems, in this case, to be more representative of the phylogenetic relationships between some Aglaopheniidae clades. Our results highlight the importance to insert new additional descriptive characters in the current taxonomy (gathering morphological, phylogenetic, ecological, metabolomic, proteomic data...). Once performed, it would be interesting to test this approach on the genus *Gymnangium* by constructing a molecular phylogeny of the whole genus and comparing it to the classification proposed by Stechow (1921), potentially resurrecting several genera.

Morphological characters of the gonosome were used in the most recent attempt to classify the family Aglaopheniidae by Calder (1997) which defined two subfamilies: Gymnangiinae Calder, 1997 and Aglaopheniinae Marktanner-Turneretscher, 1890; with two tribes within the second subfamily (Aglaopheniini Marktanner-Turneretscher, 1890; and Cladocarpini Calder, 1997). Moura

et al. (2012) already highlighted that morphological characters of the gonosome are not linked to genetic lineages, and the present study clearly supports their conclusion. With this classification criteria, three of the six Aglaopheniidae genera studied (*Aglaophenia*, *Gymnangium* and *Lytocarpia*) are composed of several highly divergent lineages, based on mitochondrial and nuclear markers: our results reveal that the phyletic diversity of genera is probably underestimated by over 50% (Table 1) and confirm the importance of evolutionary convergences in this family, a common feature in cnidarians (Belmonte *et al.* 1997; Collins 2002; Govindarajan *et al.* 2006; Leclère *et al.* 2007, 2009; Sinniger *et al.* 2008; Cartwright & Nawrocki 2010; McFadden *et al.* 2010; Nawrocki *et al.* 2010; Gittenberger *et al.* 2011; Hoeksema & Crowther 2011; Brugler *et al.* 2013; Kayal *et al.* 2013; Arrigoni *et al.* 2014; Kitano *et al.* 2014). Thus, using morphological characters to classify Aglaopheniidae has led to underestimate the family phyletic diversity by unintentionally overlooking possible morphological convergences and by confounding two different phenomena that do not necessarily occur on the same timescale during speciation: cladogenesis and anagenesis.

Aglaopheniidae species are monophyletic

It should be noted that almost all studied species form monophyletic groups. In other words, our results reveal that morphological characters, even if not representing phylogenetic relationships among species, are able to identify monophyletic groups.

This pattern is typical for adaptive radiation (i.e. the formation of a high number of species on a small geological timescale). Indeed, such radiations can lead to a loss of phylogenetic signal and thus to unresolved phylogenies above the species level (Givnish & Sytsma 1997). However, the combination of high divergence among tropical species (compared to Atlantic species) and several resolved ancient nodes (Figs 2 and 3; Fig. S2) suggest that this radiation should be extremely old (assuming no changes in mutation rates of markers across time or taxa). It further implies that multiple evolutionary convergences occurred. However, the lack of fossil records is a huge problem to test this scenario, as we cannot calibrate the trees with a real time-based molecular clock.

A plausible alternative hypothesis is that these tropical Aglaopheniidae species, whose determination depends on morphological characters (i.e. morpho-species), do not represent true species, that is monophyletic independent metapopulations (Barberousse & Samadi 2010). In other groups of organisms with problematic and complex taxonomy, recent studies using species delimitation methods combining sequences and microsatellite data with morphological and geographic data have identified several

independent species within morpho-species (Ross *et al.* 2010; Barley *et al.* 2013; Payo *et al.* 2013). Thus, each morpho-species of Aglaopheniidae could represent a complex of true species, like hypothesised for another worldwide hydrozoan morpho-species, *Plumularia setacea* (Linnaeus, 1758), by Schuchert (2014). Such an analysis of species delimitation should be performed to clarify whether these Aglaopheniidae morpho-species are composed or not of several true species.

Conclusions

These phylogenies, based on several molecular markers, reveal the high phyletic diversity of the Aglaopheniidae and are one of the necessary steps to reassess the taxonomy of this notable hydrozoan family. Our results deliver an important message: we are able to identify Aglaopheniidae species in general, but we have a poor representation of the diversity and phylogenetic relationships of the genera within this family. An integrative approach (Dayrat 2005) must be used, in addition to a more comprehensive sampling, to improve our knowledge of this family. Such method allowed the proposition of a new classification based on morphological characters representing phylogenetic relationships in the Corynidae (Johnston, 1838), another hydrozoan family with a problematic morphological classification including several poly- and paraphyletic genera (Nawrocki *et al.* 2010).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Molecular phylogeny of 18 tropical Aglaopheniidae species generated by PhyML from 16S sequences.

Fig. S2. Molecular phylogeny of 18 tropical Aglaopheniidae species generated by PhyML from CAM sequences.

Fig. S3. Molecular phylogeny of 18 tropical Aglaopheniidae species generated by PhyML from ITS sequences.

Table S1. Number of newly sampled individuals per location.

Table S2. List of individuals per species used in this study, sampling site and GenBank accession numbers of 16S, calmodulin and ITS genes sequences.