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ORIGINAL PAPER

The Parazoanthidae (Hexacorallia: Zoantharia) DNA taxonomy: description of two new genera

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Abstract The taxonomy of the hexacorallian order Zoantharia is very problematic due to the lack of easily accessible and informative morphological taxonomic characters. This is particularly true in the widespread family Parazoanthidae, members of which use a wide variety of different organisms as substrates. Recently, DNA-based studies have proven to be of great use in clarifying relationships among Parazoanthidae. Here we reconsider Parazoanthidae taxonomy based on analyses of multiple molecular markers [mitochondrial cytochrome oxidase subunit 1 (COI), 16S ribosomal DNA (mt 16S rDNA), and the nuclear internal transcribed spacer region (ITS rDNA)], coupled with ecological and morphological characteristics. Two new genera are described in this study: *Hydrozoanthus* n. gen. within the new family Hydrozoanthidae, and *Antipathozoanthus* n. gen in the family Parazoanthidae. The genetic data further suggest that the revised genus *Parazoanthus* is still polyphyletic and is composed of three distinctive subclades. However, as currently these subclades can essentially be differentiated by genetic data, these subclades should remain within *Parazoanthus* until further molecular, ecological and morphological studies help to clarify their status and relationships to each other.

Keywords Zoanthids · Molecular phylogeny · *Hydrozoanthus* · *Antipathozoanthus* · Epibiosis · Co-evolution

Introduction

Species of the hexacorallian order Zoantharia (zoanthids) are found in most marine environments from shallow tropical

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waters to the deep sea and other extreme environments (e.g. methane cold seeps—see Reimer et al. 2007a). All zoanthids have a double row of tentacles and a single siphonoglyph. Most zoanthids have a colonial way of life and incorporate particles (sand, sponge spicules, foraminifer tests) in their ectoderm and mesoglea to help make their structure. Apart from a few exceptions (the genus *Savalia*), they do not build any skeletal structure.

The order Zoantharia is traditionally separated into two suborders characterised by the fifth pair of mesenteria (complete in Macrocnemina, incomplete in Brachycnemina) (Haddon and Shackleton 1891). The suborder Macrocnemina currently consists of two families: Epizoanthidae and Parazoanthidae. The family Epizoanthidae comprises the genus *Epizoanthus* and the monospecific genus *Palaeozoanthus* [although never found again since its original description (Carlgren 1924)]. The other macrocnemic family, Parazoanthidae, currently contains five recognised genera; *Parazoanthus, Mesozoanthus, Isozoanthus, Savalia* and *Corallizoanthus*. The latest four genera are restricted, at most, to a few species each, while *Parazoanthus* currently contains many species found worldwide in a variety of habitats. Despite the surprising absence of any mention of Parazoanthidae in a recent review of Hexacorallia (Daly et al. 2007), this family is probably the most diverse within the order Zoantharia. Another monospecific zoanthid family has recently been described (Abyssoanthidae, *Abyssoanthus*) (Reimer et al. 2007a); while apparently branching within Macrocnemic zoanthids, between Epizoanthidae and Parazoanthidae, the status of its fifth pair of mesenteria remains unknown. *Abyssoanthus*, *Corallizoanthus* and most of *Isozoanthus* are restricted to deep sea. Table 1 shows the different zoanthid families and genera with the distribution, substrate indications, original diagnostic characteristics and, when relevant, some actual characteristic being used to identify the families/genera nowadays.

Until now, several studies have attempted to find new morphological or histological characters that efficiently discriminate between zoanthid genera and species. Characters such as the cnidome (Ryland and Lancaster 2003; Herberts 1972) and sphincter muscle anatomy (Lwowsky 1913) have been investigated, but none of these have proven to be efficient and applicable to zoanthids over a wide range of taxa. The sphincter information, for example, is not distinctive for many particular genera (*Palaeozoanthus*, *Savalia*, *Sphenopus* and *Acrozoanthus*, see Table 1). And recently, taxonomic studies based largely on sphincter data have led to new species descriptions with doubtful generic assignments (e.g. Swain 2009; Philipp and Fautin 2009).

Parazoanthids are often associated with other organisms used as substrate (characteristic shared by some Epizoanthidae and the brachycnemic *Acrozoanthus australiae*, however those taxa are considered only as outgroups in this study). The type species of the genus, *Parazoanthus axinellae,* usually lives closely associated to sponges. This species was one of the first epizoic zoanthids studied (Schmidt 1862; Arndt and Pax 1936). The affinities of some Caribbean *Parazoanthus* species to different sponge species have been demonstrated by Crocker and Reiswig (1981) and more recently by Swain and Wulff (2007). Potential correlation between different monophyletic parazoanthid groups and substrate specificity has recently been shown in Sinniger et al. (2005). In the same study, it was clearly demonstrated that Parazoanthidae and the genus *Parazoanthus* in particular are paraphyletic.

This research explores relationships between different Parazoanthidae by using the sequences from two mitochondrial markers [16S ribosomal DNA (mt 16S rDNA) and cytochrome oxidase subunit I (COI)] and from the nuclear internal transcribed spacer of ribosomal DNA (ITS-rDNA; consisting of ITS-1, 5.8S rDNA and ITS-2). Molecular results are combined with ecological data to reorganise and simplify the systematics of this family. In particular, we focus on two previously recognised but undescribed clades currently within Parazoanthidae, one consisting of species associated with hydrozoans, and one associated with antipatharians. The genera *Hydrozoanthus* n. gen. (in the new family Hydrozoanthidae) and *Antipathozoanthus* n. gen. can be distinguished from other zoanthid genera by substrate specificity, as well as by highly characteristic DNA sequences.

Materials and methods

Sampling

Zoanthid samples were collected either by SCUBA or by trawling during different research cruises from the Caribbean Sea, Pacific, West Indian Ocean, East Atlantic, and the Mediterranean Sea. Zoanthid specimens were fixed and conserved in ethanol (minimum 70%) after collection. Samples are kept in the authors' personal collections or were deposited in the Natural History Museum of Geneva, Switzerland (MNHG).

DNA extraction and sequencing

DNA was extracted from ethanol-preserved samples using the DNeasy Plant Minikit (QIAGEN) or the following guanidine extraction protocol: a fragment of mesenteria (about 1 mm^3) was dried and digested 30 min at 55°C, followed by 90 min at room temperature with 100 µl guanidine extraction buffer (4 M guanidinium isothiocyanate, 50 mM Tris pH 7.6, 10 mM EDTA, Sarkosyl 2% w/v,

Table 1 Summary of families and genera within the order Zoantharia as recognized until now

Suborder (characteristics)	Family (characteristics)	Genus	Estimated species number[a]	Main habitats	Substrates	Original diagnostic characteristics	Zooxanthellae	Colonial or unitary	Notes
Macrocnemina (5th mesentery from dorsal directive complete)	Epizoanthidae (mesogleal sphincter muscle)	*Epizoanthus* Gray 1867	139	Worldwide; shallow to deep	Non-living hard substrate, pagurid crabs, mollusc shells, worm tubes, free living in the sediments	Mesogleal sphincter muscle	No	Mainly colonial	Often but not always epizoic.
		Paleozoanthus Carlgren 1924	1	South Africa	Gastropod (*Fusus rubrolineatus*)	fertile micromesenteries[c]	No	colonial	Never found again since description
	Abyssoanthidae (DNA, cold seep environments)[c]	*Abyssoanthus* Reimer et al. 2007a, b, c	1	Deep sea >3,000 m chemosynthetic environments, Japan	Mudstone	DNA, ecology[c]	No	Mainly unitary	
	Parazoanthidae (endodermal sphincter muscle)	*Parazoanthus* Haddon and Shackleton 1891	62	Worldwide; shallow to deep	Sponges, hydrozoans, antipatharians, non-living hard substrate	Mesogleal lacuna and canal forming a ring sinus	Some	Colonial	Reorganized in this paper.
		Savalia Nardo 1844 (=*Gerardia* Lac. - Duth. 1864)	3	Mediterranean sea, N Atlantic 30–500 m	Gorgonians	Secretion of hard skeleton.	No	Colonial	Lacunae and canals as *Parazoanthus*.
		Isozoanthus Carlgren 1923	26	South Africa, Northern Europe and deep sea	Non-living hard substrate, Hexactinellid, tubeworms	No ring sinus, polyps solitary or weak coenenchyme	Some	Usually solitary.	Swain 2009, included a tropical shallow species in this genus, see text.
		Corallizoanthus Reimer et al. 2008a, b	1	150–300 m, Japan	Coralliidae	DNA, substrate specificity[c]	No	Mainly unitary	
		Mesozoanthus Sinniger and Häussermann 2009	1	Shallow cold water along west coast of Americas	Non-living hard substrate	DNA, absence of biological association.[c]	No	Colonial	
Brachycnemina (5th mesentery from dorsal directive incomplete)	Sphenopidae (heavy sand encrustation)	*Palythoa* Lamouroux 1816	217[b]	Subtropical and tropical shallow waters worldwide	Non-living hard substrate	Single mesogleal sphincter	Yes	Colonial	Includes former genus *Protopalythoa*.
		Sphenopus Steenstrup 1856	10	Subtropical and tropical waters, Indo-Pacific	Free-living in the sediments	Ecology (unitary, free-living polyps)	No	Unitary	Single mesogleal sphincter as *Palythoa*.
	Zoanthidae (no sand encrustation)	*Zoanthus* Lamarck 1801	156	Subtropical and tropical shallow waters worldwide.	Non-living hard substrate	Double mesogleal sphincter	Yes	Colonial	Most common tropical zoanthid with *Palythoa*.
		Acrozoanthus Saville-Kent 1893	1	Epizoic on tube worms in Australia and Indonesia.	Eunicid worm tubes.	Substrate specificity[c]	Yes	Colonial	Characteristic budding.
		Isaurus Gray 1828	3–23	Subtropical and tropical shallow waters worldwide.	Non-living hard substrate.	Single mesogleal sphincter	Yes	Colonial	Long asymmetric column, polyps open at night.
	Neozoanthidae (endodermic sphincter muscle)[c]	*Neozoanthus* Herberts 1973	1	Coral reefs in Madagascar.	Non-living hard substrate.	Endodermic sphincter muscle[c]	Yes	Colonial	Never found again since description.

[a] From references and Fautin (http://hercules.kgs.ku.edu/Hexacoral/Anemone2/). These numbers are undoubtedly incorrect, but are provided to compare taxa listed within

[b] Includes 26 species described from *Protopalythoa*

[c] Monospecific taxa, description might be modified with future discoveries

β-mercaptoethanol 1% v/v). The DNA was subsequently precipitated with 100 µl isopropanol at −20°C overnight. DNA was then centrifuged for 15 min (15,000 rpm) and the supernatant was removed. DNA was washed once with 70% ethanol and after 5 min centrifugation (15,000 rpm) and supernatant removal, it was dried and eluted in 50 µl pure H_2O. Specimens were then amplified for COI, mt 16S rDNA and ITS rDNA region using standard Taq polymerase and the primers LCOant: 5' TTTTCYACTAATCATAAAGATAT 3', COIantr: 5' GCCCACACAATAAAGCCCAATAYYCCAAT 3', 16Sant0a: 5' GAAGTAGGCTTGGAGCCAGCCA 3' as well as primers described in Folmer et al. (1994), Sinniger et al. (2005) and Reimer et al. (2007a) respectively, according to the following thermal cycle conditions: 2 min denaturation at 94°C followed by 35 cycles of 1 min at 94°C, 30 s at annealing temperature (42°C for COI, 52°C for mt 16S rDNA and ITS rDNA), 90 s elongation at 72°C, and terminated by a single final elongation step of 2 min at 72°C. The presence of *Symbiodinium* zooxanthellae in hydrozoan-associated specimens was tested by amplifying the ITS rDNA region of the symbiont according to the protocols used in Reimer et al. (2006). Direct sequencing was carried out using a BigDye Terminator Cycle Sequencing Ready Reaction Kit following the manufacturer instructions (Applied Biosystems) for both strands of each marker. Sequences were run on an ABI-3100 Avant automatic sequencer. GenBank accession numbers are reported in Table S1 (Electronic supplementary material).

Phylogenetic analyses

Sequences for both strands were manually assembled and chromatograms were checked for quality. Resulting sequences were aligned with ClustalX ver. 1.8 (Thompson et al. 1997) with subsequent manual editing of the automated alignment using BioEdit ver. 5.0.9 (Hall 1999). Alignments were analysed with the maximum likelihood (ML) method using PhyML ver. 3.0 (Guindon and Gascuel 2003). Bayesian analyses were performed using MrBayes ver. 3.0 (Ronquist and Huelsenbeck 2003). All analyses were performed with GTR nucleotide substitution matrix, a gamma 1 invariant model with six categories, estimated α-parameter and estimated frequencies of amino acids. Species belonging to the macrocnemic family Epizoanthidae and brachycnemic family Sphenopidae were used as outgroups. COI sequences of *Abyssoanthus* and *Corallizoanthus* were not included in the analyses due to their short length. Only zoanthid outgroups were used in order to keep a maximum of informative sites in the alignments (the insertion-deletion pattern of other hexacorallian orders were too divergent to allow reliable alignment construction).

Results

Systematics

Order ZOANTHARIA Gray 1870

Hydrozoanthidae n. fam. This family groups several tropical and sub-tropical macrocnemic zoanthids; including species associated with hydrozoans and also several other non-hydrozoan associated species. This family is erected to group former Parazoanthidae species sharing specific insertions and deletions in mt-16S rDNA, especially in the V5 region (as defined in Sinniger et al. 2005) of this gene. The analyses of interfamilial genetic distances among zoanthids, especially for the gene coding for cytochrome oxidase subunit 1 consistently confirms the taxonomic level of the family (Fig. 1, distance tables available from the authors on request). Phylogenetically, species in this family are more closely related to brachycnemic zoanthids (especially from the genus *Palythoa*) than to other parazoanthids.

Genus Hydrozoanthus n. gen. Type species: *Hydrozoanthus (Parazoanthus) tunicans* (Duerden 1900)

Other species/specimens: *H. (Parazoanthus) gracilis, H. (Isozoanthus) antumbrosus.*

Etymology: Named for this group's epizoic relation with hydrozoans.

Figure: Figure 2 shows various *Hydrozoanthus* species and specimens in situ.

Material examined: *H. tunicans*, Utila, Honduras (N 16° 04.759′ W 86°55.749′), depth: 15 m, 13.02.2004, coll: F. Sinniger, MNHG INVE 64730; *H. gracilis*, Izu, Japan, depth: 17 m, 11.2004, coll: J.D. Reimer; *H. gracilis* collected in Bunaken Island, Sulawesi (Indonesia), depth: 28 m, the 12.09.2003 by M. Boyer, MNHG INVE 64731; *H. antumbrosus*, Utila, Honduras (N 16°04.759′ W 86° 55.749′), depth: 15 m, 13.02.2004, coll: F. Sinniger, MNHG INVE 64732; *H. cf. gracilis*, canal Woodin, New Caledonia, depth: 25 m, 27.11.2006, coll: J.L. Menou, MNHG INVE 64733; *H. cf. gracilis*, canal Woodin, New Caledonia, depth: 33 m, 27.11.2006, coll: J.L. Menou, MNHG INVE 64734.

Diagnosis Tropical or subtropical colonial zoanthids, polyps linked together by a basal coenenchyme. Size of the expended polyps usually between 2–6 mm width and 4–15 mm high. Mesenteries have macrocnemic organisation. Column lightly incrusted with fine sediments, not completely hiding the ectoderm. Colour of different species ranging from yellow to dark brown. Always associated to hydrozoans, leaving the smallest branches of the hydrozoan colony intact and not covered (for more details on the association see Di Camillo et al. 2009). No other zoanthids have been found with such

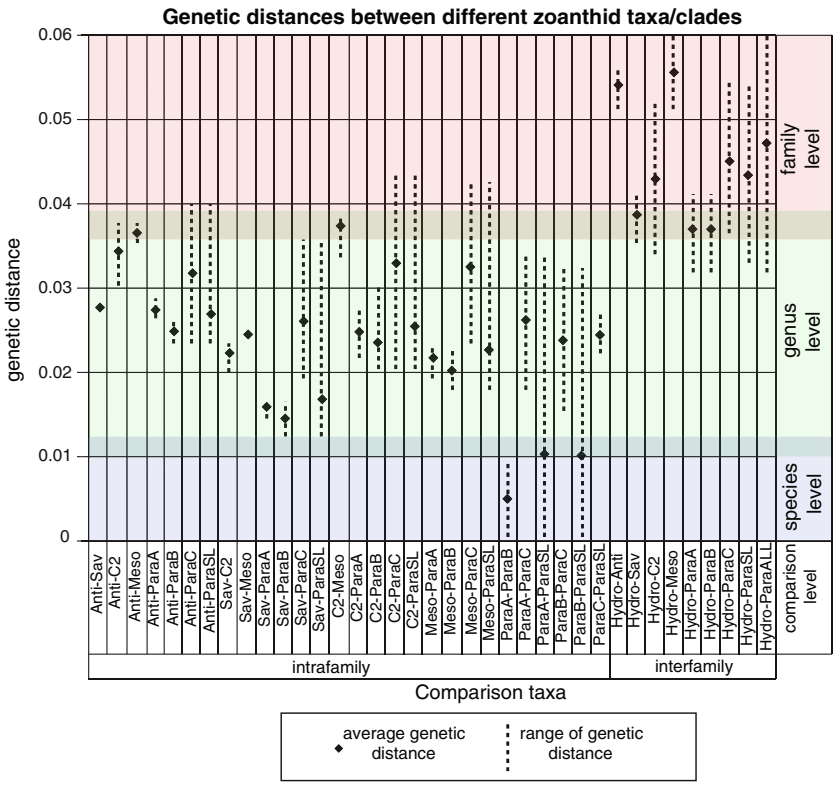

Fig. 1 Genetic distances comparisons based on COI sequences. Intrafamilial comparisons are based on the different Parazoanthid clades *Antipathozoanthus* n. gen. (*Ant*), *Savalia* (*Sav*), *Mesozoanthus* (*Meso*), "Clade 2" (*C2*) and *Parazoanthus* (*ParaSL*). Within *Parazoanthus* the distinction was made between the clades A, B and C (*ParaA*, *ParaB* and *ParaC*). The second part of the table compares *Hydrozoanthus* n. gen. (*Hydro*) and the different Parazoanthid clades. The shorter length of available *Corallizoanthus* sequences inducing a significant bias, the estimated distances are not shown here. The extreme distances between *Isozoanthus* and the other Parazoanthidae/Hydrozoanthidae n. fam. ranging between 0.2072 and 0.2265, the values are not shown here

association. Some species are zooxanthellate, the majority azooxanthellate (see below).

Remarks Among species formerly assigned to *Parazoanthus*, *P. tunicans* (Duerden 1900) from the Caribbean and *P. gracilis* (Lwowsky 1913) from the Indo-Pacific belong to *Hydrozoanthus* n. gen., with *Hydrozoanthus tunicans* becoming the type species for this genus.

In the original description of *H. tunicans* (Duerden 1900), the presence of zooxanthellae was mentioned; however, West (1979) re-examined this species from Puerto Rico and suggested that zooxanthellae were confused with pigment cells. The affirmation on the presence of zooxanthellae in the species description of *H. antumbrosus* in Swain (2009) was not supported by any data or references. Preliminary results (based on ITS sequences) would suggest the presence of *Symbiodinium* sp. in *H. tunicans* but not in any other *Hydrozoanthus* examined (including *H. antumbrosus*). The presence of zooxanthellae might also be irregular within the same species as this could explain the divergent results obtained by the different researchers.

Family Parazoanthidae Delage and Hérouard 1901

Antipathozoanthus n. gen. Type species: *Antipathozoanthus* (*Gerardia*) *macaronesicus*

Other species/specimens: *Antipathozoanthus macaronesicus* from Principe, *Antipathozoanthus* sp. from Madagascar, *Antipathozoanthus* sp. from Japan, *Antipathozoanthus* sp. from Galapagos.

Etymology: The name was chosen with regard to the substrate specificity of this genus, as it is found only on antipatharians. The ending is uniform with most other zoanthid genera.

Figures: Figure 2 shows different species and specimens of *Antipathozoanthus* in situ.

Material examined: *Antipathozoanthus macaronesicus* "CV1", Cape Verde, depth 18 m, 09.2003 coll: P. Wirtz, MHNG INVE 64735; *Antipathozoanthus macaronesicus* "CV2", depth 18 m, 09.2003 coll: P. Wirtz, MHNG INVE 64736; *Antipathozoanthus macaronesicus*, Principe, depth: 45 m, 02.2004 coll: P. Wirtz, MHNG 64737; *Antipathozoanthus* sp., Sakatia, NW Madagascar, depth:10 m, 07.12.2004, coll: F. Sinniger, MHNG 64738; *Antipathozoanthus* sp., Otsuki, Kochi, Japan, depth: 26 m, 26.01.08, coll: F. Sinniger, MNHG 64739; *Antipathozoanthus* sp., Galapagos, depth: 20 m, 11.11.2003, coll: C. Hickman

Diagnosis Colonial zoanthids, polyps linked together by a basal coenenchyme usually covering all of antipatharian substrate axis, size of expended polyps usually between 4–6 mm width and 4–15 mm high. Column lightly incrusted with fine sediments, not completely hiding ectoderm. Column and tentacles usually yellowish or pinkish. Mesenteries follow macrocnemic organisation. Grows exclusively on antipatharians. Distributed

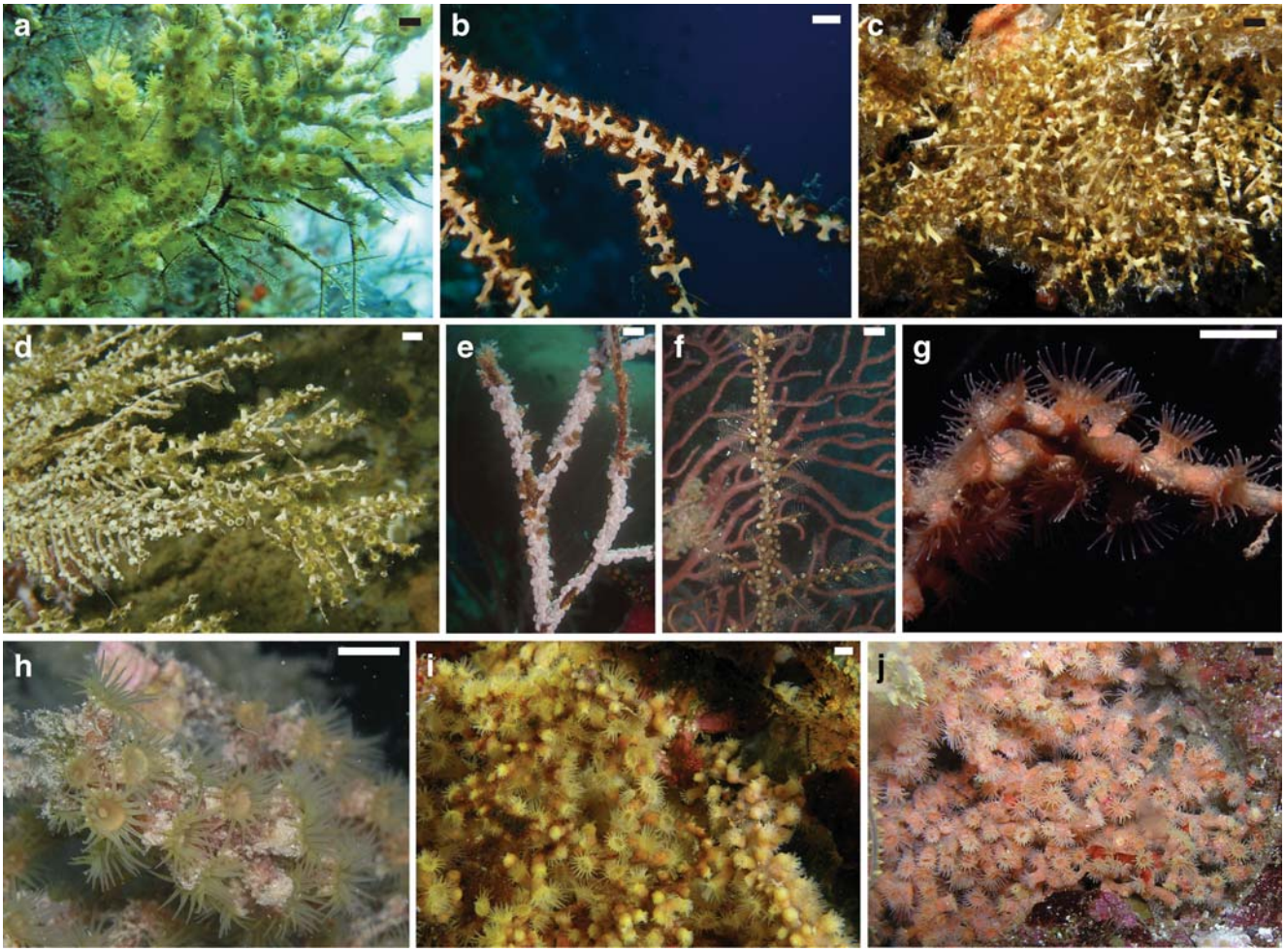

Fig. 2a–j In situ pictures of *Hydrozoanthus* n. gen. spp. and *Antipathozoanthus* n. gen. Images were taken by the first author unless otherwise mentioned. **a** *H. gracilis* from Japan (type locality) (J.D. Reimer), **b** *H. gracilis* from Sulawesi (C. Di Camillo), **c** *H.* cf. *gracilis* from New Caledonia, colony 1, **d** *H.* cf. *gracilis* from New Caledonia, colony 2, **e** *H. tunicans* from Honduras, **f** *H. antumbrosus* from Honduras, **g** *A. macaronesicus* from Cape Verde (P. Wirtz), **h** *Antipathozoanthus* sp. from Madagascar, **i** *Antipathozoanthus* sp. from Japan, **j** *Antipathozoanthus* sp. from Galapagos (J.D. Reimer). *Scale bar* on the top right of each picture represents 1 cm

in tropical and subtropical area at depths ranging from 10 m to 45 m.

Remarks Species of this genus have been collected in the East Atlantic (Cape Verde and Principe Islands), in Madagascar, in Japan and in the Galapagos. This genus is known to live in association with the black coral species *Tanacetipathes cavernicola* (*Antipathozoanthus macaronesicus*), *Antipathes* aff. *hypnoides* (Madagascar species), *Antipathes galapagensis* (Galapagos species) (Reimer et al. 2008a, b) and *Antipathes* aff. *grandiflora* (Japanese species). However, more sampling is necessary to gain a clearer picture of the true range of antipatharian species used as substrate. As observed in the gorgonian-associated genus *Savalia*, the colony can extend out a few centimetres from its substrate.

The type species *A. macaronesicus* was originally included in the description of *Savalia (Gerardia) macaronesica* (Ocaña and Brito 2003), and later the description was amended and the authors suggested the possible placement of this species in a separate genus (Ocaña et al. 2007). The species name was accorded to the genus gender. Skeletal secretion (similar to *Savalia* spp.) was advanced by Ocaña and Brito (2003) as occurring in *Antipathozoanthus macaronesicus* but no reliable evidence of such secretion has been found so far further despite the attempts to observe this.

Phylogenetic analyses

COI

The alignment obtained with 48 COI sequences contains 624 sites. The different topologies obtained using different analyses methods all showed congruent results, with main differences in the resolution at supra-specific clade levels. The tree obtained with Bayesian analyses of codons (Fig. 3)

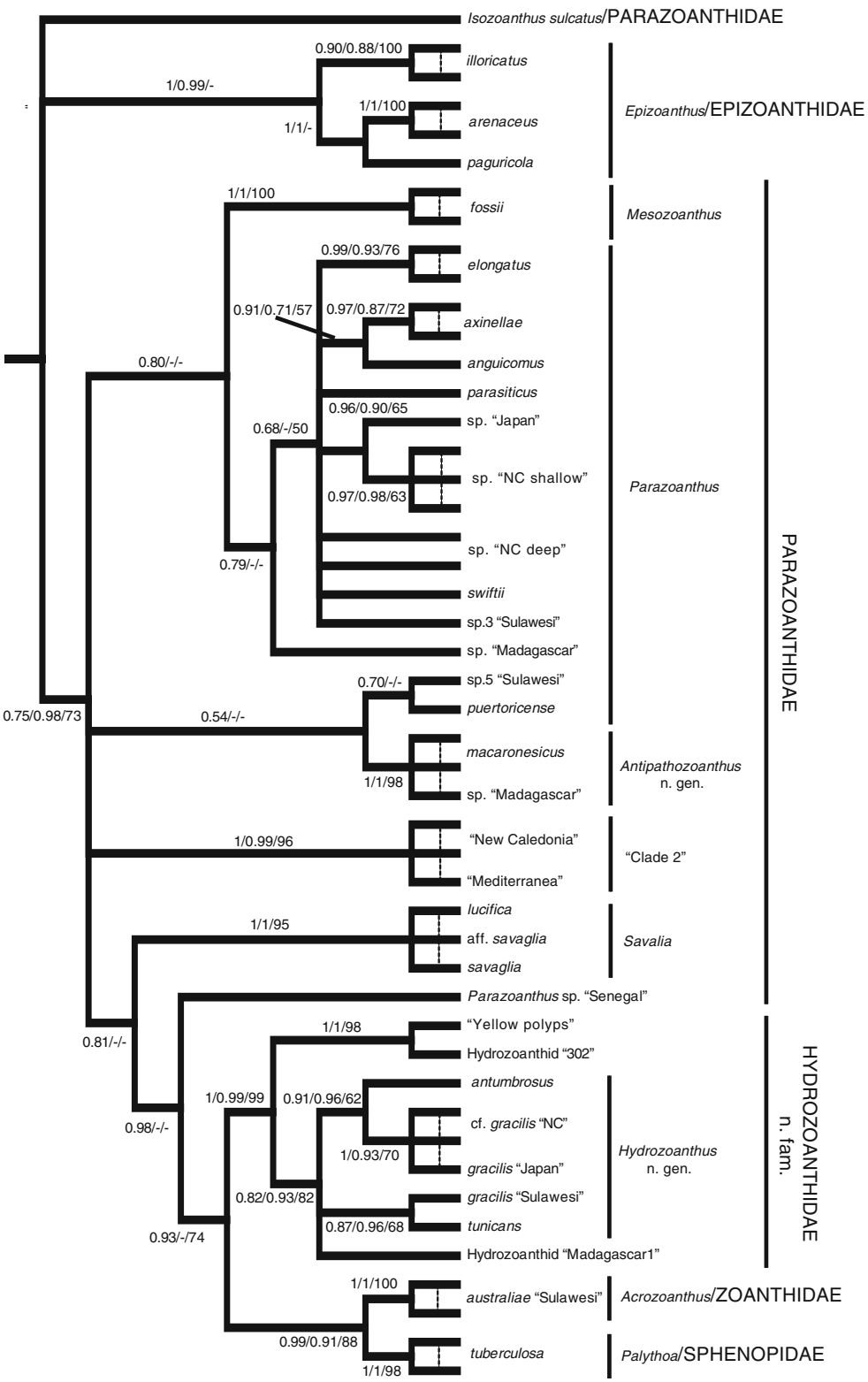

Fig. 3 Bayesian tree obtained with COI sequences using codon analysis. Values at the nodes indicate posterior probabilities with codon analysis, posterior probabilities with nucleotide analysis and ML (nucleotide) bootstrap support when >50%

showed the best resolution and, among the trees obtained with nucleotides, the ML tree showed considerable resolution when compared with the Bayesian tree. In all trees obtained, the different generic level clades were highly supported, with the exception of *Parazoanthus,* which appeared unresolved. *Epizoanthus* was used as an outgroup and *Isozoanthus sulcatus* (traditionally grouped within Parazoanthidae) also branched at the base of the tree. The remaining zoanthids, including Parazoanthidae and brachycnemic zoanthids grouped into a poorly resolved clade.

Parazoanthus appeared paraphyletic and unresolved. The grouping of *Mesozoanthus* with some *Parazoanthus* species appeared only in the codon analysis, while in the nucleotide analyses most species appeared as independent clades with unresolved positions. Most other generic level clades appeared independent in all the analyses. In the codon analysis, *Savalia* branches at the base of a clade composed of one undescribed *Parazoanthus*, Hydrozoanthidae n. fam. and Brachycnemina. The position of *Parazoanthus* sp. from Senegal in this clade might be a consequence of long branch attraction.

A highly supported monophyletic Hydrozoanthidae n. fam. sister to Brachycnemina was recovered in the analyses. The association of those two sister groups was recovered in all the analyses (posterior probabilities cod.=0.93, pp nuc.=0.91, ML=74%). One zoanthid not associated to Hydrozoa (Hydrozoanthid M1) appeared to be closely related to *Hydrozoanthus* n. gen. in all analyses.

Genetic distances between the families Hydrozoanthidae n. fam. and Parazoanthidae range between 0.0328 and 0.0616 (0.0328 and 0.0567 between *Hydrozoanthus* n. gen. and other parazoanthid genera), while distances between parazoanthid genera range between 0.0432 and 0.0129 (0.0400 and 0.0210 between *Antipathozoanthus* n. gen. and other parazoanthids). Distances between Hydrozoanthidae n. fam. and Sphenopidae range between 0.0352 and 0.0448 (Fig. 1).

mt 16S-rDNA

The length of the 51 sequences composing the alignment varied between 536 and 641 bp. Most of the length variation was located in two regions corresponding to the regions V5 and V8 described in Sinniger et al. (2005). Similar to COI trees, the resolution of the trees was poor at specific level, however most generic level clades were clearly distinct (Fig. 4).

The monophyly of the "Parazoanthidae and Sphenopidae" clade was highly supported (pp=1, ML=100%), but the relationships between most different groups within this clade remained unresolved (pp≤0.5, ML≤50%). However, mt 16S-rDNA was useful in distinguishing between some groups within this clade, although this marker is apparently too conservative to distinguish between some closely related species (e.g. *Parazoanthus axinellae* and *P. anguicomus* had identical sequences). Most generic level clades were shown to be supported monophyletic groups [*Antipathozoanthus* n. gen., parazoanthid clade 2, *Corallizoanthus* and *Mesozoanthus* (pp=1.00, ML=100%), *Savalia* (pp=1.00, ML=64%)] and *Hydrozoanthus* n. gen. (pp=0.77, ML=52%), but the phylogeny of sponge-associated *Parazoanthus* remained problematic.

The monophyletic group of *Hydrozoanthus* n. gen. and related species (pp=0.89, ML=77%) branching as a sister group of the family Sphenopidae appeared in the best ML tree but was not supported by significant bootstrap values nor by posterior probabilities (pp<0.6, ML<50%). As with COI topologies, a zoanthid not associated to hydrozoans branched at the base of the *Hydrozoanthus* n.gen..

Genetic distances between specimens of the family Hydrozoanthidae n. fam. and Parazoanthidae range between 0.0191 and 0.0611, while genetic distances between different parazoanthid genera range between 0.0133 and 0.0679 (0.0286 and 0.0679 between *Antipathozoanthus* n. gen. and other parazoanthids). Distances between *Hydrozoanthus* species range from 0 to 0.0086, while distances between *Hydrozoanthus* spp. and other Hydrozoanthidae range between 0.0139 and 0.0226.

ITS-rDNA region (ITS-1, ITS-2 and 5.8S-rDNA)

The alignment of the ITS-rDNA region contained 1019 sites and 79 sequences. Multiple copies originating from some of the 49 samples were included in the alignment. While all species were represented at least once by ITS-1, 5.8S and ITS-2 rDNA, additional copies for ITS-2 were added to the alignment for some samples. The trees obtained further supported the results obtained with the two mitochondrial markers with slightly higher resolution for relationships between genera in the Bayesian analysis (Fig. 5). However, in the ML tree no supported resolution could be obtained for most supra-generic-level clades. The main difference of the Bayesian analyses was the clade grouping Sphenopidae and Hydrozoanthidae n. fam. (pp=1, ML=71%) did not emerge within the Parazoanthidae but as a sister group to this family and that the genus *Parazoanthus* appeared monophyletic (pp=1). The monophyly of Parazoanthidae is well supported in the Bayesian topology (pp=0.99).

Hydrozoanthidae n. fam. appeared monophyletic (pp=0.98, ML=96%) and branched as a sister group to Sphenopidae. Within this clade, two samples clearly not associated to hydrozoans, (the aquarium-grown "Yellow polyps" and an undetermined zoanthid from Madagascar) branched together at the base of the clade, while "Hydrozoanthid" sp. "Madagascar1" branches at the base of *Hydrozoanthus* n.gen.. The *Hydrozoanthus* n.gen. (*H. gracilis*, *H.* cf. *gracilis*, *H. tunicans*, *H. antumbrosus*) monophyly was supported by posterior probabilities of 1.00. The different ITS-rDNA copies of the recently described species *H. antumbrosus* (Swain 2009), originally assigned to *Isozoanthus* branch together (pp=0.85, ML=72%) within *Hydrozoanthus*. *H. tunicans* copies appear distinct from the other *Hydrozoanthus* in both analyses

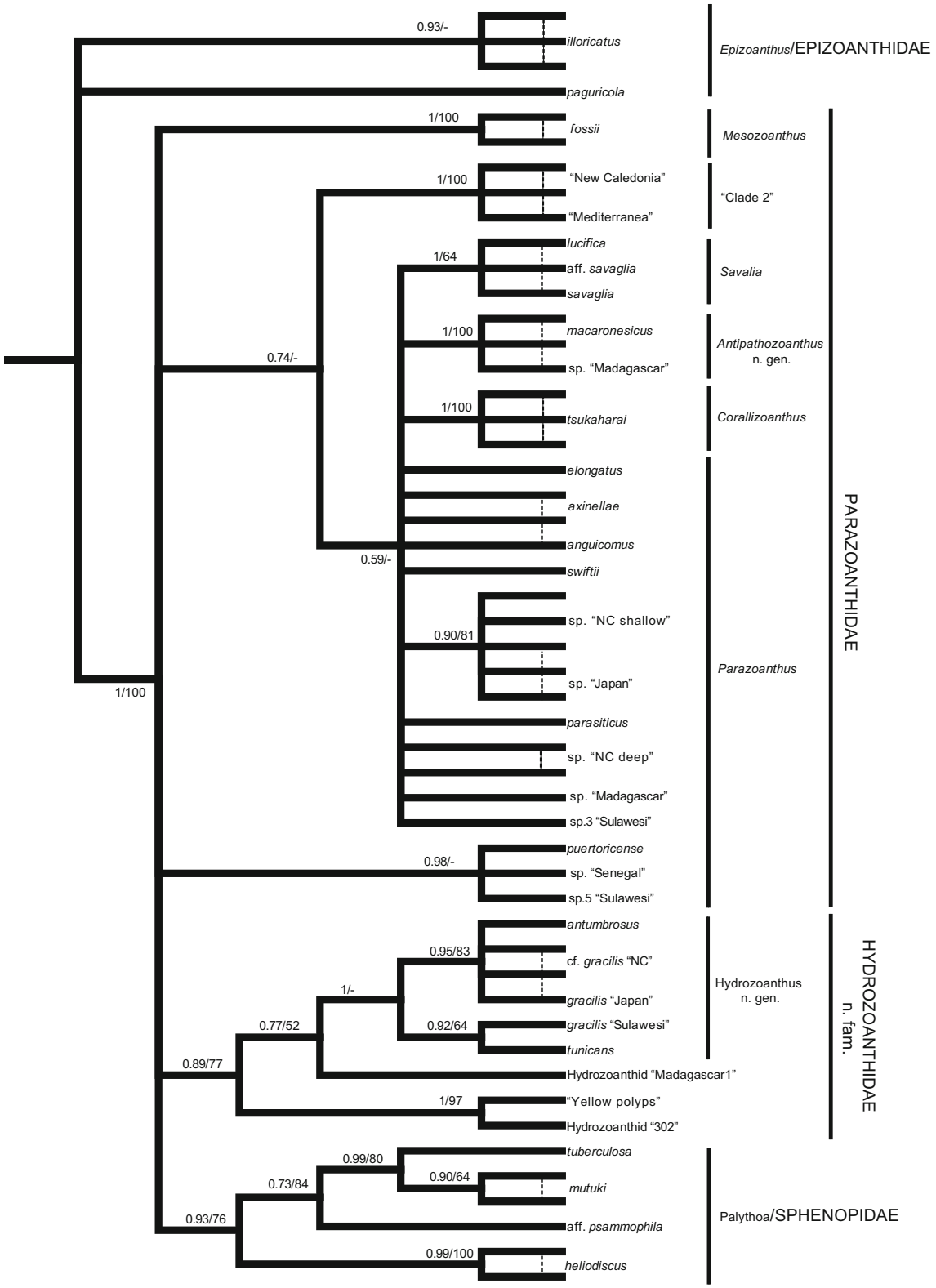

Fig. 4 Bayesian tree obtained with mt 16S rDNA data. Values at the nodes indicate posterior probabilities and ML bootstrap support when >50%

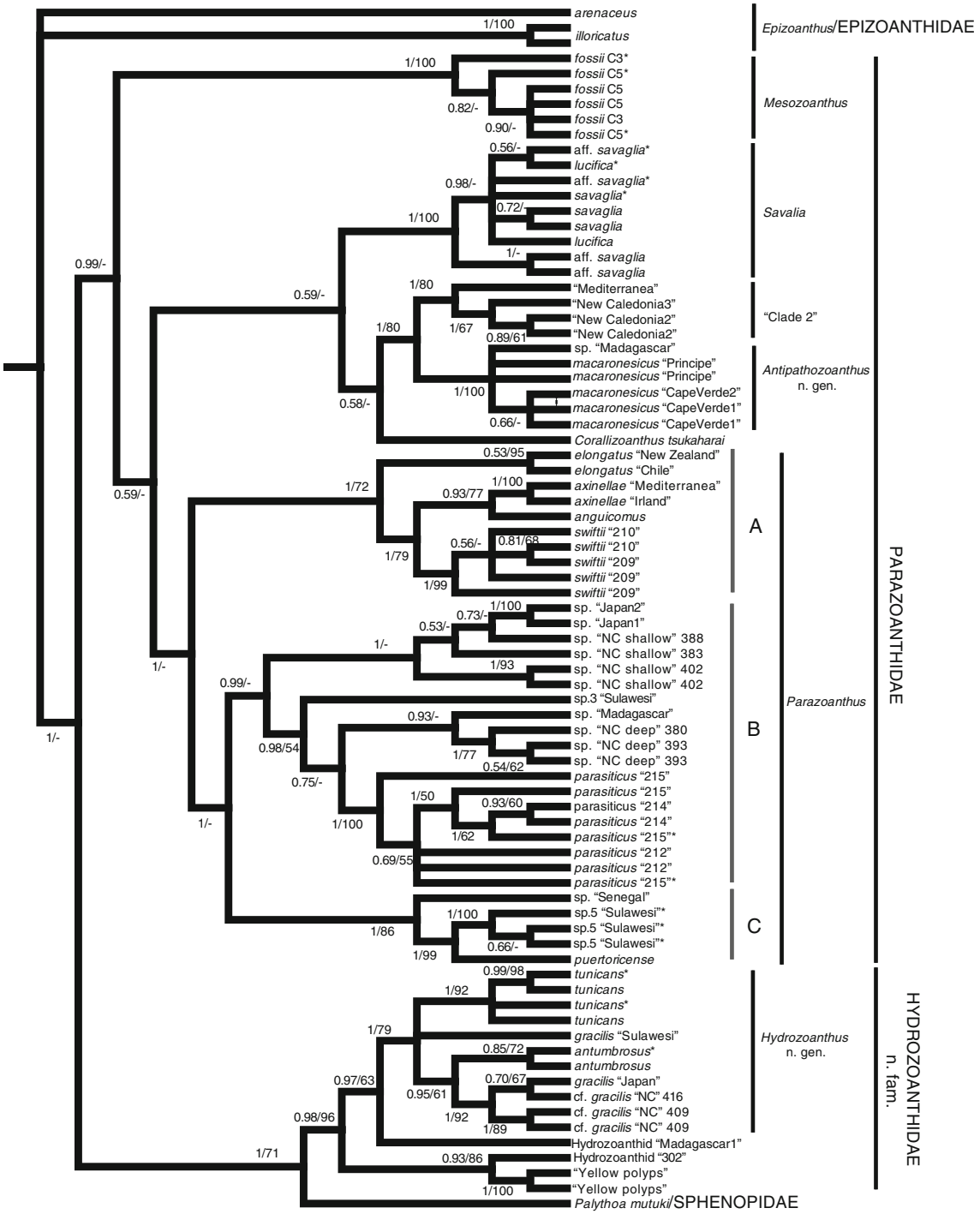

Fig. 5 Bayesian tree obtained with ITS sequences. Numbers following some species names serve to identify different specimens. Names followed by an *asterisk* indicate sequences of ITS2 only. Values at the nodes indicate posterior probabilities and ML bootstrap support when >50%. Divergent sequences do not always represent different species due to the presence of ITS copies. The three *Parazoanthus* subclades are indicated with *capital letters*

(pp=1, ML=92). However, copies from the two apparently identical *H.* cf. *gracilis* collected in the same site in New Caledonia branched at slightly different positions, one sample forming a monophyly with *Hydrozoanthus gracilis* (pp=0.7, ML=67%), while the other is sister to the *H. gracilis* monophyly (pp=1, ML=89%).

Similarly to as observed in the mitochondrial marker phylogenetic trees, generic level clades were well supported (pp=1, ML>80%), with the exception of *Parazoanthus*, which was unresolved in ML analysis (ML<50%). At the supra-generic level, the clades *Antipathozoanthus* and "Clade 2" grouped together (pp=1, ML=80%). In the

Bayesian analysis *Corallizoanthus* and *Savalia* branched at the base of the *Antipathozoanthus*- "Clade2" group with weak support (pp=0.58 and 0.59 respectively), while in the ML analysis these two genera branched independently. The genus *Parazoanthus* appeared unresolved in ML analyses with the subclades A and C branching as independent monophylies (72% and 86% bootstrap, respectively) and subclade B composed of one monophyletic group and four independent branches. In Bayesian analysis, the monophyly of *Parazoanthus* was well supported (pp=1.00) and the three *Parazoanthus* subclades also appeared monophyletic (pp=1.00 or 0.99). In the three subclades, multiple copies belonging to described species formed fully (pp=1.00) supported monophylies (subclade A: *P. elongatus*, *P. swiftii* and *P. axinellae;* subclade B: *P. parasiticus*). The third subclade contained *P. puertoricense*, a *Parazoanthus* sp. from Senegal and multiple ITS copies were obtained only from *Parazoanthus* "sp5" from Sulawesi (pp=1.00).

Discussion

Utility of different molecular markers

Zoanthids have very few reliable diagnostic morphological characters, but molecular results here and in other studies (Sinniger et al. 2005) have proven to fit well with certain ecological (substrate specificity) and biogeographical (*P. elongatus* in South Pacific and *P. axinellae* in Mediterranean and North-East Atlantic) features. This strongly suggests that a combination of these features can be successfully used to distinguish between and identify most zoanthid species and recent taxonomic studies already showed the adequacy to use DNA information combined with ecological information in species or genus descriptions (Reimer et al. 2007a; 2008a, b; Sinniger and Häussermann 2009). However, caution must be taken when using different molecular markers. Indeed, in 16S and ITS, much information is contained in indels, which are problematic to align when dealing with different genera. Our results show clearly the influence of such issues, in particular affecting accurate estimation of genetic distances. Sequence conservation issues were problematic for bootstrapping and when not considering insertion/deletion events in ITS-rDNA region and mt 16S rDNA to infer the phylogenetic relationships between species groups.

Therefore, while these two markers are the most useful to clearly distinguish the different taxa, COI appears more suitable to objectively compare genetic distances and potential relationships between the different clades. Figure 1 illustrates the efficiency to separate different taxonomical levels based on COI genetic distances. Despite some overlap exists, the average values provide a good estimation of the taxonomical level.

Generic level clades

The generic level of both *Antipathozoanthus* n. gen. and the *Hydrozoanthus* n. gen. is supported both by substrate specificity (antipatharians and hydrozoans, respectively) and molecular results (Fig. 1).

Another genus level parazoanthid clade, "Clade 2", uses hexactinellid stalks as substrate. This substrate is also characteristic for a few species of *Epizoanthus* and *Isozoanthus* (see Carlgren 1923), but the molecular results exclude clearly our specimens from *Epizoanthus* (Figs. 3, 4 and 5). COI (Fig. 3) and preliminary ITS rDNA (F. Sinniger, data not shown) sequences of *Isozoanthus sulcatus* strongly diverge from parazoanthid sequences, and thus would exclude our specimens from belonging to *Isozoanthus.* However, *I. sulcatus* is clearly distinct from other species of the genus (*I. arborescens* being the type species of the genus) and in the absence of sequence data from *I. arborescens* or other typical *Isozoanthus* species for comparison, the possibility that *I. sulcatus* is not actually within *Isozoanthus* cannot be excluded. Polychaete worms were found associated with specimens from New Caledonia and possibly to the Mediterranean specimens as structures similar to polychaete tubes were found on the small sample collected. Such an association was also found by Carlgren with *Epizoanthus fatuus*, *E. planus*, *Isozoanthus valdiviae*, *I. arenosus* and *I. africanus* (with *Eunice mindanavensis*) (Carlgren 1923). A molecular re-examination of the five species listed above is necessary to compare their relationships with our specimens and to test potential convergent evolution toward the use of hexactinellid stalks as substrate among deep sea zoanthids.

Despite their efficiency to distinguish the different clades, the analyses of data from the two mitochondrial genes were unable to resolve relationships between the different clades within *Parazoanthus*. This may reflect the high conservation of mitochondrial genes in anthozoans (Shearer et al. 2002; Huang et al. 2008).

Of the genetic markers examined here, the ITS-rDNA region was the most variable. There were a few uncertain cases regarding the specific status of different morphotypes and ecotypes (potentially different species) that have identical ITS-rDNA sequences; for example, between specimens belonging to *Antipathozoanthus* n. gen. However, detailed relationships within this new genus will be described elsewhere.

On the other hand, the opposite situation occurs in *Hydrozoanthus* n. gen. Samples classified as *H.* cf. *gracilis* collected throughout the Indo-Pacific (Indonesia, Japan and New Caledonia) showed sequence variation despite identi-

cal external appearances and distribution. *H. gracilis* from Sulawesi appeared more closely related to *Hydrozoanthus tunicans* than to other *Hydrozoanthus* specimens with both mitochondrial markers, while *H. gracilis* from Japan and *H.* cf. *gracilis* from New Caledonia branched closer to *H. antumbrosus* (Figs. 3 and 4). This suggests either the presence of different species within *H. gracilis*, or that *H. gracilis* and *H. tunicans* represent a single pantropical species or species complex. Moreover, *H. antumbrosus* recently described by Swain (2009) and assigned to the genus *Isozoanthus* also falls within this new genus. The original placement of this species was doubtful as no other *Isozoanthus* specimens were studied for comparison in the description and the morphological characters used to place the species into this genus are subject to controversial interpretation (i.e. inconspicuous sphincter). At the species level, some diagnostic characters of *I. antumbrosus* are doubtful (i.e. "holes" potentially left by the dissolution of siliceous or calcareous incrustations could be interpreted as lacunae). It is likely that several other species that are known to be associated with hydrozoans for which specimens were not available, including *Parazoanthus dichroicus* and *P. douglasi*, could belong to this genus. Recently, Di Camillo et al. (2009) reported the presence of a different hydrozoan-associated zoanthid with potential separate specific status in their detailed study of hydrozoan-zoanthid associations in Indonesian waters. A hydrozoanthid from Madagascar ("Mada1") not found on hydrozoan substrate was also shown to possess very closely related ITS-rDNA sequences to other *Hydrozoanthus* spp and branched basal to *Hydrozoanthus* spp in all three molecular analysis (altghough not clearly supported with COI). In the light of those results, more studies are necessary to understand the molecular evolution and species delimitation within this group and species descriptions/identifications should be considered with caution.

It may be of importance that *Hydrozoanthus* n. gen. branches as a sister group of the suborder Brachycnemina (grouping Zoanthidae and Sphenopidae), which is known to have relatively high inter- and intra-specific ITS-rDNA sequence variation (Reimer et al. 2007b, c). *Hydrozoanthus* n. gen. could be a transitional step in the molecular evolution from Macrocnemina towards brachycnemic zoanthids. In some brachycnemic species the mode of sexual reproduction has been suggested to explain the presence of potential reticulate evolution (Reimer et al. 2007b, c). Unfortunately, nothing is currently known about the reproduction of *Hydrozoanthus* n. gen. species.

Subclades within *Parazoanthus*

The original morphological description of *Parazoanthus* mentions several characteristics such as diffuse endodermal sphincter, encircling sinus, endodermal canals, lacunae and cell-islets in the mesoglea, continuous ectoderm and body-wall incrusted with mineral particles, often with numerous sponge spicules present in the incrustations. As shown in Sinniger et al. (2005) and here, these morphological characteristics alone do not ascertain the monophyly of *Parazoanthus.* Morphological characteristics in zoanthids can often become artifactual due to both complications encountered in making thin cuttings of heavily sediment-incrusted polyps, and in interpreting the results of such sections. In the past, the large majority of epizoic macrocnemic zoanthids were described as belonging to *Parazoanthus* despite clearly different ecologies in many cases. Thus, the results of this study strongly suggest that only zoanthid species able to associate with sponges should remain in *Parazoanthus*, as the type species of this genus, *P. axinellae* from the Mediterranean Sea, is regularly associated with demosponges.

Within the redefined *Parazoanthus,* three different monophyletic subclades can be distinguished (subclades A, B and C, Fig. 5). Subclade A contains *P. axinellae* and other species (*P. anguicomus*, *P. axinellae*, *P. elongatus, P. swiftii*) able to live on sponges but not exclusively found on sponges. Indeed, it is common to find *P. axinellae* or *P. elongatus* on rocky substrates. Polyps of this group are relatively big (up to 22 mm high and 10 mm diameter) and share a well-developed basal coenenchyme, forming dense colonies. Subclade A zoanthid species are often yellow.

Subclade B comprises species exclusively found on sponges. The polyps are small and linked together through stolons that may sometimes be absent altogether. The polyps are usually scattered on the surface of the sponge. This clade contains the well-known Caribbean zoanthid *P. parasiticus*, as well as different species from the Indo-Pacific, and mitochondrial markers place it closer to the *P. axinellae* group (Subclade A) than to subclade C, while the nuclear (ITS) marker place subclades B and C as sister group to A.

Subclade C comprises *P. puertoricense*, one undescribed species from Senegal and one undescribed species from Sulawesi. The sequences of these species are highly divergent compared with the other *Parazoanthus* and their position within *Parazoanthus* is only supported by statistical analyses for the Bayesian analyses of the ITS-rDNA region sequences (pp=1.00, ML<50%, Fig. 5), while they branch at different positions within Parazoanthidae in both COI and mt 16S-rDNA trees (Figs. 3 and 4). This clade could be an artificial grouping of divergent parazoanthids showing ecological convergence with other *Parazoanthus* regarding association with sponges as substrate. As the knowledge on this group is scarce besides molecular sequences to distinguish this clade from other *Parazoanthus* (and in particular subclade B), it was decided to leave

these species in *Parazoanthus* until new data clarify the situation, although the genetic distances (Fig. 1) would suggest the creation of a separate genus.

Consequences for taxonomy

Despite the overall paucity of data regarding zoanthid taxonomy and ecology, the taxonomic revision presented here helps clarify the taxonomic situation of many zoanthids which until now belonged to the family Parazoanthidae. It is apparent that the genus *Parazoanthus* as previously defined was a "catch-all" taxon for many macrocnemic epizoic zoanthid species. Based on phylogenetic results, it is highly possible that the different epizoic clades described here have long evolutionary histories in association with specific groups of organisms used as substrates. Our results also show that ecological and geographical parameters are valuable and accurate taxonomic characters for Parazoanthidae and other macrocnemic zoanthids. The ease of acquisition of substrate data and related distinguishing parameters (locality, environmental data, and external morphology such as size, colour, type and amount of incrustations, number of ridges or tentacles, presence or absence of symbiotic dinoflagellates) should help improve and make proper classification of zoanthids more accessible. Hopefully this will accordingly spur an increase in the overall knowledge of zoanthids.

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All the experiments reported in this study complied with the laws of the countries in which they were performed.

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