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ORIGINAL PAPER

Molecular phylogenetic hypotheses of *Zoanthus* species (Anthozoa:Hexacorallia) using RNA secondary structure of the internal transcribed spacer 2 (ITS2)

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Abstract Previously, it has been found that species of the zoanthid genus *Zoanthus* (Cnidaria: Hexacorallia) possess high levels of intrageneric ITS-rDNA sequence variation both between congeners and also within one species, *Zoanthus sansibaricus*, resulting in an uncertain internal transcribed spacer of ribosomal DNA (ITS-rDNA) phylogeny for this group. For the first time, the secondary structures of the internal transcribed spacer 2 (ITS 2) sequences were analyzed in an attempt to further clarify and solve relationships within *Zoanthus*, and also with the closely related genera *Acrozoanthus* and *Isaurus* (all in family Zoanthidae). Results show that most species' ITS2 secondary structures follow the basic four-helix-ring model proposed for most eukaryotes including anthozoans. However, not all structures had the conserved motifs present in scleractinian corals, and *Z. sansibaricus* had two different structural conformations from the two different ITS2 types present. The ITS2 secondary structures of *Zoanthus* in this study, present a well resolved and supported phylogeny that makes it an appropriate tool for solving phylogenies in this taxonomic group. Based on ITS2 secondary structure results here, we theorize that: (1) *Acrozoanthus* is a valid monophyly separate from *Zoanthus*; (2) *Z. gigantus*, *Z. sansibaricus*, and *Z. praelongus* do not have any compensatory base changes (CBCs) between them and form a related clade distinct from the more divergent *Z. kuroshio*/*Z. vietnamensis* clade. The results furthermore suggest that the presence of CBCs may exist only at much lower ratios between different species in zoanthids than in other investigated organism lineages.

Keywords ITS2 secondary structures · Zoanthids · *Zoanthus* · Cnidaria · Evolution

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Introduction

Molecular studies for closely related species have always been a challenge for lower eukaryotic phylogenetics, such as in cnidarians. However, recently the use of internal transcribed spacer 2 (ITS2), found within the nuclear ribosomal 18S–28S rDNA tandem array in eukaryotes, has allowed good phylogenetic resolution even at the interspecies level for many different lower eukaryotes (Grajales et al. 2007). As tandem repeats, ITS2 sequences are believed to have evolved through concerted evolution, which implies homogenization of all ribosomal gene copies (Elder and Turner 1995), allowing differences to be accumulated between species (Hillis and Dixon 1991).

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Despite concerted evolution, intragenomic ITS2 variation has been found in many different types of invertebrates (Fabry et al. 1999; Harris and Crandall 2000), including *Acropora* (Vollmer and Pallumbi 2004; but see Wei et al. 2006) and seafans *Pseudopterogorgia* (Sánchez and Dorado 2008), indicating consideration has to be given for intra-individual rDNA variation.

Additionally, secondary structure prediction of ITS2 has resulted in new insights in obtaining reliable phylogenetic hypotheses. ITS2 secondary structures have been used not only for low-level phylogenetic analyses (Ahvenniemi et al. 2009) but also in higher–level (e.g., generic or higher) analyses due to secondary structure homology at different levels (Schultz et al. 2005). Several studies have revealed how the dynamic four-helix-ring model of ITS2 in yeast has a fundamental role in ITS2 transcription during pre-rRNA processing. Conserved structural motifs have been found in scleractinians (Chen et al. 2004), making structure reconstruction more reliable for other related cnidarian groups. Additionally, the presence of compensatory base changes (CBCs) in the different ITS2 helices is now believed to have good utility for species differentiation (Müller et al. 2007) and is a useful tool for phylogeny reconstruction. Due to the increasing number of studies that rely on ITS2 secondary structure conformation, there have been many recent studies investigating the proper folding of ITS2 structures. One of the main concerns is the annotation of the start and end position of the ITS2, as the positions have been shown to differ between studies (Keller et al. 2009), and new homology-based models intend to standardize structure construction procedure (Wolf et al. 2005a).

Such phylogenetic tools are being used to help resolve phylogenetic and taxonomic gaps in many marine benthic taxa, for which different molecular markers previously used have sometimes resulted in unresolved phylogenies (Berntson et al. 2001). The molecular taxonomy of zoanthids (Anthozoa: Hexacorallia) is one example of such a group (Reimer et al. 2007b).

Zoanthids are an order of benthic, generally colonial cnidarians found worldwide in a variety of marine environments from intertidal waters to the deep sea. As a member of the subclass Hexacorallia, they are closely related to hard corals. Despite their relative abundance in many ecosystems, zoanthids have largely remained understudied and taxonomically neglected due to several reasons: (1) high amounts of intraspecific morphological variation; (2) a general lack of valid diagnostic morphological characteristics; (3) the incorporation of sand and other particles into the mesoglea making histology very difficult; (4) a lack of fossil record.

Initial zoanthid molecular phylogenies utilized mitochondrial markers such as cytochrome oxidase subunit 1 (COI), 12S ribosomal DNA (mt 12S rDNA) and 16S ribosomal DNA (mt 16S rDNA) (Reimer et al. 2004; Sinniger et al. 2005), which have all been shown to evolve slowly in Cnidaria (Shearer et al. 2002; Huang et al. 2008). For zoanthids, mt DNA has been shown to have good utility for examining relationships between families, genera, and most species (Sinniger et al. 2008), but the resolution of mt DNA markers cannot always determine between closely related species. For example, the zoanthid species *Palythoa mutuki* Haddon and Shackleton 1891 and *Palythoa tuberculosa* Esper 1791 cannot be distinguished from each other by COI sequences (although they are distinguishable with mt 16S rDNA sequences) despite having clearly different morphologies and ecologies (Reimer et al. 2006c).

The genus *Zoanthus* Lamarck 1801 is perhaps the best known genus within Zoantharia and being zooxanthellate is found in shallow coral reef environments in subtropical and tropical waters worldwide. *Zoanthus* species (and other members of the family Zoanthidae) do not have mesogleal encrustations to the exception of other zoanthids, but show an amazing variety of intraspecific variation with regards to their oral disk and tentacle coloration (Reimer et al. 2004). Additionally, polyp shapes and sizes can apparently change in response to the (micro)environment (Koehl 1977). Originally, species within *Zoanthus* were classified by color and other morphological characteristics alone, but recent research using molecular techniques has shown that these "traditional identifications" are often not accurate and a single *Zoanthus* species may encompass a wide variety of different morphotypes (Reimer et al. 2004, 2006b).

Previously, studies have shown primary ITS2 alignments could not always be reliably used in *Zoanthus* phylogenetic analyses due to alignment difficulties and uncertainties; different regions of ITS-rDNA were used for their systematics with no intra-species differentiation (Reimer et al. 2007b). ITS-rDNA often is present in multiple, variable copies in the genome, but thus far intragenomic variation in most *Zoanthus* ITS-rDNA appears to be at acceptable levels, e.g., <5% (as per Wörheide et al. 2004; described in Reimer et al. 2007b). Of four *Zoanthus* species investigated in Japan (Reimer et al. 2007b), one species (*Z. sansibaricus* Carlgren 1900) was previously found to have two different ITS-rDNA types present, indicating either a reticulate evolutionary history or ancestral polymorphism (Reimer et al. 2007b). Additionally, two other species (*Z.* aff. *vietnamensis* Pax and Mueller 1957, *Z. kuroshio* Reimer and Ono 2006 in Reimer et al. 2006b) showed very high levels of ITS-rDNA sequence similarity (99–100%) despite clear morphological differences, calling into question their status as separate taxonomic units (Reimer et al. 2006a). Overall, the ITS-rDNA sequences found in different *Zoanthus* species are very divergent (up to approximately 45%) with one another, making alignment of ITS-rDNA

sequences and particularly the spacers ITS1 and ITS2 very difficult (Reimer et al. 2007b). Furthermore, ITS-rDNA trees present very different topologies from mt DNA trees for *Zoanthus* species and therefore the relationships between different species within the genus *Zoanthus* remain unclear.

Thus, while ITS-rDNA alignments and phylogenetic analyses of *Zoanthus* species provide valuable information, new methods are needed to both support previous results and reexamine species' relationships in this genus. The aim of this study was to use ITS2 secondary structures as a new method in assessing the systematics of *Zoanthus* and the family Zoanthidae in order to obtain a better taxonomic understanding of this group.

Materials and methods

ITS2 sequences

Sequences of different species from the family Zoanthidae for all described genera (*Zoanthus* Lamarck 1801, *Isaurus* Gray 1828, *Acrozoanthus* Saville-Kent 1893) were either obtained from GenBank or from unpublished sequences from previous studies. *Palythoa* sp. (family Sphenopidae) ITS2 sequences were used as outgroup. A total of 16 sequences were used. A complete list of the species, sequences, and GenBank accession numbers is given in Table 1, along with their respective references. Details on primers, DNA extraction, PCR conditions, cloning, and sequencing are given in the respective references.

It should be noted that *Zoanthus sansibaricus* Carlgren 1900 has previously been shown to have two distinct types of ITS-rDNA sequences within its genome, one "normal" (= "B" in Reimer et al. 2007b) type that is closely related to other *Zoanthus* spp. sequences, and one "distant" (= "sansi" in Reimer et al. 2007b) type that is much more divergent from other *Zoanthus* spp. ITS-rDNA sequences.

ITS2 predicted secondary structures and sequence analysis

Secondary structures for the 16 zoanthid sequences were reconstructed by aligning their sequences (using Bioedit; Hall 1999) with homologous structures already published, such as *Pseudopterogorgia* spp. (Aguilar and Sánchez 2007). Moreover, visual restrictions and constraints were made in order to submit each sequence to MFOLD (Zuker 2003). RNA was folded at a default temperature of 37°C, and the structures chosen from different output files had the highest negative free energy values while maintaining conserved patterns (e.g., ring model structure, see Online Resource Table 1 for folding parameters). CT-file formats were obtained from the previous program and run on CBCAnalyzer (Wolf et al. 2005b), and the resulting output

Table 1 Nuclear internal transcribed spacer 2 (ITS2) sequences utilized in this study, source species, and references (*NA* not applicable)

Family	Genus	Species	Allele "type"/specimen or sequence name	GenBank accession number[a]	Reference(s)
Sphenopidae	*Palythoa*	*tuberculosa*	PtIrHo11-3	DQ997911	Reimer et al. 2007a
Zoanthidae	*Zoanthus*	*sansibaricus*	"normal"/ZAT5-5	AB214155	Reimer et al. 2007b
Zoanthidae	*Zoanthus*	*sansibaricus*	"normal"/ZAT5-11	AB214155	Reimer et al. 2007b
Zoanthidae	*Zoanthus*	*sansibaricus*	"distant"/ SakZ5	AB214133	Reimer et al. 2007b
Zoanthidae	*Zoanthus*	*sansibaricus*	"distant"/ SakZ2	AB214130	Reimer et al. 2007b
Zoanthidae	*Zoanthus*	*sansibaricus*	"distant"/ YakZ1	AB214144	Reimer et al. 2007b
Zoanthidae	*Zoanthus*	*sansibaricus*	"distant"/ AmamiZ2	AB214125	Reimer et al. 2007b
Zoanthidae	*Zoanthus*	*sansibaricus*	"distant"/ ZSH1	AB214139	Reimer et al. 2007b
Zoanthidae	*Zoanthus*	*sansibaricus*	"distant"/ SakZ7	AB214135	Reimer et al. 2007b
Zoanthidae	*Zoanthus*	*sansibaricus*	"distant"/ ZAT5-9	AB214153	Reimer et al. 2007b
Zoanthidae	*Zoanthus*	*gigantus*	AmamiZg4–15	AB214123	Reimer et al. 2007b
Zoanthidae	*Zoanthus*	*gigantus*	AmamiZg4–24	AB214123	Reimer et al. 2007b
Zoanthidae	*Zoanthus*	*praelongus*	WAM80	AB517553	Reimer et al. 2008; this study
Zoanthidae	*Zoanthus*	*kuroshio*	ZkYS23	DQ442480	Reimer et al. 2006a, 2007b
Zoanthidae	*Zoanthus*	*vietnamensis*	ZvSH3	AB235397	Reimer et al. 2006a
Zoanthidae	*Acrozoanthus*	sp.	NA	AB517555	This study
Zoanthidae	*Isaurus*	*tuberculatus*	NA	AB517554	This study

[a] Some GenBank accession numbers match other clones' sequences from Reimer et al. (2006a, 2007b) as sequences were identical, hence identical GenBank accession numbers in some cases

files "bracket-dot-bracket" were used for creating multiple sequence alignments based on secondary structures in 4SALE (Seibel et al. 2006). The latter program uses ClustalW and a special algorithm based on secondary structure models when creating multiple RNA alignments. In addition, a compensatory base changes (CBCs) matrix was calculated based on the alignment and the structures. All secondary structures were drawn in PseudoViewer sofware (Han and Yanga 2003).

ITS2 secondary structures, secondary alignments and primary alignments were analyzed by different phylogenetic programs placing *Palythoa* as an outgroup. A multiple sequence comparison (MAFFT version 6; Katoh and Hiroyuki 2008) was performed for the primary alignment that was used to determine the best primary ITS2 topology. Bayesian inference of phylogeny was done using MrBayes 3.1 (Ronquist and Huelsenbeck 2003) and applying the settings for the best-fit model (HKY + G) according to MrModeltest. The analyses were run for 10-million Monte-Carlo Markovian chain generations [Bayesian-Monte-Carlo simulation by MrBayes sampling every 100 simulations, burn-in 1,000, Prset statefreqpr = dirichlet (1, 1, 1, 1); Lset nst = 2 rates = gamma]. Maximum likelihood (ML) analyses were also done for the primary ITS2 alignment in PhyML 3.0 (Guindon and Gascuel 2003) using the HKY + G model as selected according to the Akaike Information Criterion (AIC) in Modeltest (Posada and Buckley 2004). Bootstrap values of 1,000 replicates were performed on ML topology (Nst=2 TRatio= 1.0758 Rates = gamma Shape=1.3647 Pinvar=0). The secondary structure alignment from 4SALE was used for two different analyses. First, an alignment without structure information (no dots and brackets) was partitioned into two: helix and no-helix. MrModeltest was run for each partition and the information [helix: Prset statefreqpr = dirichlet (1, 1, 1, 1); Lset nst = 6 rates = equal; and no-helix Prset statefreqpr = fixed(equal); Lset nst=2 rates = gamma] was used to built a matrix for MrBayes 3.1 (Ronquist and Huelsenbeck 2003) in which the different models were given to the two partitions (five helix partitions and six no-helix partitions).

Second, ProfDist (Wolf et al. 2008) phylogeny reconstruction using General Time Reversible (GTR) and a ITS2 specific model matrix was obtained for profile neighbor-joining (PNJ) analyses using secondary alignment and secondary structure information from 4SALE. Bootstraps values were obtained from 1,000 replicates. Different topologies were examined (excluding *Z. sansibaricus* "distant" sequences) to further investigate the status of *Zoanthus* and *Acrozoanthus* (topologies in Online Resource Fig. 2). Furthermore, the Kishino-Hasegawa (KH) (Kishino and Hasegawa 1989) tests were performed in PAUP* (Swofford 1998) to compare the obtained topologies.

Results

RNA secondary structures

New sequences (GenBank accession numbers AB517553-AB517555; Table 1) and predicted RNA secondary structures for ITS2 of 16 zoanthids were obtained (Fig. 1).

The ITS2 structures exhibited the highly conserved four-helix model found in 14,000 species from different taxonomic groups; and as proposed by Chen et al. (2004) using the coral *Achantastrea echinata* Dana 1846 helix I is divided into two subhelices (Ia and Ib). The proximal stem of ITS2 was conserved among all the species; except for CBCs in the *Palythoa* Lamouroux 1816 outgroup (Fig. 1b), *Z. kuroshio* and *Z.* aff. *vietnamensis*. The motif 5′-CRCGGYC-3′ in helix II was not found in any of the zoanthid specimens, unlike as found in scleractinian corals (Chen et al. 2004). Another difference was *Z. gigantus* Reimer and Tsukahara 2006 (in Reimer et al. 2006b) with a U and A insert of 12 bp at the end of this helix, which made this helix predominantly longer than the rest of helix II (Fig. 1c). Subhelix Ia in most cases presented adenine-uracil bonds. The "normal" *Z. sansibaricus* type (= "B" in Reimer et al. 2007b) structure did not have subhelix Ia, as the high ratio of adenine after the 5.8S stem enabled helix pairing (Fig. 1d). *Acrozoanthus* sp. and *Z. gigantus* were characterized by a long helix II with two to four internal bulges, which differed from the rest of the examined zoanthids. Helix IV had a variable stem loop size in all the samples. The seven *Z. sansibaricus* "distant" (= "sansi" in Reimer et al. 2007b) type structures presented differences in helix IV due to insertions.

The structure of *Isaurus* ITS2 was found to be unusual, with helix II the longest instead of helix III. The tip of helix III tip had conserved sequences in the middle of this helix. Since its conformation was not stable and did not show proper base-pairing, phylogenetic analyzes with this species were considered unreliable, and therefore we excluded *Isaurus* ITS2 sequences from the secondary structures analyses.

Compensatory base changes (CBCs)

All CBCs were found as full changes, and no hemi-CBCs were found. Full changes were of two types: pyrimidine-purine to pyrimidine-purine (e.g. C-G to U-A), or pyrimidine-purine to purine-pyrimidine (e.g. U-A to A-U). *Palythoa tuberculosa* had two CBCs in the 5.8S–28S rDNA helix compared with the remaining species (compare Fig. 1a, b), including a unique (C-G to U-A) change as found in the *Acropora* "longi" isotype (see Coleman and van Oppen 2008). The *Z. sansibaricus* "distant" type had four CBCs in helix III compared with the *Palythoa* outgroup. Helix III had

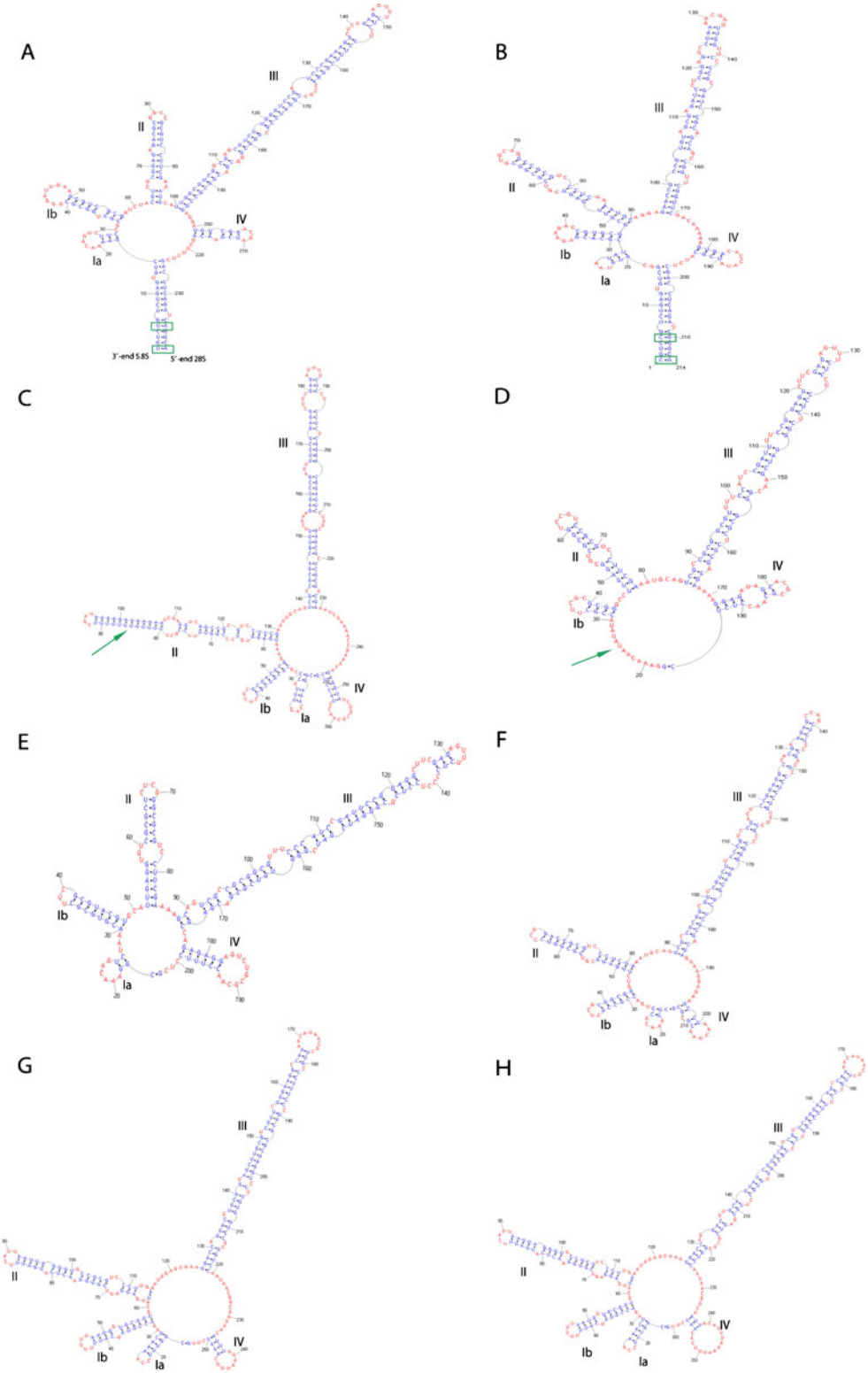

Fig. 1 ITS2 predicted RNA secondary structures for eight species of zoanthids: (**a**) *Acrozoanthus*; (**b**) *Palythoa tuberculosa*; (**c**) *Z. gigantus*3, the *arrow* points to an A-U insertion; (**d**) *Z. sansibaricus*74, the *arrow* points to where helix Ia is missing; (**e**) *Z. praelongus*; (**f**) *Z. kuroshio*; (**g**, **h**) *Z. sansibaricus* "distant" type (SakZery, Zat5-9). Numeration represents helix numbers, helix I is divided into two subhelices (Ia and Ib). The *boxes* in **a** and **b** represent CBCs in the proximal stem

the greatest number of CBC changes compared with the other helices (excluding the proximal stem), and between *Palythoa* and the "sansibaricus distant" sequences four CBCs were present in this helix. There was one CBC at the end of helix II that supported the 5′-CRCGGYC-3′ motif between *Z. praelongus* Gray 1867 (C-G) with *Z. kuroshio/Z.* aff. *vietnamensis* and *Z. sansibaricus* (the latter three species having the conserved G-C pairing).

There were no CBCs between *Z. gigantus* and three zoanthid species (*Z. sansibaricus*, *Z. praelongus* and *Acrozoanthus* sp.).

Phylogenetics analysis

The tree phylogenetic trees (Fig. 2) showed the *Z. sansibaricus* "distant" type as a highly supported [Bayes posterior probability (PP)=1.00] divergent group. Secondary phylogeny (Fig. 2b, c) placed *Acrozoanthus* (bootstrap=97; PP=1.00) as basal to the *Zoanthus* clade, contrasting with the primary phylogeny where *Acrozoanthus* was divergent from *Z. kuroshio/vietnamensis*. Basal *Acrozoanthus* sp. (Fig. 2b, c) gives support to the validity of *Zoanthus* as a monophyletic genus (differing from primary mt 16S rDNA results; Reimer et al. 2008).

The PNJ topology showed less polytomies in the *Z. sansibaricus* "distant type" clade (Fig. 2c). The main difference between the three topologies was *Z. praelongus*'s (Carlgren 1954) location; a sister taxon of *Z. gigantus* in the primary and secondary alignment but grouping with *Z. sansibaricus* in the PNJ topology, although with low bootstrap support (bootstrap=42). The three species *Z. gigantus*, *Z. praelongus* and *Z. sansibaricus* formed a

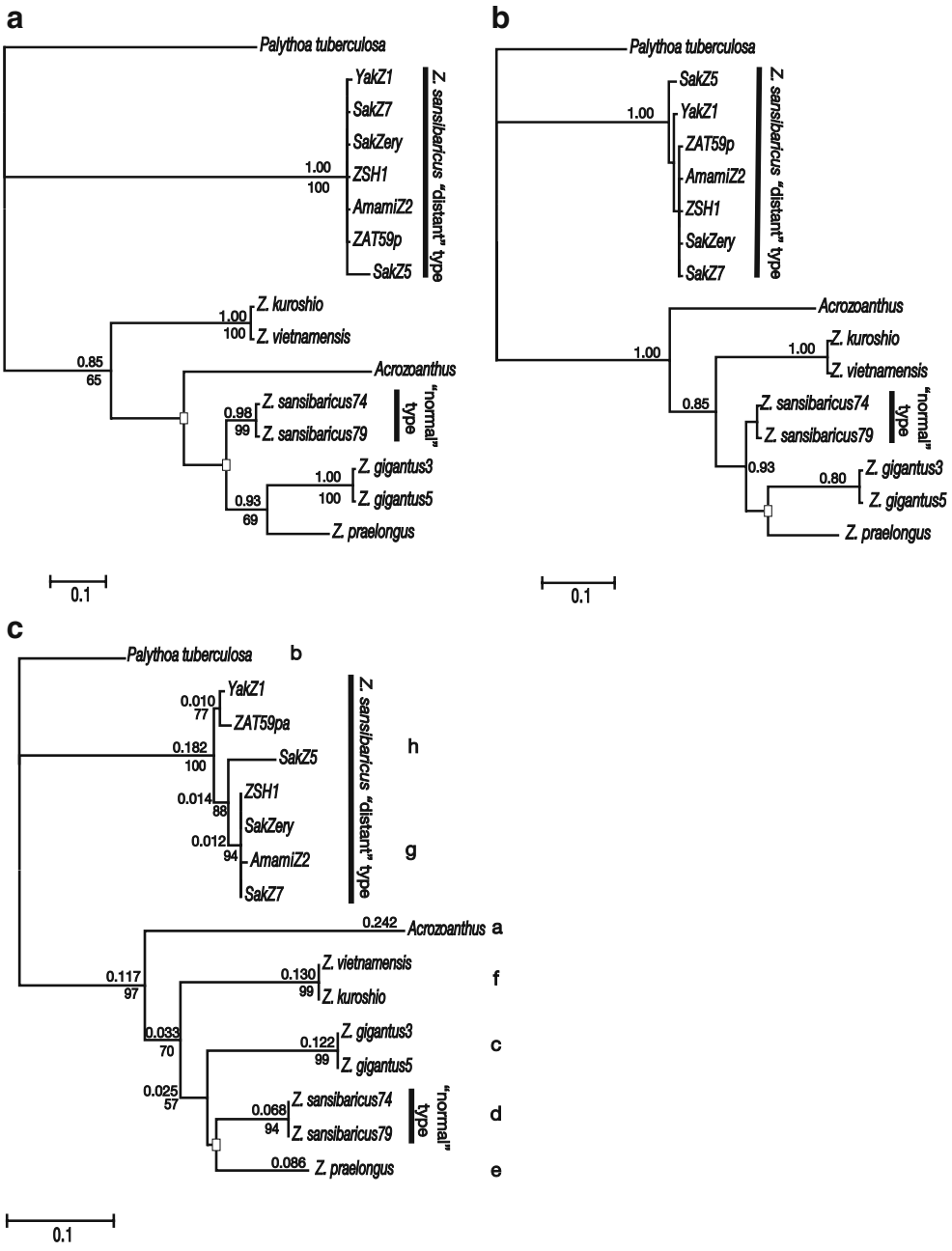

Fig. 2 Molecular phylogenetic hypotheses of ITS2 sequences of 16 species from family Zoanthidae. **a** Phylogram from Bayesian analysis of primary MAFFT alignment, values above the nodes represent Bayesian probabilities and below ML 1,000 bootstraps values. **b** Phylogram from Bayesian analysis of secondary alignment using helix partition, values represent Bayesian probabilities. **c** PNJ phylogram from secondary structure, values above the nodes represent distances estimated by ML and below 1,000 bootstraps values. *Letters* in front correspond to secondary structures in Fig. 1. The nodes denoted with a *box* represent bootstraps values below 55 and Bayesian probabilities below 0.80

consistent clade in the three phylogenies, with secondary alignment (Fig. 2b) having higher support (PP=0.93). A comparison between Bayesian primary, Bayesian secondary, and secondary structure topologies using the KH test did not show any significant differences (p value=0.28, -lnL=1,669 for the primary, -lnL=1,666 for secondary and -lnL=1,669 for the secondary structure).

The exclusion of *Z. sansibaricus* "distant" from the ML analyses placed *Z kuroshio/vietnamensis* outside the *Acrozoanthus* and *Zoanthus* clade (Online Resource Fig. 2a). With the secondary structure analyses (PNJ) *Z. kuroshio/vietnamensis* where placed as basal to *Acrozoanthus* when *Z. sansibaricus* "distant" was excluded, however low bootstrap support was given to this node. Moreover, *Z. praelongus* formed a sister clade with *Z. gigantus* but again low bootstrap support (see Online Resource Fig. 2b for the phylogram).

Discussion

Relationships within Zoanthidae based on ITS2 secondary structures

From the results of ITS2 secondary structure analyses we examine the following points. (1) Is *Acrozoanthus* a valid monophyly separate from *Zoanthus*? (2) *Z. gigantus*, *Z. sansibaricus*, and *Z. praelongus* form a related clade distinct from the divergent *Z. kuroshio*/*Z.* aff. *vietnamensis* clade. (3) The two different ITS2 types found in *Z. sansibaricus* are either the result of ancient hybridization, or, more likely are an ancestral polymorphism. Below, each theory is discussed in detail.

1. Is *Acrozoanthus* is a valid genus separate from *Zoanthus*?

In a previous study investigating the molecular phylogeny of *Isaurus*, it was proposed that *Acrozoanthus* may not be a valid genus as it was placed in a mt 16S rDNA phylogeny within the genus *Zoanthus* (Reimer et al. 2008). Despite having a unique ecology and clear differences from *Zoanthus* spp., this could be possible as *Zoanthus* spp. are quite morphologically plastic. In this study, specific analyses of *Acrozoanthus* and *Zoanthus* spp. (excluding *Z. sansibaricus* "distant" type) ITS2 show *Acrozoanthus* and *Z. kuroshio/Z.* aff. *vietnamensis* as basal to the *Zoanthus* spp., but *Acrozoanthus* has different placement in the various analyses (*Acrozoanthus* basal in the secondary structure analysis with Z. *sansibaricus* "distant" type and separated from *Z. kuroshio/Z.* aff. *vietnamensis* excluding the "distant" type, but low branch support). This may be the result of long branch attraction in the secondary structure analyses because primary alignment conserved the topology during the "distant" type exclusion (Online Resource Fig. 2), showing how variable results for this group demonstrate no straightforward conclusions can be made regarding the status of *Acrozoanthus* at this time.

Acrozoanthus ITS2 secondary structures have some important differences from the *Zoanthus* structures. Helix II is longer than seen in *Zoanthus* spp. except *Z. gigantus* (compare Fig. 1a–f), and these characters are more similar to the *Palythoa* outgroup. However, it should be noted that our analyses did not include any *Isaurus* spp., as in the original primary alignment from Reimer et al. (2008), and this may influence the analyses here. As mentioned previously, *Isaurus* ITS2 secondary structures were somewhat problematic to calculate, and clearly further research on this subject is needed, which will help further ascertain the correct position of not only *Isaurus* but also *Acrozoanthus*. For now, the question of whether *Acrozoanthus* is a valid genus separate from *Zoanthus* remains unanswered.

2. *Z. gigantus*, *Z. sansibaricus*, and *Z. praelongus* form a related clade distinct from the more divergent *Z. kuroshio/Z.* aff. *vietnamensis* clade.

Both ITS2 secondary structure analyses and the primary alignment analyses show these three species forming a clade, albeit with relatively low bootstrap support in two phylogenies (PP=0.71, Fig. 2a; bootstrap=62, Fig. 2c). It should be noted that this clade does not include the *Z. sansibaricus* "distant" type, which is discussed below.

While the overall relationships between *Zoanthus* spp. remain somewhat unclear (compare Fig. 2a, c), it is clear that the *Z. kuroshio*/*Z.* aff. *vietnamensis* clade forms a different lineage than *Z. gigantus*, *Z. sansibaricus*, and *Z. praelongus*. Additionally, *Z. gigantus, Z. sansibaricus*, and *Z. praelongus* (as well as *Acrozoanthus*) do not have any CBCs present between them, further supporting this clade's validity. According to the KH tests there were not significant differences in the topologies of primary, secondary and secondary structure analyses-generated trees (see above), but the topology based on secondary structures allowed comparisons between structures, from which more informative data could be used to explain the results. While the relationships between *Z. sansibaricus*, *Z. praelongus*, and *Z. gigantus* remain conflicting (compare Fig. 2a, c), they are clearly more related to each other than to the *Z. kuroshio/Z.* aff.*vietnamensis* clade. Given that benthic cnidarians have a slower rate of mitochondrial evolution (Shearer et al. 2002), these two branches within *Zoanthus* likely have a relatively ancient origin on the scale of millions of years, and future analyses using molecular clock analyses are underway to more clearly pinpoint this (J.D.R., unpublished data).

Of particular importance is the fact that despite being only distantly related (within *Zoanthus*), *Z. sansibaricus* and *Z. kuroshio*/*Z.* aff. *vietnamensis* are morphologically much more similar to each other than either *Z. gigantus* or *Z. praelongus*, both in terms of polyp size and shape. *Z. gigantus* is much larger than the other *Zoanthus* species discussed here, and has distinct white markings on the outside of closed polyps (Reimer et al. 2006b), while *Z. praelongus* is often mistaken for an *Isaurus* spp. due to recumbent polyps (Muirhead and Ryland 1985). On the other hand, *Z. sansibaricus* and *Z. kuroshio*/*Z.* aff. *vietnamensis* have overlapping polyp sizes (e.g. oral disk diameter), tentacle numbers, and distributions (water depth and ecological) in southern Japan, with both groups having non-recumbent polyps with no obvious external markings. This situation clearly demonstrates the simplicity of zoanthid structures, and also how convergent morphological evolution may be a common occurrence within this group.

3. The two different ITS2 types found in *Z. sansibaricus* are either the result of ancient hybridization or ancestral polymorphism.

In this study, as in previous research (Reimer et al. 2007b), primary alignments failed to resolve the *Z. sansibaricus* "distant" type group. However, secondary structure analyses were able to differentiate between the few changes present in these types, confirming ITS2's utility for solving intrapopulation variation. From ITS2 secondary structure analyses, the "distant" type is clearly Anthozoan, and appears to be most closely related to *Palythoa* ITS2 structure, as helix II is much longer than in *Zoanthus* spp. and is similar to *Palythoa* (which belongs to a different family; Sphenopidae). It is unusual to find two apparently functional yet highly divergent types. Odonnell and Cigelnik (1997) have suggested that an ancient hybridization or gene duplication event could be responsible for such a situation. If this happened with *Z. sansibaricus*, then the event could have coincided with the original radiation of *Zoanthus*, based on the "distant" type's *Palythoa*-related structure. In such a case, distinguishing between interspecific hybridization and ancestral polymorphism becomes unclear. In a previous study (Reimer et al. 2007b), interspecific hybridization was theorized to be the cause of the presence of two types over ancestral polymorphism, but based on results here it may be impossible to distinguish between the two ideas, although it appears that something unusual occurred with regards to ITS-rDNA early in *Z. sansibaricus*'s history. The alternative theory, that the "distant" type has recently (or is currently) entered *Z. sansibaricus* from another, very distantly-related (at the level of family) zoanthid becomes unlikely at best.

Only this "distant" ITS-rDNA type is found in some *Z. sansibaricus* specimens, while other *Z. sansibaricus* have only the "normal" type, and yet others have both the "distant" and "normal" type (Reimer et al. 2007b). These results indicate that both the types are functional and may have differing benefits/drawbacks to *Z. sansibaricus*. It is interesting to note that the "normal" ITS2 type is missing helix Ia while the "distant" ITS2 type has a normal Anthozoan structure, but whether this plays a role in the continued presence of types within the *Z. sansibaricus* genome is unknown.

The very small ITS-rDNA sequence differences between and within some *Z. kuroshio* and *Z.* aff. *vietnamensis* (Reimer et al. 2006a) did not result in any CBCs being present, and therefore the status of these two species remains unsolved. Despite identical ITS-rDNA structures, the two species are different morphologically and also ecologically. Further research into these two groups is needed.

Secondary structure models and the utility of secondary structure characters

ITS2 secondary structure is a variable model (hairpin and ring model) with different RNA base interactions during the ribosome assemblage (Côte et al. 2002), and there are different software programs, each with their own algorithm to predict the most accurate model (e.g. MFOLD, ITS2 database, RNAfold WebServer, etc.). Unfortunately, this makes secondary structure prediction often difficult, in particular structure comparison. However, methods that identify the start and end position of ITS2 (Keller et al. 2009) are helping to obtain more uniform secondary structures. In this study we found that the 5.8S–28S proximal stem was conserved among all zoanthid species as in most animal groups.

Aside from an accurate proximal stem, secondary structure helices' characteristics are also important for phylogenetic reconstruction, and conserved motifs such as the one found in helix II are useful for species differentiation (Oliverio et al. 2009). For the zoanthid species examined in this study, some unique features include the U-A insertion on *Z. gigantus* helix II (Fig. 1c) and the absence of helix 1a in *Z. sansibaricus* (Fig. 1d). These features may demonstrate that even though the structural model is mostly conserved, there are special features for each species that can provide information about these species' evolution (see Coleman and van Oppen (2008) for acroporid coral examples). Such features should be studied in detail in the future for a more clear understanding of their evolution and meaning.

Compensatory base changes present between different zoanthid species?

One further ITS2 secondary structure feature consists of CBCs, which are found to maintain the structure of helices

and thus allow proper ribosome assembly (Côte et al. 2002). CBCs are now currently believed to be a practical marker for eukaryotic species delineation, as in 93.11% of congeneric plants and fungi species at least one CBC was found between the species' ITS2 sequences (Müller et al. 2007). In this study we found that CBCs were not always present between different zoanthid genera (e.g. *Z. gigantus* compared to *Z. sansibaricus*, *Z. praelongus* and *Acrozoanthus*), perhaps implying that the theory of Müller et al. (2007) of "at least one CBC between species" does not always apply in animal groups and thus for eukaryotic species delineations. Moreover, the presence of the CBC change present in the 5.8S–28S basal helix in the *Palythoa* outgroup, compared with *Zoanthus* species, shows the same variability present in unique *Acropora* "longi" (Coleman and van Oppen 2008), which has a rare CBC different from the identical basal pairing in all other examined scleractinians. In order to further analyze the uniqueness of this event, more *Palythoa* ITS2 structures are needed to reach more specific conclusions in the Zoanthidae family.

As for comparison of the three methods, they gave relatively congruent results with only minor differences between them. An increase in the number of taxa could help obtain better topology resolution in further studies; moreover, it has been found that for ITS the best performance is given at higher levels of divergence (Keller et al. 2010). From our results, it can be said that zoanthid ITS2 has shown to be changing at a high rate (e.g., *Z. sansibaricus* "distant" type divergence, helix II insertion in *Z. gigantus*, and missing helix Ia in *Z. sansibaricus* "normal" type), which may interfere in obtaining more accurate results.

The ultimate utility of ITS2 remains still obscure due to the presence of intraspecific variation (Sánchez and Dorado 2008), which reflects the well-known problems with ITS2 as a DNA barcode in Anthozoa (Oliverio et al. 2009). However, ITS2 structures are a good tool for understanding species evolution at different taxonomic levels, since increasing amounts of information are now available, increasing data analyses richness, which in turn leads to better phylogeny reconstruction (e.g. CBCs, helix composition, 5.8S–28S stem, and bulge numbers). As sequence-structure based trees have been found to improve phylogenetic accuracy (Keller et al. 2010), ITS2 secondary structure phylogenetic data for zoanthids should be further utilized to solve remaining phylogenetic questions within this group, taking special care regarding intragenomic variation and comparison among different phylogenetic methods.

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References

Aguilar C, Sánchez JA (2007) Phylogenetic hypothesis of gorgoniid octocorals according to ITS2 and their predicted RNA secondary structures. Mol Phylogenet Evol 43:774–786

Ahvenniemi P, Wolf M, Lehtonen MJ, Wilson P, German-Kinnari M, Valkonen JPT (2009) Evolutionary diversification indicated by compensatory base changes in ITS2 secondary structures in a complex fungal species, Rhizoctonia solani. J Mol Evol 69:150–163

Berntson EA, Bayer FM, McArthur AG, France SG (2001) Phylogenetic relationships within the Octocorallia (Cnidaria: Anthozoa) based on nuclear 18 S rRNA sequences. Mar Biol 138:235–246

Carlgren O (1954) Actiniaria and Zoantharia from South and West Australia with comments upon some Actiniaria from New Zealand. Arkiv för Zoologi (2) 6:571–595

Chen CA, Chang CC, Wei NV, Chen CH, Lein YT, Lin HE, Dai CF, Wallace CC (2004) Secondary structure and phylogenetic utility of the ribosomal internal transcribed spacer 2 (ITS2) in scleractinian corals. Zool Stud 43:759–771

Coleman AW, van Oppen MJH (2008) Secondary structure of the rRNA ITS2 region reveals key evolutionary patterns in acroporid corals. J Mol Evol 67:389–396

Côte CA, Greer CL, Peculis BA (2002) Dynamic conformational model for the role of ITS2 in pre-rRNA processing in yeast. RNA (N Y) 8:786–797

Elder JF Jr, Turner BJ (1995) Concerted evolution of repetitive DNA sequences in eukaryotes. Q Rev Biol 70:297–320

Fabry S, Kohler A, Coleman AW (1999) Intraspecies analysis: comparison of ITS sequence data and gene intron sequence data with breeding data for a worldwide collection of *Gonium pectorale*. J Mol Evol 48:94–101

Grajales A, Aguilar C, Sánchez JA (2007) Phylogenetic reconstruction using secondary structures of internal transcribed spacer 2 (ITS2, rDNA): finding the molecular and morphological gap in Caribbean gorgonian corals. BMC Evol Biol 7:90

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52:696–704

Haddon AC, Shackleton AM (1891) Reports on the zoological collections made in Torres Straits by Professor A.C. Haddon, 1888–1889. Actiniae: I. Zoantheae. Sci Trans R Dublin Soc 4:673–701

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Han K, Yanga B (2003) PseudoViewer2: visualization of RNA pseudoknots of any type. Nucleic Acids Res 31:3432–3440

Harris DJ, Crandall KA (2000) Intragenomic variation within ITS1 and ITS2 of freshwater crayfishes (Decapoda: Cambaridae): Implications for phylogenetic and microsatellite studies. Mol Biol Evol 17:284–291

Hillis DM, Dixon MT (1991) Ribosomal DNA: molecular evolution and phylogenetic inference. Quart Rev Biol 66:411–453

Huang D, Meier R, Todd PA, Chou LM (2008) Slow mitochondrial COI sequence evolution at the base of the metazoan tree and its implications for DNA barcoding. J Mol Evol 66:167–174

Katoh K, Hiroyuki T (2008) Improved accuracy of multiple ncRNA alignment by incorporating structural information into a MAFFT-based framework. BMC Bioinform 9:212

Keller A, Schleicher T, Schultz J, Mullar T, Dandekar T, Wolf M (2009) 5.8S–28S rRNA interaction and HMM-based ITS2 annotation. Gene 430:50–57

Keller A, Forster F, Mullar T, Dandekar T, Schultz J, Wolf M (2010) Including RNA secondary structures improves accuracy and robustness in reconstruction of phylogenetic trees. Biol Direct 4 (5):1–12

Kishino H, Hasegawa H (1989) Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. J Mol Evol 29:170–179

Koehl MAR (1977) Water flow and the morphology of zoanthid colonies. Proc 3rd Int Coral Reef Symp 1:437–444

Muirhead A, Ryland JS (1985) A review of the genus *Isaurus* Gray 1828 (Zoanthidea), including new records from Fiji. J Nat Hist 19:323–335

Müller T, Philippi N, Dandekar T, Schultz J, Wolf M (2007) Distinguishing species. RNA (N Y) 13:1469–1472

Odonnell K, Cigelnik E (1997) Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Mol Phylogenet Evol 7:103–116

Oliverio M, Barco A, Modica MV, Richter A, Mariottini P (2009) Ecological barcoding of corallivory by second internal transcribed spacer sequences: hosts of coralliophiline gastropods detected by the cnidarian DNA in their stomach. Mol Ecol Resour 9:94–103

Pax F, Mueller I (1957) Zoantharien aus Viet-Nam. Mem Mus Natl Hist Nat 16:1–40

Posada D, Buckley TR (2004) Model selection and model averaging in phylogenetics: advantages of the AIC and Bayesian approaches over likelihood ratio tests. Syst Biol 53:793–808

Reimer JD, Ono S, Takishita K, Fujiwara Y, Tsukahara J (2004) Reconsidering *Zoanthus* spp. diversity: molecular evidence of conspecifity within four previously presumed species. Zool Sci 21:517–525

Reimer JD, Ono S, Iwama A, Tsukahara J, Maruyama T (2006a) High levels of morphological variation despite close genetic relatedness between *Zoanthus* aff. *vietnamensis* and *Zoanthus kuroshio* (Anthozoa: Hexacorallia). Zool Sci 23:755–761

Reimer JD, Ono S, Iwama A, Tsukahara J, Takishita K, Maruyama T (2006b) Morphological and molecular revision of *Zoanthus* (Anthozoa: Hexacorallia) from southwestern Japan with description of two new species. Zool Sci 23:261–275

Reimer JD, Ono S, Takishita K, Tsukahara J, Maruyama T (2006c) Molecular evidence suggesting species in the zoanthid genera *Palythoa* and *Protopalythoa* (Anthozoa: Hexacorallia) are congeneric. Zool Sci 23:87–94

Reimer JD, Takishita K, Ono S, Maruyama T (2007a) Diversity and evolution in the zoanthid genus *Palythoa* (Cnidaria: Hexacorallia) utilizing nuclear ITS-rDNA. Coral Reefs 26:399–410

Reimer JD, Takishita K, Ono S, Tsukahara J, Maruyama T (2007b) Molecular evidence suggesting intraspecific hybridization in *Zoanthus* (Anthozoa: Hexacorallia). Zool Sci 24:346–359

Reimer JD, Ono S, Tsukahara J, Iwase F (2008) Molecular characterization of the zoanthid genus *Isaurus* (Anthozoa: Hexacorallia) and its zooxanthellae (*Symbiodinium* spp). Mar Biol 153:351–363

Ronquist F, Huelsenbeck JP (2003) MrBayes3: Bayesian phylogenetic inference under mixed models. Bioinformatics (Oxf) 19:1572–1574

Sánchez JA, Dorado D (2008) Intragenomic ITS2 variation in Caribbean seafans. Proc 11th Int Coral Reef Symp 7–11

Schultz J, Maisel S, Gerlach D, Müller T, Wolf M (2005) A common core of secondary structure of the internal transcribed spacer 2 (ITS2) throughout the Eukaryota. RNA (N Y) 11:361–364

Seibel PN, Müller T, Dandekar T, Schultz J, Wolf M (2006) 4SALE-A tool for synchronous RNA sequence and secondary structure alignment and editing. BMC Bioinform 7:498

Shearer TL, van Oppen MJH, Romano SL, Wörheide G (2002) Slow mitochondrial DNA sequence evolution in the Anthozoa (Cnidaria). Mol Ecol 11:2475–2487

Sinniger F, Montoya-Burgess JI, Chevaldonne P, Pawlowski J (2005) Phylogeny of the order Zoantharia (Anthozoa, Hexacorallia) based on mitochondrial ribosomal genes. Mar Biol 147:1121–1128

Sinniger F, Reimer JD, Pawlowski J (2008) Potential of DNA sequences to identify zoanthids (Cnidaria: Zoantharia). Zool Sci 25:1253–1260

Swofford DL (1998) PAUP*. V Phylogenetic analysis using parsimony (*and other methods), version 4. Sinauer, Sunderland

Vollmer SV, Palumbi SR (2004) Testing the utility of internally transcribed spacer saequences in coral phylogenetics. Mol Ecol 13:2763–2772

Wei NWV, Wallace CC, Dai CF, Pillay KRM, Chen CA (2006) Analyses of the ribosomal internal transcribed spacers (ITS) and the 5.8 S gene indicate that extremely high rDNA heterogeneity is a unique feature in the scleractinian coral genus *Acropora* (Scleractinia; Acroporidae). Zool Stud 45:404–418

Wolf M, Achtziger M, Schultz J, Dandekar T, Müller T (2005a) Homology modeling revealed more than 20,000 rRNA internal transcribed spacer 2 (ITS2) secondary structures. RNA (N Y) 11:1616–1623

Wolf M, Friedrich J, Dandekar T, Müller T (2005b) CBCAnalyzer: inferring phylogenies based on compensatory base changes in RNA secondary structures. In Silico Biol 5:0027

Wolf M, Ruderisch B, Dandekar T, Müller T (2008) ProfdistS: (Profile-) Distance based phylogeny on sequence-structure alignments. Bioinformatics (Oxf) 24:2401–2402

Wörheide G, Nichols SA, Goldberg J (2004) Intragenomic variation of the rRNA internal transcribed spacers in sponges (Phylum Porifera): implications for phylogenetic studies. Mol Phylogenet Evol 33:816–830

Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 31:3406–3415