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ORIGINAL PAPER

The sands of time: rediscovery of the genus *Neozoanthus* (Cnidaria: Hexacorallia) and evolutionary aspects of sand incrustation in brachycnemic zoanthids

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Abstract The zoanthid family Neozoanthidae (Anthozoa: Hexacorallia: Zoantharia) was described in 1973 from Madagascar as a monogeneric and monotypic taxon, and never reported again in literature. In 2008–2010, numerous zoanthid specimens fitting the morphological description of *Neozoanthus* were collected in the Ryukyu Islands, Okinawa, Japan, and the Great Barrier Reef (GBR), Australia. Utilizing these specimens, this study re-examines the phylogenetic position of Neozoanthidae and analyzes the evolutionary history of sand incrustation in zoanthids through phylogenetic and ancestral state reconstruction analyses. Specimens were colonial, partially incrusted with large, irregular sand and debris, zooxanthellate, and found from the intertidal zone to depths of approximately 30 m. Phylogenetic results utilizing mitochondrial 16S ribosomal DNA and cytochrome oxidase subunit I sequences show the presence of two *Neozoanthus* species groups, one each from Japan and the GBR. Unexpectedly, the molecular results also show *Neozoanthus* to be very closely related to the genus *Isaurus*, which as a member of the family Zoanthidae, is not sand incrusted. These results suggest that during evolution zoanthids can acquire and lose the ability to incrust sand with relative rapidity.

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Introduction

Zoanthids, one order within the hexacorals (Anthozoa: Hexacorallia), are generally characterized by their colonial structure and sand incrustation, although not all zoanthids possess these features. Many zoanthid species of the suborder Brachycnemina are quite common in shallow tropical and subtropical waters, particularly in coral reef environments. Most of these coral reef zoanthid species are zooxanthellate, possessing endosymbiotic *Symbiodinium* dinoflagellates (e.g. Reimer et al. 2006).

The large majority of brachycnemic zoanthid species in coral reef environments belong either to the genera *Zoanthus* (family Zoanthidae) or to *Palythoa* (Sphenopidae), each with over 100 species mentioned in literature (Fautin 2009), although the true numbers of species in these genera are not known (Burnett et al. 1997; Reimer et al. 2004). The Zoanthidae are unique among the zoanthids in that the species within this family (in the genera *Zoanthus*, *Acrozoanthus*, *Isaurus*) do not have any sand incrustation. On the other hand, Sphenopidae species are well known for

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their heavy incrustation of sand and detritus (Haywick and Mueller 1997), rendering sectioning and internal examination virtually impossible for this group (Reimer et al. 2010b).

However, there exists a third shallow-water brachycnemic zoanthid family, the family Neozoanthidae, consisting of one species (*Neozoanthus tulearensis*) that was described in 1972 from zooxanthellate specimens found on the reef flat of southwestern Madagascar, and subsequently never reported again in scientific literature. The family Neozoanthidae is believed to be a primitive stage in the suborder Brachycnemina based on its endodermal sphincter, a feature used to define the suborder Macrocnemina. Unusually, *N. tularensis* is only partially sand incrusted, with large, irregular incrustations only lightly embedded in the outer mesoglea (Herberts 1972). Despite being zooxanthellate, this species, asides from brachycnemic mesentery arrangement, has no shared morphological features with the Zoanthidae (Herberts 1972).

In Anthozoa, several different strategies to strengthen and protect polyps have evolved in different orders. Scleractinia (hard corals) form hard calcareous skeletons, while octocorals usually produce calcareous sclerites and also often produce rubbery/strong proteins (e.g. gorgonin in holaxonians). Some sea anemones (Actinaria), which have no skeleton, can often swim or move on the sea floor, and anemones usually possess strong and fast-acting muscle tissue that allows polyps to retract rapidly, as do corallimorpharians and cerianthids. Zoanthids, however, do not calcify, do not have generally strong muscles and are not mobile, although one genus (*Sphenopus*) is found unattached to the bottom in sandy sediment. Sand incrustation in zoanthids is therefore an alternate strategy to the strengthening/protection methods described above, and species in the genus *Palythoa* can contain up to 65% sand by weight (Haywick and Mueller 1997).

The trait of sand incrustation is unique to zoanthids among cnidarians and has several advantages over both calcification and well-developed muscle formation. Under some ocean conditions (i.e. ocean acidification), it has been theorized that calcifying organisms increasingly cannot form skeletons, which negatively impacts such species' ability to compete and survive (Kurihara 2008). Zoanthids do not face such limitations, as sand and detritus is readily available in all marine environments, implying there is no lack of "building material", even under conditions harsh for corals and octocorals. By utilizing sand incrustation, zoanthids can also spend relatively less amounts of energy on muscle and body wall development. It may be that sand incrustation evolved as an alternate strategy in the zoanthids during a period when organisms with other strategies were at a relative disadvantage.

During recent Census of Coral Reef Ecosystem (CReefs) surveys at Heron Island, Australia, in the southern Great Barrier Reef, and additional surveys in Okinawa and the Nansei Islands, Japan, numerous specimens of unknown zoanthids fitting the morphology of *Neozoanthus* were discovered and collected from shallow coral reef waters. Here, we report on preliminary molecular and morphological examinations of these specimens and discuss the phylogeny and evolutionary history of *Neozoanthus* and other brachycnemic shallow water zoanthids. As the only partially sand incrusted zoanthid taxa, examinations of *Neozoanthus* may further our understanding of sand incrustation in zoanthids, and we therefore examine the evolutionary implications of the phylogenetic placement of this genus.

Materials and methods

Specimen collection and initial identification

Great Barrier Reef, Australia

A total of 15 zoanthid specimens (in this study specimens = colonies) resembling *Neozoanthus* were collected from various sites around Heron Island on the Great Barrier Reef (GBR) by SCUBA in November 2009 as part of the Census of Coral Reefs (CReefs) Australia Program (Table S1).

Okinawa and Nansei Islands, Japan

A total of 15 zoanthid specimens resembling *Neozoanthus* were collected from various sites in the Nansei (=Ryukyu) Islands by snorkeling or SCUBA between May 2008 and March 2010 (Table S1).

Specimen examination

Specimens were preliminarily identified using external morphological characteristics described in Herberts (1972); e.g. lightly sand incrusted with irregularly sized particles, absence of bractea, zooxanthellate based on in situ observation and photographs (taken with an Canon Powershot digital camera in an underwater housing) and ex situ physical examination under dissecting scope. Specimens were subsequently stored in 70–99% ethanol at ambient temperature. All samples are currently deposited at the University of the Ryukyus (Nishihara, Okinawa, Japan) in 99.5% ethanol at −20°C, except for samples FS652 (4% formalin) and FS651 (75% ethanol) placed in the Natural History Museum of Geneva, Switzerland. Specimens from Okinawa were assigned numbers MISE-(number)

(Molecular Invertebrate Systematics and Ecology Laboratory collection numbers at the University of the Ryukyus), while specimens from Heron Island were numbered HI-(number), and specimens in the senior author's collection were numbered FS-(number) (Table S1).

Decalcification

Two polyps from two specimens each from Okinawa and Australia (total $n = 8$) were decalcified by chelation with a 5% citric acid. Polyps were decalcified for 1–2 days; decalcification was stopped when bubbles no longer emitted from the polyps. Colonies were then rinsed overnight in DW, with water changed multiple (>5) times.

Histology and electron microscopy

The specimens were dehydrated through an ethanol–xylene series and then embedded in paraffin. Serial sections of 5–10 μm thickness were prepared with a rotary microtome and stained with Delafield's hematoxylin and eosin. Serial sectioning continued until the desired structures were exposed, after which the paraffin was removed by soaking in xylene. After a brief rinse with ethanol, the specimens were immersed in *t*-butanol and freeze-dried. The dried specimens were spatter-coated with gold–palladium and examined under a scanning electron microscope (SEM; JEOL JEM-6060LV).

DNA extraction, PCR amplification, cloning, and sequencing

DNA was extracted from specimen portions (tentacles and column) weighing 5–20 mg using a DNeasy Animal Extraction protocol (Qiagen, Tokyo, Japan) or guanidine protocol as described in Sinniger et al. (2010). PCR amplification using the genomic DNA as a template was performed using HotStarTaq DNA polymerase (Qiagen) according to the manufacturer's instructions. Mitochondrial (mt) 16S ribosomal DNA (mt 16S rDNA) was amplified using primers and following procedures outlined in Sinniger et al. (2005). A portion of cytochrome oxidase subunit I (COI) was amplified using zoanthid-specific primer COIZoanF (Reimer et al. 2007) and general COI primer HCO2198 (Folmer et al. 1994). COI PCR amplification was performed on the samples under the following conditions: an initial denaturing step at 95°C for 5 min, followed by 35 cycles of 1-min denaturing at 94°C, 1-min annealing at 40°C, and 90-s extension at 72°C, followed by 7-min extension at 72°C. The amplified products were visualized by 1.5% agarose gel electrophoresis. PCR products were treated with Exonuclease I and Alkaline Phosphatase (Shrimp) (Takara) prior to sequencing. Some sequencing reactions (6 specimens) used DTCS Quick Start Master Mix (Beckman Coulter), and the products were analyzed using a CEQ8800 (Beckman Coulter) automated DNA sequencing system. Other sequences (11 specimens) were outsourced and products analyzed by MacroGen Japan (Tokyo, Japan).

Phylogenetic analyses

New sequences obtained in the present study were deposited in GenBank (accession numbers HM991227-HM991257). Nucleotide sequences of mt 16S rDNA and COI from samples were manually aligned with previously published mt 16S rDNA and COI sequences from various zoanthid species representing the genera *Zoanthus*, *Acrozoanthus*, *Isaurus* (all other described genera within the family Zoanthidae), *Palythoa* (family Sphenopidae), *Hydrozoanthus*, *Terrazoanthus*, and unidentified hydrozoanthids (family Hydrozoanthidae, within suborder Macrocnemina but closely related to suborder Brachycnemina). As the genus *Acrozoanthus* may be within the genus *Zoanthus* based on recent DNA phylogenies (Reimer et al. 2010a) and previous morphological examinations (Ryland 1997), hereafter "*Zoanthus*" includes *Acrozoanthus*. No previous *Neozoanthus* sequences were available in GenBank. Outgroup sequences for both mt 16S rDNA and COI trees were from the genus *Parazoanthus* (from family Parazoanthidae, within suborder Macrocnemina).

All alignments were inspected by eye and manually edited. All ambiguous sites of the alignments were removed from the dataset for phylogenetic analyses. Consequently, two alignment datasets were generated: (1) 624 sites of 25 sequences (mt 16S rDNA) and (2) 462 sites of 26 sequences (COI). The alignment data are available on request from the corresponding author and at the webpage http://web.me.com/miseryukyu.

For the phylogenetic analyses of the two alignments, the same methods were applied independently. Alignments were subjected to analyses with maximum likelihood method (ML) using PhyML (Guindon and Gascuel 2003). PhyML was performed using an input tree generated by BIONJ with the general time-reversible (GTR) model (Rodriguez et al. 1990) of nucleotide substitution incorporating invariable sites and a discrete gamma distribution (eight categories) ($GTR + I + \Gamma$). The proportion of invariable sites, a discrete gamma distribution, and base frequencies of the model were estimated from the dataset. PhyML bootstrap trees (1,000 replicates) were constructed using the same parameters as the individual ML tree. The distances were calculated using a Kimura's 2-parameter model (Kimura 1980).

Bayesian trees were reconstructed by using the program MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) under the

GTR model. One cold and three heated Markov chain Monte Carlo (MCMC) chains with default-chain temperatures were run for 20 million generations, sampling log-likelihoods (InLs), and trees at 100-generation intervals (20,000 InLs and trees were saved during MCMC). The first 1,500,000 generations of all runs were discarded as "burn-in" for all datasets. The likelihood plots for both datasets also showed that MCMC reached the stationary phase by this time (PSRF = 1.000 for both mt 16S rDNA and COI). Thus, the remaining 185,000 trees (18.5 million generations) of mt 16S rDNA and COI were used to obtain posterior probabilities and branch-length estimates, respectively.

Ancestral reconstruction

Ancestral character state reconstructions were performed with both ML and maximum parsimony (MP) methods by tracing the character state of sand incrustation (with single parameter Markov model) over a "reduced taxa" ML tree using Mesquite 2.74 (Maddison and Maddison 2010) to examine the potential ancestral state of sand incrustation and its evolution in zoanthids. The "reduced taxa" ML tree contained only one mt 16S rDNA sequence representative of each species group (alignment = 15 taxa, 624 sites), using the same basic alignment as the mt 16S rDNA alignment in the previous section. Phylogenetic ML tree analyses were performed as in the previous section using PhyML. Species' sand incrustation characters were assigned as follows: 0 (=sand incrusted) for *Parazoanthus swiftii* (outgroup), *Terrazoanthus sinnigeri*, *T. onoi*, Hydrozoanthid sp. 302, *Hydrozoanthus* sp. "yellow polyps", *H. gracilis*, *Palythoa heliodiscus*, *P. tuberculosa*, and *P. mutuki*; 1 (=partial incrustation) for *Neozoanthus* sp. okinawa, and *N.* sp. australia; 2 (=no sand incrustation) for *Zoanthus kuroshio*, *Z. sansibaricus*, *Z. gigantus*, and *Isaurus tuberculatus*.

Results

In situ observations

Putative *Neozoanthus* specimens were found at several locations both on the Great Barrier Reef (GBR), Australia, and on the Ryukyu Islands, Japan (Table S1, Fig. 1), and were somewhat common at some locations (e.g. 2–5 colonies encountered per 1 h dive).

Neozoanthus specimens from both GBR and the Ryukyu Islands were relatively small for brachycnemic zoanthids (in situ expanded oral disk diameters of 2–5 mm) compared with other observed shallow water zoanthids (*Zoanthus* and *Palythoa* spp. diameters 3–20 mm) and were zooxanthellate. Oral disks and tentacles were generally characterized by having white flecks or stripes alternating with purple, red, black, and white markings (Fig. 1), with striped tentacles, and were clearly different from the light green to yellow mentioned in the description of *N. tulearensis*. Specimens from both regions were incrusted with relatively large (e.g. sponge sclerites >300 μm in length) and irregular grains of sand and other detritus (Fig. S1). However, the oral ends of closed polyps (=scapulus) were observed to not have incrustations, unlike the rest of the scapus (=column) (Fig. 1e). Specimens were found from intertidal areas to depths of 27.8 m, in a wide variety of environments, including reef edges, reef flats, and channels (Table S1). The only discernable common point for all localities was the presence of strong hydrodynamism,

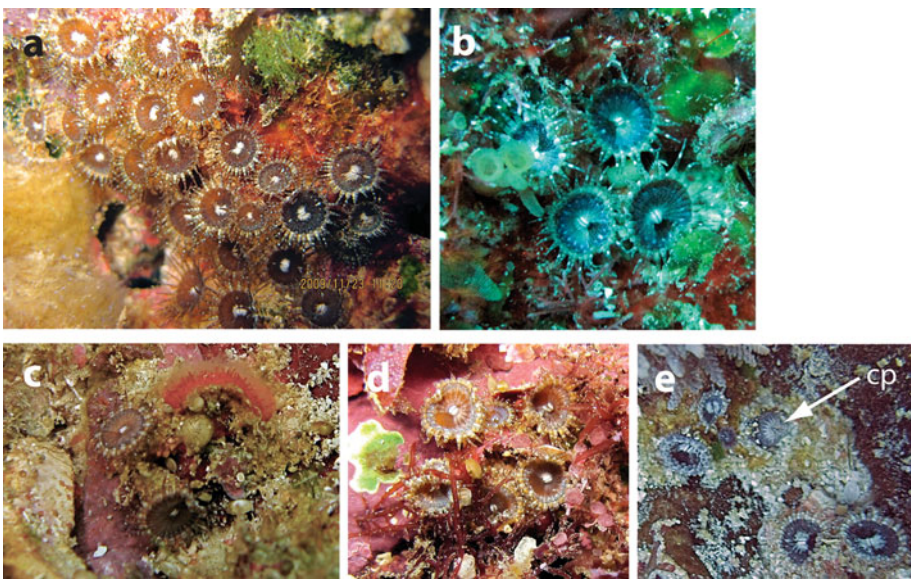

Fig. 1 *Neozoanthus* specimens in situ from the Great Barrier Reef, Australia and the Ryukyu Islands, Japan. (**a**) specimen MISE HI218 at Sykes Reef, Heron Island, Australia; (**b**) MISE HI142 at Sykes Reef, Heron Island, Australia; (**c**) MISE 1115 at Korijima, Okinawa, Japan; (**d**) MISE 1401 at San, Tokunoshima, Japan; (**e**) MISE HI209 at Sykes Reef, Heron Island, Australia. Abbreviation: *cp* closed polyp, note lack of incrustation on oral end of this polyp. *Scale bar* = 1 cm

either from waves or from tidal currents. *Neozoanthus* specimens were not found at other locations near sampling localities that were more sheltered (i.e. reef lagoons, protected harbors, etc.).

Histology

From cross-sections of specimens from Japan and Australia, the following observations could be made.

Incrustation, endoderm, mesoglea

Incrustation of specimens was primarily in the ectoderm, with very little incrustation observed in the mesoglea (Fig. S1). Incrusted detritus were observed to be of all shapes and sizes, and not within only one size class as observed previously in *Palythoa* spp. (Haywick and Mueller 1997). Occasionally, sponge spicules (Fig. S1) and other detritus were observed along with the more common coral sand grains.

No lacunae, mesogleal canals or endodermal invagination were observed in specimens.

Mesenterial arrangement and numbers

The mesenteries were observed to be brachycnemic in arrangement. In other words, the fifth mesentery from the dorsal directive was incomplete, not reaching the pharynx. Specimens from Japan were observed to have approximately 22 mesenteries, while Australian specimens had approximately 20. Additionally, possible gametes were observed in several cross-sections.

Sphincter muscle

A potential sphincter muscle was observed in the endoderm in the region of the pharynx. However, the endoderm was very thin, and this observation cannot be considered completely conclusive.

Phylogenetic analyses

mt 16S rDNA

Sequences from putative *Neozoanthus* specimens from Australia (MISE specimens HI141-145, HI200, HI209, HI214, HI218, HI224-225, HI227, HI231) and Japan (MISE specimens 548, 1115, 116) each formed moderately supported subclades (Australia ML = 85%, Bayes = 1.00; Japan ML = 63%, Bayes = 0.88, respectively), which were sisters within a very well-supported (ML = 94%, Bayes = 1.00) *Neozoanthus* and *Isaurus tuberculatus* clade (Fig. 2). This larger clade was sister to a clade of *Zoanthus* spp. sequences, and these two large clades formed one very well-supported (ML = 98%, Bayes = 1.00) large Zoanthidae-Neozoanthidae monophyly, separate from *Palythoa* and *Hydrozoanthus* sequences. All putative *Neozoanthus* mt 16S rDNA sequences had an insertion of 24 base pairs (at positions 509–533 in the mt 16S rDNA alignment) (Fig. 3) not seen in Zoanthidae or Sphenopidae. A similar insertion was previously found in two undescribed hydrozoanthids (specimen "302" and specimen "yellow polyps"), but these two hydrozoanthids also had other insertions not seen in the *Neozoanthus* sequences (Fig. 3).

COI

Sequences from putative *Neozoanthus* specimens from Australia (MISE specimens HI141-142, HI145, HI200, HI209, HI214, HI218, HI224-225, HI227, HI231) and Japan (MISE specimens 548, 550, 1115, 1116) formed, together with *Isaurus tuberculatus* sequences, a very well-supported (ML = 99%, Bayes = 0.98) clade (Fig. S2). The *Neozoanthus-Isaurus* clade was again sister to a *Zoanthus* clade, and these two clades formed a large, well-supported monophyly (ML 97% but Bayes <0.50), clearly distinct from *Palythoa* and *Hydrozoanthus* sequences.

Ancestral reconstruction

Both MP and ML analyses indicated that the common ancestor of *Zoanthus*, *Isaurus*, and *Neozoanthus* was most likely not sand incrusted (ML proportional likelihood = 0.6225) (nodes 1 and 2), while all previous ancestors were sand incrusted (Fig. 4). In the ML analyses, the ancestor of *Isaurus* and *Neozoanthus* was most likely only partially sand incrusted (=0.7506, node 3), with Isaurus re-evolving a lack of sand, while in the MP analyses, the *Isaurus-Neozoanthus* common ancestor was not sand incrusted (node 4), with *Neozoanthus* alone re-evolving partial incrustation.

Discussion

Identity of *Neozoanthus*

Based on morphological examination results, the specimens collected in this study correspond to the genus *Neozoanthus* as defined by Herberts (1972). In detail, the following morphological characteristics fit with Herberts' original description: (a) only partial incrustation not extending into the mesoglea, with various sizes of incrusted particles, (b) relatively short, conical tentacles, (c) zooxanthellate, (d) tops of closed polyps (=scapulus) lacking

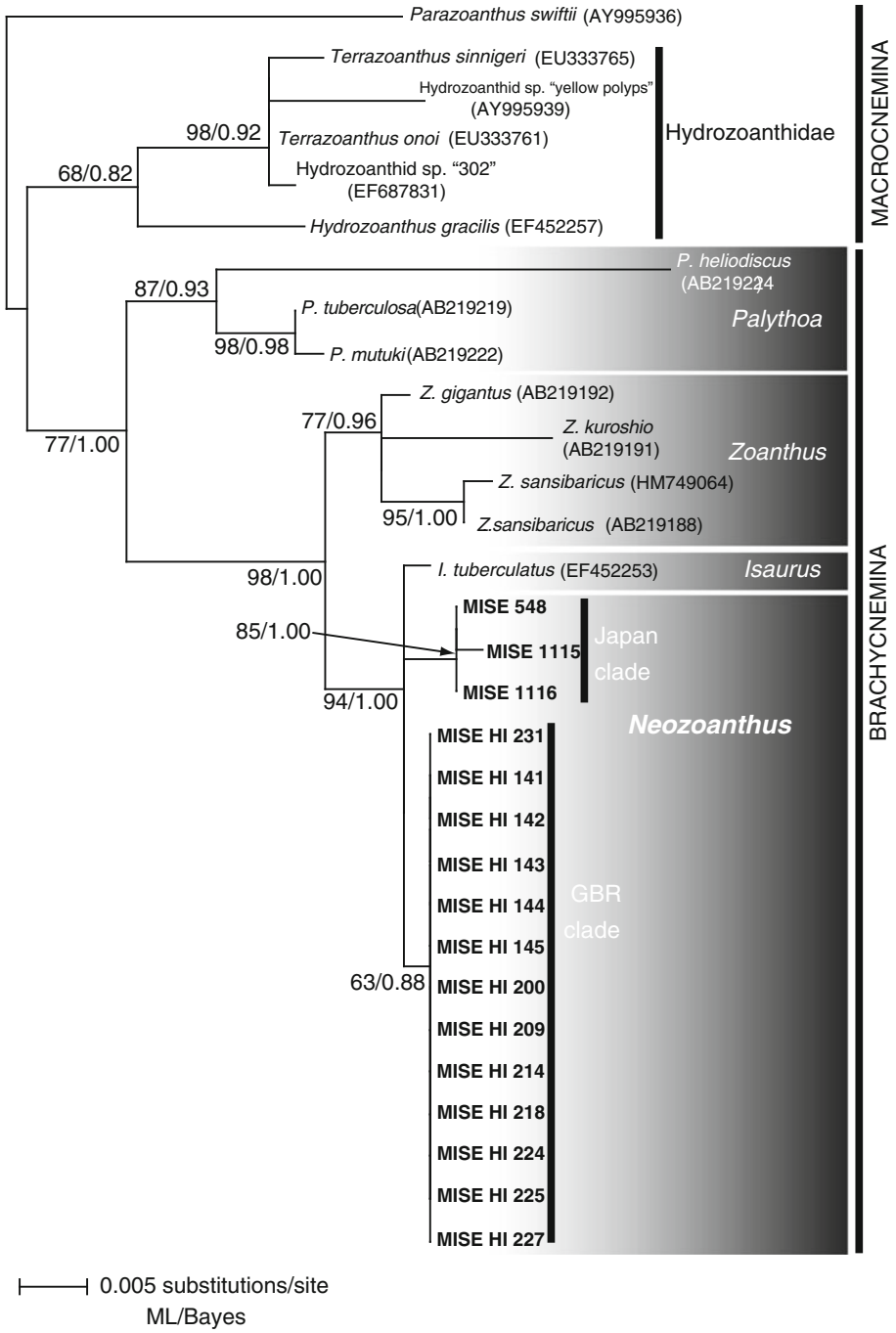

Fig. 2 Maximum likelihood (*ML*) tree of mitochondrial 16S ribosomal DNA, sequences for zoanthid specimens. Values at branches represent ML probabilities (>50%) and Bayesian posterior probabilites (>0.50), respectively. Sequences newly obtained in this study in bold. Sequences/species names from previous studies in regular font with GenBank Accession Number. For specimen information, see Table S1

incrustation, (e) brachycnemic mesentery arrangement, (f) no lacunae, mesogleal canals, or endodermal invaginations, (g) no bractae, (h) found in coral reef habitats. Additionally, we could not observe any mesogleal sphincter. The original description notes the endodermal sphincter of *Neozoanthus*, which we likely observed but could not confirm due to the narrowness of the endoderm in specimens.

The only morphological characters that did not fit with the original *Neozoanthus* description are color, which has been shown to be highly variable in many brachycnemic zoanthid species (Burnett et al. 1997; Reimer et al. 2004), and tentacle/mesentery count, which is also variable between and among species.

Thus, most morphological characteristics of specimens from this study fit with *Neozoanthus*. Additionally, although the phylogenetic position of *Neozoanthus* is somewhat unexpected (discussed below), the group is still within Brachycnemina. For these reasons, here, we designate the specimens as *Neozoanthus*. From the mt 16S

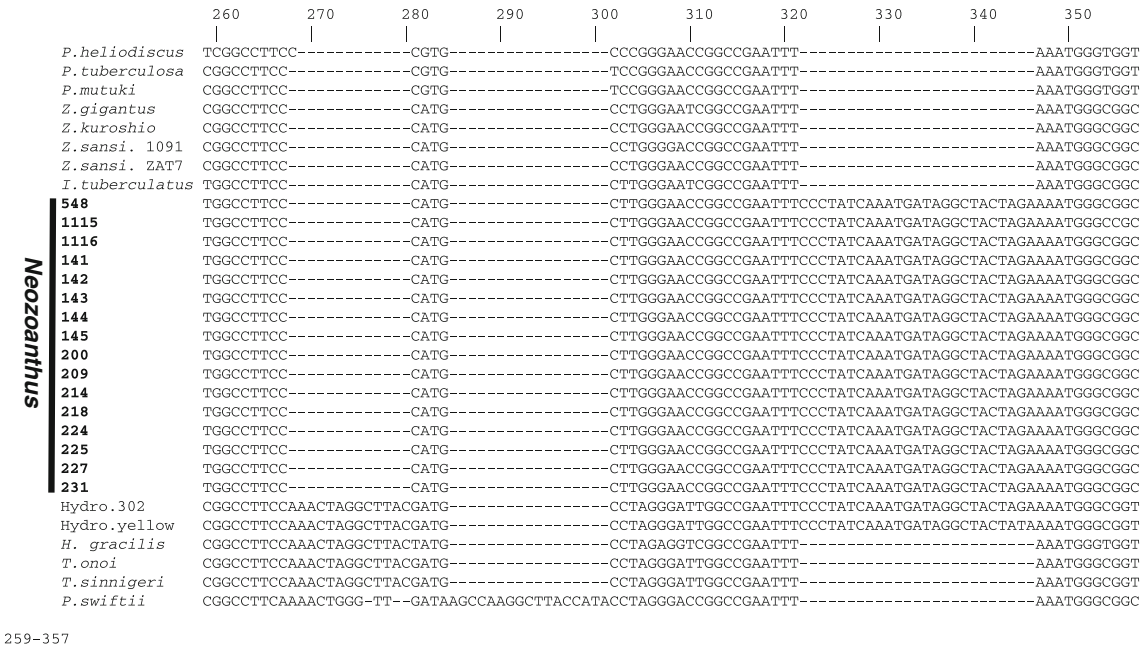

Fig. 3 Alignment of a portion of mitochondrial 16S rDNA (mt 16S rDNA) showing sequences from putative *Neozoanthus* specimens (in bold) in this study, as well as other suborder Brachycnemina sequences from families Zoanthidae (*Zoanthus*, *Isaurus*) and Sphenopidae (*Palythoa*) with suborder Macrocnemina outgroups from Hydrozoanthidae (*Hydrozoanthus*, *Terrazoanthus*, and unidentified Hydrozoanthidae) and Parazoanthidae (*Parazoanthus*). Note the indel between positions 322 and 346 shared by *Neozoanthus* and unidentified Hydrozoanthidae, while only Macrocnemina exclusively share the indel at positions 268–280. Alignment position numbers are identical to those in the mt 16S rDNA alignment used in phylogenetic analyses

rDNA phylogenetic analyses showing two clades, as well as mesentery number and collection location, it is concluded that the specimens represent two undescribed species within *Neozoanthus*, preliminarily designated as *Neozoanthus* sp. okinawa and *Neozoanthus* sp. australia.

Unexpected expanded range of *Neozoanthus* and implications on the status of zoanthid research

The rediscovery of numerous *Neozoanthus* populations from two different regions, one in each hemisphere, and thousands of kilometers away from the original type locality, demonstrates the potentially wide distributional range of *Neozoanthus*. While not documented in literature, occasionally *Neozoanthus*-like specimens also appear in the aquarium pet industry, often included with *Zoanthus* spp. from coral reefs of the Indo-Pacific (J. Sprung, personal communication), and it is therefore considered likely that *Neozoanthus* is present in the Indo-Pacific in the areas between Madagascar, the Great Barrier Reef, and Japan. However, no known specimens of *Neozoanthus* are listed in zoanthid collections in museums in Japan, Australia, or Singapore, despite the presence of most other zoanthid taxa commonly seen in coral reef ecosystems (e.g. *Zoanthus*, *Palythoa*, and *Parazoanthus* spp.). This may be due to the cryptic habitat and coloration of *Neozoanthus*, an irregular or patchy distribution, or the lack of correct identification of zoanthid specimens, or a combination of all three potential causes. In other words, it is very likely *Neozoanthus* has been ignored or not found, and/or collected but not properly identified in previous surveys. At both locations (GBR, Okinawa) where *Neozoanthus* was found in this study, colonies were not particularly rare, especially when particularly searched for, and the absence of data until now is most likely simply due to the lack of in situ examination by zoanthid experts until recently. Thus, from these new findings of *Neozoanthus*, the following conclusions regarding the status of zoanthid research can be made: (1) although research on zoanthids is increasing, there is a paucity of general zoanthid field identification knowledge and reports of zoanthids from biodiversity surveys are scarce, and this situation urgently needs to be addressed and (2) it is likely new taxa (genera and species) of shallow water (e.g. <30 m) zoanthids remain to be discovered in Indo-Pacific coral reef ecosystems. Clearly, more investigation into zoanthid biodiversity is needed to truly understand their biodiversity and biogeography.

Neozoanthus' close relationship with *Isaurus* and a redefined Zoanthidae

The most surprising result from the molecular phylogenetic analyses was the close relationship between *Neozoanthus* and *Isaurus*, despite their current classification in different

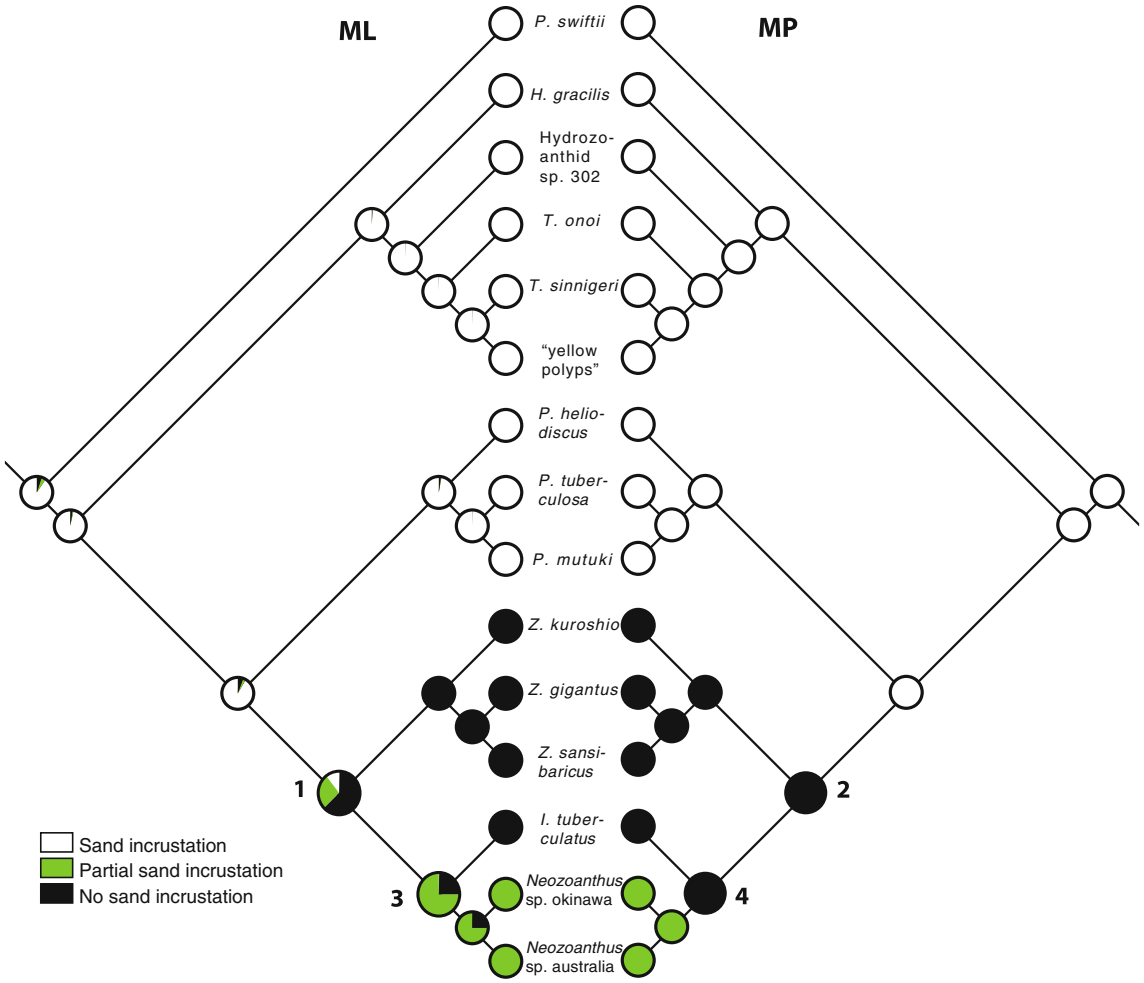

Fig. 4 Ancestral state reconstructions of sand incrustation in zoanthids utilizing maximum likelihood (ML; *left tree*) and maximum parsimony (MP; *right tree*) methods traced on an identical ML tree of mt 16S rDNA sequences. Pie chart sections represent relative likelihood of each character state at the respective node. Pie charts at some nodes enlarged. *Bold* single-digits at nodes indicate node numbers referred to in the text

families. Outwardly, these two genera are very different in appearance, as *Isaurus* is not incrusted, and has polyps with tubercules (bumps) present, while *Neozoanthus* has polyps incrusted with large sand and detritus fragments. The molecular results clearly demonstrate a close relationship and are supported with high bootstrap values, and if not for the indel present in *Neozoanthus* mt 16S rDNA, *Isaurus,* and *Neozoanthus* would be as closely related as many other zoanthid congeners are (e.g. 1–5 bp difference/902 bp total = 0.1–0.6% difference) (compare with Table 4 in Reimer et al. 2008). Phylogenetic results from COI, which does not have large indels as seen in mt 16S rDNA, further demonstrate the very close phylogenetic relationship between *Neozoanthus* and *Isaurus* (2–4 bp difference/462 bp total = 0.4–0.9% difference). A closer examination does reveal some common morphological and ecological features between these two genera.

Internally, *Neozoanthus* has no lacunae and has a simple sphincter muscle, which is also characteristic of *Isaurus* and *Palythoa*. As well, both *Neozoanthus* and *Isaurus* have no mesogleal canals, unlike *Palythoa* and *Zoanthus*. However, despite these similarities, the close phylogenetic relationship between these two groups is unexpected and somewhat surprising.

From the phylogenetic analyses in this study, *Neozoanthus* is closely related to *Isaurus*, and as *Isaurus* is a member of Zoanthidae, a reassessment of the status of the families Neozoanthidae and Zoanthidae is necessary in the near future.

The obvious morphological diagnostic character to creating a "redefined" Zoanthidae is the presence or absence of incrustation. As explained above, even though *Neozoanthus* is incrusted, this incrustation is primarily only external in nature and does not extend well into the

mesoglea as in all other incrusted zoanthids. Thus, simply defining Zoanthidae as brachycnemic zoanthids with either no incrustation or only external (e.g. "partial") incrustation that does not extend into the inner mesoglea allows for reconciliation with the DNA phylogenies. This definition also allows for relatively easy field identification and is clearly different from Sphenopidae. For these reasons, in the future creating a large comprehensive family including *Neozoanthus*, *Isaurus*, and *Zoanthus* may be the most harmonious taxonomic solution.

Considering its phylogenetic position, the most curious aspect of *Neozoanthus* is its incrustation. Interestingly, not the entire polyp is incrusted, as the oral ends of polyps are free of incrustation. This is seen clearly in the original description (Herberts 1972, page 139, Fig. 10) and also was observed in specimens in this study (Fig. 1e). In "truly" incrusted zoanthids such as *Palythoa*, the mesoglea is heavily incrusted with inclusions, while in *Neozoanthus*, there are only very few inclusions on the outside of the mesoglea (Herberts 1972; Fig. S1).

Evolutionary history of *Neozoanthus* and implications

These observations combined with molecular phylogenies suggest that Neozoanthidae, although intermediate in level of incrustation, is not an evolutionary intermediate step between Sphenopidae and Zoanthidae, nor is it a primitive state ancestral to both Sphenopidae and Zoanthidae as theorized by Herberts (1972). Based on the phylogenetic trees from this study, the most parsimonious of three possible scenarios is that the Zoanthidae evolved from ancestral incrusting zoanthids with the subsequent loss of incrustation ability, which is moderately supported by both ML and MP ancestral character state reconstruction analyses. Subsequently, after splitting into the *Acrozoanthus*/*Zoanthus* and *Isaurus*/*Neozoanthus* ancestral clades, the genus *Neozoanthus* alone partially re-evolved incrustation, which is supported by MP but not ML ancestral state analyses (Fig. 4). A less parsimonious explanation is that the ancestor of *Isaurus*/*Neozoanthus* partially regained sand incrustation, and then ancestral *Isaurus* alone again lost the ability to incrust sand, and this is the most likely scenario in ML ancestral state analyses (Fig. 4). In this theory, the loss of incrustation occurs twice, once in the original Zoanthidae ancestor and once more in ancestral *Isaurus*. The least parsimonious explanation has partial loss of incrustation as the ancestral state of Zoanthidae, with complete loss of incrustation occurring in ancestral *Zoanthus* and ancestral *Isaurus*, but this scenario was not supported by any ancestral state analyses (Fig. 4) and is considered unlikely. In all three of these scenarios, sand incrustation has appeared, been lost, or re-appeared in zoanthids more than once.

Thus, the presence or absence of sand incrustation in zoanthids may be a case of homoplasy. Homoplasy is in which unrelated species or groups develop the same features, implying that the genetic architecture involved in the expression and development of these features are different in some manner. However, given that *Neozoanthus* is very closely related to *Isaurus*, we feel the chances of the incrustation of *Neozoanthus* (if the *Neozoanthus*/*Isaurus* common ancestor was non-incrusted) or the non-incrustation of *Isaurus* (if *Neozoanthus*/*Isaurus* ancestor was incrusted) being homoplasic and evolving independently are very low. This is further supported by the fact that the loss and regain of sand incrustation has not occurred (to the best of our knowledge) in other zoanthid groups, or in any other cnidarian group, despite their relative diversity and long evolutionary history of hundreds of millions of years (e.g. Ezaki 1997) and that sand incrustation (excepting Zoanthidae) is a feature unique to zoanthids among Anthozoa. Despite their relatively basal position in Hexacorallia (Sinniger and Pawlowski 2009), we do not consider it possible that sand incrustation is an ancestral state of Hexacorallia or Anthozoa, as zoanthid larvae are not sand incrusting (e.g. Hirose et al. in press). Instead, sand incrustation is an evolved trait unique in cnidarians to the zoanthid monophyly that develops upon settlement of larvae on substrata, similar to calcification in Scleractinia. Sand incrustation occurs through "sediment assimilation", in which sand/detritus are selected based on size, and enter the mesoglea through a transepithelial transport (Haywick and Mueller 1997).

Therefore, we propose that the current situation with *Neozoanthus* and *Isaurus* is somewhat likely to be a case of a "sand incrustation switch" or mechanism being turned on and off. In the case of zoanthids, it may be that at least two genes are involved, as there are three patterns of incrustation; "total" incrustation in most zoanthids (including *Palythoa*), "partial" incrustation in *Neozoanthus*, and no incrustation in *Isaurus*, *Acrozoanthus,* and *Zoanthus*. If sand incrustation expression is truly a case of a "switch" being turned on and off, based on recent literature the exact time scale for the evolution of *Neozoanthus* and/or *Isaurus* from ancestral Zoanthidae appears to have been no greater than 10 million years (Marshall et al. 1994), and perhaps less if more genes are involved. We propose that is likely that the genetic architecture responsible for sand incrustation in *Neozoanthus* is inherently the same as in *Palythoa* and other basal zoanthid groups. For now, however, distinguishing between homoplasy and the re-enabling of sand incrustation remains impossible until the availability of genome scale data for zoanthids becomes a reality in the future.

Regardless of which theory is correct, the phylogenetic results of this study suggest that the evolution and loss of incrustation in zoanthids can occur over relatively short

Table 1 Summary of morphological characters of major brachycnemic zoanthid genera

Genus	Incrustation?	Sphincter complexity	Sphincter position	Lacunae?	Mesogleal canals?	Endodermal invagination?
Palythoa	Yes	Simple	Mesogleal	No	Yes	No
Neozoanthus	Partial	Simple	Endodermal	No	No	No
Isaurus	No	Simple	Mesogleal	No	No	Yes
Zoanthus	No	Double	Mesogleal	Yes	Yes	No

evolutionary periods. This discovery has major consequences on the definition of the order itself as the ability to incorporate mineral particles in the column and coenenchyme has been considered as one of the major distinguishing characters of the order Zoantharia, with the subsequent loss of incrustation in the family Zoanthidae being considered as a unique synapomorphy.

Future investigations into Zoanthidae, Sphenopidae, Hydrozoanthidae, and *Neozoanthus* utilizing new molecular markers and developmental characteristics, etc., should help shed further light on the evolutionary history of zoanthids. In particular, *Neozoanthus'* shared indel with some Hydrozoanthidae specimens despite being phylogenetically closer to *Isaurus* and other Zoanthidae points to an interesting evolutionary history for this group.

At the same time, it is apparent that the location of the sphincter muscle (endodermal or mesogleal) is not a consistently valid diagnostic character as previously used in zoanthid taxonomy for family-level relationships (see Table 1), at least in the suborder Brachycnemina. Similar results have been found in recent analyses of the entire order Zoantharia (Sinniger et al. 2005). The location of the sphincter muscle, the presence, partial, or complete absence of incrustations, and other characters can apparently change and evolve relatively rapidly in brachycnemic zoanthids.

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