



Discovery and description of a new species of *Abyssoanthus* (Zoantharia: Hexacorallia) at the Japan Trench: the world's deepest known zoanthid

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Abstract: Until recently, very little was known about zoanthids (Cnidaria: Hexacorallia) from deep-sea environments, with all known specimens assigned to the family Epizoanthidae. However, in June 2005 a small number of unusual samples of a zoanthid-like species were sampled during deep-sea submersible dives (Shinkai 6500 dive # 884) at a methane cold seep at 3259 m in the Nankai Trench off Muroto, Japan (32°34.945'N-134°41.545'E). Specimens were highly divergent in ecology, morphology and molecular phylogeny from all known families of zoanthids, and were thus classified as the new species *Abyssoanthus nankaiensis* Reimer & Fujiwara, 2007 belonging to the new family Abyssoanthidae. *A. nankaiensis* is distinguished by its unitary polyps, presence at methane cold seeps at extreme depths, and divergent phylogenetic status from other zoanthids. Unfortunately, due to difficulties in histology and the very small number of specimens, many questions remain on the ecology and morphology of *A. nankaiensis*. From images taken during Shinkai 6500 dive # 959 it was hypothesized that there were other potential Abyssoanthidae populations at a non-methane seep site at the Japan Trench (39°06.50'N-143°53.4'E). In October 2007, JAMSTEC cruise YK07-15 and the Shinkai 6500 dove to a depth of 5347-5360 m (dives # 1038 & 1041) at this site to confirm the presence of zoanthids. Our molecular (mitochondrial 16S ribosomal DNA) and morphological (dimensions, cnidome) findings show a large population of a new species, *Abyssoanthus convallis*, living on mudstone in an "ecological hotspot" characterized by large amounts of marine snowfall. Specimen polyps *in situ* were approximately 15-25 mm in height, 5-15 mm in diameter, and had 20-30 tentacles. This species of zoanthids represents the deepest recorded zoanthid population, and further investigations at other hadal sites will increase our knowledge of these understudied benthic cnidarians.

Keywords: *Abyssoanthus* • Deep sea • Marine snow • Zoanthid

Introduction

Zoanthids (order Zoantharia) are generally colonial benthic cnidarians that have two rows of tentacles and usually encrust sand and other debris into their structure. Known from most of the world's marine ecosystems, zoanthid taxonomy has been described as "chaotic", with true levels of species diversity in this order unknown (Burnett et al., 1997; Reimer et al., 2004). In particular, until recently, very little was known about zoanthids (Cnidaria: Hexacorallia) from deep-sea environments, with most specimens assigned to the family Epizoanthidae and its sole genus *Epizoanthus* (e.g. Muirhead et al., 1986). Zoanthids are notoriously difficult to properly classify to the species level as a result of difficulties in examining internal structure due to the aforementioned encrustation, as well as due to a lack of useful diagnostic morphological characteristics. It has been shown that many zoanthids have large amounts of intraspecific variation with regards to various morphological characteristics, including cnidae (Ryland & Lancaster, 2004), polyp and colony shape and size (Burnett et al., 1997) and color (Reimer et al., 2004).

The application of molecular methods: allozymes (see Burnett et al., 1997); DNA (see Reimer et al., 2004; Sinniger et al., 2005) to zoanthid taxonomy has resulted in the re-examination of relationships within this order, with new species (Reimer et al., 2006), new genera, and new families (Reimer et al., 2007) being described. It has become apparent that while many described species are apparently synonymous, there are also large amounts of "hidden" diversity within Zoantharia, particularly at generic and higher taxonomic levels (Sinniger et al., 2010a).

In June 2005 a small number of unusual samples of a zoanthid-like species were inadvertently sampled during deep-sea submersible dives (*Shinkai 6500* dive #884) at a methane cold seep at 3259 m in the Nankai Trough off Muroto, Japan (32°34.945'N-134°41.545'E). Specimens were different in ecology, morphology and molecular phylogeny from all known families of zoanthids, and were subsequently classified as the new species *Abysssoanthus nankaiensis* Reimer & Fujiwara, 2007 belonging to the newly erected family Abysssoanthidae.

Like most zoanthids, *Abysssoanthus* has a mesoglea encrusted with sand/debris and has two rows of tentacles, but *A. nankaiensis* was distinguished by its unitary polyps, presence at methane cold seeps at extreme depths, and divergent sequences and phylogeny from all other zoanthids. Unfortunately, due to encrusted sand causing difficulties in conducting morphological examinations, and the small number of specimens, internal morphological data were not obtained. Similarly, no detailed *in situ* images were available from dive #884, and many questions remain

on the ecology and morphology of *Abysssoanthus*. The discovery of *Abysssoanthus* plus anecdotal evidence of other deep-sea zoanthids (Reimer et al., 2007; Sinniger et al., 2010b) demonstrated the potential for the existence of many unknown zoanthids and other benthic cnidarian species from abyssal and hadal depths.

After characterization of *A. nankaiensis*, it was learned from images taken during *Shinkai 6500* dive #959 that there were other potential Abysssoanthidae populations at a site at the Japan Trench (39°06.50'N-143°53.4'E). In October 2007, cruise YK07-15 and the *Shinkai 6500* dove to a depth of 5347-5360 m (dives # 1038 & 1041) at this site to confirm the presence of zoanthids. Additional dives at the same site were conducted in September 2009 (cruise YK09-12). Here we report on the morphological and phylogenetic results of examinations of new deep-sea zoanthid specimens from the Japan Trench, and describe one new *Abysssoanthus* species.

Materials and Methods

Sample collection

Zoanthid specimens were collected by the HOV *Shinkai 6500* during cruise YK07-15 (October 2007; dives #1038 & 1041) at the Japan Trench at depths of 5347-5360 m. All zoanthid specimens were photographed *in situ*, then again on the surface, and immediately preserved in either 99.5% ethanol (for molecular analyses) or 10% SW formalin (for morphological analyses).

Morphological analyses

The external morphology of specimens was examined using both preserved specimens and *in situ* images. Polyp dimensions (oral disk diameter, polyp height) for both *in situ* and preserved specimens were obtained, as were the following data: tentacle number, color of polyp, color(s) of oral disk, relative amount of sand encrustation, associated/substrate species. Additionally, presence/absence of the coenenchyme was examined.

Nematocyst observation

Undischarged nematocysts were measured from tentacles, column, actinopharynx, and mesenterial filaments of polyps (specimens examined $n = 4$; nematocysts $n > 50$ for all specimens). 400x images of the nematocysts were obtained by optical microscope, and measured using the software ImageJ (National Institutes of Health, USA). Nematocyst nomenclature generally followed England (1991). During nematocyst observations, encrustations of the polyp body walls were also conducted.

DNA extraction and PCR amplification

DNA was extracted using a spin-column Dneasy Blood and Tissue Extraction protocol (Qiagen, Santa Clarita, CA, USA). Mitochondrial 16S ribosomal DNA (mt 16S rDNA) was amplified using primers and protocol described in Sinniger et al. (2005), and the cytochrome oxidase subunit I (COI) gene was amplified following Reimer et al. (2004 & 2007). PCR amplification procedures for each of the molecular markers were as given in the original references above. Amplified products were visualized by 1.5% agarose gel electrophoresis.

Phylogenetic analyses

New sequences obtained in the present study were deposited in DDBJ and GenBank (accession numbers HQ224862-HQ224867). By using CLUSTAL X version 1.8 (Thompson et al., 1997), the nucleotide sequences of mt 16S rDNA and COI from samples were aligned with previously published sequences (see Figure 1 for Accession Numbers) from various zoanthid species representing the genera *Abysoanthus*, *Epizoanthus*, *Palythoa*, *Zoanthus*, *Acrozoanthus*, *Isaurus*, *Savalia*, *Corallizoanthus*, *Mesozoanthus*, *Hydrozoanthus*, and *Parazoanthus*. The only zoanthid genera not included in both analyses were *Sphenopus* (Sphenopidae) and *Neozoanthus* (Neozoanthidae), which are in the suborder Brachycnemina, and *Isozoanthus* and *Antipathozoanthus* in the family Parazoanthidae, all of which are clearly divergent from *Abysoanthus*. In total, these represent the current entire range of described zoanthid genera.

The outgroup sequences for both mt 16S rDNA and COI trees were from *Epizoanthus*, previously demonstrated to be basal to all other zoanthids (Reimer et al., 2007).

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) 664 sites of 27 sequences (mt 16S rDNA); and 2) 280 sites of 45 sequences (COI). The alignment data are available on request from the corresponding author.

For the phylogenetic analyses of the two genetic markers, the same methods were independently applied. Alignments were subjected to analyses with maximum-likelihood (ML) with PhyML (Guindon & Gascuel, 2003). PhyML was performed using an input tree generated by BIONJ with the general time-reversible model (Rodriguez et al., 1990) of nucleotide substitution incorporating invariable sites and a discrete gamma distribution (eight categories) (GTR + I + G). The proportion of invariable sites, a discrete gamma distribution, and base frequencies of the model were estimated from the dataset. PhyML bootstrap trees (500 replicates) were constructed using the

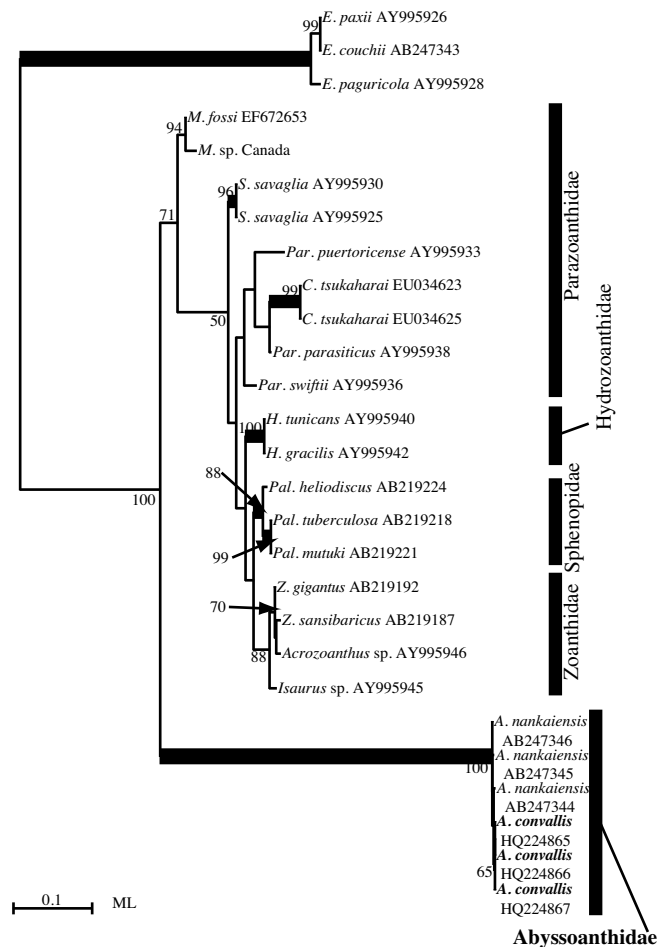


Figure 1. *Abysoanthus convallis* sp. nov. Maximum likelihood tree of mitochondrial 16S ribosomal DNA sequences from the order Zoantharia (with GenBank Accession Numbers) highlighting the phylogenetic position of Abysoanthidae (sample names in bold) and new Japan Trench specimens (in bold). Values at branches represent ML J bootstrap probability (> 50%). Thick branches signify Bayesian posterior probability > 0.95.

Figure 1. *Abysoanthus convallis* sp. nov. Arbre phylogénétique construit sur le principe du maximum de vraisemblance (ML) à partir des séquences du gène ribosomique mitochondrial 16S de l'ordre des Zoanthaires (avec numéros d'accès GenBank) montrant la position des Abysoanthidae (noms en gras) et des nouveaux spécimens de la fosse du Japon (en gras). Les valeurs au niveau des branches représentent les probabilités obtenues par bootstrap (ML) > 50%. Les branches épaisses représentent une probabilité bayésienne a posteriori > 0,95.

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 & Huelsenbeck, 2003) under the GTR model for mt 16S rDNA. One cold and three heated Markov chain Monte Carlo (MCMC) chains with default-

chain temperatures were run for 2 million generations, sampling log-likelihoods (InLs), and trees at 100-generation intervals (20,000 InLs and trees were saved during MCMC). The first 150,000 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 = 1.001). Thus, the remaining 18,500 trees (1.85 million generations) were used to obtain posterior probabilities and branch-length estimates, respectively.

Abbreviations used

- NSMT National Museum of Nature and Science, Tokyo, Japan
 USNM Smithsonian National Museum of History, Washington D.C., USA
 MHNG Natural History Museum of Geneva, Switzerland
 MISE Molecular Invertebrate Systematics and Ecology Laboratory, University of the Ryukyus, Nishihara, Okinawa, Japan

Results

Cytochrome oxidase subunit I

COI sequences acquired from Japan Trench specimens were seen to be identical to previously reported *Abysoanthus nankaiensis* COI sequences (sequences' lengths = 280–310 base pairs). As in Reimer et al. (2007), *Abysoanthus* formed a completely supported (ML = 100%) monophyly clearly divergent and sister to another clade consisting of most other known zoanthids (families Zoanthidae, Sphenopidae, Parazoanthidae, Hydrozoanthidae). These two large clades were derived from a basal clade of Epizoanthidae (data not shown).

Mitochondrial 16S ribosomal DNA

The mt 16S rDNA phylogenetic tree (Fig. 1) was very similar in topology to the COI tree. Acquired mt 16S rDNA sequences were slightly different (2/550 base pairs = 0.36%) from previously acquired mt 16S rDNA sequences from *A. nankaiensis*. One difference was in a homopolymer at position 614 in the alignment (*A. nankaiensis* = 4 Gs; new samples = 3 Gs), while the other change was at position 633 (*A. nankaiensis* = G; new samples = A). Thus, the new specimens' sequences formed a subclade (ML = 65%, Bayes = 0.93) apart from *A. nankaiensis*. These two clades formed a completely supported monophyly (ML = 100%, Bayes = 1.00), which was sister to a large clade (ML = 71%, Bayes < 0.50) of sequences from Zoanthidae, Sphenopidae, Parazoanthidae, and Hydrozoanthidae

specimens. These two large groups were directly derived from an ancestral zoanthid group, including Epizoanthidae.

Systematics

Order Zoantharia Gray, 1870

Family Abysoanthidae Reimer & Fujiwara, 2007

Diagnosis

Sand/detritus/sediment-encrusted Zoantharia with unitary (non-colonial) free-living polyps, attached to hard substrates (often mudstone) at abyssal (non-continental shelf deep-sea) depths. Polyps dully colored, coloration mostly from encrusted sand/detritus/sediment. Inhabits methane cold seeps or other chemosynthetic ecosystems.

Remarks

This diagnosis almost unchanged from Reimer et al. (2007) except for the changing of habitat from “limited to methane cold seeps” to a slightly broader meaning to take into account the Japan Trench site investigated in this study, which has not yet been confirmed to be a methane cold seep.

Genus *Abysoanthus* Reimer & Fujiwara, 2007

Type species: *Abysoanthus nankaiensis* Reimer & Fujiwara, 2007

Diagnosis

As for family above.

***Abysoanthus convallis* sp. nov.**
(Figs 1 & 2, Table 1)

Etymology

Named from the Latin for ravine or enclosed valley, to reflect the type locality of the Japan Trench.

Material examined

Holotype: NSMT-Co 1531, single polyp, collected from the Japan Trench at 39°06.36'N–143°53.56'E, during *Shinkai 6500* dive #1038, 22 October 2007, depth = 5348 m by J.D. Reimer.

Paratypes: USNM 1134416, MHNG-INVE-67703; all collection details as above.

Additional material: > 250 other MISE specimens; all collection details as above except depths 5348–5349 m; and tens of other MISE specimens collected from the Japan Trench at 39°06.25'N–143°53.46'E during *Shinkai 6500* dive #1041, 25 October 2007, depth = 5356 m by F.

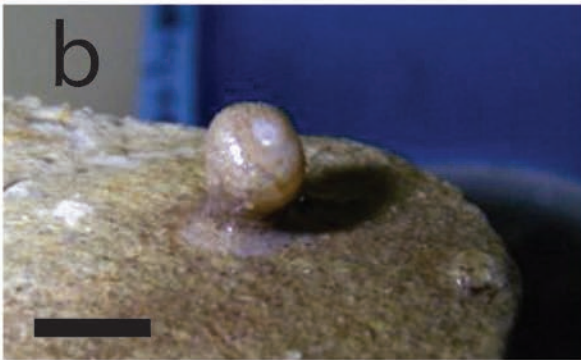
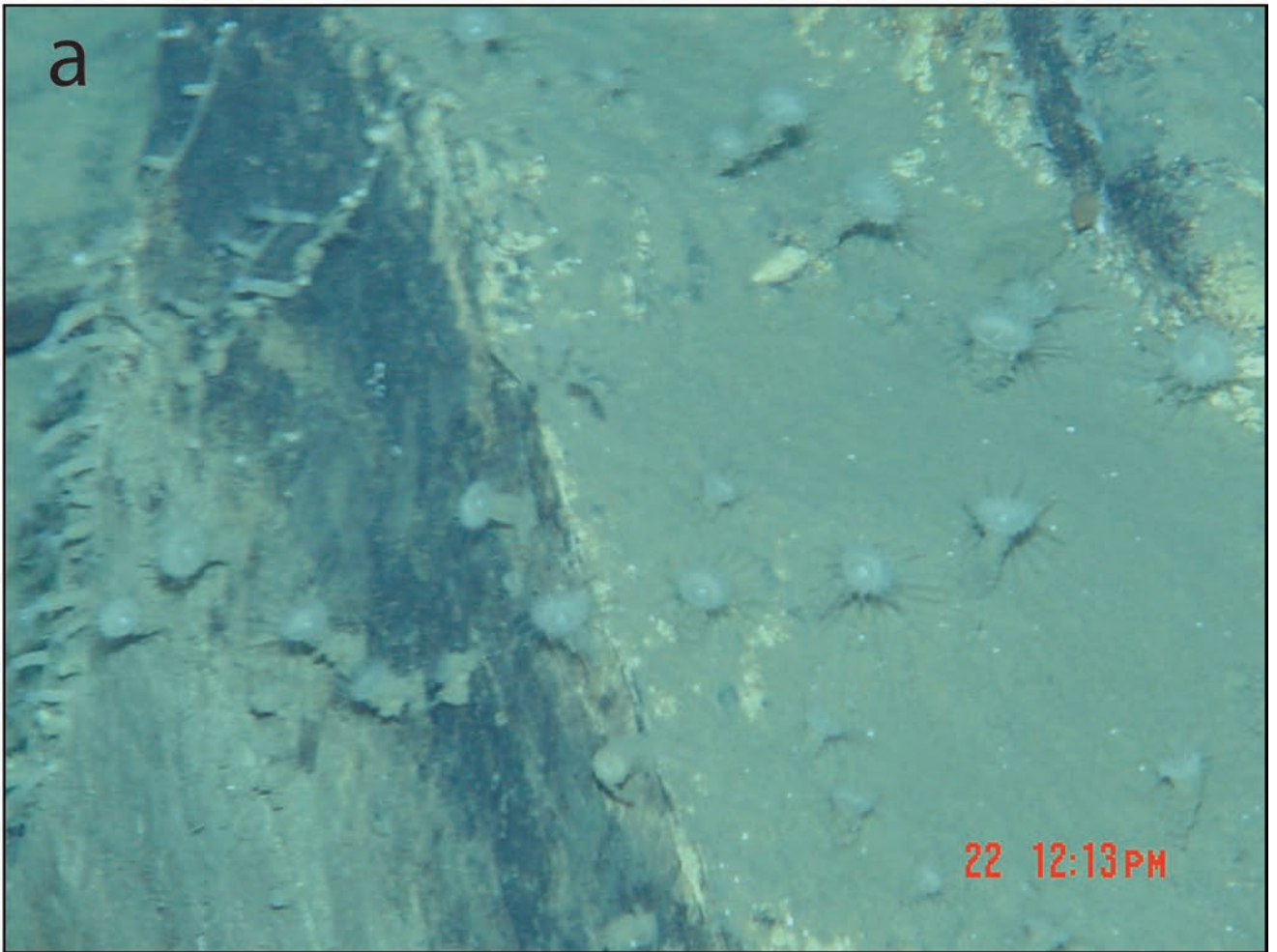


Figure 2. *Abyssoanthus convallis* sp. nov. **A.** In situ image at the Japan Trench, depth 5349 m, taken on October 22, 2007. **B.** On-board (*RV Yokosuka*) image of two closed polyps taken on September 4, 2009. Note unitary polyps in both A and B, and polyps growing in a row in A. Smaller polyps connected by a stolon on the left side of the image are an unidentified octocoral. Scale bars: 1 cm.

Figure 2. *Abyssoanthus convallis* sp. nov. **A.** Photo in situ dans la fosse du Japon, à 5349 m, prise le 22 octobre 2007. **B.** Photo prise à bord du *RV Yokosuka* de deux polypes rétractés, le 4 septembre 2009. Noter les polypes isolés en A et B ainsi que les polypes se développant en rang en A. Les plus petits polypes connectés par un stolon sur la partie gauche de la photo sont un octocoralliaire non identifié. Echelle : 1 cm.

Sinniger; and several other MISE specimens collected from the Japan Trench at 39°06.35'N-143°53.50'E during *Shinkai 6500* dive #1160, 4 September 2009, depth = 5347 m, identified by T. Fujii.

Description

Size: In situ polyps 15-25 mm in height. Expanded oral disks up to 5-15 mm in diameter. Preserved (in 99.5% EtOH) polyps 5-15 mm in height.

Morphology: Polyp body wall heavily encrusted yet smooth, of similar color (ash gray) to surrounding sea floor and mudstone, with encrustations barely visible to the naked eye (Fig. 2b). Encrustations consist of unidentified spicules, foraminifer tests, and sand and other detritus. Mouth visible as slightly white in color on expanded polyps, tentacles 20-30 in number, generally transparent or translucent (Fig. 2a & b). Capitulary ridges not clearly visible on closed polyps (Fig. 2b). Closed polyps narrow

Table 1. *Abyssoanthus convallis* sp. nov. Types (generally following nomenclature of England, 1991), relative abundances and sizes of cnidae.

Tableau 1. *Abyssoanthus convallis* sp. nov. Types (généralement suivant la nomenclature selon England, 1991), d'abondance et de taille relative des nématocystes.

Tissue	Cnidae type	Length x width (µm)	Relative abundance
Base	Holotrich L	18-27 x 8-14	Very common
	Holotrich S	9 x 4	Rare
	Spirocyst M	14-34 x 3-5	Occasional
	Spirocyst L	22 x 12	Rare
	Basitrich	19 x 6	Rare
Column	Holotrich L	18-30 x 6-11	Very common
	Spirocyst M	27 x 9	Rare
	p-mastigophore	24 x 7	Rare
	Basitrich	21-44 x 4-14	Occasional
Mesenterial filaments	Holotrich L	11-29 x 4-13	Very common
	Spirocyst M	14-15 x 5-6	Occasional
	b-mastigophore	13 x 4	Rare
	Basitrich	41 x 8	Rare
Tentacles	Holotrich L	18-24 x 9-10	Occasional
	Spirocyst M	9-27 x 3-6	Very common

towards the base, wider towards the oral end similar to a rounded club (Fig. 2b). Polyps unitary, found in rows on mudstone (Fig. 2a).

Cnidae: Holotrichs, spirocysts, p-mastigophores, b-mastigophores, basitrichs (Table 1).

Diagnosis

Very similar in external appearance (unitary, lack of bright coloration) to *A. nankaiensis*, and also found on mudstone rocks. Polyps appear smoother and less heavily encrusted than *A. nankaiensis*, size of closed polyps of *A. convallis* larger and oral end more club-like than in *A. nankaiensis* polyps (compare Fig. 2b with Fig. 2 in Reimer et al., 2007). Unlike *A. nankaiensis*, *A. convallis* is found much deeper (approx. 5350 m as opposed to 3259 m), at a site that has not been confirmed to be a methane cold seep, although it is likely a chemosynthetic habitat given the presence of *Calyptogena* clams. Additionally, *A. convallis* at the Japan Trench is over 1000 km distant and clearly separated from the Nankai Trough and *A. nankaiensis*. DNA sequences from mt 16S rDNA of *A. convallis* are also distinct from *A. nankaiensis* sequences.

Habitat and distribution

Has been found only at one location in the Japan Trench (39°06'N-143°53'E), off the east coast of the Sanriku region, Japan, at depths of 5347-5356 m. The location receives much "marine snow" fall from the water column.

Other remarks

Based on the results of the YK07-15 cruise and the previous discovery of *A. nankaiensis* at the Nankai Trough, as well as from preliminary examinations of other specimens (Sinniger et al., 2010b) it is very likely that there are other deep-sea zoanthid species and populations awaiting discovery and description in the waters around Japan and the world.

Discussion

As anthozoans have been shown to have very slow mitochondrial evolution compared to most organisms (Shearer et al., 2002), even a difference of one base pair in COI or mt 16S rDNA sequences has been shown to clearly designate different species' groups (reviewed in Sinniger et al., 2008), and thus the molecular results here can be considered to show congeners within *Abyssoanthus*.

The ecology of *Abyssoanthus*

Although it was originally believed that *Abyssoanthus* consisted of species solely found at methane cold seeps based on the type species *A. nankaiensis*, this may not be the case with *A. convallis*, as although it is very close phylogenetically and morphologically to *A. nankaiensis*, it is found in a different ecosystem that is over 2000 m deeper. The *A. convallis* site is also likely chemosynthetic (T. Maruyama & Y. Fujiwara, pers. comm.), as seen at other sites at the Japan Trench (Fujikura et al., 1999), although it

is not established if this location is a methane cold seep or not. If Abyssoanthidae species are shown to not be limited to chemosynthetic ecosystems, then it is likely that there are many more species that await discovery, perhaps on mudstone similar to the two known *Abyssoanthus* species. Images from the Atlantic (mentioned in Reimer et al., 2007) and specimens from the Mediterranean (Sinniger et al., 2010b) also point to the possible existence of Abyssoanthidae in these areas.

In Reimer et al. (2007) it was uncertain as to whether *A. nankaiensis* was chemosynthetic, acquiring energy from chemosynthetic bacteria, or free-living, acquiring nutrition from the water column. Similar to *A. nankaiensis*, *A. convallis* is not epizoic on any other organism, and thus may be free-living, perhaps acquiring energy from “marine snow” that falls in large amounts at the type locality (e.g. Sawada et al., 1998). Future planned isotope analyses and other studies will help our understanding of this group of deep-sea zoanthids.

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