Mar Biodiv (2010) 40:249–260
DOI 10.1007/s12526-010-0045-0

ORIGINAL PAPER

Unexpected diversity in Canadian Pacific zoanthids (Cnidaria: Anthozoa: Hexacorallia): a molecular examination and description of a new species from the waters of British Columbia

James Davis Reimer · Frederic Sinniger

Received: 5 October 2009 / Revised: 25 April 2010 / Accepted: 3 May 2010 / Published online: 27 May 2010

Abstract Recent investigations into the species diversity of zoanthids have demonstrated the existence of many previously unrecognized species, genera, and even families within this order. The application of molecular markers, combined with more "traditional" morphological and ecological investigations have proven highly useful in examining this taxonomically neglected group. Here, using these combined techniques on newly collected and preserved museum specimens, we examine the diversity of zoanthids in the waters of British Columbia for the first time ever. Results show the presence of one undescribed species, *Mesozoanthus lilkweminensis* n. sp., and point to the existence of two to four other ones. *Mesozoanthus lilkweminensis* is distinguished by its salmon-pink coloration, 34–38 tentacles, and is found on hard rocky substrate. It is easily distinguishable from the only other described zoanthid species from the Canadian Pacific coast, *Epizoanthus scotinus* Wood 1958, by size, coloration and tentacle number. Specimens of *M. lilkweminensis* are known from only one location thus far. Although there may still be undiscovered populations of this new species, it is expected to be relatively rare. These findings highlight the need for further investigations into the diversity of marine invertebrate biodiversity in northern temperate Pacific waters.

Keywords Zoanthid · Diversity · *Mesozoanthus* · ITS-rDNA · New species · Canadian Pacific

Electronic supplementary material The online version of this article (doi:10.1007/s12526-010-0045-0) contains supplementary material, which is available to authorized users.

J. D. Reimer (✉)
Molecular Invertebrate Systematics and Ecology Laboratory,
Rising Star Program, Trans-disciplinary Organization
for Subtropical Island Studies, University of the Ryukyus,
1 Senbaru, Nishihara,
Okinawa 903-0213, Japan
e-mail: jreimer@sci.u-ryukyu.ac.jp

J. D. Reimer
Marine Biodiversity Research Program, Institute of
Biogeosciences, Japan Agency for Marine-Earth Science
and Technology (JAMSTEC),
2-15 Natsushima, Yokosuka,
Kanagawa 237-0061, Japan

F. Sinniger
Department of Chemistry, Biology and Marine Science,
Faculty of Science, University of the Ryukyus,
1 Senbaru, Nishihara,
Okinawa 903-0213, Japan

F. Sinniger
Department of Oceanography, Florida State University,
117N. Woodward Avenue,
Tallahassee, FL 32306-4320, USA

Introduction

The year 2010 has been declared the International Year of Biodiversity by the United Nations as an effort to highlight the Earth's species richness. Part of the international efforts will combine new identification techniques, such as DNA barcoding (Hebert et al. 2003), with reassessed conservation priorities (e.g., Myers et al. 2000) to help increase taxonomic knowledge while reducing the current rate at which biodiversity is being lost. It is hoped that such projects will help alleviate the "taxonomic bottleneck" that many regions, ecosystems and understudied taxa now face (Kim and Byrne 2006). One species-rich marine region comprises the Pacific coastline of Canada, which is home to many understudied marine invertebrates (Lamb and Hanby 2005), including many cnidarians. One group of relatively understudied cnidarians is the zoanthids (Zoantharia=Zoan-

thidea), which are an order of benthic and generally colonial anthozoans that are widespread and occasionally common in many marine ecosystems. This order of cnidarians is generally characterized by having two rows of tentacles, one siphonoglyph, and using sand and detritus to help form their structure. Zoanthids remain largely understudied due to uncertain and difficult taxonomy and an overall scarcity of reliable information

The use of molecular phylogenetic techniques combined with new efforts to understand the biodiversity of marine invertebrates has proven beneficial to zoanthid taxonomy. Much recent work has focused on zoanthid diversity using allozymes (Burnett et al. 1997) and DNA markers (e.g., Reimer et al. 2004; Sinniger et al. 2005), and as a result several new zoanthid taxa have been described (Reimer et al. 2006b, 2007a, 2008a).

One region of the world for which almost no information exists on zoanthids is the northwest coast of North America (see Cutress and Pequegnat 1960), and in particular British Columbia, Canada, despite the presence of many guidebooks on the marine fauna of this region. A single species, *Epizoanthus scotinus* Wood 1958, is described from Puget Sound, Washington, USA, and is known from southern California to northern Alaska and Siberia (Lamb and Hanby 2005). Almost no other information exists for the North American northwest coast for other zoanthid species, although several other species have been described from southern California (Cutress and Pequegnat 1960; Philipp and Fautin 2009), and there have been no specific studies conducted on zoanthids in Canadian waters. Based on recent discoveries of undescribed zoanthids in other underexplored regions (Galapagos in Reimer et al. 2008b; Singapore in Reimer and Todd 2009) as well as images from the waters of British Columbia in Lamb and Hanby (2005), and considering the fact that approximately half of all marine species from northwest Pacific coast of North America are endemic (Lamb and Hanby 2005), the possibility of undescribed zoanthid species existing in this region is high. Here we examine various collected zoanthid specimens from British Columbia using both molecular and morphological techniques to address the following question: does underestimated zoanthid biodiversity exist within the waters of British Columbia?

Materials and methods

Specimen collection and preservation

Specimens were collected by SCUBA from three locations in British Columbia (Fig. 1, Table 1). Specimens from Agamemnon Channel and the Gulf Islands were collected in March 2007 and transported alive in aquaria to Vancouver Aquarium (VA) (Vancouver, B.C.) and kept in tanks for display purposes until fixation (following the protocol below) by the first author in September 2007. Images of these specimens were taken both in situ and from tanks at VA. Specimens from Jesse Island, Nanaimo were collected in September 2007 and fixed upon processing. Upon collection, morphological and ecological data (size, depth, substrate, etc.) were taken, specimens were immediately preserved in 95% ethanol (VA specimens $n=5$, Jesse Island specimens $n=15$, see Table 1) until further analyses.

Museum specimens

All zoanthid specimens ($n=11$) previously deposited in the RBC were examined morphologically (form, color, tentacle/mesentery number) and ecologically (substrate, sampling data) in October 2007 (see Table 1). Additionally, small tissue samples were collected for further DNA examination despite all samples having been originally preserved in 10% saltwater (SW) formalin and then transferred to 60% isopropyl alcohol (IPA). All RBC specimens were classified to species or generic level when possible based on observed morphological and available ecological data (colony formation, depth, habitat, coloration, etc).

DNA extraction, PCR amplification, and sequencing

DNA was extracted from tissue (5–20 mg) from collected zoanthid samples (ESM Table S1), and also attempted for all RBC museum specimens ($n=11$) following procedures outlined in Reimer et al. (2004) by using a DNEasy Tissue Kit for animals (Qiagen, Tokyo, Japan).

The mitochondrial cytochrome oxidase c subunit I (COI) gene was amplified using the zoanthid-specific primers COIZoanF (3′-TGATAAGGTTAGAACTTTCTGCCCCG GAAC-5′) and COIZoanR (3′-AGGCTAAATATAGC CATGTCCACG-5′) (Reimer et al. 2007a). The following thermal cycle conditions were utilized: 35 cycles of: 1 min at 94°C, 1 min at 40°C, 1 min 30 s at 72°C, and followed by a 7-min extension at 72°C.

Mitochondrial 16S ribosomal DNA (mt 16S rDNA) was amplified using zoanthid-specific primers described by Sinniger et al. (2005), with the following thermal cycle conditions: 40 cycles of: 1 min at 94°C, 1 min at 52°C, 2 min at 72°C, and followed by a 7-min extension at 72°C.

The nuclear internal transcribed spacer region of ribosomal DNA (ITS-rDNA) was amplified using zoanthid-specific primers previously reported in Reimer et al. (2007b). The following thermal cycle conditions were utilized: 35 cycles of: 1 min at 94°C, 1 min at 50°C, 2 min at 72°C, and followed by a 10-min extension at 72°C.

The amplified PCR products were checked by 1.5% agarose gel electrophoresis. The PCR-amplified DNA

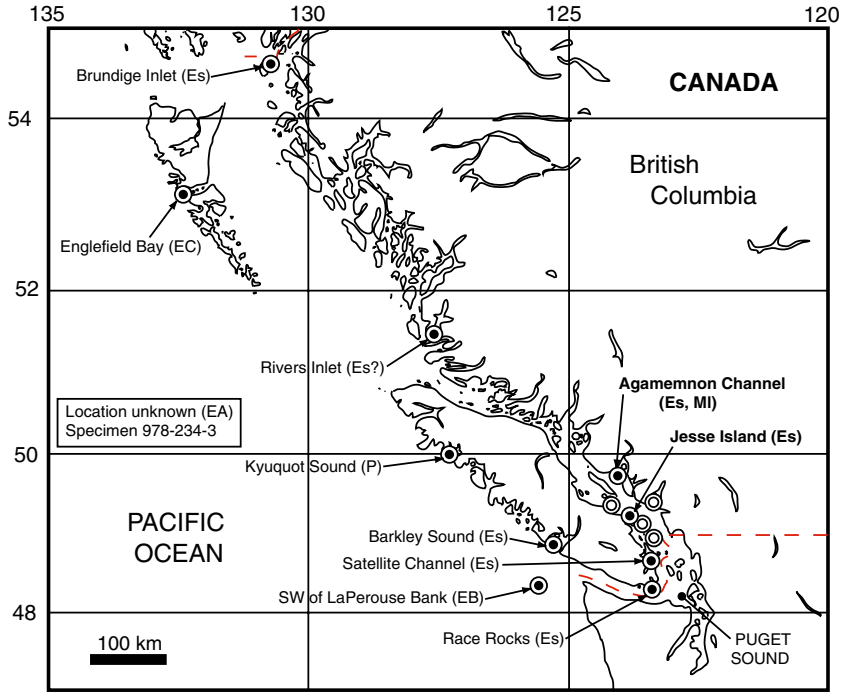

Fig. 1 Map of zoanthid specimen locations in British Columbia. Locations for specimens examined in this study represented by *closed symbols*, locations for which only photographic evidence exists by *open symbols*. Locations in *bold* indicate specimens for which molecular data are available. Species abbreviations after locations: *Es Epizoanthus scotinus*, *Es?* potentially *E. scotinus*, *EA* unknown *E.* sp. A, *EB* unknown *E.* sp. B, *EC* unknown *E.* sp. C, *Ml Mesozoanthus lilkweminensis*, *P* unknown Parazoanthidae. Note: one specimen in the *box on left* for which location is unknown. Puget Sound (*lower right*) is the type locality for *E. scotinus*

fragments were sequenced with an ABI PRISM 3700 DNA Analyzer (PE Biosystems, Foster City, Calif., USA) using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems). The sequences were analyzed using GENETYX-MAC version 8.0 (Software Development, Tokyo, Japan) and DNASIS Mac v3.6 (Hitachi Software Engineering Company, Tokyo, Japan).

Phylogenetic analyses

New sequences obtained in the present study were deposited in GenBank (accession numbers HM042363-HM042366; HM042383-HM042386). By using CLUSTAL X version 1.8 (Thompson et al. 1997) on default settings, the nucleotide sequences of the COI gene, mt 16S rDNA from samples were separately aligned with previously obtained zoanthid sequences from all known families (Epizoanthidae, Parazoanthidae, Sphenopidae, Zoanthidae, Abyssoanthidae—ESM Table S1). Phylogenetic analyses of ITS-rDNA sequences for Parazoanthidae specimens included only sequences from Parazoanthidae, as other families such as Zoanthidae and Abyssoanthidae with ITS-rDNA sequences publically available from GenBank are extremely divergent and thus very difficult to align with any confidence. All three alignments thus contained sequences from all described genera in Parazoanthidae (*Parazoanthus, Savalia, Mesozoanthus, Antipathozoanthus, Corallizoanthus*) and included members from all other potentially generic-level clades within *Parazoanthus* (see Sinniger et al. 2005). The alignments were inspected by eye and manually edited. Consequently, three alignment datasets were generated: (1) 744 sites of 68 taxa (mt 16S rDNA), (2) 311 sites of 59 taxa (the COI gene), and (3) 986 sites of 28 taxa (ITS-rDNA). The alignments are available as upon request from the corresponding author.

For the phylogenetic analyses of the mt 16S rDNA, COI gene and ITS-rDNA the same methods were independently applied. Maximum-likelihood (ML) analyses were performed using PhyML (Guindon and 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 + Γ). 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 same parameters as the individual ML trees.

Bayesian trees were also reconstructed by using the program MrBayes 3.0 (Ronquist and Huelsenbeck 2003) under GTR + I + Γ. One cold and three heated Markov chains Monte Carlo (MCMC) with default-chain temper-

Table 1 Order Zoantharia specimens from British Columbia examined in this study including sampling information (*MISE* Molecular Invertebrate Systematics and Ecology Laboratory, Nishihara, Okinawa, Japan, *RBC* Royal British Columbia Museum, Victoria, B.C., *UBC* University of British Columbia, *NA* not available or not acquired)

Specimen no.	Specimen identification	Date collected	Collector(s)	Depth (m)	Location	Lat. (N)	Long (W)	Notes
MISE 210–215	*Epizoanthus scotinus*	2007.9.12	J.D. Reimer	18	Jesse Island, Nanaimo, BC	49°12′18″	123°56′27″	On large rock overhang
MISE 216–220	*Epizoanthus scotinus*	2007.9.12	J.D. Reimer	15	Jesse Island, Nanaimo, BC	49°12′18″	123°56′27″	On large rock overhang
MISE 221–224	*Epizoanthus scotinus*	2007.9.12	J.D. Reimer	12	Jesse Island, Nanaimo, BC	49°12′18″	123°56′27″	On large rock overhang
MISE 225–226	*Mesozoanthus lilkweminensis*	2007.3.27	Vancouver Aquarium	37–43	Agamemnon Channel, BC	49°44′14″	124°02′19″	On rock
MISE 227–228	*Mesozoanthus lilkweminensis*	2007.3.27	Vancouver Aquarium	37–43	Agamemnon Channel, BC	49°44′14″	124°02′19″	On rock next to gorgonian
MISE 229	*Epizoanthus scotinus*	Summer 2007	Vancouver Aquarium	NA	Gulf Islands, BC	NA	NA	Exact sampling location unknown
RBC 973-176-1	*Epizoanthus scotinus*	1973.7.19	Gosling & P. Lambert	<15	Turret I, Barkley Sound, BC	48°53′42″	125°19′12″	On dead barnacle
RBC 973-231-4	*Epizoanthus scotinus*	1973.8.8	P. Lambert	21	Nettle I, Barkley Sound, BC	48°56′ [a]	125°15′ [a]	On dead barnacle
RBC 973-235-7	*Epizoanthus scotinus*	1973.8.14	P. Lambert	18	Treble Is, Barkley Sound, BC	48°56′ [a]	125°17′ [a]	On dead barnacle/shell
RBC 973-251-11	*Epizoanthus scotinus*	1973.9.18	P. Lambert et al.	6–10	Race Rocks, Victoria, BC	48°18′	123°32′	Slightly less massive polyps
RBC 974-236-6	*Epizoanthus scotinus*	1974.6.22	P. Lambert & Kerfoot	<43	Brundige Inlet mouth, BC	54°36′48″	130°50′24″	
RBC 974-568-1	Unknown *Epizoanthus* sp.	1974.8.14	A. Parkinson	<12	Small island entrance of Rivers Inlet, Fitz Hugh Sound, west of Bilton I, BC	51°27′6″	127°41′12″	Darker than *E. scotinus*
RBC 978-234-3	Unknown *Epizoanthus* sp.	?1978?.11.8[b]	UBC	160	NA	NA	NA	UBC sample no. 621 IOUBC
RBC 978-334-10	*Epizoanthus scotinus*	1978.12.5	P. Lambert & Green	36	Arbutus I, Satellite Channel, BC	48°[a]	123° [a]	On dead barnacle
RBC 988-259-3	*Epizoanthus* sp.	1988.2.23	Green	705	SW of LaPerouse Bank	48°27′6″	126°15′24″	On mollusc shell
RBC 990-317-21	Unknown Parazoanthidae	1990.2.2	P. Lambert et al.	90–200	Kyuquot Sound, Kyuquot Channel, BC	49°59′30″	127°13′18″	On dead sponge and polychaete tubes
RBC 991-343-10	Unknown Epizoanthidae	1991.3.26	P. Lambert et al.	100–125	Inskip Channel, Englefield Bay, Moresby I, Queen Charlotte Is, BC	53°02′	132°15′	Unitary on rocks

[a] Approximate latitude and longitude as none noted on RBC specimen cards

[b] No sample information other than shown asides from specimen collected by the UBC. Sampling year is unknown, but based on RBC specimen number the date is no later than 1978

atures were run for 1,000,000 generations, sampling log-likelihoods (InLs), and trees at 100-generation intervals (10,000 InLs and trees were saved during MCMC). The likelihood plot for mt 16S rDNA, COI and ITS-rDNA datasets suggested that MCMC reached the stationary phase after the first 30,000 generations for mt 16S rDNA and COI analyses [potential scale reduction factor (PSRF)=1.000 and 1.012, standard deviation of split frequencies= 0.012149 and 0.014053, respectively], and after 100,000 generations for ITS-rDNA (standard deviation of split frequencies=0.008640). Thus, the remaining 9700 trees of mt 16S rDNA and COI, and the remaining 9,000 trees of ITS-rDNA were used to obtain clade probabilities and branch-length estimates.

Morphological analyses

Initial observation of samples and polyp surfaces were made using a dissecting microscope. Due to the presence of detritus in the endoderm of zoanthids (except for the family Zoanthidae), obtaining complete cross-sections is unusually difficult unless potentially dangerous hydrofluoric acid (HF) is used. Rough cross-sections of polyps were made with paraffin following Ono et al. (2005), however, and mesentery numbers confirmed. Mesogleal thickness and structure were also examined.

Additional data collected for potential new species, as indicated by initial molecular analyses included tentacle number, which is approximately the same as mesentery number in zoanthids. As well, polyp dimension data (expanded polyp diameter and height, closed polyps' aboral end maximum diameter) were obtained. As specimens were preserved in ethanol, formalin or IPA, polyps were closed and tentacles retracted to varying degrees, and thus we avoided recording potentially erroneous oral end diameter data. Aboral maximum diameter is not as prone to such size changes upon polyp closure, and thus was recorded for all four preserved specimens. Only large polyps were selected to avoid selecting potentially immature polyps.

Nematocyst observation

Undischarged nematocysts were measured from tentacles, column, actinopharynx, and mesenterial filaments of polyps for potential new species based on molecular analyses. Images of the nematocysts at 400× magnification were obtained by optical microscope, and measured using the software ImageJ (National Institutes of Health, Washington, D.C., USA). Nematocyst nomenclature generally followed England (1991), Ryland and Lancaster (2004), and Sinniger and Häussermann (2009), however both Schmidt (1974) and Hidaka et al. (1987), Hidaka (1992) have previously suggested basitrichs and microbasic b-mastigophores are the same type of the nematocyst, and thus in this study, data for these two types were pooled.

Results

Systematics

Suborder Macrocnemina Haddon and Shackleton 1891

Diagnosis: Characterized by a complete fifth pair of mesenteries.

Family Parazoanthidae Delage and Hérouard, 1901

Diagnosis: *(after Sinniger and Häussermann* 2009*)* Macrocnemic zoanthids that have an endodermal sphincter muscle. Many species in this family are associated with other organisms utilized as substrate

Genus *Mesozoanthus* Sinniger and Häussermann 2009

Type species Mesozoanthus fossii Sinniger and Häussermann 2009

Diagnosis (after Sinniger and Häussermann 2009) Parazoanthidae occurring on rocky substrate, not epizoic on sponges or other living organisms. Scapus and coenenchyme incrusted with foreign material. Polyps colonial, in clusters connected by basal coenenchyme, with relatively long pointed tentacles. Present in small colonies; does not colonize demosponges. Azooxanthellate. DNA sequences (mt 16S rDNA, COI, ITS-rDNA) clearly divergent from other Parazoanthidae genera.

Mesozoanthus lilkweminensis n. sp.

Figures 2a, b and 3, Tables 1, 2 and 3

Etymology: Named after the shíshálh (Sechelt) First Nation name for the type locality, Agamemnon Channel (lílkw'émin).

Material examined: All from the type locality: Canada, British Columbia, Agamemnon Channel, depth 37–43 m, 03.27.2007. Collected by VA. Stored in 99% ethanol. Holotype: NSMT (National Museum of Nature and Science, Tokyo, Japan)-Co 1532. Paratypes: USNM (National Museum of Natural History, Smithsonian Institution, Washington, D.C., USA) 113069, MHNG (Natural History Museum of Geneva, Switzerland) INVE 67706, MISE 225.

Diagnosis: Size—Preserved polyps to 20 mm high, with column diameter of up to 6–8 mm. In situ oral disk diameter 12–14 mm with polyp column approximately half this value, height to 35 mm. Morphology and coloration—34–38 tentacles (average=36, n=4 colonies) on large polyps, less on smaller polyps. Pharynx oval, pale salmon in color. Polyps cream to pale salmon-pink in color, with outside of polyp around oral disk slightly paler than polyp column, encrusted slightly with particles of various colors (white, gray, black). Oral disk same color as polyp

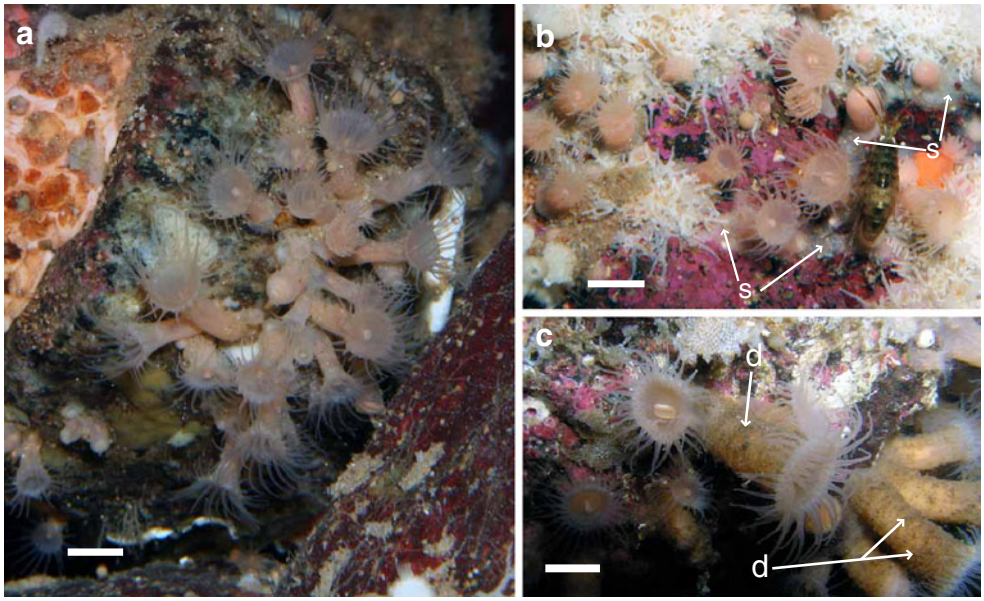

Fig. 2 *Mesozoanthus lilkweminensis* n. sp. in aquarium at VA. Holotype polyps were taken from this colony. Note the cream-pink coloration in both **a** and **b** and reduced or stoloniferous coenenchyme (*s*) in **b**. **c** *Epizoanthus scotinus* from Jesse Island, Nanaimo, British Columbia (12.7–17.2 m) shown for comparison. Note higher sedimentation/detritus (*d*) on outer surface of polyps as well as different coloration, particularly of polyp column's outer surface. Additionally, *E. scotinus* has more (44–52) tentacles than *M. lilkweminensis* (34–38). All *scale bars*=1 cm

column with paler mesentery insertions clearly visible. Tentacle length up to approximately 1.5-times oral disk diameter, pointed, thin; color cream to opaque. Coenenchyme between polyps generally reduced, often stoloniferous, creamy white or opaque, appears more heavily encrusted than polyps. Polyps generally regularly spaced (about 4–8 mm apart) with occasional smaller polyps arising from base of large polyps. Polyps often form clusters linked by basal coenenchyme. Cnidae—Spirocysts, basitrichs and microbasic b-mastigophores, holotrichs (Fig. 3, Table 2).

Differential diagnosis: Different coloration, smaller size, and reduced coenenchyme (stolon) distinguish this species from sympatric *Epizoanthus scotinus*. *Mezoanthus lilkweminensis* is similarly colored but much larger than *E. induratum* from southern California. *Mesozoanthus lilkweminensis* is very similar to *M. fossii*, but with slightly different average tentacle numbers (34–38 versus 36–46), different coloration (pale salmon opposed to grayish-brown), and differing distribution (British Columbia and Chile, respectively) (Table 3). The cnidomes between these two congeners also differ, with *M. fossi* having no small holotrichs in the tentacles, no spirocysts or basitrichs/mastigophores in the column, and *M. lilkweminensis* having no very small holotrichs in the filaments (Fig. 3, Table 2). There are also differences in frequency of some other nematocyst types (Table 2).

Habitat and distribution: Known only from Agamemnon Channel, British Columbia, Canada (Fig. 1), at depths of 37–43 m. Occurring in channels, like *M. fossii*.

***Mesozoanthus lilkweminensis* sp. n.**

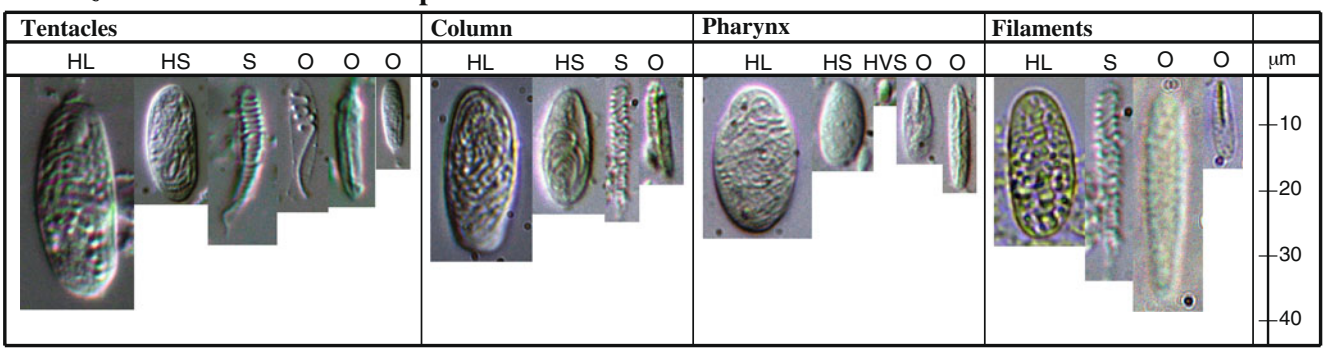

Fig. 3 Cnidae in the tentacles, column, pharynx and filament of *Mesozoanthus lilkweminensis* n. sp. *HL* large holotrich, *HS* small holotrich, *HVS* very small holotrich, *O* basitrichs or mastigophores, *S* spirocysts

Table 2 Types[a], relative abundances and sizes[b] of cnidae in species in the genus *Mesozoanthus*

Tissue	Cnidae type	*n*[c]	*Mesozoanthus lilkweminensis* n. sp.			*Mesozoanthus fossi*[d]		
			Length (max-min; avg ± SD)	Width (max-min; avg ± SD)	Frequency[e]	Length (max-min)	Width (max-min)	Frequency[e]
Tentacles	Spirocysts	44	19–53; 26.5±8.2	3–8; 4.5±1.3	V. common	20–30	3–5	V. common
	Holotrichs L	14	23–39; 31.3±5.3	10–16; 12.7±2.4	Common	26–33	16–19	Rare
	Holotrichs S	2	16–21; 18.7±4.4	4–5; 4.4	Rare			
	Basitrichs & b-mastigophores	24	15–24; 18.6±2.9	3–6; 4.2±0.8	Common	15–30	3–6	Sporadic; common
Column	Spirocysts	3	22–33; 27.4±5.6	3–4; 3.8±0.6	Rare			
	Holotrichs L	12	26–29; 28.1±1.2	11–13; 12.4±0.5	V. common	28–35	13–17	Common
	Holotrichs S	13	12–20; 15.3±2.8	8–9; 8.1±0.4	Common	15–24	8–10	Common
	Basitrichs & b-mastigophores	5	13–16; 14.6±1.4	4; 4.2±0.3	Rare			
Pharynx	Holotrichs L	5	25–34; 29.4±5.7	14–15; 14.1±0.7	V. common	32–33	14–18	Rare
	Holotrichs S	1	15	8	Sporadic	16–20	9–11	Common
	Holotrichs VS	16	4–7; 5.4±1.1	1–2; 1.9±0.4	V. common	6–9	3–4	V. common
	Basitrichs & b-mastigophores	20	10–21; 15.7±3.8	6–12; 8.4±2.0	V. common	11–23	3–5	V. common; few
Mesenterial filaments	Spirocysts	1	17	6	Sporadic	19–25	5–6	Common
	Holotrichs L	17	27–35; 31.1±2.9	10–15; 12.1±1.6	V. common	30–32	14–18	Few
	Holotrichs S	1	21	7	Rare	15–20	9–11	V. common
	Holotrichs VS					7–11	4–6	V. common
	Basitrichs & b-mastigophores	36	19–58; 33.6±12.4	5–12; 8.1±2.6	V. common	16–40	4–8	Few; sporadic
	p-mastigophores					17–21	6–7	V. common

[a] Nomenclature follows England (1991), see Materials and methods for details

[b] All sizes in µm.

[c] Total numbers of nematocysts of each type examined for *M. lilkweminensis* in this study. Two polyps each from two colonies (MISE 225, MISE 228) examined

[d] All data for *M. fossi* adapted from Sinniger and Häussermann (2009). Data for basitrichs and p-mastigophores combined

[e] Frequencies in decreasing order: very common, common, few, rare, sporadic. Frequencies for *M. fossi* given in order of basitrich, then p-mastigophores

Table 3 Comparison of zoanthid species with similar distribution and/or morphological characters to *Mesozoanthus lilkweminensis* n. sp

Species and authority	Polyp length (mm)	Oral disk diameter (mm)	Capitular ridges	Mesenteries and/or tentacles	Coenenchyme	Color	Distribution	Other notes
Mesozoanthus lilkweminensis n. sp. this study	To 35	To 12	Barely visible, to approx. 20	34–38 tentacles	Reduced and often stoloniferous	Cream to light salmon-pink, oral disk slightly pale with whitish radii	Agamemnon Channel, British Columbia	
Mesozoanthus fossi Sinniger and Häussermann 2009	To 35	To 10	20–23, whitish	36–46 tentacles	Reduced and often stoloniferous	Oral disk grayish-beige, column brownish-gray	Patagonian coast of Chile	
Epizoanthus scotinus Wood 1958	To 70	10–30	NA	40–70 mesenteries; 44–52 tentacles	Not stoloniferous	Variety of colors including brown, white, yellow.	Southern California to Kamchatka	Additional data from Lamb and Hanby 2005
Epizoanthus induratum Cutress and Pequegnat 1960	1–5	1.5–4	To 22	34–38 mesenteries	Not given	Pale salmon	Corona del Mar, California	Bioluminescent

Biology and associated species: *Mesozoanthus lilkweminensis* was found growing close to the gorgonian *Paragorgia pacifica* Verrill 1922 on rock substrate. Not in association with sponges or other living substrates.

Results

Cytochrome oxidase subunit I

The obtained cytochrome oxidase subunit I sequences from specimens in this study belonged to two groups (ESM Fig. S1). One group (MISE 210, MISE 211) formed a well-supported monophyly (NJ=81%, ML=92%, Bayes=0.95) within an *Epizoanthus* clade (NJ≤50%, ML=100%, Bayes=1.00), and the other group (MISE 225, MISE 228) formed a very highly supported monophyly (NJ=100%, ML=100%, Bayes=1.00) with *M. fossii*. These sequences were identical to previously obtained *M. fossii* COI sequences (311/311 bp). The *Mesozoanthus* clade was ambiguously within the Parazoanthidae phylogeny in all analyses with low support (<50%) (ESM Fig. S1).

mt 16S rDNA

Mitochondrial 16S ribosomal DNA (mt 16S rDNA) sequences obtained from specimens in this study are summarized in a maximum likelihood phylogenetic tree (Fig. 4). Sequences from British Columbia "*Mesozoanthus*" zoanthid specimens belonged to a clade that branched with a newly described species and genus from Chile, *M. fossii* (Sinniger and Häussermann 2009) with very high support (NJ=99%, ML=100%, Bayes=1.00). These sequences were almost identical to *M. fossi* (3-bp difference between the two groups), with similar unique mt 16S rDNA indels that are characteristic at the generic level as described in Sinniger and Häussermann (2009). The *Mesozoanthus* clade was ambiguously placed within Parazoanthidae, and was sister to *Corallizoanthus*, albeit with very weak support (NJ≤50%, ML=54%, Bayes≤0.7).

ITS rDNA

Obtained raw data (peaks) from the sequencing of ITS-rDNA showed in clear peaks with no "double peaks" or other unclear data associated with multiple ITS-rDNA alleles and intragenomic variation (discussed in Reimer et al. 2007b), and therefore all PCR products were sequenced directly.

The ITS-rDNA tree is shown in ESM Fig. S2. As ITS-rDNA has been shown to have a much faster rate of evolution than mitochondrial markers in zoanthids (Reimer et al.

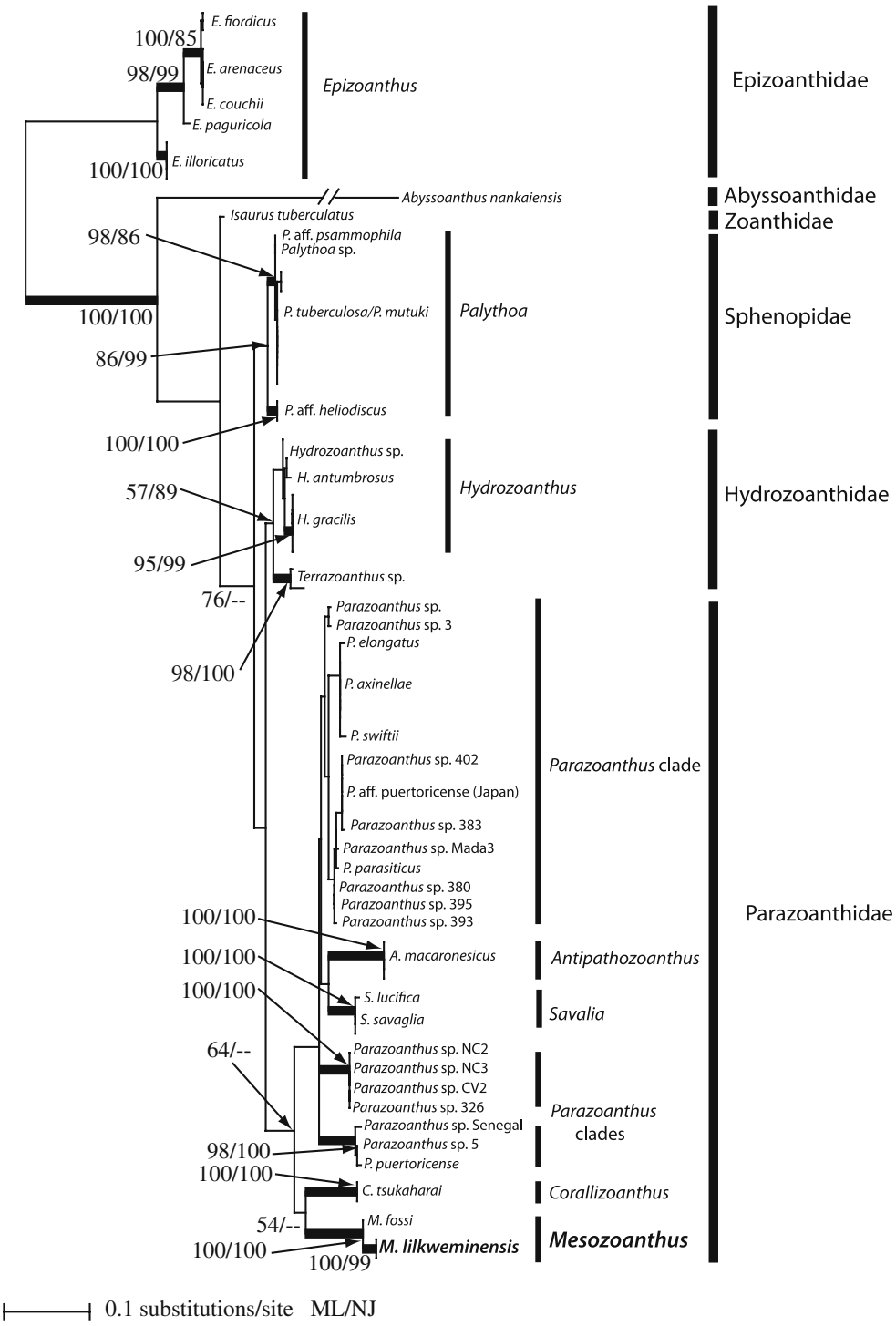

Fig. 4 Maximum likelihood tree of obtained and previous mitochondrial 16S ribosomal DNA (mt 16S rDNA) sequences for the order Zoantharia. Values at branches represent ML and neighbor joining (NJ) bootstrap probabilities (>50%). Bayesian posterior probabilities of >0.95 are represented by *thick branches*. Specimens from this study in *bold*. Note paraphyly of the genus *Parazoanthus*

2007b), acquired sequences were analyzed only with other Parazoanthidae and Hydrozoanthidae sequences (as in Reimer et al. 2008b). ITS-rDNA sequences from specimens 225, 226, and 228 in this study formed a distinct and very highly supported clade together with Chilean *Mesozoanthus fossii* sequences, (NJ=100%, ML=100%, Bayes=1.00). These "*Mesozoanthus* clade" ITS-rDNA sequences possessed distinct indels compared with other Parazoanthidae ITS-rDNA sequences (ESM Fig. S2, Fig. 5). Additionally, there were small indel differences between *M. fossii* sequences and sequences from Canadian "*Mesozoanthus*" specimens in this study (approximately 3%, 23/753 bp) (Fig. 5), and thus these sequences formed a monophyly (NJ=100%, ML=76%, Bayes=0.66) closely related but separate from *M. fossii* sequences. As well, some small variation (approx. 1.3%, 10/753 bp) was seen among the Canadian "*Mesozoanthus*"

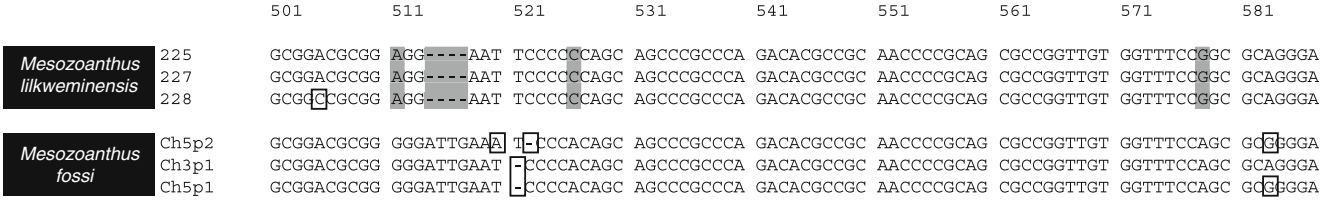

Fig. 5 Alignment of a portion of the internal transcribed spacer region of ribosomal DNA (ITS-rDNA) from ITS-2 showing sequences obtained from *Mesozoanthus* specimens. Areas in *shaded boxes* are indels unique to *Mesozoanthus lilkweminensis* n. sp., while areas in *open boxes* are areas of indels shared between only some specimens. Alignment position numbers are for an ITS-rDNA alignment of only *Mesozoanthus* species (available from corresponding author). *M. lilkweminensis* n. sp. specimen numbers are as in text. *Ch5p2* Chile specimen 5 paralogue 2, *Ch3p1* Chile specimen 3 paralogue 1, *Ch5p1* Chile specimen 5 paralogue 1 (all from Sinniger and Häussermann 2009)

sequences. The ITS-rDNA sequence from specimen 228 was slightly divergent from sequences from specimens 225 and 226, which formed a highly supported subclade (NJ=99%, ML=91%, Bayes=0.94). The *Mesozoanthus* clade was consistently sister to a *Corallizoanthus*/*Savalia* clade (NJ= 98%, ML=91%, Bayes=0.94) in the Parazoanthidae/Hydrozoanthidae phylogeny (ESM Fig. S2).

GenBank Sequence Accession Numbers: COI HM042382-HM042383, mt 16S rDNA HM042363-HM042366, ITS-rDNA HM042384-HM042386

Discussion

Zoanthid diversity on the Pacific coast of Canada

As demonstrated by both our examination of RBC zoanthid specimens and phylogenetic data from newly collected specimens, it is likely that there are several undescribed zoanthids in the waters of British Columbia, and the Pacific coast of North America. In this study we have described one new species, *Mesozoanthus lilkweminensis*, but our morphological examination of RBC samples points to the existence of at least two and perhaps four more undescribed species [one unknown Parazoanthidae (specimen 990-317-21-Table 1), potentially one to three unknown *Epizoanthus* species (991-343-10, 974-568-1, 978-234-3, 988-259-3)].

Examinations of zoanthids in other regions of the world have revealed similar previously "unknown" species diversity; this is particularly true in environments that have not yet been well explored, such as the deep sea (Reimer et al. 2007a) and insular regions (e.g., New Caledonia in Sinniger 2006; the Galapagos in Reimer et al. 2008b). Thus, there is high potential for the existence of new zoanthid species in British Columbia. Further investigations by both SCUBA and ROV would help confirm this hypothesis.

Ecology of *M. lilkweminensis*

While it is very likely that other populations of *M. lilkweminensis* exist along the Canadian Pacific coast, it is notable that no specimens from the Royal British Columbia Museum (RBC) matched morphologically with *M. lilkweminensis*, and no similar descriptions are found in the few published studies on East Pacific zoanthids. The only congener, *M. fossii*, has been shown to be relatively rare in Chile (found at only seven of 100 sites investigated—see Sinniger and Häussermann 2009), preferring steep walls in fjords and channels, and it may be that *M. lilkweminensis* is similarly rare or low in density, or perhaps found at depths below the usual range of SCUBA (>35 m). Additionally, other benthic cnidarians, such as the gorgonian *Paragorgia pacifica*, from the *M. lilkweminensis* type locality (Agamemnon Channel) have not been found elsewhere in British Columbia (T. Oyama, VA, personal communication) and it is possible that this ecosystem is very unique for this region. Thus, until further studies can be conducted, it is recommended that Agamemnon Channel be protected for containing possibly endemic and otherwise rare species

The four sampled colonies of *M. lilkweminensis* at this site were all found on rocky substrate, in contrast to Chilean *M. fossii* colonies that were often found on biogenic substrate. Further findings of *M. lilkweminensis* colonies may help expand the ecological and morphological description of this new zoanthid species in more detail.

Although *M. lilkweminensis* and *M. fossii* are very similar, both morphologically and genetically, they are readily distinguishable from each other, and are thus not "sibling species" (cf. Knowlton 1993), but instead appear to be closely related antipodal congeners. Despite their relatively close phylogenetic relatedness (with nearly identical mt 16S rDNA and identical COI sequences), their morphological differences are not surprising given previous research showing that closely related zoanthids can be very different morphologically (Reimer et al. 2006a).

Mesozoanthus lilkweminensis is found in cold temperate waters, making it a northern hemisphere congener of *M. fossii* described from Chile in similar environments. This antitropical distribution pattern has previously been seen in many marine invertebrate taxa in the eastern Pacific (reviewed in Lindberg 1991). Lindberg (1991) further suggests that temperate regions in the northern and southern

hemispheres in the eastern Pacific have been potentially temporally connected several times in relatively recent history (e.g., Pleistocene, Pliocene periods, <5.3 million years ago), allowing biotic interchange and resulting in closely related northern and southern hemisphere congeners in many taxa, similar to as seen here with *M. lilkweminensis* and *M. fossii*. Such biotic interchange would also explain the relatively close phylogenetic relationship observed between the two *Mesozoanthus* congeners. There may also be further undescribed *Mesozoanthus* species and populations in temperate waters worldwide (e.g., European waters, Atlantic Ocean), and clearly further investigations are necessary.

As many zoanthid species have wide distributions, it is possible that the two *Mesozoanthus* species are in fact one species, connected with a wide distribution along the Pacific coast of the Americas, but this possibility is unlikely for the following reasons: (1) no similar zoanthids have been discovered in regions in between Chile and British Columbia, despite the relatively detailed investigations in both California (Cutress and Pequegnat 1960; Philipp and Fautin 2009) and the Galapagos (Reimer et al. 2008a,b; Reimer and Todd 2009; Reimer and Fujii 2010), as well as unpublished data from Costa Rica (first author, data not shown); (2) the region between Chile and British Columbia does not have the type of environment (numerous fjords, cold temperate oceans) in which *Mesozoanthus* has been observed; (3) observed morphological (cnidome, tentacle numbers) and molecular (mt 16S rDNA differing by 3 bp; ITS-rDNA indels) data indicate a species-level difference between the two groups of *Mesozoanthus* specimens. In particular, mitochondrial DNA has been shown to evolve slowly in Anthozoa (Shearer et al. 2002; Huang et al. 2008), and thus even a difference of 1 bp in the mt 16S rDNA of zoanthids likely indicates a species-level difference (Reimer et al. 2006c). COI sequences longer than those acquired in this study, as utilized in Sinniger and Häussermann (2009), may also yet show such species-level differences.

Future recommendations

Any future investigation of zoanthid diversity would be best served by careful in situ data collection (location, depth, high-resolution images of zoanthids with polyps both open and closed), followed by preservation of samples in both 99.5% ethanol (for molecular examination) and 10% SW formalin (for future morphological studies). This and other studies (e.g., Reimer et al. 2004, 2006a) have demonstrated the problems of identifying zoanthids based solely on morphology, and other factors—including molecular phylogenetics and ecological data (substrate, etc.)—are also important in correct zoanthid identification. The necessity of this "combined" approach is well demonstrated by *M. lilkweminensis* as described within. Morphologically, this new species is very similar to species in another genus (*Parazoanthus*) within the family Parazoanthidae, but molecularly it is clearly divergent.

Conclusions

Implications for zoanthid phylogenetic molecular markers

It is recommended that future investigations into zoanthid diversity utilize not only mt DNA but also ITS-rDNA and other faster-evolving markers. While the two known species of *Mesozoanthus* are not sympatric, and easily distinguishable based on morphological characters, based solely on mt DNA these two species differed by only 3 bp. Thus, while "barcoding" (Sinniger et al. 2008) of these zoanthids utilizing mt DNA will result in a correct identification, in our study ITS-rDNA was able to more clearly phylogenetically distinguish between northern hemisphere *M. lilkweminensis* and southern hemisphere *M. fossii* based on unique indels. A re-examination of ITS-rDNA from many different zoanthids may help us understand "species-level" thresholds of divergence for this marker, and allow us to improve our understanding of this understudied group of cnidarians.

Marine invertebrate biodiversity in Canadian waters

Based on current understanding of marine biodiversity research (e.g., Mikkelsen and Cracraft 2001), it is very likely that many more undescribed species of not only zoanthids but also of many other marine taxa are present in Canadian waters, and that the threat of extinction may also be underestimated (Thorne-Miller et al. 1999). In order to obtain a complete and proper understanding of marine biodiversity, it is hoped more research similar to the present study is conducted on a wide variety of marine taxa in the near future.

Acknowledgements This research would have been impossible without the cooperation and advice of the following people: Tak Oyama and Danny Kent (VA) for gratefully supplying images, data and specimens of *M. lilkweminensis*, Dr. Phil Lambert (RBC) for kindly allowing access to RBC collections, Sundown Diving (Nanaimo, B.C.) for help with sample collection, Mark Kissenger (Conservation Officer, Duncan, B.C.) and Andy Lamb (Cedar Beach Ocean Lodge, Thetis Island) for additional information on zoanthids in Georgia Strait, Eric Harding, Don, Donna, Mika and Maika Reimer (Nanaimo), Denny, Masako and Kohei Probizanski (Vancouver) for logistical support, and Drs. Kiyotaka Takishita and Tadashi Maruyama (JAMSTEC) for advice on the manuscript. Sid Quinn and elders of the shíshálh nation kindly suggested the species name. This research was funded in part by a grant from the Fujiwara Natural History Foundation (Tokyo, Japan), the 21st Century Center of Excellence Program (COE) at the University of the Ryukyus, the Rising Star

Program at the University of the Ryukyus, and a grant from the Japan Society for the Promotion of Science (JSPS) ("Wakate B" no. 21770089) (all J.D.R.), as well as a post-doctoral fellowship from JSPS (no. P07812) to F.S. The constructive comments of Dr. Oscar Ocaña and three other, anonymous reviewers greatly improved this manuscript.

References

Burnett WJ, Benzie JAH, Beardmore JA, Ryland JS (1997) Zoanthids (Anthozoa, Hexacorallia) from the Great Barrier Reef and Torres Strait, Australia: systematics, evolution and a key to species. Coral Reefs 16:55–68

Cutress CE, Pequegnat WE (1960) Three new species of Zoantharia from California. Pacific Science 14:89–100

England KW (1991) Nematocysts of sea anemones (Actiniaria, Ceriantharia and Corallimorpharia: Cnidaria): nomenclature. Hydrobiologia 216(217):691–697

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

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. Proc R Soc Lond B 270:313–322

Hidaka M (1992) Use of nematocyst morphology for taxonomy of some related species of scleractinian corals. Galaxea 11:21–28

Hidaka M, Miyazaki I, Yamazato K (1987) Nematocysts characteristic of the sweeper tentacle of the coral *Galaxea fascicularis* (Linnaeus). Galaxea 6:195–207

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

Kim KC, Byrne LB (2006) Biodiversity loss and the taxonomic bottleneck: emerging biodiversity science. Ecol Res 21:784–810

Knowlton N (1993) Sibling species in the sea. Ann Rev Ecol System 24:189–216

Lamb A, Hanby BP (2005) Marine life of the Pacific Northwest. Harbour Publishing, Madeira Park

Lindberg DR (1991) Marine biotic interchange between the northern and southern hemispheres. Paleobiology 17:308–324

Mikkelsen PM, Cracraft J (2001) Marine biodiversity and the need for systematic inventories. Bull Mar Sci 69:525–534

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J (2000) Biodiversity hotspots for conservation priorities. Nature 403:853–858

Ono S, Reimer JD, Tsukahara J (2005) Reproduction of *Zoanthus sansibaricus* in the infra-littoral zone at Taisho Lava Field, Sakurajima, Kagoshima, Japan. Zool Sci 22:247–255

Philipp NA, Fautin DG (2009) Three new species of shallow water, yellow zoanthids (Hexacorallia: Zoanthidea: Epizoanthidae) from southern California, USA, and southern Australia. Zootaxa 2058:53–61

Reimer JD, Fujii T (2010) Four new species and one new genus of zoanthids (Cnidaria: Hexacorallia) from the Galápagos. ZooKeys 42:1–36

Reimer JD, Todd PA (2009) Preliminary molecular examination of zooxanthellate zoanthid (Hexacorallia, Zoantharia) and associated zooxanthellae (*Symbiodinium* spp.) diversity in Singapore. Raffl Bull Zool Suppl 22:103–120

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, Hirano S, Fujiwara Y, Sinniger F, Maruyama T (2007a) Morphological and molecular characterization of *Abyssoanthus nankaiensis*, a new family, new genus and new species of deep-sea zoanthid (Anthozoa: Hexacorallia: Zoantharia) from a northwest Pacific methane cold seep. Invert Systemat 21:255–262

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, Nonaka M, Sinniger F, Iwase F (2008a) Morphological and molecular characterization of a new genus and new species of parazoanthid (Anthozoa: Hexacorallia: Zoantharia) associated with Japanese red coral (*Paracorallium japonicum*) in southern Japan. Coral Reefs 27:935–949

Reimer JD, Sinniger F, Hickman CP Jr (2008b) Zoanthid diversity (Anthozoa: Hexacorallia) in the Galapagos Islands: a molecular examination. Coral Reefs 27:641–654

Rodriguez F, Oliver JL, Marin A, Medina JR (1990) The general stochastic model of nucleotide substitution. J Theor Biol 142:485–501

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

Ryland JS, Lancaster JE (2004) A review of zoanthid nematocyst types and their population structure. Hydrobiologia 530 (531):179–187

Schmidt H (1974) On evolution in the Anthozoa. Proc 2nd Int Coral Reef Symp 1:533–560

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 (2006) Zoanthids of New Caledonia. In: Richier de Forges B (ed) Compendium of marine species from New Caledonia. IRD Editions, Nouméa, pp 127–128

Sinniger F, Häussermann V (2009) Zoanthids (Cnidaria: Hexacorallia: Zoantharia) from shallow water of the southern Chilean fjord region, with descriptions of a new genus and two new species. Org Divers Evol 9:23–36

Sinniger F, Montoya-Burgos JI, Chevaldonné 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

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Thorne-Miller B et al (1999) The living ocean: understanding and protecting marine biodiversity, 2nd edn. Island Press, Washington DC

Wood RL (1958) Identification and microanatomical study of a new species of *Epizoanthus* (Zoanthidea). Dissertation, University of Washington, Seattle