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RESEARCH ARTICLE

Molecular characterization of the zoanthid genus *Isaurus* (Anthozoa: Hexacorallia) and associated zooxanthellae (*Symbiodinium* spp.) from Japan

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Abstract The zoanthid genus *Isaurus* (Anthozoa: Hexacorallia) is known from both the Indo-Pacific and Atlantic Oceans, but phylogenetic studies examining *Isaurus* using molecular markers have not yet been conducted. Here, two genes of markers [mitochondrial cytochrome oxidase subunit I (COI) and mitochondrial 16S ribosomal DNA (mt 16S rDNA)] from *Isaurus* specimens collected from southern Japan (n = 19) and western Australia (n = 3) were sequenced in order to investigate the molecular phylogenetic position of *Isaurus* within the order Zoantharia and the family Zoanthidae. Additionally, obtained sequences and morphological data (polyp size, mesentery numbers, mesogleal thickness) were utilized to examine *Isaurus* species diversity and morphological variation. By comparing our obtained sequences with the few previously acquired sequences of genera *Isaurus* as well as with *Zoanthus*, *Acrozoanthus* (both family Zoanthidae), and *Palythoa* spp. (family Spenophidae) sequences, the phylogenetic position of *Isaurus* as sister to *Zoanthus* within the Family Zoanthidae was suggested. Based on genetic data, *Isaurus* is most closely related to the genus *Zoanthus*. Despite considerable morphological variation (in particular, polyp length, mesentery numbers, external coloration) between collected *Isaurus* specimens, all specimens examined are apparently conspecific or very closely related based on molecular data and observed morphological variation within colonies. Additionally, obtained internal transcribed spacer of ribosomal DNA (ITS-rDNA) sequences from symbiotic zooxanthellae (*Symbiodinium* spp.) from all *Isaurus* specimens were shown to be subclade C1-related *Symbiodinium*.

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Introduction

The order Zoantharia (Anthozoa: Hexacorallia) currently consists of five recognized families, including two mainly zooxanthellate families (possessing symbiotic zooxanthellae of the dinoflagellate genus *Symbiodinium*); the sand-encrusted Sphenopidae, and the non-encrusted Zoanthidae. Species from both families are often seen in sub-tropical and tropical shallow coral reef habitats worldwide.

Zoanthidae is currently organized into three genera: *Zoanthus*, *Acrozoanthus*, and *Isaurus*.

Isaurus spp. are known from both the Atlantic and Indo-Pacific, and are generally found in intertidal or shallow subtropical and tropical waters. Currently, three species are recognized; *I. tuberculatus* Gray from Hawaii, Fiji, Australia, East Africa, and the Caribbean (pan-tropical distribution); *I. maculatus* Muirhead and Ryland described from Fiji; and *I. cliftoni* Gray from western Australia (see Muirhead and Ryland 1985), although 22 species have been historically listed in the literature (Fautin 2006). In Japan, *Isaurus* spp. are believed to be very rare, and have been reported from only a handful of locations (Uchida 2001; Uchida and Iwase personal communication; see also Fig. 1). The observed rarity of *Isaurus* may be partially due to its cryptic appearance (see Fig. 2) and/or its preference for habitats on rocky shores facing the open ocean that are often difficult to access.

The status of *Isaurus* as separate from *Zoanthus* is somewhat confused when examining past literature. Many researchers placed nominal *Isaurus* spp. samples in *Zoanthus* (discussed in Muirhead and Ryland 1985), and even in more recent literature the extreme similarity in external morphology between *Zoanthus praelongus* and *I. maculatus* has been noted (Muirhead and Ryland 1985). *Isaurus* has been defined to be different from *Zoanthus* by having recumbent, non-erect polyps (although *Z. praelongus* shares this characteristic with *Isaurus*) and the presence of tubercules on the polyps (although *I. cliftoni* does not have tubercules), but otherwise shares many morphological characters [e.g. not sand-encrusted, zooxanthellate, colonial, generally "liberae" polyps (see Pax 1910)] with *Zoanthus*, making phylogenetic placement of this genus as separate to *Zoanthus* open to speculation.

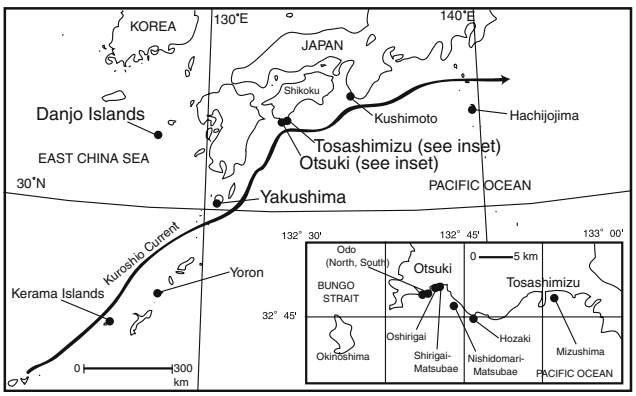

Fig. 1 Map showing sampling locations of *Isaurus* specimens from Japan examined in this study, with *inset* showing locations in southern Shikoku. Locations in large font from this study, locations in small font previous show previously reported locations of *Isaurus* (main map only)

Recent genetic studies investigating the genera *Zoanthus* (Reimer et al. 2006a) and *Palythoa* (Reimer et al. 2006b, 2007b) have demonstrated that relatedness in many zoanthids is difficult to judge based solely on morphology. This is largely due to zoanthids often being very morphologically plastic with regards to their external morphology (polyp and colony shape, etc.) (Karlson 1982; Larson and Larson 1982). DNA sequencing and analyses have often

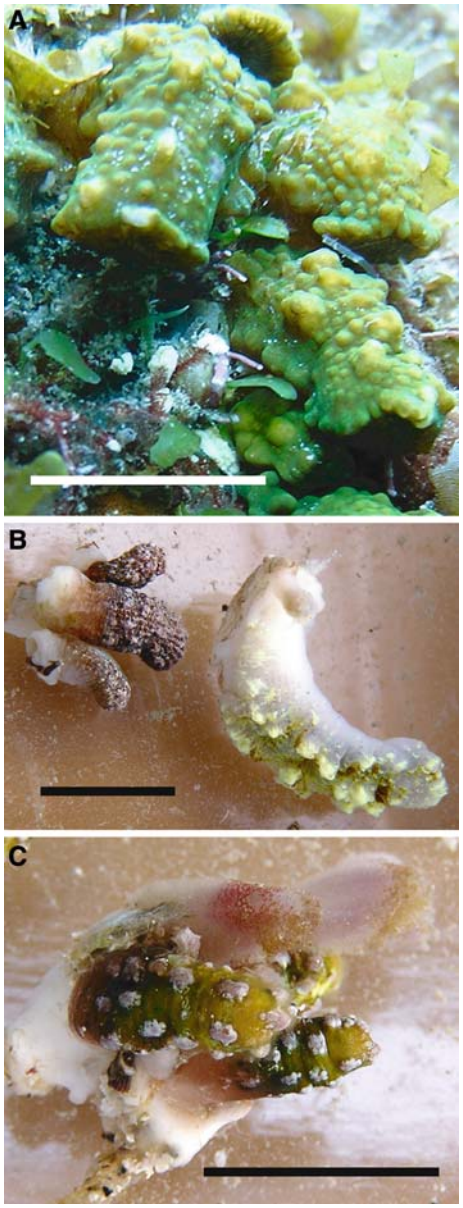

Fig. 2 Various *Isaurus* morphotypes nominally identified as *Isaurus tuberculatus* **a** specimen IOtsH6 in situ at Hozaki site (depth = 4.0 m), **b** two polyps from different colonies at Oshirigai-Matsubai (IOtsOM5 *left* and IOtsOM6 *right*) showing polyp size and color variation in a tank at BIK. **c** Colony with *Isaurus* sp. B morphology from Oshirigai-Matsubai (IOtsOM4) in a tank at BIK. All scale bars = 1 cm. All images originally from Reimer (2007). Note in in situ images the recumbent, non-erect polyps characteristic of *Isaurus*

resulted in taxonomic revision of species (Reimer et al. 2006b) and genera (Reimer et al. 2006c).

However, until now only three single genetic sequences from *Isaurus* spp. have been deposited in GenBank; one mitochondrial (mt) 16S ribosomal DNA (mt 16S rDNA) sequence from *I. tuberculatus* (AF398919—Burnett unpublished), and two sequences [mt 16S rDNA (AY995945) and 12S ribosomal DNA (AY995922)] from Sinniger et al. (2005), who examined a single unidentified *Isaurus* sp. sample of origin unknown acquired from the aquarium trade. These sequences suggest the specimens examined are closely related to the genus *Zoanthus*. However, due to the limited number of *Isaurus*, *Zoanthus*, and *Palythoa* sequences examined in Sinniger et al. (2005), as well the overall lack of research conducted on *Isaurus*, questions remain about the phylogenetic position of *Isaurus* within Zoanthidea.

Another method that may aid in characterizing zooxanthellate zoanthid species is by identifying their symbiotic dinoflagellates from the genus *Symbiodinium* (order Suessiales). This is primarily achieved through sequencing of the internal transcribed spacer of ribosomal DNA (ITS-rDNA) of *Symbiodinium.* While some zooxanthellate zoanthid species are known to host more than one type of *Symbiodinium* within individuals (LaJeunesse 2002), the majority of zooxanthellate zoanthids examined thus far host one type/subclade of *Symbiodinium* per colony/individual (see Reimer et al. 2006d, e). Additionally, particular zoanthid species often specifically associate with certain *Symbiodinium* types (Reimer et al. 2006d, 2007a). Thus, identification of *Symbiodinium* may often help in facilitating host zoanthid species identification, although symbiont data alone should not be used for any identification. However, no data on *Symbiodinium* distribution patterns in *Isaurus* spp. have been reported as of date.

In this study, two molecular markers [mitochondrial cytochrome oxidase subunit I (COI) and mt 16S rDNA] previously shown to be able to discern between zoanthid genera and species (see Reimer et al. 2004, 2006b, c; Sinniger et al. 2005) were utilized to confirm the position of the genus *Isaurus* within the family Zoanthidae, and to investigate potential species diversity within *Isaurus* specimens in Japan. In addition, identification of *Symbiodinium* spp. from all collected samples was performed using ITS-rDNA to aid in potential *Isaurus* species identification.

Materials and methods

Sampling

Samples of *Isaurus* spp. (n = 19) representing a variety of morphotypes were collected from several sites in Kochi and Kagoshima, Japan (Fig. 1) between August 2004 and October 2006 (Table 1), and stored in 100% ethanol at –20°C at the Biological Institute on Kuroshio (BIK) until further utilization. Despite large variation in polyps (external coloration, tubercule shape, polyp dimensions), most samples were nominally classified as *Isaurus tuberculatus* sensu Muirhead and Ryland (1985) (Fig. 2a, b). Uchida (2001) lists specimens from Tatsukushi, Japan as *I. assymmetricus*, but *I. assymmetricus* was included in *I. tuberculatus* (along with three other putative species) by Muirhead and Ryland (1985). Photographs of *I. assymmetricus* from Tatsukushi are morphogically similar to *I. tuberculatus*, and thus we have nominally classified samples from Japan collected here as *I. tuberculatus* following Muirhead and Ryland (1985). Two colonies (IOtsOM4, IOtsNM1) were observed to be morphologically different [having smaller polyps, different polyp coloration (see Fig. 2), smaller tubercules, larger (thousands of polyps) colony sizes] from the majority of samples, and were nominally classified as *Isaurus* sp. B (Fig. 2c). Additionally, a sample preserved in formalin (thus no genetic examinations were possible) received from Dr. H. Uchida from the Danjo Islands (sample IND1; see Table 1) (collected 26 October 1983 by F. Iwase) also matched the external morphology of *Isaurus* sp. B. As samples were collected in situ photographs were taken to assist in identification and for collection of morphological data (oral disk/polyp diameter, color, polyp form). Unlike *Zoanthus* and *Palythoa* spp., during collection *Isaurus* polyps in situ were observed to always be closed, and no tentacle count data were obtained, although it is believed to be close to the mesentery number (data shown in Results). Five western Australian zoanthid samples with external morphology consistent with either *Isaurus* (n = 3) or *Zoanthus praelongus* (n = 2) [see Muirhead and Ryland (1985) for a discussion on identification problems between these two groups] loaned from the Western Australian Museum (WAM) were also included in subsequent DNA analyses (Tables 1, 2). Sample nomenclature is explained in Tables 1 and 2.

Morphological analyses

Digital in situ photographs of all collected *Isaurus* specimens were examined, and the following morphological data were collected: polyp and coenenchyme form, polyp external dimensions and color. Internal morphological examinations followed Ono et al. (2005), with specimens first fixed in formalin and then fixed with Bouin's fluid and embedded in paraffin. Samples were cross-sectioned into 8-µm thick sections, stained with Azan, and observed under the microscope. Data were collected on polyp diameter

Table 1 *Isaurus* specimens and sequences utilized in this study

Morphological identification	Sample name[a]	Location[b]	Depth (m)	Year and month sampled	Collected by	Algal polyp coloring	Polyp length (cm)	Colony size[c]	mt 16S rDNA Accession number	COI Accession number	*Symbiodinium* ITS-rDNA Accession number	*Symbiodinum* type
Isaurus tuberculatus	IYS1	Sangohama, Yakushima	+0.5	2005.12	JDR	Red and white	1.0–3.0	Small	EF452239	EF452258	EF452277	C1/C3
Isaurus sp. B	IND1	Danjo Islands, Nagasaki	5.0	1983.10	FI	Gray-green	0.5–1.5	Medium	NA	NA	NA	NA
Isaurus tuberculatus	IOtsOs1	Oshirigai, Otsuki	1.5	2006.9	JDR	Purple and white	0.7–2.0	Small	EF452240	EF452259	NA	C1/C3
Isaurus tuberculatus	IOtsOM1	Shirigai-Matsubae, Otsuki	2.0	2006.9	JDR	Green-brown with fl. green tubercules	1.0–3.5	Small	EF452241	EF452260	EF452278	C1/C3
Isaurus tuberculatus	IOtsOM2	Shirigae-Matsubae, Otsuki	2.0	2006.9	JDR	Purple and white	0.7–2.0	Small	EF452242	NA	EF452279	C1/C3
Isaurus tuberculatus	IOtsOM3	Shirigai-Matsubae, Otsuki	2.5	2006.9	JDR	Mottled white	1.0–2.5	Small	EF452243	EF452261	EF452280	C1/C3
Isaurus sp. B	IOtsOM4	Shirigai-Matsubae, Otsuki	1.5	2006.9	JDR	Dark green with purple tubercules	1.0	Large	EF452244	EF452262	EF452281	C1/C3
Isaurus tuberculatus	IOtsOM6	Shirigai-Matsubae, Otsuki	2.0	2006.9	JDR	White with fl. green tubercules	4.0	Small	EF452245	EF452263	EF452282	C1/C3
Isaurus tuberculatus	IOtsB1	Bentenjima, Otsuki	3.0	2006.10	JDR	Purple and white	1.0–2.5	Small	EF452246	EF452264	EF452283	C1/C3
Isaurus sp. B[f]	IOtsNM1	Nishidomari-Matsubai, Otsuki	0.5	2004.10	FI	light green	1.5	Large	EF452247	AB247631[f]	EF452284	C1/C3
Isaurus tuberculatus	IOtsO1	Odo South, Otsuki	0.5	2006.9	JDR	White, green	1.5–2.5	Medium	NA	NA	NA	C1/C3
Isaurus tuberculatus	IOtsO2	Odo North, Otsuki	0.5	2006.9	JDR	White, green	1.0–2.0	Medium	NA	NA	NA	C1/C3
Isaurus tuberculatus	IOtsH1	Hozaki, Otsuki	0.5	2006.9	JDR	Brown	1.5	Small	EF452248	EF452265	EF452285	C1/C3
Isaurus tuberculatus	IOtsH2	Hozaki, Otsuki	0.5	2006.9	JDR	Brown	1.5	Small	EF452249	EF452266	EF452286	C1/C3
Isaurus tuberculatus	IOtsH3	Hozaki, Otsuki	1.0	2006.9	JDR	Brown	1.5	Small	EF452250	EF452267	EF452287	C1/C3
Isaurus tuberculatus	IOtsH4	Hozaki, Otsuki	1.0	2006.9	JDR	Brown	2.0	Small	EF452251	EF452268	EF452288	C1/C3
Isaurus tuberculatus	IOtsH5	Hozaki, Otsuki	0.5	2006.9	JDR	Brown	2.0	Small	EF452252	EF452269	EF452289	C1/C3
Isaurus tuberculatus	IOtsH6	Hozaki, Otsuki	2.5	2006.9	JDR and KT	Light green	2.0–3.5	Small	NA	EF452270	EF452290	C1/C3
Isaurus tuberculatus	IToM1	Mizushima, Tosashimizu	0.5	2006.9	JDR	White	2.0–3.0	Medium	EF452253	EF452271	EF452291	C1/C3
Isaurus tuberculatus[d]	NA	Unknown	NA	NA	NA	NA	NA	NA	AF398919[d]	NA	NA	NA
Isaurus sp.[e]	FS-2005	aquarium trade (presumably Indonesia)	NA	2003.9	NA	Red-brown	6.5	NA	AY995945[e]	NA	NA	NA

Table 1 continued

Morphological identification	Sample name[a]	Location[b]	Depth (m)	Year and month sampled	Collected by	Algal polyp coloring	Polyp length (cm)	Colony size[c]	mt 16S rDNA Accession number	COI Accession number	*Symbiodinium* ITS-rDNA Accession number	*Symbiodinium* type
Isaurus sp.	WAMZ 40074	North Essex, Jurien Bay, W. Australia	3.0–4.5	2005.4	GC, RB, AS	NA	NA	NA	NA	EF452272	NA	NA
Isaurus sp.	WAMZ 40075	North Essex, Jurien Bay, W. Australia	3.0–4.5	2005.4	GC, RB, AS	NA	NA	NA	EF452254	EF452273	EF452292	C1/C3
Isaurus sp.	WAMZ 40081	Booker Rocks, Jurien Bay, W. Australia	4.8	2005.4	GC, RB, AS	NA	NA	NA	NA	EF452274	NA	NA

JDR J. Reimer, *FI* F. Iwase, *KT* K. Tanaka, *GC* G. Clapin, *RB* R. Babcock, *AS* A. Sampey

* NA = data not acquired or not available

[a] New specimens collected in this study were assigned names based on genus and sampling site. Thus, IOtsH1 is *Isaurus* sp. from Otsuki, Hozaki, specimen number 1 at this site. Specimens collected in previous studies or by other institutions retain sample names assigned by the original collector/institution

[b] All locations in Japan unless otherwise noted

[c] Colony sizes: small = <100 polyps, medium = 100–1,000 polyps, large = >1,000 polyps

[d] From Burnett (unpublished)

[e] From Sinniger et al. (2005)

[f] From Reimer et al. (unpublished)

(maximum and minimum), mesogleal thickness (maximum and minimum), and number of mesenteries, as well as maximum tubercule dimensions (when available).

DNA extraction, PCR amplification, and sequencing

The DNA was extracted from samples weighing 5–20 mg using a spin-column Dneasy Animal Extraction protocol (Qiagen, Santa Clarita, CA, USA). PCR amplification using the genomic DNA as a template was performed using HotStarTaq DNA polymerase (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions. Mitochondrial (mt) 16S rDNA was amplified following procedures outlined in Sinniger et al. (2005). COI was amplified following procedures outlined in Reimer et al. (2004). The ITS-rDNA region of *Symbiodinium* was amplified following procedures outlined in Reimer et al. (2006e). The amplified products were visualized by 1.5% agarose gel electrophoresis.

Phylogenetic analyses

New sequences obtained in the present study were deposited in GenBank (accession numbers EF452239-EF452292) (Table 1). By using CLUSTAL X version 1.8 (Thompson et al. 1997), the nucleotide sequences of mt 16S rDNA from samples were aligned with previously published mt 16S rDNA sequences from *Isaurus* (AF398919—Burnett unpublished; AY995945—Sinniger et al. 2005), as well as *Palythoa* (AB 219218, AB219223, AB219225; Reimer et al. 2006c), *Zoanthus* (AB219187, AB219191, AB219192—Reimer et al. 2006b; AB235407—Reimer et al. 2006a) and *Acrozoanthus* (AY995946, AY995947—Sinniger et al. 2005) sequences, with a newly obtained sequence from *Parazoanthus gracilis* sensu Uchida 2001 as the outgroup (Table 2). Obtained COI sequences were aligned with previously published *Palythoa* (AB219199, AB219214, AB219217; Reimer et al. 2006c) and *Zoanthus* (AB214166, AB214175, AB214177; Reimer et al. 2004 supplemental), with a previously obtained sequence [AB214178 (Reimer et al. 2004 supplemental)] from *Parazoanthus gracilis* sensu Uchida 2001 as the outgroup (Table 2). Obtained *Symbiodinium* ITS-rDNA sequences (Table 1) were aligned with previously obtained clade C1/C3 and C1/C3-related *Symbiodinium* sequences [AY186567 (Rodriguez-Lanetty; Hoegh-Guldberg 2003); AF195144 (Baillie et al. 2000); AY237296, DQ072720, DQ068036 (Bui et al. unpublished); DQ335255, DQ335271, DQ335284, DQ335319, DQ335325, DQ335366, DQ335367. DQ335376, DQ335396 (Reimer et al. 2007a)], with *Symbiodinium* subclade C15/C91 and related sequences

Table 2 Zoanthid specimens and sequences used in this study (excluding *Isaurus* spp.)

Species	Sample name[a]	Sampling location[b]	Depth (m)	Date collected	Collected by [c]	mt 16S rDNA Accession No.	COI Accession no.
Zoanthus praelongus	WAMZ 40080	Favourite I., Jurien Bay, W. Australia	4.0–7.3	2005.4	GC, RB, AS	EF452255	EF452275
Zoanthus praelongus	WAMZ 40082	Escape I., Jurien Bay, W. Australia	6.9	2005.5	GC, RB, AS	EF452256	EF452276
Zoanthus sansibaricus	ZSH23[e]	Hakamagoshi, Sakurajima	9.0	2004.7	JDR	AB219187[e]	AB214166[f]
Zoanthus kuroshio	ZkYS1[e]	Sangohama, Yakushima	1.5	2004.7	JDR	AB219191[e]	AB214175[f]
Zoanthus gigantus	ZgYS1[e]	Sangohama, Yakushima	1.5	2004.7	JDR	AB219192[e]	AB214177[f]
Zoanthus vietnamensis	ZvSH2[h]	Hakamagoshi, Sakurajima	3.0	2005.7	JDR	AB235407[h]	NA
Acrozoanthus sp.	"Sulawesi" [g]	N. Sulawesi, Indonesia	9.0	2003.9	MB	AY995947[g]	NA
Acrozoanthus sp.	"shop" [g]	aquarium trade (presumably Indonesia)	NA	2003.11	NA	AY995946[g]	NA
Palythoa mutuki	PmMiI1[d]	Izushita, Miyakejima	0.0	2005.5	JDR	AB219225[d]	AB219217[d]
Palythoa tuberculosa	PtMiI1[d]	Izushita, Miyakejima	2.0	2005.5	JDR	AB219218[d]	AB219199[d]
Palythoa heliodiscus	PhSaiLL1[d]	Lau Lau, Saipan	3.0	2004.12	JDR	AB219223[d]	AB219214[d]
Parazoanthus gracilis sensu Uchida (2001)	PgChK1	Kamogawa, Chiba	15.0	2006.11	JDR and FI	EF452257	NA
Parazoanthus gracilis sensu Uchida (2001)	PgIJ1[f]	Jogasaki, Izu	17.0	2004.11	JDR	NA	AB214178[f]

* NA = Data not acquired, not available, or not used in this study

[a] Specimens collected in by JDR were assigned names based on genus and sampling site. Thus, ZSH23 is *Zoanthus* sp. from Sakurajima-Hakamagoshi, specimen number 23 at this site. Samples collected in previous studies retain sample names assigned by the original collector/institution

[b] All locations in Japan unless otherwise noted

[c] *JDR* J. Reimer, *FI* F. Iwase, *MB* Marcel Boyer, *GC* G. Clapin, RB = R. Babcock, AS = A Sampey

[d] From Reimer et al. (2006c)

[e] From Reimer et al. (2006b)

[f] From Reimer et al. (2004, and supplemental sequences)

[g] From Sinniger et al. (2005)

[h] From Reimer et al. (2006a)

[AF195157 (Baillie et al. 2000); AJ291514, AJ291519 (Pawlowski et al. 2001); AJ311944 (Pochon et al. 2001), AB190278, AB190279, AB190284 (Reimer et al. 2006e)] as the outgroup (Table 2). The alignments were inspected by eye and manually edited. All ambiguous sites of the alignments were removed from the dataset for phylogenetic analyses. Consequently, three alignment datasets were generated: (1) 790 sites of 24 sequences (*Isaurus* mt 16S rDNA); (2) 302 sites of 27 sequences (*Isaurus* COI); and (3) 422 sites of 31 sequences (*Symbiodinium* ITS-rDNA). The alignment data are available on request from the corresponding author.

The alignments of mt 16S rDNA, COI and ITS-rDNA were tested for optimal fit of various nucleotide substitution models using the MODELTEST version 3.06 (Posada and Crandall 1998). The base frequencies, proportion of invariable sites and a gamma distribution were estimated from the datasets. For the mt 16S rDNA and ITS-rDNA datasets, the TN model (Tamura and Nei 1993) incorporating variable sites (TN + I) was selected by MODELTEST. For the COI dataset, the Hasegawa, Kishino and Yano (HKY) model (Hasegawa et al. 1985) incorporating a discrete gamma distribution (four categories) (HKY + Γ) was selected by MODELTEST. The maximum likelihood (ML) analyses with PhyML (Guindon and Gascuel 2003) of these datasets were independently performed using an input tree generated by BIONJ (Gascuel 1997) with the models selected by MODELTEST. PhyML bootstrap trees (500 replicates) were constructed using the same parameters as the individual ML trees.

ML distances of the three datasets were calculated under the optimal models described above with PAUP* Version 4.0 (Swofford 1998). Distance trees were constructed using the neighbour-joining (NJ) method (Saitou and Nei 1987). The ML distance bootstrap analyses with 1,000 replicates were also performed.

Bayesian trees were reconstructed by using the program MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) under the general time reversible (GTR) model (Rodriguez et al. 1990) of nucleotide substitution for the mt DNA 16S rDNA dataset, and under HKY for the COI and ITS-rDNA datasets [all models selected by MrModeltest (Nylander 2004. MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University)]. One cold and three heated Markov chain Monte Carlo (MCMC) chains with default-chain temperatures 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 40,000, 30,000 and 30,000 generations, respectively. Thus, the remaining 960,000, 970,000 and 970,000 trees of mt 16S rDNA, COI and ITS-rDNA were used to obtain posterior probabilities and branch-length estimates, respectively.

Results

Morphological analyses

Morphological data acquired from cross-sections (shown in Fig. S1) are shown in Table 3. Collected *Isaurus* specimens were shown to have large amounts of variation not only in external coloration and polyp length (0.7–4.0 cm) (see Table 1), but also in number of mesenteries (36–42), mesogleal thickness (130–920 μm), and polyp diameter (3,200–4,400 μm). Putative *Isaurus* sp. B specimens had morphological dimensions and counts both greater and lesser than putative *Isaurus tuberculatus* specimens (Table 3), excepting polyp length.

Genetic analyses

mt 16S rDNA

The mt 16S rDNA tree (Fig. 3) showed all obtained *Isaurus* sequences from Japan forming a highly supported [ML

Table 3 Morphological data from examined *Isaurus* spp. specimens

Sample (putative species)	Approx. average polyp length[a] (cm)	Polyp diameter[b] (μm)	Mesogleal thickness[b] (μm)	Mesentery count[b]	Tubercule size[b] (μm)
IND1 (*Isaurus* sp.B)	1.0	4,100–4,400	470–920	42	560
IOtsNM1 (*Isaurus* sp.B)	1.5	4,000–4,300	500–700	40	675
IOtsOM4 (*Isaurus* sp.B)	1.0	3,200–3,500	130–420	36	NA
IOtsOM6 (*Isaurus tuberculatus*)	4.0	3,600–4,400	340–710	42	NA

[a] For entire colony in situ

[b] For examined polyps [minimum–maximum, except for tubercule size (maximum only)]

NA Data not available

bootstrap support = 99%, NJ bootstrap support = 97%, posterior probability of Bayesian inference (PP) = 0.89] monophyly together with AY995945 and WAMZ40075. In fact, these sequences were identical over the 790 bp length of the alignment analyzed. Additionally, truncated mt 16S rDNA sequences from other *Isaurus* samples not included in the alignment (IOtsOS1, IOtsOM2, IOtsH2) also were identical over their entire length to the sequences in the monophyletic group of *Isaurus*. The mt 16S rDNA sequence from an *Isaurus tuberculatus* specimen (Burnett unpublished) from a location unspecified (AF398919) differed from the other *Isaurus* samples by 1 bp over its 464 bp length, but was not included in the alignment as the sequence was substantially shorter than our alignment.

The *Isaurus* monophyly was sister to a moderately supported (ML = 76%, NJ = 77%, PP = 0.73) *Zoanthus* clade. The two *Acrozoanthus* sequences radiated within the *Zoanthus* clade, sister to the *Zoanthus vietnamensis*/*Z. kuroshio* clade. Samples WAMZ40080 and WAMZ40082 were also within the *Zoanthus* radiation, basal to both the *Acrozoanthus* and *Zoanthus vietnamensis*/*Z. kuroshio* clades and to *Z. sansibaricus*. *Palythoa* sequences formed a well-supported clade (ML = 91%, NJ = 87%, PP = 0.73) separate from the *Zoanthus* and *Isaurus* clades. The *Isaurus* and *Zoanthus*/*Acrozoanthus* clades formed one large clade (=Family Zoanthidae) with high support (ML = 99%, NJ = 100%, PP = 1.00).

COI

The COI tree (Fig. 4) showed all collected *Isaurus* specimens from Japan and western Australia forming a highly supported (ML = 99%, NJ = 96%, PP = 1.00) monophyly together with AB247631, separate from the *Zoanthus* sequences. The *Isaurus* monophyly was derived from *Zoanthus* sequences, which did not form a monophyly. However, together the *Zoanthus* and *Isaurus* sequences (=Family Zoanthidae) formed a highly supported monophyly (ML = 99%, NJ = 99%, PP = 1.00). *Palythoa* sequences formed a poorly supported clade (ML = <50%, NJ = <50%, PP = <0.50) separate from Zoanthidae.

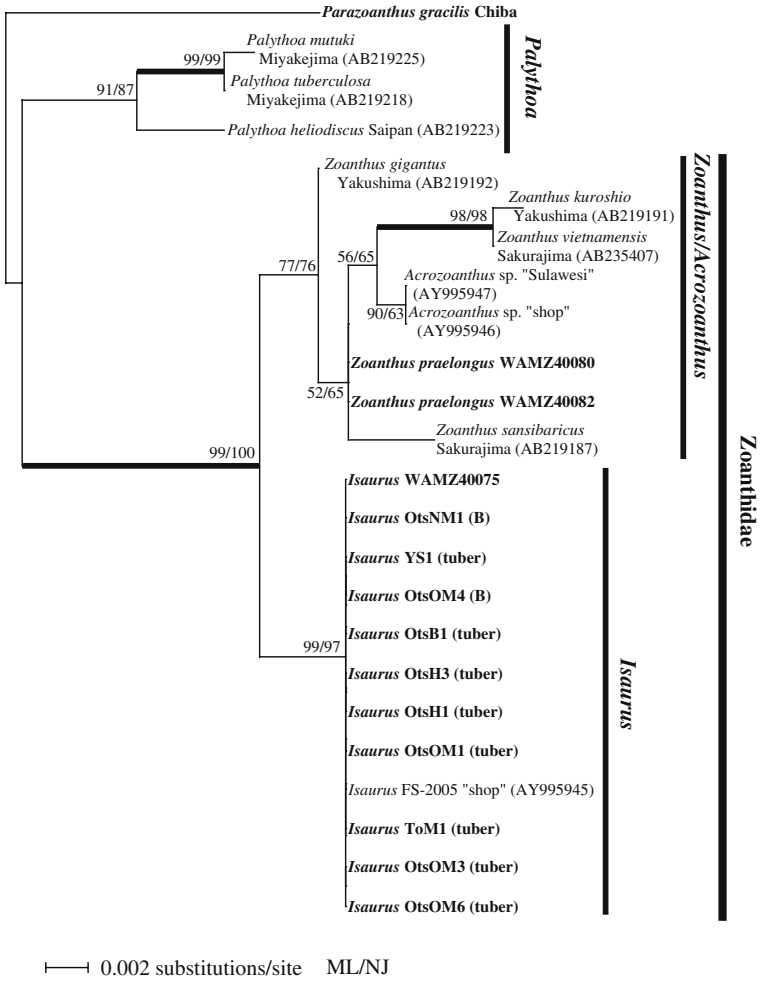

Fig. 3 Maximum likelihood tree of mitochondrial 16S ribosomal DNA (mt 16S rDNA) sequences. Values at branches represent ML and NJ bootstrap probabilities, respectively (>50%). Bayesian posterior probabilities of >95% are represented by *thick branches*. For sample name abbreviations see Tables 1 and 2. Sample names with Accession Numbers are from previous studies (see Table 2). "tuber" (=*Isaurus tuberculatus*) and "B" (=*Isaurus* sp. B) in *parentheses* after sample names show the morphological identity/type of each specimen

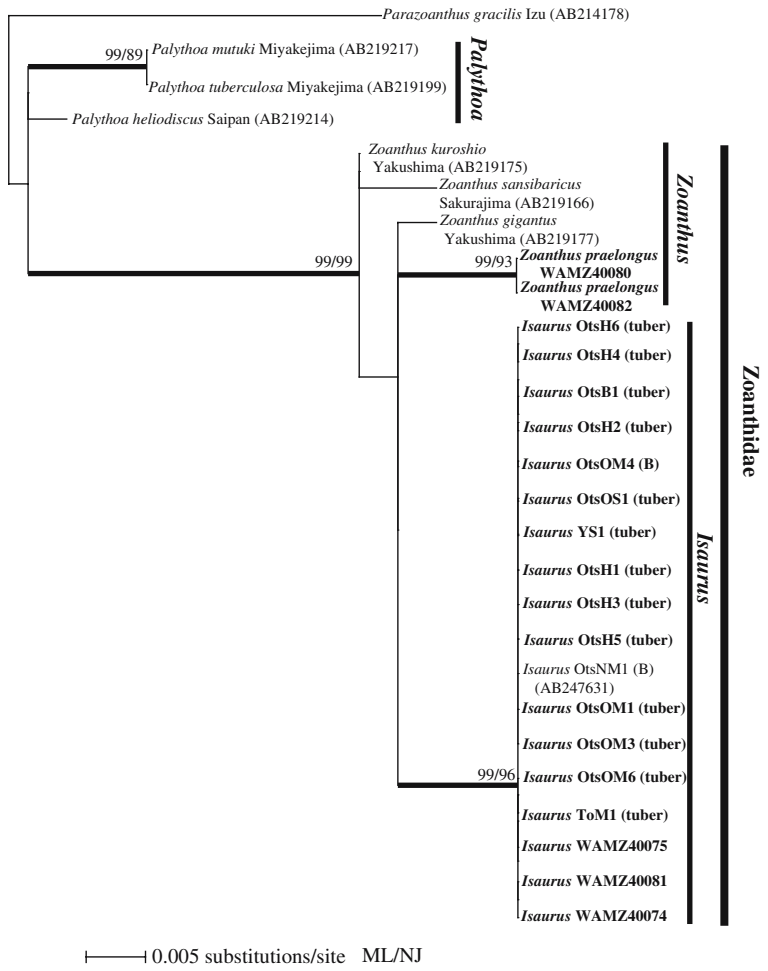

Fig. 4 Maximum likelihood tree of mitochondrial cytochrome oxidase subunit I (COI) sequences. Values at branches represent ML and NJ bootstrap probabilities, respectively (>50%). Bayesian posterior probabilities of >95% are represented by *thick branches*. For sample name abbreviations see Tables 1 and 2. Sample names with Accession Numbers are from previous studies (see Table 2). "tuber" (=*Isaurus tuberculatus*) and "B" (=*Isaurus* sp. B) in *parentheses* after sample names show the morphological identity/type of each specimen

Symbiodinium *ITS-rDNA*

Based on phylogenetic analyses, no ITS-rDNA sequences from *Isaurus* specimens belonged to any *Symbiodinium* clade other than clade C. All *Symbiodinium* ITS-rDNA sequences from collected *Isaurus* specimens from Japan were within the very highly supported (ML = 100%, NJ = 100%, PP = 1.00) clade consisting of types C1 and C3 (sensu LaJeunesse 2004) and related types (Fig. 5). While most sequences from *Isaurus* were identical to sequences previously noted as *Symbiodinium* types C1/C3 sensu LaJeunesse (2004) (DQ068036, DQ072720, AF195144—see Reimer et al. 2007a), five obtained ITS-rDNA sequences had small differences (SymIOtsH5, SymITaM1, SymIOtsOM4, SymIOtsB1, SymIOtsH6), and radiate from the basal C1/C3 branch. These sequences are, however, still clearly related to types C1/C3. Additionally, shorter ITS-rDNA sequences from other *Isaurus* samples not included in the alignment (IOtsH1, IOtsH2, IOtsH3) also were clearly within the C1/C3 related radiation based on our phylogenetic analyses (data not shown).

No *Symbiodinium* ITS-rDNA sequences from *Isaurus* specimens were seen to cluster within a separate and moderately well supported (ML = 70%, NJ = 64%, PP = 0.94) clade of *Symbiodinium* sequences from *Zoanthus sansibaricus* (Reimer et al. 2006e, 2007a) derived from C1/C3 types.

Discussion

Species diversity of examined *Isaurus* specimens

Cnidarian mitochondrial DNA is known to evolve at a very slow rate when compared to most other groups of animals (Shearer et al. 2002), and thus it is possible that the two mitochondrial genetic markers used here are not sensitive enough to distinguish between *Isaurus* spp. However, as shown in Table 4, sequence divergence rates for our *Isaurus* spp. specimens are much lower than observed previously between other congeneric zoanthids within Zoanthidae and Sphenopidae. COI sequences have been shown to be unable

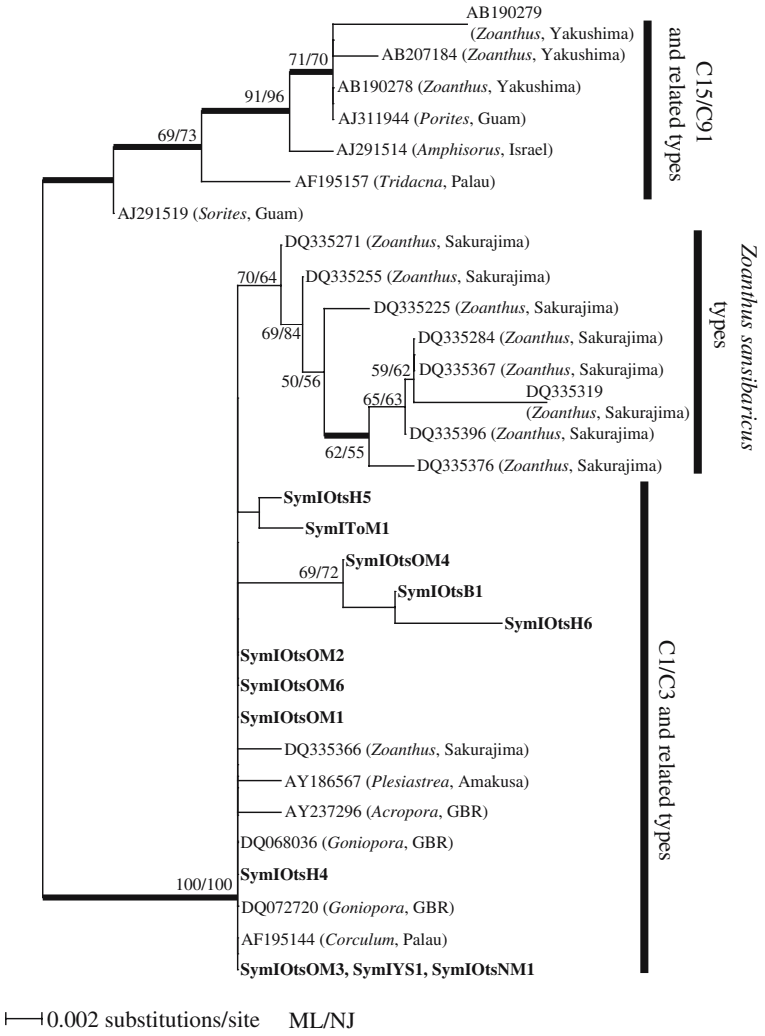

Fig. 5 Maximum likelihood tree of internal transcribed spacer ribosomal DNA (ITS-rDNA) sequences from *Symbiodinium*. Values at branches represent ML and NJ bootstrap probabilities, respectively (>50%). Bayesian posterior probabilities of >95% are represented by *thick branches*. For sample name abbreviations see Tables 1 and 2. Sample names with Accession Numbers are from previous studies (see Table 2)

to distinguish between *Palythoa tuberculosa* and *P. mutuki* (see Fig. 4 and Reimer et al. 2006c), but mt 16S rDNA did delineate (see Fig. 3; Table 4) between these two closely related species (Reimer et al. 2006c). Similarly, while COI sequences did not differ between *Zoanthus kuroshio* and *Z. vietnamensis* sensu Uchida (2001), mt 16S rDNA did show a low level of variation (Table 4) between these two putative species groups (Reimer et al. 2006a), and thus far there have been no cases where the combined mt 16S rDNA and COI sequences have been unable to distinguish between any examined Zoanthidae or Sphenopidae congeners. If the specimens nominally identified as *Isaurus* sp. B (based on external morphology) are in fact a separate species from *I. tuberculatus*, then based on our phylogenetic results these two groups must be very closely related. Further studies on these specimens using genetic markers that are faster evolving than mitochondrial DNA (such at nuclear ITS-rDNA) may help reconfirm the results seen in this study, Unfortunately, previously reported zoanthid-specific ITS-rDNA primers (Reimer et al. 2007b, c) did not reliably work on *Isaurus* specimens in this study. Preliminary nuclear 18S rDNA and 5.8S rDNA data indicate specimens of both *I. tuberculatus* and putative *I.* sp. B (sequences $n = 2$ each, data not shown) form a monophyly separate from *Zoanthus* and *Palythoa* spp., but due to the unreliability of the primer sets used and small number of sequences obtained, further investigation is needed on this subject before phylogenetic conclusions based on these markers can be confirmed.

However, our phylogenetic results combined with morphological data suggest that despite wide variation in some morphological characteristics (see Fig. 2; Tables 1, 3) that all *Isaurus* spp. specimens examined in this study were conspecific (*I. tuberculatus*). Furthermore, morphological data showing putative *Isaurus* sp. B specimens having morphological characteristics within the range of *I. tuberculatus* (excepting polyp length, see Tables 1, 3) make the case for conspecificity even stronger. These results are very similar to both Larson and Larson (1982) and Muirhead and Ryland (1985), who noted the large amounts of not only intraspecific variation in *Isaurus*, but also intra-colony

Table 4 DNA sequence variation within the zoanthid genera *Isaurus*, *Zoanthus*, and *Palythoa*

Comparison	COI sequence variation (%)	mt 16S rDNA variation (%)	Notes	Reference(s)
Isaurus specimens	0.0	0.0	Morphological variation (mesentery numbers, mesogleal thickness, external coloration) within *Isaurus* monophyly.	This study
Zoanthus sansibaricus–Zoanthus gigantus	0.7	1.1	Separate species. Evidence of reticulate evolutionary history between these groups based on ITS-rDNA.	Reimer et al. (2006b, 2007c)
Zoanthus sansibaricus–Zoanthus kuroshio	1.3	0.8	Separate species.	Reimer et al. (2006b)
Zoanthus kuroshio–Zoanthus vietnamensis	0.0	0.1	Separate species?—not yet determined. Morphological data suggest separate species, genetic data do not.	Reimer et al. (2006a)
Palythoa tuberculosa–Palythoa heliodiscus	0.9	1.1	Separate species	Reimer et al. (2006c)
Palythoa tuberculosa–Palythoa sp. sakurajimensis	NA	0.4–0.7	Separate species	Reimer et al. (2007b)
Palythoa tuberculosa–Palythoa mutuki[a]	0.0–0.2	0.1–0.2	Separate species. Evidence of reticulate evolutionary history between these groups based on ITS-rDNA.	Reimer et al. (2006c, 2007c)

NA=data not available

[a] *P. mutuki* here includes both *P. mutuki* 1 and *P. mutuki* 2 groups as noted in references

variation of polyps due to differing habitats and apparent degrees of exposure. As seen in Fig. 2b, we also observed large amounts of intra-colony polyp variation, with large amounts of variation in polyp coloration and size even in small colonies.

The *Isaurus* specimens examined here from western Australia (WAMZ40074, WAMZ40075, WAMZ40081) also had identical mt 16S rDNA and COI sequences to the Japanese *Isaurus* specimens, supporting the hypothesis that *I. tuberculatus* is distributed over a wide range (Muirhead and Ryland 1985). Furthermore, the results here may lend support to the assertion made by Muirhead and Ryland (1985) that *I. tuberculatus* consists of several previously presumed species, as zoanthid species were traditionally identified and described by their locality and morphology, which has shown to be highly variable.

Placement of *Isaurus* within Zoanthidae

Our phylogenetic results suggest the placement of *Isaurus* within the family Zoanthidae as a separate monophyly from *Zoanthus*. While COI analyses show *Isaurus* as derived from non-monophyletic *Zoanthus*, the COI sequences here are much shorter than mt 16S rDNA sequences. As discussed above, mt 16S rDNA has been shown previously with both *Palythoa* spp. and *Zoanthus* spp. to be a slightly more accurate marker than COI (both at the species and higher-taxa levels), and it is likely that the mt 16S rDNA tree topology is more reflective than the COI tree of the phylogeny of Zoanthidae.

Preserved specimens of *Z. praelongus* obtained from western Australia (WAMZ40080, WAMZ40082) were very difficult to morphologically distinguish from *Isaurus* specimens (see Muirhead and Ryland 1985); both *Z. praelongus* and *Isaurus* spp. have recumbent, 'liberae' (see Pax 1910) polyps and are of similar size and external morphology. However, the mt 16S rDNA and COI results clearly showed *Z. praelongus* samples within the *Zoanthus* radiation, to the exclusion of *Isaurus* sequences (Figs. 3, 4). Levels of sequence divergence between *Zoanthus* spp. and *Isaurus* spp. as well as the topology of both the COI and mt 16S rDNA trees support the separation of these two groups into separate genera.

One further result of note from the mt 16S rDNA data is that *Acrozoanthus* specimens were located within the *Zoanthus* clade, most closely related to the *Z. kuroshio*/*Z. vietnamensis* group (Fig. 3). Clearly more research utilizing more samples and genetic markers is needed to confirm whether *Acrozoanthus* is congeneric with *Zoanthus*.

Regardless of the placement of *Acrozoanthus*, the results here clearly demonstrate the monophyly of the Family Zoanthidae, further supporting the observations made in Sinniger et al. (2005) on the validity of this family.

Symbiodinium spp. in *Isaurus*

Based on our *Symbiodinium* ITS-rDNA phylogeny (Fig. 5), Japanese *Isaurus* specimens examined in this study apparently specifically associate with *Symbiodinium* of types C1/C3 sensu LaJeunesse (2004) or very closely related *Symbiodinium* types. These results are very similar to data seen in *Palythoa* spp. in Japan, which also apparently specifically associate with C1/C3. C1/C3 has been theorized to be both a host-generalist (LaJeunesse 2004) as it associates with many different host species, and also an environmental generalist (Reimer et al. 2006d), as it is found over a wide environmental range.

Such a specific association with one or a few closely related types of *Symbiodinium* does not conclusively demonstrate conspecificity of the specimens examined, particularly since C1/C3 is very common in the Indo-Pacific (LaJeunesse 2004). Therefore, further data on *Symbiodinium* distribution patterns from the other two *Isaurus* species (*I. maculatus* and *I. cliftoni*) are needed. Previous examples of specific associations seen in zooxanthellate zoanthids include the *Z. kuroshio*/*Z. vietnamensis* group that associates with *Symbiodinium* of C15 and related types (Reimer et al. 2006e); and *Z. sansibaricus* that harbors either C1/C3-derived *Symbiodinium* unique to *Zoanthus* or A1 *Symbiodinium* (Reimer et al. 2006e, 2007a).

How rare are *Isaurus* spp.?

In the past, little research has been conducted on *Isaurus* spp., in part due to difficulty in finding specimens (F. Iwase, personal observation). In Japan it has been believed until now that *Isaurus* spp. are quite rare. Our field studies here show that this may not be the case even though local frequency of colonies appears to be very low. Several (14) new colonies of *Isaurus* spp. were found in the Tatsukushi-Otsuki region during the course of this study, as well as one colony on Yakushima, where no previous records for *Isaurus* spp. exist (Fig. 1). All specimens collected in this study were found at locations not easily accessible; on or near rocky shorelines in areas that experience high and consistent levels of current and/or waves. As well, *I. tuberculatus* specimens in this study usually had coloration that closely resembles the rock and seaweed surrounding the colony (see Fig. 2), providing camouflage and making the colonies very difficult to spot unless specifically searched for. Based on these observations, it may be that *Isaurus* spp. distribution worldwide is wider than currently known.

Conclusions

The molecular and morphological data strongly suggest all *Isaurus* specimens examined in this study are conspecific *I. tuberculatus*, although it remains to be conclusively (i.e. hybridization experiment results, etc.) shown if putative *Isaurus* sp. B specimens are in fact *I. tuberculatus*. Our results support the hypotheses suggested by Larson and Larson (1982) and Muirhead and Ryland (1985) that *I. tuberculatus* has considerable intraspecific morphological variation (in particular external coloration). Even if the nominal *Isaurus* sp. B specimens are in fact a different species from *I. tuberculatus* specimens (although data here do not suggest this), we observed high levels of morphological variation (in particular coloration and polyp size) between both different *I. tuberculatus* specimens and within individual colonies. Despite the apparent low species diversity of *Isaurus* specimens here, our results also highlight the morphologically variable and cryptic nature of *Isaurus* spp., and suggest that despite relatively low frequency that this genus may be more widespread than previously believed. In the future, utilization of faster genetic markers (such as ITS-rDNA from *Isaurus*) combined with more *Symbiodinium* distribution data and ecological studies (reproduction characteristics, etc.) will help further increase our understanding of this enigmatic zoanthid genus.

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