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Different zooxanthellae types in populations of the zoanthid *Zoanthus sansibaricus* along depth gradients in Okinawa, Japan

Minaho Kamezaki · Marie Higa · Mamiko Hirose · Shoichiro Suda · James Davis Reimer

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Abstract Zooxanthellate zoanthid colonies of the species *Zoanthus sansibaricus* (Cnidaria: Anthozoa: Hexacorallia) are present both in the intertidal zone and at depths greater than 7 m at three locations (Manza, Zanpa, Sunabe) along the west coast of Okinawa, Japan. In this study, the identity of *Z. sansibaricus* colonies from various depths as the same species was confirmed using morphological analyses of tentacle numbers, polyp diameter, and nematocyst examination. In addition, molecular analyses of sequences of mitochondrial cytochrome oxidase subunit I and the internal transcribed spacer region 2 of ribosomal DNA (ITS2-rDNA) were performed. Surveys from 0 to 35 m depths at Manza indicated that the populations of *Z. sansibaricus* were discontinuous in their bathymetrical distribution, with few or no *Z. sansibaricus* colonies found at depths of 4–7 m. Examination of *Symbiodinium* (=zooxanthellae) types within *Z. sansibaricus* colonies by phylogenetic analyses of *Symbiodinium* ITS2-rDNA showed clear differences between the two populations, with some (=27 %) intertidal colonies having *Symbiodinium* clade A, while all deeper subtidal (>7 m depth) colonies possessed *Symbiodinium* clade C (n=18). Detailed phylogenetic analyses further indicated significant differences within *Symbiodinium* clade C populations over depth. From this study, it is clear that the zoanthid *Z. sansibaricus* has some variation in its symbiosis with *Symbiodinium*, potentially allowing the species to colonize different depths in the subtropical coral reef environment of Okinawa, and that its range extends far past the shallow subtidal zone as previously believed.

Keywords Zoanthid · Zooxanthellae · Okinawa · ITS-rDNA · *Symbiodinium*

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M. Kamezaki · M. Higa · M. Hirose · S. Suda
Department of Marine Science, Biology and Chemistry, University of the Ryukyus,
Senbaru 1,
Nishihara, Okinawa 903-0213, Japan

M. Hirose · J. D. Reimer
Molecular Invertebrate Systematics and Ecology Laboratory, Rising Star Program, Transdisciplinary Research Organization for Subtropical Island Studies (TRO-SIS), University of the Ryukyus,
Senbaru 1,
Nishihara, Okinawa 903-0213, Japan

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
e-mail: jreimer@sci.u-ryukyu.ac.jp

Introduction

Many benthic cnidarians found in shallow subtropical and tropical waters are in symbiosis with zooxanthellae, endosymbiotic photosynthetic dinoflagellates of the genus *Symbiodinium*. In recent years, the phenomenon of coral bleaching, in which the host animal loses its *Symbiodinium* symbionts due to stress, has become a serious problem in coral reef ecosystems worldwide (Hoegh-Guldberg 1999). Therefore, much research has focused on identifying and quantifying the diversity of *Symbiodinium* to provide baseline data in understanding coral bleaching and its impact (e.g., LaJeunesse 2002). Currently, *Symbiodinium* is divided into nine "clades" (designated A to I; Pochon and Gates 2010) of unknown taxonomic rank, with numerous subclades or types within the clades. It has been shown that

different types have different physiologies and responses to environmental factors (Tchernov et al. 2004), and thus the typing of *Symbiodinium* within different hosts is an important and necessary step when examining their bleaching ecology.

While most research into *Symbiodinium* diversity has focused on the hard corals (Anthozoa: Hexacorallia: Scleractinia), other investigations have examined *Symbiodinium* in related benthic cnidarians such as sea anemones (Actiniaria) (LaJeunesse and Trench 2000), soft corals (Octocorallia: Alcyonacea) (Carlos et al. 1999), and zoanthids (Zoantharia) (Burnett 2002; LaJeunesse 2002; Reimer et al. 2006c), but overall the understanding of the symbiont–host relationship for such animals remains incomplete when compared to hard coral–symbiont systems. However, an understanding of groups asides from hard corals is also critical to understanding bleaching, as the various responses of different benthos will undoubtedly shape future coral reef benthic communities. One group for which *Symbiodinium* data are still relatively sparse is the zoanthids. In shallow subtropical and tropical areas, the genera *Zoanthus* and *Palythoa* are usually dominant taxa on reef crests and the shallow reef front area (Irei et al. 2011).

One of the zoanthid species that has been most closely examined in terms of symbionts is *Zoanthus sansibaricus* Carlgren 1900. In southern Japan, it has been shown to associate with different *Symbiodinium* types based on its environment, with most specimens containing type C1z, which is unique to *Z. sansibaricus*, while colonies on reef flats exposed to strong light often contain potentially light-resistant type A1 (Reimer et al. 2006c). In addition, it has recently been shown that *Z. sansibaricus* from the oceanic Ogasawara Islands does not contain types C1z or A1, but instead the more common C1 (Reimer et al. 2011). Thus, *Z. sansibaricus* as a species may have a somewhat variable association with specific *Symbiodinium* types, although individual colonies appear to be in specific stable symbioses with a single symbiont type (Reimer et al. 2007b).

Zoanthus sansibaricus has been reported to exist in shallow waters (e.g., <10 m) in southern Japan (Reimer et al. 2004, 2006a, 2006c), with the largest numbers found on the reef crest at shallow subtidal or intertidal depths (Irei et al. 2011), but during recent investigations, numerous colonies were found at depths between 10 and 52 m (F. Sinniger, personal communication) on the west coast of Okinawa Island. At several sites, it appeared that this species had a discontinuous distribution, with a shallow, mainly intertidal population (hereafter referred to as "intertidal") and a deep population from 7 to 35+ m ("subtidal"). Thus, based on previous research showing that *Z. sansibaricus* has a variable yet specific association with *Symbiodinium* in Japanese waters, in this study, we have investigated these potentially discontinuous populations using field surveys, morphological examination of specimens and molecular techniques in order to answer the following questions:

1. Are the intertidal and deep subtidal populations of *Z. sansibaricus* disjunct in distribution at sites along the west coast of Okinawa?
2. Are the two populations of *Z. sansibaricus* truly conspecific? And do morphological or genetic differences exist between the two populations?
3. Do the two populations associate with different *Symbiodinium* types?

Materials and methods

Sampling location and procedure

The coral reef topographies of Cape Manza (26°30'N, 127°50'E) and Cape Zanpa (26°26'N, 127°42'E) on the west coast of Okinawa Island are similar in depth zonation, with a narrow intertidal ledge, followed by a short (usually 20–100 m width) platform or fringing reef of 3–10 m depth. After this, the reef drops off steeply to a sandy/rubble bottom of 20–50 m depth. Another site on the west coast, Sunabe (26°19'N, 127°44'E), is similar in zonation, although the 3–10 m platform is much wider and more well developed, and the reef's outer steep wall is rarely deeper than 25 m. In SCUBA surveys at Manza in 2007–2008, *Z. sansibaricus* colonies were observed both in the intertidal zone and along the steep wall below 10 m. Depths of observed *Z. sansibaricus* colonies were recorded during subsequent dives (n =31; 2008–2011; Supplementary Table S1). For the two populations, the term "intertidal" includes both intertidal and very shallow subtidal specimens (<4 m depth), while "subtidal" refers to subtidal colonies at >7 m depth.

Over the course of the experiment, 90 *Z. sansibaricus* colonies were collected (Table S1). Sampling was conducted at Manza, Zanpa, and Sunabe between September 2008 and December 2010, with additional deep subtidal populations confirmed from several other locations on Okinawa Island over the same period (Fig. 1). Specimens (n= 78) were photographed in situ for later use in morphological analyses (e.g. Fig. 2), and their depth was recorded. For intertidal colonies, the relative shading of each specimen or subsample was recorded as "shaded" (=completely shaded), "partially shaded", or "exposed", based on their exposure to sunlight at midday. For some colonies (n=15), multiple subsamples were collected from different parts of the colonies that had different amounts of sunlight exposure to investigate if different parts of colonies contained similar or different symbionts. All specimens were placed into individual Ziploc bags or plastic jars to prevent cross-contamination. Samples were fixed in either 99.5 % ethanol

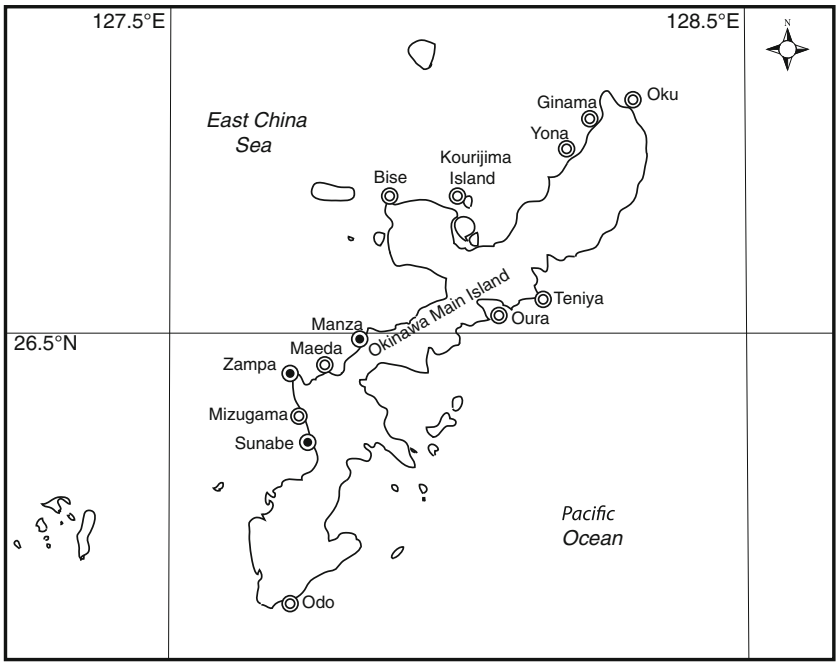

Fig. 1 Okinawa Island showing sampling locations where *Zoanthus sansibaricus* and its *Symbiodinium* were investigated in this study. Locations with names symbolized by *closed circles* were investigated in depth, while locations with *open circles* are where only "subtidal" populations (>7 m) of *Z. sansibaricus* were observed

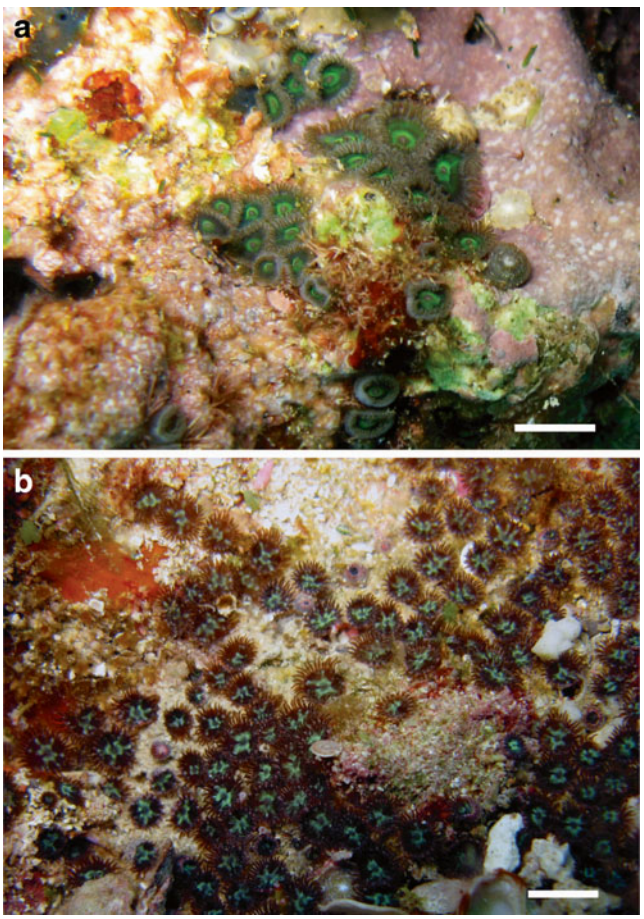

Fig. 2 **a** Intertidal population specimen of *Zoanthus sansibaricus* at 0 m (low intertidal), specimen MH11, at Manza, Onna Village, Okinawa. **b** Subtidal specimen of the same zoanthid species taken at the same location, depth=18 m, specimen MH10

for molecular analyses or 5–10 % seawater (SW) formalin for nematocysts analyses upon return to shore, and stored for further examination.

Finally, to provide an example of observed distributions, limited field surveys examining *Z. sansibaricus* distribution were conducted on August 4, 2009 at Manza by the belt transect method at two locations separated by approximately 50 m. Transect lines were made at a perpendicular (90°) angle to the shoreline, and were extended out until they reached 30 m depth. At each 2-m length interval along the measuring tape, the depth was recorded. By plotting the depths for each 2-m length of measure, a relief profile of each transect was plotted.

At the same time, the presence or absence of *Z. sansibaricus* colonies was checked using the methods described in Irei et al. (2011). A width of 0.5 m from each side of the measuring line was examined, and thus a 1-m width was examined from intertidal to 35 m depth. In this study, *Z. sansibaricus* colony numbers were recorded at 2-m depth intervals (e.g., 0–2 m, 2–4 m, etc.), except in two cases were bottom topography made it difficult to record in 2-m intervals and instead 3-m intervals were used (transect 1 at 0–3 m and 21–24 m). Polyps clearly connected by coenenchyme (common tissue) were counted as the same colony, as were polyps of the same oral disk color directly adjacent (within 5 cm) to each other unless clear gaps in coenenchyme were observed. In the surveys, counting of colonies proved to be relatively easy due to the bright oral disk coloration of *Z. sansibaricus*, and also because most *Z. sansibaricus* colonies in Okinawa are relatively small (<100 cm^2; Irei et al. 2011).

Morphological analyses of *Z. sansibaricus* specimens from intertidal and subtidal populations were conducted in order to confirm that both populations were conspecific, and

not a case of cryptic speciation. External morphological characters such as polyp size (oral disk diameter) and tentacle numbers have often been used as diagnostic characters for zoanthids (Ryland and Lancaster 2003), more recently in combination with DNA sequencing (e.g., Reimer et al. 2006a). In situ images of *Z. sansibaricus* colonies from intertidal and subtidal colonies at Manza and Sunabe were examined (total $n=78$; Table 1). The expanded oral disk diameter of fully open polyps was measured (largest polyp/colony), and tentacle numbers (five polyps/colony) were recorded. The disk diameter and tentacle number results were compared by *t* test between intertidal and subtidal populations, and between sites.

Nematocysts of *Z. sansibaricus* specimens from Manza and Sunabe were examined ($n=6$ for each population at each site) following methods described in Sinniger and Häussermann (2009), with mesenteries and body walls of one polyp per colony isolated and squashed on a glass microscope slide. Nematocysts were classified following Ryland and Lancaster (2003). Holotrichs were specifically focused on, as they are by far the most common nematocyst in *Z. sansibaricus*, and often the only type present in non-tentacle tissues. They were examined with a light microscope, and their length and width were recorded. Variance among sites in both holotrich length and width was tested using a Kruskal–Wallis test and Dunn's multiple comparison test since some data were not normal. Statistical analyses were performed using Instat 3 for Macintosh (GraphPad Software, La Jolla, CA, USA).

Molecular analyses

DNA was extracted from specimens using the guanidine method described in Sinniger et al. (2010). PCR amplification was performed with genomic DNA as template and using HotStarTaq DNA polymerase (Qiagen, Tokyo, Japan) following the manufacturer's instructions.

Zoanthus cytochrome oxidase subunit 1 [=COI] sequences ($n=4$) were amplified using the zoanthid-specific primer COI-ZoanF and the general COI primer HCO2198 (Reimer et al. 2007a; Folmer et al. 1994, respectively) following procedures as described in Reimer et al. (2011). Internal transcribed spacer of ribosomal DNA (=ITS-rDNA) sequences ($n=4$) were amplified according to Reimer et al. (2007c). For *Symbiodinium* phylogenetic analyses, sequences of the internal transcribed spacer region 2 of nuclear ribosomal DNA (ITS2-rDNA) ($n=52$, 26 each from i thentertidal and subtidal populations) were amplified following Reimer et al. (2006c). Amplified products were visualized by 1.5 % agarose gel electrophoresis, and positive PCR products were then treated with Exonuclease I and Shrimp Alkaline Phosphotase (SAP). Sequencing reactions and sequencing were performed by MacroGen Japan (Tokyo).

Novel sequences acquired in this study were deposited in GenBank (Accession Numbers JQ762312-JQ762359) (Table S1). Eight sequences from specimens in this study (COI and ITS-rDNA=4 sequences each) were compared with previously acquired *Z. sansibaricus* sequences from other studies to confirm the identity of these specimens as *Z. sansibaricus*. No other phylogenetic analyses were performed with these sequences, as DNA "barcoding" of zoanthids using COI (in combination with mitochondrial 16 S ribosomal DNA; Sinniger et al. 2008) or ITS-rDNA (Bo et al. 2012) appears to work well.

For *Symbiodinium* ITS2-rDNA sequences obtained in this study, two alignments were generated and phylogenetic analyses performed. Different *Symbiodinium* clade ITS2-rDNA sequences are highly divergent from each other, and a global "eight clade" *Symbiodinium* alignment of ITS2-rDNA was downloaded from the Scott Santos laboratory homepage (http://www.auburn.edu/~santosr/), and previously obtained sequences from other recent studies were added, including clades A and C sequences from *Z. sansibaricus* (Reimer et al. 2006c; 2007b). This global alignment was subsequently split into two alignments, one each for clades A and C, and each aligned manually using the software SeAl v2.0a11 (University of Edinburgh). The alignments consisted of mainly the second internal ribosomal spacer of ribosomal DNA (ITS2), which has been shown to have great utility in identifying *Symbiodinium* types (e.g., LaJeunesse 2002). Ambiguous sites (total seven sites) were edited in the case of "singletons" (four sites) or retained if present in multiple sequences (three sites, each in two to four sequences) from the datasets for subsequent phylogenetic analyses, and were only seen in the Clade C alignment. Clade A and clade C alignments consisted of 226 sites and 26 sequences, and 281 sites and 47 sequences, respectively. Alignment

Table 1 Comparison of oral disc diameter and tentacle number of *Zoanthus sansibaricus* colonies at Manza and Sunabe between sites, and between intertidal and subtidal populations

Site	Population	Average oral disc diameter ± SD (mm)	Average number of tentacles ± SD
Manza	Intertidal ($n=12$)	2.50±0.50	42.7±4.0
	Subtidal ($n=54$)	2.48±0.52	42.8±3.4
Sunabe	Intertidal ($n=3$)	2.00±0	45.6±1.0
	Subtidal ($n=9$)	2.22±0.78	42.7±2.2

data are available from the corresponding author and at http://web.me.com/miseryukyu/.

The alignments were analyzed with maximum-likelihood (ML) with PhyML (Guindon and Gascuel 2003), performed using an input tree generated by BIONJ under the general-time reversible (GTR) model of nucleotide substitution with invariable sites and eight categories (GTR+I). Frequencies of the model were estimated from the dataset, and bootstrap trees (1000 replicates) were constructed using the same parameters as the individual ML tree. Distances were calculated using Kimura's 2-parameter model (Kimura 1980).

An analysis of the molecular variance (AMOVA; Excoffier et al. 1992) was performed using the Arlequin version 3.0 program package (Excoffier et al. 2005) to estimate the total percentage variance attributable to among-morphotype differences, among-location differences, and differences among individuals within populations. The population differentiation was estimated from pairwise population comparisons of values of F_{ST} (Reynolds et al. 1983), and the significance of variance components was tested using 16,000 permutations of the data set.

Results

Observation and specimen collection of *Zoanthus sansibaricus*

The belt transect field data (Fig. 3) show that in both transects, *Z. sansibaricus* colonies were common on the steep wall at Manza at depths of 8–30 m (n=110 and 127 in each transect), with some colonies present in the intertidal and shallow subtidal zone (n=0 and 4), and only one colony found at depths between intertidal and subtidal populations (4 to 7 m).

Between 2008 and 2011, 31 dives were conducted at Manza (n=22), Zanpa (n=3) ,and Sunabe (n=6). During this time, numerous *Z. sansibaricus* colonies were observed at all three locations. The species is known as a primarily intertidal/shallow subtidal organism (Reimer et al. 2006c). Many colonies were observed in the intertidal to depths of 4 m or less, most of which were sampled (Table S1). It should be noted that most colonies did not cover large areas as seen at other sites on Okinawa Island with more developed shallow coral reefs (e.g., Teniya, Mizugama). The species was also seen to be abundant at depths >7 m along walls or steep gradients and again, many specimens were collected (Table S1). Only one colony each at Sunabe (November 9, 2009) and Manza (August 4, 2009) were noticed at depths between 4 and 7 m. Both specimens were photographed, one of which was collected (Table S1). A total of 105 specimens (including 15 colonies with 2 subsamples) were collected (Manza, n=56; Sunabe n=34; Zanpa n=15; Table S1), with additional (n=63) colonies only photographed in situ. Specimens are currently curated in the corresponding author's laboratory at the University of the Ryukyus.

Morphological analyses

Oral disk diameters and tentacle numbers of *Z. sansibaricus* polyps did not significantly vary between the intertidal and subtidal populations.There were no statistical differences between Sunabe and Manza sites (Table 1). At Manza, intertidal population holotrich mean length was 21.1±2.0 μm and width was 9,2±1.1 μm (n=120; from 6 colonies). Subtidal population means were 19.0±1.6 and 8.6±1.2 μm, respectively (n=204; from 6 colonies). At Sunabe, intertidal population holotrich length means was 19.9±2.3 μm and width was 9.0±1.1 μm (n=85; from 6 colonies). Subtidal population means were 17.8±2.0 and 8.6±1.0 μm, respectively (n=163; from 6 colonies). Holotrich length varied, and variation among all four populations (Manza intertidal, Manza subtidal, Sunabe intertidal, and Sunabe subtidal) was significant (Kruskal–Wallis test, p<0.001). Holotrich width also varied, and variation among all four populations was also significant (Kruskal–Wallis test, p<0.001). Significant differences were found between data sets of Manza intertidal and subtidal, Manza intertidal and Sunabe subtidal, and Shunabe intertidal and Manza subtidal, but not between the combinations of Sunabe intertidal and Sunabe subtidal, Sunabe intertidal and Manza intertidal, and Manza reef and Sunabe subtidal (Dunn's multiple comparison test, p<0.05).

Phylogenetic analyses

Symbiodinium ITS2-rDNA sequences from *Z. sansibaricus* specimens were directly sequenced. No sequences showed "double peaks" or mixed signals over the ITS2-rDNA region, and thus the sequences obtained were interpreted as representing the only or dominant *Symbiodinium* type in each specimen. However, we cannot discount the possibility that minority (e.g., <5–10 % total) populations of other *Symbiodinium* types exist in specimens (Mieog et al. 2007). Based on the ITS2-rDNA sequences obtained, specimens were seen to associate with at least two clades of *Symbiodinium* (Table S1; Fig. 4). In intertidal specimens, 6 of 19 total specimens in exposed (no shade) areas associated with clade A, and the remaining 13 colonies associated with clade C. In partially or completely shaded intertidal colonies, 20 of 21 specimens possessed clade C, with only one colony having clade A. Overall, 11 of 41 specimens in the intertidal possessed clade A, and 30 specimens had clade C (note these totals are different from shaded/partially shaded/exposed totals, as no degree of shading was noted for some colonies; Table S1).

All acquired novel clade C ITS2-rDNA sequences from *Symbiodinium* in *Z. sansibaricus* were seen to be identical

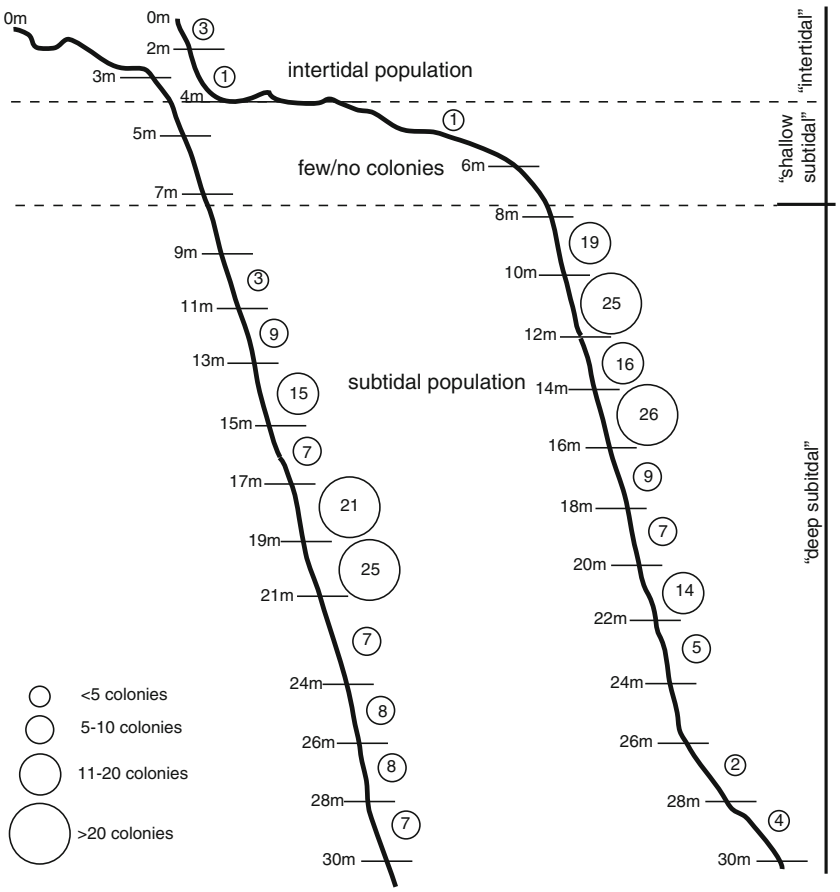

Fig. 3 Results of vertical transects of *Zoanthus sansibaricus* colony frequency at Manza, Onna, Okinawa, while collecting specimens MH15-MH20, to demonstrate "intertidal" (intertidal to 4 m depth) and "subtidal" (>7 m depth) populations. *Left* transect 1, *right* transect 2. Relative numbers of colonies within *circles*. *Dotted line* indicates boundary between intertidal and reef populations. Note narrow reef platform, particularly in transect 2, at 4–8 m. Horizontal and vertical scales identical

or closely related to those previously reported (reported in Reimer et al. 2006c; designated type C1z sensu Reimer 2008) sequences, as well as types C69a, C67, C39, C22, C49, and C31, and together these groups formed a well supported clade (ML=100 % in Fig. 4a). There was some variation (approximately 4–5 base pairs) in clade C sequences from *Symbiodinium* in *Z. sansibaricus*, with a total of at least 15 different haplotypes observed from a total of 36 clade C sequences phylogenetically analyzed (Fig. 4a). Several small subclades formed within the large clade, but all had bootstrap support of <50 %. However, it should be noted that one subclade consisted only of sequences from intertidal sequences as well as type C1z from intertidal/shallow *Z. sansibaricus* from previous studies, while another subclade consisted of only subtidal sequences (Fig. 4a).

Based on phylogenetic analyses, clade A *Symbiodinium* was seen to be identical to clade A previously reported from intertidal, non-shaded *Z. sansibaricus* colonies on Amami Oshima Island (ML=66 %) (Reimer et al. 2006c), and closely related to sequences "A1" (AF427466), "A4" (AF427465), "Odo06" (EU106365), and "A3" (AF427467), which all together formed a strongly supported clade (ML=97 % in Fig. 4; 99.6 % in Fig. 4b). All (n=18) subtidal specimens were only in symbiosis with clade C (Table S1). The analysis of molecular variance (AMOVA), based on acquired clade C ITS2-rDNA sequences, indicated that the estimated F_{ST} value between intertidal and subtidal populations was 0.10272 (p<0.01). A pairwise test for genetic differentiation between the two different populations revealed significant F_{ST} values (F_{ST}=0.10272, p<0.01).

Zoanthus sansibaricus COI sequences in this study were seen to be identical to those previously acquired, regardless of whether the sequences were from intertidal or subtidal colonies (n=4). For *Z. sansibaricus* ITS-rDNA sequences, as shallow specimens have previously been examined (Reimer et al. 2007c; Y. Sakamoto and J.D. Reimer, unpublished data), we examined only subtidal specimens (n=4). Results showed sequences matching 97-–8 % with previously acquired *Z. sansibaricus* sequences, well within the intraspecific level of variation for this species (Reimer et al. 2007c). Additional microsatellite data (D. Wham, T. LaJeunesse and J.D. Reimer, unpublished data) confirm that the intertidal and subtidal populations are panmictic.

Discussion

Intertidal and subtidal *Zoanthus*: same species?

Results from the numerous dives at Manza, Zampa, and Sunabe, and from the two transects at Manza, clearly

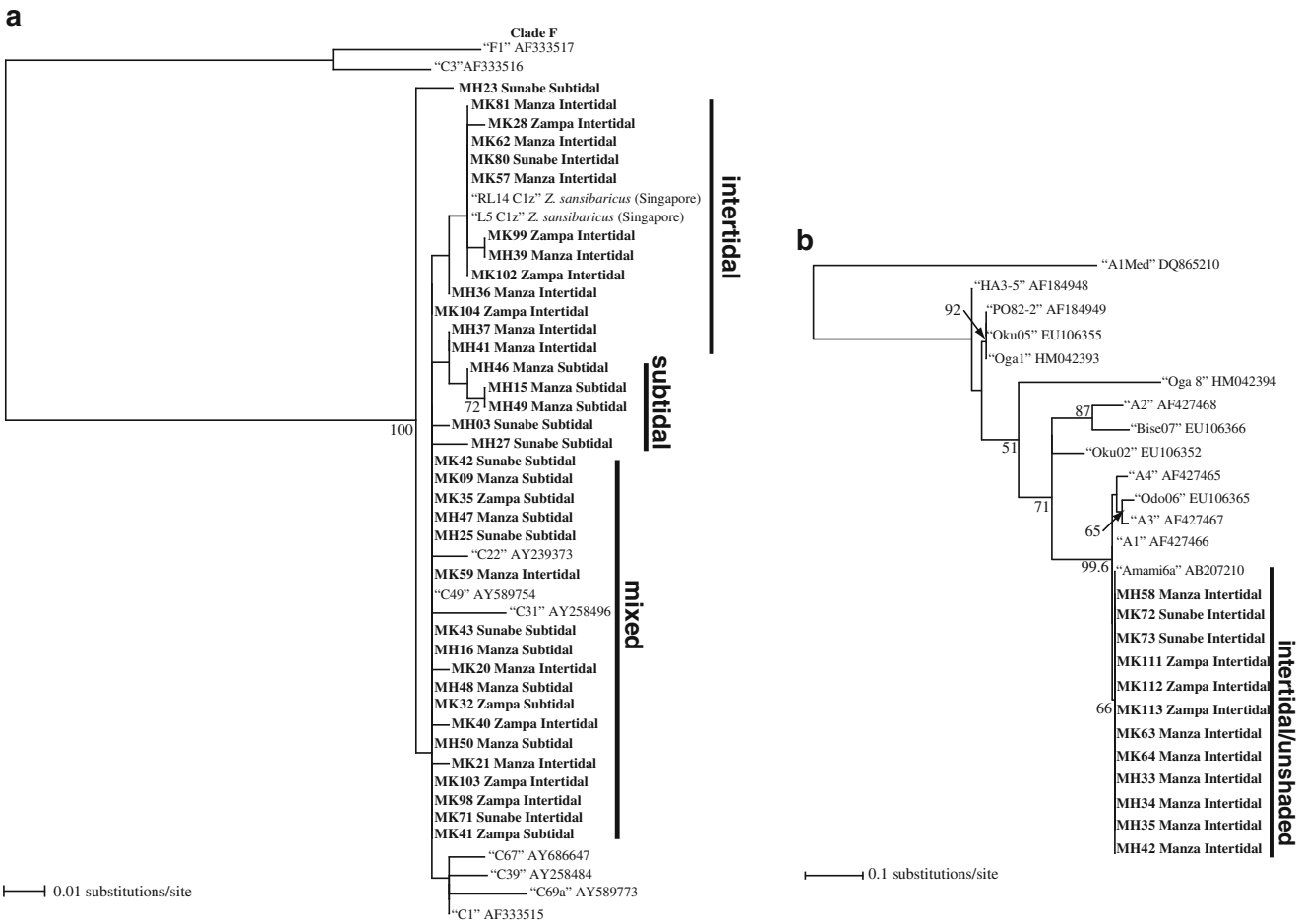

Fig. 4 Maximum likelihood trees of the **a** clade C, and **b** clade A alignments of internal transcribed spacer 2 (ITS-2) region sequences of ribosomal DNA of *Symbiodinium* including new sequences from *Zoanthus sansibaricus* specimens in this study (in *bold* with "intertidal" or "subtidal" population designations as defined in "Materials and methods"). Values at branches represent maximum likelihood bootstrap probabilities. For previously reported sequences, type names are in *parentheses* followed by GenBank Accession numbers

demonstrate that the intertidal and subtidal *Zoanthus sansibaricus* populations do not overlap. Shallow colony numbers were lower than in a previous study in Okinawa (Irei et al. 2011), but this is likely due to the lack of a well-developed shallow reef at the three investigated locations in this study. Despite the apparent discontinuous distribution of intertidal and subtidal populations in this study, the morphological and molecular data strongly suggest that the zoanthid populations are conspecific. No differences were observed in polyp sizes or in tentacle numbers between intertidal and subtidal populations, and when taken with the host DNA data showing both intertidal and subtidal colonies were *Z. sansibaricus*, these results seem conclusive that both populations are conspecific.

However, there were significant differences in nematocyst sizes between some of the intertidal and subtidal populations, and this potentially could indicate genetic differences between the two populations, or perhaps even cryptic or incipient speciation. Nematocyst sizes have previously been used to help distinguish between *Palythoa* spp. (Ryland and Lancaster 2003), but generally the size of nematocysts has not been utilized as a diagnostic character, and instead the presence or absence of different types of nematocysts in various zoanthid tissues has been used (e.g., Sinniger and Häussermann 2009; Reimer and Fujii 2010). Furthermore, large intraspecific variations in nematocyst sizes have been observed in corallimorpharians (Acuña and Garese 2008). In this study, no differences in nematocyst types present were noticed between intertidal and subtidal specimens. Furthermore, significant size differences were observed between holotrichs in *Z. sansibaricus* specimens from Manza and Sunabe, suggesting that physical environment (e.g., nutrition, light levels or current) may somehow influence the size of holotrichs. Finally, although significantly different, sizes of holotrichs from this study fit within the range of holotrich sizes from *Z. sansibaricus* specimens sampled from various locations around Okinawa (lengths 15–21 μm, widths 5–10 μm; Y. Sakamoto and J.D. Reimer, data not shown). Thus, given the information above, we conclude here that the size differences of

holotrichs in *Z. sansibaricus* from intertidal and subtidal populations are not indicative of speciation, and that the two populations are the same species. From this study, it appears that environmental factor(s) may influence holotrich size. In the future, the acquisition of *Z. sansibaricus* microsatellite data will help clarify the genetic identity of intertidal and subtidal populations to a more refined degree.

A variable but specific symbiotic association?

Zoanthus sansibaricus on the west coast of Okinawa associates with three types of *Symbiodinium*. In intertidal locations with little or no shade, colonies sometimes associated with clade A (6/19 colonies). This has previously been observed from the intertidal zone in Amami Oshima Island (Reimer et al. 2006c), and as clade A has been speculated to be tolerant of higher levels of light (Banaszak et al. 2000), this appears to help some colonies survive at <7 m depth in non-shaded locations, although other (13/19) colonies in non-shaded locations had clade C *Symbiodinium*.

However, most colonies from the intertidal and subtidal shallow zones were found in crevices that were partially shaded from direct sunlight at midday, and these colonies almost exclusively contained type C1z (sensu Reimer 2008) or closely related types (20/21 colonies, 1 colony with A). Similar reports for shallow water (e.g., <10 m) *Z. sansibaricus* have been reported from many locations in Japan, including the Ryukyu Archipelago (Reimer et al. 2006c). C1z is closely related to type C1, which is a common, "generalist" Indo-Pacific *Symbiodinium* type known from multiple hosts and environments (LaJeunesse 2005; Reimer et al. 2006b) but not usually found in *Z. sansibaricus*. On the other hand, C1z apparently is a unique type of *Symbiodinium* found only (thus far) in *Z. sansibaricus*.

The AMOVA analyses in this study clearly show significant differences between intertidal and subtidal *Symbiodinium* C sequences in *Z. sansibaricus*, and this is also shown in the phylogenetic tree, with certain genotypes specific to each population (Fig. 4a). We interpret these differences as being indicative of very closely related but separate types of C1z. As with the host genetic data mentioned above, future research utilizing microsatellite data should help clarify the differences between the theorized "intertidal" and "subtidal" C1z types. The fact that *Z. sansibaricus* in different environments apparently associates with specific but different *Symbiodinium* types indicates that co-evolution may be occuring, and yet the symbiont system has some kind of variable specificity to allow *Z. sansibaricus* to inhabit various environments. The development of *Z. sansibaricus* larvae is still unknown, although it is likely that this species is an external broadcast spawner based on unpublished data (T. Mezaki, personal communication), with larvae (=zoanthina) that may have an extended (=weeks) planktonic stage (Ryland et al. 2000). It is not known, however, when larvae acquire *Symbiodinium*, although it is known that eggs of this species do not contain *Symbiodinium* just before spawning while still inside polyps (Ono et al. 2005). Based on Ono et al.'s (2005) and our present observations, it seems most likely that *Z. sansibaricus* acquires symbionts upon settlement, as colonies at different depths and light levels associate with different and specific types adapted to each environment. The theory of symbiont acquisition upon settlement is further supported by recent data from the oceanic Ogasawara Islands, where colonies were found to associate with generalist C1, and no C1z colonies were found (Reimer et al. 2011). Such results suggest that, although *Z. sansibaricus* is present in these oceanic islands, C1z *Symbiodinium* is not, and therefore *Z. sansibaricus* associates with C1 instead.

Although such specific and variable associations between zoanthids and *Symbiodinium* spp. have not been reported on in detail apart from in *Z. sansibaricus*, similar observations have been reported from coral species. In particular, it has been demonstrated that *Montastrea annularis* from the Caribbean associates with different *Symbiodinium* at different depths, which is theorized to be primarily due to different light levels (Rowan and Knowlton 1995). Furthermore, this association has been demonstrated to be flexible, with changes in symbiont type observed in experimental bleaching/transplant experiments (Toller et al. 2001). It may be that the *Z. sansibaricus–Symbiodinium* association is similarly flexible. However, it does appear that many cnidarian–symbiont systems are not as flexible as in *M. annularis*, and have a higher degree of stability and specificity with a single type of *Symbiodinium* (Smith et al. 2009).

Similarly, *Madracis pharensis* in Curaçao has also been shown to have "depth-related symbiont variation" (Frade et al. 2008a), suggesting that their different *Symbiodinium* niches are not only based on host specificity but also on "light spectral niches" for zooxanthellae (Frade et al. 2008b). This explanation appears to be valid for our observed results here, although we did not see any partitioning of symbiont type based on *Z. sansibaricus* color morphs, unlike in *M. pharensis* (Frade et al. 2008b).

It is clear from this study that the range of *Z. sansibaricus* extends far deeper past the intertidal and shallow subtidal zone, showing that this species can be widely distributed in many different environments and light regimes. Large amounts of data now exist for *Symbiodinium* in *Z. sansibaricus* from southern Japan to the Izu Islands, as well as for the Ogasawara Islands. This host–symbiont system would make an appropriate model system to examine how zooxanthellate benthic cnidarians along the Kuroshio Current can acclimatize to the environmental variation in this region. Future research into the *Symbiodinium* of *Z. sansibaricus* from Taiwan and the Sea of Japan (Shimane Prefecture) are

planned to further examine the diversity of this symbiotic system in this region.

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