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ORIGINAL PAPER

Phylogenetic analyses of potentially free-living *Symbiodinium* spp. isolated from coral reef sand in Okinawa, Japan

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Abstract The existence of "free-living" *Symbiodinium* that can form symbioses with hosts is implied by the presence of hosts that produce *Symbiodinium*-free gametes and expulsion and/or expelled symbiotic algae from host. However, it is still unclear if potentially symbiotic *Symbiodinium* are found "free-living" in the coral reef environment. Sixteen *Symbiodinium* strains were established from samples taken from three sampling locations of coral reef sand in Okinawa, Japan. Phylogenetic analyses of the partial large subunit ribosomal DNA (28S-rDNA) and the internal transcribed spacer of ribosomal DNA (ITS-rDNA) conclusively showed that all 16 isolates belonged to *Symbiodinium* clade A *sensu* Rowan and Powers (1991). The lack of other *Symbiodinium* clades besides clade A in this study may be due to other clades not being readily culturable under culture conditions used here. The new isolates could be phylogenetically divided into four groups, though no sequences were identical to previously reported *Symbiodinium*. Two of the four groups were closely related to symbiotic *Symbiodinium* clade A isolated from a variety of host species. One isolate group formed a highly supported monophyly with *Symbiodinium* types that have previously been characterized as "free-living". The remaining isolate group, although within clade A, was quite divergent from other clade A *Symbiodinium*. These results indicate that novel diversity of free-living *Symbiodinium* exists in coral sand.

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Introduction

Zooxanthellae are symbiotic dinoflagellates (genus *Symbiodinium*) that form mutual associations with foraminifers and a wide range of marine invertebrates such as giant clams, anemones, jellyfish, zoanthids, and in particular reef-building corals (e.g., Trench 1987). Until the 1970s, all symbiotic dinoflagellates were considered members of a single pandmic species, *Symbiodinium microadriaticum* Freudenthal (Taylor 1974). However, molecular genetic studies have revealed that the genus *Symbiodinium* is a highly diverse group of dinoflagellates (cf. Coffroth and Santos 2005). Currently *Symbiodinium* is divided into eight large clades (*Symbiodinium* clades A–H, cf. Coffroth and Santos 2005) and within each clade numerous closely related "types" or "subclades" exhibit distinctive host, biogeographic, and/or environmental distributions (e.g., LaJeunesse 2004; Pochon et al. 2006).

One important but unanswered question concerning *Symbiodinium* is if potentially symbiotic *Symbiodinium* exist outside of hosts in the coral reef environment. Here, the term "free-living" is defined as *Symbiodinium* that have ability to associate with but are living outside hosts. The existence of "free-living" *Symbiodinium* is implied by aposymbiotic host larvae that acquire these symbionts from the environment in Scleractinia (Little et al. 2004), Zoantharia (Ono et al. 2005), Octocorallia (Coffroth et al. 2006),

Scyphozoa (Thornhill et al. 2006), and Bivalvia (Hirose et al. 2006). Additionally, Baghdasarian and Muscatine (2000) have suggested that dividing algal cells are preferentially expelled from their host for regulation of algal population density. Thus *Symbiodinium* spp. in the environment (outside of hosts) are implied by regular expulsion of excess *Symbiodinium* (e.g., Stimson and Kinzie 1991; Maruyama and Heslinga 1997). Additionally, it has been shown many times that *Symbiodinium* are often expelled under stress conditions (e.g., Gates et al. 1992; Ralph et al. 2001; Bhagooli and Hidaka 2004). However, laboratory isolations of *Symbiodinium* dinoflagellates from both sand and the water column are rare (Loeblich and Sherley 1979; Chang 1983; Carlos et al. 1999; Gou et al. 2003; Coffroth et al. 2006). To examine the potential "outside of host" *Symbiodinium* from the coral reef environment, *Symbiodinium* dinoflagellates were isolated from coral reef sand in Okinawa (Japan) and identified using the internal transcribed spacer of ribosomal DNA (ITS-rDNA) and large subunit ribosomal DNA (28S-rDNA) molecular markers.

Materials and methods

Symbiodinium isolation and culture conditions

Symbiodinium were isolated from coastal wet sand samples collected from three locations in Okinawajima Island, Japan; Oku, Kunigami (northern Okinawajima), in August 2003; Bise, Motobu (west coast, mid-Okinawajima), and Odo, Itoman (southern Okinawajima), in August 2004 (Fig. 1). All sand samples were collected from coral reef lagoon sand at a depth of 30–50 cm with a scoop. There were coral (*Porites* spp.) at Oku and Odo, and sea grass (*Thalassia hemprichii*) and coral (*Montipora digitata*) at Bise, but the sand was collected from points at least 3 m away from coral and sea grass. Sand was collected from a depth of 1 cm below the sea floor surface. Collected samples (about 0.5 ml volume) were put in plastic petri dishes (diameter = 9 cm), and animals (e.g., molluscs, copepods, lugworms) and other detritus were removed. The sizes of inoculated sand grains were 0.2–2 mm. The sand samples were enriched by 20 ml IMK medium (Nippon Pharmaceutical Co.) with 5 mg l^{-1} of GeO_2 for inhibition of diatom growth. Unialgal clonal strains were established from 3 days to 3 week-old cultures by single cell isolation using micropipettes. A total of 16 individual *Symbiodinium*-like swimming dinoflagellate cells were randomly picked up by micropipettes after 20 days incubation of coral sand samples from Oku (collected in August 2003), and 14 unialgal strains were established. One strain each from the Odo and Bise sand samples (collected in August 2004) was established by single cell isolation of *Symbiodinium*-like cells as

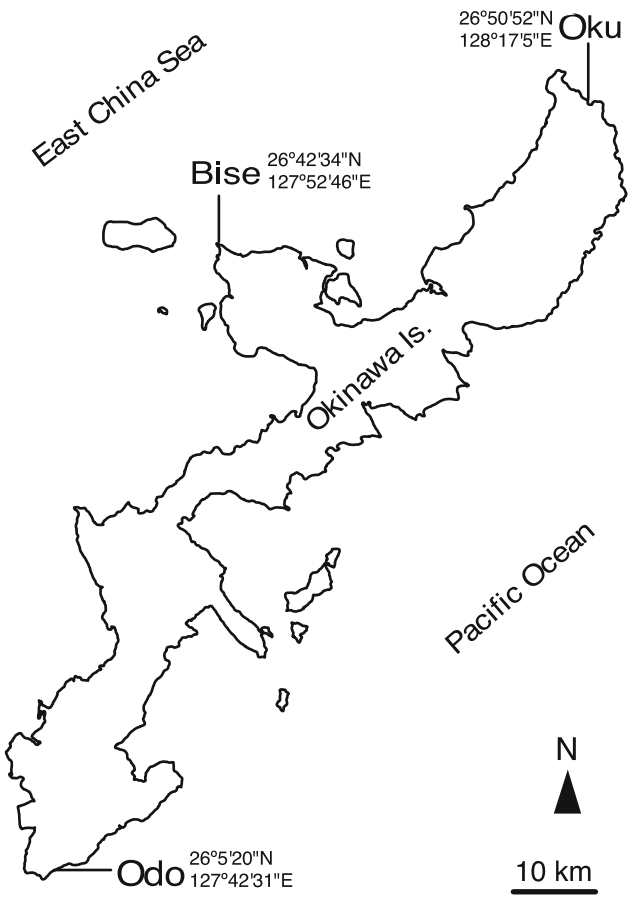

Fig. 1 Map of Okinawa Island and sampling locations

described after 3 and 7 days incubation, respectively. The strains were subcultured with natural seawater based IMK medium and maintained at 22 ± 1°C, under a 14:10 h light/dark cycle at approximately 40 µmol photons m^{-2} s^{-1} provided by cool-white fluorescent lamps.

DNA extraction, PCR amplification, and sequencing

Algal cells from 3 week-old clonal cultures of each strain were harvested. DNA extraction followed the protocols outlined by Coffroth et al. (1992) after dissolving the cells in guanidine thiocyanate solution following Fukami et al. (2004). ITS-rDNA and partial 28S-rDNA were amplified using primers ZITSUP (Santos et al. 2001) and lsu-URP1 (Zardoya et al. 1995). To estimate the genetic variation of the 16 strains, PCR products (ITS-1-5.8S rDNA-ITS-2 partial 28S rDNA region, about 1400 bp) were digested with restriction enzymes *Taq* I and *Sau* 3A. Digests were separated by electrophoresis through an approximately 4% agarose gel (1.6% Synergel™ (Diversified Biotech, Boston) and 0.7% Seakem GTG agarose (Lonza, Basel), and were stained with ethidium bromide. Subsequently, the ITS-rDNA (ITS-1-5.8S rDNA-ITS-2) region of the 16 *Symbi-*

odinium strains were sequenced to examine the phylogenetic position of strains established in this study at a higher resolution. The purified PCR-amplified DNA fragments were cloned into pT7Blue Vectors (Novagen) using a DNA ligation kit (Mighty Mix, Takara). Additional primers [ZITSDN (Santos et al. 2001), lsu-UFP1 (Zardoya et al. 1995)] were used for sequencing. Cycle sequencing reactions were carried out using DTCS Quick Start Master Mix (Beckman Coulter) and products were analyzed using a CEQ8800 (Beckman Coulter) automated DNA sequencing system.

Phylogenetic analyses

The sequences derived from this study are referred to in Table 1. New sequences obtained in the present study were deposited in GenBank (accession numbers EU106351–EU106366). Previously reported ITS-rDNA sequences of *Symbiodinium* clade A (Table 1) were retrieved from the DNA data bank of Japan (DDBJ) and were aligned with our present 28S-rDNA and ITS-rDNA data using ClustalW. The alignments were inspected by eye and manually edited. All ambiguous sites of the alignments were removed from the dataset for phylogenetic analyses. The alignment datasets of 28S-rDNA and ITS-rDNA (28S-rDNA = 11 taxa/423 sites; ITS-rDNA = 24 taxa/562 sites) are available on request from the corresponding author.

For the phylogenetic analyses of the 28S rDNA sequences and the ITS-rDNA sequences the same methods were independently applied. Maximum-likelihood (ML) analyses were performed using PhyML (Guindon and Gascuel 2003). Parameters for analyses followed Reimer et al. (2006). PhyML bootstrap trees (500 replicates) were constructed using the same parameters as the individual ML trees. Using the same datasets, Bayesian trees were also reconstructed by using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) with input trees generated by BIONJ with the general time-reversible model (Rodriguez et al. 1990) incorporating invariable sites and a discrete gamma distribution (eight categories) (GTR + I + Γ). 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-generations intervals (10,000 InLs and trees were

Table 1 List of *Symbiodinium* ITS-rDNA and 28S-rDNA sequences for phylogenetic tree reconstruction

ITS type	Isolation source (host organism)	Geographic origin	Strain	ITS-rDNA accession number	28S-rDNA accession number	References
A1	*Cassiopea xamachana*	Jamaica	Cx	AF427466	AF427454	Santos et al. (2002)
A2	*Zoanthus sociatus*	Jamaica	Zs	AF427468	AF427456	Santos et al. (2002)
A3	*Tridacna gigas*	Indo-Pacific	T	AF427467	AF427455	Santos et al. (2002)
A4	*Aiptasia pallida*	Florida Keys	FLA#4	AF427465	AF427453	Santos et al. (2002)
A5	*Tridacna aquamosa*	Palau	#315	AF333508		LaJeunesse (2001)
A7	*Millepora platyphylla*	GBR	A7	AY239388		LaJeunesse et al. (2003)
	Amphisorus hemprichii	Palau	P082-2	AF184949		Baillie et al. (2000)
	On sand (free living)	Hawaii	HA3-5	AF184948		Baillie et al. (2000)
	No data	No data	Z1	DQ174725	DQ174745	Moore (2006)
	Coral reef sand	Okinawa, Oku	Oku01	EU106351		This study
	Coral reef sand	Okinawa, Oku	Oku02	EU106352		This study
	Coral reef sand	Okinawa, Oku	Oku03	EU106353	EU106353	This study
	Coral reef sand	Okinawa, Oku	Oku04	EU106354		This study
	Coral reef sand	Okinawa, Oku	Oku05	EU106355	EU106355	This study
	Coral reef sand	Okinawa, Oku	Oku07	EU106356		This study
	Coral reef sand	Okinawa, Oku	Oku08	EU106357		This study
	Coral reef sand	Okinawa, Oku	Oku09	EU106358		This study
	Coral reef sand	Okinawa, Oku	Oku10	EU106359		This study
	Coral reef sand	Okinawa, Oku	Oku11	EU106360		This study
	Coral reef sand	Okinawa, Oku	Oku12	EU106361		This study
	Coral reef sand	Okinawa, Oku	Oku15	EU106362		This study
	Coral reef sand	Okinawa, Oku	Oku16	EU106363		This study
	Coral reef sand	Okinawa, Oku	Oku17	EU106364		This study
	Coral reef sand	Okinawa, Odo	Odo06	EU106365	EU106365	This study
	Coral reef sand	Okinawa, Bise	Bise07	EU106366	EU106366	This study

saved during MCMC). The likelihood plots for the two datasets suggested that MCMC reached the stationary phase after the first 30,000 generations (28S-rDNA) and 50,000 generations (ITS-rDNA) [both potential scale reduction factors (PSRF) = 1.000]. Thus, the remaining 9,700 and 9,500 trees were used to obtain clade probabilities and branch-length estimates, respectively.

The neighbor-joining (NJ) method (Saitou and Nei 1987) was performed using PAUP* Version 4.0 (Swofford 2000), with ML distances (GTR + I + Γ). NJ bootstrap trees (1,000 replicates) were constructed using the same model.

Results

A total of 16 cells were individually transferred to test tubes filled with culture medium. Subsequently, 14 unialgal strains were established from the Oku samples. One strain each from the Odo and Bise sand samples (collected in August 2004) was established by single cell isolation of *Symbiodinium*-like cells as described above after 3 and 7 days incubation, respectively. We obtained ITS-rDNA region sequences from all 16 strains.

The partial 28S rDNA region (about 650 bp) of four selected strains (Oku03, Oku05, Bise07, and Odo06) were selected and sequenced as they respectively represented one of the four RFLP patterns observed (Fig. 2) and thus representative of the total variation in the 16 strains. When the four 28S-rDNA sequences from this study were analyzed with previously reported 28S-rDNA sequences of *Symbiodinium* clades A–H, all four sequences were seen to belong to a highly supported *Symbiodinium* clade A *sensu* Rowan and Powers (1991) monophyly [Maximum Likelihood (ML) = 100%; data not shown].

The 16 *Symbiodinium* ITS rDNA sequences from this study fell into four "groups" (Fig. 3). The topology of the clade A phylogenetic tree based on ITS-rDNA (Fig. 3a) resembled the topology of the clade A 28S-rDNA tree (Fig. 3b).

The 14 strains from Oku were divided into two groups. Five strains (Oku05, 09, 11, 12, and 17) formed a very highly supported monophyly with *Symbiodinium* HA3-5 (free-living, AF184948) and *Symbiodinium* P082-2 (from *Amphisorus hemprichii*, AF184949) [ML = 100%, neighbor-joining (NJ) = 100%, Bayes posterior probability (B) = 1.00]. The remaining nine strains from Oku (Oku01, 02, 03, 04, 07, 08, 10, 15, and 16) formed another highly supported monophyly (ML = 100%, NJ = 87%, B = 1.00), separate from all other previously found *Symbiodinium* clade A types. Strain Bise07 formed a highly supported monophyly with *Symbiodinium* type

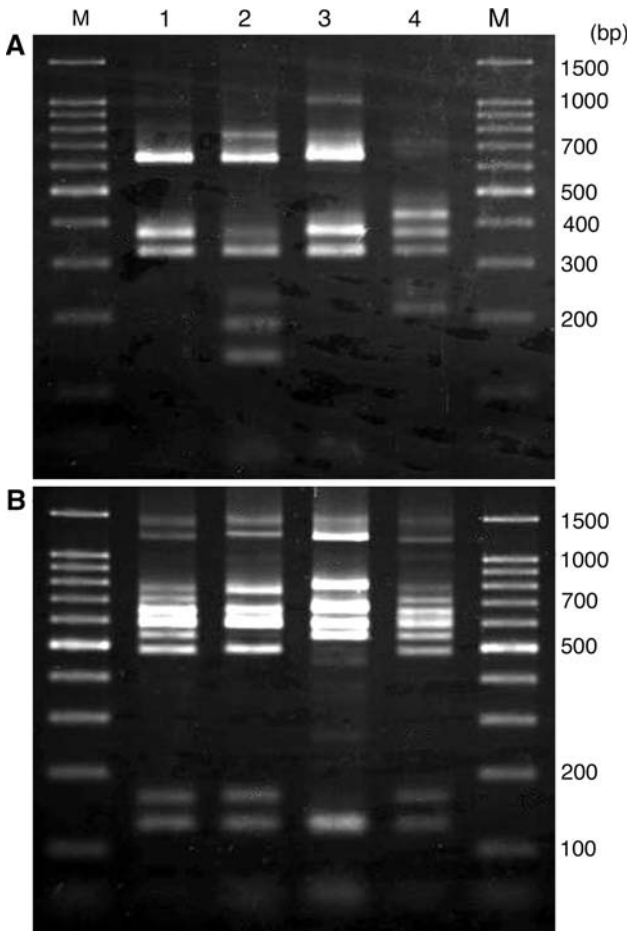

Fig. 2 RFLP genotyping of *Symbiodinium* strains employed in this study. PCR products of the partial 28S rDNA region (about 1,400 bp) were digested with either *Taq* I (**a**), or *Sau* 3A (**b**). Lanes at both ends of the gels labeled with M are DNA fragment size standards of a 100 bp DNA ladder. *Lane 1* Oku03, *Lane 2* Oku05, *Lane 3* Bise07, *Lane 4* Odo06

A2 isolated from *Zoanthus sociatus* in the Caribbean (AF333506 and AF427468) (ML = 99%, NJ = 99% B = 1.00). In the ITS-rDNA tree, strain Odo06 was part of a very highly supported monophyly including *Symbiodinium* types A1, A3, A4, and A5 (*sensu* LaJeunesse 2001) (ML = 100%, NJ = 100%, B = 1.00). This group contains *Symbiodinium* isolated from various hosts (e.g., Carlos et al. 1999; LaJeunesse 2001; Santos et al. 2002; Reimer et al. 2006).

Discussion

Why was only clade A *Symbiodinium* observed?

In this study, all 16 strains of *Symbiodinium* collected from coral reef sand samples all belonged to clade A, and these strains may not represent the true diversity of environmen-

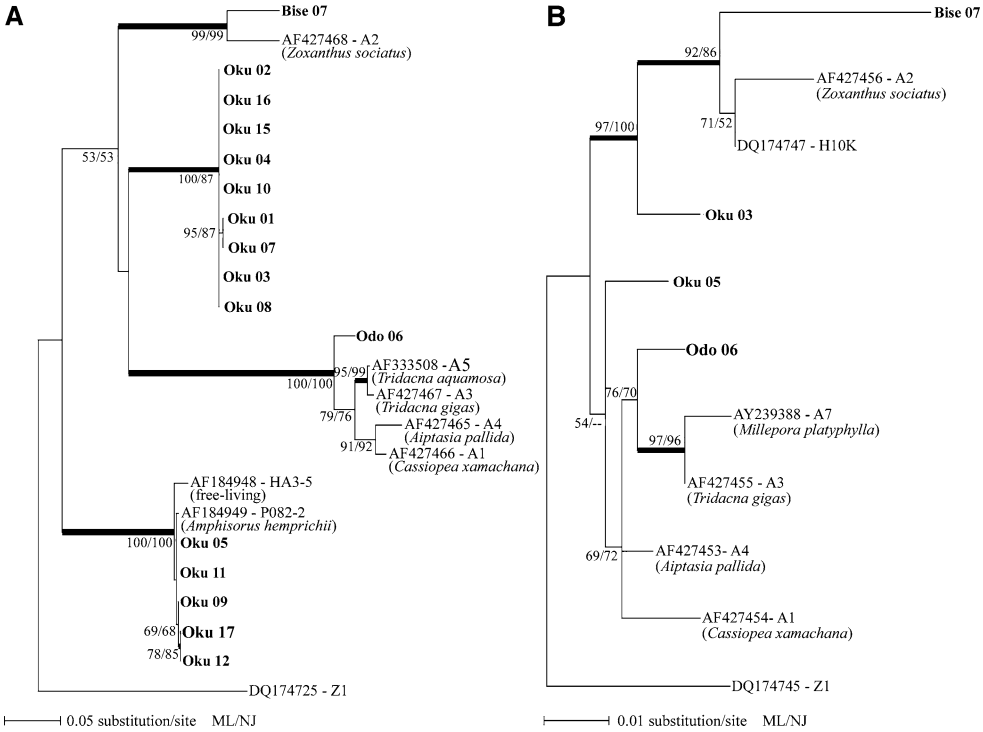

Fig. 3 **a** Maximum likelihood tree of obtained internal transcribed spacer of ribosomal DNA (ITS-rDNA) sequences of *Symbiodinium* clade A. **b** Maximum likelihood tree of obtained large subunit ribosomal DNA (28S rDNA) sequences of *Symbiodinium* clade A. Values at branches represent ML and NJ bootstrap probability, respectively (>50%). Monophylies with more the 95% Bayesian posterior probability are shown by thick branches. Isolates established in this study and their ITS-rDNA sequences are given in *bold* face. Sequences from previous studies are designated with accession numbers, followed by clade/subclade/strain identities, and host species (in parentheses)

tal populations of *Symbiodinium* in coral reef sand. Many scleractinian corals in the Indo-Pacific mainly associate with *Symbiodinium* clades C and D (e.g., Baker 2003; LaJeunesse et al. 2003, 2004a, 2004b; Little et al. 2004). The lack of other *Symbiodinium* clades besides clade A in this study may be due to other clades not being as readily culturable under normal default culture conditions. Ishikura et al. (2004) succeeded in the isolation of two new *Symbiodinium* strains belonging to clades C and D utilizing culture mediums containing giant clam tissue homogenate. To culture other *Symbiodinium* of various types in the future it will be necessary to further examine different culture conditions (e.g., varying growth mediums, light intensities, temperatures, etc.). These potential problems demonstrate that knowledge of the diversity of *Symbiodinium* in the environment is still in its nascent stage.

New strains of *Symbiodinium*

In this study, new *Symbiodinium* strains that were closely related to other clade A *Symbiodinium* found in a variety of hosts and environments were obtained from coral sand from three sampling locations in southern Japan. At least three previously unrecorded strains were cultured (Oku03, Bise07, and Odo06), and the novelty of these strains has not only been confirmed with widely-used ITS-rDNA (e.g., van Oppen et al. 2001; LaJeunesse 2001, 2004), but also with more conservative 28S rDNA sequences (e.g., Baker and Rowan 1997; Pochon et al. 2006). A monophyletic group was formed by five Oku strains (Oku05, 09, 11, 12, and 17) and two closely related strains (HA3-5 and PO82-2). HA3-5 was isolated from beach sand in Hawaii as a "free-living" strain, and P082-2 was isolated from foraminifera in Palau (*A. hemprichii*) (Carlos et al. 1999). Additionally, sequences of *Symbiodinium* from newly settled polyps of *Briareum* spp. and of those collected from reef rubble and from several grass bed and hard bottom sites in the Caribbean (Coffroth et al. 2006) were also within this monophyletic group. The differences between ITS-rDNA sequences within five Oku strains (Oku05, 09, 11,12, and 17) and between the same five Oku strains and PO82-2 were very low (0–2/529 total bp) whereas sequence differences between HA3-5 and the same five Oku strains were higher (7–9/529 bp). It is interesting that the *Symbiodinium* strains isolated from coral sand in Okinawa are closely related with a strain isolated from Palauan foraminifera and other "free-living" strains. If the number of sampling sites is increased, numbers of members of this interesting group may also increase.

Previous molecular studies investigating *Symbiodinium* spp. diversity in Ryukyu Islands have largely focused on Scleractinia. Previous studies on *Symbiodinium* associated with 71 scleractinian species from Okinawa (Loh et al. 2001; LaJeunesse et al. 2004b; Magalon et al. 2007; Suwa et al. 2008) showed that 67 coral species harbor mainly clade C *Symbiodinium*. On the other hand, reports of clade A *Symbiodinium* from Ryukyu Islands are relatively rare (Baillie et al. 2000; Ishikura et al. 2004; LaJeunesse et al. 2004b; Reimer et al. 2006; Magalon et al. 2007). In scleractinian species, *Pocillopora damicornis* from Bise (in the

northern part of Okinawa Island, 127°52′E, 26°42′N, see Fig. 1) was reported to harbor clade A (type A1) (Magalon et al. 2007). Among the strains established in this study, the strain Odo06 was most closely related to a group that included type A1, though it was not identical with type A1 (7.1% difference over the ITS-1 5.8S rDNA ITS-2 region compared to AF427466, see Fig. 3). LaJeunesse et al. (2004b) also reported that one mollusc from Zamami (*Tridacna* sp.) harbored *Symbiodinium* clade A (type A6). Two *Symbiodinium* clade A cultures have previously been established from Okinawa, Tc2FIZ (AF195146, Baillie et al. 2000) and OTcH (AB097464, Ishikura et al. 2004). Both strains originate from *Tridacna crocea* and are identical in sequence over the ITS-2 region (accession number AY686646) with *Symbiodinium* type A6 *sensu* LaJeunesse et al. (2004b). *Symbiodinium* type A6 was not identical to any other isolates in this study over the ITS-2 region (6.2, 27.4, 32.0, and 33.7% sequence differences from isolates Odo06, Oku 03, Oku 05, and Bise 07, respectively). Because there are few reports regarding *Symbiodinium* clade A in Okinawa, it is unclear whether the *Symbiodinium* types found in this study are harbored by living host animals in this area. Future studies should examine *Symbiodinium* from a variety of host animals (e.g., Hydrozoa (*Myrionema*), Anthozoa (*Aiptasia*), Zoantharia (*Palythoa*, *Isaurus*, *Zoanthus*), Octocorallia (soft coral), and Nudibranchia (*Pteraeolidia*) etc.).

Does clade A include "free-living" *Symbiodinium*?

It is possible that clade A includes many free-living strains as Coffroth et al. (2006) have demonstrated that the some *Symbiodinium* strains isolated from environment samples were capable of establishing symbioses with newly settled polyps of aposymbiotic cnidarians. However, they also reported that not all *Symbiodinium* spp. isolated from the environment were capable of establishing symbioses. They suggested that potentially both "free-living" and "non-symbiotic" *Symbiodinium* exist in the water column and benthic environment. However, it was not confirmed whether *Symbiodinium* isolates in this study were potentially symbiotic or not. Future studies should examine if these newly isolated *Symbiodinium* in this study can form symbioses with host species.

It has been shown that different *Symbiodinium* clades and/or types potentially have different physiologies (e.g., thermal sensitivity, cell growth, and photosystem II (PSII)) (Tchernov et al. 2004; Robinson and Warner 2006). This is likely one important reason that distribution patterns of symbiotic *Symbiodinium* are affected by several environmental factors (i.e., temperature and light intensity) (e.g., Rodriguez-Lanetty et al. 2001; Reimer et al. 2006). Therefore, in order to explore the widest possible diversity of *Symbiodinium* (both symbiotic and free-living), sampling over a wider geographical and environmental range and throughout the seasons is necessary. A broad sampling of *Symbiodinium* combined with usage of new culture techniques should help further our understanding of the potential diversity of free-living *Symbiodinium* in the future.

The construction of a clone library derived from environmental samples should also reveal more *Symbiodinium* diversity. However, to show that a reservoir of *Symbiodinium* exists in environment, it must be demonstrated that environmentally-derived isolates are able to establish symbioses with host animals. The cultured *Symbiodinium* cells established here will enable additional studies (e.g., morphological, physiological, etc.) and help clarify the diversity of the *Symbiodinium*.

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