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ORIGINAL PAPER

Preliminary analyses of cultured *Symbiodinium* isolated from sand in the oceanic Ogasawara Islands, Japan

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Abstract The dinoflagellate genus *Symbiodinium* is generally found in many tropical and subtropical marine invertebrates. Recently, reports have focused on free-living types. We examined free-living *Symbiodinium* from the Ogasawara (Bonin) Islands, a group of oceanic islands south of Japan. Examining sand samples, seven of eight initial isolates were successfully cultured. Genetic analyses of 18S, 28S and internal transcribed spacer (ITS) ribosomal DNA regions reveal that one isolate cultured with only IMK was identical to clade A isolated from coral reef sand in Okinawa, and four additional isolates cultured with only IMK comprised a new clade A lineage. Additionally, two isolates cultured with IMK and soil extract were closely related to a little-known divergent lineage within clade D. Our results demonstrate some free-living *Symbiodinium* types may have very wide distributions, and that utilizing different culturing techniques will further discovery of unique *Symbiodinium* lineages from environmental samples.

Keywords Zooxanthellae · *Symbiodinium* · Free-living · Ogasawara (Bonin) Islands · Sand

Introduction

Zooxanthellae are photosynthetic dinoflagellates found in symbioses with a wide variety of subtropical and tropical marine invertebrates, including reef-building corals (Rowan and Knowlton 1995), zoanthids (Reimer et al. 2006), giant clams and sponges (Carlos et al. 1999). Most zooxanthellae

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belong to the genus *Symbiodinium* (Order Sussiales) and, based on molecular phylogenies of the internal spacer of ribosomal DNA (ITS-rDNA), this genus has been shown to be highly diverse, with eight different "clades" of unknown taxonomic level each containing numerous "subclades" or "types" (Pochon et al. 2004). Many of these types have been demonstrated to specifically associate with different host species (LaJeunesse 2002). As well, many host organisms with *Symbiodinium* have been demonstrated to acquire new zooxanthellae every generation, and this horizontal acquisition may allow some host organisms the ability to acquire multiple different *Symbiodinium* types, allowing some physiological flexibility of the holobiont (host + symbiont) (LaJeunesse et al. 2004). Thus, an understanding of free-living *Symbiodinium* diversity, especially in light of climate change and associated bleaching, is critically important to coral reef marine science.

Despite horizontal acquisition, the presence of *Symbiodinium* outside of hosts (hereafter called "free-living") has only been confirmed and reported on very recently. *Symbiodinium* specimens have been isolated from the outside of marine algae and parrot fish feces in the Caribbean (Porto et al. 2008), seawater above coral reefs in Hawaii (Manning and Gates 2008) and sand from Hawaii (Carlos et al. 1999) and Okinawa, Japan (Hirose et al. 2008). Another recent study has shown that *Acropora monticulosa* coral larvae exposed to sand acquire a more diverse suite of different *Symbiodinium* clades and types compared with larvae exposed only to seawater (Adams et al. 2009). While much progress has been made, free-living *Symbiodinium* data are still limited and it is clear more investigations are needed to fully understand these enigmatic dinoflagellates.

The Ogasawara Islands (or Bonin Islands) are a chain of small oceanic islands that stretch over 1,000 km south of Tokyo (Fig. 1). Isolated from the rest of the Japanese Archipelago, the islands are also isolated from the effects of the warming Kuroshio Current, and are justifiably called the "Galapagos of the Orient" due to their unique terrestrial flora and fauna and extremely high levels of endemism. The marine fauna of the islands has been much less examined, although some subtropical coral communities exist (Tsuchiya et al. 2004). While the presence of coral communities and other coral reef animals is documented, no formal examination of the *Symbiodinium* diversity of these islands has yet been conducted.

Here, we have conducted the first examination of *Symbiodinium* diversity in the Ogasawara Islands to answer the following questions:

1. Are there free-living *Symbiodinium* in the sands of Ogasawara?
2. If so, how do free-living *Symbiodinium* in Ogasawara compare with that previously observed in Okinawa and other locations?

Materials and methods

Sampling

Symbiodinium specimens were isolated from coastal wet sand samples collected from Miyano-hama, Chichi-jima Island, Tokyo, Japan in May 2009 (Fig. 1). All sand samples (approximately 15 ml of sand in 40 ml of SW) were collected from sand near a local coral reef community at a depth of approximately 2 m with new and sterile plastic 50-ml tubes. At Miyano-hama some coral (mainly massive scleractinians, but also massive colonies of *Lobophyllia* and some acroporids (Tsuchiya et al. 2004) and zoanthids (*Zoanthus* and *Palythoa* spp.; JD Reimer and F Sinniger, unpublished data)) were observed, but the sand was collected from points at least 3 m away from benthic cnidarians. Sand was collected from a depth of approximately 1 cm below the sea floor surface. Collected samples were transported to the University of the Ryukyus, Okinawa, and 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 mainly 0.2–2 mm. Each petri dish contained between 2 and 5 ml of sediment.

Symbiodinium isolation and culture conditions

Isolation generally followed procedures outlines in Andersen and Kawachi (2005). Sand samples were enriched by 20 ml IMK medium (Nippon Pharmaceutical Co.) + S medium (for 1 l IMK medium containing 5 ml of soil extact (Kasai et al. 2009). Enrichment culture was started on May 15, 2009 and isolations were made on May 21, 2009. Unialgal clonal strains were established from cultures by single cell isolation using micropipettes. Single cells were inoculated into screw-capped glass tubes (150 mm × 15 mm diameter) that contained 8 ml of medium. A total of eight individual *Symbiodinium*-like swimming dinoflagellate cells were randomly picked up by micropipettes after 7 days' incubation of coral sand samples from Chichi-jima Island, and seven unialgal strains were successfully established. The strains were subcultured with natural seawater based IMK medium or IMK + S medium and maintained in 24 ± 2°C, under a 14/10 h light/dark cycle at approximately 40 µmol photons $m^{-2}s^{-1}$ provided by cool-white fluorescent lamps.

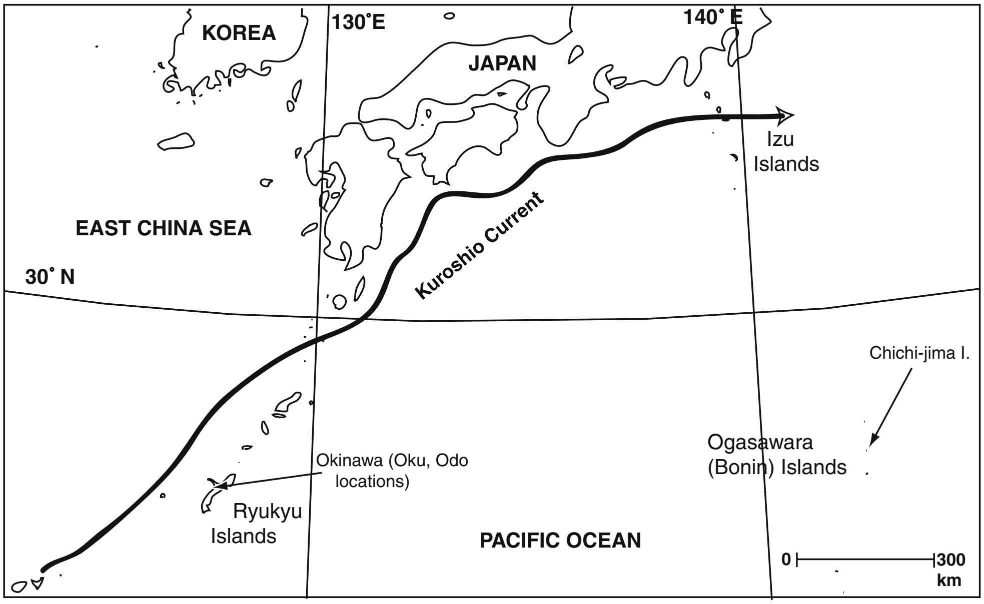

Fig. 1 Map showing the position of Ogasawara Islands and the sampling location for the current study, and Okinawa (referred to in Hirose et al. 2008)

DNA extraction, PCR amplification and sequencing

Algal cells from 1-month-old clonal cultures of each strain were harvested. DNA extractions were made by a DNeasy Plant Minikit (QIAGEN Sciences, Md., USA) and purified with a Geneclean II Kit (MP Biomedicals, LLC, Ohio, USA) according to the manufacturer's instructions. Amplification of 18S, 28S and ITS-rDNAs was obtained using 10–100 ng total genomic DNA, with primers and PCR cycles according to Takano and Horiguchi (2005). The PCR cycles were: 93°C for 1 min, followed by 35 cycles of denaturation at 93°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min (Takano and Horiguchi 2005). Subsequently, the ITS-rDNA (ITS-1, 5.8S rDNA, ITS-2) region of seven *Symbiodinium* strains was sequenced to examine their phylogenetic position at a higher resolution. The purified PCR-amplified DNA fragments were cloned into TOPO TA Cloning Kit (Invitrogen, Carlsbad, Calif., USA). Cycle sequencing reactions were carried out using DTCS Quick Start Master Mix (Beckman Coulter) and products (three clones per sample; see Table 1) were analyzed using a CEQ8800 (Beckman Coulter) automated DNA sequencing system.

Phylogenetic analyses

The sequences derived from this study as well as other sequences used in alignments are listed in Table 1. New sequences obtained in the present study were deposited in GenBank (accession numbers HM042367-HM042381 and HM042387-HM042402). Previously reported 18S rDNA, 28S rDNA and ITS-rDNA sequences of all *Symbiodinium* clades, including free living strains (Table 1), were retrieved from the DNA Data Bank of Japan (DDBJ) and were aligned with our present 18S rDNA, 28S rDNA and ITS-rDNA data using ClustalW. For ITS-rDNA, two alignments were made, a "global" alignment including sequences from all known clades (based on an alignment from the Santos Laboratory at Auburn University's homepage; http://www.auburn.edu/~santosr/sequencedatasets.htm), and a "clade A only" alignment. 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 18S rDNA, 28S rDNA, "global" ITS-rDNA and "clade A only" ITS-rDNA (18S rDNA=23 taxa/898 sites; 28S rDNA=25 taxa/424 sites; "global" ITS-rDNA=56 taxa/339 sites; "clade A only" ITS-rDNA=28 taxa/264 sites) are available on request from the corresponding author.

For the phylogenetic analyses of all alignments the same maximum likelihood (ML), Bayesian, and neighbor joining (NJ) (Saitou and Nei 1987) methods were independently applied. The "clade A only" ITS-rDNA alignment was additionally subjected to a maximum-parsimony (MP) analysis conducted in PAUP* Version 4.0 (Swofford 2000) with indels treated as a fifth character state. For all alignments, 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

Table 1 List of *Symbiodinium* and outgroup 18S rDNA, 28S rDNA, and ITS-rDNA sequences and GenBank accession numbers for phylogenetic tree reconstruction, with sequences from this study in **bold** (*NA* not available)

Clade/ subclade	Isolation source (host organism)	Geographic origin	Strain	18S rDNA accession number	28S rDNA accession number	ITS-rDNA accession number	References
A	No data	No data	Z1		DQ174745		Moore 2006
A	No data	No data	H10K		DQ174747		Moore 2006
A	Coral reef sand	Okinawa	Oku03		EU106353	EU106353	Hirose et al. 2008
A	Coral reef sand	Okinawa	Oku05		EU106355	EU106355	Hirose et al. 2008
A	Coral reef sand	Okinawa	Odo06		EU106365	EU106365	Hirose et al. 2008
A	Coral reef sand	Okinawa	Bise07		EU106366	EU106366	Hirose et al. 2008
A	Coral reef sand	Okinawa	Oku01			EU106351	Hirose et al. 2008
A	Coral reef sand	Okinawa	Oku02			EU106352	Hirose et al. 2008
A	Coral reef sand	Okinawa	Oku04			EU106354	Hirose et al. 2008
A	Coral reef sand	Okinawa	Oku07			EU106356	Hirose et al. 2008
A	Coral reef sand	Okinawa	Oku08			EU106357	Hirose et al. 2008
A	Coral reef sand	Okinawa	Oku09			EU106358	Hirose et al. 2008
A	Coral reef sand	Okinawa	Oku10			EU106359	Hirose et al. 2008
A	Coral reef sand	Okinawa	Oku11			EU106360	Hirose et al. 2008
A	Coral reef sand	Okinawa	Oku12			EU106361	Hirose et al. 2008
A	Coral reef sand	Okinawa	Oku15			EU106362	Hirose et al. 2008
A	Coral reef sand	Okinawa	Oku16			EU106363	Hirose et al. 2008
A	*Amphisorus hemprichii*	Palau	PO82-2			AF184949	Baillie et al. 2000
A	Sand	Hawaii	HA3-5			AF184948	Baillie et al. 2000
A	**Sand**	**Ogasawara**	**Oga01**			**HM042393**	**This study**
			Oga01-c1	**HM042374**			
			Oga01-c2				
			Oga01-c3	**HM042375**			
A1	*Anemonia* sp.	E. Med. Sea	A1med			DQ865210	Hunter et al. 2007
A1	*Cassiopea xamachana*	Jamaica	Cx		AF427454	AF427466	Santos et al. 2002
A2	*Zoanthus sociatus*	Jamaica	Zs		AF427456	AF427468	Santos et al. 2002
A3	*Tridacna gigas*	Indo-Pacific	T		AF427455	AF427467	Santos et al. 2002
A4	*Aipstasia pallida*	Florida Keys	FLA#4		AF427453	AF427465	Santos et al. 2002
A7	*Millepora platyphylla*	GBR	A7		AY239388		LaJeunesse et al. 2003
Novel A	**Sand**	**Ogasawara**	**Oga03**			**HM042396**	**This study**
			Oga03-c1	**HM042371**			
			Oga03-c2	**HM042376**			
			Oga03-c3				
Novel A	**Sand**	**Ogasawara**	**Oga04**			**HM042397**	**This study**
			Oga04-c1				
			Oga04-c2				
			Oga04-c3	**HM042377**			
Novel A	**Sand**	**Ogasawara**	**Oga05**			**HM042395**	**This study**
			Oga05-c1	**HM042378**			
			Oga05-c2	**HM042379**			
			Oga05-c3	**HM042380**			
Novel A	**Sand**	**Ogasawara**	**Oga08**			**HM042394**	**This study**
			Oga08-c1	**HM042372**			
			Oga08-c2	**HM042381**			
			Oga08-c3	**HM042373**			
B	NA	NA	Culture702			EU449065	in Correa & Baker 2009

Table 1 (continued)

Clade/ subclade	Isolation source (host organism)	Geographic origin	Strain	18S rDNA accession number	28S rDNA accession number	ITS-rDNA accession number	References
B	*Aipstasia pulchella*	Taiwan	6-2		GQ984273		Keshavmurthy et al. unpubl.
B4	*Anthopleura elegantissima*	E. Pacific	B4			AF333510	LaJeunesse 2001
B11	NA	NA	B11			AY239361	in Hunter et al. 2007
B19	*Briareum asbestinum*	Florida	B19			AF360560	Santos et al. 2001
B21	*Briareum* sp.	Florida	B21			DQ865213	Hunter et al. 2007
C	*Pocillopora* sp.	Mozambique	Clone 12	EF419283			Ruiz Sebastian et al. 2009
C	*Euphyllia ancora*	Taiwan	EAK47-3		GQ984275		Keshavmurthy et al. unpubl.
C	NA	Australia	Ulstrup		EF205017		Moestrup & Daugbjerg 2007
C22	*Lobophyllia corymbosa*	GBR	C22			AY239373	LaJeunesse et al. 2003
C31	*Montipora turtlensis*	GBR	C31			AY258496	LaJeunesse et al. 2004
C39	*Mussidae, Diploastrea*	Red Sea	C39			AY258484	LaJeunesse 2005
C49	*Mycetophyllia*	W. Caribbean	C49			AY589754	LaJeunesse 2005
C67	*Heteractis magnifica*	GBR	C67			AY686647	LaJeunesse et al. 2004
C69a	*Stichodactyla gigantea*	GBR	C69a			AY589773	LaJeunesse et al. 2004
D	*Montastraea franksi*	Panama		DQ838542			Garren et al., 2006
D	*Marginopora kudakajimensis*	Japan		U10893			Lee et al. 1995
D	*Pocillopora* sp.	Mozambique	AF30	EF419281			Ruiz Sebastian et al. 2009
D	*Haliclona koremella*	Palau	PSP1-05	AB016578			Carlos et al. 1999
D	*Haliclona koremella*	Palau	PSP1-05		AJ308899		Pochon et al. 2001
D	*Marginopora vertebralis*	Guam	GIII86_2189x		AJ830923		Garcia et al. unpubl.
D	*Isopora palifera*	Taiwan	IP740-4		GQ984265		Keshavmurthy et al. unpubl.
D	*Palythoa mutuki*	Singapore	K11			EU333714	Reimer & Todd 2009
D	*Entacmaea quadricolor*	Okinawa	Ua#2			AF396631	Santos et al. 2003
D	*Corculum cardissa*	Okinawa	Cc-19-07			AB294667	Kii et al. unpubl.
D	*Acropora* sp.	Guam	1655			AJ311948	Pochon et al. 2001
D1a	cultured	NA	D1a_2040			EU074900	Thornhill et al. 2007
D1a	cultured	NA	D1a_2068			EU074906	Thornhill et al. 2007
D4	*Lithophyton* sp.	Red Sea	D4			DQ865214	Hunter et al. 2007
Novel D	**Sand**	**Ogasawara**	**Oga02-c1**^{a}	**HM042367**	**HM042402**	**HM042389**	**This study**
			Oga02-c2^{a}	**HM042368**	**HM042401**	**HM042390**	

Table 1 (continued)

Clade/ subclade	Isolation source (host organism)	Geographic origin	Strain	18S rDNA accession number	28S rDNA accession number	ITS-rDNA accession number	References
Novel D	**Sand**	**Ogasawara**	**Oga02-c3**[a]			**HM042387**	**This study**
			Oga06[a]			**HM042392**	
			Oga06-c1[a]	**HM042370**	**HM042400**	**HM042391**	
			Oga06-c2[a]		**HM042399**		
			Oga06-c3[a]	**HM042369**	**HM042398**	**HM042388**	
E	*Montastraea faveolata*	Caribbean	E:0–1	AF238261			Toller et al. 2001
E	*Montastraea faveolata*	Caribbean	E:0–2	AF238262			Toller et al. 2001
E	*Siderastrea siderea*	Caribbean	E:0–3	AF238263			Toller et al. 2001
E	*Anthopleura elegantissima*	E. Pacific	E #383			AF334659	LaJeunesse 2001
F	*Amphisorus hemprichii*	Australia	Ro39_2093x		AJ830925		Garcia et al. unpubl.
F	*Montipora verrucosa*	Culture 135	F1			AF333517	LaJeunesse 2001
G	*Amphisorus* sp.	Guam	1582			AJ291537	Pawlowski et al. 2001
H	*Sorites* sp.	Guam	1678J		AJ291520		Pawlowski et al. 2001
H	*Sorites* sp.	E. Pacific, Caribbean	Fr1			AJ291513	Pochon et al. 2004
Gymnodium	NA	NA	CCMP423		EF205004		Moestrup & Daugbjerg 2007

[a] Nomenclature of strains from this study: Cultures were numbered Oga01 to Oga08. For novel clade A and D strains, cloning was performed; these are numbered with "-c1" for clone 1, etc. Strains with no such endings are products of direct sequencing

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 plots for the two datasets suggested that MCMC reached the stationary phase after the first 100,000 generations (18S rDNA) and 30,000 generations (28S rDNA, "global" ITS-rDNA, and "clade A only" ITS-rDNA) [potential scale reduction factors (PSRF)=1.008, 1.000, 1.000, and 1.009, respectively]. Thus, the remaining 9,000, 9,700, 9,700 and 9,700 trees were used to obtain clade probabilities and branch-length estimates, respectively.

The NJ method 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

18S rDNA analyses

Figure 2 shows the ML phylogenetic tree for the aligned 18S rDNA sequences. Novel sequences from cultured specimens isolated from the sands of Ogasawara (Oga02-c1, Oga02-c2, Oga06-c1 and Oga06-c3) clustered with AB016578, a *Symbiodinium* type isolated from a sponge (Carlos et al. 1999), and this group formed a moderately well supported subclade (ML=88%, NJ=91%, but Bayes=0.50), sister to another more weakly supported suclade (ML=66%, Bayes=0.54, NJ=67%) containing four sequences (EF419281, AF238261, AF238262, AF238263) from clade D + E (*sensu* Toller et al. 2001) *Symbiodinium*. These two groups formed a weakly supported monophyly (ML=65%, Bayes≤0.50, NJ=69%) separate from other clade D (DQ838542 and U10893) and clade C (EF419283) *Symbiodinium* 18S rDNA sequences. All of these sequences were sister to a completely supported clade (ML=100%, Bayes=1.00, NJ=100%) formed by clones from Oga01, Oga03, Oga04, Oga05, and Oga08. Within this clade some subclades were observed, with weak to strong support.

28S rDNA analyses

Figure S1 shows the ML phylogenetic tree for the aligned 28S rDNA sequences. Novel sequences from this study formed a weakly to moderately supported subclade (ML=80%, Bayes=0.82, NJ=59%) within clade D *Symbiodi-*

nium. Clade D, which also contained sequences AJ308899, AJ830923, and GQ984265, was a moderately supported monophyly (ML=75%, Bayes=0.80, NJ=94%) within the *Symbiodinium* radiation, sister to clades B, C, F, and H, which altogether were sister to clade A.

"Global" ITS-rDNA analyses

Figure S2 shows the ML phylogenetic tree for the aligned "global" ITS-rDNA sequences. Some novel ITS-rDNA sequences from this study (Oga02 and Oga06 sequences) formed a totally supported monophyly (ML=100%, Bayes=1.00, NJ=100%) sister to a group of sequences including other *Symbiodinium* clade D and one clade G sequence. However, overall the grouping of clades D and G as well as our novel sequences, despite forming a monophyly, was not well supported (ML=57%, Bayes≤ 0.50, NJ≤50%). Clades B, C, D, F, G and H formed a very well supported (ML=99%, Bayes=0.99, NJ=100%) monophyly separate from a group that included *Symbiodinium* clades A and E. Several other novel sequences acquired in this study were placed within clade A. One sequence (Oga01) was within a moderately well supported clade (ML=83%, Bayes=0.98, NJ=100%) that included free-living isolates from Okinawa as well as AF184948 and AF1841949, while four other sequences (Oga03, Oga04, Oga05, Oga08) formed a totally supported novel subclade (ML=100%, Bayes=1.00, NJ=100%) within clade A.

"Clade A only" ITS-rDNA

Figure 3 shows the MP phylogenetic tree for the aligned "clade A only" ITS-rDNA sequences. One sequence (Oga01) was within a well supported clade (ML=98%, Bayes=0.94, NJ=99%) that included free-living isolates from Okinawa as well as AF184948 and AF1841949, while

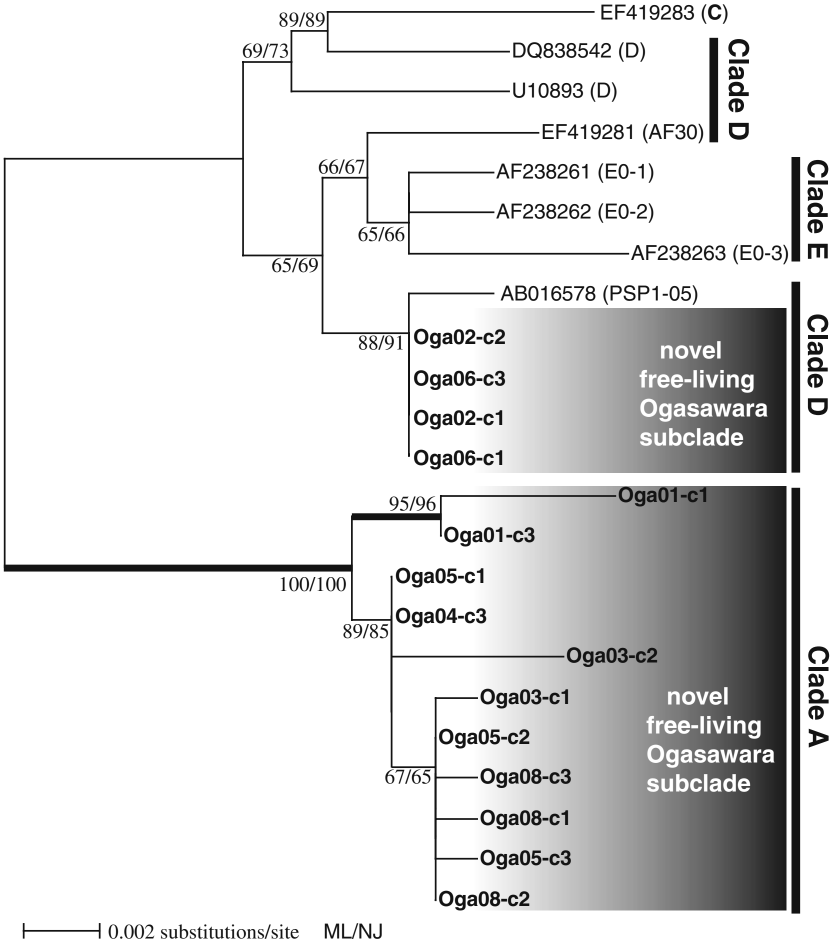

Fig. 2 ML tree of 18S rDNA sequences for *Symbiodinium* including specimens from this study. Values at branches represent ML and NJ bootstrap probabilities, respectively. Monophylies with more than 95% Bayesian posterior probabilities are shown by *thick branches*. New isolates and sequences from this study in *bold*. Sequences used in the alignment for this tree are in Table 1

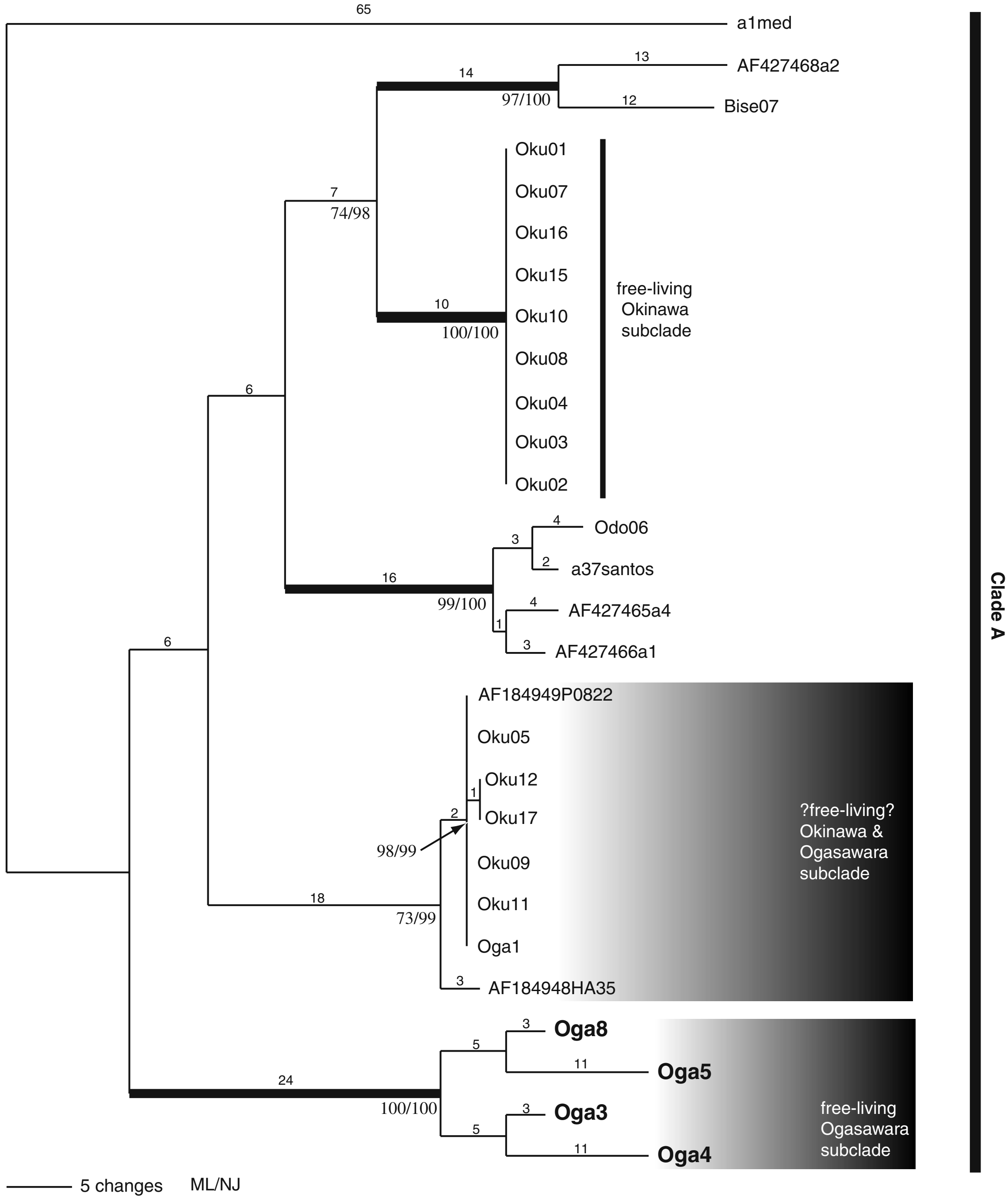

Fig. 3 MP tree of the ITS-rDNA sequences for clade A *Symbiodinium*, including specimens from this study. *Values above branches* represent the minimum number of changes. *Values below branches* represent ML and NJ bootstrap probabilities, respectively. Monophylies with more than 95% Bayesian posterior probabilities are shown by *thick branches*. New isolates and sequences from this study in *bold*. Sequences used in the alignment for this tree are in Table 1

four other sequences (Oga03, Oga04, Oga05, Oga08) formed a totally supported new novel subclade (ML=100%, Bayes=1.00, NJ=100%) within clade A.

Overall, despite the number of taxa for the three markers being disparate, no major topological differences between the *Symbiodinium* clades in phylogenetic trees were seen, excepting the placement of the novel D subclade (placed separately from all other sequences in the "global" ITS-rDNA topology), and results were in general agreement with *Symbiodinium* phylogenies in other recent papers showing eight clades (e.g., Pochon et al. 2004).

Discussion

Although many recent reports on free-living *Symbiodinium* have appeared in literature (Table 2), our overall knowledge of them is scarce when compared with the numerous studies performed on *Symbiodinium* isolated from invertebrate hosts. This study is the second report after Manning and Gates (2008) from Hawaii to demonstrate the existence of free-living *Symbiodinium* on oceanic islands. Both of these studies along with other literature (Table 2), clearly show that the presence of free-living *Symbiodinium* is undoubtedly common in shallow tropical and sub-tropical waters, and our knowledge of them is hindered primarily by both a lack of studies and difficulties in successfully establishing cultures.

This study is the second demonstration of putative *Symbiodinium* clade D *sensu* LaJeunesse (2002) found from non-host samples. However, the clade D specimens found here are different from D1 found in Manning and Gates (2008), and are most similar to a divergent type reported from a sponge in Carlos et al. (1999). These unusual types likely represent a new and previously unknown *Symbiodinium* lineage. This is particularly demonstrated by the ITS-rDNA phylogenetic analyses, which show the novel sequences as clearly divergent from all known clade D types. In fact, as shown in Fig. S2, clade D *Symbiodinium* does not form a monophyly in ITS-rDNA analyses, although it is monophyletic in the more limited 18S and 28S rDNA analyses. Further phylogenetic analyses, in particular of 18S and 28S rDNA, as well as cp23 and other recently reported molecular markers utilized in *Symbiodinium* phylogenies, will help place the novel D isolates from Ogasawara more definitively within *Symbiodinum* at either the subclade or clade level.

Our observation of another additional novel group of free-living *Symbiodinium* in clade A (isolates Oga03, Oga04, Oga05, Oga08) further demonstrates that it is very likely many new novel free-living *Symbiodinium* types await discovery. Another Ogasawara isolate (Oga01) was identical or nearly identical to previously reported potentially free-living clade A *Symbiodinium* from coral sands in Okinawa, demonstrating that at least this type of *Symbiodinium* has a wide distribution and can reach oceanic islands. Overall, it remains to be seen if the two novel subclades of free-living *Symbiodinium* observed here are endemic to oceanic Ogasawara, or are more widely distributed such as the Okinawa/Ogasawara subclade. Investigations into *Symbiodinium* diversity at other isolated locations may help shed light on this topic.

Additionally, as shown in Table 2, it appears that there are many *Symbiodinium* clades that do have either some free-living types, or types that can be found outside of

Table 2 Summary of research into free-living *Symbiodinium* in previous studies compared to results of this study (in **bold**)

Reference	Free-living type(s) detected	Location	Substrates isolated from	Notes
Carlos et al. 1999	A	Hawaii	Coral reef sand sediment	
Baillie et al. 2000	A	Hawaii	Coral reef sand sediment	
Gou et al. 2003	E	China	Water column	Originally placed in clade "F"
Coffroth et al. 2006	A	Florida Keys	Coral reef sand sediment	
Koike et al. 2007	unknown	Ishigaki Island, Okinawa	Water column (surface + above sand)	Focused on detection methodology
Porto et al. 2008	A, B184, C	Colombia	Coral reef sand sediment, macroalgae (*Halimeda*, *Lobophora*, *Amphiroa*, *Dictyota*)	Parrotfish theorized as *Symbiodinium* dispersers
Hirose et al. 2008	A	Okinawa	Coral reef sand sediment	Four types detected
Littman et al. 2008	Not given	GBR, Australia	Coral reef sand sediment, water column	Focused on detection methodology
Manning and Gates 2008	B1, C3, C15, C21, D1, H, novel B, C	Hawaii, Mexican Caribbean	Water column	
Adams et al. 2009	A (inferred)	Okinawa Island	Coral reef sand sediment	Based on experiments of larval uptake of *Symbiodinium*
This study	**A, novel A, D subclades**	**Ogasawara Islands**	**Subtropical sand sediment**	**D subclade matches closely with isolate from sponge in Carlos et al. (1999)**

hosts. Previously, it was theorized by LaJeunesse that clade A2 (*Symbiodinium pilosum* Trench and Blank 1987; corresponds to AF427456 in Fig. 2 and AF427468 in Fig. S2) could either be a free-living strain or form cryptic populations in hosts due to having never been found in *Symbiodinium* surveys (LaJeunesse 2001, 2002). In this study, clade A2 appears to be very closely related to putative free-living specimens from Okinawa described in Hirose et al. (2008). Thus, we suggest that most or all clades (A–H) have types that can occasionally be found in the water column or sand. It remains to be seen if symbiotic *Symbiodinium* types are commonly found outside of their hosts, as suggested by Porto et al. (2008), who suggested dispersal by parrotfish as a possible mechanism to explain the spread of *Symbiodinium*.

In the future, a combination of sampling of various coral reef substrates (sand, macroalgae, water column) on a global scale will help ascertain more accurate estimates of free-living *Symbiodinium* diversity and numbers. Culture-independent methods, such as metagenomic studies, are often utilized in examining the diversity of *Symbiodinium* but, as demonstrated here with the finding of a unique type of Clade D through a different culture technique (IMK + S), the application of various culturing techniques is equally important in order to successfully establish clonal lineages. The utilization of culture-focused techniques will allow more biochemical and physiological research of *Symbiodinium* to be conducted. Overall, the continued combination of wide-ranging sampling and culturing will undoubtedly lead us to a more complete understanding of free-living *Symbiodinium*.

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