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Specificity in communities of *Symbiodinium* in corals from Johnston Atoll

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ABSTRACT: The diversity of endosymbiotic dinoflagellates (*Symbiodinium*) in corals at Johnston Atoll in the central Pacific Ocean was assessed using both the internal transcribed spacer 2 (ITS2) region of the nuclear rDNA and chloroplast 23S rDNA. More sequences were recovered from corals using the ITS2 primers than with the chloroplast 23S primers, a finding that reflects both the higher taxonomic resolution and level of intragenomic variation in ITS2 in eukaryotes as compared to chloroplast 23S. Parsimony network analysis, Bray-Curtis coefficient of similarity and 1-way analysis of similarity resolved coral species- and/or genus-specific lineages and/or groupings of *Symbiodinium* that were generally congruent between the 2 genetic markers. Comparison of coral–*Symbiodinium* assemblages at Johnston Atoll with those in corals sampled on other reefs in the Pacific reveals differences that include novel host–symbiont unions and a *Symbiodinium* lineage previously reported to be Caribbean-specific in *Acropora* from Johnston Atoll.

KEY WORDS: *Symbiodinium* · Coral · Dinoflagellate · Symbiosis · ITS2 · Chloroplast

INTRODUCTION

Dinoflagellates in the genus *Symbiodinium* are found in symbiosis with a diverse range of marine invertebrate taxa and some protists (Lobban et al. 2002, reviewed in Coffroth & Santos 2005, Stat et al. 2006). In corals, *Symbiodinium* reside inside host gastroderm cells and supply the animal with organic carbon fixed via photosynthesis, a resource that underpins the growth and formation of coral reefs (Muscatine et al. 1981). The *Symbiodinium* genus is currently differentiated into 8 phylogenetic groups, clades A to H, based on nuclear 18S and 28S rDNA (Rowan & Powers 1991, Carlos 1999, LaJeunesse & Trench 2000, LaJeunesse 2001, Pawlowski et al. 2001, Pochon et al. 2001, 2004) and the chloroplast 23S domain V (Santos et al. 2002, Pochon et al. 2006). Each of these clades is further divided into subclade genetic types based on more variable markers such as the internal transcribed spacer regions of the nuclear rDNA (ITS1 and ITS2, e.g. van Oppen et al. 2001, 2005, LaJeunesse 2002, 2005, LaJeunesse et al. 2003, 2004a, Pochon et al. 2007). Patterns in *Symbiodinium* subclade types associate with biogeography, host species, light environment and *Symbiodinium* acquisition strategy (e.g. Rowan & Knowlton 1995, Baker et al. 1997, Rowan et al. 1997, Rodriguez-Lanetty et al. 2001, Ulstrup & van Oppen 2003, Iglesias-Prieto et al. 2004, LaJeunesse 2005, Stat et al. 2008a). The taxonomy of *Symbiodinium* and the specificity of the association between hosts and *Symbiodinium* clades and subclade types can often explain differences in coral physiology such as growth rate, thermal tolerance, photophysiology and bleaching and disease susceptibility (Rowan & Knowlton 1995, Rowan et al. 1997, Baker et al. 2004, Little et al. 2004, Rowan 2004, Tchernov et al. 2004, Sampayo et al. 2008, Stat et al. 2008b, Correa et al. 2009).

The eukaryotic nuclear rDNA region is repeated multiple times within a genome (e.g. the dinoflagellate *Prorocentrum micans* contains 200 copies; Long & Dawid 1980, Herzog & Maroteaux 1986). The ITS regions of the rDNA are more variable than the 18S,

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28S and 5.8S regions and are often used for distinguishing more closely related taxa (Coleman 2003); however, the rapid rate of evolution in the ITS regions combined with high levels of repetition in rDNA drive intragenomic and intraspecific variation (Adachi et al. 1996, Le Blancq et al. 1997, Lott et al. 1998, Coleman 2003, Denboh et al. 2003, Litaker et al. 2003, 2007, Orsini et al. 2004, Rynearson & Armbrust 2004, Shankle et al. 2004, Godhe et al. 2006, Aktas et al. 2007, Chen et al. 2007, Kawahata et al. 2007, Cantacessi et al. 2008). Intragenomic variation has been demonstrated in cultured *Symbiodinium* cells and is likely a confounding factor in accurately assessing diversity in symbiotic communities (Thornhill et al. 2007). An endosymbiotic lifestyle may also influence mutation rates for *Symbiodinium* as compared to free-living dinoflagellates, resulting in less divergence between species (Litaker et al. 2007). The evolutionary time required for concerted evolution to act on the genome and patterns in the ecological distribution of some *Symbiodinium* ITS2 types have been used as evidence to suggest that the ITS2 provides species-level taxonomic resolution (e.g. LaJeunesse 2001, Sampayo et al. 2009). However, whether single base changes within the dominant sequence across the ITS2 region of the rDNA consistently represent different *Symbiodinium* species remains contentious and clusters of closely related sequences have also been interpreted as ecotypes (Correa & Baker 2009). In other species of diatoms and dinoflagellates, patterns in the biogeography and ecology of different ITS2 sequences have been interpreted as variation at the subspecies level (Rynearson & Armbrust 2004, Shankle et al. 2004, Godhe et al. 2006). This genetic diversity and accompanying physiological diversity within a species is explained as plasticity that allows the species to occupy a wide geographic and environmental gradient.

Genes in the chloroplast genome of dinoflagellates exist on individual plastid minicircles (Gray 1999, Zhang et al. 1999, Green 2004). The copy number of these minicircles is unknown; however, some dinoflagellates show evidence of multiple copies (Zhang et al. 2002, Koumandou & Howe 2007). The chloroplast 23S rDNA is considered to have a relatively fast rate of DNA evolution and is therefore a potentially useful marker for investigating *Symbiodinium* diversity; however, to date it has not been applied broadly to the genus (but see Santos et al. 2002, Pochon et al. 2006).

In the present study, the ITS2 and chloroplast 23S rDNA markers were used to explore *Symbiodinium* diversity in corals from Johnston Atoll. Johnston Atoll, situated southwest of the main Hawaiian Islands in the central Pacific Ocean, represents one of the most isolated reef ecosystems in the world and an area where *Symbiodinium* diversity has not been examined before. The aims of the present study were to: (1) assess the diversity of *Symbiodinium* in 12 coral species using ITS2 and chloroplast 23S, (2) examine host specificity in coral–*Symbiodinium* associations from this area, (3) compare the *Symbiodinium* diversity recovered from corals at Johnston Atoll with the diversity found in corals elsewhere in the Pacific, and (4) compare the utility of the ITS2 and chloroplast 23S markers in assessing *Symbiodinium* diversity and patterns of specificity.

MATERIALS AND METHODS

Sample collection. Five colonies of each of 12 coral species (n = 60 colonies) were sampled from 9 sites around the remote coral reef ecosystem at Johnston Atoll in June 2006 (Fig. 1, Table 1). Coral fragments (≈2 mm of tissue) were stored in 400 µl of DNA extraction buffer (50% w/v guanidinium isothiocyanate; 50 mM Tris pH 7.6; 10 µM EDTA; 4.2% w/v sarkosyl; 2.1% v/v β-mercaptoethanol) until further processing.

DNA extraction, PCR, cloning and sequencing. For extraction of nucleic acids, samples were incubated at 72°C for 10 min, centrifuged at 16 000 × *g* for 5 min and the resulting supernatant mixed with an equal volume of isopropanol and incubated at –20°C overnight. The DNA was precipitated by centrifugation at 16 000 × *g* for 15 min and the DNA pellet washed in 70% ethanol and resuspended and stored in Tris buffer (0.1 M pH 9).

Partial 5.8S, the entire ITS2 and partial 28S rDNA genes in *Symbiodinium* were amplified in PCR using itsD (Pochon et al. 2001; forward; 5′-GTG AAT TGC AGA ACT CCG TG-3′, 5 pmol) and its2rev2 (reverse; 5′-CCT CCG CTT ACT TAT ATG CTT-3′, 5 pmol) primers and 0.5 U of Immolase (Bioline) in a 25 µl reaction using the following cycling conditions: denaturation at 95°C for 7 min followed by 35 cycles of 45 s at 95°C, 45 s at 52°C, 45 s at 72°C and a final extension at 72°C for 5 min. The 23S-rDNA domain V region of the *Symbiodinium* chloroplast was amplified in PCR using the 23S1 (forward; 5′-GGC TGT AAC TAT AAC GGT CC-3′,

Table 1. Collection sites at Johnston Atoll where corals were sampled for *Symbiodinium* genotyping

Site	Depth (m)	Latitude	Longitude
JA-HIMB3	3–7	16°44.576 N	169°32.269 W
JOH-1AP	15–20	16°47.162 N	169°27.710 W
JOH-20	5–10	16°45.670 N	169°31.618 W
JOH-22	10–15	16°45.858 N	169°31.535 W
JOH-23	2–4	16°44.838 N	169°30.559 W
JOH-25	7–12	16°46.615 N	169°29.502 W
JOH-4P	3–7	16°46.260 N	169°30.336 W
JOH-5P	2–4	16°46.449 N	169°29.821 W
JOH-6P	3–7	16°43.111 N	169°33.080 W

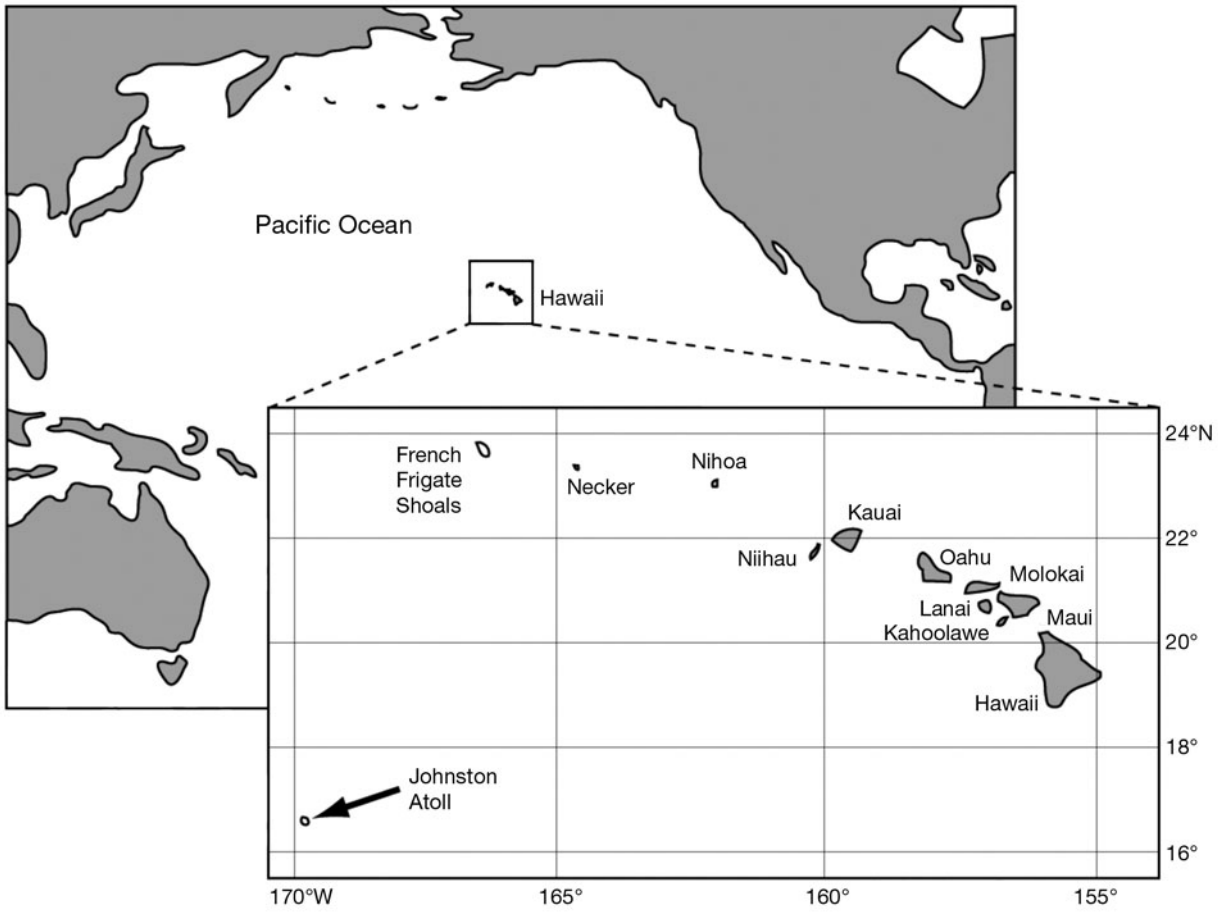

Fig. 1. Location of Johnston Atoll (indicated by arrow) where corals were sampled for *Symbiodinium* genotyping

5 pmols) and 23S2 (reverse; 5′-CCA TCG TAT TGA ACC CAG C-3′ 5 pmols) primers (Zhang et al. 2000) and 0.5 U of Immolase (Bioline) in a 25 µl reaction as described above with an annealing temperature of 55°C. PCR amplicons were purified using the QIAquick® PCR Purification Kit (Qiagen), ligated into the pGEM®-T Easy vector (Promega), transformed into α-select gold efficiency competent cells (Bioline) and grown overnight in Circlegrow® (MP Biomedicals). Plasmids were purified using the Perfectprep® Plasmid Isolation Kit (Eppendorf) and at least 10 clones from each PCR product cycle-sequenced using BigDye Terminators (PerkinElmer) on an ABI-3100 automated sequencer at the University of Hawaii. Ambiguous sequences were confirmed by alignment to the reverse complement.

Sequence and phylogenetic analyses. Sequences were inspected, aligned and edited using the software MacVector® 10. Identical sequences obtained from more than one clone library and singletons matching existing GenBank entries were used in downstream analysis. The remaining clone singletons were assumed to be artifacts or rare intragenomic repeats not useful for diversity analysis, and the nucleotide at the site of polymorphism converted to the consensus. In addition, IT2S folding was conducted using previously published *Symbiodinium* ITS2 structures as templates (Hunter et al. 2007, Thornhill et al. 2007) in the ITS2 database interactive website (Schultz et al. 2006, Selig et al. 2008) and manually edited using the software 4SALE (Seibel et al. 2006, 2008). Potential pseudogenes were characterized by significant changes to the 5.8S sequence not observed in *Symbiodinium* or other closely related dinoflagellates (Thornhill et al. 2007) and changes to the secondary structure of the ITS2 RNA molecule that likely disrupt the functional fold. As for singletons above, sequences representing putative pseudogenes were converted to the consensus at the site of polymorphism. Editing singletons and potential pseudogenes in this way collapsed minor sequence variants to the closest dominant sequence present in the data set determined from statistical parsimony. Published *Symbiodinium* ITS2 sequences were named as in previous studies (LaJeunesse 2001,

2002, 2005, LaJeunesse et al. 2003, 2004b). Novel ITS2 sequences were assigned a unique specifier reflecting the closest relationship to a published dominant subclade type. Novel sequences were assigned a decimal and a numeral to distinguish them from the published type and each other. For example, 3 different sequences closely related to C15 were named C15.1, C15.2, and C15.3. For chloroplast 23S, sequences were named numerically with a letter corresponding to the *Symbiodinium* clade followed by a *p* to reflect the plastid origin of the sequence, and numbered sequentially.

Statistical parsimony networks were run using the software TCS v.1.21 (Clement et al. 2000). The cladogram estimation was performed under a 95% connection limit and gaps were treated as a 5th state with the alignment edited so that the entire indel was considered a single mutation.

***Symbiodinium* sequence signatures and community analyses.** All edited *Symbiodinium* sequences recovered from an individual coral colony are referred to here as the sequence signature of that colony. *Symbiodinium* sequence signatures from all coral colonies were compared using the Bray-Curtis coefficient of similarity (*S*) in the software package PRIMER v.6 (Clarke & Warwick 2001), which ranges between 0 and 100, where a value of 100 indicates an identical assemblage (Bray & Curtis 1957). For both the ITS2 and chloroplast 23S rDNA, the *Symbiodinium* sequence signature in each coral was first standardized by square-root transforming the relative abundance of each sequence in that colony prior to calculation of *S*. To test for the relationship of *Symbiodinium* sequence signatures in the coral colonies from Johnston Atoll, Bray-Curtis similarities were analyzed by hierarchical clustering in PRIMER, and the SIMPROF test conducted to establish the significance of the dendrogram nodes.

Symbiodinium sequence signatures were grouped by coral species to obtain a community sequence signature for each coral species. To test for host specificity, 1-way analysis of similarities (ANOSIM) was calculated in PRIMER to detect significant differences in the *Symbiodinium* community sequence signature for each marker (ITS2 and chloroplast 23S) grouped by coral species (global test) and between coral species (pairwise test). The test statistic (R) in ANOSIM ranges between +1 to –1, where a value of 0 indicates no differences between groups, values approaching +1 indicate partitioning of variation by group and values approaching –1 indicate partitioning of variation within each group is greater than between groups.

RESULTS

Symbiodinium sequence diversity

A total of 615 *Symbiodinium* ITS2 sequences and 602 *Symbiodinium* chloroplast 23S sequences were recovered from the coral colonies sampled at Johnston Atoll and the same *Symbiodinium* clades were detected using both markers (Table 2). *Symbiodinium* clade A represented 2.8 and 3.5%, clade C 85.2 and 83.2%, and clade D 12.0 and 13.3% of the ITS2 and chloroplast 23S clone

Table 2. *Symbiodinium* ITS2 and chloroplast 23S sequences identified in the corals sampled at Johnston Atoll and from other studies in the Pacific. Numbers superscript and in parentheses indicate the frequency of that sequence recovered from the coral. Numbers subscript refer to source. AS: American Samoa; GBR: Great Barrier Reef; NWHI: Northwestern Hawaiian Islands

Coral host	Site	ITS2 signature	23S signature	Pacific-wide
Acroporidae				
Acropora cytherea	JOH-20	C1$^{(2)}$, C3$^{(4)}$, C3.2$^{(2)}$, C3.8$^{(1)}$, C3.9$^{(1)}$, C3.11$^{(1)}$, C3b$^{(1)}$	C*p*4$^{(10)}$	*Acropora:*
	JOH-23	C1$^{(3)}$, C3$^{(7)}$, C3.2$^{(1)}$	C*p*4$^{(10)}$	GBR$_{1, 2, 3, 4}$
	JOH-4P	C1$^{(2)}$, C3$^{(3)}$, C3.2$^{(2)}$, C3.7$^{(1)}$, C3.8$^{(1)}$, C3.9$^{(1)}$, C3.10$^{(1)}$	C*p*4$^{(10)}$	A1, C1, C3, C3h,
	JOH-5P	C1$^{(1)}$, C3$^{(8)}$, C3.2$^{(1)}$, C3.8$^{(1)}$	C*p*4$^{(11)}$	C3i, C3k, D1, D2
	JOH-6P	C1$^{(2)}$, C3$^{(5)}$, C3.10$^{(1)}$, C3.11$^{(1)}$, C21.13$^{(2)}$	C*p*4$^{(11)}$	
Acropora nasuta	JOH-1AP	C1$^{(1)}$, C3$^{(7)}$, C3.2$^{(1)}$, C3.8$^{(1)}$, C21.12$^{(2)}$	C*p*4$^{(10)}$	Japan$_2$
	JOH-20	C1$^{(5)}$, C3$^{(3)}$, C3.7$^{(1)}$, C3b$^{(1)}$	C*p*4$^{(10)}$	C1, C3, C3i
	JOH-4P	C1$^{(3)}$, C3$^{(4)}$, C3.2$^{(1)}$, C3b$^{(1)}$, C21.12$^{(1)}$, C21.13$^{(1)}$	C*p*4$^{(10)}$	
	JOH-4P	C1$^{(3)}$, C3$^{(3)}$, C3.8$^{(1)}$, C3b$^{(2)}$, C21.13$^{(1)}$	C*p*4$^{(10)}$	NWHI$_5$
	JOH-4P	C1$^{(1)}$, C3$^{(4)}$, C3.2$^{(2)}$, C3.8$^{(1)}$, C3.10$^{(1)}$, C3.11$^{(1)}$, C3b$^{(1)}$, C21.12$^{(1)}$	C*p*4$^{(10)}$	A1, C1c, C27
Montipora capitata	JA-HIMB3	C17$^{(2)}$, C17.2$^{(2)}$, C31$^{(5)}$, D1a$^{(1)}$	C*p*7$^{(5)}$, D*p*1$^{(5)}$	*Montipora:*
	JA-HIMB3	D1$^{(5)}$, D1a$^{(5)}$	D*p*1$^{(10)}$	GBR$_{1, 2, 4}$
	JOH-20	C17.2$^{(3)}$, C21$^{(1)}$, C31$^{(4)}$, D1$^{(1)}$, D1a$^{(1)}$	C*p*7$^{(2)}$, D*p*1$^{(8)}$	C15, C17, C21, C31
	JOH-20	C17$^{(1)}$, C17.2$^{(4)}$, C21$^{(1)}$, C31$^{(4)}$	C*p*7$^{(10)}$	
	JOH-6P	D1$^{(3)}$, D1a$^{(7)}$	D*p*1$^{(10)}$	Hawaii$_6$ C31, D1a

Table 2 (continued)

Coral host	Site	ITS2 signature	23S signature	Pacific-wide
Montipora patula	JA-HIMB3	C21[3], C31[7]	C*p*8[10]	
	JA-HIMB3	D1[10]	D*p*1[10]	Japan$_2$
	JOH-20	D1[4], D1a[6]	D*p*1[10]	C31
	JOH-23	D1[7], D1a[3]	D*p*1[10]	
	JOH-4P	D1a[10]	D*p*1[10]	
Agariciidae				
Pavona varians	JA-HIMB3	C1[3], C1ca[3], C1h[1], C21.14[1], C45[3]	C*p*1[10]	*Pavona:*
	JA-HIMB3	C27[8], C27.1[2]	C*p*1[1], C*p*9[9]	GBR$_{1, 2}$
	JA-HIMB3	C1[4], C3[2], C21.14[1], C27[2], C27.1[1], C45[3]	C*p*1[10]	C1, C1ca, C27
	JOH-20	C27[8], C27.1[2]	C*p*9[10]	
	JOH-4P	C21[1], C21.14[1], C27[7], C27.1[1]	C*p*9[10]	Hawaii$_6$
				C27
Faviidae				
Leptastrea sp.	JOH-1AP	C1[1], C1.7[1], C1ca[5], C1f[2], C3[1]	C*p*1[10]	*Leptastrea:*
	JOH-1AP	C1[2], C1.6[1], C1ca[2], C1f[4], C3.2[1]	C*p*1[9], C*p*3[1]	GBR$_2$
	JOH-1AP	C1[1], C1ca[6], C1f[3]	C*p*1[10]	C1, C1ca
	JOH-5P	C1[1], C1ca[5], C1f[2], C3[1], C3.2[1]	C*p*1[10]	
	JOH-5P	C1[1], C1ca[5], C1f[1], C3[3]	C*p*1[10]	Hawaii$_6$
				C1f
Fungiidae				
Fungia scutaria	JA-HIMB3	C1[4], C1ca[4], C1f[1], C3[1]	C*p*1[10]	*Fungia:*
	JOH-4P	C1[2], C1.6[1], C1ca[5], C1f[2]	C*p*1[10]	GBR$_{1, 2}$, C1
	JOH-4P	C1.6[3], C1ca[6], C1f[1]	C*p*1[10]	
	JOH-4P	C1[2], C1ca[4], C1f[2], C3[1], C3.2[1]	C*p*1[10]	Hawaii$_6$, C1f
	JOH-5P	C1[7], C1.7[1], C1.8[2]	C*p*1[10]	
				Japan$_2$, C1
Pocilloporidae				
Pocillopora	JOH-1AP	C1[2], C1.7[1], C42[5], C45[2]	C*p*6[10]	*Pocillopora:*
damicornis	JOH-23	C1[1], C1ca[2], C3.12[2], C21[1], C42[3], C45[1]	C*p*6[10]	AS$_7$
	JOH-23	C42[10]	C*p*1[3], C*p*6[7]	C1, C1c, C42, D, D1a
	JOH-23	C1.5[1], C3[3], C3.12[1], C42[3], C45[3]	C*p*1[4], C*p*2[1], C*p*6[5]	GBR$_{1, 2, 4, 8}$
	JOH-25	C3.12[2], C42[7], C45[1]	C*p*1[9], C*p*6[1]	C1, C1ca, C42, C45
Pocillopora eydouxi	JA-HIMB3	A1[4], A1.2[1], C1[1], C42[4]	A*p*1[5], A*p*2[1], C*p*1[4]	Hawaii$_6$
	JA-HIMB3	A1[6], C1[1], C42[2], C45.3[1]	A*p*1[2], C*p*1[7], C*p*3[1]	C45
	JA-HIMB3	A1[4], C1.8[1], C42[4], C45.3[1]	A*p*1[2], A*p*2[4], C*p*1[4]	
	JOH-1AP	C1[2], C42[7], C45[1]	C*p*1[10]	Japan$_2$
	JOH-22	C1[2], C42[7], C45[1]	C*p*1[10]	C1ca, C45
Pocillopora	JA-HIMB3	A1[2], C1[3], C42[1], C45[3], D1[1]	A*p*1[1], A*p*2[6], C*p*1[2], D*p*1[1]	
meandrina	JA-HIMB3	C1[2], C42[8]	C*p*1[10]	
	JA-HIMB3	C1[2], C42[5], C45[3]	C*p*1[10]	
	JOH-1AP	D1[2], D1a[8]	C*p*1[4], D*p*1[6]	
	JOH-22	C1[2], C42[3], C45[5]	C*p*1[10]	
Poritidae				
Porites sp.	JOH-5P	C15[3], C15.3[1], C15.4[3], C15.5[1], C15a[2]	C*p*5[10]	*Porites:*
	JOH-5P	C15[2], C15.1[2], C15.4[5], C15.5[1]	C*p*5[10]	AS$_7$
	JOH-5P	C15[2], C15.1[1], C15.2[1], C15.4[6]	C*p*5[10]	C15
	JOH-5P	C15[3], C15.2[1], C15.3[1], C15.4[4], C15a[1]	C*p*5[10]	GBR$_{1, 2, 4}$
	JOH-5P	C15[7], C15.4[2], C15.5[1]	C*p*5[10]	C15
Porites lobata	JOH-1AP	C15[10]	C*p*5[10]	Hawaii$_{6, 9}$
	JOH-1AP	C15[10]	C*p*5[10]	C15
	JOH-23	C15[10]	C*p*5[10]	Japan$_2$
	JOH-23	C15[10]	C*p*5[10]	C15
	JOH-23	C15[10]	C*p*5[10]	

[1]Lajeunesse et al. (2003); [2]LaJeunesse et al. (2004a); [3]LaJeunesse et al. (2009); [4]Stat et al. (2008a); [5]Stat & Gates (2008); [6]LaJeunesse et al. (2004b); [7]Smith et al. (2008); [8]Sampayo et al. (2007); [9]Apprill & Gates (2007)

Table 3. GenBank accession numbers for *Symbiodinium* ITS2 and chloroplast 23S sequences identified in the present study

Symbiodinium Clade	Subclade sequence	Accession number	Source
ITS2			
A	A1	AF333505	LaJeunesse (2001)
A	A1.2 (Amami6a)	AB207210	Reimer et al. (2006)
C	C1	AF333515	LaJeunesse (2001)
C	C1.5 (DongshanCS)	EF634157	Z. Dong et al. unpubl. data
C	C1.6	FJ461493	Present study
C	C1.7	FJ461494	Present study
C	C1.8 (culture 152)	EU074955	Thornhill et al. (2007)
C	C1ca (C1b/C1e)	EU449104	T. C. LaJeunesse unpubl. data
C	C1f	AY258490	LaJeunesse et al. (2004b)
C	C1h	AY258473	LaJeunesse (2005)
C	C3	AF499789	LaJeunesse (2002)
C	C3.2	FJ461497	Present study
C	C3.7	FJ461502	Present study
C	C3.8	FJ461503	Present study
C	C3.9	FJ461504	Present study
C	C3.10	FJ461505	Present study
C	C3.11	FJ461506	Present study
C	C3.12	FJ461507	Present study
C	C3b	AF499791	LaJeunesse (2002)
C	C15	AY239369	LaJeunesse et al. (2003)
C	C15.1	FJ461508	Present study
C	C15.2	FJ461509	Present study
C	C15.3	FJ461510	Present study
C	C15.4	FJ461511	Present study
C	C15.5	FJ461512	Present study
C	C15a	AY258476	LaJeunesse (2005)
C	C17	AY239370	LaJeunesse et al. (2003)
C	C17.2	FJ461513	Present study
C	C21	EU449102	LaJeunesse et al. (2003)
C	C21.12	FJ461514	Present study
C	C21.13	FJ461515	Present study
C	C21.14	FJ461516	Present study
C	C27	AY239379	LaJeunesse et al. (2003)
C	C27.1	FJ461517	Present study
C	C31	AY258496	LaJeunesse et al. (2004b)
C	C42	AY765402	LaJeunesse (2005)
C	C45	EU449103	LaJeunesse (2005)
C	C45.3	FJ461522	Present study
D	D1	AF334660	LaJeunesse (2001)
D	D1a	AF499802z	LaJeunesse (2002)
23S			
A	A*p*1	FJ461476	Present study
A	A*p*2	FJ461477	Present study
C	C*p*1	FJ461478	Present study
C	C*p*2	FJ461479	Present study
C	C*p*3	FJ461480	Present study
C	C*p*4	FJ461481	Present study
C	C*p*5	FJ461482	Present study
C	C*p*6	FJ461483	Present study
C	C*p*7	FJ461484	Present study
C	C*p*8	AY035425	Santos et al. (2002)
C	C*p*9	FJ461486	Present study
D	D*p*1	FJ461475	Present study

libraries, respectively. A higher diversity of sequences was recovered using the ITS2 as compared to the chloroplast 23S marker. Two subclade A, 36 subclade C and 2 subclade D sequences were recovered using ITS2 (secondary structures for these ITS2 sequences are given in supplement 1 available at www.int-res.com/articles/suppl/m386p083_app.pdf) and 2 subclade A, 9 subclade C and 1 subclade D sequences were recovered with chloroplast 23S (Tables 2 & 3).

Of the corals sampled, ITS2 sequences representing a single *Symbiodinium* clade were recovered from 90% of the colonies (clade C: n = 47; clade D: n = 7), 8.3% contained 2 clades (clades C and D in *Montipora capitata*: n = 2; clades A and C in *Pocillopora eydouxi*: n = 3) and 1.7% contained 3 clades (clades A, C and D in *Pocillopora meandrina*: n = 1). Similarly, chloroplast 23S sequences representing a single *Symbiodinium* clade were recovered from 88.3% of the colonies (clade C: n = 47; clade D: n = 6), 10% contained 2 clades (clades C and D in *M. capitata*: n = 2, and *P. meandrina*: n = 1; clades A and C in *P. eydouxi*: n = 3) and 1.7% contained 3 clades (clades A, C and D in *P. meandrina*: n = 1). All coral colonies harbored diverse assemblages of *Symbiodinium* ITS2 sequences, except for *Porites lobata*, which contained only C15. In contrast, in the majority of corals where a single *Symbiodinium* clade was detected, this was represented by a single chloroplast 23S sequence.

Symbiodinium ITS2 and chloroplast 23S sequences resolve into a single network for each clade except for the 2 clade A chloroplast 23S sequences (Fig. 2). The network structure for clade C *Symbiodinium* is similar between the 2 markers. The ITS2 and chloroplast 23S clade C networks both show shared sequences present in several coral species that are inferred as the ancestral sequence (C1 for ITS2 and C*p*1 for chloroplast 23S) with multiple genus-specific sequences that have evolved from these. Specific *Symbiodinium* clade C sequences, resolved using both markers, were found associated with the coral genera *Porites*, *Pocillopora*, *Montipora* and *Pavona*. The coral genus *Acropora* contained multiple genus-specific *Symbiodinium* ITS2 sequences in addition to C3. In contrast, a single *Acropora*-specific *Symbiodinium* sequence was resolved using the chloroplast 23S rDNA (C*p*4) that was a single base change from the shared dominant sequence type C*p*1 harbored by corals with *Symbiodinium* ITS2 sequences C1, C3, C1f, C1h and C1ca.

Analyses of *Symbiodinium* sequence signatures and community signatures

Cluster analysis of the *Symbiodinium* sequence signature from each coral colony largely grouped into

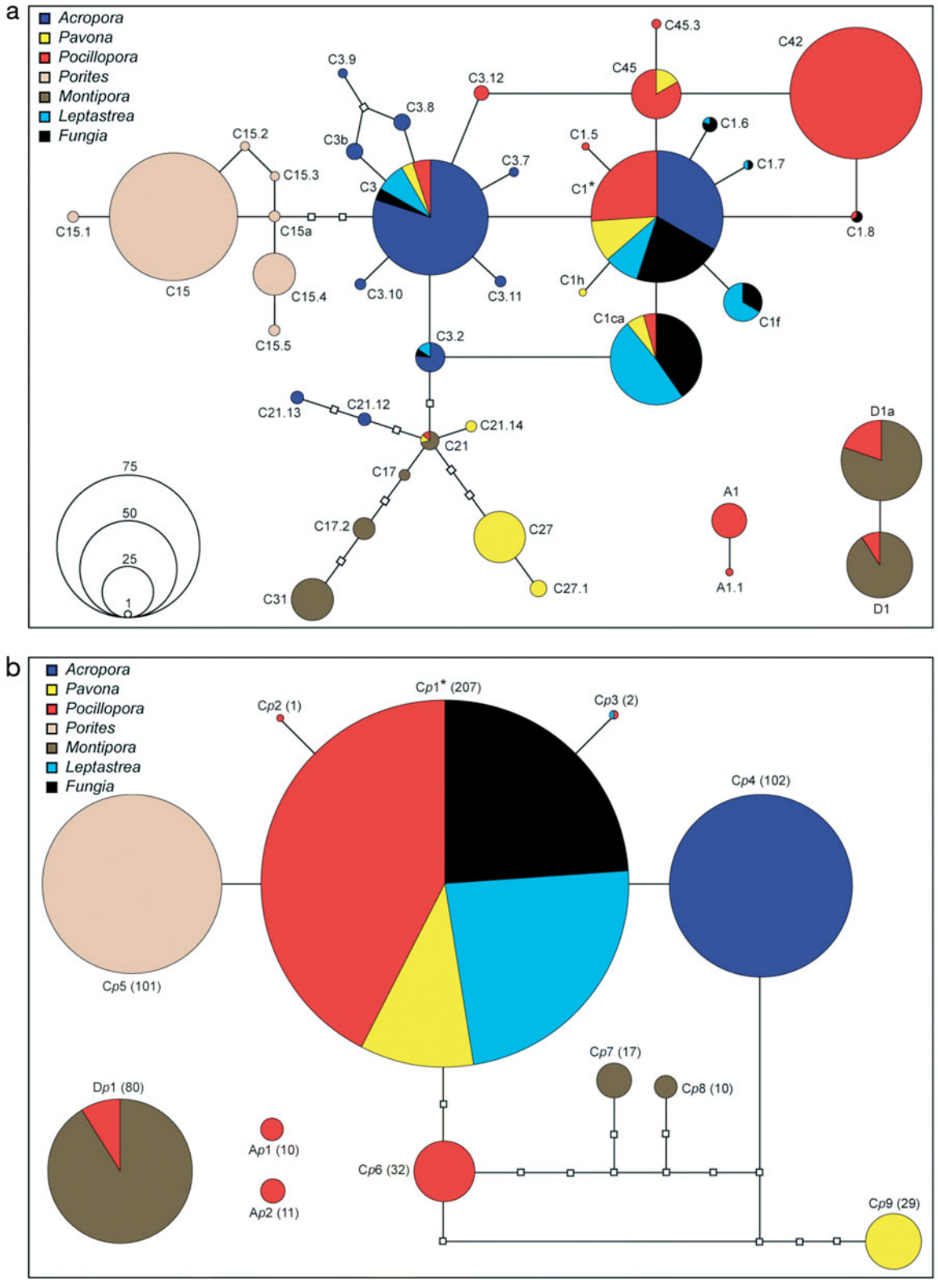

Fig. 2. Statistical parsimony networks for *Symbiodinium* clades A, C and D for (a) ITS2 and (b) chloroplast 23S sequences retrieved from corals sampled at Johnston Atoll. The size of each pie chart represents the frequency of a given sequence in the corals sampled. Open squares joining pie charts represent a single mutational step and an asterisk denotes the inferred ancestral sequence. Numbers in parentheses indicate the frequency of that sequence in the study

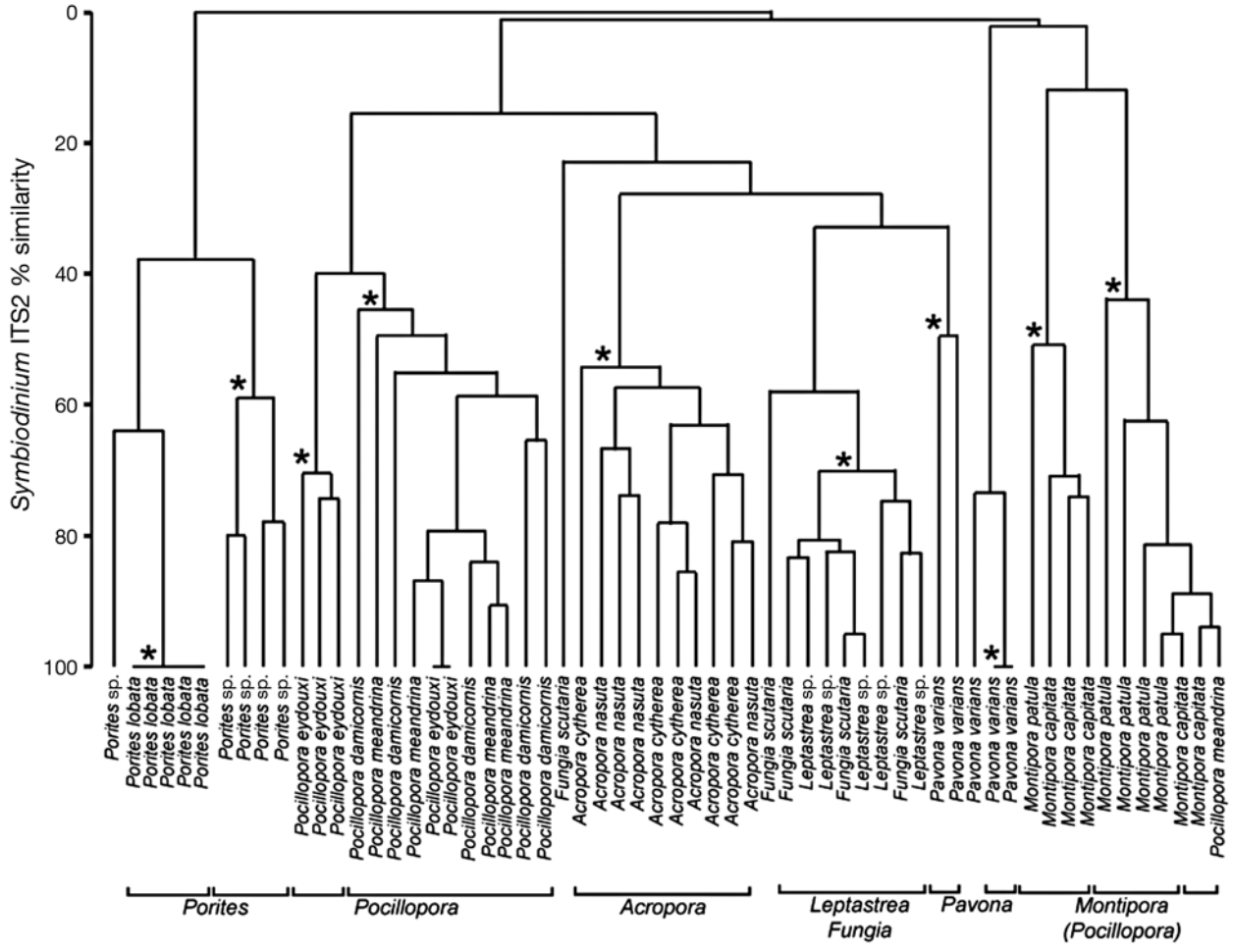

Fig. 3. UPGMA cluster dendrogram of Bray-Curtis similarities calculated from square-root transformed relative abundances of *Symbiodinium* ITS2 sequences in coral colonies sampled at Johnston Atoll. Asterisks at nodes indicate significant groupings of *Symbiodinium* ITS2 sequence signatures from the coral colonies ($p < 0.05$) calculated from the SIMPROF test

species- or genus-specific assemblages for both markers (Figs. 3 & 4). For example, the *Symbiodinium* sequence signatures from colonies of *Porites* grouped into a significant cluster in both the ITS2 and chloroplast 23S dendrograms. *Symbiodinium* from some coral species (e.g. *Leptastrea* spp. and *Fungia* spp.) grouped into a single significant cluster in both the ITS2 and chloroplast 23S dendrograms. The presence of clade D *Symbiodinium* in coral colonies obscured significant groupings by species or genus in some instances (e.g. *Pocillopora meandrina* grouping with *Montipora*). The higher *Symbiodinium* diversity recovered using the ITS2 compared to the chloroplast 23S marker is also reflected in the higher number of significant clusters identified in the ITS2 compared to the chloroplast 23S dendrogram. For example, the *Symbiodinium* sequence signatures from colonies of *Pocillopora* grouped into 3 significant clusters in the ITS2 dendrogram and only 2 in the chloroplast 23S dendrogram.

Differences among the *Symbiodinium* community sequence signature for coral species sampled at Johnston Atoll resolved using both genetic markers were supported by significant results in the ANOSIM global test (ITS2: $R = 0.770$, $p < 0.05$; chloroplast 23S: $R = 0.734$, $p < 0.05$), and pairwise comparisons showed that the *Symbiodinium* community sequence signature for most coral species were significantly different from one another (Table 4). Significant differences in the pairwise comparisons of *Symbiodinium* community sequence signatures were mostly congruent with the hierarchical clustering analysis for significant groupings of *Symbiodinium* sequence signatures from individual colonies. Overall, these data show that the host taxonomy influences the community of *Symbiodinium* harbored by corals at Johnston Atoll.

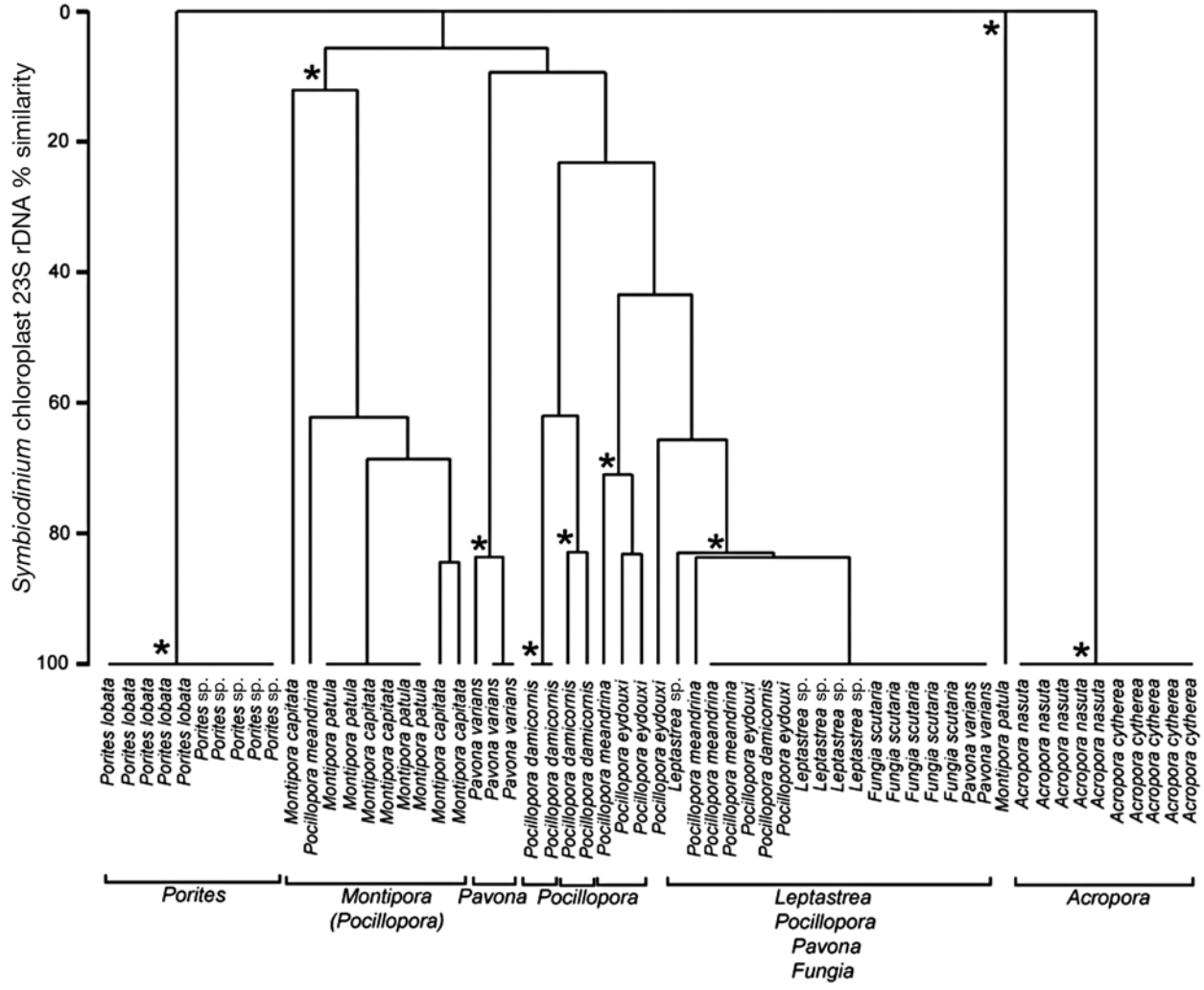

Fig. 4. UPGMA cluster dendrogram of Bray-Curtis similarities calculated from square-root transformed relative abundances of *Symbiodinium* chloroplast 23S sequences in coral colonies sampled at Johnston Atoll. Asterisks at nodes indicate significant groupings of *Symbiodinium* chloroplast 23S sequence signatures from the coral colonies ($p < 0.05$) calculated from the SIMPROF test

DISCUSSION

The diversity of *Symbiodinium* obtained using the nuclear ITS2 and chloroplast 23S rDNA for corals at Johnston Atoll reveals similar patterns in the host–dinoflagellate assemblages. The large sequence diversity generated with ITS2, much of which can arguably be attributed to intragenomic and/or intraspecific variation, is largely resolved into distinct chloroplast 23S sequences. As such, even though chloroplast 23S provides less taxonomic resolution than ITS2, the apparent lack of intragenomic variation makes it easier to interpret and thus a good marker for characterizing *Symbiodinium* diversity.

Symbiodinium diversity and host specificity at Johnston Atoll

A high level of *Symbiodinium* ITS2 diversity was recovered from the coral population at Johnston Atoll, consistent with the findings of Apprill & Gates (2007). *Symbiodinium* assemblages are largely species- or genus-specific and those shared among coral species are generally associated with hosts that acquire their *Symbiodinium* anew each generation from the environment (horizontal acquisition). This is consistent with patterns in the coral–dinoflagellate assemblages in the southern Great Barrier Reef (LaJeunesse et al. 2003, Stat et al. 2008a). *Symbiodinium* ITS2 types within

Table 4. Results of ANOSIM pairwise comparisons of *Symbiodinium* ITS2 and chloroplast 23S community sequence signatures associated with each coral species sampled at Johnston Atoll. Numbers are the R-statistic for each pairwise comparison (ITS2 sequence comparison/23S sequence comparison) and significant values ($p < 0.05$) are in **bold**

	Acropora cytherea	*Acropora nasuta*	*Montipora capitata*	*Montipora patula*	*Pavona varians*	*Leptastrea* sp.	*Fungia scutaria*	*Pocillopora damicornis*	*Pocillopora eydouxi*	*Pocillopora meandrina*	*Porites* sp.
Acropora nasuta	0.132/0.000										
Montipora capitata	**0.900/0.900**	**0.900/0.900**									
Montipora patula	**0.750/0.800**	**0.750/0.800**	−0.024/0.052								
Pavona varians	**0.812/0.800**	**0.796/0.800**	**0.776/0.700**	**0.632/0.600**							
Leptastrea sp.	**1.000/1.000**	**0.990/1.000**	**0.900/0.900**	**0.750/0.800**	**0.796**/0.240						
Fungia scutaria	**0.774/1.000**	**0.758/1.000**	**0.850/0.900**	**0.700/0.800**	**0.636**/0.260	0.006/0.000					
Pocillopora damicornis	**1.000/0.900**	**1.000/0.900**	**0.892/0.800**	**0.740/0.700**	**0.796/0.456**	**0.960/0.604**	**0.960/0.608**				
Pocillopora eydouxi	**1.000/1.000**	**1.000/1.000**	**0.900/0.900**	**0.750/0.800**	**0.796**/0.320	**1.000**/0.208	**1.000**/0.240	0.308/**0.602**			
Pocillopora meandrina	**0.670/1.000**	**0.682/1.000**	**0.536/0.728**	0.360/**0.624**	**0.554**/0.256	**0.690**/0.002	**0.690**/0.050	0.122/**0.496**	0.098/0.052		
Porites sp.	**1.000/1.000**	**1.000/1.000**	**0.900/0.900**	**0.750/0.800**	**0.900/0.800**	**1.000/1.000**	**1.000/1.000**	**1.000/0.900**	**1.000/1.000**	**0.085/1.000**	
Porites lobata	**1.000/1.000**	**1.000/1.000**	**0.900/0.900**	**0.750/0.800**	**0.900/0.800**	**1.000/1.000**	**1.000/1.000**	**1.000/0.900**	**1.000/1.000**	**0.085/1.000**	**0.900**/0.000

clade C are thought to have evolved from pandemic generalist types C1 and C3 that are found globally and interact with a diverse range of marine invertebrate hosts (LaJeunesse 2005). Here, *Symbiodinium* C1 was inferred as the ancestral sequence in the statistical parsimony network analysis, with C3 situated a single mutational step from C1. These 2 sequences were found in multiple host genera including *Acropora*, *Pavona*, *Pocillopora*, *Leptastrea* and *Fungia*. Clusters of sequences specific to the coral genera *Porites*, *Pocillopora*, *Pavona*, *Montipora* and *Acropora* surround C3, C1 and C1ca. In addition, the most abundant sequences generally occupy a central position within the network, with less common sequences radiating from them, a finding consistent with Thornhill et al. (2007). The chloroplast 23S network reduces the complexity of *Symbiodinium* diversity by collapsing most of the rare ITS2 sequences into a single sequence type and also combining some abundant ITS2 sequences (e.g. C1, C3 and C1f), while maintaining a network structure largely consistent with that of the ITS2 rDNA. Comparisons of the *Symbiodinium* community sequence signatures found in each coral species are also similar between the 2 markers, with most forming significant clusters specific to a coral genus or symbiont acquisition strategy.

The coral genera *Pocillopora*, *Porites* and *Montipora* all associate with specific *Symbiodinium* sequences that are consistent with other studies that used the ITS2 marker (LaJeunesse et al. 2004a,b, LaJeunesse 2005, Sampayo et al. 2007, Stat et al. 2008a). Here, we show that genus-specific lineages for the chloroplast 23S rDNA mimic those of ITS2 rDNA. Also, *Pavona* and *Acropora* harbored specific *Symbiodinium* sequences in addition to the generalist ITS2 C1 that both genera are known to associate with on other Pacific reefs (LaJeunesse et al. 2003, 2004a,b). The extent to which these specific associations occur will be evident as more studies are performed using these markers across different reefs in the Pacific. Although coevolution and reciprocal speciation has not been confirmed between coral and *Symbiodinium*, the specificity in host–symbiont associations is likely the result of coadaptation and the evolution of ecologically successful partnerships that have evolved from generalist associations. It is therefore likely that these specific symbiotic partnerships have enabled a functionally advantageous holobiont to become more widespread across the Pacific.

Biogeography of coral–*Symbiodinium* assemblages

In addition to most corals harboring multiple *Symbiodinium* ITS2 sequences, novel sequences, new host–symbiont assemblages and/or new biogeogra-

phic patterns in *Symbiodinium* were revealed. Comparison of the host–symbiont assemblages described here using chloroplast 23S is limited due to the low number of studies that have used this marker in the past, and hence the only shared sequence is that between *Symbiodinium* from *Pocillopora damicornis* from Johnston Atoll and a culture originating from an unidentified anemone from Japan (Santos et al. 2002). A comparison of the patterns in host–symbiont assemblages at Johnston Atoll with other reefs in the Pacific described using ITS2 reveals both similarities and differences. For example, *Acropora cytherea* at Johnston Atoll associates with *Symbiodinium* ITS2 C1, C3 and C3b, while in the Northwestern Hawaiian Islands, the closest reef system to Johnston Atoll, *A. cytherea* associates with A1, C1c and C27 (Stat & Gates 2008) and in the Great Barrier Reef with just C3 (LaJeunesse et al. 2003, 2004a). Similarly, *A. nasuta* harbors C3 and C3b at Johnston Atoll, but in the Great Barrier Reef associates with C3, C3k and C3i (LaJeunesse et al. 2003, 2004b). *Symbiodinium* ITS2 C3b has also previously been reported as Caribbean-specific (LaJeunesse 2005). Other novel symbioses recovered at Johnston Atoll include C21, C3, A1 and D1a with *Pocillopora*, C1h, C3 and C45 with *Pavona*, C3 with *Leptastrea* and C3 with *Fungia*. The novel assemblages identified for Johnston Atoll and differences in the *Symbiodinium* assemblages with locations in the Pacific likely reflect specificity driven by geographic location and isolation, local host–symbiont adaptation and fine-scale niche partitioning of symbionts.

Intragenomic nature of rDNA and analyses using community signatures

Intragenomic variation in rDNA is common in dinoflagellates and other single-celled protists (Litaker et al. 2003, 2007, Aktas et al. 2007, Thornhill et al. 2007). Furthermore, in *Symbiodinium*, ITS2 sequence divergence within a genome has been found to be greater than that between sequences labeled as ecologically distinct types (van Oppen et al. 2001, Thornhill et al. 2007). This makes distinguishing between intragenomic, interspecific and intraspecific variation in the ITS2 sequences recovered from a single coral extremely challenging. Also, a rare sequence variant in one *Symbiodinium* cell may be the dominant copy in a different cell, making it difficult to determine the ecological importance of these ITS2 variants. For example, *Symbiodinium* ITS2 C1 and C3 (the inferred ancestral sequences in clade C) are dominant types in a wide range of corals, but these sequences are likely present in many different cells where the dominant rDNA copy has evolved to a different sequence (e.g. C42). These interpretational problems are inherent when working with a multicopy marker.

Identifying dominant *Symbiodinium* ITS2 sequences within a coral using DGGE analysis has been used as a method to screen for the putative ecologically significant type (e.g. LaJeunesse 2002, LaJeunesse et al. 2003, 2004a,b, Thornhill et al. 2006a,b, Pochon et al. 2007, Sampayo et al. 2007, Stat et al. 2008a). This methodology has been widely used and has significantly increased our understanding of *Symbiodinium* diversity and biogeography. However, detection limits of the technique and interpretation of DGGE banding profiles can underestimate diversity (Thornhill et al. 2006b, Stat et al. 2008a) and misidentification of ITS2 types due to comigration of different sequences can occur (Apprill & Gates 2007, Pochon et al. 2007, Sampayo et al. 2008).

In the present study we used an extremely conservative approach to selecting cloned sequence data to describe the *Symbiodinium* diversity in individual corals and used statistical treatments to detect patterns in the assemblages of *Symbiodinium* associated with different coral species and genera. This analysis resolved high ITS2 sequence diversity that formed significantly different groupings by host species or genus. This approach circumvents the need to assign the ecological importance of a given sequence by naming it a type and more accurately reflects the complexity of the rDNA and the heterogeneity of coral–dinoflagellate symbiosis. However, exploring *Symbiodinium* diversity in coral hosts would be made much easier using a marker with no intragenomic variation.

With this in mind, we explored the diversity of *Symbiodinium* sequences recovered using chloroplast 23S rDNA in parallel with ITS2. Lower sequence diversity was recovered using chloroplast 23S rDNA as compared to ITS2 (usually a single *Symbiodinium* sequence per coral host), but the patterns in host–symbiont assemblages were still detectable. This marker is considered diverse enough to delineate species (Barbrook et al. 2006), and although multiple copies of the chloroplast 23S plastid minicircle are present in dinoflagellates and the copy number changes with the growth phase of the cell, the transcribed region appears homogenous within cells (Zhang et al. 2002, Koumandou & Howe 2007). The data presented here certainly support the broader application of chloroplast 23S rDNA as an easily interpretable and informative marker for assessing *Symbiodinium* diversity.

In conclusion, corals from Johnston Atoll in the central Pacific host diverse *Symbiodinium* communities that are specific to host species and genera. Some coral species conform to the Pacific-wide pattern of general and specific symbioses, while others engage in novel unions with *Symbiodinium*. The chloroplast 23S mar-

ker resolves most of the patterns in host–symbiont assemblages detected with ITS2 and exhibits lower intragenomic variation, making the *Symbiodinium* diversity data collected using this marker much easier to interpret. These findings set the stage to explore the functional implications of the distinct patterns in *Symbiodinium* diversity found in corals from Johnston Atoll and provide ample rationale to more broadly apply chloroplast 23S in studies of *Symbiodinium* diversity.

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