Proc 8th Int Coral Reef Sym 2:1599–1602. 1997

THE UTILITY OF ITS SEQUENCES IN ASSESSING RELATIONSHIPS AMONG ZOOXANTHELLAE AND CORALS

Cynthia L. Hunter[1], Clifford W. Morden[1,2] and Celia M. Smith[1]

[1]Botany Department, [2]Hawai'i Evolutionary Biology Program, University of Hawai'i, Honolulu, HI 96822, U.S.A.

ABSTRACT

Sequence data from the internal transcribed spacer (ITS) region of nuclear ribosomal DNA has been used to identify variation within zooxanthellae and host coral genomes of *Porites* species from the Pacific and Caribbean, as well as lab-cultured strains of zooxanthellae derived from *Montipora verrucosa*, *Pocillopora damicornis*, *Cassiopeia xamachana*, and *Zoanthus pacificus*. Our preliminary zooxanthellae sequences sorted into three deeply branched clades, but showed little genetic differentiation within or among the Hawai'ian *Porites* species. For *Porites* corals, ITS sequences clearly resolved species-level differences among the five taxa examined, as well as geographic differences among populations within two *Porites* species. Molecular assessment of the phylogeny within this diverse genus of Hawai'ian corals is currently underway, in conjunction with expansion of the data library to include ITS sequences from the rarer *Porites* species and additional populations from other islands within the Hawai'ian archipelago.

INTRODUCTION

There are a number of important ecological and evolutionary questions in contemporary coral biology for which molecular tools promise to hold great utility. Clonal species--those with multiple representatives of the same genotype, and cryptic species--those in which distinctive morphologies are lacking, highlight the need for readily applicable methods to resolve both genotypic identity and the full range of variability among their component members. Here, we describe preliminary results of a molecular approach to questions of genetic diversities in *Porites* corals and their symbiotic microalgae in the genus *Symbiodinium*.

There are currently ten described species of *Porites* in Hawai'i, nearly one fourth of the total scleractinian diversity in this island chain. Of these ten species, six are considered to be endemic and four occur commonly throughout tropical Pacific reefs. Three species (*P. lobata*, *P. compressa*, and *P. evermanni*) dominate Hawai'ian reef by percent cover and abundance (Maragos 1977). In his 1907 monograph of Hawai'ian *Porites*, Vaughan described dozens of discrete formae and subformae of these species based on skeletal morphology, indicating the range of morphological variation that exists within any one species. Confounding variation also exists among species. Although nearly identical in calical architecture, there is a continuum in colony morphology ranging from finely branched *P. compressa* to massive *P. lobata*; intermediate forms occur commonly. Additionally, an undescribed branching form of *Porites* has a calical structure similar to *P. evermanni*. Because of their extreme range of variation, Bernard (1905), in his early treatise on *Porites*, proposed abandoning the species concept altogether for this group.

As reported for numerous other coral species, individual genotypes of Hawai'ian *Porites* show often stunning differential susceptibility to bleaching. Most colonies of *P. compressa* previously identified through allozyme and histocompatibility assays as Morphotype 5 (Hunter 1988) bleached in October, 1988, while neighboring colonies of other genotypes retained normal pigmentation (C.L. Hunter and R.A. Kinzie, pers. obs.). For *P. evermanni*, within-colony variation in bleaching occurs biannually in spring and fall coincident with periods of greatest change in ambient seawater temperature (Hunter, unpubl. data). As proposed by Buddemeier and Fautin (1993), bleaching may represent an adaptive mechanism in which loss and replacement of algal symbionts ("zooxanthellae") results in an acquisition by the coral host of algae better suited to particular environmental conditions. What types of algae might be available for such "reshuffling" under natural conditions? And what tools might distinguish them?

Nine species of endosymbiotic algae in the genus *Symbiodinium* cultured from widely divergent hosts including corals, sea anemones, zooanthids, jellyfish, and giant clams have been described to date (Trench and Blank 1987). Rowan and Powers (1991) compared 18S rRNA sequence and RFLP data from algae freshly isolated from a broad range of Caribbean, Atlantic, and Pacific taxa; these algae grouped into three clades ("A, "B", and "C"). It is now apparent that algal types belonging to all three of these clades can be found in single colonies of *Montastrea* spp. and other Caribbean taxa (Rowan and Knowlton 1995; Baker and Rowan 1996). However, algae from all Hawai'ian corals sampled by Rowan and Powers, as well as those from the Eastern Pacific (Baker and Rowan 1996) fall into Clade C as defined by variation within the conservative 18S and 24S rRNA regions.

We examined the utility of the ITS (internal transcribed spacer) region of rRNA to delimit phylogenetic relationships and provide genetic markers in *Porites* corals and their symbiont algae. ITS is variable at or below the species level in a number of animal, plant, and algal taxa (e.g., Porter and Collins 1991; Baldwin 1992; Bakker et al. 1995) . It exists in multiple copies of tandem repeats in the nuclear genome; despite concerted evolution, there is the potential for intra-genomic variation among these copies (Rogers and Bendich 1988). Secondary structure of ITS has been recently described (Bakker et al. 1995), but as a non-coding spacer region it is predicted to have much less selective constraint than adjacent (18S, 24S) coding regions (Schlötterer et al. 1994). The power of ITS sequence comparisons in coral-algal studies is enhanced by the availability of a zooxanthellae-specific 5' PCR primer and the presence of the highly conserved 5.8S rRNA embedded between ITS-1 & ITS-2 for anchoring of sequence alignments.

METHODS

Taxa for which ITS sequences were obtained are listed in Table 1. For field collected material (*Porites* coral and symbiont algae), coral- or zooxanthellae-enriched fractions were obtained by repeated differential centrifugation of tissue slurries removed by waterpiking approximately 10 cm^2 of coral surface. Lysis buffers for coral fractions contained 1% v:v SDS and 2% v:v Sarcosyl for algal-enhanced fractions or pelleted zooxanthellae cultures. Coral ITS fragments were PCR amplified with universal 5' (GGA AGT AAA AGT CGT AAC AAG) and 3' (TCC TCC GCT TAT TGA TAT GC) ITS primers (White et al. 1990); algal ITS fragments were amplified with a zooxanthellae-specific 5' primer (CCG GTG AAT TAT TCG GAC TGA CGC AGT GCT); reverse-complement of the 3' 18s primer of Rowan and Powers 1991) and the universal 3' primer. PCR products were gel- or plasmid-purified prior to sequencing with an ABI autosequencer. Preliminary sequence alignments were obtained using the PILEUP program of UWGCG (University of Wisconsin Genetic Computer Group, version 8, 1994) and further aligned by eye. Insertions and deletions were coded and included as character states. Maximum parsimony trees were constructed by PAUP version 3.1.1; bootstrapping was performed using the branch-and-bound search option for 100 replicates (Swofford 1993).

To ascertain whether sequences obtained from PCR products were consistent with zooxanthellae or coral origins, we constructed a deep phylogeny using only the 5.8s regions and comparing our sequences with those of various other selected plant and animal taxa obtained from GenBank (*Cladophora* Z38134, *Drosophila* Z28416, MungBean X14337, Rat J00781, Urchin X00350); fungus (*Leptosphaeria maculans*; L07735) and yeast (*Saccharomyces cerevisiae*; K01048) were used as the outgroups. *Prorocentron micans* (M14649), a non-symbiotic dinoflagellate, was used as the outgroup for algal comparisons and *Balanophyllia elegans*, an azooxanthellate coral, was used as the outgroup for coral comparisons.

Table 1. Zooxanthellae and coral taxa from which ITS sequences were obtained and collection localities.

Zooxanthellae from host:		Corals:	
Field Collections		Zooxanthellate	
Porites compressa	Lanikai, O'ahu, Hawai'i	*Porites compressa*	Lanikai, O'ahu, Hawai'i
Porites evermanni	Lanikai, O'ahu, Hawai'i	*Porites evermanni*	Lanikai, O'ahu, Hawai'i
	Hanauma Bay, O'ahu, Hawai'i		Hanauma Bay, O'ahu, Hawai'i
Porites lobata	Lanikai, O'ahu, Hawai'i	*Porites lobata*	Lanikai, O'ahu, Hawai'i
			Hanauma Bay, O'ahu, Hawai'i
Porites astreoides	Key Largo, Florida	*Porites astreoides*	Key Largo, Florida
Porites porites	Key Largo, Florida	*Porites porites*	Key Largo, Florida
Laboratory Cultures		Azooxanthellate	
Cassiopeia xamachana	(=*S. microadriaticum*)	*Tubastrea coccinea*	Kane'ohe Bay, O'ahu
Montipora verrucosa	(=*S. kawagutii*)	*Balanophyllia elegans*	Monterey, California
Pocillopora damicornis			
Zoanthus sociatus	(=*S. pilosum*)		

RESULTS AND DISCUSSION

Coral and zooxanthellae 5.8S sequences were most closely related to animal and algal taxa, respectively (Figure 2). From this initial confirmation, we assumed that universal ITS primers selectively amplified coral DNA and the zooxanthellae-specific 5' primer selectively amplified zooxanthellae DNA from field collected material.

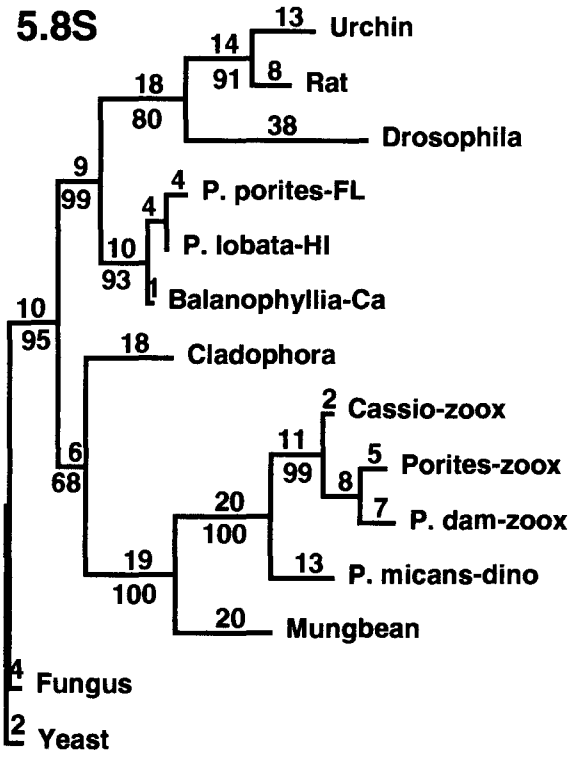

Fig. 1. Molecular phylogram of 5.8S rRNA region from representative corals and zooxanthellae aligned with sequences from various animal, plant, and algal taxa. Branch lengths are shown above and bootstrap values below branches.

Partial sequences were obtained for three of the coral species (*P. evermanni*-Lanikai, *P. astreoides*, and *P. porites*). The total ITS sequence length of coral species varied from 627 to 654 base pairs (bp) (Table 2), with 117 informative characters (PAUP defined as having at least two nucleotide states each shared by at least two sequences). Variation in coral sequences was fairly evenly distributed between ITS-1 (49%) and ITS-2 (48%), with 3% in the 5.8s region. *Balanophyllia elegans* and *Tubastrea coccinea* were alignable but distinctly different from the *Porites* group (Figure 3; alignments available upon request). Hawai'ian *Porites* species differed from the Florida species by 5 to 21%, and the two Florida species differed by 12%.

Porites evermanni was distinct (11%) from *P. lobata* and *P. compressa*, but there was little variation (2%) to distinguish the two genotypes within this species collected from widely different depth regimes and separated by 18 km of coastline. In contrast, differences between *P. compressa* and *P. lobata* (2 to 3%) were less than those between genotypes of *P. lobata* collected from the two sites (6%). These results support morphologically-based observations suggesting that *P. compressa* and *P. lobata* may form a very closely-related species complex.

Zooxanthellae ITS sequence lengths of varied from 625 to 673 bp with 206 informative sites (Table 3). The greatest proportion of variation occurred in ITS-1 (49%) relative to ITS-2 (42%) or the 5.8s region (9%). As in 18S rRNA comparisons, ITS sequences fell into three deeply branched clades (Figure 4), with cultured zooxanthellae forming two major groups and algae from the five *Porites* species forming a distinct third clade. However, while deep branches within the 18S tree varied by 2 to 3%, ITS-based clades varied by 20 to 30%. Although negligible difference were seen between two sequences obtained from cultured *S. microadriaticum*, sequences from two clones of cultured *Montipora verrucosa* (=*S. kawagutii*) varied by 3%.

ITS sequences from cultured zooxanthellae of *Montipora verrucosa* (=*S. kawagutii*) aligned most closely with cultured zooxanthellae of *Cassiopeia xamachana* (=*S. microadriaticum*) and *Zoanthus sociatus* (=*S. pilosum*); the *Pocillopora* sequence was distinct, and more closely related to the *Porites* group (Figure 4). In contrast, while fresh field-collected isolates of zooxanthellae from *Cassiopeia* grouped with algae from *Zoanthus* in Clade A as defined by 18s rRNA (Rowan and Powers 1991), zooxanthellae from *Montipora* aligned loosely with Clade C.

Table 2. Number of nucleotides and informative PAUP characters (total, ITS-1, 5.8s and ITS-2) and percent GC content in ITS sequences obtained from seven coral species. Lan=Lanikai samples, HB=Hanauma Bay samples, FL=Key Largo sample; bp=# of basepairs.

	Length (bp)				
	Total	ITS-1	5.8s	ITS-2	% GC
Porites evermanni-HB	639	250	168	221	42
P. evermanni-Lan	--	241	--	--	39
P. compressa-Lan	651	254	168	229	41
P. lobata-Lan	641	254	168	219	40
P. lobata-HB	640	256	168	216	41
P. astreoides-FL	--	250	158	--	41
P. porites-FL	--	236	169	--	42
Tubastrea	654	266	183	205	47
Balanophyllia	627	257	171	199	48
Informative chars.	117	61	3	53	

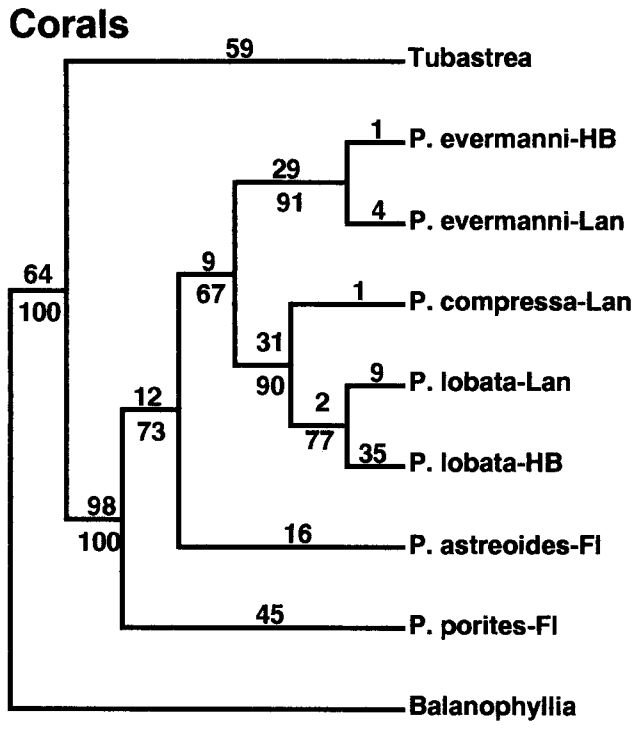

Fig. 2. Maximum parsimony cladogram generated from coral ITS sequences. Branch lengths are shown above and bootstrap values below branches.

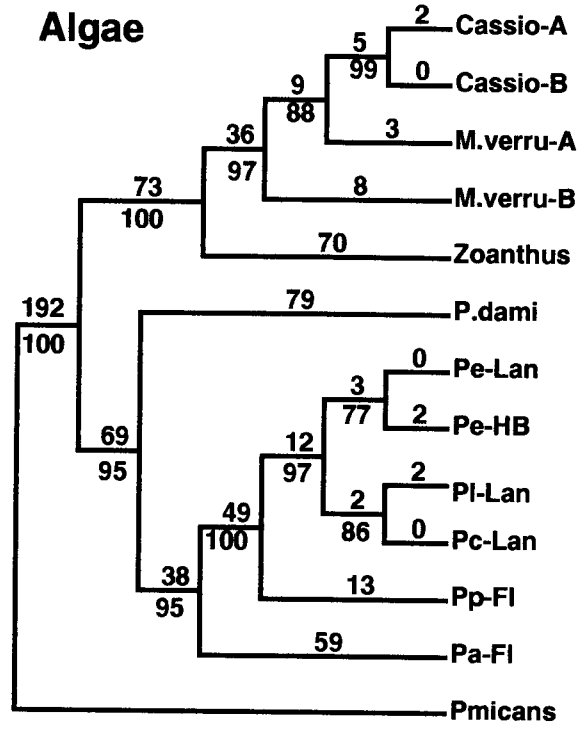

Fig. 3. Maximum parsimony cladogram generated from zooxanthellae ITS sequences. Branch lengths are shown above and bootstrap values below branches.

ITS sequences of zooxanthellae from *Porites* spp. in Hawai'i and Florida varied from 4 to 16%. There was a small divergence (1%) between algae from *P. evermanni* and *P. compressa/P. lobata* group; variation within the *P. compressa/P. lobata* group ranged from 0.2 to 0.3%.

A comparison of trees generated by coral host and zooxanthellae symbiont ITS comparisons revealed a high degree of congruence, except that the Florida *Porites* corals appeared to be more closely related to Hawai'ian taxa than were their algal symbionts.

Table 3. Numbers of nucleotides and informative PAUP characters (total, ITS-1, 5.8s and ITS-2) and percent GC content in ITS sequences obtained from zooxanthellae isolated from nine host species. *Cassiopeia* (Clones A & B), *Montipora* (Clones A & B), *Zoanthus*, and *Pocillopora* samples were from lab cultures. *Porites* zooxanthellae were freshly isolated from field-collected colonies. *P. micans* sequence obtained from GenBank. Lan=Lanikai samples, HB=Hanauma Bay samples, FL=Key Largo sample; bp=# of basepairs.

	Length (bp)				
	Total	ITS-1	5.8s	ITS-2	% GC
Cassiopeia-A	626	254	162	210	40
Cassiopeia-B	625	253	162	210	40
Montipora-A	628	256	161	211	40
Montipora-B	627	256	160	211	39
Zoanthus sociatus	637	264	162	211	42
Pocillopora	657	253	160	244	46
Porites evermanni-Lan	673	274	161	238	43
P. evermanni-HB	673	274	161	238	43
P. lobata-Lan	673	274	161	238	44
P. compressa-Lan	672	273	161	238	43
P. porites-FL	672	273	161	238	44
P. astreoides-FL	671	274	159	238	43
P. micans	667	262	162	243	43
Informative chars.	299	146	26	127	

SUMMARY

As in other studies, ITS sequence variability is taxon specific, and there appears to be no *a priori way* of assessing the utility of this region in comparisons of various taxonomic ranks. For *Porites* corals, ITS appeared to be very useful for inter-specific comparisons with great promise for construction of a molecular phylogeny within this diverse genus. ITS was more helpful in distinguishing clonal genotypes in some species (e.g., *P. lobata* and *P. compressa*) than in others (e.g., *P. evermanni*).

For zooxanthellae, ITS has the potential to be a fairly good marker for inter-specific comparisons, but will probably not be useful at lower taxonomic ranks (genotypes). However, the present study represents only a first look at variation within this region. As always, we would like to have more sequences from more taxa. The ease with which such data can now be obtained and the critical need for phylogenetic and genotypic markers will promote further investigation of this and other promising gene regions.

ACKNOWLEDGEMENTS

We thank the University of Hawai'i Biotechnology Center for use of the autosequencer; B. Batinga, R.A. Kinzie III, E. Tong, S. Santos, and G.J. Smith for algal cultures or culturing assistance; N. Phillips, S. Romano, S. Palumbi and lab, G. Roderick and lab, and R. Rowan for philosophical discussions, and K. Beauchamp for *Balanophyllia* DNA. This work was supported by ONR Grant #N000-14-92-J-1852 to CMS.

REFERENCES

Baker AC, Rowan R (1996) Biogeography of symbiotic dinoflagellates (zooxanthellae) inhabiting scleractinian corals. Proc 8th Inter Coral Reef Congress. Abstract.

Bakker FT, Olsen JL, Stam WT (1995) Evolution of nuclear rDNA ITS sequences in the *Cladophora albida/sericea* clade (Chlorophyta). J Mol Evol 40:640-651.

Baldwin BG (1992) Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: an example from the compositae. Mol Phylo Evol 1(1):3-16.

Bernard HM (1905) *Porites* of the Indo-Pacific Region. British Mus. (Natural History) Catalogue Madreporarian Corals (London), Vol. 5.

Buddemeier RW, Fautin DG (1993) Coral bleaching as an adaptive mechanism: a testable hypothesis. Bioscience 43:320-326.

Hunter CL (1988) Genotypic diversity and population structure of the Hawai'ian reef coral, *Porites compressa*. Ph.D. Dissert. Univ. of Hawai'i.

Maragos JE (1977) Order Scleractinia, Stony Corals. In: Devaney, DM Eldredge LG, Reef and Shore Fauna of Hawai'i. Section 1: Protozoa through Ctenophora. BP Bishop Mus Special Pub 64(1):158-241

Porter CH, Collins FH (1991) Species-diagnostic differences in a ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles hermis* (Diptera:Culicidae) Am J Trop Med Hyg 45:271-279

Rowan R, Powers DA (1991) A molecular genetic classification of zooxanthellae and the evolution of animal-algal symbioses. Science 251:1348-1351.

Rowan R, Knowlton N (1995) Intraspecific diversity and ecological zonation in coral-algal symbiosis. Proc Nat Acad Sci 92:2850-2853.

Schlötterer C, Hauser MT, Von Haeseler A, Tautz D (1994) Comparative evolutionary analysis of rDNA ITS regions in *Drosophila*. Mol Biol Evol 11:513-522.

Swofford DL (1993) PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1.1. Computer program distributed by the Illinois Natural History Survey, Champaign, Illinois.

Trench RT, Blank RJ (1987) *Symbiodinium microadriaticum* Freudenthal, *S. goreauii*, sp. nov., *S. kawagutii*, sp. nov, and *S. pilosum*, sp. nov.: Gymnodiniod dinoflagellate symbionts of marine invertebrates. J Phycol 23:469-481.

Vaughan TW (1907) Recent Madreporaria of the Hawai'ian Islands and Laysan. Bull U.S. Nat Mus No. 59. 427 pp.

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosoamal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols, a guide to methods and applications. Academic Press Inc, San Diego, California