Is the ITS Region the Solution to the 'Species Problem' in Corals? Intragenomic Variation and Alignment Permutation in *Porites*, *Siderastrea* and Outgroup Taxa

Zac H. FORSMAN[1,*], Cynthia L. HUNTER[1], George E. FOX[2], and Gerard M. WELLINGTON[2]

[1]Biology Program, University of Hawaii at Manoa, Dean 2, 2450 Campus Rd., Honolulu, HI 96822
[2]Department of Biology and Biochemistry, University of Houston, Houston, Texas 77204
[*]Corresponding author: Z.Forsman FAX: 1- 808-956-4745, e-mail address: zac@hawaii.edu

Abstract Two widely acknowledged problems with the use of the ribosomal ITS region as a phylogenetic marker were examined in scleractinian coral: intragenomic variation and alignment ambiguities. Sequences from Caribbean and Eastern Pacific *Porites* and *Siderastrea* species were examined, as well as the nearest available outgroup sequences from GenBank (*Tubastrea*, *Balanophyllia*, *Scapophyllia* and *Montastrea*). Intragenomic variation was considerably lower than previously reported in corals, and *Porites* species were fully resolved. Despite a patchwork of conserved sequence motifs among all taxa, objective alignment between higher taxonomic levels became difficult as gaps and ambiguities increased. To examine the potential for the ITS region to resolve deeper level relationships, fifty alternative alignments were generated by gap-penalty permutation, and examined for consensus. The tree topologies were remarkably congruent, suggesting strong phylogenetic signal at several taxonomic levels. Alternative topologies only rarely occurred at alignment parameter extremes, therefore mid-range parameters were chosen for phylogenetic analysis. The resulting phylogeny was generally consistent with the fossil record, and with previous molecular studies while yielding higher resolution.

Keywords *Porites*, *Siderastrea*, ITS region, ribosomal spacers, intragenomic variation, alignment gaps

Introduction

Reef building corals are notoriously difficult to identify at the species level. Scleractinian taxonomy has traditionally relied on skeletal morphology, which is often highly variable (Veron 1995;2000). The 'species problem' in corals is well exemplified by the circumtropical and abundant genus *Porites* (Bernard 1902;Brakel 1977); only a third of the approximately 122 named species are currently considered valid (Veron and Pichon 1982;Cairns et al. 1999). An additional problem in coral systematics is that many of the well-studied and widely used molecular markers appear to have insufficient levels of polymorphism for resolution below the genus level. Mitochondrial DNA evolves at a slow rate in corals relative to other metazoans (Romano and Palumbi 1997; van Oppen et al. 1999; Shearer et al. 2002). Molecular markers such as the nuclear 28S rRNA gene and the mitochondrial 16S rRNA gene have been uninformative at or below the genus level (Chen et al. 1995; Veron et al. 1996; Romano and Palumbi 1997). The transcribed spacers of nuclear ribosomal genes (ITS-1 and ITS-2) are the most widely used molecular markers in scleractinian coral (Hunter et al. 1997; Lopez and Knowlton 1997; Odorico and Miller 1997; Takabayashi et al. 1998a, 1998b; Medina et al. 1999; van Oppen et al. 2000, 2002; Diekmann et al. 2001; Rodriguez-Lanetty and Hoegh-Guldberg 2002; Forsman 2003,in press; Márquez et al. 2003; Chen et al. 2004; but see Vollmer and Palumbi 2004).

There is a considerable precedent for the use of ITS sequence divergence to infer relationships at or below the species level in a wide variety of other taxonomic groups, most notably plants and fungi (e.g. Baldwin et al. 1995; Sang et al.1995; Kuninaga et al. 1997); however, there are several widely acknowledged problems with the marker that can confound phylogenetic studies (see Alvarez and Wendel 2003 for a review). Ribosomal genes and associated spacers are arranged in one or more large arrays consisting of hundreds or thousands of tandemly repeated copies. Variation between copies within a species has been observed to be orders of magnitude lower than between species (multicopy genes evolving as though they are one, is known as concerted evolution) and recombinant processes such as gene conversion and unequal crossover are thought to be the homogenizing mechanisms (Hillis and Dixon 1991;Elder and Turner 1995). Significant variation between copies within a species has been discovered in some taxa, that has been attributed to introgression from hybridization, pseudogenes, or separately evolving chromosomal lineages (e.g. Wendel et al. 1995; Sang et al. 1995; O'Donnell and Cigelnik 1997; Polanco et al. 2000; van Oppen et al. 2000; Muir et al. 2001). A second potential problem with the ITS region as a phylogenetic marker is that the sequences can be hypervariable and prone to insertions and deletions, which can result in alignment ambiguities. These potential problems are rarely explicitly addressed and are necessary to examine, due to the lack of adequate molecular markers in scleractinian

corals. The goals of this study are: (i) to examine the nature of variability in the ITS region at individual, population, and higher taxonomic levels, (ii) to examine the phylogenetic signal from species to family level by comparisons of alternative ITS alignments, and (iii) to examine ITS resolution between several prominent species, genera and families of coral.

Materials and Methods

Small fragments, 20-50 grams (dry weight) of tissue and skeleton, were collected at least 100 meters apart to avoid collecting colonies that originated from fragmentation or budding. Samples were divided into two pieces in the laboratory: a small piece was preserved in 95%-100% ethanol stored at -20°C for genetic analysis and a voucher piece was bleached and dried (see Table 1 for geographic locations). A few milligrams of tissue and skeleton were dried and then homogenized in 250 µl of 50 mM tris-HCL (pH 8.0) and 10 mM EDTA with a micro-pestle, followed by 5-minute RT incubation in 250 µl of 20 mM NaOH and 1% SDS, and a 5-minute incubation on ice in 350 µl of 3.0 M potassium acetate (pH 5.5) followed by centrifugation. The top 500 µl of the cleared lysate was precipitated in 1 ml isopropanol, dried and resuspended.

The ITS region (spanning a partial sequence of the 5' end of the 18S rRNA gene, the complete sequence of ITS-1, 5.8S rRNA gene and ITS-2, and a partial sequence of the 3' end of the 28S rRNA gene) was amplified using the eukaryotic 'universal' primers, ITS-1 (5' -TCC GTA GGT GAA CCT GCG G-3'), and ITS-4 (5' -TCC TCC GCT TAT TGA TAT GC-3') (White et al. 1990), with the following PCR profile: 96˚C for 2 minutes, 30 cycles of: 96˚C for 10 seconds, 50˚C for 30 seconds, and 70˚C for 4 minutes. Reactions produced a single clear band (approx. 650 bp in *Siderastrea* and 700 bp in *Porites* species). PCR products were ligated into the PgemT-EZ cloning vector (Promega Inc.) and transformed into JM109 competent cells, followed by blue-white colony screening. White colonies were screened for inserts by colony PCR using the M13 vector primers. An average of three molecular clones from each individual were sequenced using the M13 vector primers in both the forward and reverse direction. Sequencing was performed with ABI cycle sequencing chemistry. Sequences were confirmed by sequences from *Porites* (Hunter et al. 1997; AF180112-AF180118), and other coral ITS sequences from a BLAST query of the National Center for Biological Information's (NCBI) sequence database. The sequences have been deposited in GenBank accession numbers: **AY320289-AY320352**, **AY322575-AY322612**, and **AY458021-AY458063**.

Nucleotide diversity was estimated in MEGA 2.1 (Kumar et al. 2001) based on pairwise distances (Table 2 - 3). Representative sequences (two sequences with the largest pairwise distance for each species) were used to construct a total of 50 separate alignments generated by altering the Gap Opening Penalty [GOP], and the Gap Extension Penalty [GEP] (after McFadden et al. 2001, see Fig. 1) in ClustalW (Thompson et al. 1994). All pairwise combinations of the following values were used: GOP= 0.1, 1, 2, 4, 8, GEP = 0.1, 0.3, 1, 2, 4. Twenty-five separate alignments were constructed for the *Porites* sequences, in addition to twenty-five separate alignments for the *Porites* and 'outgroup' taxa (including *Siderastrea* sequences and the closest hits from a BLAST search of GenBank; [*Tubastraea coccinea* (Dendrophylliidae AF180110), *Balanophyllia elegans* (Dendrophylliidae AF180110), *Scapophyllia cylidrica* (Faviina; Merulinidae SCU65479), *Montastaea faveolata* (Faviina; Faviidae AB065353)]. A tree was constructed for each of the 50 representative alignments using the Maximum Likelihood method implemented in PHYLIP version 3.6 (Felsenstein 2002). The program DAMBE v4.1.19 (Xia and Xie 2001) was used to examine mutational saturation in transitions and transversions as genetic distance (K80) increased. Phylogenic trees were estimated in PAUP 4.10b10 (Swofford et al. 2002). Distance trees were estimated with the HKY85 distance measure, and Maximum Parsimony trees were estimated with the fast-heuristic method, both with 1000 bootstrap replicates. The likelihood ratio test as described by Felsenstein (1988) was performed on the *Porites* taxa separately, and then with the successive addition of outgroups, in PHYLIP 3.6 (Felsenstein 2002).

Results

One hundred and twenty-seven contiguous sequences were assembled and compared from forty-six individuals and eleven species (Table 1). The length and GC content of ITS-1 and ITS-2 indicated differentiation between taxa (Table 1). Multiple sequences collected from the same individual differed on average by 0.56%±0.49% (Table 2). Comparisons between separate individuals collected from the same population were possible using 90 sequences from 34 individuals in 6 species (*P.lobata, P.*cf.*lutea*, *P.astreoides*, *P.sverdrupi*, *P.divaricata*, and *S.siderea*); intrapopulation nucleotide diversity averaged 0.95%±0.51%. Two species (*P.lobata* and *P.astreoides*) were sampled across a large geographic range, allowing interpopulation comparisons among 64 sequences from 21 individuals. The average interpopulation nucleotide diversity was 1.18%±0.45%. The same hierarchical patterns (intragenomic < intrapopulation < interpopulation) were evident when *P.lobata* or *P.astreoides* ITS nucleotide diversity was examined independently (data not shown).

Average interspecific differences were generally at least an order of magnitude larger than intraspecific nucleotide diversity (Table 2 - 3). All samples that were collected from the Pacific side of Panamá were originally identified as *P.lobata;* however, they were genetically distinct (differing on average 6.2%±0.9%) from *P.lobata* collected from all other locations (Galápagos, Tahiti, Easter Island, Fiji, Rarotonga, and Australia).

Table 1. Samples, region, collector, length, and G+C content of ITS sequences used in this study. Abbreviations: N, number of individuals; n, number of sequences; EP, Eastern Pacific; CP, Central Pacific; ATL, Atlantic; GOM, Gulf of Mexico (Flower Gardens Marine Sanctuary); GOC, Gulf of California (San Sebastian and Punta Chivato); WP, Western Pacific. Collectors and dates are represented by numbers in superscript: 1 = H. Guzman (2001), 2 = J. Mate and H. Guzman (2001), 3 = E. Neves (2000), 4 = T. Snell (2000), 5 = G. Wellington (2000), 6 = C. Guevara (2001), 7 = B. Victor (2001), 8 = G. Wellington (1999), 9 = M. Takabayashi (1998), 10 = Z. Forsman (1998). Samples in bold letters indicate that a skeletal voucher specimen was also collected.

		ITS-1			ITS-2				
		length			length				
Species	**region**	**bp**	**%GC**	**SE**	**bp**	**%GC**	**SE**	**N**	**n**
S. siderea	Panamá (ATL)[2]	305	44.22	0.35	192-193	53.41	0.58	3	14
S. radians	Panamá (ATL)[2]	307	43.65	0.12	192	55.70	0.24	2	6
S. stellata	**Brazil (ATL)[3]**	307-308	44.43	0.12	192	55.60	0.35	1	3
P. astreoides	Texas (GOM)[4]	304	42.40	0.00	234	43.47	0.23	1	3
" "	**Belize (ATL)[5]**	304	42.30	0.17	231-234	43.53	0.21	1	3
" "	**Brazil (ATL)[3]**	304-305	42.16	0.55	231-236	44.41	0.72	3	7
" "	**Panamá (ATL)[6]**	304	41.87	0.40	231-233	44.63	0.40	2	3
P. divaricata	**Belize (ATL)[5]**	298-300	41.17	0.24	223-228	45.16	0.57	3	7
P. furcata	**Panamá (ATL)[6]**	300	41.70	0.00	227-230	44.97	0.57	1	3
P. sverdrupi	**Mexico (GOC)[7]**	317-318	43.23	0.21	229-230	48.37	0.26	3	7
P. panamensis	**Panamá (EP)[1]**	317-318	43.67	0.45	233-236	49.30	0.10	1	3
P. colonensis	**Panamá (ATL)[2]**	286	43.00	0.00	248-249	44.15	0.10	2	4
P. cf. lutea	**Panamá (EP)[8]**	303-311	43.27	0.19	228-229	44.91	0.23	4	7
P. lobata	**Easter Isl.(CP)[8]**	303-312	42.02	0.47	215-226	43.89	0.22	4	10
" "	Australia (WP)[9]	305-325	42.17	0.43	210-223	43.91	0.75	2	7
" "	Rarotonga (WP)[8]	306-309	42.27	0.26	207-226	44.16	0.45	3	9
" "	**Tahiti (CP)[8]**	306-309	41.96	0.43	207-231	43.89	0.43	3	9
" "	**Galápagos (EP)[10]**	306-307	42.29	0.34	209-225	44.04	0.49	4	15
" "	**Fiji (CP)[8]**	305-309	41.91	0.41	215-223	43.81	0.83	3	7
							Total	46	127

Table 2. Intragenomic, intrapopulation, and interspecies average pairwise sequence diversity and standard error.

	N species	N individuals	n sequences	Avg. distance	SE
Intragenomic	10	33	125	0.005579	0.004979
Intrapopulation	6	34	90	0.009486	0.005053
Interspecies	2	21	64	0.011817	0.004501

Table 3. Intraspecies (first column in bold) and interspecies average pairwise sequence diversity, and standard errors.

Species	Intra-species	*S. siderea*	*S. radians*	*S. stellata*	*P. astreoides*	*P. divaricata*	*P. furcata*	*P. sverdrupi*	*P. panamensis*	*P. colonensis*	*P. cf.lutea*
S.siderea	**0.008 ±0.002**										
S.radians	**0.001 ±0.001**	0.034 ±0.007									
S.stellata	**0.002 ±0.002**	0.035 ±0.007	0.005 ±0.003								
P.astreoides	**0.011 ±0.002**	0.280 ±0.024	0.295 ±0.025	0.297 ±0.025							
P.divaricata	**0.003 ±0.001**	0.311 ±0.026	0.319 ±0.027	0.322 ±0.027	0.202 ±0.019						
P.furcata	**0.006 ±0.002**	0.317 ±0.026	0.326 ±0.027	0.329 ±0.027	0.207 ±0.020	0.013 ±0.004					
P.sverdrupi	**0.005 ±0.002**	0.351 ±0.028	0.355 ±0.028	0.358 ±0.028	0.215 ±0.021	0.133 ±0.014	0.134 ±0.014				
P.panamensis	**0.004 ±0.002**	0.361 ±0.028	0.365 ±0.029	0.367 ±0.029	0.219 ±0.021	0.145 ±0.014	0.147 ±0.015	0.011 ±0.003			
P.colonensis	**0.002 ±0.001**	0.336 ±0.026	0.346 ±0.027	0.349 ±0.027	0.223 ±0.020	0.190 ±0.018	0.197 ±0.018	0.154 ±0.017	0.163 ±0.017		
P.cf.lutea	**0.003 ±0.001**	0.274 ±0.024	0.281 ±0.024	0.283 ±0.025	0.093 ±0.012	0.210 ±0.020	0.206 ±0.020	0.209 ±0.020	0.215 ±0.021	0.223 ±0.021	
P.lobata	**0.012 ±0.002**	0.270 ±0.024	0.280 ±0.024	0.282 ±0.024	0.094 ±0.012	0.203 ±0.019	0.197 ±0.019	0.202 ±0.020	0.208 ±0.020	0.202 ±0.020	0.062 ±0.009

These specimens can also be morphometrically distinguished by principal component analysis (Forsman 2003), and appear quite similar to *P.lutea* specimens housed at the Bishop Museum (pers. obs.). Since there is no record of *P.lutea* occurring in Panamá, we will refer to this species as *P.*cf.*lutea* until the identity can be confirmed.

Despite patches of conserved regions throughout ITS-1 and ITS-2, alignment became problematic as genetic distance increased. The sensitivity of tree topology to alternative alignments was evaluated by altering alignment parameters that influence alignment length (Fig. 1 A). Tree topology was robust to all but extreme values (Fig. 1B, C). The choice of outgroup can greatly influence the tree topology through long branch attraction (Felsenstein 1988), therefore unrooted phylogenies of *Porites* taxa were estimated first. The subsequent addition of outgroup taxa did not alter the topology of the ingroup taxa (Fig. 1 C). This procedure, and the addition of multiple outgroup taxa was employed to avoid inherent problems associated with outgroup choice and long branch attraction (Swofford et al. 1996).

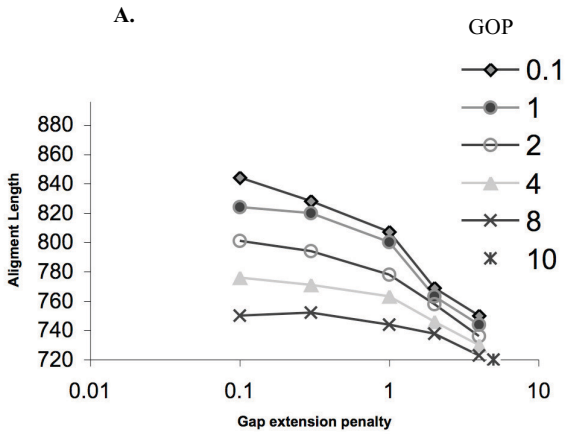
Transitions did not appear to reach mutational saturation as genetic distance increased (there was no asymptote when transitions were plotted against genetic distance—data not shown); however, transversions were highly influenced by the choice of alignment parameters. The parameters GOP = 2, GEP = 1 were chosen for further analysis due to the similarity in ts/tv ratios to taxa, or parts of the alignment with no alignment ambiguity (transversions were also linear as genetic distance increased under these parameters). Distance and parsimony methods yielded similar tree topologies (Fig. 2 - 3), with similar bootstrap support for most nodes. The likelihood ratio test of the molecular clock hypothesis (Felsenstein 1988) could not be rejected for *Porites* taxa (likelihood ratio chi-square = 14.89; 10 d.f., $p = 0.136$), but was rejected when additional taxa were included. The tree in Fig. 2 was linearized assuming a rate of evolution of 0.4% per million years, based on previously published rates for the ITS region in birch trees (Savard et al. 1993). This rate was chosen from several alternative previously published rate estimates e.g. 0.011–0.012 in Drosophilla (Schlotterer et al. 1994), and 0.003625–0.00725 in Cucurbitaceae (Jobst et al. 1998), because birch trees have more similar life history traits to corals than the available alternatives, and it has been shown that rates of evolution correlate with a suite of characteristics that affect nucleotide generation time, such as body size, metabolism, or generation time (Martin and Palumbi 1993).

Discussion

Intragenomic ITS sequence diversity in *Porites* and *Siderastrea* individuals averages approximately

half of one percent (0.56%±0.5%), which is in contrast to most other studies on scleractinians, which have described values ranging from 2% to as high as 29% (Lopez and Knowlton 1997; Odorico and Miller 1997; Medina et al. 1999; van Oppen et al. 2000, 2002; Diekmann et al. 2001; Vollmer and Palumbi 2004; but see Forsman 2003, Forsman et al. in press, and Chen et al. 2004). Previous studies have focused on species groups that are highly likely to hybridize (such as *Acropora*). High levels of ITS intragenomic variation may be due to introgression from hybridization, or slowed rates of lineage sorting of ancestral alleles. This study provides an example of two Scleractinian genera with rates of concerted evolution that appear faster than the rate of speciation. Intraspecific differences were never larger, and usually an order of magnitude smaller, than interspecific differences, and most species were fully resolved with strong bootstrap support.

The robustness of the tree topology to alternative alignments is likely due to a strong underlying phylogenetic signal that overrides noise caused by ambiguous positions in the alignment. The lack of mutational saturation (especially in transitions) indicates that the marker may be informative for deeper level phylogenetic comparisons in this group of corals. Although taxonomic sampling is currently too sparse at the genus and family level to make any firm conclusions, the results were generally consistent with previous molecular studies (Veron et al. 1996; Romano and Cairns 2000), and clearly warrant further investigation. A recent study by Chen et al. (2004) used predicted secondary structural models of the ITS-2 region to guide sequence alignment between several genra (*Cyphastrea*, *Montipora*, *Madracis,* and *Porites*), and found the region to be quite informative at several taxonomic levels. Our results echo similar findings, although derived from an alternative and completely independent approach.



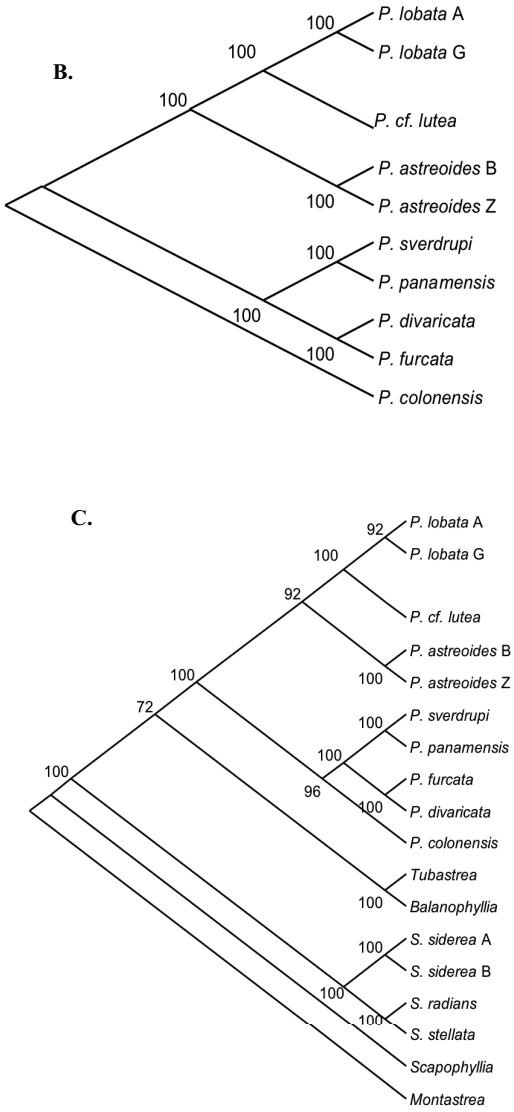


Fig. 1. The effects of gap opening and gap extension penalties on alignment length. **A,** Each line represents a gap opening penalty of 0.1, 1, 2, 4, and 8. Gap extension penalty is in log scale. The asterisk represents the default value in ClustalW (Thompson *et al*. 1994), GOP = 10, GEP = 5. **B**, Consensus of the 25 maximium likelihood cladograms generated for each alternative alignment for the *Porites* taxa. **C,** Consensus cladogram of the 25 permuted alignments for *Porites* and outgroup taxa. Values at each node are not bootstrap statistics, but rather they represent the percentage of alignments that yielded the same node (out of 25 alignments for each analysis). Abbreviations after the species name are as follows; for *P.lobata*, A = Australia, G = Galápagos, for *P.astreoides*, B = Belize and Z = Brazil, for *S.siderea*, A and B represent the two most distinct sequence types collected from the Caribbean side of Panama.

Fig. 2. A neighbor-joining tree of *Porites* and outgroup taxa. Distances were calculated with the HKY85 method, with 1000 bootstrap replicates (indicated by regular script). Values in bold script indicate Maximum Parsimony bootstrap values from 1000 replicates. Only bootstrap values above 70% are shown. The width of each triangle base is proportional to the number of sequences in the clade (approximately 4 pixels/taxon). The height (depth in time) of the triangle is proportional to the variability within the group. The scale is proportional to the number of nucleotide substitutions per site, and to estimated time of divergence, assuming a constant rate of 0.004 substitutions per site, per million years. *This node was not resolved by Maximum Parsimony. **Maximum Parsimony grouped Dendrophyllidae with Poritidae, not with Siderastridae.

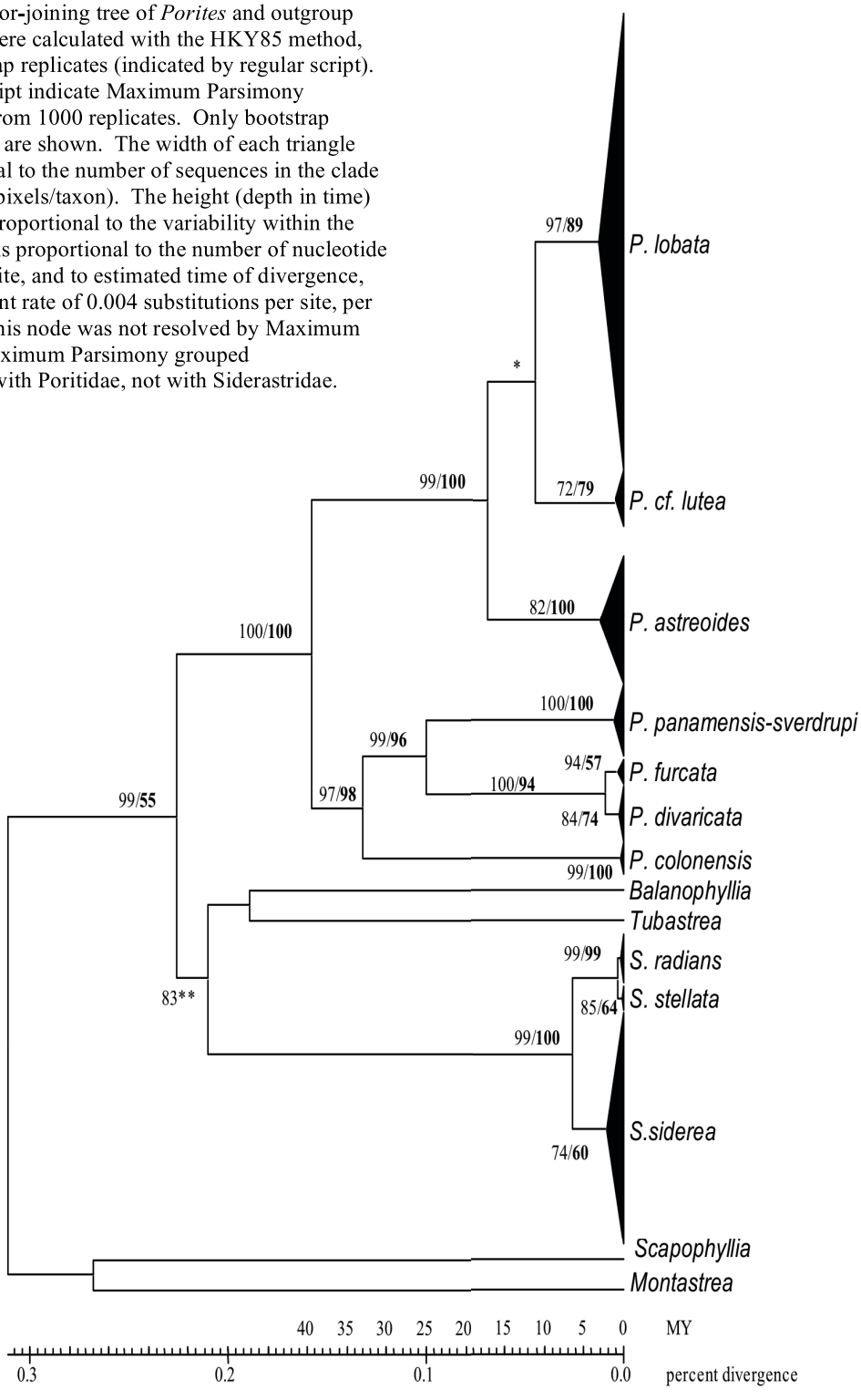
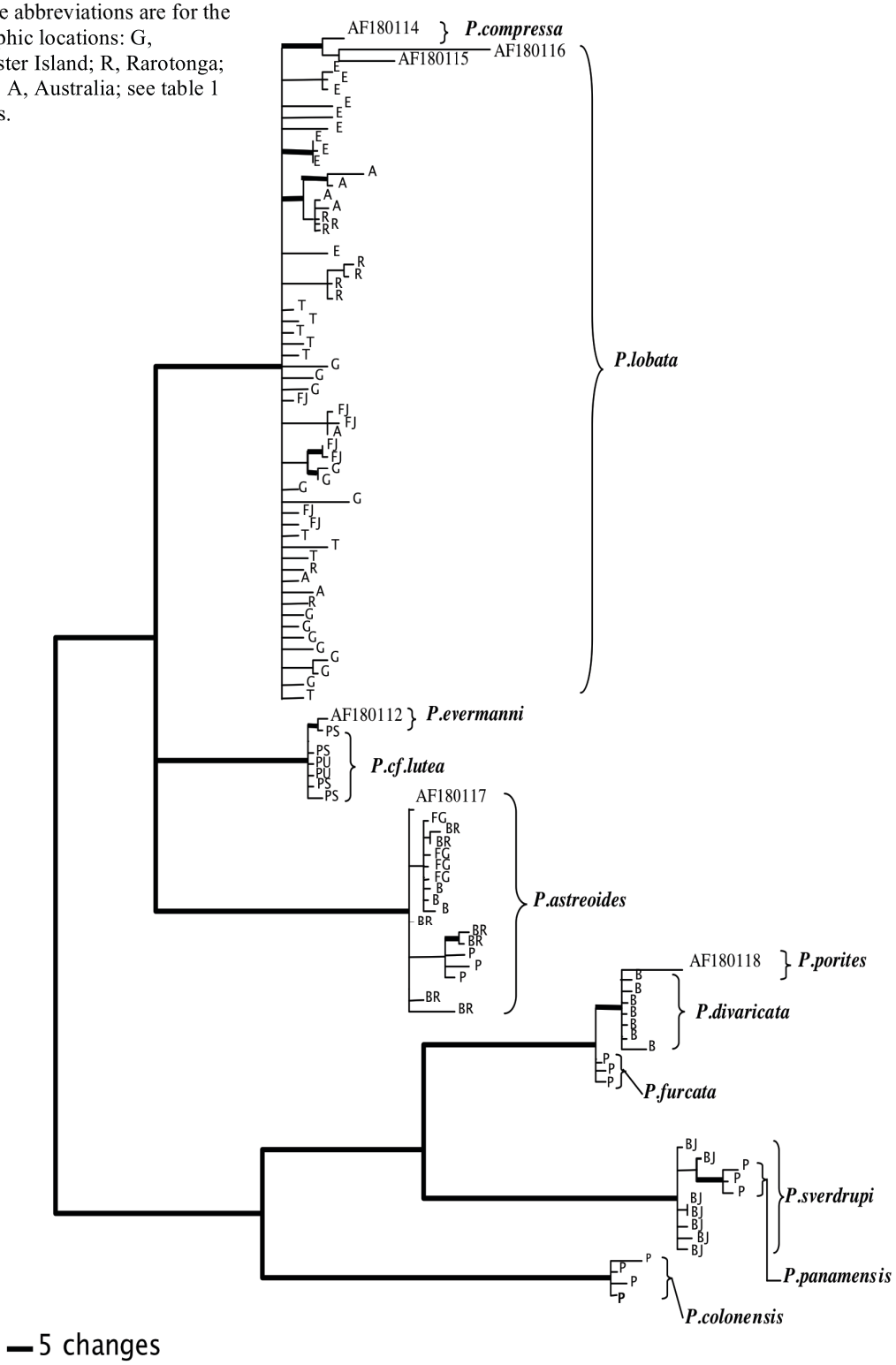


Fig. 3. A Maximum Parsimony tree of *Porites* sequences, including sequences from GenBank (Hunter et al. 1997). Thick lines indicate nodes that are supported by larger than 70% bootstrap support (see Fig. 3 for values). Sequence abbreviations are for the following geographic locations: G, Galapagos; E, Easter Island; R, Rarotonga; T, Tahiti; FJ, Fiji; A, Australia; see table 1 for other locations.



Weil (1992) and Weil et al. (1994) examined 11 polymorphic allozyme loci in many of the same *Porites* species examined in this study. The allozyme data indicate similar genetic distances between species, although the hypothesis that *P.panamensis* and *P.colonensis* are geminate species (separated by the closure of the Central American Seaway, 3.5-3.8 Ma) is not supported by this study. The results of this study confirm the results of Hunter et al. 1997 (Fig. 3), despite differences in sequence length and sequencing methods (direct sequencing of the PCR product, as opposed to cloning and sequencing in two directions in the present study).

The scleractinian fossil record is one of the most extensive of any group of organisms (Veron et al. 1996; Romano and Cairns 2000). Taxonomists have grouped Faviidae with Merulinidae, and Poritidae with Siderastreidae or Dendrophyllidae, all originating in the mid- to late Cretaceous (Wells 1956; Roniewicz and Morycowa 1993; Veron et al. 1996; Veron 2000). The earliest appearance of *Porites* in the fossil record was in the Eocene in the Caribbean and the Tethys (Veron 2000). Budd et al. (1994) divided the Caribbean *Porites* into two groups: (1) *Porites* I, which consists of primarily mounding colony morphology, and reproduction by broadcast spawning (*P. astreoides* is the only extant member). (2) *Porites* II, which consists of primarily branching colony morphology, and reproduction by brooding (*P.furcata, P.divaricata,* and *P.colonensis* are extant). According to Potts et al. (1993), the *Porites* I group diverged from II between 30-40 million years ago, and these divisions and their timings are consistent with the ITS phylogeny (Fig. 2). More recently, *P.divaricata* first appeared in Jamaican formations between 1.6 and 2.5 ma, and in Florida formations between 1.6 and 1.8 ma (Budd et al. 1994), which is also consistent with Fig. 2.

The genetic differentiation of *Porites* samples from Panamá (Figs. 1, 2, and 3) was unexpected; all mounding *Porites* (n = 20) collected from Uva and Saboga Panamá, were also morphometrically distinct from *P.lobata* collected from the Galapagos, Easter Island, Tahiti, Rarotonga, and Fiji (Forsman 2003). The ITS sequences of *P.*cf.*lutea* from Panamá, are indistinguishable from *P.evermanni* sequences from Hawaii (Forsman et al. unpublished data). *P.evermanni* has recently been independently synonomized with *P.lutea* (Fenner 2005), based on the original species descriptions.

As more specimens and more sequence data are collected, the difficult taxonomy of *Porites* is likely to become increasingly resolved. The elusive *Porites* species problem remains far from a solution; however, this study establishes important first steps, by examining the properties of a molecular marker that can resolve relationships between species. The characteristics of the ITS region in this study suggests that the marker may be very useful at several taxonomic levels. Additional studies are needed to determine if ITS variation in corals is more commonly typified by patterns seen in the *Acropora* or in *Porites* and *Siderastrea* species.

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