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Analyses of the Ribosomal Internal Transcribed Spacers (ITS) and the 5.8S Gene Indicate that Extremely High rDNA Heterogeneity is a Unique Feature in the Scleractinian Coral Genus *Acropora* (Scleractinia; Acroporidae)

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Nu-Wei Vivian Wei, Carden C. Wallace, Chang-Feng Dai, Kamla Ruby Moothien Pillay, and Chaolun Allen Chen (2006) Analyses of the ribosomal internal transcribed spacers (ITS) and the 5.8S gene indicate that extremely high rDNA heterogeneity is a unique feature in the scleractinian coral genus *Acropora* (Scleractinia; Acroporidae). *Zoological Studies* **45**(3): 404-418. One characteristic of ribosomal DNA (rDNA) sequences in staghorn corals, *Acropora* spp., is the extremely high levels of intragenomic heterogeneity and interspecific variation. This high genomic diversity is ascribed to incomplete lineage sorting that predated the divergence of species or to recent introgressive hybridization. In order to elucidate whether the high heterogeneity of rDNA is a unique feature of *Acropora* or a general pattern applicable to scleractinian corals, we examined the molecular evolution of the internal transcribed spacers (ITS) and 5.8S rDNA sequences from 78 species, representing 28 genera, and 12 families of scleractinian corals. Genetic distances (measured by *p*-distances) and frequency distribution analyses revealed that both extremely high intra- and interspecific heterogeneities of the ITS-5.8S rDNA are specific to the genus *Acropora*. The 5.8S rDNA phylogeny clearly showed a significantly long branch length leading to the cluster containing the genus *Acropora*. The molecular-clock hypothesis tested using the likelihood ratio test indicated a highly significant difference in the global evolutionary rate of scleractinian 5.8S rDNA. The relative rate tests showed that the rDNA of *Isopora*, Caribbean *Acropora*, and Indo-Pacific *Acropora* all evolved at constant tempos, indicating that the highly divergent rDNA was present in *Acropora* before it split into these three lineages. In contrast, rate constancy was rejected for most comparisons between *Acropora*/*Isopora* and other coral genera, suggesting that the rates of evolution of 5.8S differed between *Acropora*/*Isopora* and the other lineages, and that the evolutionary rate of *Acropora*/*Isopora* has accelerated since divergence from the common ancestor of scleractinian corals.
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Key words: *Acropora*, Hybridization, Ribosomal DNA, Concerted evolution, Ancient divergence.

Ribosomal DNA (rDNA) has long been used as a potential marker for phylogenetic studies (reviewed in Avise 2004). rRNA genes are organized in clusters of tandemly repeated units, each of which consists of coding regions (18S, 5.8S, and 28S) and 2 internal transcribed spacers (ITS) and 1non-transcribed spacer (NTS) region. While the coding regions are evolutionarily conserved and have been utilized for phylogenetic inferences for major phyla (reviewed in Hills and Dixon 1990),

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the 2 ITS regions are appropriate for detecting differences between conspecific individuals and are hence potentially useful markers to study the relationships of populations and closely related species in fungal, plant, and animal taxa due to their relatively rapid evolutionary rates (Baldwin 1992, Schlötterer et al. 1994, Mai and Coleman 1997, Weekers et al. 2001, Oliverio et al. 2002, Chen et al. 2002 2004).

One of the major concerns with the use of the rDNA locus in phylogenetic analyses is the existence of polymorphisms among repeated units, which may cause extensive differentiation even within a single individual. However, concerted evolution, a process resulting in the homogenization of individual repeats, is assumed to produce a mostly uniform sequence in all repeats of a given species (Li 1997). Two mechanisms, unequal crossover and gene conversion, have been proposed for the process of concerted evolution. Unequal crossing-over is caused by a recombination among tandem repeats either within (homologous) or between (heterologous) chromosomes, resulting in the stochastic elimination of variations in individuals and populations. In contrast, nonstochastic processes such as directed gene conversion assume that selection drives the homogenization of tandem repeats (Dover 1982, Hillis et al. 1991).

Phylogenetic studies based on fragments of rDNA ITS-5.8S have provided novel insights into scleractinian coral evolution (Hunter et al. 1997, Lopez and Knowlton 1997, Odorico and Miller 1997, Medina et al. 1999, van Oppen et al. 2000 2002 2004, Diekmann et al. 2001, Forsman 2003, Forsman et al. 2005a b, Lam and Morton 2003, Marquez et al. 2003, Chen et al. 2004, Fukami et al. 2004, Vollmer and Palumbi 2004, Moothien Pillay et al. 2005). Some of those studies indicated that individual coral colonies host a high degree of intragenomic variation, and coral ITS phylogenies in several cases are polyphyletic among closely related congeners (Odorico and Miller 1997, Hatta et al. 1999, Medina et al. 1999, Fukami et al. 2000, van Oppen et al. 2000 2002 2004, Diekmann et al. 2001, Marquez et al. 2003). Most of these conclusions were derived from studies of the genus *Acropora*. For example, variation in the ITS-5.8S fragment was estimated to be as high as 40% (*p*-distance) at the interspecific level for *Acropora* spp. (Odorico and Miller 1997, van Oppen et al. 2001 2002) and as low as < 8% among species of *Madracis* in the Caribbean (Diekmann et al. 2001). Despite the extreme disparity in the divergence patterns of the ITS-5.8S regions between these 2 coral groups, both phylogenetic studies concluded that the evolutionary patterns of potentially hybridizing corals are consistent with reticulation. On the contrary, phylogenetic analyses of the ITS-5.8S fragment demonstrated clear boundaries for species in the genera *Pavona*, *Platygyra*, *Porites*, and *Siderastrea* (Forsman 2003, Lam and Morton 2003, Forsman et al. 2005, Moothien Pillay et al. 2005). These contrasting results imply that the rate of concerted evolution (i.e., homogenization) among tandem repeats of ITS-5.8S is variable in different lineages of scleractinian corals.

Recent analyses of ITS-5.8S regions have revealed that the phylogenetic signature of recent introgressive hybridization is obscured in the Caribbean *Acropora* because they shared ancient rDNA lineages that predated divergence of the species (Vollmer and Palumbi 2004). It was concluded that nuclear rDNA should be abandoned as a species- and population-level phylogenetic marker for scleractinian corals due to its complicated and undistinguishable characteristics of molecular evolution (Vollumer and Palumbi 2004). Chen et al. (2004) reevaluated this proposal by examining the phylogenetic utility and secondary structure of the ITS2 from 54 species of scleractinian corals, representing 25 genera and 11 families. The comparative analysis showed that the extremely high ITS intragenomic divergence of *Acropora* appears to be an exception rather than the rule for the evolutionary history of scleractinian corals, suggesting that ITS2 DNA sequences are still applicable, with adequate adjustment of the secondary structures, to the primary sequence alignment of different levels of phylogenetic analyses (from populations to genera) in scleractinian corals.

In this study, we extended the examination of the molecular divergence of complete rDNA ITS-5.8S regions from 78 species, representing 28 genera, and 12 families of scleractinian corals. We then constructed phylogenetic trees based on 5.8S rDNA and applied the likelihood ratio test (LRT) and relative rate test (RRT) to examine the molecular evolutionary rate constancy. The results indicate that acceleration of the rDNA ITS-5.8S region occurred in the *Acropora* lineage after divergence from a common ancestor of scleractinian corals, and that the extremely high ITS rDNA diversity is a unique molecular feature of the genus *Acropora*.

Table 1. Taxonomic information, GenBank accession numbers, and data sources

Taxon	No. of species	No. of seq.	Accession numbers onGenBank	Codes of data sources
Order Scleractinia				
Family Acroporidae				
Genus *Acropora*				
Subgenus *Acropora*	19	156	AF228164-AF538598	van Oppen *et al*. (2002), Marquez *et al*. (2003), van Oppen *et al*. (2000), and this study
Subgenus *Isopora*	4	14	AF538561-AF538567	Marquez et al. (2003) and this study
Genus *Montipora*	7	7	AY722772-AY722780	Chen *et al*. (2004)
Genus *Astreopora*	1	1	AY722742	Chen *et al*. (2004)
Genus *Anacropora*	1	1	AY722747	Chen *et al*. (2004)
Family Pocilloporidae				
Genus *Pocillopora*	2	2	AY722785, AY139815	Chen *et al*. (2004) and Genbank data (unpublished)
Genus *Stylophora*	1	1	AY722795	Chen *et al*. (2004)
Genus *Seriatopora*	1	1	AY722794	Chen *et al*. (2004)
Family Faviidae				
Genus *Montastraea*	4	68	AB065299-AB065364, AY722774, AY722774	Chen *et al*. (2004) and Genbank data (unpublished)
Genus *Oulastrea*	1	1	AY722781	Chen *et al*. (2004)
Genus *Platygyra*	2	12	AF481893-AF481905	Lan and Morton (2003)
Genus *Plesiastrea*	1	11	AF483804-AF483813	Rodriguez-Lanetty and Hoegh-Guldberg (2002)
Genus *Cyphastrea*	1	3	AY722749-AY722751	Chen *et al*. (2004)
Genus *Favites*	1	2	AY722755, AY722756	Chen *et al*. (2004)
Genus *Goniastrea*	2	6	AY722759-AY722763	Chen *et al*. (2004)
Genus *Cladocora*	1	3	AY722752-AY722749	Chen *et al*. (2004)
Family Merulinidae				
Genus *Hydnophora*	1	3	AY722769-AY722771	Chen *et al*. (2004)
Family Poritidae				
Genus *Porites*	9	110	AY720289-AY722788	Chen *et al*. (2004), Forsman *et al*. (2005)
Genus *Alveopora*	1	1	AY711746	Chen *et al*. (2004)
Family *Agariciidae*				
Genus *Pavona*	2	38	AB217876-AB217913	Pillay *et al*. (2005)
Family Siderastreidae				
Genus *Psammocora*	1	3	AY722782-AY722784	Chen *et al*. (2004)
Genus *Pseudosiderastrea*	1	2	AY722789-AY722790	Chen *et al*. (2004)
Genus *Siderastrea*	4	38	AY322575-AY322612	Forsman *et al*. (2005)
Family Dendrophylliidae				
Genus *Tubastraea*	1	1	AY722796	Chen *et al*. (2004)
Family Astrocoeniidae				
Genus *Stylocoeniella*	1	3	AY722793-AY722791	Chen *et al*. (2004)
Genus *Madracis*	5	88	AF251847-AF251936	Diekmann *et al*. (2001)
Family Fungiacyathidae				
Genus *Fungiacyathus*	1	2	AY722757-AY722758	Chen *et al*. (2004)
Family Oculinidae				
Genus *Galaxea*	1	2	AY722765-AY722764	Chen *et al*. (2004)
Family Mussidae				
Genus *Acanthastrea*	1	1	AY722739-AY722740	Chen *et al*. (2004)
Order Alcyonacea				
Family Alcyoniidae				
Genus *Alcyonium*	5	6	AF262342-AF262351	McFadden *et al*. (2001)

MATERIALS AND METHODS

DNA sequence database

The published complete DNA sequences containing the ITS-5.8S rDNA region were retrieved from GenBank based on either original publications or unpublished sources (Table 1). In addition, 47 new DNA sequences, mainly from *Acropora* and *Isopora*, were obtained in this study. Coral samples were stored in 90%-95% EtOH. DNA extraction, PCR, cloning, and DNA sequencing were described in our previous works (Chen et al. 2000 2002 2003 2004). Target segments containing the ITS1-5.8S-ITS2 region were amplified using the "anthozoan-universal" primer pairs, 1S: 5'-GGTACCCTTTGTACACACCGCCCGTCGCT-3' and 2SS: 5'-GCTTTGGGCGGCAGTCCCAAG-CAACCCGACTC-3', as described in Odorico and Miller (1997). PCR was performed in a PC-9606 thermal sequencer (Corbett Research, Sydney, NSW, Australia) using the following thermal cycle: 1 cycle of 95°C for 4 min; 4 cycles of 94°C for 30 s, 50°C for 1 min, and 72°C for 2 min; and 30 cycles of 94°C for 30 s, 55°C for 1 min, and 72 °C for 2 min. The amplification reaction used 50-200 ng of template and BRL *Taq* polymerase in a 50 μl reaction volume, using the buffer supplied with the enzyme, under conditions recommended by the manufacturer. The PCR products were electrophoresed in a 1% agarose (FMC Bioproduct, Rockland, ME, USA) gel in 1X TAE

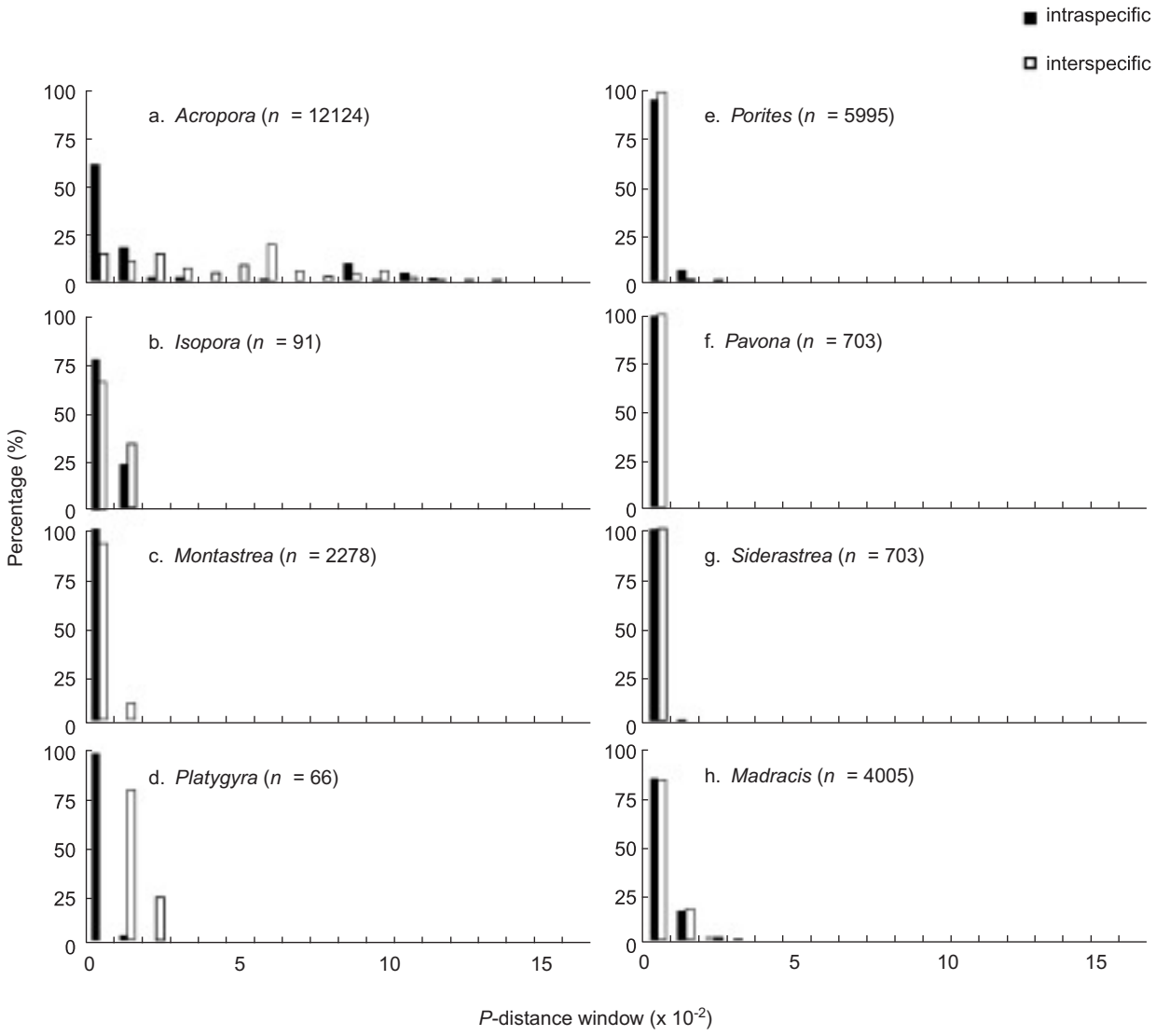

Fig. 1. Frequency distributions of intra- and interspecific genetic distances for 5.8S rDNA. The number of pairwise comparisons (*n*) is indicated.

buffer to assess the yield. Amplified DNA was extracted once with chloroform, precipitated with ethanol at -20°C, and resuspended in TE buffer. PCR products were cloned using the pGEM-T system (Promega, Madison, MI, USA) under conditions recommended by the manufacturer. Nucleotide sequences were determined for complementary strands of at least 2 clones from each sample using an ABI 377 Genetic Analyzer. The sequences obtained were submitted to GenBank under the accession numbers listed in table 1.

Molecular evolutionary analysis

Due to the extreme divergence of the 2 ITS regions between *Acropora* and non-*Acropora* corals (Chen et al. 2004), it is impossible to produce a consistent alignment of ITS-5.8S among all scleractinian corals for molecular evolutionary analyses. However, reliable alignment of ITS can be obtained at inter- and intraspecific levels using the secondary structure as a guide. DNA sequences were initially aligned using CLUSTAL X (Thompson et al. 1994), and default gap and extension penalties were used followed by manual editing using SeqApp 1.9 (Gilbert 1994). Alignments were then adjusted by eye following guidance of the predicted secondary structure by Odorico and Miller (1997) and Chen et al. (2004). The uncorrected pairwise *p*-distances (Li 1997) were calculated for the alignments from the default options of CLUSTAL for 8 genera that contained sequence data from more than 2 species (Tables 2, 3). Genetic variations at the intraspecific and interspecific levels were visualized by frequency distributions of pairwise *p*-distances. Genetic distances were divided into different categories, e.g., 1-2 x 10^{-2} (Fig. 1). Genetic distances derived from pairwise comparisons that fit into each category were counted. If the intraspecific variation is as high as the interspecific variation, the 2 distributions will highly overlap. On the contrary, the frequency plots are separated into 2 clear distributions when the former is smaller than the latter. Frequency distributions of pairwise *p*-distances were respectively conducted for ITS1, 5.8S, and ITS2 of 8 genera for which both intraspecific and interspecific variations were available. The significant difference of these 2 distributions was examined by Chi-squared test using Statview 5.1. Phylogenetic trees based on 5.8S rDNA sequences were constructed using PAUP 4.0b10 (Swofford 2002). The LRT implemented in the program MODELTEST vers. 3.6 (Posada and Crandall 1998) indicated that the Kimura 2-parameter (K2P) model (Kimura 1980) was the best-fit model of sequence evolution for 5.8S rDNA under the criterion of the hierarchical LRT. Neighbor-joining (NJ) analysis was performed using the K2P model estimated by Modeltest. Branch lengths leading to the major clades in the NJ tree were calculated. The robustness of the NJ phylogeny was assessed by 1000 bootstrap replicates. A molecular clock LRT, $2\Delta = \log L_{\text{no clock}} - \text{Log } L_{\text{clock}}$, which is distributed as X^2 with (n - 2) degrees of freedom where n is the number of sequences (Felsenstein 1981, Muse and Weir 1992), was performed using TREE-PUZZLE 5.1 (Schmidt et al. 2002) to determine whether there was a statistical difference in the global evolutionary rate for 5.8S rDNA. To examine the evolutionary rate constancy among the major clades in the 5.8S phylogeny, a modified RRT (Wu and Li 1985, Li and Bousquet 1992) introducing a phylogenetic weighting scheme (Robinson et al. 1998) was carried out using RRTree 1.1 (Robinson et al. 1998).

RESULTS

The complete sequence data set consisted of 587 sequences of ITS-5.8S from 78 species, representing 28 genera, and 12 families of scleractinian corals (Table 1). In addition, 6 sequences of 5 *Alcyonium* species were used as out groups in the 5.8S gene phylogenetic construction (see below). Eight genera/subgenera, including *Acropora*, *Isopora*, *Montastrea*, *Platygyra*, *Porites*, *Pavona*, *Siderastrea*, and Madracis, for which rDNA sequences were available for intraspecific and interspecific comparisons, were used for genetic distance estimation and the frequency distribution analysis.

Genetic distances

Intraspecific genetic distances (*p*-distances) for 5.8S, ITS1, and ITS2 were respectively calculated from 38 species (Table 2). The 5.8S was relatively conserved with 14 species showing identical (*p*-distance = 0) DNA sequences at the intraspecific level. The intraspecific genetic distances of *Acropora* 5.8S were comparatively higher than those of other corals with the highest *p*-distance value found in *A. longicyathus* (0.059 ± 0.049). The ITS1 intraspecific variations of *Acropora* were larger than those of the other genera examined, with *p*-distances ranging from 0.019

± 0.0165 in *A. digitifera* to 0.1991 ± 0.1569 in *A. pulchra*. Similarly, ITS2 variations were consistently higher in *Acropora* than in the other genera examined, with the largest genetic distance of 0.2021 ± 0.1306 in *A. digitifera*.

The interspecific genetic distances of the 5.8S varied from 0 in *Pavona* spp. to 0.0491 ± 0.0353 in *Acropora* spp. Two *Platygyra* species were moderately divergent in 5.8S with a genetic distance of 0.0196 ± 0.0026. Interspecific genetic distances of the ITS1 were also unusually higher in *Acropora* (0.2354 ± 0.1151) and *Isopora* (0.2109 ± 0.0614) compared to those of the other corals, except for large genetic distances observed in *Porites* (0.1942 ± 0.0805) and *Platygyra* (0.1955 ± 0.0183). Unexpectedly, the largest genetic distance for the ITS2 was seen in *Porites* (0.3031 ± 0.1232), probably reflecting deeper divergence

Table 2. Intraspecific *p*-distances of the 3 ribosomal regions of ITS1, 5.8S, and ITS2 in scleractinian corals. For data sources, refer to table 1. The number of sequences used for the *p*-distance calculations is indicated. The number of sequences used for calculating the *p*-distances is indicated in parentheses Please insert the correct file

Taxa	ITS1	5.8S	ITS2
Acropora *cerealis* (6)	0.115 ± 0.084	0.0393 ± 0.0501	0.0255 ± 0.0145
A. cytherea (6)	0.0945 ± 0.0486	0	0.0292 ± 0.0154
A. digitifera (6)	0.019 ± 0.0165	0.0416 ± 0.0317	0.2021 ± 0.1306
A. gemmifera (7)	0.185 ± 0.1347	0.0155 ± 0.01	0.1587 ± 0.0762
A. hyacinthus (10)	0.1704 ± 0.0892	0.0013 ± 0.0026	0.0254 ± 0.0111
A. longicyathus (9)	0.094 ± 0.0545	0.059 ± 0.049	0.0719 ± 0.0155
A. millepora (11)	0.0826 ± 0.0717	0.0014 ± 0.0027	0.1422 ± 0.0983
A. muricata (22)	0.1486 ± 0.1095	0.0413 ± 0.047	0.1384 ± 0.0969
A. papillare (7)	0.1154 ± 0.0887	0.0056 ± 0.0043	0.0138 ± 0.0073
A. pulchra (9)	0.1991 ± 0.1569	0.0158 ± 0.0097	0.1738 ± 0.0986
A. spathulata (14)	0.1855 ± 0.1239	0.0111 ± 0.0101	0.1898 ± 0.0831
A. spicifera (6)	0.1158 ± 0.1288	0.0022 ± 0.0032	0.02 ± 0.011
Isopora *cuneata* (8)	0.0585 ± 0.0527	0.0047 ± 0.0059	0.0186 ± 0.0096
Madracis *decatis* (27)	0.014 ± 0.0102	0.0048 ± 0.005	0.008 ± 0.0053
M. formosa (16)	0.0073 ± 0.0056	0.0054 ± 0.006	0.0212 ± 0.0153
M. mirabilis (14)	0.0048 ± 0.0046	0.0072 ± 0.0086	0.0092 ± 0.0057
M. pharensis (21)	0.0211 ± 0.0155	0.0031 ± 0.0038	0.0092 ± 0.0057
M. senaria (12)	0.0107 ± 0.0065	0.0011 ± 0.0024	0.0132 ± 0.0082
Platygyra *pini* (3)	0.0089 ± 0.0057	0	0.0039 ± 0.0034
P. sinensis (9)	0.0332 ± 0.0032	0.0027 ± 0.0033	0.0084 ± 0.0054
Montastrea *annularis* (25)	0.0075 ± 0.0054	0	0.0114 ± 0.0067
M. faveolata (18)	0.0099 ± 0.0064	0	0.0076 ± 0.0034
M. franksi (23)	0.0058 ± 0.0039	0	0.0087 ± 0.0049
Pavona *cactus* (13)	0.027 ± 0.0164	0	0.0029 ± 0.0039
P. decussata (25)	0.0172 ± 0.008	0	0.004 ± 0.0052
Siderastrea *glynni* (15)	0.006 ± 0.016	0.0016 ± 0.0028	0.0014 ± 0.0025
S. radians (6)	0	0	0.0018 ± 0.0026
S. siderea (14)	0.0083 ± 0.0064	0	0.0178 ± 0.0126
S. stellata (3)	0	0	0.0036 ± 0.0031
Porites *astreoides* (16)	0.0119 ± 0.0067	0.0015 ± 0.0027	0.0183 ± 0.0124
P. colonensis (4)	0.0052 ± 0.0057	0	0.0064 ± 0.0045
P. divaricata (7)	0.0028 ± 0.0025	0	0.0053 ± 0.0045
P. furcata (3)	0	0.006	0.006 ± 0.0027
P. lobata (64)	0.021 ± 0.0139	0.0028 ± 0.0036	0.0502 ± 0.0542
P. lutea (3)	0.0254 ± 0.0074	0	0.0165 ± 0.0035
P. panamensis (3)	0.0122	0.003 ± 0.0026	0
P. rus (3)	0	0	0
P. sverdrupi (7)	0.0025 ± 0.0027	0.0035 ± 0.0036	0.0109 ± 0.0053

times or a broader taxonomic sampling (Forsman 2003, 2005b). *Acropora* and *Isopora* still had relatively high interspecific genetic distances (0.1507 ± 0.0911 and 0.188 ± 0.0444, respectively) compared to the other corals.

Frequency distribution analyses of intraspecific and interspecific genetic distances

Frequency distribution analyses of intraspecific and interspecific genetic distances of ITS-5.8S rDNA revealed that *Acropora* possesses relatively high heterogeneity of ITS-5.8S rDNA (Figs. 1-3, Table 4). Over 75% of the genetic distances of 5.8S rDNA were < 0.02 at both the intra- and interspecific levels in *Isopora*, *Montastrea*, *Platygyra*, *Porites*, *Pavona*, *Siderastrea*, and *Madracis*, reflecting the conservative nature of this gene fragment (Fig. 1). For *Acropora*, the intraspecific genetic distances were dispersed and overlapped with those of the interspecific comparisons, although these 2 distributions statistically significantly differed (X^2-test = 84.07, $p < 0.001$). In contrast, the distribution of intraspecific genetic distances in *Platygyra* 5.8S was clearly separated from that at the interspecific level (X^2-test = 199, $p < 0.001$).

The frequency distribution of ITS1 intraspecific genetic distances (Fig. 2) significantly differed from those of the interspecific comparisons in *Isopora*, *Pavona*, *Platygyra*, *Porites*, and *Siderastrea* (X^2-test, $p < 0.0001$, Table 4), suggesting that the ITS1 contains signals for species phylogeny. However, in *Madracis* and *Montastrea*, the difference in frequency distributions was less significant (X^2-test, $p < 0.05$). In *Acropora*, the distribution of intra- and interspecific genetic distances highly overlapped and did not statistically significantly differ.

For ITS2, highly significant differences between intra- and interspecific genetic distances (Fig. 3) were detected in *Isopora*, *Pavona*, *Platygyra*, *Porites*, *Madracis*, and *Siderastrea* (X^2-test, $p < 0.001$, Table 4), and separate distributions suggest phylogenetic signals. Highly overlapping frequency distributions between intra- and interspecific genetic distances of both the 5.8S and ITS1 were similarly observed for the ITS2 of

Table 3. Interspecific *p*-distances of the 3 ribosomal regions of ITS1, 5.8S, and ITS2 in 8 major groups of scleractinian corals. For data sources, refer to table 1

Taxa	ITS1	5.8S	ITS2
Acroporoa	0.2354 ± 0.1151	0.0491 ± 0.0353	0.1507 ± 0.0911
Isopora	0.2109 ± 0.0614	0.0082 ± 0.0041	0.1880 ± 0.0444
Montastrea	0.0272 ± 0.0628	0.0013 ± 0.0043	0.0310 ± 0.0708
Platgyra	0.1955 ± 0.0183	0.0196 ± 0.0026	0.1460 ± 0.0074
Porites	0.1942 ± 0.0805	0.0054 ± 0.0058	0.3031 ± 0.1232
Pavona	0.0437 ± 0.0156	0	0.0231 ± 0.0042
Siderastrea	0.0465 ± 0.0363	0.0006 ± 0.0018	0.0218 ± 0.0123
Madracis	0.0204 ± 0.0088	0.0044 ± 0.0058	0.0195 ± 0.0102

Table 4. Chi-squared tests of intraspecific and interspecific variations based on the frequency distribution of figures. 1-3. The degree of freedom is indicated in parentheses. -, Chi-squared test not available

	ITS1		5.8S		ITS2	
Taxon	Chi-square	*P* value	Chi-square	*P* value	Chi-square	*P* value
Acropora	35.40 (12)	0.05	84.07 (23)	<0.001	32.25 (15)	0.006
Isopora	160.45 (14)	<0.001	2.48 (1)	0.12	187.44 (10)	<0.001
Pavona	138.15 (3)	<0.001	-	-	169.89 (1)	<0.001
Porites	167.1 (13)	<0.001	3.71 (2)	0.16	152.48 (19)	<0.001
Siderastrea	73.97 (2)	<0.001	-	-	48.62 (2)	<0.001
Madracis	11.61 (2)	0.003	0.04 (1)	0.84	21.26 (2)	<0.001
Platygyra	200 (9)	<0.001	199 (3)	<0.001	199 (3)	<0.001
Montastrea	9.53 (3)	0.02	8.33 (1)	0.003	9.3 (4)	0.05

Acropora, but the statistical test was significant (X^2-test, $p < 0.05$).

Molecular phylogenetic analysis and evolutionary rate tests of the 5.8S phylogeny

The NJ tree of 5.8S rDNA, estimated using the Kimura 2-parameter model, clearly showed a significantly long branch length leading to the clade of the genus *Acropora*, including both subgenera (Fig. 4). In contrast, the 5.8S rDNA was highly conserved among the 24 genera and 11 families of scleractinian corals, thus forming an unresolved polytomy with a short branch length in the NJ tree. The branch length leading to the *Acropora*/*Isopora* clade (0.15) was 4-6 times longer than those of other branches leading to the major clades (0.024-0.026).

The molecular-clock hypothesis tested by the LRT was rejected for 5.8S rDNA (-log $L_{\text{no clock}}$ = 858.29, -Log L_{clock} = 1016.12, d.f. = 154, $p < 0.000001$), indicating a highly significant difference in the global evolutionary rate for scleractinian 5.8S rDNA. The RRTs in table 5 show that the rDNA of *Isopora*, Caribbean *Acropora*, and Indo-Pacific *Acropora* evolved at constant tempos (Fisher's exact test, $p > 0.05$), indicating that the divergent rDNA existed before these 3 lineages split. In contrast, rate constancy was rejected for most of the comparisons between *Acropora*/*Isopora* and the other coral genera (Fisher's exact test, $p < 0.01$), suggesting that the

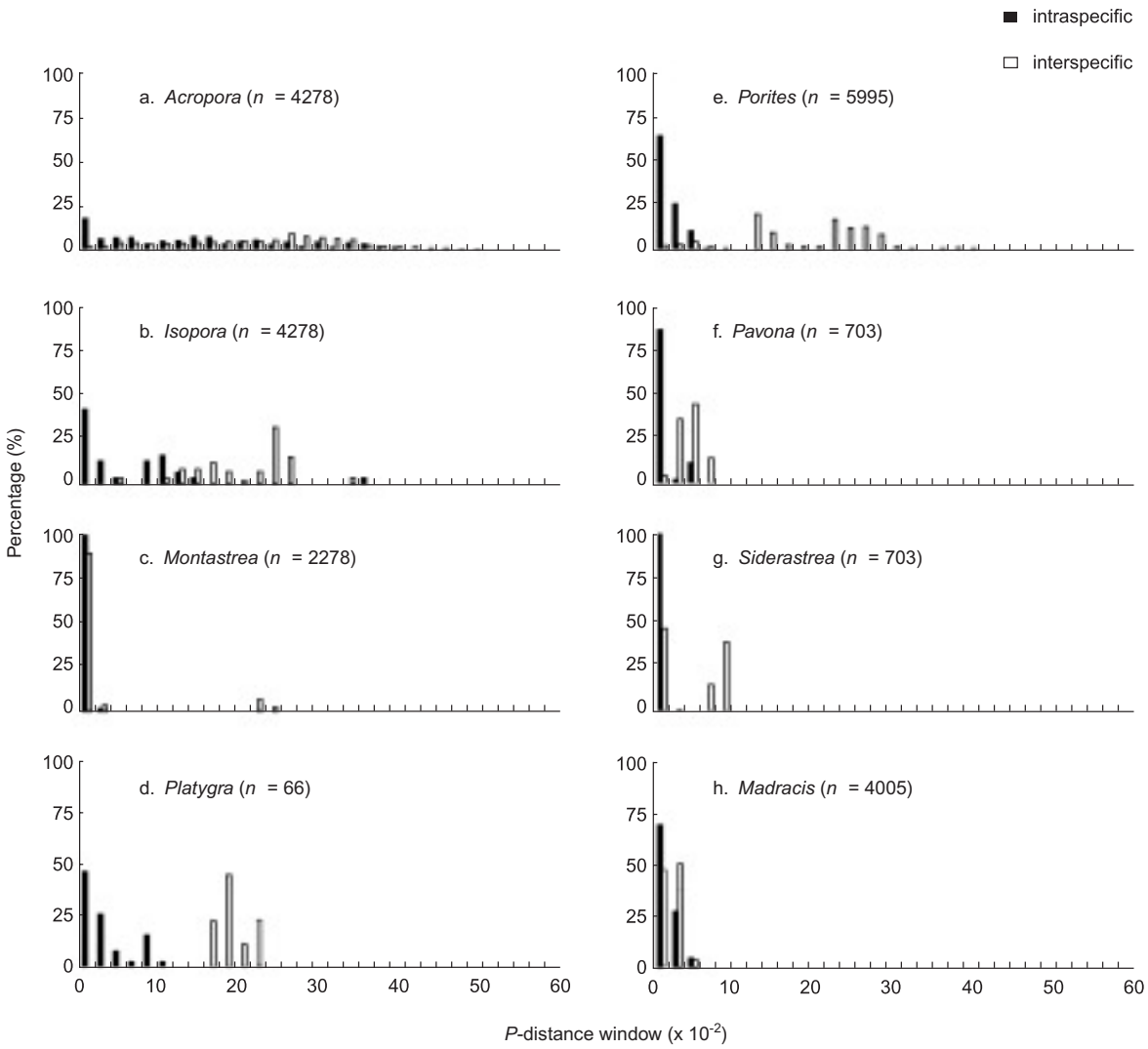

Fig. 2. Frequency distributions of intra- and interspecific genetic distances for ITS1 rDNA. The number of pairwise comparisons (*n*) is indicated.

rates of evolution of 5.8S differ between *Acropora*/*Isopora* and the other lineages, and that the evolutionary rate of *Acropora*/*Isopora* accelerated after divergence from the common ancestor of scleractinian corals.

DISCUSSION

Molecular characteristics of the rDNA ITS-5.8S region in scleractinian corals

Our analyses confirm that the molecular evolutionary pattern of the rDNA ITS-5.8S region of *Acropora*, including the subgenus *Isopora*, is the most heterogeneous at the levels of both species and individuals among scleractinian corals (Marquez et al. 2003, Chen et al. 2004). In addition to the extreme divergence, 2 other molecular characteristics, namely the shortest length of the ITS and the unusual ITS2 secondary structure, make the rDNA of *Acropora* unique. *Acropora* has the shortest ITS (ITS1 of 70-94 bp and ITS2 of 107-141 bp) not only among scleractinian corals but also among all metazoans examined to date (Odorico and Miller 1997, Marquez et al. 2003, Chen et al. 2004). For the other scleractinian corals, the length of the ITS is relatively comparable among genera (Hunter et al. 1997, Lopez and Knowlton 1997, Medina et al. 1999, Diekmann et al. 2001, Rodriguez-Lanetty and Hoegh-Guldberg 2001, Forsman et al. 2003 2005, Lam and Morton

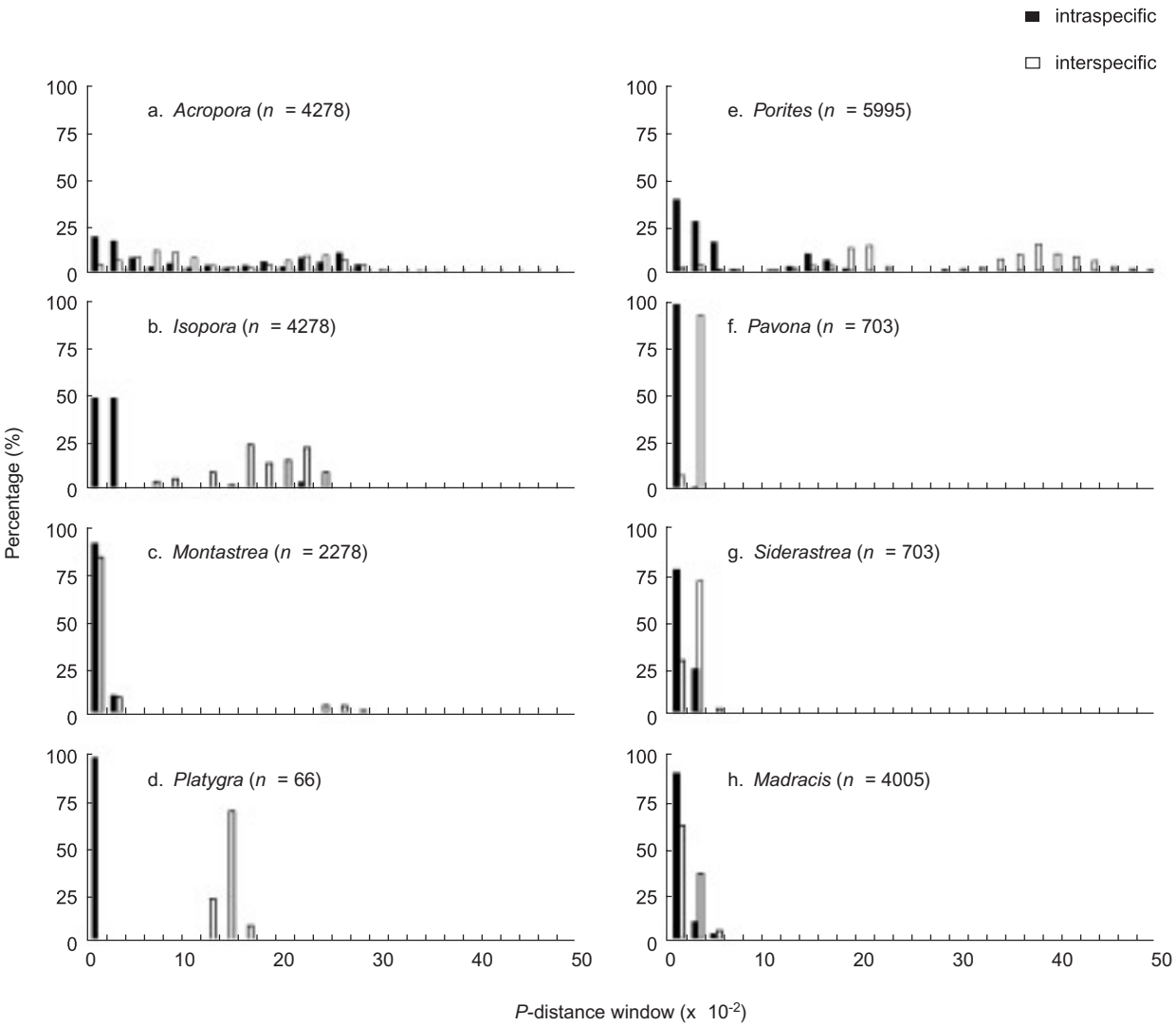

Fig. 3. Frequency distributions of intra- and interspecific genetic distances for ITS2 rDNA. The number of pairwise comparisons (*n*) is indicated.

2003, Chen et al. 2004, Fukami et al. 2004, Moothien Pillay et al. 2005). The mechanism causing such short ITS sequences in species of *Acropora* remains unknown. Furthermore, *Acropora* ITS2 forms a unique but stable 5-domain secondary structure that differs from that of other scleractinian corals. Chen et al. (2004) examined the secondary structure of ITS2 from 54 species of scleractinian corals, representing 25 genera and 11 families of both the complex and robust clades previously defined in molecular phylogenetic analyses (Fukami et al. 2004). A standard of 4 domains was observed in 17 species of corals, while 23 species had a modified number of 5 domains with domain I divided into 2 subdomains. These 2 types of secondary structures were observed across 11 coral families. The 3rd type, 5 domains with domain III divided into 2 subdomains, was only seen in the genus *Acropora*.

Extreme rDNA diversity in *Acropora*

The extreme rDNA diversity in *Acropora* may be due to the presence of pseudogenes that have been maintained through recent introgressive hybridization and slow concerted evolution as a result of frequent asexual propagation (Marquez et al. 2003). In *Acropora*, up to nine rDNA types may be present in a single colony (Odorico and Miller 1997), and distinct ITS types are often shared by species (van Oppen et al. 2000 2001 2002). Marquez et al. (2003) analyzed the RT-PCR of 5.8S rDNA expressed in *A. millepora*, examined the pattern of methylation that may indicate silencing caused by nucleolar dominance, and looked for mutations that could disrupt the secondary structure and functionality of the rRNA. These analyses consistently indicated that 1 rDNA sequence type present in a broad range of Indo-Pacific *Acropora* is likely to consist primarily of pseudogenes. It was suggested that interspecific hybridization may have brought together divergent rDNA copies into a single genome, as high divergence in the ITS region may have suppressed recombination across the entire rDNA array, slowing down concerted evolution (Muir et al. 2001). In addition, asexual reproduction, such as fragmentation by *Acropora*, may also limit concerted evolution and cause slow homogenization of divergent rDNA copies. Consequently, some of the rDNA types combined by hybridization may have been silenced by nucleolar dominance, causing them to evolve as pseudogenes, thus increasing sequence heterogeneity (Marquez et al. 2003).

Although these patterns are thought to be consistent with the occurrence of interspecific hybridization, incomplete lineage sorting and incomplete concerted evolution may also be alternative explanations (Vollmer and Palumbi 2002 2004). Vollmer and Palumbi (2004) analyzed sequence divergence rates between rDNA and single-copy nuclear genes of the Caribbean *Acropora* and suggested that the Caribbean *Acropora* rDNA lineages were quite ancient and predated the split of the species. By comparison, the most divergent ITS lineages in the Caribbean *Acropora* occurred approximately 40 million years ago (mya) (Vollmer and Palumbi 2004) which is roughly 6 times older than a conservative time of 6.6 mya for *Acropora* divergence in the Caribbean (Budd et al. 1994, van Oppen et al. 200, Miller and van Oppen 2003). Molecular evolutionary rate tests of the 5.8S conducted in our study clearly indicated that persistence of ancient rDNA could be the case in the Indo-Pacific *Acropora*, and that it is older than the split of the Caribbean and Indo-Pacific *Acropora*, or even older than the common ancestor of *Acropora* and *Isopora*. First, rate consistency was rejected by the LRT for the global molecular phylogeny of the scleractinian 5.8S. The significantly longer branch leading to the clade *Acropora*/*Isopora* suggests that the unusually highly divergent rDNA persisted in the common ancestor of *Acropora* and *Isopora* after divergence from other scleractinian corals (Fig. 4). Second, the RRT showed that *Isopora*, Caribbean *Acropora*, and Indo-Pacific *Acropora* evolved at constant tempos, and that rDNA in each of these 3 lineages is highly divergent (Table 5). Interestingly, *Isopora*, a subgenus of *Acropora*, is composed of 4 described species (Wallace 1999). Unlike *Acropora*, species of *Isopora* are distributed less sympatrically. Moreover, they brood larvae which would considerably reduce the opportunity for cross-species hybridization. However, species of *Isopora* still possess highly divergent rDNA which evolved at a similar rate to that of *Acropora*, supporting the argument that recent introgressive hybridization alone cannot account for the divergence patterns found in *Acropora* (Vollmer and Palumbi 2002 2004). In plants, ancient divergent paralogous rDNA lineages and the persistence of these lineages predating speciation events are commonly observed in many lineages (Buckler et al. 1997, Muir et al. 2001, Alvarez and Wendel 2003).

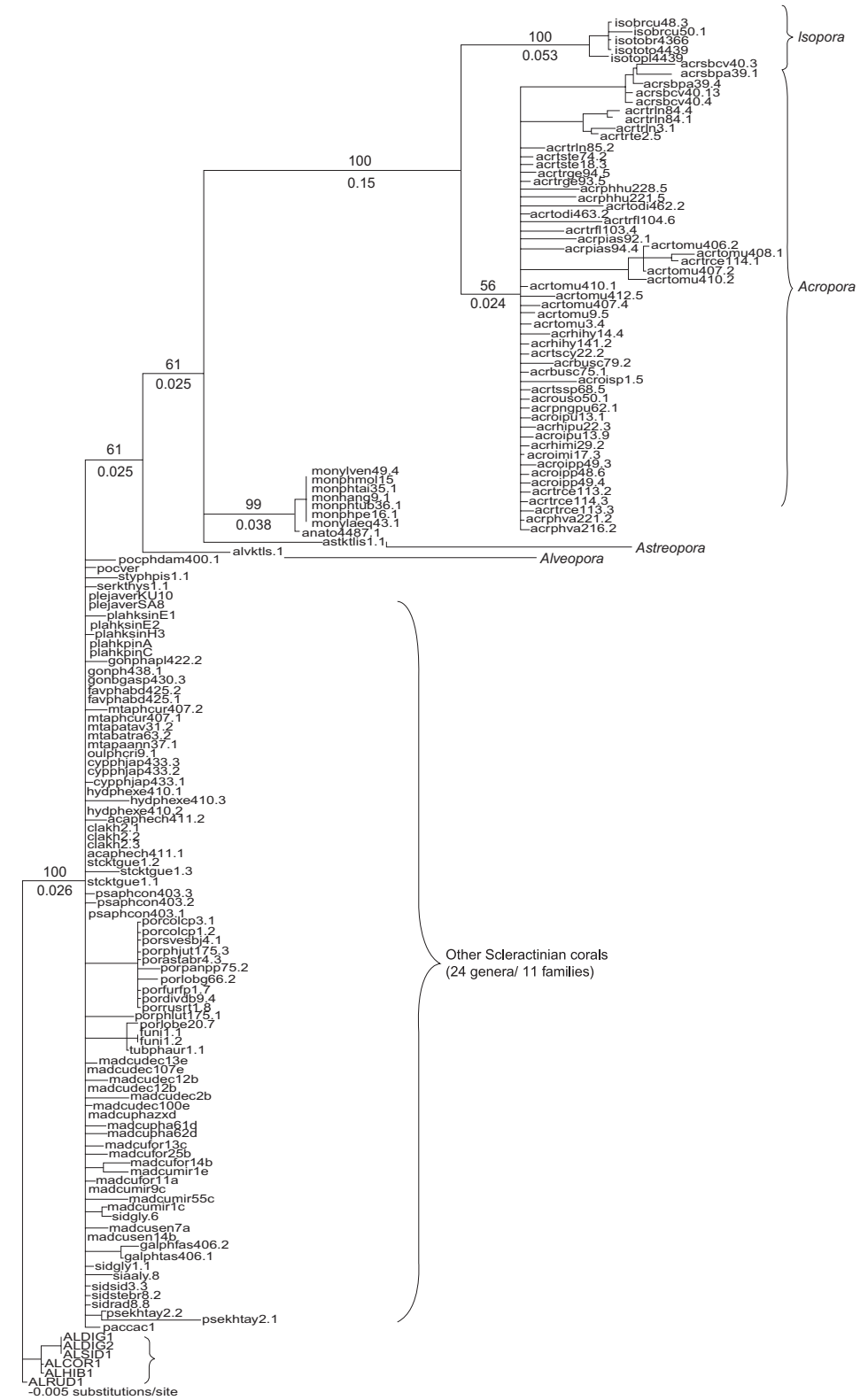

Fig. 4. Phylogenetic analysis derived from the Neighbor-joining (NJ) algorithms of scleractinian 5.8S rDNA. Numbers above and below the branches indicate the bootstrap values for the NJ (1000 replicates) analysis and the branch length of each major clade. The sample codes are the abbreviation of the genus name, locality, species name, sample number, and the clone that was sequenced (e.g., acrophva216.2 is the number 2 clone of sample 216 of *Acropora valida* collected from Penghu). The complete 5.8S dataset is available upon request from the senior author.

Table 5. Relative-rate test results for 5.8S clades in the Neighbor-joining phylogeny

[a]Clade$_A$	Clade$_B$	K_A	K_B	dK	s.d.	dK/s.d.	*P*-value
Isopora	*Acropora*-C[b]	0.2148	0.2380	-0.0232	0.0333	-0.6956	0.4867
Isopora	*Acropora*-I[c]	0.2148	0.1998	0.0150	0.0295	0.5100	0.6101
Isopora	*Montipora*	0.2148	0.0893	0.1255	0.0443	2.8312	**0.004**
Isopora	*Anacropora*	0.2148	0.0893	0.1255	0.0443	2.8312	**0.0046**
Isopora	*Astreopora*	0.2148	0.1399	0.0750	0.0452	1.6573	**0.0975**
Isopora	*Alveopora*	0.2148	0.0705	0.1443	0.0455	3.1705	**0.0015**
Isopora	*Platygyra*	0.2148	0.0433	0.1715	0.0465	3.6862	**0.0002**
Isopora	*Montastrea*	0.2148	0.0452	0.1697	0.0466	3.6400	**0.0003**
Isopora	*Porites*	0.2148	0.0625	0.1523	0.0487	3.1252	**0.0018**
Isopora	*Madracis*	0.2148	0.0496	0.1653	0.0466	3.5483	**0.0004**
Isopora	*Siderastrea*	0.2148	0.0374	0.1774	0.0466	3.8050	**0.0001**
Isopora	*Pavona*	0.2148	0.0433	0.1715	0.0465	3.6862	**0.0002**
Acropora-C	*Acropora*-I	0.2380	0.1998	0.0382	0.0219	1.7476	0.0805
Acropora-C	*Montipora*	0.2380	0.0893	0.1487	0.0488	3.0461	**0.0023**
Acropora-C	*Anacropora*	0.2380	0.0893	0.1487	0.0488	3.0461	**0.0023**
Acropora-C	*Astreopora*	0.2380	0.1399	0.0982	0.0494	1.9872	**0.0469**
Acropora-C	*Alveopora*	0.2380	0.0705	0.1675	0.0489	3.4271	**0.0006**
Acropora-C	*Platygyra*	0.2380	0.0433	0.1947	0.0499	3.9018	**0.0001**
Acropora-C	*Montastrea*	0.2380	0.0452	0.1929	0.0500	3.8576	**0.0001**
Acropora-C	*Porites*	0.2380	0.0625	0.1755	0.0520	3.3729	**0.0007**
Acropora-C	*Madracis*	0.2380	0.0496	0.1885	0.0499	3.7749	**0.0002**
Acropora-C	*Siderastrea*	0.2380	0.0374	0.2006	0.0500	4.0106	**0.0001**
Acropora-C	*Pavona*	0.2380	0.0433	0.1947	0.0499	3.9018	**0.0001**
Acropora-I	*Montipora*	0.1998	0.0893	0.1105	0.0425	2.5977	**0.0094**
Acropora-I	*Anacropora*	0.1998	0.0893	0.1105	0.0425	2.5977	**0.0094**
Acropora-I	*Astreopora*	0.1998	0.1399	0.0599	0.0448	1.3367	0.1813
Acropora-I	*Alveopora*	0.1998	0.0705	0.1292	0.0426	3.0335	**0.0024**
Acropora-I	*Platygyra*	0.1998	0.0433	0.1564	0.0428	3.6569	**0.0003**
Acropora-I	*Montastrea*	0.1998	0.0452	0.1546	0.0429	3.6061	**0.0003**
Acropora-I	*Porites*	0.1998	0.0625	0.1373	0.0449	3.0570	**0.0022**
Acropora-I	*Madracis*	0.1998	0.0496	0.1502	0.0429	3.5029	**0.0005**
Acropora-I	*Siderastrea*	0.1998	0.0374	0.1624	0.0429	3.7856	**0.0002**
Acropora-I	*Pavona*	0.1998	0.0433	0.1564	0.0428	3.6569	**0.0003**
Montipora	*Astreopora*	0.0893	0.1399	-0.0505	0.0295	-1.7140	0.0865
Montipora	*Alveopora*	0.0893	0.0705	0.0188	0.0208	0.9043	0.3659
Montipora	*Platygyra*	0.0893	0.0433	0.0460	0.0243	1.8938	0.0583
Montipora	*Montastrea*	0.0893	0.0452	0.0441	0.0244	1.8113	0.0701
Montipora	*Porites*	0.0893	0.0625	0.0268	0.0276	0.9704	0.3318

[a]Clade$_A$	Clade$_B$	K_A	K_B	dK	s.d.	dK/s.d.	*P*-value
Montipora	*Madracis*	0.0893	0.0496	0.0398	0.0244	1.6317	0.1028
Montipora	*Siderastrea*	0.0893	0.0374	0.0519	0.0226	2.2988	**0.0215**
Montipora	*Pavona*	0.0893	0.0433	0.0460	0.0243	1.8938	0.0583
Anacropora	*Astreopora*	0.0893	0.1399	-0.0505	0.0295	-1.7140	0.0865
Anacropora	*Alveopora*	0.0893	0.0705	0.0188	0.0208	0.9043	0.3659
Anacropora	*Platygyra*	0.0893	0.0433	0.0460	0.0243	1.8938	0.0583
Anacropora	*Montastrea*	0.0893	0.0452	0.0441	0.0244	1.8113	0.0701
Anacropora	*Porites*	0.0893	0.0625	0.0268	0.0276	0.9704	0.3318
Anacropora	*Madracis*	0.0893	0.0496	0.0398	0.0244	1.6317	0.1028
Anacropora	*Siderastrea*	0.0893	0.0374	0.0519	0.0226	2.2988	0.0215
Anacropora	*Pavona*	0.0893	0.0433	0.0460	0.0243	1.8938	0.0583
Astreopora	*Alveopora*	0.1399	0.0705	0.0693	0.0312	2.2199	0.0264
Astreopora	*Platygyra*	0.1399	0.0433	0.0965	0.0312	3.0940	**0.0020**
Astreopora	*Montastrea*	0.1399	0.0452	0.0947	0.0313	3.0263	**0.0025**
Astreopora	*Porites*	0.1399	0.0625	0.0774	0.0342	2.2649	**0.0235**
Astreopora	*Madracis*	0.1399	0.0496	0.0903	0.0312	2.8912	**0.0038**
Astreopora	*Siderastrea*	0.1399	0.0374	0.1025	0.0328	3.1227	**0.0018**
Astreopora	*Pavona*	0.1399	0.0433	0.0965	0.0341	2.8320	**0.0046**
Alveopora	*Platygyra*	0.0705	0.0433	0.0272	0.0202	1.3471	0.1780
Alveopora	*Montastrea*	0.0705	0.0452	0.0254	0.0203	1.2502	0.2112
Alveopora	*Porites*	0.0705	0.0625	0.0080	0.0240	0.3351	0.7376
Alveopora	*Madracis*	0.0705	0.0496	0.0210	0.0204	1.0307	0.3027
Alveopora	*Siderastrea*	0.0705	0.0374	0.0332	0.0182	1.8245	0.0681
Alveopora	*Pavona*	0.0705	0.0433	0.0272	0.0202	1.3471	0.1780
Platygyra	*Montastrea*	0.0433	0.0452	-0.0018	0.0018	-1.0036	0.3156
Platygyra	*Porites*	0.0433	0.0625	-0.0192	0.0131	-1.4669	0.1424
Platygyra	*Madracis*	0.0433	0.0496	-0.0062	0.0017	-3.6665	**0.0002**
Platygyra	*Siderastrea*	0.0433	0.0374	0.0060	0.0092	0.6497	0.5159
Montastrea	*Porites*	0.0452	0.0625	-0.0173	0.0132	-1.3126	0.1893
Montastrea	*Madracis*	0.0452	0.0496	-0.0044	0.0025	-1.7451	0.0810
Montastrea	*Siderastrea*	0.0452	0.0374	0.0078	0.0094	0.8302	0.4064
Montastrea	*Pavona*	0.0452	0.0433	0.0018	0.0128	0.1422	0.8869
Porites	*Madracis*	0.0625	0.0496	0.0129	0.0132	0.9765	0.3288
Porites	*Siderastrea*	0.0625	0.0374	0.0251	0.0157	1.6034	0.1088
Porites	*Pavona*	0.0625	0.0433	0.0192	0.0180	1.0660	0.2864
Madracis	*Siderastrea*	0.0496	0.0374	0.0122	0.0093	1.3075	0.1911
Madracis	*Pavona*	0.0496	0.0433	0.0062	0.0128	0.4848	0.6278
Siderastrea	*Pavona*	0.0374	0.0433	-0.0060	0.0092	-0.6497	0.5159

Note- K_A and K_B are mean Kimura 2-parameter distances; dK= K_A - K_B; s. d.: standard deviation Fisher's exact test: significant *p* values are labeled in bold. [a]Ocotorals were assigned as outgroups in all calculations. [b]Caribbean *Acropora*. [c]Indo-West Pacific *Acropora*.

Phylogenetic utility of coral rDNA ITS-5.8S regions: *Acropora* is an exception

Our study confirms the previous hypothesis that the extremely high diversity of rDNA is unique to *Acropora* and is not a common feature of all scleractinian corals (Chen et al. 2004). The suggestion that ITS rDNA should be abandoned as a species- and population-level phylogenetic marker due to its complicated and undistinguishable characteristics of molecular evolution (Vollmer and Palumbi 2004) should be treated with caution since *Acropora* has several atypical and unusual characteristics that are significantly distinct from other scleractinian corals (Chen et al. 2004, this study). Both genetic distance and frequency distribution analyses of the rDNA clearly showed that the extremely high and overlapping heterogeneity is characteristic only of the subgenus *Acropora*. Even though high genetic diversity was observed in the subgenus *Isopora*, frequency analyses of ITS1 and ITS2 showed significantly non-overlapping genetic distance distributions between intra- and interspecific comparisons (Figs. 2b, 3b), suggesting that phylogenetic signals are still informative, at least for specific phylogenetic inferences (Chen et al. unpubl. data). Similar patterns were also observed in *Pavona*, *Platygyra*, *Porites*, and *Siderastrea*, but their specific phylogenies were successfully resolved using the ITS-5.8S region (Lam and Morton 2003, Forsman et al. 2003 2005a b, Moothien Pillay et al. 2005). For *Madracis* and *Montastrea*, the ITS1 and ITS2 were not as informative as for those coral genera described above, although they could be used to resolve the phylogenetic relationship of each lineage to a certain extent (see Diekmann et al. 2001, Fukami et al. 2004). In addition, guided by the homologous secondary structure, the aligned ITS2 successfully provided a concordant pattern (Chen et al. 2004) with the phylogenies based on mitochondrial, nuclear ribosomal, and protein-coding genes which showed the families Faviidae, Merulindae, and Mussidae to be monophyletic within the suborder Faviina (except for *Oulastrea*), although relationships at the family level are apparently not monophyletic (Romano and Cairns 2000, Chen et al. 2002, Fukami et al. 2004).

In summary, genetic distance and frequency distribution analyses demonstrated that the extremely high diversity of the rDNA ITS-5.8S region is unique to *Acropora*. Molecular phylogenetic inferences and evolutionary rate tests support the scenario that the divergent rDNA has been maintained since ancient times and probably predated the speciation of the common ancestor of *Acropora*.

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