Variation in the Ribosomal Internal Transcribed Spacers and 5.8S rDNA Among Five Species of Acropora (Cnidaria; Scleractinia): Patterns of Variation Consistent with Reticulate Evolution

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The ITS sequences of Acropora spp. are the shortest so far identified in any metazoan and are among the shortest seen in eukaryotes; ITS1 was 70–80 bases, and ITS2 was 100–112 bases. The ITS sequences were also highly variable, but base composition and secondary structure prediction indicate that divergent sequence variants are unlikely to be pseudogenes. The pattern of variation was unusual in several other respects: (1) two distinct ITS2 types were detected in both A. hyacinthus and A. cytherea, species known to hybridize in vitro with high success rates, and a putative intermediate ITS2 form was also detected in A. cytherea; (2) A. valida was found to contain highly (29%) diverged ITS1 variants; and (3) A. longicyathus contained two distinct 5.8S rDNA types. These data are consistent with a reticulate evolutionary history for the genus Acropora.

Introduction

Acropora is the largest extant genus of scleractinian corals, comprising over 370 nominal and at least 150 true species (Wallace and Willis 1994). The possession of specialized axial corallites from which the radial polyps bud, together with its light skeletal characters and fast growth rate, have seen Acropora become the dominant coral on most Indo-Pacific reefs (Veron 1986, p. 126; Wallace and Willis 1994).

Despite its numerical and ecological importance, systematic relationships within the genus Acropora remain unclear. A contributing factor to this is the morphological plasticity, reflected in within-species variability and between-species similarities, that is typical of the genus (Veron and Wallace 1984, p. 134). Three species are placed in the subgenus Isopora, and are clearly distinct from the remaining taxa (subgenus Acropora). Based on gross skeletal morphology and fundamental similarities in skeletal detail, 14 species groups have been defined within the subgenus Acropora (Veron and Wallace 1984, pp. 134–424). However, these groups have no real taxonomic status (Veron 1995, p. 244). Although the application of a wide variety of morphological and biochemical techniques has corroborated or contradicted aspects of this species group scheme (Harrison et al. 1998, pp. 16–119; McMillan et al. 1991; Wallace et al. 1991), overall the contributions that these studies have made to Acropora systematics have been relatively minor.

Synchronous mass spawning of corals (Harrison et al. 1984), resulting in the mixing of gametes from a wide range of species, often over long distances, creates widespread opportunities for hybridization. Although naturally occurring hybridization events have been documented in a range of terrestrial and freshwater animals and are thought to be common in plants, there have been few reports of hybridization in marine invertebrates (for reviews, see Arnold 1992; Bullini 1994). Hybridization between taxonomically diverse coral species (including Acropora spp.) has been demonstrated in vitro (Wallace and Willis 1994; Willis et al. 1997) and results in viable embryos that can live for several years (Willis et al. 1997) (although the reproductive capacity of hybrid colonies is presently unknown). The extent and significance of hybridization events in the field are unknown, but clearly these experiments have major implications for both the evolution and systematics of corals. Hybridization events create the potential for reticulate evolution or introgression (hybrids backcrossing with parental species; Arnold 1992).

Molecular markers are required to evaluate the possible contribution of hybridization events in the evolutionary history of the genus Acropora, as well as to establish subgeneric relationships. The internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA (rRNA) transcription unit (the rDNA) have been shown to be appropriate for addressing relationships at or below the genus level for a wide range of plant and animal groups (e.g., Lee and Taylor 1992; Wesson, Porter, and Collins 1992; Ritland, Ritland, and Straus 1993; Vogler and Desalle 1994), including anhydrozooan cnidarians (Chen and Miller 1996; Chen, Willis, and Miller 1996). Several recent studies (Sang, Crawford, and Stuessy 1995; Buckler and Holtsford 1996a, 1996b) demonstrate the usefulness of ITS sequence data in tracking hybridization events and reticulate evolution. Multigene families, such as the rRNA genes, undergo concerted evolution (Dover 1982). A number of mechanisms (unequal crossing over and gene conversion) usually lead to the rapid homogenization of such multicopy sequence families within a (freely interbreeding) species. However, rates of homogenization are dependent on a number of factors, including whether individual repeat units are on the same chromosome (Schlotterer and Tautz 1994). In variable regions such as the ITS, novel variants that arise may rapidly become fixed; thus, in many organisms, the ITS regions differ significantly between genetically discrete units (species or popula-
Divergent and reticulate evolution result in very different outcomes with respect to multigene families. If speciation proceeds in a divergent fashion, each species is predicted to be homogenous in ITS sequences but distinct to other species, except where speciation has occurred very recently. Reticulate evolution, on the other hand, may result in a single species harboring several divergent but homologous sequences (originating from the parental species), or classes of repeat that are intermediate between those of the parental species.

Here, we report the results of a preliminary study of ITS sequence variation within and between five *Acropora* species. Two of these species, *A. hyacinthus* and *A. cytherea*, fall within a single species group (the *A. hyacinthus* group) sensu Veron and Wallace (1984, pp. 298–318) and are known to hybridize in vitro with high success rates (Willis et al. 1997). The other species each belong to different species groups. The results show that the ITS regions of *Acropora* species are unique in two respects: they are the shortest ITS regions identified to date in any metazoan, and they contain an unprecedented level of variation. The pattern of variation found is consistent with a reticulate evolutionary history for the genus.

### Materials and Methods

DNA was prepared from sperm (McMillan et al. 1988) collected from six individual colonies of five species of *Acropora* (order Scleractinia; family Acroporidae; genus *Acropora*; subgenus *Acropora*). *A. hyacinthus* (two colonies), *A. cytherea*, *A. longicyathus*, *A. valida*, and *A. formosa* collected from Geoffrey Bay, Magnetic Island (19°10′S, 146°51′E) on the Great Barrier Reef.

The forward primer, GGTACCATTTTGATACACGGCCGCTGCT, annealing at positions 1621–1645 in the 18S rDNA of *Anemonia sulcata* (Hendrikx et al. 1990; GenBank accession number X53498) was used in conjunction with the reverse primer, GCCTTTGGCTGCAAGTCCCAAGCCGCAGCCTC, which anneals to the 28S rDNA to amplify a ~820-bp region spanning the 3' terminus (~175 bp) of the 18S rDNA through to the 5' end (~260 bp) of the 28S rDNA. Polymerase chain reaction (PCR) conditions, cloning, and sequencing were as described in Chen et al. (1995), with the exception that products were cloned into pGEM-T (Promega). For each species, a minimum of four clones were sequenced in both directions using universal and the following internal sequencing primers: GCGGACCCGCTGAAATCCAGCATAT and TATGCTTAAATTCGCGGGTG, annealing to the 28S rDNA; GTTGTTAACAGTTTCCGTA, annealing to the 18S rDNA; and AGCTTGTGCGTCTTCTCATCG, annealing to the 5S rDNA.

### Table 1

<table>
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<tr>
<th>Species</th>
<th>ITS1</th>
<th>5.8S</th>
<th>ITS2</th>
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<td>%T</td>
<td>%C</td>
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<td>27.4</td>
<td>16.4</td>
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<td>25.0</td>
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<td>14.7</td>
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Sequences were aligned using CLUSTAL W 1.6 (Thompson, Higgins, and Gibson 1994), followed by manual editing using SeqApp 1.9 (Gilbert 1992a). The boundaries of the various regions were deduced by comparison with data for a range of other cnidarians (Chen, Willis, and Miller 1996; Odorico and Miller 1997). Base composition data were generated using MEGA 1.02 (Kumar, Tamura, and Nei 1993). Pairwise sequence comparisons were conducted with CLUSTAL W 1.6 (Thompson, Higgins, and Gibson 1994) using the default settings (gaps included), based on the alignment submitted to EMBL and available from them (at ftp.ebi.ac.uk in the /pub/databases/embl/align directory) via anonymous ftp as alignment number DS28409. Maximum-likelihood analyses were conducted using DNAML (global rearrangements with 10 random additions) in PHYLIP 3.572 (Felsenstein 1993). Distance analyses were also performed on a bootstrapped data set (1000 replicates; SEQBOOT) using DNADIST (Kimura two-parameter) followed by the neighbor-joining method implemented by NEIGHBOR (PHYLIP 3.572c; Felsenstein 1993). Split decomposition analysis (1,000 bootstrap replicates) based on the Hamming distances was conducted using SplitsTree 2.0d5 (Huson 1996; Dress, Huson, and Moulton 1997). Gaps were included in all phylogenetic analyses.

Folding of the 5.8S rDNA was conducted using MulFold (Gilbert 1990). For the ITS2 regions, secondary structures were predicted using the Mfold server (Zuker 1989) located at http://ibc.wustl.edu/~zuker/rna/, to identify common patterns between species. After common patterns were identified between the five species, the remaining sequences were compared manually for common folding. Structures were drawn with the aid of MulFold (Gilbert 1990) and LoopDloop (Gilbert 1992b).

**Results**

**Characteristics of the ITS and 5.8S rDNA**

PCR experiments using template DNA from a wide variety of anthozoans implied that the ITS sequences of *Acropora* spp. were unusually short. PCR products given by other anthozoans, including other members of the family Acroporidae, *Montipora digitata* and *Astreopora* sp., were more than 200 bases larger than for any *Acropora* species studied. The compact nature of the ITS regions in *Acropora* spp. was confirmed by DNA sequencing of cloned PCR products. A total of 25 clones, yielding 18 distinct sequence types, were sequenced across the range of species studied. The sequences of the individual clones were submitted to GenBank, under accession numbers U82719–U82736. Overall, the size of the ITS1 varied from 70 to 80 bases, that of the ITS2 varied from 100 to 112 bases, and that of the 5.8S rDNA varied from 155 to 158 bases (table 1). The base composition of each of the three regions did not vary significantly within species.

The sequence data revealed unexpected patterns and levels of diversity. As well as the predicted interspecific heterogeneity, high levels of intraspecific vari-
variation were also detected. Intraspecific heterogeneity was highest in the ITS1 region of *A. valida* (29% divergence between Ava.2/13 and Ava.3), but was significant in all species for both ITS1 and ITS2. In *A. longicyathus*, intraspecific heterogeneity extended to the 5.8S rDNA. In this species, two distinct 5.8S rDNA types were detected, differing from each other at five (six in one case) positions (i.e., substitutions and indels). By contrast, 5.8S rDNA sequences were identical within each of the other species, with the exception of a single base change in one *A. cytherea* clone (possibly a PCR artifact).

Whereas in *A. longicyathus* ITS variants were clearly more similar to each other than to variants in other species, this distinction was not so marked for the other *Acropora* species studied. In the case of *A. hyacinthus* and *A. cytherea*, inter-versus intraspecific similarities were blurred (table 2). In these species, two distinct ITS variants were detected. Clones Ahy.9, Ahy.2, Ahy.5, Acy.2, and Acy.8 were >98% identical in the ITS2 (Ahy.2, Acy.2, and Acy.8 were also identical in the ITS1) and are hereafter referred to as type A. Clones Ahy.1 and Acy.4 appear to represent a second ITS2 variant (type B) which differs by 14% from type A. Clone Acy.3 appears to be intermediate between the types; while being overall more like type B, it has several substitutions in common with type A (at positions 297, 328, and 387 in the alignment). While some of the substitutions present in single clones may be PCR artifacts, the frequent occurrence of common patterns between clones indicates that most of the sequence variation reflects real ITS heterogeneity.

**Phylogenetic Analyses of the ITS Regions**

Phylogenetic analyses were conducted on individual ITS sequences (ITS1 only, ITS2 only, and combined data sets), using both maximum-likelihood and distance methods, with the aim of evaluating relationships between individual sequences. Because the ITS2 sequences could be aligned with higher confidence than could the ITS1 data, results of analysis of the former region only are shown in figure 1. However, phylogenetic trees based on ITS1 alone and on combined (ITS1 + ITS2) datasets were very similar in overall topology to those based on ITS2 alone. In the resulting trees, *A. longicyathus* was always clearly resolved from the other four species of *Acropora*. Both repetitive sequence (McMillan et al. 1991) and sperm ultrastructure (Harrison 1988, pp. 16-119) data support this distinction. These ITS analyses also resolved *A. formosa* and *A. valida*, but indicated a close relationship between these species. However, clones from *A. cytherea* and *A. hyacinthus* formed a single cluster, resolved from the other species but not from each other. Split decomposition analyses supported most aspects of the topology of the distance tree shown in figure 1a; the splits graph shown in figure 1b has a splitability index of 60.8%, indicating that most of the splits are significant (Dress, Huson, and Moulton 1997).

**Secondary Structure Prediction**

The level of variation detected implied that there might be consequences for the secondary structure of the ITS and 5.8S rDNA regions. The size and level of variation within the ITS1 effectively precluded the identification of common folding patterns for this region. However, the lower level of variation and larger size of the ITS2 permitted the identification of common features in predicted secondary structures for this region across the range of species studied. For these analyses, the 3' end of the 5.8S rDNA was paired with the 5' end of the 28S rDNA according to the ITS2 model for yeast (Yeh and Lee 1990). With this constraint, all of the individual sequences gave similar predicted secondary structures, each with six helical domains (fig. 2; helix I representing the region of complementarity between the 5.8S and 28S rDNAs). Similarity at the primary-structure level between the two ITS2 sequence types in the *A. hyacinthus* species group was reflected in the inferred secondary structures for this region, which show only minor differences (see fig. 2). For other species, more widespread, though still minor, intraspecific variation was detected. Helix IV was highly conserved across all clones.

The 5.8S rDNA was assessed in terms of the effects of the polymorphic sites on predicted secondary structure. Figure 3 shows the 5.8S rDNAs of *A. hyacinthus* and *A. longicyathus* folded according to the 23S-like rRNA model proposed by Gutell, Gray, and Schnare (1993). When G-T (as well as Watson-Crick) base pairing was permitted, most nucleotide substitutions could be shown to be compensatory. However, at two sites (positions 180 and 245 in the alignment), C<->T transitions resulted in C=O base pairing being required to maintain the secondary structure.

**Discussion**

The ITS regions of *Acropora* spp. are the shortest characterized to date in the Metazoa and are among the shortest known in eukaryotes (see Hausner, Klassen, and Reid 1993; Katiyar, Visvesvara, and Edlind 1995). *Acropora* spp. appear to be atypical of the Anthoza (Chen, Willis, and Miller 1996), even other members of the same scleractinian family having significantly larger ITS regions. In yeast, the secondary structure of these spacers is essential for correct processing of the rRNA primary transcript (e.g., Van Nues et al. 1995a). However, in a group of amitochondrial protozoans, it has...
Fig. 2.—Predicted secondary structures for the ITS2 region of a representative range of Acropora clones. The figure shows predicted structures for type A (Ahy.2), type B (Aht.1), and the putative intermediate type (Acy.3) clones from A. hyacinthus and A. cytherea, as well as representative clones from each of the three other Acropora species (Ava.2, Alo.4, and Afo.2). Free energy (ΔG) values for each structure are expressed in kcal/mol. Helix I represents 5.8S-28S rDNA complementarity (see text). The 3' end of the 5.8S rDNA and the 5' end of the 28S rDNA are identified by "*" and "**" respectively. Aht = A. hyacinthus (colony A); Ahy = A. hyacinthus (colony B); Acy = A. cytherea; Ava = A. valida; Afo = A. formosa; Alo = A. longicythus.

Fig. 3.—5.8S rDNA sequences from A. hyacinthus (clone Ahy.2) and A. longicythus (clone Alo.2) folded according to the secondary structure model for the 23S-like rDNA of Gutell, Gray, and Schnare (1993).
been suggested that it is the lack of secondary structure in the spacers that may facilitate processing (Katiyar, Visvesvara, and Edlind 1995). Like Acropora spp., these protists have very short ITS regions. However, whereas in the protists the spacers have little potential for secondary structure formation, our analyses (fig. 2) clearly demonstrate that this is not the case in Acropora spp. The compact nature of the Acropora ITS regions should facilitate the identification of those features required for processing of the rRNA primary transcript. Highly conserved regions, such as helix IV (fig. 2), are good candidate processing elements (Van Nues et al. 1995b). In addition, as in many respects cnidarians are “mainstream” metazoans (see Berghammer et al. 1996), Acropora may be highly informative with respect to the general principles of rRNA processing in animals.

While it is not unusual for a single individual to harbor more than one ITS sequence variant, the pattern and level of rDNA heterogeneity in Acropora spp. appear to be without precedent. Similarity in base composition within species and in predicted secondary structure argues against any of these variants representing pseudogenes (Buckler and Holtsford 1996a, 1996b). As the normal range of structural constraints presumably applies, the extreme brevity of these regions in Acropora makes the high level of heterogeneity more remarkable. The only other study of cnidarian ITS sequences contrasts markedly with the results for Acropora spp.; only minor intraspecific differences in the ITS sequences were detected across wide geographic ranges in tropical corallimorphs (Chen and Miller 1996; Chen, Willis, and Miller 1996), and ITS sequences were more similar between a range of Rhodactis spp. than within a single individual of (for example) A. valida.

For several other animal and plant species (e.g., Wesson, Porter, and Collins 1992), higher mutation than homogenization rates (Ohta and Dover 1983; Schlötterer et al. 1994) can account for observed levels of sequence heterogeneity. However, estimates for rates of divergence for the same region in Drosophila are in the region of 1.2% per Myr (Schlüttter et al. 1994). Therefore, the extreme level of intraspecific variation observed in Acropora spp. (up to 29% divergence in the ITS1 in A. valida; table 2) is inconsistent with explanations based on simple rate imbalances.

The detection of two ITS2 sequence types common to both A. cytherea and A. hyacinthus may be explained by the operation of differential maintenance mechanisms (see Ritland, Ritland, and Straus 1993) if the variants were present before these species diverged from a common ancestor. However, consideration of the known hybridization potential of A. cytherea and A. hyacinthus (Willis et al. 1997) leads us to believe that the presence of sequence variants that are shared between species is evidence of recent interspecific hybridization. Note that in addition to the sequences included in the alignment shown, two other clones identical with Ahy.2 and one other identical with Acy.2 were detected. Therefore, of the total of 11 clones sequenced for A. hyacinthus and A. cytherea, eight had type A and two had type B ITS2 sequences, with one clone (Acy.3) being intermediate.

Our interpretation of these results is that the ITS2 is undergoing homogenization in favor of type A at the level of a “metaspecies” (Veron 1995, pp. 210–239) comprising a minimum of A. cytherea and A. hyacinthus, and that clone Acy.3 represents an intermediate stage in this process. The origin of the type B sequence may be more clear when ITS data are available for other species known to hybridize in vitro with this group (Willis et al. 1997). The presence of highly diverged ITS1 sequence variants in A. valida and of distinct 5.8s rDNA types in A. longicyathus are both also suggestive of recent hybridization events.

Based on this model, several testable predictions can be made with respect to Acropora rDNA data. First, other ITS2 variants intermediate between types A and B may be present in the A. hyacinthus/A. cytherea complex. Second, there may be spatial variation in the distribution of ITS types, arising from different distribution ranges of hybridizing species. Third, there may be ITS1 and 5.8s rDNA types in A. valida and A. longicyathus respectively, that are intermediate between those found here. Fourth, it may be possible to identify other species that contain only one of the sequence variants. Finally, similar phenomena are predicted to occur in other Acropora species.

In summary, the ITS and 5.8s rDNA data suggest a reticulate evolutionary history for the genus Acropora. In peonies (Paeonia spp.), the evidence for reticulate evolution is that some species show patterns of ITS substitutions that are additive with respect to the putative parental species; the hybridization events that gave rise to these species occurred relatively recently (during the late Pleiocene and Pliocene), and concerted evolution has not completely homogenized the ITS sequences (Sang, Crawford, and Stuessy 1995). Levels of inter- and intraspecific variation in the ITS regions of peonies contrast markedly with those seen in Acropora spp. This may reflect the rather limited opportunities for hybridization in peonies compared with corals; the majority of Paeonia spp. are allopatric, whereas Acropora species capable of hybridizing in vitro frequently occur sympatrically, and fertilization can occur over relatively long ranges. Therefore, in corals, hybridization may occur on biological, rather than geological, time scales.

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LITERATURE CITED


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