Atypically low rate of cytochrome *b* evolution in the scleractinian coral genus *Acropora*

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Unexpectedly low levels of mitochondrial DNA (mtDNA) cytochrome *b* sequence divergence are found between species of the scleractinian coral genus *Acropora*. Comparison of 964 positions of the cytochrome *b* gene of two out of the three Caribbean *Acropora* species with seven of their Pacific congeners shows only 0.3–0.8% sequence difference. Species in these biogeographic regions have been evolving independently for at least three million years (since the rise of the Isthmus of Panama) and this geological date is used to estimate nucleotide divergence rates. The results indicate that the *Acropora* cytochrome *b* gene is evolving at least 10–20 times slower than the ‘standard’ vertebrate mtDNA clock and is one of the most slowly evolving animal mitochondrial genes described to date. The possibility is discussed that, unlike higher animals, cnidarians may have a functional mtDNA mismatch repair system.

Keywords: mtDNA; cytochrome *b*; substitution rate; corals; *Acropora*

1. INTRODUCTION

Mitochondrial DNA (mtDNA) sequences are often used as population-level genetic markers and have also been used to estimate absolute divergence times between closely related taxa. The comparison of mtDNA sequences (mainly based on RFLP analysis of the entire mitochondrial genome) among a range of vertebrates has led to the assumption that nucleotide substitutions are accumulated linearly at a clock-like rate of approximately 1% per million years (Ma) in vertebrates (Brown *et al.* 1979, 1982; Shields & Wilson 1987). Consequently, it is generally accepted that mtDNA provides a reliable molecular clock. Several recent studies, however, clearly demonstrate variations in the absolute rate of mtDNA evolution. For example, the mitochondrial rDNAs of Antarctic notothenioid fishes evolve at a rate that is 10–20 times slower than the vertebrate norm (Bargelloni *et al.* 1994). In perciform fishes the cytochrome *b* rate of evolution is 3–5 times lower than in mammals (Cantatore *et al.* 1994) and in sharks the cytochrome *b* and cytochrome oxidase rate is 7–8 fold lower than in primates and ungulates (Martin *et al.* 1992). Factors such as generation time (Li *et al.* 1987; Wu & Li 1985), exposure to mutagens (Schlötterer *et al.* 1991), rate of cell division (Wu & Li 1985) and metabolic rate (Martin & Palumbi 1993) are generally believed to be responsible for inter-lineage variations in nucleotide substitution rate.

Within the mitochondrial genome, different regions evolve at different rates, although rate differences between protein-encoding genes are more pronounced at the amino acid level than at the nucleotide level (Simon *et al.* 1994). In testudine turtles, for instance, the overall

rate of mtDNA divergence is eightfold slower than the ‘standard’ clock rate (2% Ma⁻¹ divergence rate; Avise *et al.* 1992), whereas cytochrome *b* evolves at approximately one-third of the ‘standard’ rate (Bowen *et al.* 1993). In general, the most rapidly evolving region is the D-loop (also called control region or AT-rich region) but, when present, intergenic regions may also display rapid sequence divergence. For example, an intergenic region in ambystomatid salamanders evolves three times faster than the control region (McKnight & Shaffer 1997). The most slowly evolving mitochondrial genes are those encoding the two rRNAs and the 22 tRNAs, and the protein-coding genes accumulate base changes at an intermediate pace. Most of the mitochondrial protein-coding genes have been used in phylogenetic or population genetic studies (see examples in Avise 1994; Simon *et al.* 1994). Here we investigate the use of the cytochrome *b* gene as a marker to study interspecific hybridization and reticulate evolution in the scleractinian coral genus *Acropora*.

Acropora (Acroporidae, Scleractinia) is one of the most widespread scleractinian genera, spanning the Indian and Pacific Oceans and the Caribbean. With over 370 nominal and probably around 150 valid species (Veron & Wallace 1984), *Acropora* is by far the largest extant genus of reef-building coral. The occurrence of approximately 70 *Acropora* species in sympatry (Veron 1993) and their participation in synchronous mass spawning events (Harrison *et al.* 1984; Willis *et al.* 1985; Babcock *et al.* 1986) creates major opportunities for interspecific hybridization and hence introgression. Recent studies imply that hybridization may play an important role in the evolution of the genus *Acropora* (Willis *et al.* 1997; Odorico & Miller 1997). Hybridization frequently results in the nuclear genome having a mosaic structure, complicating analyses of nuclear sequence data. However, the maternal

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inheritance and clonal transmission behaviour of the mitochondrial genome means that mtDNA markers can be used to directly trace hybridization events (e.g. Avise (1994) and references therein). To address the issue of natural hybridization in *Acropora*, we are presently characterizing the mitochondrial genome of *Acropora tenuis*. Based on the available sequence for *A. tenuis*, we investigated rates of divergence of the (mitochondrial) cytochrome *b* gene in the genus *Acropora*. The results indicate that the *Acropora* cytochrome *b* gene is among the most slowly evolving mitochondrial genes described to date. Together with the recent detection of a gene encoding a putative mutS homolog (MHS) in the mitochondrial genome of the soft coral *Sarcophyton* (Pont-Kingdon *et al.* 1998), the extremely low rate of cytochrome *b* divergence in *Acropora* suggests that, unlike higher animals, cnidarians may have a functional mtDNA mismatch repair system.

2. MATERIAL AND METHODS

(a) Samples

Sperm or eggs of *Acropora tenuis*, *A. millepora*, *A. formosa*, *A. valida*, *A. latistella*, *A. cerealis*, *A. digitifera* and *Montipora aquituberculata* (Acroporidae) were collected during the mass spawning event of 1987 at Magnetic Island and Orpheus Island at the Great Barrier Reef, Australia. Sperm and eggs were directly frozen in liquid nitrogen and kept in liquid nitrogen until DNA extraction. Small pieces of the Caribbean species *A. palmata* and *A. cervicornis* were collected at San Blas Island off Panama and stored in 80% EtOH.

(b) DNA extraction and PCR

DNA was extracted from eggs and sperm as described in McMillan *et al.* (1988). DNA was extracted from somatic tissue as follows. Approximately 1 cm³ of branch was ground in a pestle and mortar using liquid nitrogen. The powder was added to 3 ml of extraction buffer (75 mM NaCl, 25 mM EDTA pH 8.0, 2% SDS) containing 25 µl of proteinase-K (20 mg ml⁻¹) and incubated overnight in a shaker/incubator at 50 °C. NaCl to a final concentration of 1.5 M as well as an equal volume of chloroform were then added. The mixture was gently shaken for 15–30 min and centrifuged (3500 rpm for 30 min) to collect the denatured proteins at the interface. The aqueous phase was transferred to a new tube and the DNA was precipitated. DNA was resuspended in 100–200 µl TE.

One µl of 10 × or 50 × diluted DNA was added to 24 µl of PCR-master mix consisting of: 1 µl of each primer at a concentration of 20 µM (forward primer Atcytb-FP1: 5'-ATGC-CATTGCGTAAAGAGAATCC-3'; reverse primer Atcytb-RP2: 5'-GATTATTTTCCACTTCCCCCAAC-3' or Atcytb-RP3 (80 nt downstream of cytochrome *b*: 5'-CAATTAGCCCCACCTCC TCTTG-3')), 2.5 µl dNTPs (2 mM), 2.5 µl 10 × reaction buffer (Promega), 2 µl MgCl₂ (25 mM), 0.13 µl Taq polymerase (5 u µl⁻¹, Promega) and 14.87 µl H₂O. The cytochrome *b* gene was amplified in a PTC-100 Thermocycler (MJ Research, Inc.) using the following temperature profile: an initial denaturation step of 5 min at 95 °C, followed by 5 cycles of 30 s at 94 °C, 30 s at 50 °C and 90 s at 72 °C, followed by 26 cycles as before but with an annealing temperature of 56 °C instead of 50 °C. Finally, the mixture was incubated at 72 °C for 10 min. PCR primers were designed from the *A. tenuis* cytochrome *b* sequence obtained from cloned mtDNA fragments of *A. tenuis* (M. J. H. van Oppen, N. R. Hislop and D. J. Miller, unpublished results).

(c) Cloning

Initially PCR products were cloned before sequencing to check for the presence of different mitotypes within individuals (i.e. heteroplasmy or the presence of nuclear copies). 5 µl of 6 × loading dye was added to the PCR product and the whole sample was loaded on to a 0.8% TAE-agarose gel containing EtBr and electrophoresed. PCR products were excised from the gel and purified using the Jetsorb gel extraction kit (Genomed, Inc.) according to the manufacturer's protocol. These purified products were ligated into a T-vector using standard procedures (Sambrook *et al.* 1989). Positive colonies were lightly touched with a sterile tooth pick which was rinsed in 1 µl of sterile H₂O in a PCR tube. The tube was placed in a thermocycler and heated to 95 °C for 5 min. This was followed by PCR amplification of the insert by adding the master mix described above but with the SK and KS vector primers instead of the cyt *b* primers. The PCR profile was: 30 cycles of 30 s at 95 °C, 30 s at 52 °C and 40 s at 72 °C, followed by 2 min at 72 °C. The products were run on a 0.8% TAE-agarose gel, excised and purified as before. 60–90 ng of product was used for a 10 µl sequencing reaction consisting of 1 µl primer at a concentration of 3.3 µM (either Atcytb-FP1, Atcytb-RP2 or Atcytb-RP3), 4 µl of Perkin Elmer BigDye terminator mix (PE Applied Biosystems) and 5 µl of DNA solution. Sequencing products were separated on an ABI 310 Genetic Analyser. Once it had been ascertained that individual clones yielded identical sequences which were also identical to the directly sequenced PCR products, the cloning step was omitted and PCR products were sequenced directly from gel-purified fragments. The *Acropora* and *M. aquituberculata* sequences have been submitted to GenBank (accessions AF099650 to AF099659).

(d) Sequence analyses

Sequences were assembled and aligned manually. Unweighted parsimony analysis was performed in PAUP 3.1.1 (Swofford 1993) using the heuristic search option. Bootstrap analysis was replicated 500 times.

3. RESULTS

Of the nine *Acropora* and one *Montipora* species, 964 nucleotides of the cytochrome *b* gene were obtained (figure 1a), starting at nucleotide position 121. Within the *Acropora* species, 15 positions were polymorphic, but among each pair of species the sequences varied at only zero to eight positions (figure 1b). Uncorrected pairwise distances (table 1) range between 0 and 0.8% within *Acropora*, and 4.8 and 5.5% between *Acropora* and *Montipora*. Sequences were translated using the *Metridium senile* mtDNA code (Pont-Kingdon *et al.* 1994) and compared. Three nucleotide changes in *A. valida* led to amino acid changes compared to the other eight *Acropora*, one from asparagine to aspartic acid (AAT to GAT), one from phenylalanine to leucine (TTT to CTT) and one from tyrosine to histidine (TAT to CAT). In *A. cerealis* one base change led to an amino acid replacement from tyrosine to phenylalanine (TTT to TAT). All but one (from phenylalanine to leucine) are conservative changes. In *Montipora* seven amino acid replacements were found compared to *Acropora*. *Montipora* is very rarely recorded in fossil faunas and could therefore not be used to calibrate substitution rates. Veron (1995) states that it may have been present in the Eocene of the Pacific and the Oligocene of the Tethys.

(a)

GCTATGCAAATCGTAACGGGGTCTTTTGTCCATGCATTATTGTGCAGAGGTCGGCTTGGCTTTTGCATCGGTGGGACATATTATGCGCGATGTAACT 100
 ATGGGTTTTTATTAAATATTTTCATGCTAATGGGGCTTCTCTGTTTTTTTATGTCTTTATTTTCATATTGGGAGAAAGTTTGTATTATGGGGGTTATTT 200
 GAAAGGTCGGGTTTGGCGAGTTGGCATTGTAATATTCTTTTGACGATGGCGACGGCTTTTCTGGGCTATGTCTACCTTGAGGTCAAATGTCTTTCTGG 300
 GGGGCGACGGTTATTACAAATCTATTGTCCGCAATACCCTATGTGGGGACAGATGTGTGCAATGAGTTTGAGGGGGTTTGTATTGTTCTGGGGCCACGC 400
 TCACTAGATTTTTTAGTCTGCACCTTTCTTTCCCTTTATTTTAGCAATTTTAGTTGTGGTTCATTTAATATTTTTACATATAGAGGGATCCAATAGTCC 500
 TGTGGGGTCGAAGACTCCTGTGGACGATGTGGTATTTCATGTTTATATACATCTAAAGACTGATATGGAATAGTAGTTACGCTTATGTTATTGAGTATA 600
 GTTGTGTATTTAATGCCTAATTTATTGGGCGATCCAGAAAATTTTATTCAAGCTAACTCATTAGTAACCCAGTGCACATTGAGCTGAGTGATACTTTT 700
 TATTTGCTTATGCAATTTTGCCTCAATACCGAATAAATTTGGGGGGGTTGTGTCTATGTTTTAAGTATATTAATTTTATTTTCCCACTACTACA 800
 CCGGAGTTGATTAAGGGGGTTCCTTTCTGTCATTTGGACGATGGCTTTTGTATCTTTTGTGTGAATTTTGTCTCTTACCTGAATCGGGTCTTTG 900
 GTTGTAGAAGAGCCTTTTATAATAATAGGGCAGCTGGTTGCCTATACTATTTTTTCTACTTTT 964

(b)

1111222333333333444445555566666777777888888888999999
 Site 3456936770288233467789235790134579005691144689122445689000134
 29579903776925236092876926324043868194964717598602098221036795
A. tenuis CTTCACATAAGTATCAATTATCTCTTCGGATAAAGTTCCATTGACAGTGATTGTCGTATTA
A. palmataGC.....C.....CG...
A. cervicornisGC.....A.....C.....C.....C.....
A. cerealisC.....G.....C.....C.....C.....
A. digitiferaC.....G.....C.....C.....C.....
A. formosaC.....G.....C.....C.....C.....
A. latistellaNN...C.....C.....G.....C.....C.....
A. milleporaC.....G.....C.....C.....C.....C.....
A. validaGC.....C.....C.....G.....CC.....C.....
M. aquituberculata T..CTCT...CGCTTC..CG..CGCTCAA..CTCG..GA..CTTC..CATTTACCGCCACTAC..GG

Figure 1. (a) Nucleotide sequence of the partial cytochrome *b* gene of *Acropora tenuis*. (b) Variable positions among the nine *Acropora* and one *Montipora* species. Non-synonymous changes are underlined. Numbers indicate the positions of the variable sites.

Table 1. Proportional differences (%) between the nine *Acropora* and one *Montipora* species based on the alignment of 964 nt of the cytochrome *b* gene (see figure 1)

(Note: 2 and 3 are Caribbean species, 1 and 4–10 are Indo-Pacific species.)

	1	2	3	4	5	6	7	8	9
1. <i>A. tenuis</i>									
2. <i>A. palmata</i>	0.5								
3. <i>A. cervicornis</i>	0.6	0.3							
4. <i>A. cerealis</i>	0.5	0.4	0.5						
5. <i>A. digitifera</i>	0.5	0.4	0.5	0.2					
6. <i>A. formosa</i>	0.4	0.3	0.4	0.1	0.1				
7. <i>A. latistella</i>	0.5	0.4	0.5	0.2	0.2	0.1			
8. <i>A. millepora</i>	0.4	0.3	0.4	0.1	0.1	0.0	0.1		
9. <i>A. valida</i>	0.8	0.7	0.8	0.5	0.5	0.4	0.5	0.4	
10. <i>M. aquituberculata</i>	5.1	5.0	5.1	5.0	5.0	4.9	4.8	4.9	5.3

The number of phylogenetically informative positions in the data set was too small to resolve the relationships between the coral species investigated. Parsimony analysis of the nucleotide sequences showed a polytomy of all species, except the two Caribbean species which formed a clade supported by a bootstrap value of 60% (tree not shown).

Of all the Caribbean and Indo-Pacific *Acropora* species studied, *A. valida* had the most divergent cytochrome *b* sequence (figure 1 and table 1). A number of other studies have also found *A. valida* to be atypical. The usual somatic chromosome number of *Acropora* spp. is 28, as in other corals (Heyward 1985), whereas *A. valida* has 42 chromosomes (Kenyon 1997); the most likely explanation for this is that *A. valida* is triploid. *A. valida* may have originated by allopolyploidy followed by backcrossing between the tetraploid and a diploid species. *A. valida*

contains two highly (29%) diverged rDNA ITS1 variants (Odorico & Miller 1997), which is consistent with the presence of an extra set of chromosomes. However, the presence of an extra set of chromosomes does not explain the high number of non-synonymous substitutions (37.5–75%) in the mtDNA cytochrome *b* gene. No ambiguities in the sequences were encountered at the variable sites, indicating that heteroplasmy or nuclear copies (pseudogenes) are unlikely to explain our findings.

4. DISCUSSION

(a) Hybridization and cytochrome *b* homogeneity

One interpretation of the low level of sequence variation detected in the *Acropora* cytochrome *b* gene is that extensive introgression of the mitochondrial genome has occurred. Although the mechanisms by which this might occur exist (Willis *et al.* 1997), this hypothesis requires that there is a strong selective advantage in possession of one specific mitochondrial type—otherwise one would expect to see sharing of a particular mtDNA type between a subset of species, while at the same time other subsets of species would each be characterized by a different mtDNA type. In the absence of any evidence supporting selective advantages of specific mtDNAs, it is more parsimonious to assume that the high level of cytochrome *b* sequence identity between species is due to a slow substitution rate. Moreover, the Caribbean species of *Acropora* have been physically isolated from those of the Indo-Pacific for at least 3 Ma (see below), making it impossible for them to hybridize.

(b) Slowdown in *Acropora* cytochrome *b* evolution

The Isthmus of Panama closed approximately 3.0–3.5 Ma ago (e.g. Keigwin 1982; Duque-Caro 1990; Coates & Obando 1996), indicating that no exchange between

Caribbean and Pacific *Acropora* species has been possible for at least 3 Ma. Because relationships between the Caribbean and Indo-Pacific *Acropora* species are not clear, it is not possible to accurately estimate the divergence rate of the *Acropora* cytochrome *b* gene. The available genetic distance data—the smallest (0.3%), modal (0.55%) or average (0.5%) distances between Caribbean and Indo-Pacific species—indicate a maximum divergence rate of 0.1–0.18% Ma⁻¹, which is 10–20 times slower than the ‘standard’ clock (2% Ma⁻¹) and similar to the pace of mitochondrial rDNA divergence in Antarctic notothenioid fishes (Bargelloni *et al.* 1994). True divergence rates may be much lower in *Acropora*, because the divergence time between Caribbean and Indo-Pacific taxa is probably significantly greater than 3 Ma. Knowlton *et al.* (1993) studied divergence in allozymes, mtDNA and reproductive compatibility among seven closely related, transisthmian pairs of snapping shrimps and found that isolation of pairs was staggered rather than simultaneous, with divergence times between Caribbean and eastern Pacific taxa ranging from 3.0–9.1 Ma. Hence, our estimated cytochrome *b* divergence rate of 0.1–0.18% Ma⁻¹ for the *Acropora* lineage is conservative and the actual tempo of evolution in this gene may be considerably slower. This finding was unexpected, because levels of sequence divergence in the rDNA ITS regions are high among *Acropora* species from the two regions. ITS1 sequences are largely unalignable between Caribbean and Indo-Pacific species, while the uncorrected genetic distance between the two groups ranges from 0.210 to 0.434 for ITS2 (M. J. H. van Oppen, T. van Rheede, B. L. Willis and D. J. Miller, unpublished results).

Several factors appear to influence mitochondrial nucleotide substitution rates, including metabolic rate (Martin *et al.* 1992) and the maintenance of a constant body temperature (e.g. Larson & Wilson 1989; Kocher *et al.* 1989; Avise *et al.* 1992; Martin *et al.* 1992; Martin & Palumbi 1993; Cantatore *et al.* 1994; Rand 1994), base composition at silent codon positions (Wolfe *et al.* 1989), effective population size and generation time (Li *et al.* 1987; Wu & Li 1985). Another possibility is that cnidarian mitochondria contain a functional mismatch repair (Pont-Kingdon *et al.* 1998).

Species with higher metabolic rates experience higher rates of oxidative DNA damage and, among vertebrates, endotherms (homeotherms) accumulate nucleotide substitutions at a higher rate than do ectotherms (poikilotherms). Rates of mtDNA evolution have been shown to be higher in mammals than in fish, amphibians and a range of invertebrates (sea urchins, insects and nematodes; Lynch & Jarrell 1993). Based on these data, we might expect a divergence rate somewhat lower than 2% Ma⁻¹ for *Acropora*. But simple metabolic rate differences are unlikely to account for the observation that the *Acropora* cytochrome *b* mutation rate is among the lowest described for any ectotherm, comparable to or lower than the rate of mtDNA rDNA evolution in Antarctic fishes that have extremely low metabolic rates (Bargelloni *et al.* 1994). Water temperatures on coral reefs fluctuate between approximately 18 and 33 °C, and *Acropora* are among the fastest-growing coral species, suggesting that their metabolic rate is relatively high.

In mammals, the rate of substitution at silent sites is related to base composition at these sites (Wolfe *et al.* 1989), high GC content correlating with low substitution rates, and low GC content with highly mutable sites. The base composition of the *A. tenuis* cytochrome *b* gene is 34% (G+C). Based on the data for mammals, this relatively low (G+C)-content suggests that we should expect a high mutation rate at synonymous sites, whereas the opposite was observed.

Individual factors discussed above are unlikely to account for the observed rate of mitochondrial sequence divergence in *Acropora*. While a combination of factors may be responsible, the presence in the *Sarcophyton* mitochondrial genome of an ORF encoding a protein resembling part of the bacterial DNA mismatch repair system (Pont-Kingdon *et al.* 1998) suggests an alternative explanation—an artificially low divergence rate in *Acropora* due to mtDNA repair. Whereas mammals (and presumably other animals) lack mitochondrial mismatch repair systems, in yeast, one of the four MSH homologues (MSH1) is targeted to the mitochondria (where it functions in the mismatch repair pathway) and the rate of nucleotide substitution in mitochondrial protein-coding genes is lower than in nuclear protein-coding genes (Clark-Walker 1991). A comparison of the divergence rates of mitochondrial and nuclear genes may be informative in terms of the likelihood of mtDNA mismatch repair activity in cnidarians.

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