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## Analysis of complete mitochondrial DNA sequences of three members of the *Montastraea annularis* coral species complex (Cnidaria, Anthozoa, Scleractinia)

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**Abstract** Complete mitochondrial nucleotide sequences of two individuals each of *Montastraea annularis*, *Montastraea faveolata*, and *Montastraea franksi* were determined. Gene composition and order differed substantially from the sea anemone *Metridium senile*, but were identical to that of the phylogenetically distant coral genus *Acropora*. However, characteristics of the non-coding regions differed between the two scleractinian genera. Among members of the *M. annularis* complex, only 25 of 16,134 base pair positions were variable. Sixteen of these occurred in one colony of *M. franksi*, which (together with additional data) indicates the existence of multiple divergent mitochondrial lineages in this species. Overall, rates of evolution for these mitochondrial genomes were extremely slow (0.03–0.04% per million years based on the fossil record of the *M. annularis* complex). At higher taxonomic levels, patterns of genetic divergence and synonymous/nonsynonymous substitutions suggest non-neutral and unequal rates of evolution between the two lineages to which *Montastraea* and *Acropora* belong.

**Keywords** *Montastraea* · Species complex · Coral · Mitochondrion · Cnidaria

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### Introduction

Members of the *Montastraea annularis* complex (*M. annularis*, *Montastraea faveolata*, and *Montastraea franksi*) are dominant reef-builders in the Caribbean whose species status has been disputed for many years (e.g., Knowlton et al. 1992, 1997; van Veghel and Bak 1993, 1994; Weil and Knowlton 1994; Szmant et al. 1997; Medina et al. 1999; Fukami et al. 2004a; Levitan et al. 2004). The fossil record suggests that *M. franksi* most closely resembles the morphology of the ancestral lineage, that *M. faveolata* diverged from this ancestral lineage about 3–4 million years ago (mya), and that *M. annularis* and *M. franksi* diverged much more recently (0.5 mya; Pandolfi et al. 2002).

Genetic studies have played a central role in the analysis of this complex (Fukami et al. 2004a and references therein), but progress has been hindered by the poorly understood genetics of corals and anthozoans generally, and by low levels of mitochondrial genetic variability (Medina et al. 1999; van Oppen et al. 1999; Shearer et al. 2002). For example, *M. cavernosa* and the *M. annularis* complex have been isolated for over 30 million years (recent studies indicate that they are not in fact congeneric; Fukami et al. 2004b), but differ by only 2.4% in partial DNA sequences of the cytochrome oxidase subunit I (*cox1*) (Medina et al. 1999).

To provide a comprehensive picture of mitochondrial DNA in this species complex, we determined entire mitochondrial genome sequences for two individuals of each of the three members of the group, thereby allowing both intra- and interspecific analyses of variation and divergence within this closely related assemblage. We also compare these DNA sequences with those of *Acropora tenuis* (van Oppen et al. 2002) [a distantly related coral (Romano and Palumbi 1996, 1997; Romano and Cairns 2000; Veron 2000; Chen et al. 2002)], the sea anemone *Metridium senile* (Beagley et al. 1998), and other cnidarians.

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## Material and methods

### DNA extraction, PCR amplification, and sequencing

Total DNA was extracted from eggs stored in guanidine solution (see Fukami et al. 2004a) by conventional phenol/chloroform extraction and ethanol precipitation methods. The corals used were a subset of Panamanian corals previously analyzed (samples Pa01-23, M01-4, Pa00-3, Pa01-5, Pa00-9, Pa01-28 of Fukami et al. 2004a).

Initially, mitochondrial DNA from *M. annularis* was amplified by the long-PCR method using four primers: 5' GAC AGA GAA ACT TTC GTG ACA CCA TTC ATA 3' (MTLNF3) and 5' GTA AGA CGA GAA GTC CCC ATG GAG CTT TAC 3' (MTLNR3) [designed from partial DNA sequences of the large-subunit ribosomal RNA (*rnl*) of *Acropora nasuta* (Fukami et al. unpublished data)], and 5' GTC CCA ATT AGA CCT GCT CCA ACA CC 3' (COI-LOF) and 5'CAA CGA TTT TCA ACA TGC GAG CCC CTGG 3' (COI-LOR) [designed from partial DNA sequences of *cox1* of *A. nasuta* (Fukami et al. unpublished data)]. Long-PCR bands (more than 5 kb) were obtained using the following primer combinations: MTLNF3×MTLNR3, COI-LOF×MTLNF3, and COI-LOF×COI-LOR. The PCR protocol was 10 cycles at 94°C for 15 s, 60°C for 30 s, 68°C for 5 min, followed by 20 cycles at 94°C for 15 s, 60°C for 30 s, 68°C for 5 min with 20 s increments in the extension times for each cycle using *Taq* polymerase (SIGMA). Generally, the amplified fragments were purified using GeneClean (Promega) from pieces of gel cut after observing the PCR products in a 1.0% agarose/TAE gel. Alternatively, the amplified fragments separated by electrophoresis on 0.7% low melting-point agarose/TAE gel were cut from the gel and treated by GELase (Biocompare) at 42°C overnight to digest the agarose. The recovered fragments were digested with *EcoRV* and *SmaI*, cloned in pGEM-T Easy Vector (Promega) after being treated at 72°C for 10 min by *Taq* polymerase to add adenine to make the fragments insert easily into the vector, and sequenced using an ABI automated sequencer.

Based on the nucleotide sequences obtained, the remainder of the mitochondrial DNA sequence of *M. annularis* was determined using primer walking methods. Mitochondrial sequences from *M. franksi* and *M. faveolata* were subsequently determined using several primers designed based on the complete mitochondrial nucleotide sequence of *M. annularis*. All sequences are available in DDBJ (accession numbers AP008973-AP008978). Open Reading Frames (ORFs) were translated in DNASIS version 2 (Hitachi Software Engineering, Inc.) and MEGA Program version 2.1 (Kumar et al. 2001) using the *A. tenuis* genetic code (van Oppen et al. 2002). Genes and their start and stop codons were identified by comparison with mitochondrial DNA sequences of *A. tenuis* (van Oppen et al. 2002) and

*Metridium senile* (Pont-Kingdon et al. 1994; Beagley et al. 1998). Secondary structure of tRNA was estimated using tRNAscan-SE (<http://lowelab.ucsc.edu/tRNAscan-SE/>). Base composition bias, numbers of transitions (Ts) and transversions (Tv), and pair-wise nucleotide divergences based on the Kimura-2 parameter model (Kimura 1980) were calculated using PAUP\* 4.05 (Swofford 2002). The number of non-synonymous substitutions per non-synonymous site (dN) and the number of synonymous substitutions per synonymous site (dS) of the translated sequences were calculated using Nei and Gojobori's method (Nei and Gojobori 1986) in MEGA. A Z test was used to determine whether dN and dS differed significantly among *M. annularis*, *A. tenuis* and *Metridium senile*. Gap regions were excluded from analyses except for calculations of base composition.

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## Results and discussion

### Overall structure of the *Montastraea annularis* complex mitochondrial genome

The mitochondrial DNA of the three members of the *M. annularis* complex is a circular molecule, as is typical of anthozoans but not other cnidarians (Bridge et al. 1992; van Oppen et al. 2002). Sequences were 16,137 or 16,138 bp in length (Table 1), and thus slightly shorter than those of the four other anthozoans whose mtDNAs have been completely sequenced: two in the subclass Alcyonaria or Octocorallia [the soft coral *Sarcophyton glaucum*, 18,453 bp (Beaton et al. 1998); the sea pen *Renilla kolikeri*, 18,911 bp (Beagley et al. 1995)] and two in the subclass Zoantharia or Hexacorallia [the sea anemone *Metridium senile*, 17,443 bp (Beagley et al. 1998); the scleractinian coral *A. tenuis*, 18,338 bp (van Oppen et al. 2002)]. The AT content of the whole mitochondrial genome of the *M. annularis* complex (66%) was slightly higher than that of other cnidarians (e.g., 62% for *A. tenuis*) both including and excluding non-coding regions. As in *Acropora*, cytosine was the least common and thymine the most common nucleotide in *Montastraea* mitochondrial DNA (25.0% A, 13.2% C, 20.5% G, 41.3% T). Base frequency for specific genes differed significantly ( $P < 0.005$ ) for one or more pair-wise comparisons in ATP synthase subunit 6 (*atp6*), ATP synthase subunit 8 (*atp8*), cytochrome oxidase b (*cob*), *cox1*, cytochrome oxidase subunit 3 (*cox3*), NADH hydrogenase subunit 2 (*nad2*), NADH hydrogenase subunit 4 (*nad4*), NADH hydrogenase subunit 5 (*nad5*), NADH hydrogenase subunit 6 (*nad6*), short-subunit ribosomal RNA (*rns*), and *rnl* (Table 1).

As in other cnidarian mitochondrial genes, ATG was the start codon, and both TAA and TAG were stop codons. ATA and GTG also acted as start codons for some genes, as is the case in *Acropora* (van Oppen et al. 2002). There were five cases of gene overlap (indicated by negative values for intergenic regions in Table 1)

**Table 1** Comparison of the whole mitochondrial genome among the scleractinian corals *Montastraea annularis* complex (Ma) and *Acropora tenuis* (Ac) (van Oppen et al. 2002) and the sea anemone *Metridium senile* (Ms) (Beagleby et al. 1998)

Region	Length (bp)			Genetic distance (%)		
	Ma	At	Ms	Ma-At	Ma-Ms	At-Ms
<i>trnM</i>	72	71	71	42.9	81.8	36.8
<i>rnl</i>	1,973	2,261	2,220	32.5*	38.9*	25.1*
IGR	0	102	N.A.	N.A.	N.A.	N.A.
<i>Nad5-5'</i>	711	720	717	36.8	36.1	29.9
<i>Nad5</i> Intron5'	109	323	202	N.A.	N.A.	N.A.
<i>Nad1</i>	948	984	1,005	30.2	35.4	26.2
IGR	2	108	N.A.	N.A.	N.A.	N.A.
<i>cob</i>	1,140	1,155	1,182	29.6*	36.4*	39.2
IGR	25	521	N.A.	N.A.	N.A.	N.A.
<i>nad2</i>	1,287	1,098	1,158	38.3*	50.3*	32.9*
IGR	1	32	N.A.	N.A.	N.A.	N.A.
<i>nad6</i>	561	594	609	35.1*	47.4*	32.0
IGR	-1	68	N.A.	N.A.	N.A.	N.A.
<i>atp6</i>	678	699	690	31.7	38.4*	29.2
IGR	-1	151	N.A.	N.A.	N.A.	N.A.
<i>nad4</i>	1,440	1,476	1,476	37.3	44.5*	29.0*
IGR	138	52	N.A.	N.A.	N.A.	N.A.
<i>rns</i>	903	1,176	1,082	24.6*	34.8*	22.5
IGR <sup>1</sup>	137	1,086	N.A.	N.A.	N.A.	N.A.
<i>cox3</i>	780	789	789	28.3	39.5	32.9*
IGR	2	56	N.A.	N.A.	N.A.	N.A.
<i>cox2</i>	708	744	747	33.0	38.2	25.5
IGR	-19	32	N.A.	N.A.	N.A.	N.A.
<i>nad4L</i>	300	300	300	27.2	39.4	28.8
IGR	2	32	N.A.	N.A.	N.A.	N.A.
<i>nad3</i>	342	357	357	35.3	38.2	27.4
<i>nad5</i> Intron3'	58	95	111	N.A.	N.A.	N.A.
<i>nad5-3'</i>	1,104	1,116	1,086	39.2*	45.9*	32.4*
<i>nad5</i> all	1,815	1,836	1,803	38.1*	41.8*	31.2*
IGR	-2	30	N.A.	N.A.	N.A.	N.A.
<i>trnW</i>	71	70	69	25.6	44.9	12.9
IGR	3	32	N.A.	N.A.	N.A.	N.A.
<i>atp8</i>	198	219	219	49.2	59.0*	41.2
IGR	-1	-19	N.A.	N.A.	N.A.	N.A.
<i>cox1</i>	1,578	1,602	1,593	19.9	25.0	25.0*
IGR	891	206	N.A.	N.A.	N.A.	N.A.
Total	16,138	18,338	17,443			
Total coding	14,794	15,431	15,370	33.1*	40.1*	29.7*
Total non-coding	1,368	2,926	2,073			
Overlap	24	19	0			

<sup>1</sup> IGR between *rns* and *cox3* was identified as the control region in *A. tenuis*.

Pair-wise genetic distances were calculated based on the Kimura 2-parameter method using PAUP. Gaps were excluded from calculations. Asterisks mean that base frequency of the pairs differs significantly ( $P < 0.005$ ). IGR: Intergenic region (minus indicates gene overlap). N.A.: not applicable.

ranging from 1 bp (the most common situation) to 19 bp. This degree of overlap is comparable to that of higher animals but larger than that reported for other cnidarians: one overlap in *A. tenuis* (*atp8* with *cox1* by 19 bp), two overlaps in *S. glaucum* [*nad5* with the NADH hydrogenase subunit 2 (*nad2*) and *nad4* genes by 13 and 19 bp, respectively], one overlap in *R. kolikeri* (*nad5* with *nad2* by 13 bp), and one overlap in *Hydra attenuata* [*atp8* with *atp6* by 1 bp (although only partial sequences are available for this species, Pont-Kingdon et al. 2000)]. The mitochondrial gene order, gene orientation and number of genes of the *M. annularis* complex were as described for *A. tenuis* (van Oppen et al. 2002) and *A. nasuta* (gene map only; Fukami et al. 2000). The coding regions of the *M. annularis* complex

and *A. tenuis* differed in length by only 4% (*M. annularis* complex the shortest), but their non-coding regions differed in length by 214% (*M. annularis* complex again the shortest) (Table 1). The large length difference between these two corals was primarily due to the difference in length of the intergenic region described as the purported control region for *A. tenuis*. However, *A. tenuis* also had a greater number of intergenic regions than members of the *M. annularis* complex (13 vs 8, respectively) (Table 1). Although the lengths of the non-coding regions were more similar between members of the *M. annularis* complex and the anemone *Metridium senile* than between the *M. annularis* complex and *A. tenuis*, there are a number of differences in gene order, orientation and number between the sea anemone and

the two corals (as discussed with respect to *A. tenuis* by van Oppen et al. 2002).

#### Comparisons of specific regions within the mitochondrial genome

The presumed secondary structures of the two tRNA genes of the *M. annularis* complex, tRNA<sup>met</sup> (*trnM*) and *trnW*, were very similar to those reported for other hexacorals (*A. tenuis* and *Metridium senile*) (data not shown) and exhibited the major conserved features of standard prokaryotic and eukaryotic nuclear- and chloroplast- encoded tRNAs (data not shown). Octocorals (Alcyonaria), in contrast, have only *trnM* (Beaton et al. 1998).

The intergenic region between the *rns* and *cox3* genes was identified as the control region in *A. tenuis* (van Oppen et al. 2002), but not in other cnidarians. This region in the *M. annularis* complex is only 137 bases long (as compared to 1,086 bp in *A. tenuis*) (Table 1) and does not contain the latter's specific control region characters.

The longest non-coding region in the *M. annularis* complex (891 bp) occurs in the *cox1-trnM* intergenic region. Interestingly, the last 40 bases of *cox1* and first 40 bases of the *cox1-trnM* intergenic region had 94.7% similarity. Also, about 120 bases between positions 108–221 in *cox1* had 73% similarity with positions 590–708 in the *cox1-trnM* intergenic region. These patterns suggest that this intergenic region represents a past partial duplication, or a complete duplication of *cox1* followed by partial deletions. In *Acropora*, the *cox1-trnM* region is much shorter and lacks evidence of a *cox1* duplication event.

Also of note is the intron observed in *nad5* at the same insertion site as reported for *Acropora* (van Oppen

et al. 2002) and *Metridium senile* (Beagley et al. 1996, 1998). In all three, it appears to be a group I intron, supporting the suggestion that it was acquired by a common ancestor of the Scleractinia and Actiniaria (van Oppen et al. 2002). On the other hand, gene composition within this intron differs between the sea anemone and the scleractinian corals; in the *M. annularis* complex and *A. tenuis* it contains many genes whereas in *Metridium senile* it contains only two genes. This suggests that these gene rearrangements occurred early in the history of scleractinians but after their split from the actinarians.

#### Evidence for non-neutral and unequal rates of evolution at higher taxonomic levels

Scleractinian corals are separated into two genetically distinct groups: complex corals (which include *Acropora*) and robust corals (which include *Montastraea*) (Romano and Palumbi 1996, 1997; Romano and Carins 2000; Chen et al. 2002). As outlined below, several puzzling aspects of our results on first examination would support a closer relationship between *Acropora* and *Metridium* than between the two scleractinian corals, but this is highly unlikely based on the substantial differences in mitochondrial gene order and structure between the anemone and the two coral species (van Oppen et al. 2002; this study) and other phylogenetic analyses (Berntson et al. 1999; Chen et al. 2002; Daly et al. 2003). An alternative explanation is that numerous mitochondrial genes are under strong selective pressures, and that the robust coral lineage is evolving more rapidly than the complex coral lineage, as was suggested by an earlier analysis of the mitochondrial *rns* gene for several scleractinian genera (Chen et al. 2002).

Analyses of dN and dS values (Table 2) showed dN values significantly lower than dS for all but one coding

**Table 2** Non-synonymous (dN) and synonymous (dS) substitutions of the protein-coding genes among the scleractinian corals *Montastraea annularis* complex (Ma) and *Acropora tenuis* (Ac) (van Oppen et al. 2002) and the sea anemone *Metridium senile* (Ms) (Beagley et al. 1998)

Region	dN			dS			dN/dS		
	Ma-At	Ma-Ms	At-Ms	Ma-At	Ma-Ms	At-Ms	Ma-At	Ma-Ms	At-Ms
<i>nad5</i> *	0.22	0.23	<b>0.14</b>	<b>0.56</b>	0.60	0.60	0.39	0.39	0.24
<i>nad1</i> *	0.16	0.18	<b>0.11</b>	<b>0.56</b>	0.65	0.58	0.28	0.28	0.19
<i>cob</i> *	<b>0.16</b>	0.19	0.18	<b>0.57</b>	0.62	0.71	0.27	0.31	0.25
<i>nad2</i> *	0.24	0.27	<b>0.15</b>	<b>0.54</b>	0.68	0.64	0.44	0.40	0.23
<i>nad6</i>	0.19	0.23	<b>0.15</b>	<b>0.57</b>	0.71	0.60	0.33	0.32	0.24
<i>atp6</i> *	0.15	0.19	<b>0.12</b>	0.61	0.68	<b>0.59</b>	0.24	0.28	0.20
<i>nad4</i> *	0.21	0.23	<b>0.12</b>	<b>0.57</b>	0.67	0.62	0.37	0.35	0.19
<i>cox3</i> *	<b>0.13</b>	0.20	0.16	<b>0.60</b>	0.71	0.62	0.22	0.27	0.26
<i>cox2</i> *	0.19	0.20	<b>0.11</b>	<b>0.60</b>	0.71	<b>0.60</b>	0.31	0.29	0.19
<i>nad4L</i> *	<b>0.14</b>	0.21	0.16	0.53	0.62	<b>0.52</b>	0.26	0.35	0.31
<i>nad3</i> *	0.20	0.20	<b>0.13</b>	<b>0.58</b>	0.60	<b>0.58</b>	0.34	0.34	0.24
<i>atp8</i>	<b>0.30</b>	0.39	<b>0.30</b>	<b>0.50</b>	0.53	0.56	0.60	0.73	0.53
<i>cox1</i> *	<b>0.06</b>	0.08	0.08	<b>0.54</b>	0.63	0.65	0.12	0.13	0.12
Total coding*	0.17	0.20	<b>0.13</b>	<b>0.56</b>	0.65	0.62	0.30	0.31	0.21

Genes with significant differences ( $P < 0.0001$ ) in all comparisons of three hexacorallians in selection (Z) tests are indicated by asterisks. Lowest values of the three pair-wise comparisons for dN and dS for each gene are in bold; in the absence of selection we would expect most bold values to occur in the Ma-At (coral vs coral) comparison.

gene (*atp8*) in comparisons among *Metridium senile*, *M. annularis* and *A. tenuis*. Selection (*Z*) tests rejected the null hypothesis of neutrality ( $P < 0.00001$ ) in all cases except between *M. annularis* and *Metridium senile* in *atp8*, suggesting that purifying selection is occurring in almost all mitochondrial protein-coding genes. In addition, for most protein-coding genes the lowest dN values were found between *A. tenuis* and *Metridium senile*, and for all genes, the highest dN values were observed in comparisons between *M. annularis* and *Metridium senile*. Thus amino acid substitutions were more common be-

tween the two scleractinian corals than between the coral *A. tenuis* and the anemone *Metridium senile* (amino acid P-distances showed similar results, data not shown). In contrast to the dN values, dS values from 10 of 13 protein-coding genes were lowest between the two scleractinians (Table 2). Because synonymous substitutions are more likely to be neutral than non-synonymous substitutions, dS values may better reflect relationships among these three hexacorallians.

Ts/Tv ratios showed high variability for mitochondrial genes among the three genera, suggesting different

**Table 3** Nucleotide substitutions in the mitochondrial genomes of the three taxa in the *Montastraea annularis* complex

Region	Position	Species names and colony identifications						Genetic distances between colonies (Minimum–maximum)
		<i>M. annularis</i>		<i>M. franksi</i>		<i>M. faveolata</i>		
		Pa01-23	M01-4	Pa00-3	Pa01-5	Pa00-9	Pa01-28	
<i>trnM</i>	-	-	-	-	-	-	-	
<i>rnl</i>	143	T	T	T	<i>A</i>	T	T	0–0.05
<i>nad5-5'</i>	2594	T	T	T	<i>C</i>	T	T	0–0.14
<i>nad5</i> Intron5'	-	-	-	-	-	-	-	
<i>nad1</i>	2,889	G	G	G	<i>A</i>	G	G	0–0.21
	3,090	C	C	C	<i>T</i>	C	C	
IGR	-	-	-	-	-	-	-	
<i>cob</i>	4,505	C	C	C	<i>T</i>	C	C	0–0.09
IGR	-	-	-	-	-	-	-	
<i>nad2</i>	-	-	-	-	-	-	-	
IGR	-	-	-	-	-	-	-	
<i>nad6</i>	-	-	-	-	-	-	-	
<i>atp6</i>	-	-	-	-	-	-	-	
<i>nad4</i>	7,619	G	G	G	<i>A</i>	G	G	0.07–0.21
	7,812	T	T	T	T	<i>G</i>	T	
	8,498	<i>C</i>	T	<i>G</i>	T	T	T	
	8,528	G	<i>C</i>	G	G	G	G	
	8779	T	C	T	C	T	C	
IGR	-	-	-	-	-	-	-	
<i>rns</i>	9,841	C	C	C	<i>T</i>	C	C	0–0.11
IGR	10,045	<i>G</i>	T	T	T	T	T	0–1.48
	10,057	C	C	C	<i>T</i>	C	C	
	10,086	T	T	<i>C</i>	T	T	T	
<i>cox3</i>	10,420	T	T	T	<i>C</i>	T	T	0–0.26
	10,732	A	A	A	<i>C</i>	A	A	
IGR	-	-	-	-	-	-	-	
<i>cox2</i>	11,099	G	G	G	<i>T</i>	G	G	0–0.28
	11,226	G	G	G	<i>A</i>	G	G	
<i>nad4L</i>	-	-	-	-	-	-	-	
IGR	-	-	-	-	-	-	-	
<i>nad3</i>	-	-	-	-	-	-	-	
<i>nad5</i> Intron3'	12,247	G	G	G	*	G	G	N.A.
<i>nad5-3'</i>	-	-	-	-	-	-	-	
( <i>nad5</i> total)								0–0.06
<i>trnW</i>	-	-	-	-	-	-	-	
IGR	-	-	-	-	-	-	-	
<i>atp8</i>	-	-	-	-	-	-	-	
<i>cox1</i>	-	-	-	-	-	-	-	
IGR	15,372	A	A	A	A	<i>C</i>	A	0–0.45
	15,373	T	T	T	T	T	<i>G</i>	
	15,512	T	<i>G</i>	T	T	T	T	
	15,636	G	G	G	<i>A</i>	G	G	
	15,670	A	A	A	<i>C</i>	A	A	
	16,053	T	T	T	<i>C</i>	T	T	

Pair-wise genetic distances were calculated based on the Kimura 2-parameter method using PAUP. Comparisons with no nucleotide substitutions are shown by hyphen (-). Asterisk indicates a deletion. Nucleotide substitutions are shown in bold and italic. IGR: Intergenic region. N.A.: not applicable.

evolutionary rates among genes. Although Ts/Tv ratios are typically lower for more divergent lineages (e.g., Chen et al. 2002), Ts/Tv ratios were higher in comparison between *A. tenuis* and *Metridium senile* (0.91–2.27 at first and second codons, 0.67–1.90 at third codon, 1.42 for all codons) than they were between the two scleractinian corals (0.45–1.69 at first and second codons, 0.59–1.29 at third codon, 0.96 for all codons). Similarly, the extent of genetic divergence among genes of the three hexacorallians (Table 1) was often less between the coral *A. tenuis* and the anemone *Metridium senile* than between the two scleractinian corals. Across all coding regions, divergence values were 32.4% between the *M. annularis* and *A. tenuis*, 30.4% between *A. tenuis* and *Metridium senile*, and 39.7% between *M. annularis* and *Metridium senile* (Table 1). These patterns also suggest unequal rates of molecular evolution between the *M. annularis* complex and *A. tenuis*.

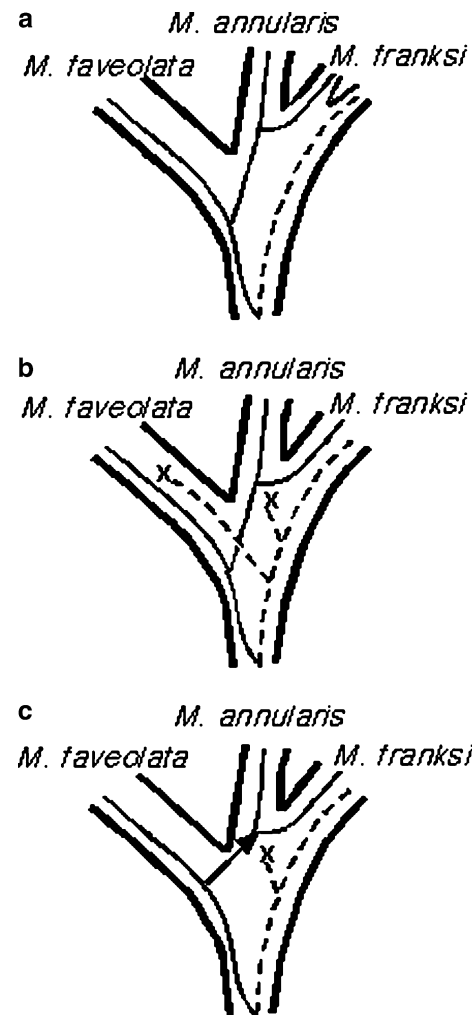
#### Patterns of genetic variability and divergence within the *M. annularis* complex

The three members of the *M. annularis* complex exhibit extraordinarily low levels of variability. Across 16,134 bp, only 25 variable positions (including one indel) were observed among the six colonies (Table 3). Pair-wise genetic divergences estimated using Kimura's 2-parameter model ranged from 0 to 0.12%. Using a minimum divergence time of 3–4 mya between *M. franksi* and *M. faveolata* based on the fossil record (Pandolfi et al. 2002) and comparing the most divergent sequences yields a maximum divergence rate of only 0.03–0.04% per million years. By comparison, the divergence rate of *cob* in *Acropora* was estimated at 0.1–0.18% per my (van Oppen et al. 1999), although the authors assumed that Pacific and Atlantic *Acropora* were isolated by the closure of the Isthmus of Panama, which almost certainly overestimates rates.

Substitutions were observed in 11 out of 28 genes/regions (Table 3). The intergenic region between *rns* and *cox3* had the highest substitution rate. Among coding genes, *cox3* had the highest substitution rate. Sixteen out of 25 variations occurred in just one colony (Pa01-5) of *M. franksi*, whereas the other five colonies [including another *M. franksi* colony (Pa00-3)] had just 2–3 variable sites. This pattern appears to reflect the presence of two divergent mitochondrial lineages within *M. franksi* from Panama, as it was also seen when 68 individuals were analyzed at just the *cox1-trnM* intergenic region (Fukami et al. 2004a; note that the *cox1-trnM* intergenic region in this paper is identical to the “non-coding region between COI and lrRNA” in Fukami et al. 2004a). In that analysis, half of the *M. franksi* individuals had sequences that differed by three or four base pairs from *M. annularis* and *M. faveolata*, and half had sequences that were similar or identical to these taxa. This previous analysis of more individuals at just the *cox1-trnM* intergenic region also indicated that for the lineage shared

among the three species, the most common haplotypes were identical in *M. annularis* and *M. franksi* and differed by a single substitution from the common haplotypes in *M. faveolata*.

There are several possible explanations for this pattern of one clade of haplotypes occurring in all three taxa (mt-clade 1) and another (mt-clade 2) just in *M. franksi*. First, *M. franksi* as currently defined could consist of two cryptic species (Fig. 1a). However, there is no correspondence between the mitochondrial lineages and several nuclear genes (Fukami et al. 2004a), and no evidence of reproductive barriers between these



**Fig. 1** Alternative scenarios to explain the presence of two highly divergent mitochondrial clades in *M. franksi* (mt-clade 1 in solid line, mt-clade 2 in dotted line, see text for details) and one mitochondrial clade (mt-clade 1) in *M. faveolata* and *M. annularis*. X indicates clade extinction and arrow indicates clade introgression. **a** Both *M. faveolata* and *M. annularis* arise from lineages with just mt-clade 1, whereas *M. franksi* retains ancestral polymorphism. **b** All three species initially retain ancestral polymorphism, but mt-clade 2 is subsequently lost in *M. faveolata* and *M. annularis*. **c** *M. franksi* and *M. annularis* lineages initially have mt-clade 2 and *M. faveolata* has mt-clade 1, but mt-clade 2 is lost in *M. annularis* and mt-clade 1 is introgressed from *M. faveolata* to *M. franksi* and *M. annularis*.

mitochondrially defined lineages (Levitan et al. 2004). Second, this pattern could reflect polymorphism in the ancestor of the *M. annularis* complex (which arose from a more diverse group, Budd and Klaus 2001). This would require the independent derivation of both *M. annularis* and *M. faveolata* from an ancestor that contained just mt-clade 1 (as for the cryptic species scenario illustrated in Fig. 1a), or the independent loss of mt-clade 2 in those species subsequent to divergence (Fig. 1b). Alternatively, the pattern could reflect an initial divergence between *M. faveolata* (with mt-clade 1) and the lineage leading to *M. annularis* and *M. franksi* (with mt-clade 2), followed by mitochondrial introgression of mt-clade 1 from *M. faveolata* back into the ancestor of the modern *M. franksi* and *M. annularis* and the loss of mt-clade 2 from *M. annularis* (Fig. 1c). Additional analyses (including Bahamian corals which, based on short sequences, lack mt-clade 2 but have additional divergent haplotypes, Fukami et al. 2004a) would be required to further refine these interpretations.

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