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ORIGINAL ARTICLE

Comparative Analyses of Coding and Noncoding DNA Regions Indicate that *Acropora* (Anthozoa: Scleractina) Possesses a Similar Evolutionary Tempo of Nuclear vs. Mitochondrial Genomes as in Plants

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Abstract Evidence suggests that the mitochondrial (mt) DNA of anthozoans is evolving at a slower tempo than their nuclear DNA; however, parallel surveys of nuclear and mitochondrial variations and calibrated rates of both synonymous and nonsynonymous substitutions across taxa are needed in order to support this scenario. We examined species of the scleractinian coral genus *Acropora*, including previously unstudied species, for molecular variations in protein-coding genes and noncoding regions of both nuclear and mt genomes. DNA sequences of a calmodulin (*CaM*)-encoding gene region containing three exons, two introns and a 411-bp mt intergenic spacer (*IGS*) spanning the cytochrome *b* (*cytb*) and NADH 2 genes, were obtained from 49 *Acropora* species. The molecular evolutionary rates of coding and noncoding regions in nuclear and mt genomes were compared in conjunction with published data, including mt cytochrome *b*, the control region, and nuclear *Pax-C* introns. Direct sequencing of the *mtIGS* revealed an average interspecific variation comparable to that seen in published data for mt *cytb*. The average interspecific variation of the nuclear genome was two to five times greater than that of the mt genome. Based on the calibration of the closure of Panama Isthmus (3.0 mya) and closure of the Tethy Seaway (12 mya), synonymous substitution rates ranged from 0.367% to 1.467% Ma^{-1} for nuclear *CaM*, which is about 4.8 times faster than those of mt *cytb* (0.076–0.303% Ma^{-1}). This is similar to the findings in plant genomes that the nuclear genome is evolving at least five times faster than those of mitochondrial counterparts.

Keywords Molecular evolution · Nuclear genes · Mitochondrial genes · Scleractinian corals · *Acropora* · Calmodulin

Introduction

A growing body of evidence suggests that the mitochondrial (mt) DNA of anthozoans is evolving at a slow tempo (Medina et al. 1999; van Oppen et al. 1999; Chen and Yu 2000; Shearer et al. 2002; Fukami and Knowlton 2005; Tseng et al. 2005; Hellberg 2006). A comparison of 964 positions of the cytochrome b (*cytb*) gene between two of the three Caribbean *Acropora* species and seven Pacific species showed only 0.3–0.8% sequence divergence, equal to a maximum divergence rate of 0.1–0.18% Ma^{-1}, which is at least ten to 20 times slower than the "standard" vertebrate mtDNA clock (van Oppen et al. 1999). Estimates of sequence divergence within the family Acroporidae

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showed that mt 12S ribosomal RNA (rRNA) is ten times slower (<0.01% Ma^{-1}) than the *cytb* gene (Chen and Yu 2000; Tseng et al. 2005). An even slower evolutionary tempo was observed among three members of the *Montastraea annularis* complex, for which only 0.03–0.04% Ma^{-1} was observed in complete mt genomes (Fukami and Knowlton 2005). Shearer et al. (2002) concluded that slow evolution and unique genomic characteristics may be common in primitive metazoans and that their patterns of mtDNA evolution differ from those in other animal systems.

This slow characteristic, however, requires parallel surveys of nuclear variations and calibrated rates of both synonymous and nonsynonymous substitutions across taxa in order to put it in a phylogenetic framework. Information on scleractinian mt genomes is increasing (van Oppen et al. 2001; Fukami and Knowlton 2005; Tseng et al. 2005; Medina et al. 2006; Chen et al. 2008). The available data for comparing nuclear variations are confined to ribosomal RNA genes and their spacers, for which it is difficult to estimate particular synonymous and nonsynonymous substitutions of protein-coding genes (reviewed in Shearer et al. 2002). To overcome this limitation, Hellberg (2006) compared the molecular evolution of 630-bp mt cytochrome oxidase I (COI) DNA sequences of 18 populations of *Balanophyllia elegans*, a solitary coral species known from a population genetic study to have relatively high allozyme variations (Hellberg 1994, 1995). His result was consistent with the characteristics of slow evolution in anthozoan mt genomes, showing no variations but a low synonymous substitution rate (0.05% Ma^{-1}), found in *B. elegans* (Hellberg 2006), however, relative rates and evolutionary tempos between mt and nuclear genomes were not estimated, since allozymes are the summation of protein variations, which cannot be directly converted to nucleotide substitutions. Additionally, the noncoding regions of nuclear genomes, such as introns, were not estimated.

In this study, we examined the evolutionary tempos of coding and noncoding regions of both mt and nuclear genomes in the scleractinian genus *Acropora*. This is by far the largest extant genus of reef-building corals with over 113 valid species (Wallace 1999) and also one of the most widespread, spanning the Indian and Pacific Oceans and the Caribbean. The occurrence of up to 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) create major opportunities for interspecific hybridization and, hence, introgression (Willis et al. 1997). Because of these ecological and evolutionary characteristics, the molecular evolution of *Acropora* has been studied extensively (Wei et al. 2006; reviewed in Willis et al. 2006). Previous studies of interspecific divergences among *Acropora* species revealed rates of mtDNA sequence evolution two–three times slower than those of the single-copy minicollagen nuclear gene and its intron from the same species (Fukami et al. 2000; van Oppen et al. 2001). Further comparisons of rates of synonymous and nonsynonymous substitutions are needed to reveal the full landscape of differentiation in evolutionary tempos between mt and nuclear genomes in *Acropora*.

In order to survey nucleotide variations from nuclear protein-coding genes, we characterized the gene structure of the calmodulin (*CaM*)-encoding genea calcium (Ca^{2+})-binding protein which plays an important role in many cellular organization and signal transduction pathways (Friedberg and Rhoads 2001) in *Acropora muricata*. This showed that *A. muricata CaM* is a single-copy gene which possesses a four-intron structure, the ancestral organization for eumetazoans (Chiou et al. 2008). The exons and introns of *CaM* provided the molecular information necessary for comparing the evolutionary rates of the nuclear and mt genomes. In addition, published data of *Pax-C* intron were used in comparison as well. For the mt genome counterpart, we sequenced the intergenic spacer (*IGS*), the largest spacer spanning cytochrome *b* (*cytb*) and NADH 2 (*NAD2*) in the *Acropora* mt genome (van Oppen et al. 2001) for noncoding region comparisons and also used in published data on the *cytb* and mt control regions.

Materials and Methods

Sample Collection

The study used 48 species from 15 species groups including four major clades of *Acropora* (Wallace 1999) plus *A. pichoni*, *A.* cf *divaricata*, *A. dendrum*, *A. derawanensis*, and *A. kirstyae*, with four species of *Isopora* as the outgroup because *Isopora* is sister group to *Acropora*, shown in phylogenetic analyses using morphological, reproductive, and molecular characters (Wallace et al. 2007; Table 1). Samples of *A. valida* were from Penghu, Taiwan and *A. palmata* and *A. cervicornis* were from Panama; all other species were from the Great Barrier Reef of Australia or Indonesia, with voucher specimens deposited in the Museum of Tropical Queensland, Australia.

DNA Extraction, Amplification, Cloning, and Sequencing

Coral DNA was extracted from preserved *Acropora* branches using a plant genomic DNA extraction miniprep system (VIOGENE, Taipei). Small pieces of skeleton containing tissue (1–2 g) were ground into powder using liquid nitrogen. The extraction process followed the manufacturer's protocol. The extraction efficiency was examined with 1% LE Agarose (SAMBREX) gel electrophoresis in

Table 1 *Acropora* species groups, species, length of calmodulin and mitochondrial intergenic spacer characterized in this study, and the GenBank accession numbers

Species group	Species	*CaM* (bp)				GenBank	*IGS* (bp)	GenBank
		Exons	Intron 2	Intron 3	Total length	Accession no.	Total length	Accession no.
A. humilis A	*A. digitifera*	410	383–384	128–404	922–1197	EU534169–70	–	–
A. nasuta A	*A. cerealis*	–	–	–	–	–	410	EU533979
	A. valida	410	369–380	403–405	1184–1193	EU534165–68	410	EU534047
	A. nasuta	–	–	–	–	–	410	EU534032
	A. lutkeni	410	377	403	1190	EU534141–44	410	EU534023
A. divaricata A	*A. divaricata*	410	376	403	1189	EU534103–07	410	EU533985
	A. solitarynesis	–	–	–	–	–	410	EU534039
A. lovelli B	*A. bushyensis*	–	–	–	–	–	410	EU533975
A. cervicornis A	*A. palmata*	410	377	128	915	EU534133–40	410	EU533962–63
	A. cervicornis	410	380	128	918	EU534127–32	410	EU533960–61
A. muricata B	*A. grandis*	410	–	–	–	–	410	EU533994
	A. acuminata	410	375	128	913	EU534049–51	410	EU533969
	A. muricata	410	375	403	1188	EU534145–49	410	EU534030
A. rubusta B	*A. intermedia*	–	–	–	–	–	410	EU533965, EU533968
	A. polystoma	–	–	–	–	–	410	EU533964
	A. listeri	–	–	–	–	–	410	EU533966
A. selago C	*A. yongei*	–	–	–	–	–	410	EU534048
	A. tenuis	–	–	–	–	–	410	EU534046
	A. selago	410	378–379	555	1343–1344	EU534158–59	410	EU534035–38
A. florida C	*A. florida*	–	–	–	–	–	410	EU533993
	A. sarmentosa	–	–	–	–	–	410	EU534034
A. aspera C	*A. spicifera*	–	–	–	–	–	410	EU534041
	A. millepora	410	378–379	554	1342–1343	EU534160–63	410	EU534029
	A. spathulata	–	–	–	–	–	410	EU534040
	A. papillare	410	376	553	1340	EU534150–52	–	–
	A. pulchra	410	378–80	409–555	1197–1343	EU534153–57	410	EU533967
A. hyacinthus C	*A. anthocercis*	410	369–378	409–416	1182–1198	EU534052–55	410	EU533970
	A. hyacinthus	–	–	–	–		410	EU534002
	A. cytherea	410	379–380	403	1192–1193	EU534120–23	–	–
	A. microclados	–	–	–	–		410	EU534024–25
	A. indonesia	410	374–380	403	1187–1193	EU534116–19	410	EU534003
A. latistella C	*A. nana*	–	–	–	–	–	410	EU534031
	A. aculeus	410	379–383	128–403	915–1193	EU534108–12	–	–
A. horrida D	*A. horrida*	–	–	–	–	–	410	EU533998–4001
	A. microphthalma	410	374–376	403	1187–1189	EU534113–15	410	EU534026–28
	A. abrolhosensis	–	–	–	–	–	410–411	EU533959
A. elegans D	*A. elegans*	–	–	–	–	–	410	EU533988–90
A. loripes D	*A. granulosa*	410	376–382	403–406	1189–1195	EU534078–84	410	EU533995–97
	A. jacquelineae	410	382–387	403–414	1195–1211	EU534072–77	410	EU534012
	A. loripes	410	369–388	403	1181–1202	EU534092–98	409–410	EU534019–22
	A. chesterfield	410	378–384	403	1187–1193	EU534066–68	409–410	EU533981–82
	A. caroliniana	410	383–384	403–406	1193–1195	EU534069–71	–	–
A. echinata D	*A. longicyathus*	–	–	–	–	–	410	EU534017–18
	A. elseyi	410	380–390	403	1193–1203	EU534087–91	410	EU533991–92
	A. carduus	–	–	–	–	–	410	EU533976–78
	A. subglabra	410	376–380	403	1188–1192	EU534061–65	410	EU534042–45
	A. echinata	–	–	–	–	–	410–411	EU533986–87
	A. batunai	–	–	–	–	–	410	EU533971–74
	A. pichoni	410	369	403	1182	EU534056–60	410	EU534033
	A. cf divaricata	410	380	403	1193	EU534124–26	410	EU533980
	A. dendrum	410	377	128	915	EU534099–102	410	EU533983
	A. derawanensis	–	–	–	–	–	410–411	EU533984
	A. kirstyae	–	–	–	–	–	410	EU534013–15
	A. sp.	–	–	–	–	–	410	EU534016
Isopora	*I. brueggemanni*	410	345–353	403–462	1187–1234	EU534171–72, EU534177–80	410	EU534004–5
	I. palifera	410	364	462	1236	EU534173	410	EU534010–11
	I. togianensis	410	364	456–460	1230–1234	EU534181–83	410	EU534008–9
	I. cuneata	410	364	461–462	1235–1236	EU534174–6, EU534184	410	EU534006–7

A, B, C, and D are the clades classified in Wallace (1999)
CaM Calmodulin, *IGS* intergenic spacer, – data not available

one time Tris-acetate-EDTA (TAE) buffer. A full-length cDNA clone coding for the *CaM* gene of *A. muricata* and its genomic organization were isolated and characterized (Chiou et al. 2008). A Southern blot assay indicated that the *A. muricata CaM* gene is a single copy. A primer pair, CaME×23F: 5′-CTG ATC AAC TTA CAG AGG AAC A-3′ and CaME×23R: 5′-GTT GAC TTG ACC ATC AC CGT CA-3′, was designed to amplify the region spanning exons 2 to 4 of the *CaM* gene. Polymerase chain reaction (PCR) profiles for *CaM* were one cycle of 94°C for 3 min; followed by 30 cycles of 94°C for 30 s, 50°C for 45 s, and 72°C for 1 min 45 s; then 1 cycle of 72°C for 5 min. For *IGS*, the profile was one cycle of 94°C for 3 min; followed by 30 cycles of 94°C for 30 s, 50°C for 1 min, and 72°C for 1 min 30 s; then one cycle of 72°C for 10 min. PCR reactions consisted of one time PCR buffer (Invitrogen), 3 mM $MgCl_2$ (Invitrogen), 0.2 mM of dNTP (Promega), 0.04 μM of each primer (Mission Biotech), 1% of dimethylsulfoxide (Merck), 0.02 unit *Taq* DNA polymerase (Invitrogen), 10 ng coral DNA, and double-distilled H_2O to 50 μl. The PCR products were electrophoresed in 1% TAE-agarose gels, using one time TAE buffer to assess their appropriate sizes. Then the PCR products were cloned into a TA cloning system (Promega) and transformed into *Escherichia coli* DH5α (Promega). At least three positive colonies for each species were sequenced on an ABI PRISM 3700 DNA Analyzer (Applied Biosystems) with M13 forward and reverse primers in two directions. The mitochondrial intergenic spacer (*mtIGS*) spanning between the *cytb* and *NADH* 2 was amplified and directly sequenced following the protocols described in Tseng et al. (2005). DNA sequences obtained from this study have been submitted to the GenBank with their accession numbers listed in Table 1.

Data Processing and Analysis

Sequences of insert PCR fragments were assembled and manually proofread with the program SeqMan (DNASTAR). *Acropora Pax-C* intron sequences (van Oppen et al. 2001), mt control region sequences (van Oppen et al. 2001), and cytochrome *b* sequences (van Oppen et al. 1999; Fukami et al. 2000) were obtained from GenBank for further analyses and comparisons.

Whole DNA sequences were aligned using MegAlign (DNASTAR) and were manually readjusted in MacClade (Sinauer Associates). The *p*-distances of within and among *Acropora* species, within *Acropora*, and *Acropora* vs. *Isopora* were calculated using MEGA 3.1 (Kumar et al. 2004). And the pairwise distances for the noncoding region under the Kimura 2-parameter model (Kimura 1980) were determined using MEGA 3.1. The numbers of substitutions per synonymous and nonsynonymous sites (K_S and K_A, respectively; Nei and Gojobori 1986) over protein-coding regions were analyzed using DnaSP 4.10.9 (Rozas and Rozas 1999). The numbers of transitions and transversions for noncoding regions were analyzed using MEGA 3.1 (Kumar et al. 2004). The range of evolution rates (% Mya^{-1}) from the predicted divergence time of 3.0 Mya, as the date of final closure of the Isthmus of Panama (Keigwin 1982; Duquecaro 1990; Coates and Obando 1996), to 12 Mya, the closure of Tethys sea (Bellwood et al. 2002), were used for the Caribbean and Indo-West Pacific species of *Acropora* in this study. The mt*IGS* region was highly conserved containing little information for phylogenetic construction. Phylogenetic analyses were performed using only the intron 2 and 3 regions of *CaM* gene. The *CaM* intron sequences were aligned using MEGA 4.0 (Tamura et al. 2007) and manually edited. The aligned introns that contain a region of large indels between the position 727 and position 985 was excluded from the following phylogenetic analyses. In total, 553 nucleotides of introns were used for tree construction based on the maximum likelihood (ML) and Bayesian analyses. Maximum likelihood tree were constructed using online server MultiPhyl (Thomas et al. 2007). Model was generated by the Modelgenerator (Thomas et al. 2006) and TVM+G model was shown as the best model. One hundred bootstrapping replicates were used to evaluate the statistic support of the ML tree. Bayesian analysis was performed using MrBayes 3.12 (Ronquist and Huelsenbeck 2003). The Markov chain Monte Carlo search was run four chains for 650,000 generations. Trees sampled every 100 generation and first 1,000 trees were discarded.

Results

Molecular Characterization of *CaM* and mt*IGS* and Genetic Distances

A PCR fragment containing the partial exon 2 and exon 4, the full length of exon 3, and two introns spanning the calmodulin-encoding gene was amplified for 27 species of *Acropora* and four species of *Isopora* (Table 1). The total length of exons amplified in the *CaM* was 410 bp. The intron length was highly variable with the shortest length of 128 bp and the longest of 555 bp both found in intron 3. The large fragment of insertions and deletions (indels) in the introns caused distinct length variation among the PCR products ranging from 913 bp in *A. acuminata* to 1,344 in *A. selago* (Table 1). The mitochondrial intergenic spacer was highly conserved at 409 and 411 bp in length. The base compositions of nuclear *CaM* introns and mt*IGS* were biased toward AT with 66.9% for *CaM* introns and 62.5%

for mt*IGS*. No apparent base composition bias was observed for *CaM* exons (56.4%).

Interspecific genetic distances (*p*-distances) were larger than those at the intraspecific level in all pairwise comparisons for both nuclear and mt genomes of *Acropora* (Table 2). The nuclear introns of both *CaM* and *PaxC* were two–four times more diverse than the exons. The interspecific genetic distance of the *CaM* exon (1.12) was about 2.87 times that of the mt *cytb* (0.39), which is 2.43 times that of *mtIGS* but 3.56 times slower than that of mt control region (3.98). In contrast, the nuclear *CaM* introns (3.19) evolved at a rate similar to that of the mt control region but 6.93 times faster than that of *mtIGS* and 8.18 times faster than that of mt *cytb*. Similar patterns were observed when comparing the *PaxC-intron* with the mt regions (Table 2). Transitional (ts) substitutions accumulated faster than transversional (tv) substitutions in all pairwise comparisons in both nuclear and mitochondrial noncoding regions, with the largest mean ts/tv ratio being 5 in *CaM* introns and the smallest being 1.15 in the *PaxC* intron (Fig. 1).

Evolutionary Rate

Separation of the Caribbean and Indo-West Pacific marine biota can be calibrated using either the closure of the Isthmus of Panama at 3 mya (Knowlton et al. 1993) or closure of the Tethyan Sea at 12 mya (Wallace 1999). Evolutionary rates based on these two calibrations are listed in Table 3. As reflected by genetic distances, evolutionary rates of nuclear introns were faster than those of exons and mt regions, with the exception of the mt control region. Overall, the evolutionary rate of nuclear (n)DNA was two–five times faster than that of mtDNA (Table 3). While the nonsynonymous substitution rate (K_A) evolved at similar tempos in *CaM* exons (0.006–0.023% Mya^{-1}) and *cytb* (0.003–0.013% Mya^{-1}), the synonymous substitution rates (K_S) of *CaM* exons (0.367–1.467% $Mya^{-1)}$ were 4.8 times faster than those of *cytb* (0.076–0.303% Mya^{-1}; Table 4; Fig 2).

CaM Introns Phylogeny

Figure 3 shows the ML tree based on the *CaM* intron 2 and intron 3. Bayesian analysis yielded a similar topology to that of ML tree. Two major clades can be identified with 100% bootstrapping and 100 Bayesian probability supports. Clade I is small and can be divided in two subclades. Subclade I contains only the two Caribbean *Acropora—A. palmata* and *A. cervicornis.* This is the only cluster which reflects a species group based on the morphological characters (Wallace 1999). Subclade II in clade I contains *A. aculeus*, *A. accuminata*, *A. dendrum*, and *A. digitifera.* Clade II contains the remaining *Acropora* species used in this study. Within clade II, most species contain diverse alleles which cause the *CaM* intron phylogeny to be not monophyletic, except for *A. jacquelineae* and *A. divaricata*. Note that the sample sizes were small for each species so that polyphyletic patterns could possibly be found for these species with the addition of new samples. Overall, species in clade II did not show concordance with groupings based on the morphological species groups.

Discussion

Our study provides parallel surveys of DNA variations in coding and noncoding regions and calibrated rates of synonymous substitutions of mt and nuclear genomes of

Table 2 Intraspecific, interspecific, and intergeneric mean *p*-distances (%) and ranges (in parentheses) for coding and noncoding regions of *Acropora* nuclear and mitochondrial genomes

p distance (%)	nDNA					MtDNA		
	CaM exons	*CaM* intron2	*CaM* intron3	*CaM* intron2+ intron3	*Pax-C*[a] intron	*cytb*[b]	*CR*[c]	*IGS*
Acropora								
Intraspecific	0.44 (0–1.6)	0.76 (0–5.3)	0.81 (0–11.5)	1.15 (0–8.4)	1.75 (0–8.9)	–	0.93 (0–4.5)	0.21 (0–0.6)
Interspecific	1.12 (0–3.2)	3.07 (0–6.9)	3.53 (0–11.9)	3.19 (0–22.2)	5.09 (0–12.6)	0.39 (0–0.8)	3.98 (0.1–7.2)	0.46 (0–1.7)
Intergeneric								
Acropora vs. Isopora	4.3	21.3	44.1	25.3	21.5	1.8	17.2	3.1

– Data not available

[a] *Acropora Pax-C* intron sequences (GenBank accession nos. AF344337–AF344353, AF3443355–AF3443379, AF3443381–AF3443391, AF3443394–AF3443398, AF344400–AF344408, and AF344410–344423) published in van Oppen et al. (2001)

[b] *Acropora* and *Isopora* cytochrome *b* sequences (GenBank accession nos. AF099650–AF099656, AF099658–AF099659, AB033178–AB033179) published in van Oppen et al. (1999) and Fukami et al. (2000)

[c] *Acropora* mt control region (GenBank accession nos. AY02618–AY026460) published in van Oppen et al. (2001)

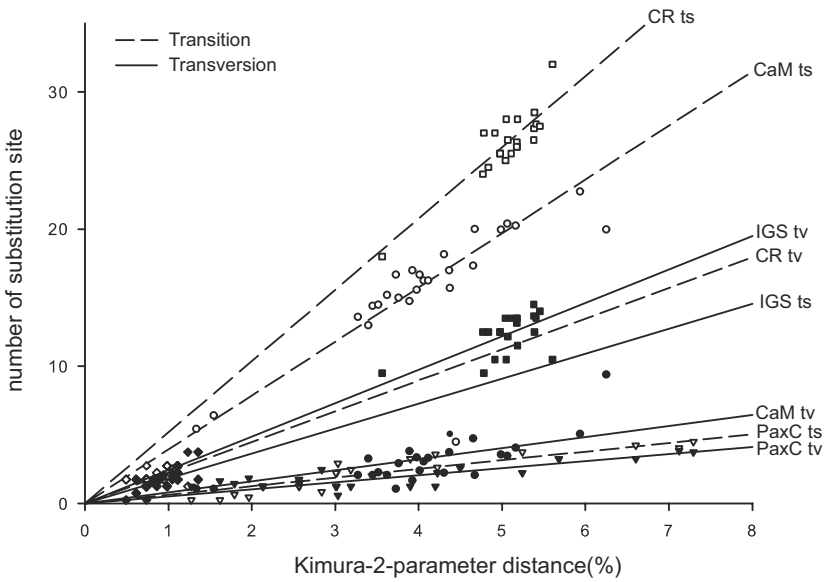


Fig. 1 Number of transitions (*filled circles*) and transversions (*empty circles*) plotted against Kimura-2-parameter (K2P) pairwise distances for the noncoding region (intron II and intron III) of *CaM*. Sequences of the *Pax-C* intron, mitochondrial (mt) control region, and cytochrome *b* are from GenBank that were published in van Oppen et al. (1999, 2001; GenBank accession nos. AF344337–AF344353, AF3443355–AF3443379, AF3443381–AF3443391, AF3443394–AF3443398, AF344400–AF344408, AF344410–344423; AY02618–AY026460; AF099650–AF099656, and AF099658–AF099659). Regression lines between transition (*dash line*) and transversion (*solid line*) against K2P distances for every coding and noncoding DNA sequences were indicated

scleractinians, showing not only that coral mt genomes evolved two–five times more slowly than their nuclear genomes but also that the rate difference of synonymous substitutions between these two genomes was similar to that of flowering plants (Wolfe et al. 1987; Palmer et al. 2000).

Slow Evolution and Low-Level Variation of mtDNA

Low mitochondrial variation has been noted in previous studies, in which genetic variations in coding regions of coral mtDNA show either no variation (Snell et al. 1998; van Oppen et al. 1999; Chen and Yu 2000; Fukami et al. 2000; Hellberg 2006) or very little variation restricted to nonsynonymous sites (Medina et al. 1999). A more striking pattern of slow evolutionary tempos was observed among three members of the *Montastraea annularis* complex of only 0.03–0.04% Ma^{-1} for their complete mt genomes (Fukami and Knowlton 2005). The low variability of the coral mt genome limits its applicability for phylogeographic surveys and phylogenies of closely related species. However, the coding genes, *cytb* and *cox1*, have successfully been utilized to infer the deep divergences of Atlantic and Pacific scleractinian families and genera (Fukami et al. 2004b).

Such a low level of phylogenetic variation in the coral mt genome is even distinct when compared to that of the nuclear genome. While vertebrate mtDNA generally is evolving up to ten times faster than single-copy genes, invertebrates exhibit similar evolutionary rates between nDNA and mtDNA (reviewed in Li 1997). In contrast, previous studies and ours indicated that coral nDNA is evolving two–five times faster than mtDNA (Fukami et al. 2000; van Oppen et al. 2001; reviewed in Shearer et al. 2002). This scenario is also supported by the comparison between allozyme heterozygosity and mtDNA sequence divergence in *Banophyllia* and *Tubastrea* corals (Hellberg 2006), microsatellites (Baum et al. 2005), and amplified fragment length polymorphisms (Fukami et al. 2004a). With the comparison between *Acropora* mt *cytb* and nuclear *CaM* exons in this study, we confirm that the low

Table 3 Divergence rate (% Mya^{-1}) of nuclear (n)DNA and mitochondrial (mt)DNA coding and noncoding regions based on the calibrations of formation of the Panama Isthmus (3 mya) and the closure of the Tethyan Sea (12 mya)

DNA region	nDNA			MtDNA		
	CaM exons	*CaM* introns	*Pax-C* intron	*cytb*	*IGS*	*CR*
Divergence rate % Mya^{-1}	0.081–0.325	0.178–0.709	0.138–0.550	0.021–0.083	0.036–0.143	0.237–0.948

Table 4 Numbers of synonymous (K_S) and nonsynonymous (K_A) substitutions per site and the estimated rate (% Mya^{-1}) of nonsynonymous substitutions per million years for nuclear (n)DNA *CaM* and mitochondrial (mt)DNA *cytb* of *Acropora*

Acropora	Site	L_S	K_S ×100	L_A	K_A ×100	K_A/K_S	K_S rate (% Mya^{-1})[a]	K_A rate (% Mya^{-1})[a]
CaM	408	84.08	8.80	323.92	0.14	0.016	0.367–1.467	0.006–0.023
cytb[a]	954	231.74	1.82	722.26	0.08	0.044	0.076–0.303	0.003–0.013

L_S and L_A are the numbers of synonymous and nonsynonymous sites, respectively
T Divergent time
[a] (K_S or $K_A/2T$), where T is 3.0–12.0 Mya of the divergence time for Caribbean and Indo-West Pacific species of *Acropora*

variation in both coding and noncoding DNA appears to be a characteristic of the coral mt genome.

Our estimation of the rate difference between nDNA and mtDNA might be still low. In this study, the *CaM* and its introns served as the nuclear candidate for the comparison. The *CaM*, a calcium-binding protein, exists widely in eukaryotic organisms and is one of the ancient genes that has been highly conserved at the amino acid level in eukaryotes (Friedberg and Rhoads 2001). Thus, our estimation of the nDNA evolutionary rate might have been conservative. In contrast, a recent study indicated that *Acropora* possesses extremely highly heterogeneity in its ribosomal RNA genes (Wei et al. 2006), suggesting that the faster-evolving genes remain to be scanned from the nuclear genome. Once these genes are found, the rate difference of nDNA compared to that of mtDNA is expected to increase. An available EST library for *A. millepora* (Kortschak et al. 2003) can serve this purpose.

Low Rates of Synonymous Substitution in the Coral mt Genome

The slow rate of synonymous substitutions (K_S) has been documented in the dendrophilid corals for two genera, *Tubastrea* and *Balanophyllia*, with independent fossil records extending back over 50 million years showing the rate of COI K_S of 0.056% Mya^{-1} (Hellberg 2006). *Acropora cytb* K_S (0.076% Mya^{-1}) is compatible with that of dendrophilid COI K_S if divergence of the Caribbean and Indo-West Pacific *Acropora* is calibrated by the closure of the Tethyan before 12 Mya. It increases to 0.303% Mya^{-1} if the divergence time is calibrated by the closure of the Isthmus of Panama at 3 Mya (van Oppen et al. 1999). Although there is a four-fold difference in these two calibrations, *Acropora cytb* K_S is still 50 to 100 times slower than those reported for most animals (Govindarajan et al. 2005; Hellberg 2006). In contrast, the nonsynon-

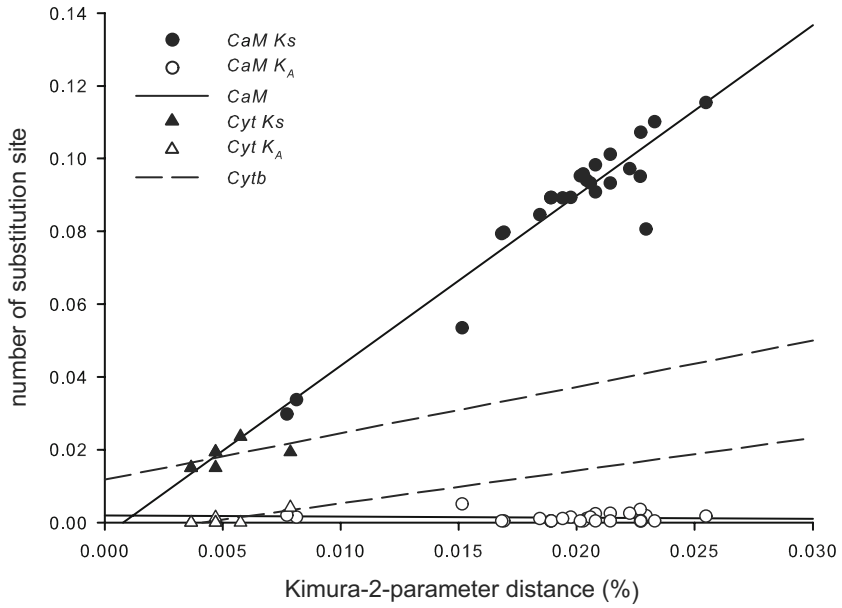
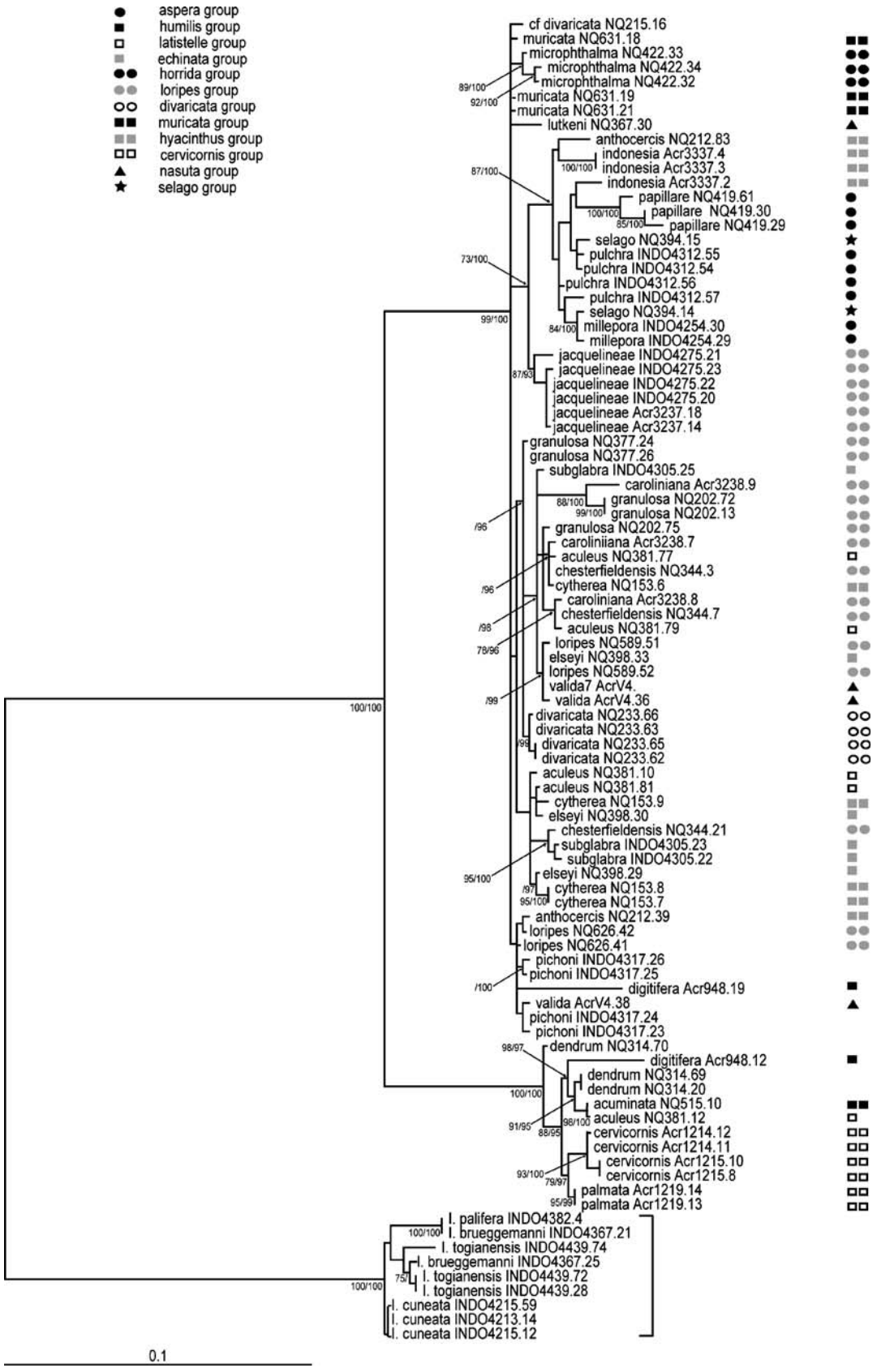


Fig. 2 Number of synonymous (K_S) and nonsynonymous (K_A) substitutions per site for *CaM* and *cytb* plotted against Kimura-2-parameter pairwise distances. *Circles* with a rigid regression line and *triangles* with a dotted regression line are for *CaM* and *cytb*, respectively. *Filled symbols* are K_S, and the *empty ones* are K_A. Regression lines of *cytb*'s K_S and K_A (*dash line*) and *CaM*'s K_S and K_A (*solid line*) against K2P distances were indicated


aspera group
humilis group
latistelle group
echinata group
horrida group
loripes group
divaricata group
muricata group
hyacinthus group
cervicornis group
nasuta group
selago group
cf divaricata NQ215.16
muricata NQ631.18
microphthalma NQ422.33
microphthalma NQ422.34
microphthalma NQ422.32
muricata NQ631.19
muricata NQ631.21
lutkeni NQ367.30
anthocercis NQ212.83
indonesia Acr3337.4
indonesia Acr3337.3
indonesia Acr3337.2
papillare NQ419.61
papillare NQ419.30
papillare NQ419.29
selago NQ394.15
pulchra INDO4312.55
pulchra INDO4312.54
pulchra INDO4312.56
pulchra INDO4312.57
selago NQ394.14
millepora INDO4254.30
millepora INDO4254.29
jacquelineae INDO4275.21
jacquelineae INDO4275.23
jacquelineae INDO4275.22
jacquelineae INDO4275.20
jacquelineae Acr3237.18
jacquelineae Acr3237.14
granulosa NQ377.24
granulosa NQ377.26
subglabra INDO4305.25
caroliniana Acr3238.9
granulosa NQ202.72
granulosa NQ202.13
granulosa NQ202.75
caroliniiana Acr3238.7
aculeus NQ381.77
chesterfieldensis NQ344.3
cytherea NQ153.6
caroliniana Acr3238.8
chesterfieldensis NQ344.7
aculeus NQ381.79
loripes NQ589.51
elseyi NQ398.33
loripes NQ589.52
valida7 AcrV4.
valida AcrV4.36
divaricata NQ233.66
divaricata NQ233.63
divaricata NQ233.65
divaricata NQ233.62
aculeus NQ381.10
aculeus NQ381.81
cytherea NQ153.9
elseyi NQ398.30
chesterfieldensis NQ344.21
subglabra INDO4305.23
subglabra INDO4305.22
elseyi NQ398.29
cytherea NQ153.8
cytherea NQ153.7
anthocercis NQ212.39
loripes NQ626.42
loripes NQ626.41
pichoni INDO4317.26
pichoni INDO4317.25
digitifera Acr948.19
valida AcrV4.38
pichoni INDO4317.24
pichoni INDO4317.23
dendrum NQ314.70
digitifera Acr948.12
dendrum NQ314.69
dendrum NQ314.20
acuminata NQ515.10
aculeus NQ381.12
cervicornis Acr1214.12
cervicornis Acr1214.11
cervicornis Acr1215.10
cervicornis Acr1215.8
palmata Acr1219.14
palmata Acr1219.13
l. palifera INDO4382.4
l. brueggemanni INDO4367.21
l. togianensis INDO4439.74
l. brueggemanni INDO4367.25
l. togianensis INDO4439.72
l. togianensis INDO4439.28
l. cuneata INDO4215.59
l. cuneata INDO4213.14
l. cuneata INDO4215.12
0.1

◀ **Fig. 3** Maximum likelihood tree using TVM+G model of the *CaM* introns. Value before the *slash* on the branch indicated the bootstrap values (1,000 replicates) and values below 70 was not shown. Value after the *slash* on the branch indicated the credibility values of Bayesian method and value below 95 was not shown

ymous substitution (K_A) of coral mtDNA (dendrophilids, 0.019% Mya^{-1}; *Acropora*: 0.003–0.013% Mya^{-1}) is similar to those of other animals and plants (0–0.056% Mya^{-1}; Hellberg 2006). In a summary by Hellberg (2006), the low synonymous substitution rate in the mt genome evolved in two different modes: one is slow relative to nDNA and with little bias toward transitions, while the other is faster relative to nDNA and often exhibits a high-transition bias. The mt genomes of flowering plants, fungi, sponges, and anthozoans belong to the former mode, and the rest of the animals belong to the latter mode. The direction of the rate of evolution always switches from the slow to the fast mode and has occurred at least four times, twice in flowering plants, once in cnidarians, and once in bilateral animals (Hellberg 2006).

Constraints on codon usage in mitochondria from the reduced rate of synonymous substitutions can be explained by constraints on synonymous codon choices (Sharp and Li 1987). Nevertheless, constructions for the low variation of mitochondrial noncoding regions (about 3.8 times less than nDNA in this study) are intriguing. The lack of variation and the slow divergence rate in coral mitochondria are probably due to anthozoans' mitochondrial functionally mismatched repair system (Brown et al. 1982; Clayton 1982; Moritz et al. 1987; Pont-Kingdon et al. 1998) and functional constraints, which influence and decrease the substitution rate and the accumulation of mitochondrial genomic mutations. Several factors may also play roles in influencing the mitochondrial nucleotide substitution rate such as the metabolic rate (Martin et al. 1992), the base composition of silent codon positions (Wolfe et al. 1987), the effective population size, and the generation time (Wu and Li 1985; Li et al. 1987). The small effective population size of mtDNA genes may have suffered a bottleneck resulting in the founder effect (Wright 1931; Nei et al. 1975), which would lead to the phenomenon of low variations within the mitochondrial gene especially with the long generation time of corals. This scenario is exemplified by *Tubastrea coccinea* collected from the Hawaii and Caribbean that their *coxI* gene differs by only a single nonsynonymous substitution (Hellberg 2006). The Caribbean *T. coccinea* might represent a recently introduced species from the Pacific (Cairns 2000). This can also explain the low genetic diversity of mtDNA and the maternally inherited and haploid genome, relative to nDNA diversity, in the three Caribbean *Acropora* species, which might have suffered a bottleneck since last isolation at least 3 Mya due to the final closure of the Isthmus of Panama (Coates and Obando 1996). Nevertheless, this is unlikely to be the major explanation for the wide range of *Acropora* taxa in the Indo-Pacific.

Phylogenetic Inference of *CaM* Introns

Phylogenetic analyses using *CaM* intron DNA sequences produced two major clades for *Acropora*, one contained two Caribbean species, *A. cervicornis* and *A. palmata*, and four other Indo-Pacific species, and the other clades included rest 26 species. Most of the species examined in the present study were not included in the two published phylogenies by minicollagen intron (Hatta et al. 1999; Fukami et al. 2003) and *PaxC46/47* intron (van Oppen et al. 2001), thus providing little information for comparison. Nevertheless, reproductive isolation by spawning time and divergence of the Caribbean vs. Indo-Pacific *Acropora* may be still elucidated further. First, the basal group of the *Pax-C*46/47 intron tree was composed of *A. longicyathus*, *A. intermedia*, and *A. tenuis* (van Oppen et al. 2001). These three species have been proposed to be the early spawning species which provide the reproductive isolation to the other *Acropora* (van Oppen et al. 2001). Fukami et al. (2003) also observed *A. tenuis*, *A. donei*, *A. yongi*, *A. austere*, *A. verweyi*, and *A. vaughani* as the early spawners using minicollagen intron. Unfortunately, these species were not assessed by the *CaM* intron, thus, their possible positions in the *CaM* phylogeny remains uncertained. Future examining *CaM* intron of these species provides the opportunity to examine the scenario reproductive isolation through differentiation of spawning. Second, the position of the Caribbean species is quite different between the *CaM* intron tree and that of the *PaxC46/47* intron. While the Caribbean *Acropora* stand at the basal position of the genus *Acropora* in *CaM* intron tree, they were clustered with *A. cytherea* in the third clade in the *Pax-C46/47* intron tree (van Oppen et al. 2001). Interestingly, the basal position of the Caribbean *Acropora* is supported by the phylogeny of mitochondrial control region (van Oppen et al. 2001). Although incongruence between nuclear and mitochondrial gene trees are ascribed to the incomplete lineage sorting of markers, some implications of effect of major vicariant event on the evolution of *Acropora* can still be elucidated.

Similar Tempos of Mitochondrial vs. Nuclear Evolutionary Rates between Acropora and Flowering Plant Genomes: Similar Life History Traits between *Acropora* and Plants

Although mt genomes of corals (and anthozoans in general) have group I introns and intergenic spacers like those in some plants and fungi, the mt genomes of corals and

flowering plants differ in several aspects. Firstly, the anthozoan mt genome, like that of most other animals, is small (16–20 kb; van Oppen et al. 1999; Fukami and Knowlton 2005; Tseng et al. 2005; Medina et al. 2006; Chen et al. 2008), while the plant mt genome is relatively large (40–250 kb; Li 1997). Secondly, the coral mt genome contains relatively consistent gene numbers (13 protein-coding, two rRNA, and two tRNA genes) and arrangements (Medina et al. 2006; Chen et al. 2008), while plant mt genomes undergo frequent rearrangements and contain large numbers of potential genes: for example, the liverwort (*Marchantia polymorpha*) contains 94 possible genes (Li 1997). Nevertheless, the synonymous substitution rate of the *Acropora* mt *cytb* gene is evolving 4.8 times more slowly than *CaM* exons in the nuclear genome, a tempo similar to that reported in comparisons of flowering plant mt and nuclear genomes (Wolfe et al. 1987; reviewed in Li 1997), where the synonymous substitution rate of the mt *cytb* gene is at least five times slower than *CaM* exons in the nuclear genome. Plants mt genome was documented evolving slowly in DNA sequences (Palmer and Herbon 1988). Wolfe et al. (1987) showed synonymous substitution rate in plants mtDNA is slower than in mammalian mt DNA and only as half as faster in plants nuclear DNA. Substitution rate in plants mtDNA slower than nuclear DNA has been confirmed in subsequent studies (Gaut 1988; Muse 2000).

This coincidence is unexpected and might reflect a similarity in life history traits between *Acropora*, or corals in general, and plants. Recent studies have confirmed that hermaphroditic coral species with broad dispersal potentials are either completely or almost completely self-incompatible, while species with limited dispersal potentials have high, but variable, rates of self-fertilization (Heyward and Babcock 1986; Black et al. 1991; Miller and Mundy 2003). This interspecific variation in coral mating systems is similar to that found in terrestrial plants (reviewed in Carlon 1999). The influence of life history traits on the rate of molecular evolution is exemplified by a correlation between body size (i.e., metabolic rate) and rate of molecular evolution in vertebrates: smaller species tend to have faster rates of molecular evolution (Martin and Palumbi 1993). However, a recent study showed no evidence of any influence of body size on invertebrate substitution rates, and it was concluded that the vertebrate body size effect is a special case, which cannot simply be extrapolated to the rest of the animal kingdom (Thomas et al. 2006). The scenario of correlating mating systems and rates of molecular evolution in corals can be further tested by examining more genes across different taxa. The challenge now is to consider whether this similarity is coincidental or actually reflects genuine similarities in genetically programmed life functions of corals and plants. Both have modular growth and lifestyle, in which growth, reproduction and many physiological functions are performed by repeated and interconnected modules. Coordination among modules is an important aspect of the way that geometry and architecture of both plants and corals are achieved, and physiological processes are maximized (Dauget 1991; Donoghue 2005). In *Acropora*, in particular, the development of an axial mode of growth leading to various shape possibilities has been noted (Dauget 1991; Vermeij 1973). Plants and corals share some of the features such as this, which have been described as "key innovations", leading to enhance evolutionary rates in higher taxa (Vermeij 1973).

In conclusion, our study provides comparative DNA sequences of protein-coding and noncoding regions of coral mt and nuclear genomes and supports evidence from other coral studies that the mt genome is evolving at a slower tempo compared to the nuclear genome. This is similar to findings in plant genomes that nuclear genomes are evolving the fastest, followed by chloroplast genomes, and last mt genomes. Furthermore, the evolutionary tempos are also similar between corals and plants in that nuclear genomes are evolving at least five times faster than the mitochondrial counterparts.

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