The complete mitochondrial genomes of needle corals, *Seriatopora* spp. (Scleractinia: Pocilloporidae): An idiosyncratic *atp8*, duplicated *trnW* gene, and hypervariable regions used to determine species phylogenies and recently diverged populations

Chienhsun Chen ^{a,b}, Chang-Feng Dai ^a, Sakanan Plathong ^c, Chih-Yung Chiou ^b,
Chaolun Allen Chen ^{a,b,*}

^a Institute of Oceanography, National Taiwan University, Taipei 106, Taiwan

^b Research Center for Biodiversity, Academia Sinica, Nankang, Taipei 11529, Taiwan

^c Department of Biology, Prince of Songkla University, Hat Yai, Songkla, Thailand

Received 29 November 2006; revised 11 September 2007; accepted 16 September 2007

Available online 21 September 2007

Abstract

Complete DNA sequences were determined for the mitochondrial (mt) genomes of the needle corals, *Seriatopora caliendrum* (17,011 bp) and *S. hystrix* (17,060 bp). Gene arrangement of the *Seriatopora* mt genomes is similar to the 14 currently published scleractinian mitogenomes with three unusual features, including an idiosyncratic *atp8*, a duplicated *trnW* (*tRNA*^{TRP}), and a putative control region located between *atp6* and *nad4*. *Atp8*, located between duplicate *trnW* genes, showed relatively low amino acid similarity (25.6–34.6%) with those of published scleractinian corals. A reverse-transcription polymerase chain reaction confirmed the transcription of this novel *atp8* gene in *Seriatopora*. A duplicated *trnW* was detected in the region close to the *cox1* gene and shares the highly conserved primary and secondary structure of its original counterpart. The intergenic spacer between *atp6* and *nad4*, which contains several distinct repeated elements, is being designated as the putative control region in the *Seriatopora* mt genomes. Evaluation of the molecular evolution of several protein-coding genes and intergenic spacers showed 3- to 4-fold higher divergence rates among populations or between species than those published for scleractinian mt genomes. This study not only successfully revealed the phylogenies of *S. hystrix* and *S. caliendrum* from the West Pacific Ocean by mtDNA, but also highlighted the potential utilities of mt hypervariable regions in phylogenetic construction below the species level for *Seriatopora*.

© 2007 Published by Elsevier Inc.

Keywords: Complete mitochondrial genomes; *Seriatopora*; Idiosyncratic ATP8; Control region; tRNA duplication; Scleractinia

1. Introduction

In the last decade, attempts to sequence complete mitochondrial (mt) genomes from diverse taxa have challenged the concept of mitogenomic evolution of several lower invertebrates (Boore, 1999; Burger et al., 2003; Dellaporta

et al., 2006). Increasing evidence has shown that the mt genomes of many invertebrates do not agree with the standard knowledge of mitochondrial characterization (reviewed in Wolstenholme, 1992). For example, mt genomes of scleractinian corals include extensive intergenic spacers between genes, only two of the typical 22 tRNA genes, and contain a group I intron that bisects the NADH dehydrogenase subunit 5 gene (*nad5*) (Fukami and Knowlton, 2005; Medina et al., 2006; Tseng et al., 2005; van Oppen et al., 2002). A smaller subset of Scleractinia also contain a group I intron in the cytochrome oxidase I gene

* Corresponding author. Address: Research Center for Biodiversity, Academia Sinica, Nankang, Taipei 11529, Taiwan. Fax: +886 2 2785 8059.

E-mail address: cac@gate.sinica.edu.tw (C.A. Chen).

(*cox1*), which may contain signals indicative of a homing endonuclease gene (Fukami et al., 2007; Goddard et al., 2006). Despite these unusual features, evolutionary rates in corals or anthozoans in general are considered to be relatively slow in comparison to those of higher animals (Hellberg, 2006; Shearer et al., 2002). A phylogenetic analysis showed that the evolutionary rate of the *Acropora* cytochrome b gene (*cob*) is 10–20 times slower than the standard molecular clock rate of 2% per million years (Ma^{-1}) (van Oppen et al., 1999). Among sibling species of the *Montastraea annularis* complex, the evolutionary rate of their mitogenomes is only 0.03% Ma^{-1} (Fukami and Knowlton, 2005). This value corresponds to only 25 variable sites across 16,134 bp, 16 of which occurred in one of the three species analysed. Fossil record data places the divergence of the *Montastraea annularis* complex at approximately 3–4 million years ago (Fukami and Knowlton, 2005; Pandolfi et al., 2002). Most surprisingly, Tseng et al. (2005) noted invariance in DNA sequences between corresponding mt protein-coding genes of *Anancopora matthai* and *Montipora cactus* (0–0.91% differences in p-distances). The most divergent mt region (the 3rd intergenic spacer) between these two genera of the Acroporidae was only 1.12% in pairwise distances for over 30 million years of divergence time (Frost, 1977; Gregory and Trench, 1916; Tseng et al., 2005).

The observed slow evolution may limit the applications of mtDNA sequences to the elucidation of species phylogenies and population genetics of scleractinian corals. Previous studies of population genetics in coding regions of mtDNA revealed little to no variation among conspecifics; even geographically distant or potentially isolated populations are invariant (Hellberg, 2006; Medina et al., 1999; Snell, 2000; Snell et al., 1998). For example, there was no variation within *cox1* among populations over 3000 km apart in the range of the coral, *Ballanophyllia elegans* (Hellberg, 2006). Watanabe et al. (2005) examined the polymorphisms of the *cob-nad2* intergenic spacer in *Galaxea fascicularis* in Ryukyu Archipelago, and observed significant differences in the haplotype frequencies among three sampling sites. Their result, however, may be explained by sympatric occurrence of two cryptic species rather than genetic differentiation among the three populations. Whether this conclusion is applicable to all scleractinian coral remain uncertain, since patterns of evolutionary rates can vary greatly among genes or regions across different coral lineages. Further evaluation of complete scleractinian mt genomes is needed before we can determine which mtDNA sequences will be useful for species and/or population-level phylogenies.

1.1. Study organism

The genus *Seriatopora* is a member of the Pocilloporidae, and known for wide variations in colony morphologies (Veron and Pichon, 1976). Two major species groups, or six valid species, are recognized according to dif-

ferences in colony morphology and branch diameter, respectively (Veron, 2000; Veron and Pichon, 1976). In this study, the complete mt genome sequences of *S. hystrix* and *S. caliendrum* were characterized and compared to those of *Acropora tenuis* and the *Montastraea annularis* complex. Different mtDNA regions were evaluated by analysing variations and divergences within and between populations of the same species and by comparisons between two sister *Seriatopora* species.

2. Materials and methods

2.1. Sample collection and DNA extraction

Both *Seriatopora caliendrum* and *S. hystrix* were collected from Tiaoshi (21°57'27"N; 120°45'56"E), southern Taiwan. Apical fragments (1–2 cm long) of corals were narcotized in calcium-free seawater, and the attached epifauna were removed by fine tweezers under a dissecting microscope within 2 h to prevent further coral tissue dissociation (Domart-Coulon et al., 2004, 2001), before preserving in 70% ethanol.

Total genomic DNA of the preserved sample was extracted with an optimized protocol by including CTAB (Cetyl trimethylammonium bromide) to remove mucus and by modification of the standard phenol-chloroform extraction techniques (Sambrook and Russell, 2001). The terminal branch (0.5–1 cm) of the preserved sample was ground into power under liquid nitrogen with a mortar and pestle. The powder was lysed with DNA isolation buffer, containing 400 mM NaCl, 1% (w/v) sodium dodecyl sulphate SDS and 50 mM EDTA, pH 8, for 1 h at 65 °C, and digested with proteinase K (500 $\mu\text{g}/\text{ml}$) at 55 °C for 6–8 h. RNase was added to a final concentration of 20 $\mu\text{g}/\text{ml}$ and the lysate was incubated at 37 °C for 30 min in order to eliminate any RNA molecules. Subsequently, the lysate was treated with 800 mM NaCl and 1% (w/v) CTAB and incubated at 65 °C for 30 min. After incubation, solution was extracted once by adding an equal volume of chloroform and its supernatant was extracted again after transferring to a new tube with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1). The DNA of the supernatant from the 2nd extraction was precipitated by adding an equal volume of isopropanol. The precipitated DNA was washed with 70% EtOH and air-dried for 24 h with the tube inverted. The DNA was resuspended and dissolved in an appropriate volume of double-distilled water.

2.2. Molecular protocols

The complete mt genome was amplified by means of two long overlapping PCR reactions (Cheng et al., 1994) from the total DNA of both *S. caliendrum* and *S. hystrix*. To accomplish this, partial sequences of *rns* and *rnl* were initially obtained by a standard polymerase chain reaction (PCR) using coral-specific primers (Chen and Yu, 2000;

Romano and Palumbi, 1996). From *rns* and *rnl* sequences, we designed primers for the long PCR, 5'-GAC TTG GCT GTT CGG TTG TTA ATT AGA GGA GCG CG-3' (F25) and 5'-CGC GCT CCT CTA ATT AAC AAC CGA ACA GCC AAG TC-3' (R21), and 5'-TAC CCT GGG GAT AAC AGC GCA ATA ACG-3' (F02) and 5'-AAG GCC CAA TAA CCT TCC ATT GCA TCC GGT AGC-3' (R06), respectively. Long PCRs were performed using the Long PCR Enzyme Mix (Fermentas) under the conditions recommended by the manufacturer, in a PTC-200 Thermocycler (MJ Research). Long PCR reactions were setup in a volume of 50 μ l: 1 $\times$ PCR buffer, 1.5 mM MgCl₂, 0.2–0.4 mM of each dNTP, 0.5 μ M of each primer, 2% of DMSO, 1.875 U of the PCR enzyme mix, and approximately 0.5 μ g of genomic DNA. Long PCR profiles were 1 cycle of 94 °C for 2 min; 10 cycles of 96 °C for 20 s, 65 °C for 30 s, and 68 °C for 8 min; followed by 25 cycles of 96 °C for 20 s, 65 °C for 30 s, and 68 °C for 8 min, with a 10 s extension per cycle; then 1 cycle of 68 °C for 10 min. PCR products were electrophoresed in a 0.8% agarose gel to inspect their quantity, and purified using the Montage PCR purification kit (Millipore). If necessary, PCR products were recovered from the agarose gels using the gel purification kit under manufacturer's protocol (Topo XL gel purification, Invitrogen). Purified long PCR products were cloned into a pGEM-T vector system (Promega) and transformed into *Escherichia coli* (DH5 α). The sequence for each fragment was obtained in both directions by primer walking on the same purified PCR product. The M13 forward and reverse primers were used to obtain the initial sequences from the ends of each fragment. The consensus sequences from 2 to 6 sequenced clones were present for each species. The sequences obtained were submitted to GenBank under Accession No. EF633600 and EF633601.

To examine whether the *Seriatopora* ATP8 gene was functional, a reverse-transcription PCR (RT-PCR) was conducted within the predicted *atp8* from the amplified cDNA library. Total RNA was prepared from approximately 5 cm² of *S. hystrix* using the TRIzol reagent (Invitrogen), according to the manufacturer's suggestion. In general, coral samples were preserved in 2 ml TRIzol reagent for 10–20 min, and total RNA was extracted once by chloroform. The RNA was precipitated in isopropanol with the addition of sodium acetate (0.25 M), and dissolved in DEPC-treated water. The first-strand cDNAs of the total RNA were synthesized and amplified by RT-PCR using SuperScript III Reverse Transcriptase (Invitrogen) under the conditions recommended by the manufacturer, in a PTC-200 Thermocycler (MJ Research). RT-PCRs were setup in a volume of 50 μ l: 1 $\times$ RT-PCR buffer, 1 μ l of RNase block ribonuclease inhibitor (40 U/ μ l), 1 mM of each dNTP, and 1 μ M of primer (oligo-dT). RT-PCR profiles consisted of 1 cycle of 60 °C for 10 min and 1 cycle of 42 °C for 1.5 h.

Aliquots of first-strand cDNA were used in the PCRs (with the same reagents as above) using the PF1ST (5'-AGT GCC TCA GTT AAA AGT AA-3') and PR1SE

(5'-ATT TCT TTT TGT AAC CAA ATT TT-3') primers, while the negative control used the F30 and PR1SE primers. PCR reactions were setup in a volume of 50 μ l: 1 $\times$ PCR buffer, 2.5 mM MgCl₂, 1 mM of each dNTP, 0.2 μ M of each primer, 1 U of *Taq* polymerase, and 5 μ l of cDNA aliquots. The PCR profiles consisted of 1 cycle of 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 46 °C for 30 s, and 72 °C for 1 min; then 1 cycle of 72 °C for 5 min. The PCR products were electrophoresed and inspected in 1.5% agarose gels, purified using the Montage PCR purification kit (Millipore) then cloned into a Topo TA cloning system (Invitrogen) and transformed into *E. coli* (Top10). The sequences of inserts were determined in both directions by M13 forward and reverse primers on an ABI 377 automated DNA sequencer.

2.3. Sequence analysis

The DNA sequences were assembled using Sequencher 4.2 (Gene Codes Corporation), then analysed in Vector NTI 6.0 (InforMax, Invitrogen life science software). Open reading frames (ORFs) of considerable length (>50 amino acids) in the sequences were initially translated using cnidarian mt genetic codes (NCBI translation Table 4), then compared with the databases using the BLASTX program (Beagley et al., 1998; Gish and States, 1993). The identified protein coding and rRNA genes were aligned with the corresponding genes of previously published scleractinian mitogenomes for final recognition (Fukami and Knowlton, 2005; Tseng et al., 2005; van Oppen et al., 2002), using MEGA v3.1 (Kumar et al., 2004) with the weighted matrix of ClustalW (Thompson et al., 1994) and Gonnet (Gonnet et al., 1992). In addition to comparisons of the nucleotide and amino acid sequences, the hydropathy profiles of the unassigned ORFs were also compared with those of the identified *atp8* of scleractinian corals (Kyte and Doolittle, 1982). In order to assess the stability of the potential secondary structures of the intergenic spacers, free energies of the predicted secondary structures and their random sequences were examined using the DNA Mfold server (<http://bioinfo.math.rpi.edu/~mfold/dna/>; Zuker, 2000). Intergenic spacers were also examined for the presence of canonical tRNAs with tRNAscan-SE search server v1.21 (Lowe and Eddy, 1997), using the default search mode and specifying mt/chloroplast DNA as the source and the mold and protozoan mt genetic codes for tRNA structure prediction. The nucleotide composition was calculated using BioEdit 7.01 (Hall, 1999), while tandem repeat sections in the intergenic spacer were identified using the Tandem Repeat Finder 4.0 (Benson, 1999).

2.4. Utility of mtDNA for species phylogeny and population genetics

For evaluating the phylogenetic utility of the mtDNA regions of *Seriatopora*, an additional total 29 coral colonies were sampled from Taiwan, Taiping Island (Itu Aba Island) in the Spratly Islands (10°43'20"N; 115°49'32"E),

South China Sea, and Similan Island (8°39'17"N; 97°39'85"E), Andaman Sea, Thailand (Fig. 1, Table 1).

Specimens sampled from different oceans were arbitrarily considered to be different populations, due to the differences in oceanography, geography, and far distances among locations (2000–4000 km, Fig. 1). Amongst them, Taiwan is located in the middle of Ryukyu-Philippine Archipelago; the oceanography of its east side is dominated by the northward Kuroshio Current. The South China Sea is a marginal sea of the Pacific Ocean, and is semi-enclosed between the West Pacific and the Indian Oceans. Its surface currents are more complex than the nearby oceans and have dramatic seasonal variability driven by the monsoon (Morton and Blackmore, 2001). In winter, an anticlockwise gyre is created by the influence of the Northeast Monsoon; but, a clockwise gyre and a northeasterly current are established by the Southwest Monsoon during the summer (Chern and Wang, 2003; Chu and Guihua, 2003). The Andaman Sea is part of the Indian Ocean and located to the west of Malay Peninsula. Studies of the physical oceanography indicated that the current system of Andaman Sea is also driven by seasonal monsoons; it develops a clockwise and an anticlockwise gyre during the summer and winter, respectively (Varkey et al., 1996). Hence, our sampling represented 1 population of *S. caliendrum* and 3 populations *S. hystrix* corals (Table 1).

These coral samples were preserved in the field in 70% EtOH, and total DNA extractions were completed follow-

ing the methods described previously. The partial or complete nucleotide sequences of *atp6*, *cox1*, the 9th intergenic spacer (IGS9, an intergenic spacer spanning the region between *trnW* and *cox1*), and the putative control region were chosen for evaluation of their phylogenetic utilities, after genetic variations of each region was evaluated between mt genomes of *Seriatopora* spp. Table 2 illustrates the PCR primers and the optimized annealing temperatures of these PCR reactions. The PCR was setup in a volume of 50 μ l consisting of 1 $\times$ PCR buffer, 2.0–2.5 mM $MgCl_2$, 0.075 mM of each dNTP, 0.17 μ M of each primer, 1% DMSO, 1.0 U of HPTTM HotStart *Taq* (Protech Technology), and 0.5 μ g of genomic DNA. The PCR profiles consisted of 1 cycle of 95 °C for 11 min; 35 cycles of 93 °C for 1 min, an optimized annealing temperature for 40 s, and 72 °C for 1–2 min; then 1 cycle of 72 °C for 2–3 min. The PCR products were electrophoresed and inspected in 1.2% agarose gels, and their nucleotide sequences were determined for both strands with the same primers in the PCR amplification or with additional internal primers on an ABI 377 automated DNA sequencer.

The DNA sequences were assembled in Sequencher 4.2 (Gene Codes Corp.), aligned in ClustalW 1.6 (Thompson et al., 1994) then manually edited in MEGA v3.1 (Kumar et al., 2004). The alignment parameters of IGS9 were the same as those of the putative control region. Their gap opening and extension penalties were set to the values of 15 and 6, respectively. Changing the penalty values does

Fig. 1. Map of the Indo-West Pacific region. Stars and dot lines with arrows indicate sampling locations of *Seriatopora* populations and the directions of major ocean currents in the region, respectively.

Table 1

Sampling locations, number of samples collected, and details of successfully amplified sequences for *Seriatopora caliendrum* and *S. hystrix*

Species	Sample locations (Ocean)	No. of coral samples	No. of sequences ^a			
			<i>atp6</i>	<i>cox1</i>	IGS9 ^b	Putative control region
<i>S. caliendrum</i>	Taiwan (Pacific)	10	6	5	10	6
<i>S. hystrix</i>	Similan Is. (Indian)	8	5	3	4	5
	Taiping Is. (Pacific)	4	4	3	2	4
	Taiwan (Pacific)	7	6	7	7	7
Total counts		29	21	18	23	22

^a Accession Nos.: *atp6*, EF633533-EF633554; *cox1*, EF633514-EF633532; IGS9, EF633555-EF633577; putative control region, EF633578-EF633599.^b IGS9: the 9th intergenic spacer, spanning the region between the duplicated *trnW* (*trnW'*) and *cox1* in *Seriatopora* mt genome; see also Fig. 2 and Table 3.

Table 2

PCR primer pairs and annealing temperature applied for different mitochondrial loci

Loci	PCR reaction		Sequence primers
	Primer pairs	<i>T_m</i> (°C) ^a	
<i>atp6</i> and control region	F18 + R17	57–59	F18, R16, R17
IGS9 and <i>cox1</i>	F30 + R28 or F30 + R27 and F31 + R28 ^b	55–65, 61, 64	F30, F31, R27, R28
	F32 + R29	64–59	F32, R29

Primer sequence (5'–3')

F18: GTCCTTACGTCTTTACACCGAC

F30: TGGTTATCCCCCTCAGGTGGTC

F31: TCAACGATGTAGGTGCAAGTCC

F32: AAGTAGAGGCAGGGTCATGGTC

R16: CTACATACATTGCCTCGG

R17: AAAGACCACTCTAAAGCCCGCT

R27: AACTCTAGCACGCTTACG

R28: CCCCCCAATCATAACCGGCATAACC

R29: AACCGGTACATAAACCCGGCAC

^a Touch-down PCR was performed for all primer pairs, except F30 + R27 and F31 + R28.^b Due to poor DNA quality, overlapping DNA fragments were amplified for some coral samples.

not affect the aligning result. The repetitive regions within the putative control region of the mtDNA were excluded from the analysis, because the processes by which these repeats evolve are not well understood for scleractinian corals. Pairwise genetic distances (uncorrected p-distances) of each mtDNA region were calculated between species, and between and within populations of the same species using MEGA v3.1 (Kumar et al., 2004). The Mann–Whitney *U*-test or Kruskal–Wallis test was applied to examine the differences in p-distances among the three hierarchical categories using Stateview 5.0 (SAS Institute).

Phylogenetic relationships among sequences were constructed on the basis of neighbor-joining (NJ), maximum-parsimony (MP), and maximum-likelihood (ML) methods with the use of PAUP*4.0b10 (Swofford, 1999), and Bayesian (BAY) analysis in the program MrBayes 3.12 (Huelsenbeck and Ronquist, 2001). Similar tree topologies were generated using methods that included and excluded indels. Thus, analyses were run with indels treated as missing data, according to suggestions in Nei and Kumar (2000). The NJ analysis was performed with the Kimura 2-parameter model of nucleotide substitution (Kimura, 1980). In the MP and ML analyses, heuristic searches with TBR branch swapping and 10 random additions of sequences were per-

formed. For the ML and BAY analyses, the best-fitting model of DNA substitution and parameter estimates were performed by the Akaike information criterion (AIC) in PAUP*4.0b10 (Swofford, 1999) and Modeltest 3.7 (Posada and Crandall, 1998). The best-fitting evolutionary models were TrN for *atp6* and *cox1*, K81uf for IGS9 and GTR+G for the putative control region. Five-hundred bootstrapping values were used to evaluate support for the NJ, MP and ML trees. In the BAY analysis, the Markov Chain Monte Carlo search was run with 4 chains for 1×10^6 generations, with trees sampled every 100 generations. The first 2500 trees were discarded as the “burn-in”, after which the likelihood scores had stabilized.

3. Results

3.1. Characterization of the *Seriatopora* mt genomes

The entire mitochondrial (mt) genomes of *Seriatopora caliendrum* and *S. hystrix* were 17,011 and 17,060 bp in length, respectively. These are smaller than those of acroporids (17,887–18,338 bp; Tseng et al., 2005; van Oppen et al., 2002), but larger than those of the *Montastraea ann-*

ularis complex (about 16,138 bp; Fukami and Knowlton, 2005).

The *Seriatopora* mt genomes contained 12 identified open reading frames (ORFs), the small (*rns*) and large (*rnl*) subunits of ribosomal RNA genes, and 3 tRNA genes, all of which were transcribed on the same strand (Fig. 2). Except for *atp8*, these identified 12 ORFs included 7 NADH dehydrogenases (*nad*), 3 cytochrome oxidases (*cox*), ATPase (*atp6*), and cytochrome b (*cob*) (Table 3). The arrangements of the protein-coding genes of *Seriatopora* species were similar to those of other scleractinian corals (Fukami and Knowlton, 2005; Medina et al., 2006; Tseng et al., 2005; van Oppen et al., 2002). The *nad5* of both *Seriatopora* species was also interrupted by an intron, which contains 10 protein-coding genes, *rns*, and the putative control region (Fig. 2). The A + T base composition varied among different regions of both *Seriatopora* mitogenomes, ranging from 58% to 86%, with the highest value for the *nad5* intron (86%) and the lowest for the 9th intergenic spacer (IGS9) and the putative control region (Table 3). The mean A + T content of mt genomes of both *Seriatopora* species was significantly higher than those of other scleractinian corals ($p < 0.01$, Chi-square test; 60% and 66% for acroporids and the *M. annularis* complex, respectively).

3.2. Codon usage

Thirty-eight hundred and twenty-three amino acids were encoded for the 12 protein coding genes, and no significant

differences in codon usage were detected between these 2 *Seriatopora* species ($p > 0.05$, Chi-square test). Leucine was the most frequent amino acid, followed by phenylalanine; arginine was the least frequent. UUU (phenylalanine) was the most frequently used codon (57.5%), and UUA (leucine) was the second most common codon (38.0%). Although leucine and the UUU codon are also the most frequently used in acroporids and *Montastraea* corals, codon usages significantly differed among *Seriatopora*, acroporids, and the *M. annularis* complex ($p < 0.01$, Chi-square test for each comparison).

Translation initiation and termination codons of the *Seriatopora* mt protein-coding genes were similar to those of the *M. annularis* complex ($p = 0.07$, paired Sign test), and no differences existed between *S. hystrix* and *S. caliendrum* (Table 3; except for the termination of *nad4*). For *Seriatopora* corals, 10 of the 12 protein-coding genes used methionine (AUG) as the start codon, while *nad2* and *cob* use isoleucine (AUU) and valine (GUG), respectively. Most of the protein-coding genes were terminated by a UAA codon, except for *cox2* and *nad4* genes (UAG). Start/stop codon usage is in contrast with the majority of start/stop codons of acroporids ($p < 0.01$, paired Sign test), where start codons (AUG and GUG) and stop codons (UAA and UAG) were in equal use (Tseng et al., 2005; van Oppen et al., 2002).

3.3. Idiosyncratic *atp8* gene

ORFs corresponding to 12 of 13 typical protein-coding genes were detected in the mt genomes for *Seriatopora*, except for *atp8*. The search strategies of BLASTN and BLASTX detected no *atp8* coding sequences or *atp8*-like motifs. However, an unidentified ORF found in both mt genomes may correspond to the *atp8* gene of other scleractinian corals. This ORF, located between the 2 *trnW* genes, had low amino acid similarity to other scleractinian coral *atp8* genes (25.6–34.6%), but was significantly larger than its counterparts in other scleractinian corals (79 sense codons in *Seriatopora*, 66 in the *M. annularis* complex, 73 in acroporids, and 66–78 in other scleractinian corals). The transcription of the idiosyncratic *atp8* was confirmed by the fact that a RT-PCR product could be amplified in the predicted partial *atp8* using the total RNA prepared from *S. hystrix* as the template (Fig. 3). The identity of the RT-PCR product was also confirmed by DNA sequencing (data not shown).

In 9 of 14 released scleractinian mt genomes, the start of *atp8* was inferred by the presence of a methionine (Fig. 4). In *Seriatopora*, *atp8* begins with a valine, which is the same as in *Agaricia humilis* and *Pavona clavus* (Fig. 4). High similarities were observed at the beginning of the aligned sequences, and this resulted in a well-conserved N-terminal motif (Fig. 4). In addition to sequence similarities, the hydropathy profile of the ORF-encoded protein was similar to those of *atp8* in other scleractinian corals (Fig. 5). However, the hydropathy varied at the C-terminals, which became negatively charged at the 58th amino-acid position

Fig. 2. Gene map of the *Seriatopora caliendrum* mitogenome. Protein-coding genes, tRNA, and rRNA genes are abbreviated as in the text. The black and striped light grey arrows indicate the rRNA and the protein-coding genes. Arrow indicates the direction of transcription. The black and solid grey portions indicate the tRNA genes and the putative control region. *trnW'* indicate a duplicated *trnW*. The *nad5* intron is denoted by a black curve. The 9th intergenic spacer (IGS9) corresponds to a large intergenic spacer between *trnW'* and *cox1*. There are no differences in the gene orientations between *S. caliendrum* and *S. hystrix*.

Table 3
Mitogenomic organizations of *Seriatopora caliendrum* and *S. hystrix*

Region	Position		Length (bp)	AT %	Start/stop codon	Intergenic nucleotides ^a
	<i>S. caliendrum</i>	<i>S. hystrix</i>				
<i>trnM</i>	1–71	1–71	71	59.2		0
<i>rnl</i>	72–1973	72–1975	1902 (1904)	72.5 (72.8)		0
<i>nad5</i> (5')	1974–2684	1976–2686	711	71.6 (71.7)	ATG	0
Group I intron(5')	2685–2808	2687–2810	124	85.5		0
<i>nad1</i>	2809–3786	2811–3788	978	67.3	ATG/TAA	19, IGS1
<i>cob</i>	3806–4945	3808–4947	1140	72.8 (73.1)	GTG/TAA	220, IGS2
<i>nad2</i>	5166–6257	5168–6259	1092	74.7	ATT/TAA	1, IGS3
<i>nad6</i>	6259–6822	6261–6824	564	72.7 (72.9)	ATG/TAA	–1
<i>atp6</i>	6822–7499	6824–7501	678	71.4 (71.2)	ATG/TAA	0
Putative control region	7500–8494	7502–8524	995 (1023)	62.8 (62.0)		0
<i>nad4</i>	8495–9940	8525–9970	1446	68.7 (69.0)	ATG/TAG (TAA)	12, IGS4
<i>rns</i>	9953–10,868	9983–10,898	916	65.8 (66.6)		0
<i>cox3</i>	10,869–11,648	10,899–11,678	780	66.4	ATG/TAA	36, IGS5
<i>cox2</i>	11,685–12,443	11,715–12,473	759	69.7 (69.6)	ATG/TAG	–19
<i>nad4L</i>	12,425–12,724	12,455–12,754	300	77.3 (76.7)	ATG/TAA	–1
<i>nad3</i>	12,724–13,068	12,754–13,098	345	70.8 (71.0)	ATG/TAA	0
Group I intron(3')	13,069–13,122	13,099–13,152	54	81.5		0
<i>nad5</i> (3')	13,123–14,250	13,153–14,280	1128	74.0 (73.5)	TAA	12, IGS6
<i>trnW</i>	14,263–14,332	14,293–14,362	70	62.9 (64.3)		1, IGS7
Putative <i>atp8</i>	14,334–14,570	14,364–14,600	237	75.5	GTG/TAA	128 (91), IGS8
<i>trnW</i>	14,699–14,768	14,692–14,761	70	64.3 (62.9)		657 (713), IGS9
<i>cox1</i>	15,426–16,973	15,475–17,022	1548	64.7 (64.9)	ATG/TAA	38, IGS10

Different values and codons of *S. hystrix* are given in parentheses.

^a Intergenic nucleotides refer to intergenic bases between the gene on the same line and the gene on the line underneath, with a negative number indicating an overlap of that length. The names of the intergenic spacers (IGS1–10) are designated after the intergenic nucleotides.

of the presumptive *atp8*, then gradually positively charged in both *Seriatopora* species (Fig. 5). This negatively charged region at the C-terminal is shorter in other scleractinian corals; it was about 4 codons in length, starting 8 codons upstream from the end of the *atp8*. Considering the results of the analysis of amino acid similarity and hydropathy together suggested that the putative *atp8* had a greatly extended 3' end.

3.4. Duplicated *trnW* (*tRNA*^{TRP})

To date, only two tRNAs have been found in scleractinian mt genomes (*trnM* and *trnW*). However, an additional tRNA, namely *trnW'*, was identified in the region close to the *cox1* gene in both *Seriatopora* genomes (Fig. 2). All three tRNA genes could be folded into typical secondary structures (Fig. 6). These structures were 70 or 71 nucleotides in length, and had an aminoacyl stem of 7 bp, a dihydrouridine (DHU) stem of 3 bp (4 bp in *trnW*), a D-loop of 6 or 9 nt, an anticodon stem of 5 bp, an anticodon loop of 7 nt, a T Ψ C stem of 4 bp, and a T Ψ C loop of 7 nt. The sequence similarity was high between *trnW* and *trnW'* of *Seriatopora* corals with only 1 nucleotide substitution observed at position 1 for *S. hystrix* and position 9 for *S. caliendrum*, suggesting that *trnW'* is a duplicated gene of its counterpart (Fig. 6).

3.5. Intergenic spacers and the putative control region

As in other scleractinian mt genomes, intergenic spacers also existed between most mt genes of *Seriatopora*. Only 5

protein-coding genes overlapped with each other: *nad6* with *atp6* by 1 bp, *cox2* with *nad4L* by 19 bp, and *nad4L* with *nad3* by 1 bp (Table 3). Intergenic spacers, excluding the putative control region (see below), totalled 1124 and 1143 bp in *S. caliendrum* and *S. hystrix*, respectively. These regions varied in length from 1 to 713 bp. Approximately 48% and 52% of the intergenic spacers were attributed to the IGS of *trnW*–*cox1* (IGS9), and another 704 and 667 bp were located in the other nine IGSs. Total lengths of *Seriatopora* coral IGSs were smaller than those of acroporids (2508 bp) and the *M. annularis* complex (about 1201 bp).

The *atp6*–*nad4* intergenic spacer, containing distinct repeated elements, was identified as a putative control region for *Seriatopora* (Fig. 7). The tandem repeats in both species began forming at the same position, 290-bp downstream from the 3'-end of *atp6*. In the aligned sequences, 4 and 5 copies of the 51-bp repeated fragments (consensus unit: 5'-BYA GAA AGT AKH GVV RAY TTR AGR GAG DGT GWM RHT ARS GYA WTA MGT SAG-3') were, respectively, recognized for *S. caliendrum* and *S. hystrix*. This predicted control region was also supported by several potential hairpin structures (Fig. 8). The analysis predicted 2 stable hairpins with a long stem corresponding to positions 614–692 and 764–825 in the alignment of both *Seriatopora* species. The stabilities of the structures predicted from the real data were compared with those predicted from 10 randomized sequences. All of the randomized sequences had the potential to fold into hairpin structures, but their predicted structures were shorter than those of the actual

Fig. 3. Identification of the *atp8* by RT-PCR analysis. (a) Nucleotide and predicted amino acid sequence of the *Seriatopora* ATP8 gene. The positions of primers used in the RT-PCR experiment are indicated. Numbering refers to the nucleotide position in the peculiar *atp8* coding sequence. (b) Agarose gel electrophoresis analysis of the RT-PCR product. (Lane 1) Low-molecular-weight standards (Fermentas pUC19 MspI marker); (lane 2) RT-PCR product and its size estimated by comparison with the standards corresponded well with that predicted from the sequence (186 bp); (lane 3) negative control of RT-PCR by the primers spanning the *nad5*(3') and *atp8*; (lanes 4 and 5) PCR products of genomic DNA of *S. hystrix* (positive controls) by the corresponding primer pairs of lanes 2 and 3, respectively; (lane 6) PCR reaction without DNA or RNA templates; (lane 7) low-molecular-weight standards (Promega 100-bp ladder DNA marker).

sequences (data not shown). In addition, while the free energies of the secondary structures of these two hairpins were -12.2 and -13.2 kcal/mol, respectively, the free energies of the potential secondary structures obtained by the randomized sequences were -1.9 to -6.1 and -0.6 to -5.4 kcal/mol. These predicted secondary structures for the randomized sequences were significantly less stable than those predicted from the actual data ($p < 0.01$,

Sign test), implying the biological functions of these two hairpin structures.

3.6. Evaluating the utilities of mtDNA for species-level phylogeny and population genetics

Overall, there were 78 variable nucleotides between the corresponding protein-coding and rRNA genes, and 13

Fig. 4. Comparison of amino acids of the *Seriatopora caliendrum* and *S. hystris* putative mitochondrial *atp8* with the corresponding amino acid sequences of 11 other scleractinian corals, identified by their database accession numbers as follows: *Agaricia humilis* NC008160, *Astrangia* sp. NC008161, *Colpophyllia natans* NC008162, *Mussa angulosa* NC008163, *Pavona clavus* NC008165, *Porites porites* NC008166, *Siderastrea radians* NC008167 (Medina et al., 2006); *Montastraea annularis* complex NC007224–NC007226 (Fukami and Knowlton, 2005); *Anacropora matthai* NC006898, *Montipora cactus* NC006902 (Tseng et al., 2005); and *Acropora tenuis* NC003522 (van Oppen et al., 2002). Under the sequences, an arrow indicates an amino acid conserved in all 12 sequences; solid and open circles indicate amino acids conserved in 10–11/12 sequences that include and did not include the *Seriatopora* sequences, respectively. Dots indicate identical amino acids against coral alignments, and dashes indicate indels.

Fig. 5. Comparisons of *Seriatopora* spp., *Montastraea annularis*, and *Acropora tenuis* ATP8 hydropathy profiles; (a) window size of 9, (b) window size of 13. Numbers below the profiles designate amino acid positions. (—) *Seriatopora* spp.; (····) *Montastraea annularis*; (---) *Acropora tenuis*.

variable amino acids between the corresponding protein-coding genes of these 2 *Seriatopora* species (Table 4). Among protein-coding genes, *atp6* was the most divergent gene, followed by *nad5*(3'), while *cob* and *nad2* were identical in terms of their nucleotide and amino acid sequence similarity. No amino acid differences were detected between the corresponding *nad1*, *nad2*, *nad5*(5'), *nad6*, *nad4L*, and *cob* of these two species (Table 4). Based on these comparisons, we chose mt *atp6*, *cox1*, IGS9, and the putative control region to evaluate the phylogenetic

utilities of the *Seriatopora* mt genomes, specifically for species phylogeny and population genetics.

For *atp6*, 11 variable sites were observed among the analysed 334-bp sequence for 21 individuals (Table 1). Genetic distances of the partial *atp6* sequences ranged from 0 to 0.041 [mean = 0.014 ± 0.014 (SD), $n = 231$], and the differences among these three hierarchical categories were significant ($p < 0.01$, Mann–Whitney *U*-test for each comparison). The highest divergences were in the interspecific category with a *p*-distance of 0.029 ± 0.004 , which was

Fig. 6. Predicted secondary structures of tRNAs encoded by the mitogenomes of *Seriatopora caliendrum*. Circles indicate the substituted nucleotides of corresponding tRNA genes in *S. hystrix*. (a) *trnM*, (b) *trnW* near the *nad5*(3') region, and (c) *trnW* near *cox1* (*trnW*).

about 7.5- and 30-times higher than those of the interpopulational and intrapopulational categories (Fig. 9). For *cox1*, 9 variable sites were observed among the aligned 775 nt sequences. The pairwise genetic distances (p-distances) ranged from 0 to 0.01 (mean = 0.002 ± 0.002 , $n = 153$). The genetic divergence of *cox1* of the interspecific category was 0.004 ± 0.002 and was significantly higher than those of the intraspecific categories ($p < 0.01$, Mann–Whitney *U*-test for each comparison, Fig. 9). Among the 713-bp of IGS9 sequences from 23 individuals, 25 substitutions and 3 indels were observed. The p-distances of the IGS9 sequences ranged from 0 to 0.038 (mean = 0.020 ± 0.019 , $n = 253$). The genetic distance of the interspecific category was 0.038 and was significantly higher than that of the intraspecific categories ($p < 0.01$, Mann–Whitney *U*-test for each comparison, Fig. 9). For the non-repeating regions of the control region, alignment consisted of 792 positions, of which 736 bp was constant and 37 sites were parsimoniously informative. The pairwise genetic distances of the non-repeating regions ranged from 0 to 0.044 (mean = 0.021 ± 0.016 , $n = 231$), and the differences among the 3 hierarchic categories were significant ($p < 0.01$, Kruskal–Wallis test). The highest divergences were in the interspecific category with a p-distance of 0.039 ± 0.003 , which was 3.3- and 10-times higher than those of the interpopulational and intrapopulational categories (Fig. 9).

3.7. Phylogenetic implications

The significance of the observed hierarchical genetic divergences was also revealed in the phylogenetic analysis (Fig. 10). All of the phylogenetic trees suggested that these *Seriatopora* corals were divided into 2 major lineages, supported by bootstrap values exceeding 80%.

Phylogenetic analysis of *atp6* and the control region provided greater resolution among populations of *S. hystrix*

a		<i>S. caliendrum</i> (51 bp x 4)	
7789	TCAGAAAGTATCGCGAATTTGAGAGAGTGTGTAGATAGGCAATACGTCAG	7839	
7840	.T.....GA.AA.....G...A.AC...C...T..A..G..	7890	
7891	GA.AC.....G...A.....C..AC...T..A..G..	7941	
7942	GA.GCG....A..G...A....AC.....T.T....G..	7992	
b		<i>S. hystrix</i> (51 bp x 5)	
7791	CCAGAAAGTATTGAGAACTTGAGAGAGTGTGTCGTTAGGGCATTACGTCAG	7841	
7842	TT.....GA.....T.....G...AAAC...C.....A..G..	7892	
7893	T.....GA.....T.....G...AAAC...C.....A..G..	7943	
7944	G.....GA..C..T....G...A....A.C...C.....A..G..	7994	
7995	T.....GA.GCG..T..A..G...A....AAC.....T.....G..	8045	

Fig. 7. Alignment of the tandem repeats in the putative control regions of (a) *Seriatopora caliendrum* and (b) *S. hystrix*. Dots indicate sequence identity with the top line. Numbers indicate the positions in mitochondrial genomes.

Fig. 8. Putative secondary structures in the *Seriatopora* candidate control region. (a) Hairpin structure at position 614–692. (b) Hairpin structure at position 764–825.

than did those of *cox1* and IGS9 (Fig. 10). The ML tree for the 22 control region sequences and 21 *atp6* sequences, with the homologous region of *Stylophora pistillata* as an out-group, showed two distinct lineages, each with high support (>80% bootstrap support; Fig. 10a and b). One clade was comprised of all individuals of *S. caliendrum* from Taiwan. All populations of *S. hystrix* belonged to two separate subclades, and these subclades were joined as a sister lineage to the *S. caliendrum* clade. The *S. hystrix*-Andaman Sea subclade was monophyletic, containing only individuals from the Andaman Sea. Another subclade represented *S. hystrix* of the Pacific Ocean, which contained individuals from Taiwan and the South China Sea. The tree topology showed that the most recent diversification was the split between the Andaman Sea and West Pacific *S. hystrix*. The previous split was between the

Table 4

Nucleotide (NT) and amino acid (AA) sequences identities (%) and numbers of variable sites (Vn) between the corresponding mtDNA loci of *Seriatopora caliendrum* and *S. hystrix*

Loci	NT		AA	
	Identity	Vn	Identity	Vn
Protein-coding				
<i>nad5</i>	99.3	12	98.8	7
(5')	99.8	1	100	0
(3')	99.0	11	98.1	7
<i>nad1</i>	99.8	1	100	0
<i>cob</i>	100	0	100	0
<i>nad2</i>	100	0	100	0
<i>nad6</i>	99.6	2	100	0
<i>atp6</i>	98.8	8	99.5	1
<i>nad4</i>	99.5	7	99.1	0
<i>cox3</i>	99.3	5	99.6	1
<i>cox2</i>	99.6	3	99.2	2
<i>nad4L</i>	99.3	2	100.0	0
<i>nad3</i>	99.7	1	99.1	1
<i>cox1</i>	99.8	3	99.8	1
rRNA				
<i>rnl</i>	98.4	20		
<i>rns</i>	98.2	14		
Intergenic spacer				
IGS1	100	0		
IGS2	99.5	1		
IGS3	100	0		
IGS4	58.3	5		
IGS5	100	0		
IGS6	100	0		
IGS7	100	0		
IGS8	69.5	2		
IGS9	88.6	25		
IGS10	100	0

Fig. 9. Interspecific (inter-spp) and intraspecific (inter-pop and intra-pop) p-distances of different mtDNA loci of *Seriatopora caliendrum* and *S. hystrix*. Error bar represents $\pm$ SD; (○) intra-pop comparisons; (□) inter-pop comparison; (●) inter-spp comparison.

S. caliendrum clade and the ancestor of *S. hystrix*. Nearly identical topologies were generated by the NJ, MP, ML and BAY analyses, except for moderate bootstrap support at the node between the Andaman Sea and West Pacific *S. hystrix* for *atp6* (Fig. 10a). Relationships between species

Fig. 10. Phylogenies of *Seriatopora caliendrum* and *S. hystrix* using mt genes and the intergenic spacer (a) *atp6*, (b) putative control region, (c) *cox1*, and (d) IGS9. Bootstrap values (500 replicates) based on the neighbor joining, maximum likelihood, maximum parsimony, and Bayesian analyses with probabilities greater than 60% are shown on the branches. *Stylophora pistillata* was used as an outgroup in (a) and (c); midpoint rooting was applied in (b) and (d). Scale bar: substitution per site.

were resolved by the phylogenetic analysis of both *cox1* and IGS9 datasets with *S. caliendrum* and *S. hystrix* clustering into 2 distinct clades, but polytomically among different populations of *S. hystrix* (Fig. 10c and d).

4. Discussion

4.1. Molecular evolution of the *Seriatopora* mitochondrial genomes

The *Seriatopora* mt genomes contain three unique features that differ from the published mt genomes of scleractinian corals: (1) a novel *atp8*, (2) a duplicated *trnW*, and (3) the location of the putative control region between *atp6* and *nad4*.

The peculiar *atp8* is presumably a functional gene in *Seriatopora* mt genomes, although it highly differs at both the nucleotide and amino acid levels from those of other

scleractinian corals. The *atp8* is quite heterogeneous in length among diverse organisms, e.g., 52 amino acids (aa) in *Drosophila* and 68 aa in human. This protein is more conserved at the level of its secondary structure and the chemical character of the identified amino acids (Papakonstantinou et al., 1996b). Comparisons of the amino acid sequences from diverse organisms indicate that *atp8* is an intrinsic membrane protein composed of 3 domains (Papakonstantinou et al., 1993, 1996a). The N-terminal domain is located in the intermembrane space and shows a conserved Met-Pro-Gln-Leu motif, a central membrane-spanning hydrophobic domain, and a positively charged C-terminal region exposed in the matrix space (Devenish et al., 1992).

The presence of a unique *atp8* was also discovered in a nematode, *Trichinella spiralis*, and an ascidian, *Ciona intestinalis*, while an *atp8* deficiency was previously considered an evolutionary constraint of these organisms (He et al.,

2005; Hu et al., 2003; Le et al., 2000; Yokobori et al., 1999). Analyses of sequence and hydropathic similarities support their 3-domain compilation and their likeness to *atp8* in *Limulus* and humans (Gissi et al., 2004; Lavrov and Brown, 2001). The transcribed ORF between the 2 *trnW* genes in the *Seriatopora* mitogenomes also bears similar chemical compilations to *atp8* of other scleractinian corals. All of them contain the conserved N-terminal domain and a central hydrophobic domain, but are more variable at the C-terminal positively charged region (Fig. 5). Despite its low global similarity to other *atp8* genes, the ORF analysed here can be unambiguously regarded as a novel *atp8* form with a longer C-terminal domain when compared to other scleractinian corals.

Duplication of an additional tRNA gene has been reported in other metazoan mt genomes. For example, 2 isoacceptors of *trnM* have been mentioned in the mt genomes of the mussels, *Mytilus edulis* and *M. californianus* (Beagley et al., 1999; Hoffmann et al., 1992), and the ascidian, *Ciona intestinalis* (Gissi et al., 2004). The additional *trnM* is thought to originate from its isoacceptor and functionally differs from it. In *M. edulis*, similarities at the 5' end of the 2 *trnM* genes suggest that they arose by gene duplication (Hoffmann et al., 1992). It has also been suggested that *trnM*(AUG) and *trnM*(AUA) are used as an initiator and elongator, respectively, although there is no experimental evidence to support this hypothesis (Beagley et al., 1999).

In *Seriatopora*, high similarities between the 2 *trnW* genes of the same species also suggest their origin by recent gene duplication of a common ancestor. By folding the *trnW*, the difference of nucleotide does not change the secondary structure of the loop region, which indicates that *trnW* genes are functional in both *Seriatopora* species. These 2 *trnW* genes in *Seriatopora* are coded by the same 5'-UCA-3' anticodon, and no supplemental function can be deduced. The additional *trnW* is supported by a postulated model of tRNA molecule replication which hypothesizes their origin simply by direct duplication of a molecule housing double-hairpin structures (Di Giulio, 1992, 2004). Nevertheless, it is not clear why tRNA was duplicated in *Seriatopora* mtDNA but not in other scleractinian corals. Further comparative data of other species and related genera are needed to elucidate the evolutionary process for this duplication.

Mitochondrial control regions are not well-defined and may be highly variable among scleractinian corals. The presence of tandem repeats and functional secondary structures indicate that the control region is located between *atp6* and *nad4* in *Seriatopora* and other members of pocilloporid corals (Chen et al., 2006). However, they are located between *rns* and *cox3* in the Acroporidae (Tseng et al., 2005; van Oppen et al., 2002), and between *cob* and *nad2* in *Siderastraea* (Chuang, 2006), but not yet recognized for other published scleractinian mt genomes (Fukami and Knowlton, 2005; Medina et al., 2006).

The observed differences in the mt control region of diverse Scleractinia may reflect their independent origins. If the control region had been rearranged soon after the origin of scleractinian corals, they would be close to each other in nucleotide sequences and structural composition. However, there are relatively high similarities in nucleotide sequences and in the molecular organization among congeners or genera of the same family, but the same does not hold true among families. In addition, no rearrangement or recombination of genes has been reported among the 14 published scleractinian mt genomes. Hence, the most parsimonious explanation for disparities in the control region is that it evolved independently among the different scleractinian lineages.

Taken together with the divergent *atp8* and duplicated *trnW*, these distinctive features of *Seriatopora* mt genomes reveal profound implications for the evolution of the family Pocilloporidae in scleractinian phylogeny. Similar structures were also observed in the confamilial genera, *Stylophora* and *Pocillopora* (Chen et al., 2006). The Pocilloporidae has been regarded as a monophyletic group of the robust clade in scleractinian phylogeny, as inferred by mitochondrial ribosomal DNA sequences (Chen et al., 2002; Romano and Palumbi, 1996, 1997), but could be regarded as a distinct lineage and apart from the robust clade based on our study. This hypothesis is also supported by a new phylogenetic analysis based on the nucleotide sequences of mitochondrial *cob* and *cox1* and nuclear β -tubulin and ribosomal genes (Fukami et al., 2006).

4.2. Applications of the hypervariable regions to the phylogeny of coral species and population genetics

Our study provides evidence that several protein-coding genes and intergenic spacers in scleractinian corals, at least in pocilloporids, are potentially useful for species phylogenetic construction or population genetic analyses. This contradicts the general impression that anthozoan mt genomes are slowly evolving and have little potential for phylogenetic utility below the species level (revealed in Shearer et al., 2002). The genetic distance (p-distance) between these 2 populations (Andaman Sea and Pacific Ocean) of *S. hystrix* ranged 0.3–0.9% and 0.9–1.7% for *atp6* and the putative control region, respectively. These variations provide enough information to successfully recover the reciprocal monophyly between the Andaman Sea and Pacific Ocean *S. hystrix* populations (Fig. 10a and b), which is concordant with the biogeographic patterns observed in several marine organisms distributed in both the Indian and Pacific Oceans (reviewed in Benzie, 1998). Benzie (1999) suggested that although the Indo-Pacific has a complicated geographic and climatic history, recent repeated periods of lowered sea levels during the Pleistocene are the suggested major vicariance events initiating the genetic differences of separated populations for most of the last 3 Ma. If we calibrate the divergent time in isolating these 2 populations based on this scenario, the maximum diver-

gence rate would be 0.1–0.3% and 0.3–0.57% Ma^{-1} for *atp6* and the putative control region, respectively. These divergences are about 2- to 3-times faster than that of the *cob* divergence rate of *Acropora* congeners (0.1–0.18% Ma^{-1}). However, a lower resolution was observed for *cox1* and IGS9, which were successfully used to recover the species phylogeny between *S. caliandrum* and *S. hystrix* (Fig. 10c and d). Our findings indicate that the evolutionary rate of the mt genomes could be highly variable among different genes and intergenic spacers, and in different lineages of scleractinian corals. Thus, the generalization of slow evolution and limitations of phylogenetic utility in coral mt genomes should be treated with caution before the molecular evolution of each coral lineage is evaluated.

Acknowledgments

We thank the Minister of Domestic Affairs and Minister of National Defence for providing field trips to the Taiping Island (Itu Aba Island) in the Spratly Islands; Drs. Hiro-nobu Fukami and Nancy Knowlton for the initiation and encouragement of this study, members of the Evolutionary and Ecology Genetics of Coral Reef Discussion Group, Research Center for Biodiversity, Academia Sinica (RCBAS) and three anonymous referees for constructive comments. This work was supported by the Academia Sinica Thematic Grants (2002–2004), a Grant from the Genomic Research Center, Academia Sinica (2006–2007), and the National Science Council (NSC) Grant (NSC-94-2621-B-001-005), Taiwan to C.A.C., and a NSC Grant (NCS-94-EPA-Z002-004) to C.-F. Dai. This is the Evolutionary and Ecology Genetics of Coral Reef Group, RCBAS contribution no. 42.

References

- Beagley, C.T., Okimoto, R., Wolstenholme, D.R., 1998. The mitochondrial genome of the sea anemone *Metridium senile* (Cnidaria): introns, a paucity of tRNA genes, a near-standard genetic code. *Genetics* 148, 1091–1108.
- Beagley, C.T., Okimoto, R., Wolstenholme, D.R., 1999. Mytilus mitochondrial DNA contains a functional gene for a tRNA^{Ser} (UCN) with a dihydrouridine arm-replacement loop and a pseudo-tRNA^{Ser} (UCN) gene. *Genetics* 152, 573–652.
- Benson, G., 1999. Tandem repeats finder: a program to analyze DNA sequences. *Nucleic Acids Research* 27, 573–580.
- Benzie, J.A.H., 1998. Genetic structure of marine organisms and SE Asian biogeography. In: Hall, R., Holloway, J.D. (Eds.), *Biogeography and Geological Evolution of SE Asia*. Backhuys Publishers, The Netherlands, pp. 197–209.
- Benzie, J.A.H., 1999. Genetic structure of coral reef organisms: ghosts of dispersal past. *American Zoologist* 39, 131–145.
- Boore, J.L., 1999. Animal mitochondrial genomes. *Nucleic Acids Research* 27, 1767–1780.
- Burger, G., Gray, M.W., Lang, B.F., 2003. Mitochondrial genomes: anything goes. *Trends in Ecology and Evolution* 19, 709–716.
- Chen, C., Dai, C.-F., Fukami, H., Knowlton, N., Chen, C.A., 2006. Evolutionary mitogenomics of pocilloporid corals: ATP8 gene loss, a novel open reading frame (ORF) and phylogenetic utility. *First Asia Pacific Coral Reef Symposium*, Hong Kong, M39.
- Chen, C.A., Wallace, C.C., Wolstenholme, J., 2002. Analysis of the mitochondrial 12S rRNA gene supports a two-clade hypothesis of the evolutionary history of scleractinian corals. *Molecular Phylogenetics and Evolution* 23, 137–149.
- Chen, C.A., Yu, J.-K., 2000. Universal primers for amplification of mitochondrial small subunit ribosomal RNA-encoding gene in scleractinian corals. *Marine Biotechnology* 2, 146–153.
- Cheng, S., Chang, S.Y., Gravitt, P., Respass, R., 1994. Long PCR. *Nature* 369, 684–685.
- Chern, C.-S., Wang, J., 2003. Numerical study of the upper-layer circulation in the South China Sea. *Journal of Oceanography* 59, 11–24.
- Chu, P.C., Guihua, W., 2003. Seasonal variability of the thermohaline front in the central South China Sea. *Journal of Oceanography* 59, 65–78.
- Chuang, Y.-Y., 2006. Mitogenomics and molecular evolution of the group I intron in the cytochrome oxidase I gene of *Siderastrea* (Cnidaria; Scleractinia; Siderastreidae). Master's thesis. Institute of Oceanography. National Taiwan University, Taipei, Taiwan.
- Dellaporta, S.L., Xu, A., Sagasser, S., Jakob, W., Moreno, M.A., Buss, L.W., Schierwater, B., 2006. Mitochondrial genome of *Trichoplax adhaerens* supports Placozoa as the basal lower metazoan phylum. *Proceedings of the National Academy of Sciences of the United States of America* 103, 8751–8756.
- Devenish, R.J., Papakonstantinou, T., Galanis, M., Law, R.H., Linnane, A.W., Nagley, P., 1992. Structure/function analysis of yeast mitochondrial ATP synthase subunit 8. *Annals of the New York Academy of Sciences* 671, 403–414.
- Di Giulio, M., 1992. On the origin of the transfer RNA molecule. *Journal of Theoretical Biology* 159, 199–214.
- Di Giulio, M., 2004. The origin of the tRNA molecule: implications for the origin of protein synthesis. *Journal of Theoretical Biology* 226, 89–93.
- Domart-Coulon, I., Tambutté, S., Tambutté, E., Allemand, D., 2004. Short term viability of soft tissue detached from the skeleton of reef-building corals. *Journal of Experimental Marine Biology and Ecology* 309, 199–217.
- Domart-Coulon, I.J., Elbert, D.C., Scully, E.P., Calimlim, P.S., Ostrander, G.K., 2001. Aragonite crystallization in primary cell cultures of multicellular isolates from a hard coral, *Pocillopora damicornis*. *Proceedings of the National Academy of Sciences of the United States of America* 98, 11885–11890.
- Frost, S.H., 1977. Oligocene reef coral biogeography, Caribbean and western Tethys. *Bureau de Recherches Géologiques et Minières (France)* 89, 342–352.
- Fukami, H., Chen, C.A., Chiou, C.-Y., Knowlton, N., 2007. Novel group I introns encoding a putative homing endonuclease in the mitochondrial *cox1* gene of scleractinian corals. *Journal of Molecular Evolution* 64, 591–600.
- Fukami, H., Chen, C.A., Knowlton, N., 2006. Chaos of the scleractinian coral family. *First Asia Pacific Coral Reef Symposium*. Hong Kong, pp M40.
- Fukami, H., Knowlton, N., 2005. Analysis of complete mitochondrial DNA sequences of three members of the *Montastraea annularis* coral species complex (Cnidaria, Anthozoa, Scleractinia). *Coral Reefs* 24, 410–417.
- Gish, W., States, D.J., 1993. Identification of protein coding regions by database similarity search. *Nature Genetics* 3, 266–272.
- Gissi, C., Iannelli, F., Pesole, G., 2004. Complete mtDNA of *Ciona intestinalis* reveals extensive gene rearrangement and the presence of an *atp8* and an extra *trnM* gene in ascidians. *Journal of Molecular Biology* 58, 376–389.
- Goddard, M.R., Leigh, J., Roger, A.J., Pemberton, A.J., 2006. Invasion and persistence of a selfish gene in the Cnidaria. *PLoS ONE* 1, e3. doi: 10.1371/journal.pone.0000003.
- Gonnet, G.H., Cohen, M.A., Benner, S.A., 1992. Exhaustive matching of the entire protein sequence database. *Science* 256, 1443–1445.

- Gregory, J.W., Trench, J.B., 1916. Eocene corals from the Fly River, New Guinea. *Geological Magazine* 6 (481–488), 529–536.
- Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41, 95–98.
- He, Y., Jones, J., Armstrong, M., Lamberti, F., Moens, M., 2005. The mitochondrial genome of *Xiphinema americanum sensu stricto* (Nematoda: Enopleia): considerable economization in the length and structural features of encoded genes. *Journal of Molecular Biology* 61, 819–883.
- Hellberg, M.E., 2006. No variation and low synonymous substitution rates in coral mtDNA despite high nuclear variation. *BMC Evolutionary Biology* 6, 24, doi:10.1186/1471-2148-1186-1124.
- Hoffmann, R.J., Boore, J.L., Brown, W.M., 1992. A novel mitochondrial genome organization for the blue mussel, *Mytilus edulis*. *Genetics* 131, 397–412.
- Hu, M., Chilton, N.B., Gasser, R.B., 2003. The mitochondrial genome of *Strongyloides stercoralis* (Nematoda)—idiosyncratic gene order and evolutionary implications. *International Journal for Parasitology* 33, 1393–1408.
- Huelsenbeck, J.P., Ronquist, F.R., 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17, 754–755.
- Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16, 111–120.
- Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* 5, 150–163.
- Kyte, J., Doolittle, R.F., 1982. A simple method for displaying the hydrophobic character of a protein. *Journal of Molecular Biology* 157, 105–142.
- Lavrov, D.V., Brown, W.M., 2001. *Trichinella spiralis* mtDNA: a nematode mitochondrial genome that encodes a putative ATP8 and normally structured tRNAs and has a gene arrangement relatable to those of coelomate metazoans. *Genetics* 157, 621–637.
- Le, T.H., Blair, D., Agatsuma, T., Humair, P.-F., Campbell, N.J.H., Iwagami, M., Littlewood, D.T.J., Peacock, B., Johnson, D.A., Bartley, J., Rollinson, D., Herniou, E.A., Zarlenga, D.S., McManus, D.P., 2000. Phylogenies inferred from mitochondrial gene orders – a cautionary tale from the parasitic flatworms. *Molecular Biology and Evolution* 17, 1123–1125.
- Lowe, T.M., Eddy, S.R., 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Research* 25, 955–964.
- Medina, M., Collins, A.G., Takaoka, T.L., Kuehl, J.V., Boore, J.L., 2006. Naked corals: Skeleton loss in scleractinia. *Proceedings of the National Academy of Sciences of the United States of America* 103, 9096–9100.
- Medina, M., Weil, E., Szmant, A.M., 1999. Examination of the *Montastraea annularis* species complex (Cnidaria: Scleractinia) using ITS and COI sequences. *Marine Biotechnology* 1, 89–97.
- Morton, B., Blackmore, G., 2001. South China Sea. *Marine Pollution Bulletin* 42, 1236–1263.
- Nei, M., Kumar, S., 2000. *Molecular Evolution and Phylogenetics*. Oxford University Press, Oxford.
- Pandolfi, J.M., Lovelock, C.E., Budd, A.F., 2002. Character release following extinction in a Caribbean reef coral species complex. *Evolution* 56, 479–501.
- Papakonstantinou, T., Galanis, M., Nagley, P., Devenish, R.J., 1993. Each of three positively-charged amino acids in the C-terminal region of yeast mitochondrial ATP synthase subunit 8 is required for assembly. *Biochimica et Biophysica Acta* 1144, 22–32.
- Papakonstantinou, T., Law, R.H., Nagley, P., Devenish, R.J., 1996a. Non-functional variants of yeast mitochondrial ATP synthase subunit 8 that assemble into the complex. *Biochemistry and Molecular Biology International* 39, 253–260.
- Papakonstantinou, T., Law, R.H.P., Nesbitt, W.S., Nagley, P., Devenish, R.J., 1996b. Molecular genetic analysis of the central hydrophobic domain of subunit 8 of yeast mitochondrial ATP synthase. *Current Genetics* 30, 12–18.
- Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14, 817–818.
- Romano, S.L., Palumbi, S.R., 1996. Evolution of scleractinian corals inferred from molecular systematics. *Science* 271, 640–642.
- Romano, S.L., Palumbi, S.R., 1997. Molecular evolution of a portion of the mitochondrial 16 S ribosomal gene region in scleractinian corals. *Journal of Molecular Evolution* 45, 397–411.
- Sambrook, J., Russell, D.W., 2001. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York.
- Shearer, T.L., van Oppen, M.J.H., Romano, S.L., Worheide, G., 2002. Slow mitochondrial DNA sequence evolution in the Anthozoa (Cnidaria). *Molecular Ecology* 11, 2475–2487.
- Snell, T.L., 2000. Low levels of mitochondrial sequence variation in scleractinian corals. In: Hopley, D., Hopley, P., Tamelander, J., Done, T. (Eds.), *Ninth International Coral Reef Symposium*. Smithsonian Tropical Research Institute, Bali, Indonesia, p. 21.
- Snell, T.L., Sammarco, P.W., Foltz, D.W., 1998. Variation in morphology vs conservation of a mitochondrial gene in *Montastraea cavernosa* (Cnidaria, Scleractinia). *Gulf of Mexico Science* 16, 188–195.
- Swofford, D.L., 1999. *PAUP: phylogenetic analysis using parsimony (and other methods)*. Sinauer Associates, Sunderland, MA.
- Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22, 4673–4680.
- Tseng, C.-C., Wallace, C.C., Chen, C.A., 2005. Mitogenomic analysis of *Montipora cactus* and *Anacropora matthai* (cnidaria; scleractinia; acroporidae) indicates an unequal rate of mitochondrial evolution among Acroporidae corals. *Coral Reefs* 2005, 502–508.
- van Oppen, M.J.H., Catmull, J., McDonald, B.J., Hislop, N.R., Hagerman, P.J., Miller, D.J., 2002. The mitochondrial genome of *Acropora tenuis* (Cnidaria; Scleractinia) contains a large group 1 intron and a candidate control region. *Journal of Molecular Evolution* 55, 1–13.
- van Oppen, M.J.H., Willis, B.L., Miller, D.J., 1999. Atypical low rate of cytochrome b evolution in the scleractinian coral genus *Acropora*. *Proceedings of the Royal Society B: Biological Sciences* 266, 179–183.
- Varkey, M.J., Murty, V.S.N., Suryanarayana, A., 1996. Physical oceanography of the Bay of Bengal and Andaman Sea. *Oceanography and Marine Biology* 34, 1–70.
- Veron, J.E.N., 2000. *Corals of the world*. Australian Institute of Marine Science, Queensland, Australia.
- Veron, J.E.N., Pichon, M., 1976. Scleractinia of Eastern Australia. Part I. Thamnasteriidae, Astrocoeniidae and Pocilloporidae. *Australian Institute of Marine Science Monograph Series* 1, 1–86.
- Watanabe, T., Nishida, M., Watanabe, K., Wewengkang, D.S., Hidaka, M., 2005. Polymorphism in nucleotide sequence of mitochondrial intergenic region in scleractinian coral (*Galaxea fascicularis*). *Marine Biotechnology* 7, 33–39.
- Wolstenholme, D.R., 1992. Animal mitochondrial DNA: Structure and evolution. *International Review of Cytology* 141, 173–216.
- Yokobori, S.-i., Ueda, T., Feldmaier-Fuchs, G., Pääbo, S., Ueshima, R., Kondow, A., Nishikawa, K., Watanabe, K., 1999. Complete DNA sequence of the mitochondrial genome of the ascidian *Halocynthia roretzi* (Chordata, Urochordata). *Genetics* 153, 1851–1862.
- Zuker, M., 2000. Calculating nucleic acid secondary structure. *Current Opinion in Structural Biology* 10, 303–310.