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Seventy-four Universal Primers for Characterizing the Complete Mitochondrial Genomes of Scleractinian Corals (Cnidaria; Anthozoa)

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Mei-Fang Lin, Katrina S. Luzon, Wilfredo Y. Licuanan, Maria Carmen Ablan-Lagman, and Chaolun A. Chen (2011) Seventy-four universal primers for characterizing the complete mitochondrial genomes of scleractinian corals (Cnidaria; Anthozoa). *Zoological Studies* **50**(4): 513-524. Use of universal primers designed from a public DNA database can accelerate characterization of mitochondrial (mt) genomes for targeted taxa by polymerase chain reaction (PCR) amplification and direct DNA sequencing. This approach can obtain large amounts of mt information for phylogenetic inferences at lower costs and in less time. In this study, 88 primers were designed from 13 published scleractinian mt genomes, and these were tested on *Euphyllia ancora*, *Galaxea fascicularis*, *Fungiacyathus stephanus*, *Porites okinawensis*, *Goniopora columna*, *Tubastraea coccinea*, *Pavona venosa*, *Oulastrea crispata*, and *Polycyathus* sp., representing 7 families of complex and robust corals. Seventy-four of the 88 primers (84.1%) successfully amplified completed mt genomes of these 9 corals. Several unique features were identified, including a group I intron insertion in the cytochrome oxidase subunit I (COI) genes of *Por. okinawensis*, *Gon. columna*, *T. coccinea*, and *F. stephanus* and an extended length of the 3'-end of the COI gene of *E. ancora*. Preliminary tests using a subset of primers successfully obtained the COI 3'-end of *Euphyllia* representatives, and the resulting species phylogeny is in agreement with corallite characters and tentacle shapes. The universal primers provided herein effectively decoded scleractinian mt genomes, and can be used to reveal different levels of molecular phylogenetic inferences in scleractinian corals. http://zoolstud.sinica.edu.tw/Journals/50.4/513.pdf

Key words: Scleractinian corals, Universal primers, Mitochondrial genomes, Phylogeny.

Mitochondrial (mt) genomes of most metazoan animals consist of a circular molecule (13-20 kilobase pairs (kb)) that encodes 37 genes, including 13 protein-coding subunits, plus 2 ribosomal (r)RNA subunits, 22 transfer (t)RNA genes, and 1 major noncoding control region (Boore 1999). However, increasing evidence shows that mt genomes of lower invertebrates do not follow this pattern; specifically, mitochondria of scleractinian corals (subclass Anthozoa, phylum Cnidaria) have a number of unique characteristics. These include the existence of many noncoding regions between genes, the presence of complete stop codons in all protein-coding genes (van Oppen et al. 1999a b 2002, Fukami and Knowlton 2005, Tseng et al. 2005), fewer tRNA genes (van Oppen

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et al. 2002, Fukami and Knowlton 2005, Tseng et al. 2005), and protein-coding and RNA genes engulfed by an ND5 group I intron (van Oppen et al. 2002, Fukami and Knowlton 2005, Tseng et al. 2005, Medina et al. 2006). In some cases, novel group I introns encoding a putative homing endonuclease inserted in the cytochrome oxidase subunit I (COI) gene, an idiosyncratic ATP8, and duplicated trn*W* gene were also discovered in coral mt genomes (Medina et al. 2006, Fukami et al. 2007, Chen et al. 2008a b).

In addition to these unique molecular features, the scleractinian mt genome is estimated to be evolving 10-20 times more slowly than the "standard" molecular clock of vertebrate mt genomes (van Oppen et al. 2002), and 5 times more slowly than its nuclear scleractinian counterpart, a characteristic similar to that of flowering plants (Chen et al. 2009). It was suggested that slow evolution limits the application of mtDNA to species phylogeny and population genetics of scleractinian corals (Shearer et al. 2002, Hellberg 2006). However, exceptions were observed in some peculiar coral lineages, such as the Pocilloporidae in which mt genes or intergenic spacers (IGSs) can resolve closely related species and detect population differentiation (Chen et al. 2008a, Stenfani et al. 2008). Nonetheless, more mt genomes are needed to explore whether the exceptional features found in pocilloporids exist in other lineages of scleractinian corals.

Complete scleractinian mt genome sequences are usually obtained by a shotgun method of purified mitochondria (van Oppen et al. 1999b 2002) or by a polymerase chain reaction (PCR) of large fragments (Fukami and Knowlton 2005, Tseng et al. 2005, Medina et al. 2006), followed by cloning and sequencing processes, which are time-consuming and costly. In order to facilitate characterization of mt genomes, a series of "universal" primers which can amplify mt genomes of scleractinian corals were designed from published data. By rapidly overlapping the gene or spacer regions, this approach was successfully utilized to characterize mt genomes of fishes, birds, and insects for phylogenetic constructions (Miya and Nishida 1999, Sorenson et al. 1999, Simon et al. 2006). In this study, universal primers were designed from 13 published scleractinian mt genomes retrieved from GenBank. These primers were then subjected to a series of tests by amplifying approximately 1000 bp in length of each primer pair, allowing PCR products to be directly sequenced. Among the 88 primers designed, 74 successfully amplified the targeted mt genes or regions of 9 coral species, enabling the rapid characterization of scleractinian mt genomes and their utilization for different levels of phylogenetic constructions.

MATERIALS AND METHODS

Sample collection

Nine species of scleractinian corals, including *Pavona venosa*, *Tubastraea coccinea*, *Euphyllia ancora*, *Fungiacyathus stephanus*, *Porites okinawensis*, *Galaxea fascicularis*, *Goniopora columna*, *Oulastrea crispata* and *Polycyathus* sp., were collected to test the efficacy of the universal primers designed for the PCR and direct mt genome sequencing. *Pavona venosa*, *T. coccinea*, *E. ancora*, *Por. okinawensis*, *Gal. fascicularis*, *Gon. columna*, and *O. crispata* were collected from coral reefs or coral communities by scuba diving around Taiwan (Table 1). *Fungiacyathus stephanus* is a deep-sea species collected by bottom-trawling fishing vessels from the Dashi fishing port. *Polycyathus* sp. is a recently described species only found in shallow waters (< 3 m) of an uplifted Pleistocene reef in Chaishan, Kaohsiung, Taiwan (Lin et al. unpubl. data). These 9 species represent 7 families of robust and complex clades of scleractinian corals (Fukami et al. 2008, Dai and Horng 2009a b).

In order to examine the potential application of mt genes in a species phylogeny of scleractinian corals, 5 species of *Euphyllia*, including *E. ancora*, *E. paraencora*, *E. divisa*, *E. glabrescens*, and *E. paraglabrescens*, were collected from reefs in Chinwan Outer Bay, Taiwan (23°32'07"N, 119°32'43"E) and Talim Bay, the Philippines (14°00'15"N, 120°37'01"E).

Molecular methods

Eighty-eight primers were designed from the resulting alignment of 13 scleractinian mt genomes, including those of *Acropora tenuis*, *Agaricia humilis*, *Anacropora matthai*, *Montipora cactus*, *Por. porites*, *Pav. clavus*, *Siderastrea radians*, *Astrangia danae*, *Montastraea franksi*, *Mon. faveolata*, *Mon. annularis*, *Colpophyllia natans*, and *Mussa angulosa* (van Oppen et al. 2002, Tseng et al. 2005, Fukami and Knowlton 2005, Medina et al. 2006). These 13 coral species represent 2 diverse scleractinian groups,

Table 1. Universal primers for scleractinian mitochondrial (mt) genome amplification/sequencing. The position and predicated length were based on reference sequences of *Acropora tenuis*. Cs and Rs indicate primer pairs specifically designed from the mt genomes of complex and robust corals respectively

Primer	Sequence	Position (bp)	Length (bp)
Cs-F1	5'-AAGCCATGTTAGTTAATCGAGTG	497-519	984
Cs-R1	5'-GATCAACCCAATCGAAACTTCA	1493-1514	
Rs-F1	5'-TGAATAGAGTTGGTGACATTGGC	513-535	702
Rs-R1	5'-GAAATCAAGAATCAGCCCAATCG	1293-1215	
Cs-F2	5'-CCATTGCTTATCACAGTAGCT	1095-1115	1175
Cs-R2	5'-TAATGCATGGACAAAAAGCACC	2275-2296	
Cs-F2-a	5'-CCCTTTGATTTAACAGAAGGAGA	1657-1676	857
Cs-R2-a	5'-GACCTCAAGGTAACACATAGG	2518-2538	
Cs-F2-b	5'-CAGCTTATGGCTCTGTTATGAA	1923-1944	1121
Cs-R2-b	5'-CCTCTTAATCAACTTCGATGAAG	3048-3070	
Rs-F2	5'-GTTTGGGGGATTCTTCCTTATG	1105-1126	1085
Rs-R2	5'-CATAACCCATAAAAGCGGTCGC	2211-2232	
Rs-F2-a	5'-GATATTTACATGCTAATGGGGC	2061-2082	786
Rs-R2-a	5'-CCAATTCAAGTCAAAAGACC	2868-2889	
Cs-F3	5'-CATGTAGAGGGGTCAAATAGTCC	2730-2752	1266
Cs-R3	5'-CCAGATGAAAGTGCACCTAA	3965-3984	
Rs-F3	5'-GCTTATGCCATTTTGCGTTCAA	2698-2721	762
Rs-R3	5'-CCAATTCCACACAACAAAGCAC	3483-3504	
Cs-F4	5'-GTGGCATTAGGAAGTCTTTGT	3815-3835	1031
Cs-R4	5'-ATGGGCTAATTGCAACCATA	4866-4885	
Cs-F5	5'-TTTGGGTAAGTGGTTGGTT	4612-4630	1019
Cs-R5	5'-GAATTAGTCAAGGCGATCAGA	5642-5662	
Cs-F5-a	5'-TATGGTTGCAATTAGCCCAT	4866-4885	1190
Cs-R5-a	5'-ACTCCTAATGAAATAGCCCTG	6065-6085	
Rs-F5	5'-GTCTGGTTATAGTGCAGAAGC	3406-3427	1150
Rs-R5	5'-GCAATAGCCCCCACATAAACAAG	4577-4599	
Rs-F5-a	5'-GTTGTTTCTGCGTTAAACCCTG	4463-4484	876
Rs-R5-a	5'-ACAATAATATGAGTTGTCGGGG	5318-5339	
Cs-F6	5'-TATGATCATCTTCATGGTGTCG	5760-5781	1220
Cs-R6	5'-GGGATCAATATGCCCTCAAA	6889-6908	
Rs-F6	5'-GTCCTTATGTCTTTACCCCGAC	5180-5201	887
Rs-R6	5'-CTTCTCGAGAGCCTCAAATACC	6046-6067	
Cs-F7	5'-GGCTTTTGATTTAGAGGGACA	6612-6632	1123
Cs-R7	5'-CTGCCCCAAAACTAATTCGA	7755-7774	
Cs-F8	5'-TTATGTTGGGTGTTGTAGCTG	7304-7324	994
Cs-R8	5'-TCTGCTGGCACTTAATTTGACG	8343-8364	
Cs-F9	5'-GGACCACCTTGCTTATGATG	8188-8207	1164
Cs-R9	5'-GGATGACATAAAACAGTTCGCA	10,245-10,266	
Rs-F9	5'-GTTGCAATTTTGTTGGGGAG	6760-6779	1685
Rs-R9	5'-CAATTAAAGAATGGTGCCCTTG	8424-8445	
Cs-F10	5'-GTCGTAACATAGTGAGGGTGA	9122-9142	1110
Cs-R10	5'-TCTTGCAAACCCAAGTGTCA	11,149-11,168	
Rs-F10	5'-CATTTAGCGGAGCCTTCTCC	8247-8266	1313

Table 1. (continued)

Primer	Sequence	Position (bp)	Length (bp)
Rs-R10	5'-AAACATCCGCCCCTGTAACC	9539-9560	
Cs-F11	5'-CGAGTTGGTATTGGCATTTTG	10,959-10,979	1098
Cs-R11	5'-CGCAACCATAATAGCTAAACCA	12,086-12,107	
Rs-F11	5'-GGTTGGACATCAGTGATATTG	9361-9381	1036
Rs-R11	5'-AACCCTAACGTATCCCCTTC	10,378-10,397	
Cs-F12	5'-CCAGGGACGTTTTATGGTCA	11,707-11,726	1228
Cs-R12	5'-GACCCCGCACTTAAGAACAATA	12,944-12,965	
Rs-F12	5'-GATCGTCATTTAATCGATGG	10,177-10,196	1126
Rs-R12	5'-TAGTTCAAAACCCAAACGGG	11,284-11,303	
Cs-F13	5'-GGTAAAACAACCGGATCGAG	12,286-12,305	968
Cs-R13	5'-ACTAAATTCCAAGAACCCTCATG	13,267-13,289	
Rs-F13	5'-AGGTTTCTGCTTATGAGTGTG	11,133-11,153	1044
Rs-R13	5'-ACTTCCCAAAGCCAATATCCC	12,158-12,177	
Cs-F14	5'-ATGGGGTTTCCTTATTTAACAGG	13,072-13,094	726
Cs-R14	5'-TTGAAGGCTAACGGTCTACT	13,810-13,829	
Cs-F15	5'-CATGAGGGTTCTTGGAATTTAGT	13,267-13,289	1036
Cs-R15	5'-TAACATACTGAAGGCTGTACCG	14,192-14,213	
Rs-F15	5'-GCGGGATCTTTAATCCATGC	11,744-11,763	1322
Rs-R15	5'-AAGCATACTAAAAGCAGTCC	13,047-13,066	
Cs-F16	5'-TTAGGTTAAAGTAGACCGTTAGCC	13,801-13,824	1018
Cs-R16	5'-ATCCGTTAAAAGCATGGTTATGG	14,719-14,741	
Rs-F16	5'-GCCACAGTTAAACACAATGATG	12,751-12,772	1241
Rs-R16	5'-ACAAACCCCATAGCCCAAAG	13,973-13,992	
Cs-F17	5'-GGATGAACGGTTTATCCTCCT	14,478-14,498	1508
Cs-R17	5'-GCAGTAAAATATGCTCTTGTGTCC	15,002-15,025	
Cs-F17-a	5'-CCATAACCATGCTTTTAACGGATA	14,719-14,742	1421
Cs-R17-a	5'-TGCTAATACAACTCCAGTCAAACC	15,159-15,182	
Cs-F18	5'-GGACACAAGAGCATATTTTACTG	15,002-15,024	976
Cs-R18	5'-CTACTTACGGAATCTCGTTTGA	16,141-16,162	
Rs-F18	5'-GTTTACTGTTGGGATGGATG	13,834-13,853	1966
Rs-R18	5'-CCATTATACAAAAGGTACGC	15,781-15,800	
Cs-F19	5'-GTGAGTCATCGGGCTCATG	15,922-15,940	1065
Cs-R19	5'-ACAGTCTGTTCTACTACCAAGC	16,941-16,962	
Cs-F19-a	5'-TTAGACAATGAAGAGGTAGGCTT	16,806-16,828	873
Cs-R19-a	5'-TCCAATCCCAAAGACATAAGT	17,655-17,676	
Rs-F19	5'-CGAGATTCCGAGAGTAGTGG	15,617-15,636	1196
Rs-R19	5'-TTTCGGGACACCATTCATAC	16,794-16,813	
Cs-F20	5'-GCTTGGTAGTAGAACAGACTGT	16,941-16,962	1013
Cs-R20	5'-AACATCGAGGTCGCAAACAT	17,908-17,927	
Cs-F20-a	5'-GGTATGAATGGTGTCACGAG	17,440-17,459	724
Cs-R20-a	5'-CCCTTCAAAAAGTATCCGAGT	18,133-18,153	
Cs-F21	5'-AAAGCGTGGTAACACAGCTT	18,006-18,025	1062
Cs-R21	5'-CAACTGTGCAGACTTTCCAA	665-684	
Rs-F21	5'-CTTCGACTGTTTACCAAAAGC	16,621-16,641	962
Rs-R21	5'-CTAATTGAGCAGACTTACCC	658-677	

a complex clade and a robust clade, at the superfamily level (Chen et al. 2002, Fukami et al. 2008, Dai and Horng 2009a b, reviewed in Budd et al. 2010). These scleractinian mt genomes were aligned using the software, MEGA vers. 4.0 (Tamura et al. 2007), and conserved DNA sequence blocks were visually identified. Primers with an optimum annealing temperature of 50°C were developed using the software, FastPCR (Kalendar 2008), and totally 54 complex- and 34 robust-clade-specific primers were named Cs-F/R or Rs-F/R, respectively. Primer sequences and estimated sizes of the PCR products are given in table 1. PCR product sizes were around 1 kb in length (Fig. 1) enabling direct sequencing from both ends using Sanger's method (Sanger et al. 1977). Overlapping sequences between each adjacent primer set ranged 70-670 bp.

Total genomic DNA was extracted using a method described by Chen and Yu (2000). The PCR used 50-μl mixtures containing 10 μl of 10x reaction buffer (200 mM Tris-HCl (pH 8.4) and 500 mM KCl), 50 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μM of each primer, 2.5 units of *Taq* DNA polymerase (Invitrogen, San Diego, CA, USA), and approximately 0.5 μg of genomic DNA. Cycling conditions consisted of 3 min at 95°C; then 30 cycles of 30 s at 94°C, 45 s at 50°C, and 1.5 min at 72°C; followed by a final extension at 72°C for 10 min. The PCR was performed with a PxE Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA), and resulting products were visualized and their sizes were estimated under ultraviolet light after gel electrophoresis (Fig. 2). Subsequently, PCR products were directly sequenced using an ABI 3773 automated DNA sequencer (Applied Biosystems, Foster, CA, USA).

Sequence alignment and phylogenetic analyses

All sequences obtained in the present study were confirmed using the NCBI BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and assembled using SeqManII software (DNAstar

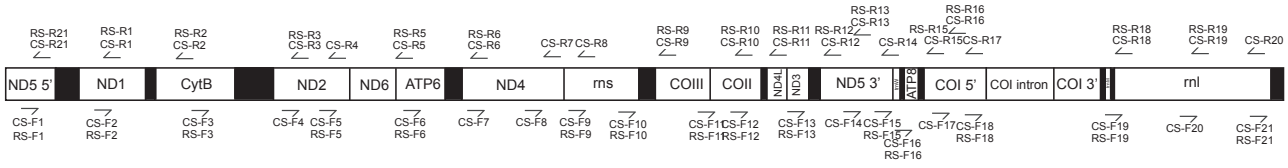

Fig. 1. Map of the scleractinian mitochondrial genome and locations of the universal primers. The direction of the primers (5' → 3') is indicated by arrows.

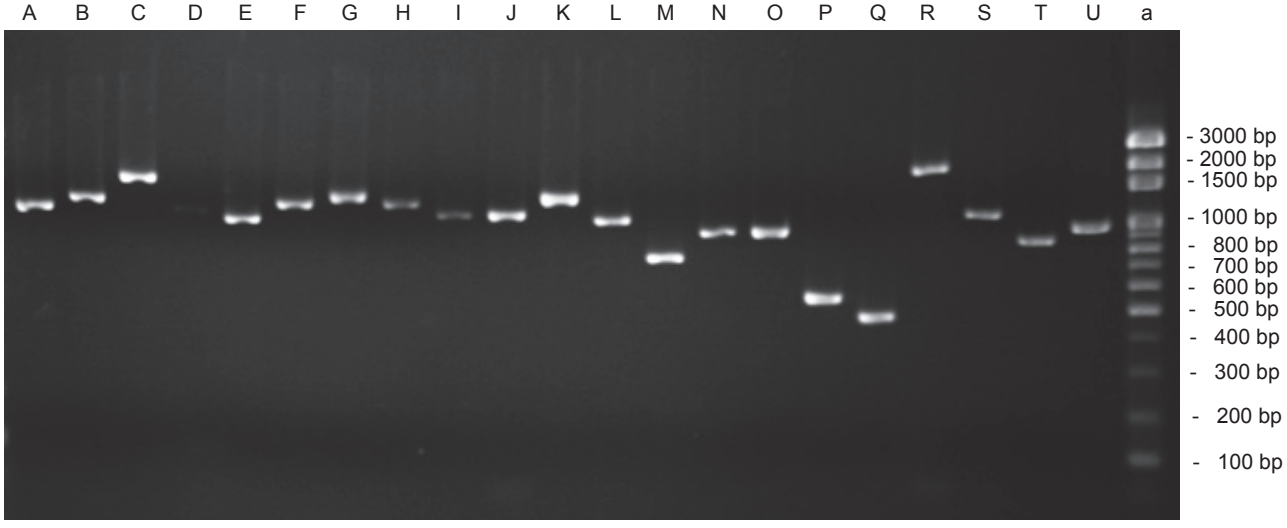

Fig. 2. One percent agarose gel electrophoresis of PCR products of *Euphyllia ancora* representing the mitochondrial (mt)DNA region from a variety of primer combinations. Lane a, 100-bp DNA ladder (BioKit); A, Cs-F1, Cs-R1; B, Cs-F2, Cs-R2; C, Cs-F3, Cs-R3; D, Cs-F4, Cs-R4; E, Cs-F5, Cs-R5; F, Cs-F6, Cs-R6; G, Cs-F7, Cs-R7; H, Cs-F8, Cs-R8; I, Cs-F9, Cs-R9; J, Cs-F10, Cs-R10; K, Cs-F11, Cs-R11; L, Cs-F12, Cs-R12; M, Cs-F13, Cs-R13; N, Cs-F14, Cs-R14; O, Cs-F15, Cs-R15; P, Cs-F16, Cs-R16; Q, Cs-F17, Cs-R17; R, Cs-F18, Cs-R18; S, Cs-F19, Cs-R19; T, Cs-F20, Cs-R20; U, Cs-F21, Cs-R21.

5.0, DNASTAR, Inc. Madison, WI, USA). Vector NTI vers. 9.0 (InforMax, Frederick, MD, USA) was used to annotate and illustrate the sequenced mt genomes. Open reading frames (ORFs) of considerable length (> 50 amino acids) were initially translated using cnidarian mt genetic codes and compared to the database using the BLAST X program (Gish and States 1993). The program, MEGA vers. 4.0 (Tamura et al. 2007), with a weighted matrix of ClustalW (Thompson et al. 1994) was used to align the identical putative ORFs, group I introns, and rRNA genes with published scleractinian mt genomes. The tRNA structure was predicted by tRNAscan-SE search server vers. 1.21 (Lowe and Eddy 1997). The mt genomes obtained in this study were deposited in GenBank with accession JF825138-JF825142.

The phylogeny of scleractinian mt genomes was constructed by aligning nucleotide sequences of 13 protein-coding genes. The best-fit model was estimated using the program MrModeltest vers. 2.3 (Nylander 2004) with the Akaike information criterion (AIC) (Akaike 1974) of a gamma distribution. A Bayesian analysis of the scleractinian family phylogeny was performed using the program, MrBayes vers. 3.1.2, with a variant of the Markov chain Monte Carlo method (Ronquist and Huelsenbeck 2003). For the phylogenetic construction of *Euphyllia* species, nucleotide sequences of the 3'-end of the COI gene were aligned using ClustalW (Thompson et al. 1994), and a phylogenetic tree was constructed based on the Neighbor-joining (NJ) algorithm with 500 bootstrapping replicates in MEGA vers. 4.0 (Tamura et al. 2007).

RESULTS AND DISCUSSION

Utility of universal primers for characterizing scleractinian mt genomes

Among the 88 primers designed in the present study, 74 (84%) successfully amplified targeted regions enabling the assembly of the mt genomes of 9 distantly related coral species. These species belong to 7 scleractinian families across the 2 major scleractinian lineages (complex and robust clades), which diverged at least 300 million years ago (Mya) (Romano and Palumbi 1996 1997, Romano and Cairns 2000).

Among the 74 primers, those 16 targeting the DNA regions of ND1-CytB, CytB-ND2, the 3'-end of ND4, COIII-COII, COI, and COI-rnl were highly conserved across both complex and robust corals, which resulted in amplification of mt genome fragments ranging 547-1254 bp long (Fig. 2). Forty-five of the 54 Cs primers (83.3%) successfully amplified the targeted DNA regions of 7 species belonging to the complex clade, including *Pav. venosa*, *T. coccinea*, *Gal. fascicularis*, *E. ancora*, *F. stephanus*, *Por. okinawensis*, and *Gon. columna*. Twenty-seven of the 34 Rs primers (79.4%) amplified the targeted DNA regions of *O. crispata* and *Polycyathus* sp., both of which belong to the robust clade. The high success rate of PCR amplifications suggests that the universal primers designed in this study have the potential to amplify mt genomes and to sequence a wide range of scleractinian taxa, including shallow- and deep-water representatives (Lin et al. unpubl. data, Kitahara et al. unpubl. data).

Molecular characteristics of scleractinian mt genomes

Complete mt genomes of *Por. okinawensis* (18,647 bp), *Gon. columna* (18,766 bp), *E. ancora* (18,875 bp), *Polycyathus* sp. (15,357), and *F. stephanus* (19,381 bp) in addition to over 10 kb of mt DNA sequences (data not shown) of *O. crispata*, *Gal. fascicularis*, *Pav. venosa*, and *T. coccinea* were obtained using the 74 universal primers, and these revealed several unique features of mt genomes of scleractinian corals (Table 1).

These 5 new mt genomes have both common and new features compared to previously published scleractinian mt genomes. The G+C contents, gene arrangement, numbers of protein-coding genes, and rRNA and tRNA genes (Table 2) were conserved in all scleractinian mt genomes (van Oppen et al. 2002, Fukami and Knowlton 2005, Tseng et al. 2005, Medina et al. 2006, Chen et al. 2008a b). Interestingly, the mt genome of *E. ancora* has an extra nucleotide of 700 bp at the 3'-end of the COI gene (amplified from the primer set of COI-rnl region). In the amino acid alignment of this gene with other scleractinians, the COI gene finishes with TAA or TAG (stop codons) between sites 542 and 551, whereas in *Euphyllia* the stop codon is at site 767.

All genes but ATP8, COI, trn*M*, trn*W*, and rnl are engulfed by the group I intron in the ND5 gene. A 2nd group I intron was found inserted in the COI gene of *Porites*, *Goniopora*, *Fungiacyathus*, and *Siderastrea* which increased their mt genomes to over 18 kb in length (Table 2). The COI intron was

Table 2. Genome size, biogeographic distribution, AT content, presence of tRNA*W* duplications, putative control region, intergenic spacers (IGSs), cytochrome oxidase subunit I (COI) intron, and GenBank accession number of scleractinian mt genomes. NA, not available, in progress

Family[1]	Species	Biogeographic distribution	Genome size (bp)	A+T (%)	tRNA*W* Duplication
Robust clade					
Caryophylliidae	*Polycyathus* sp.	North Pacific	15,357	70.9	-
Faviidae	*Oulastrea crispata*	Indo-Pacific	> 11,176	NA	-
	Colpophyllia natans	Caribbean	16,906	66.4	-
	Montastraea annularis[2]	West Atlantic	16,138	66.4	-
Mussidae	*Mussa angulosa*	Caribbean	17,245	66.3	-
Pocilloporidae	*Madracis mirabilis*	Caribbean	16,951	68.3	-
	Pocillopora damicornis	Indo-Pacific	17,426	69.8	-
	Seriatopora hystrix	Indo-Pacific	17,060	69.9	+
	Stylophora pistillata	Indo-Pacific	17,178	70.1	+
Rhizangiidae	*Astrangia* sp.	North Atlantic	14,853	68.1	-
Complex clade					
Acroporidae	*Acropora tenuis*	Indo-Pacific	18,338	62.0	-
	Anacropora matthai	Indo-Pacific	17,888	61.6	-
	Montipora cactus	West Pacific	17,887	61.6	-
Agariciidae	*Agaricia humilis*	Caribbean	18,735	59.6	-
	Pavona venosa	Indo-Pacific	> 10,050	NA	-
	Pavona clavus	Indo-Pacific	18,315	59.5	-
Dendrophylliidae	*Tubastraea coccinea*	Indo-Pacific, Atlantic	> 13,923	NA	-
Euphylliidae	*Euphyllia ancora*	Indo-Pacific	18,875	62.2	-
	Galaxea fascicularis	Indo-Pacific	> 17,408	NA	-
Fungiacyathidae	*Fungiacyathus stephanus*	Indo-West Pacific	19,381	62.2	-
Poritidae	*Goniopora columna*	Indo-Pacific	18,766	62.9	-
	Porites okinawensis	North Pacific	18,647	63.8	-
	Porites porites	Caribbean	18,648	63.7	-
Siderastreidae	*Siderastrea radians*	Caribbean	19,387	63.1	-

Family[1]	Species	Putative control region[3]	IGS (bp)	COI intron (bp)	GenBank no.
Robust clade					
Caryophylliidae	*Polycyathus* sp.	*cytb-nad2* (B)	890	-	This study
Faviidae	*Oulastrea crispata*	NA	NA	-	This study
	Colpophyllia natans	*cox3-cox2* (B)	2316	-	NC008162 (D)
	Montastraea annularis[2]	*rns-cox3* (C)	1231	-	NC007224-26 (B)
Mussidae	*Mussa angulosa*	*cox3-cox2* (B,C)	4294	-	NC008163 (D)
Pocilloporidae	*Madracis mirabilis*	*atp6-nad4* (A,B)	2120	-	NC011160 (G)
	Pocillopora damicornis	*atp6-nad4* (A,B)	2475	-	NC009797 (E)
	Seriatopora hystrix	*atp6-nad4* (A,B)	2346	-	NC010244 (F)
	Stylophora pistillata	*atp6-nad4* (A,B)	2393	-	NC011162(F)
Rhizangiidae	*Astrangia* sp.				
Complex clade		*cox1-trnM* (B)	1478	-	NC008161 (D)
Acroporidae	*Acropora tenuis*	*rns-cox3* (A,B,C)	2909	-	NC003522 (A)
	Anacropora matthai	*rns-cox3* (B,C)	2479	-	NC006898 (C)
	Montipora cactus	*rns-cox3* (B,C)	2495	-	NC006902 (C)
Agariciidae	*Agaricia humilis*	*rns-cox3* (C)	4077	-	NC008160 (D)
	Pavona venosa	NA	NA	NA	This study
	Pavona clavus	*cox1-trnM* (B)	2887	-	NC008165 (D)
Dendrophylliidae	*Tubastraea coccinea*	NA	NA	970	This study
Euphylliidae	*Euphyllia ancora*	*rns-cox3* (C)	2692	-	This study
	Galaxea fascicularis	NA	NA	-	This study
Fungiacyathidae	*Fungiacyathus stephanus*	*cytb-nad2* (B)	3858	958	This study
Poritidae	*Goniopora columna*	*cytb-nad2* (B)	3455	964	This study
	Porites okinawensis	*cytb-nad2* (B)	3354	965	This study
	Porites porites	*cytb-nad2* (B)	3348	965	NC008166 (D)
Siderastreidae	*Siderastrea radians*	*cytb-nad2* (B,C)	3862	988	NC008167 (D)

[1]Based on the classification of Dai and Horng (2009a b) and Cairns (1999). [2]The species complex includes 3 sibling species of *Montastrea annularis*, *M. franksi*, and *M. faveolata*. [3]Criteria of a putative control region: functional secondary structure, largest non-coding region, and presence of tandem repeats.

shown to encode a putative homing endonuclease in robust Indo-Pacific corals, and based on the similarity of translated amino acid sequences and insertion sites, this intron was suggested to be related to an "invasion" from sponges (Fukami et al. 2007). In contrast, the COI intron found in the 5 complex corals showed different insertion sites and low similarities to those reported from robust Indo-Pacific corals (data not shown), suggesting that the COI intron in these 2 major coral clades might have different donors and invasion histories (Chuang et al. in prep.). Nevertheless, the COI introns found in both *Por. okinawensis* (Indo-Pacific) and *Por. porites* (Caribbean) were highly conserved (with a sequence similarity of 98%), suggesting that the group I intron invasion in the COI gene of complex corals might have occurred before the separation of the Caribbean and Indo-Pacific coral fauna at least 3.5 Mya (due to the closure of the Isthmus of Panama) to 12 Mya at the closure of the Tethys Ocean (Coates et al. 1992, Veron 2008).

The mt genomes obtained in this study provide new insights into mt genome size comparisons between robust and complex corals. The COI intron and longer IGSs contributed to the larger mt genome size of complex corals. The mt genome of robust corals ranged in size from 14,853 bp (*Astrangia* sp.) to 17,426 bp (*Pocillopora damicornis*), whereas complex corals have mt genomes of 17,887 bp (*Montipora cactus*) to 19,387 bp (*Siderastrea radians*) (Table 2). The mean length of IGSs of complex corals (3219.64 ± 566.076 bp) was significantly longer than that of robust corals (2000.36 ± 956.906 bp) (Fig. 3, Mann-Whitney *U*-test = -3.25, $p < 0.001$), and the mt genome size was significantly correlated with the length of the IGS ($r^2 = 0.702$, $p < 0.001$, Fig. 4), indicating that the size of the IGSs directly reflects the mt genome size in complex corals.

The phylogenetic analysis of scleractinian corals and other anthozoans implied that there is a trend of an mt genome size reduction in robust corals (Fig. 5). Although the relationship among corallimorpharians, and complex and robust corals remains controversial (Medina et al. 2006, Fukami et al. 2008, Budd et al. 2010, Kitahara et al. 2010a), the phylogeny based on both nuclear and mitochondrial genes strongly supported the monophyly of both complex and robust corals with corallimorpharians as a sister group to scleractinians (Fukami et al. 2008, Kitahara et al. 2010a, Fig. 5). When the mt genome size was superimposed on the phylogeny, it was clear that corallimorpharians had the largest mt genomes (> 20 kb), robust corals had the smallest (14,853-17,426 bp), and complex coral mt genomes were between those 2 (17,887-19,387 bp) (Fig. 5). It was proposed that a reduction in the mt genome size through replicate advantages or metabolic efficiency was contributed by gene transfer from mitochondria to nuclei (Selosse et al. 2001). Regardless of whether or not these mechanisms have driven the reduction in mt genome sizes in scleractinians, the divergence in size between complex and robust clades during scleractinian evolution deserves further investigation.

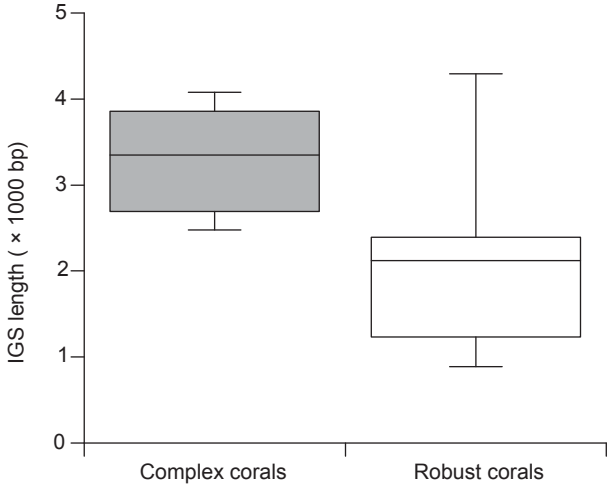

Fig. 3. Box plot of the length of intergenic spacers (IGSs) of mitochondrial genomes in the complex and robust clades.

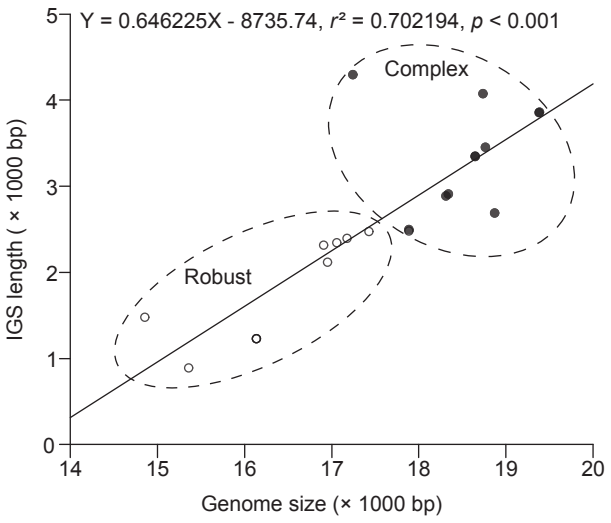

Fig. 4. Linear regression of mitochondrial genome size and intergenic spacer (IGS) length.

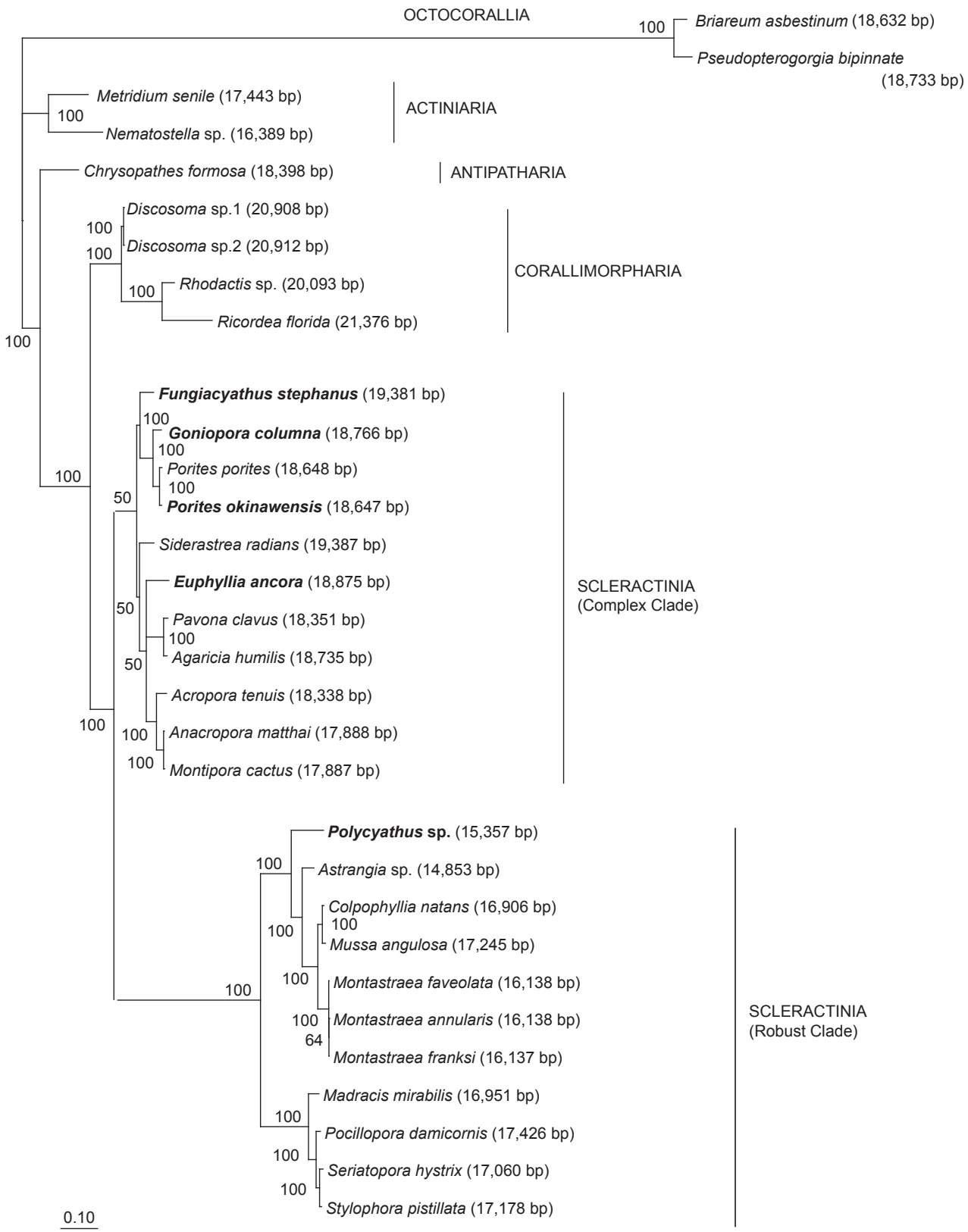

Fig. 5. Phylogenetic analyses of the mitochondrial (mt) genomes of scleractinian corals. The topology was reconstructed under the GTR+I+G model of nucleotide evolution in MrBayes. Bayesian posterior probabilities are shown at each node. The scale unit is 0.1 substitutions per site. The mt genome size of each scleractinian is indicated in parentheses after the species name.

Molecular phylogenetic inferences using mt genomes

A molecular phylogeny was constructed using nucleotide (nt) sequences of 13 protein coding genes (11,388 bp) of 22 scleractinians, 4 corallimorpharians, and selected anthozoan mt genomes available from GenBank (Fig. 5). The monophyly of complex and robust corals was strongly supported by Bayesian posterior probabilities, with corallimorpharians as a sister branch to the scleractinian lineage. This result is consistent with the tree topologies based on mt and nuclear genes (Fukami et al. 2008, Kitahara et al. 2010a b), but opposite to the topology constructed by amino acid (aa) sequences of protein-coding genes which showed a grouping of corallimorpharians and the complex clade, supporting the "naked coral hypothesis" (Medina et al. 2006). Comparison between the nt and aa phylogenies clearly showed that the latter suffered from (1) a small number of coral taxa sampled, and (2) missing "critical taxa", such as the family Poclliporidae, which is a unique monophyletic group within the robust clade. In contrast, the stability of the nt-based phylogenies increased when more taxa were sampled and critical taxa were included. In fact, when the aa sequences of pocilloporid representatives were included, the Bayesian tree supported the monophyly of scleractinian corals (Lin et al. unpubl. data). The argument of critical taxa was also supported by a recent study that included azooxanthellate scleractinians into the evolutionary reconstruction of the order, and based on the COI gene, showed that the deep-sea families, Gardineriidae and Micrabaciidae, represent a basal lineage to both complex and robust clades, supporting the monophyly of scleractinian corals (Kitahara et al. 2010b) and consequently rejecting the naked coral hypothesis (Medina et al. 2006).

The topology based on the complete mt genomes presented herein also support the phylogenetic grouping of each clade based on mt and nuclear genes (Fukami et al. 2008, Kitahara et al. 2010a b). For example, *Euphyllia*, *Siderastrea*, and *Fungiacyathus* grouped within the complex clade which is consistent with the phylogenetic tree based on COI gene, cytochrome *b* (Cyt*b*) gene, intertranscribed spacer (ITS), and β-tubulin intron (Fukami et al. 2008), but challenges radioimmunoassay results that grouped *Fungiacyathus* with corallimorpharians (Fautin and Lowenstein 1992). In addition, *Polycyathus* grouped with representatives of the robust clade (Fig. 5), corroborating the phylogeny based on the COI and 16S rRNA genes from a variety of deep-sea azooxanthellate corals (Kitahara et al. 2010a b).

Species phylogeny of *Euphyllia*

An advantage of sequencing complete mt genomes is the ability to reveal genetic variations. Additionally, it provides data enabling specific primer designs for different levels of phylogenetic inferences. However, due to its slowly evolving global characteristics, it was advocated that mt genes are of limited application for species and population diagnoses (Shearer 2002). Nonetheless, exceptional cases of rapidly evolving regions in mt genomes were recently discovered in Pocilloporidae lineages (Chen et al. 2008b). Several genes or spacer regions successfully diagnosed *Seriatopora* species (Chen et al. 2008b), differentiated populations from distinct geographic ranges (Takabayashi et al. 2003), and revealed cryptic species of *Stylophora* (Stefani et al. unpubl. data, Yang et al. in prep.).

Euphyllia is one of a few scleractinian genera for which skeletal and polyp morphology (i.e., tentacle length and shape) are together used in species identification (Veron 2000). These 5 *Euphyllia* species are colonial, with exsert septa and tubular tentacles with globular or anchor-like tips. *Euphyllia ancora* has flabelloid colonies, glabrous or finely dentate septa, and retractable tentacles with anchor-shaped tips. *Euphyllia divisa* has a skeleton identical to that of *E. ancora*, but has large tubular tentacles with smaller tubular branches. *Euphyllia paraancora*, *E. glabrescens*, and *E. paraglabrescens* all have phaceloid colonies with almost identical skeletal structures, that are only distinguished from each other by the characters of the polyp tentacles: *E. paraencora* has tubular and retractable tentacles with anchor-shaped (T-shaped) ends, and tentacle tips that often form concentric circles; *E. glabrescens* has straight, tubular, retractable tentacles with knob-like tips; and *E. paraglabrescens* has short, bubble-like, retractable tentacles (Veron 2000, Dai and Horng 2009a).

In the present study, the extra length of the COI 3'-end (of around 700 bp, amplified from the primer set Cs-F18 and Cs-R18) was selected to examine *Euphyllia* species phylogeny. A PCR survey of *E. paraencora*, *E. divisa*, *E. paraglabrescens*, and *E. glabrescens* showed the

same length for this region, suggesting that this COI gene portion is a common feature among *Euphyllia* mt genomes. The phylogenetic tree constructed based on the NJ algorithm retrieved 2 distinct groups. One was composed of *E. ancora*, *E. divisa*, and *E. paraencora*, which have flabello-meandroid (*E. ancora* and *E. divisa*) or phaceloid corallites (*E. paraencora*), and T-shaped tentacle tips (Veron 2000). The other group was composed of *E. glabrescens* and *E. paraglabrescens*, which have phaceloid corallites and elongated tentacle tips (Fig. 6). These results are in concordance with corallite characters and tentacle shapes proposed by Veron (2000), suggesting that the 3' end of the mt COI gene could be a potential marker to resolve phylogenetic relationships among *Euphyllia* species.

In conclusion, the 74 universal primers designed in the present study enabled accurate sequencing of entire mt genomes of scleractinian corals. They provide resources for future mitogenomic studies and show the potential for the use of select mt genes and mt spacer regions to examine different phylogenetic levels in scleractinian evolution.

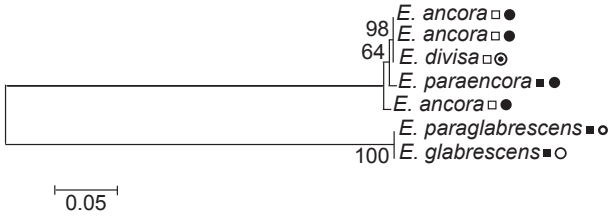

Fig. 6. Phylogenetic relationships of Euphyllia species based on the 3' end of the cytochrome oxidase I (COI) gene. The topology was reconstructed based on 500 replicates of the Neighbor-joining (NJ) algorithm using nucleotide sequences. The bootstrap support of the NJ analysis is shown at the top of each branch. Scale bar: 0.05 substitutions. ■, Colony phaceloid; □, colony flabello-meandroid; ●, tubular tentacles with T-shaped tip; ○, straight and tubular tentacles with knob-like tip; ⊙, tentacles short and bubble like; ⊛, tentacles large and tubular with small tubular branches.

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