Zoological Studies **56:** 9 (2017)
doi:10.6620/ZS.2017.56-09

Zoological Studies

Loss and Gain of Group I Introns in the Mitochondrial *Cox1* Gene of the Scleractinia (Cnidaria; Anthozoa)

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(Received 19 January 2017; Accepted 12 April 2017; Published 4 May 2017; Communicated by Benny K.K. Chan)

Yaoyang Chuang, Marcelo Kitahara, Hironobu Fukami, Dianne Tracey, David J. Miller, and Chaolun Allen Chen (2017) Group I introns encoding a homing endonuclease gene (HEG) that is potentially capable of sponsoring mobility are present in the cytochrome oxidase subunit 1 (*cox1*) gene of some Hexacorallia, including a number of scleractinians assigned to the "robust" coral clade. In an effort to infer the evolutionary history of this *cox1* group I intron, DNA sequences were determined for 12 representative "basal" and "complex" corals and for 11 members of the Corallimorpharia, a sister order of the Scleractinia. Comparisons of insertion sites, secondary structures, and amino acid sequences of the HEG implied a common origin for *cox1* introns of corallimorpharians, and basal and complex corals, but *cox1* introns of robust corals were highly divergent, most likely reflecting independent acquisition. Phylogenetic analyses with a calibrated molecular clock suggested that *cox1* introns of scleractinians and corallimorpharians have persisted at the same insertion site as that in the common ancestor 552 million years ago (mya). This ancestral intron was probably lost in complex corals around 213 to 190 mya at the junction between the Trassic and Jurassic. The coral *cox1* gene remained intronless until new introns, probably from sponges or fungi, reinvaded different positions of the *cox1* gene in robust corals around 135 mya in the Cretaceous, and then it subsequently began to lose them around 65.5 mya in some robust coral lineages coincident with the later Maastrichtian extinction at the Cretaceous-Tertiary boundary.

Key words: Group I intron, Cytochrome oxidase I, Scleractinian, Corallimorpharian, Mass extinction.

BACKGROUND

While mitochondrial genomes of anthozoan cnidarians resemble those of other animals in a number of respects, they are unique among metazoans in typically containing one or more self-splicing group I introns (Beagley et al. 1996). NADH dehydrogenase subunit 5 (*nad5*) genes of all anthozoans so far examined contain a large group I intron that may be the result of a single transfer event (Beagley et al. 1998; Boore 1999; Fukami and Knowlton 2005; Lavrov and Lang 2005; Medina et al. 2006; Tseng et al. 2005; van Oppen et al. 2002). However, in some but not all anthozoans, a second group I intron is present in the cytochrome c oxidase subunit 1 (*cox1*) gene (Lin et al. 2011; Medina et al. 2006). The *cox1* intron differs from that in the *nad5* gene in that the

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former encodes an LAGLI-DADG family homing endonuclease (HE) that is potentially capable of mobilizing the intron (Beagley et al. 1996; Fukami et al. 2007), whereas the latter does not. Group I introns containing an HE gene (HEG) can create a double-strand break at specific nucleotide sequences and invade themselves or with introns, invade other genes (Belfort 1990; Dujon 1989; Perlman and Butow 1989). A homing cyclical model of parasitic genetic elements describes the life cycle of these elements and their strategies to avoid purifying selection (Goddard and Burt 1999; Gogarten and Hilario 2006). The distribution of introns in the mitochondrial *cox1* genes of anthozoans is extremely patchy -- for example, introns are present in 13 of 41 genera (20 of 73 species) of "robust" corals, conventionally assigned to the suborder Faviina. With one exception, phylogenies of the robust coral *cox1* gene and its intron are concordant, suggesting at most 2 insertions and many subsequent losses (Beagley et al. 1996; Fukami et al. 2007).

The source(s)/donor(s) of introns in anthozoan *cox1* genes are unclear. Beagley et al. (1996) suggested a common origin from a symbiotic dinoflagellate (genus *Symbiodinium*) or endolithic fungus living in close association with corals (Bentis et al. 2000; Raghukumar and Raghukumar 1991). In contrast, a separate origin (from a sponge or fungal donor) was proposed for *cox1* group I introns in the suborder Faviina, the major group of "robust" corals (Fukami et al. 2007). While the hypothesis of 2 insertions and many subsequent losses accounts for the limited available data on anthozoan *cox1* introns, the extent to which it is more generally applicable remains to be tested, as data are available for few members of the "basal" and "complex" lineages of scleractinians or their sister group, the corallimorpharians (Fukami et al. 2008; Kitahara et al. 2010; Lin et al. 2014; Medina et al. 2006). Corallimorpharians, a small order of Anthozoa composed of 40-45 species, are morphologically similar to scleractinians but lack a calcareous skeleton, and understanding their evolutionary relationship with corals has been a challenging endeavor for decades (Fukami et al. 2008; Kitahara et al. 2010; Kitahara et al. 2014; Lin et al. 2014; Medina et al. 2006). However, recent study based on 291 orthologous single copy protein-coding nuclear genes reveals a topology consistent with scleractinian monophyly and corallimorpharians as the sister clade of scleractinians (Lin et al. 2016)

To better understand the evolutionary history of group I introns, *cox1* intron data were obtained for 12 species of Scleractinia, from 7 genera representing 6 families in the "complex" and "basal" clades (Fukami et al. 2008; Kitahara et al. 2010), and from 11 species of Corallimorpharia, including 8 genera representing 3 families. Analyses of insertion sites, primary DNA sequences, and secondary structures, together with comparisons of inferred phylogenies for introns and their host *cox1* genes, were consistent with a common origin for *cox1* introns of corallimorpharians, actiniarians, anthipatharians, and some basal and complex scleractinians, but also the loss of this intron and subsequent reinvasion of the *cox1* locus by distinct group I introns in robust corals.

MATERIALS AND METHODS

Cox1 exon and group I intron sequences

Samples and sources of *cox1* sequences used in this study are summarized in table 1. Samples were assigned to groups of corallimorpharians, and "basal", "complex", and "robust" scleractinian corals based on Fukami et al. (2008) and Kitahara et al. (Fukami et al. 2008; Kitahara et al. 2010). In addition, *cox1* genes belonging to sponges (Porifera) and other anthozoans were included in the molecular evolution analyses. Total genomic DNA was extracted using the CHAOS buffer method (Fukami et al. 2004). A primer set (COX1COMF: 5'-GGT ACG TTA TAT TTA GTA TTT GGG ATT GG-3' and COX1COMR: 5'-GGA GGA GAA ACA TGA ACC CAT TCT AAG-3') was designed to amplify complete *cox1* exon fragments. A polymerase chain reaction (PCR) was performed with the following thermal cycle: 5 min at 95°C, followed by 5 cycles of 30 s at 94°C, 30 s at 50°C, and 90 s at 72°C, and then by 30 cycles as just described but with an annealing temperature of 55°C instead of 50°C, with a final extension at 72°C for 10 min. An internal primer set (CO1in-F: 5'-CCA TGC TTT TAA CGG ATA GAA ATT-3' and CO1in-R: 5'-GCA CAT AAT GAA AAT GGG CTA CAA-3') was designed to assist DNA sequencing if an intron existed.

Sequence analysis, open reading frame (ORF), and secondary structure prediction of *cox1* group I introns

In order to examine the characteristics and origins of group I introns in scleractinians and

corallimorpharians, *cox1* exons and introns of other anthozoan orders (including the Zoantharia, Actiniaria, and Antipatharia) and Porifera, and of *Cinachyrella levantinensis* and *Plakortis angulospiculatus*, were retrieved from GenBank. In total, 94 *cox1* sequences with 42 containing group I introns were used for the following analyses. Sequence alignment of *cox1* exons and putative

Table 1. Sequence information in this study. Information includes the name, location, whether or not it contains an intron, the NCBI accession number, and the reference

		Species	Location	*cox1* intron	NCBI Accession
Porifera		*Cinachyrella levantinensis*	Israel	+	AM076987
		Plakortis angulospiculatus	Florida	+	EU237487
Octocorallia		*Acanella eburnea*	New England	-	NC_011016
		Briareum asbestinum	Florida	-	DQ640649
		Dendronephthya gigantea	Korea	-	NC_013573
		Keratoisidinae sp.	New England	-	NC_010764
		Pseudopterogorgia bipinnata	Florida	-	DQ640646
Zoanthidea		*Palythoa* sp.	Florida	+	DQ640650
		Savalia savaglia	France	+	NC_008827
Ceriantharia		*Ceriantheopsis americana*	Florida	-	DQ662399
Antipatharia		*Chrysopathes formosa*	California	-	NC_008411
		Leiopathes glaberrima	Greece	+	FJ597644
Actiniaria		*Metridium senile*	California	+	NC_000933
		Nematostella sp.	Florida	-	NC_008164
Corallimorpharia		*Amplexidiscus fenestrafer*	Taiwan	+	KP938435
		Corallimorphus profundus	Taiwan	+	KP938440
		Corynactis califirnica	Hawaii	+	KP938436
		Discosoma nummiformis	Taiwan	+	KP938434
		Discosoma sp.	Bali	+	NC_008072
		Pseudocorynactis sp.	Hawaii	+	KP938437
		Rhodactis indosinesis	Taiwan	+	KP938438
		Rhodactis mussoides	Taiwan	+	KP938439
		Rhodactis sp.	Bali	+	DQ640647
		Ricordea florida	Florida	+	NC_008159
		Ricordea yuma	Taiwan	+	KP938441
Scleractinia	Basal	*Gardineria hawaiiensis*	New Caledonia	+	GQ868677
	Complex	*Acropora tenuis*	Australia	-	NC_003522
		Agaricia humilis	Florida	-	NC_008160
		Alveopora sp.	Taiwan	-	KJ634271
		Anacropora matthai	Indonesia	-	NC_006898
		Astreopora explanata	Taiwan	-	NC_024090
		Astreopora myriophthalma	Taiwan	-	NC_024092
		Dendrophyllia sp.	Japan	+	KY887482
		Euphyllia sp.	Taiwan	-	KY887483
		Fungiacyathus stephanus	Taiwan	+	JF825138
		Galaxea sp.	Taiwan	-	KY887484
		Goniopora columna	Taiwan	+	JF825141
		Goniopora sp.	Taiwan	+	KY887485
		Isopora palifera	Indonesia	-	KJ634270
		Isopora togianensis	Indonesia	-	NC_024089
		Leptoseris cucullata	Panama	-	AB441221

Table 1. (Continued)

	Species	Location	*cox1* intron	NCBI Accession
	Montipora cactus	Taiwan	-	NC_006902
	Pachyseris sp.	Taiwan	-	KY888878
	Pavona clavus	Panama	-	NC_008165
	Porites compressa	Taiwan	+	KY888879
	Porites okinawanesis	Japan	+	JF825142
	Porites porites	Florida	+	NC_008166
	Siderastrea radians	Florida	+	NC_008167
	Pseudosiderastrea formosa	Taiwan	+	NC_026530
	Stephanocoenia michelinii	Panama	+	AB441228
	Tubastrea sp.	Taiwan	+	AB441238
Robust	*Acanthastrea echinata*	Taiwan		AB117250
	Anthemiphyllia patera	New Caledonia	-	HM018604
	Astrangia sp.	Florida	-	DQ643832
	Blastomussa wellsi	Palau	+	AB289563
	Caulastraea furcata	Japan	+	AB289579
	Cynarina lacrymalis	Pacific	+	AB289568
	Cyphastrea serailia	Japan		AB117257
	Deltocyathus suluensis	Australia	-	HM018631
	Diploastrea heliopora	Japan	+	AB289567
	Diploria clivosa	Panama	-	AB117226
	Echinophyllia aspera	Japan	+	AB289572
	Echinophyllia echinoporoides	Palau	+	AB289573
	Echinopora pacificus	Japan	-	AB117261
	Favia fragum	Brazil	-	AB117223
	Favia stelligera	Japan	-	AB117264
	Hydnophora grandis	Palau	-	AB117286
	Lobophyllia corymbosa	Japan	+	AB117241
	Madracis mirabilis	Panama	-	EU400212
	Madrepora oculata	Taiwan	-	JX236041
	Meandrina braziliensis	Brazil	-	AB117297
	Montastraea annularis	Panama	-	AB117260
	Montastraea cavernosa	Panama	-	AB117288
	Montastraea magnistellata	Japan	-	AB117279
	Mussismilia harttii	Brazil	-	AB117232
	Mycedium elephantotus	Palau	+	AB289582
	Mycetophyllia aliciae	Panama	-	AB117235
	Oulastrea crispata	Taiwan	-	AB441197
	Oulophyllia bennettae	Palau	+	AB289581
	Oxypora lacera	Palau	+	AB289571
	Paulastrea sp.	Taiwan	-	KY887486
	Pectinia paeonia	Palau	+	AB289584
	Physogyra lichtensteini	Palau	+	AB289562
	Platygyra lamellina	Japan	-	AB117282
	Pocillopora eydouxi	Taiwan	-	KY887487
	Polycyathus chaishanensis	Taiwan	-	NC_015642
	Scolymia cubensis	Brazil	-	AB117237
	Scolymia sp.	Palau	+	AB289570
	Scolymia vitiensis	Palau	+	AB289569
	Seriatopora caliendrum	Taiwan	-	NC_010245
	Seriatopora hystrix	Taiwan	-	NC_010244
	Solenastrea bournoni	Panama	-	AB117291
	Stylocoeniella sp.	Japan	-	AB441225
	Stylophora pistillata	Taiwan	-	NC_011162
	Symphyllia radians	Japan	+	AB289578
	Trachyphyllia geoffroyi	Florida	-	AB117287

ORFs were performed using MEGA 5.05 and Gblocks (Talavera and Castresana 2007; Tamura et al. 2007). ORFs were translated in Vector NTI using the *Acropora tenuis* genetic code to detect ORFs of longer than 100 amino acids in the intron (van Oppen et al. 1999). Secondary structures of group I introns were estimated using the DNA Mfold server (http://mfold.bioinfo.rpi.edu/; (Zuker 2003) and our current understanding of the group I intron recognition process (Lisacek et al. 1994). Numbers of nonsynonymous substitutions (Ka) and synonymous substitutions (Ks) were calculated using DnaSP vers. 5 software to estimate the selection forces on exon and intron sequences (Librado and Rozas 2009).

Phylogeny construction and comparisons

All *cox1* exon sequences used to reconstruct the phylogenetic relationship of scleractinian corals and the relative anthozoan taxa were listed in table 1. The best-fitting models in the maximum-likelihood (ML) analyses were evaluated using ModelTest vers. 3.7 (Posada and Crandall 1998). A general time-reversible substitution model with a proportion of invariance and gamma distribution model (GTR+I+G) of a DNA evolution model were determined using the Akaike Information Criterion (AIC) test for the ML analysis. The ML analysis with Shimodaira and Hasegawa (SH)-like branch support (Guindon et al. 2010) was conducted using the PhyML 3.0 online server. The Bayesian (BA) phylogeny was constructed using Mrbayes (Ronquist and Huelsenbeck 2003) with a substitution model evaluated with Mrmodeltest ver. 3.7 (Nylander 2004). The Bayesian tree was constructed with 6 simultaneous Markov chains for 10^7 generations with trees sampled every 1000 generations and 2500 initial trees discarded as burn-in.

In order to test the consistency of *cox1* exon and intron evolution, phylogenetic analyses of co-speciation comparisons between exons and introns were reconstructed with the ML and BA algorithms. Phylogenetic relationships of corallimorpharians, and the basal, complex, and robust clades of scleractinians were separately estimated because of the difficulty of aligning HEG sequences among basal, complex, and robust corals. The GTR+G model was determined using the AIC test for the *cox1* exon and the HEG of corallimorpharians and the basal and complex corals, while the Hasegawa, Kishino, and Yano and gamma distribution (HKY+G) model and GTR+I were determined for the *cox1* exon and HEG of robust corals.

Patristic distance correlations between exon and intron phylogenies were estimated using Mesquite vers. 2.75 to compare the similarity of tree topologies of exons and HEGs (Maddison 2011). The Kishino-Hasegawa (KH) test, Shimodaira-Hasegawa (SH) test, and Approximately Unbiased (AU) test were conducted in the Consel program to estimate the confidence level of topological differences (Shimodaira and Hasegawa 2001).

Molecular dating of the *cox1* exon tree

The divergence time of every clade was calculated using Beast vers. 1.6.1 which allows a relaxed molecular clock among different lineages (Drummond and Rambaut 2007). The Yule birthrate process was chosen as prior, and the distribution of the divergence on each node was set to a normal distribution with a 5% standard error. The GTR+I+G model was selected as the most appropriate evolutionary model to evaluate likelihood ratio tests for molecular clock estimates. In total, 5×10^8 generations were performed and saved every 5×10^4 generations to calculate their phylogenetic relationships. The first 2500 of 10^4 topologies were discarded as burnin, while the remainder was saved to calculate posterior probabilities. Four reference points were chosen to evaluate the divergence time of each clade. Time of reference points were as follow: Dendrophylliidae (127 million years ago, mya), *Acropora* (59 mya), *Stylophora* (68 mya; (Baron-Szabo 2006), and *Astrangia*/*Solenastrea* (70 mya). In order to avoid over-evaluation of time, we constrained the origin of scleractinians to 460 mya (Stolarski et al. 2011).

RESULTS

Molecular characteristics of group I introns in *cox1* loci

Lengths of introns, putative open reading frames (ORFs), noncoding regions, locations and start/stop codons of ORFs in scleractinians, corallimorpharians, and other members of the Anthozoa and Porifera are summarized in table 2. Eleven species, representing 8 genera in 3 families of corallimorpharians all contained the *cox1* intron. Among scleractinian corals, the “basal” coral, *Garderneria hawawiiensis*, and 11 “complex” coral

Table 2. Molecular characteristics of *cox1* introns and their open reading frames (ORFs), including the length of the intron, length of the ORF, length of the non-coding region, location of the ORF in the *cox1* intron, and its start/stop codon according to predictions from Kitahara et al. (2014) and Lin et al. (2014)

Taxon	Length of Intron	Length of ORF	Length of Noncoding	Location of ORF	Start/Stop Codon
Porifera					
Cinachyrella levantinensis	1143	1029	114	1-1029	TTA/TAA
Plakortis angulospiculatus	1503	1038	195	359-1396	TTT/TAA
Zoanthidea					
Palythoa sp.	1308	747	561	391-1137	ATG/TAA
Savalia savaglia	1250	723	527	441-1163	ATG/TAG
Actiniaria					
Metridium senile	853	672	181	155-826	ATG/TAA
Corallimorpharia					
Amplexidiscus fenestrafer	1206	1005	201	71-1075	ATA/TAA
Corallimorphus profundus	1182	786	396	47-832	ATA/TAG
Corynactis califirnica	1265	1008	257	71-1078	ATA/TAG
Discosoma nummiformis	1208	711	497	71-781	ATA/TAA
Discosoma sp.	1206	1005	201	71-1075	ATA/TAA
Pseudocorynactis sp.	1177	993	184	71-1063	ATA/TAA
Rhodactis indosinesis	1204	726	478	71-796	ATA/TAA
Rhodactis mussoides	1206	1005	201	71-1075	ATA/TAA
Rhodactis sp.	1206	1029	177	71-1075	ATA/TAA
Ricordea florida	1215	1017	198	90-1106	ATA/TAA
Ricordea yuma	1198	975	223	110-1084	GTG/TAA
Scleractinia (Basal complex group)					
Gardineria hawaiiensis	1140	981	159	48-1028	ATA/TAA
Scleractinia (Complex group)					
Dendrophyllia sp.	972	831	141	49-879	ATA/TAA
Fungiacyathus stephanus	970	828	142	48-875	ATA/TAA
Goniopora sp.	970	831	139	48-878	ATA/TAA
Porites porites	971	831	140	48-878	ATA/TAA
Porites compressa	970	831	139	48-878	ATA/TAA
Siderastrea radians	994	855	139	48-902	ATA/TAA
Pseudosiderastrea formosa	970	855	115	48-902	ATA/TAA
Stephanocoenia michelinii	940	792	148	47-838	ATA/TAG
Tubastrea sp.	970	831	139	48-878	ATA/TAA
Scleractinia (robust group)					
Blastomussa wellsi	1107	933	174	49-981	ATA/TAA
Caulastraea furcata	1128	1005	123	27-1031	ATA/TAA
Cynarina lacrymalis	1078	933	145	49-981	ATA/TAA
Diploastrea heliopora	1076	933	143	49-981	ATA/TAA
Echinophyllia aspera	1077	954	123	27-980	ATA/TAA
Echinophyllia echinoporoides	1077	954	123	27-980	ATA/TAA
Lobophyllia corymbosa	1077	954	123	27-980	ATA/TAA
Mycedium elephantotus	1128	1005	123	27-1031	ATA/TAA
Oulophyllia bennettae	1128	1005	123	27-1031	ATA/TAA
Oxypora lacera	1078	933	145	49-981	ATA/TAA
Pectinia paeonia	1128	1005	123	27-1031	ATA/TAA
Physogyra lichtensteini	1077	933	144	49-981	ATA/TAA
Scolymia sp.	1120	996	124	27-1022	ATA/TAA
Scolymia vitiensis	1078	933	145	49-981	ATA/TAA
Symphyllia radians	1077	954	123	27-980	ATA/TAA

species, representing 7 genera of 6 families, had introns inserted at the same nucleotide position (nt) 893 in *cox1* (herein called I893) as those of corallimorpharians, actinarians, and antipatharians (Table 3). In "robust" corals, 15 species, representing 13 genera of 4 families, had introns inserted at nt position 729 in *cox1* (herein called I729), which is identical to the insertion position of sponges (Table 3). In zoanthids, *cox1* introns were inserted at nt position 876 (herein called I876).

Lengths of introns and ORFs ranged from 853/672 bp in the actiniarian, *Metridinum senile*, to 1503/1039 bp in the sponge, *Plakortis angulospiculatus* (Table 2). Intron and ORF lengths of "complex" corals were significantly shorter than those of "robust" corals (Mann-Whitney U-test $p < 0.01$) but no significantly different between complex corals and corallimorpharians (Mann-Whitney U-test, $p = 0.09$). Start and stop codons of the *cox1* intron ORF were ATA and TAA, respectively, for most corallimorpharians and scleractinians, except those of *Ricordea yuma* (start codon = GTG) and *Stephenoconia* sp. (stop codon = TAG). An ORF composed of a specific domain of the LAGLI-DADG HE was identified, although nucleotide sequence alignments among different anthozoans were low. As observed by Fukami et al. (2007), amino acid sequences of the HE in robust corals were highly similar to those of sponges (mean p-distance = 22.2%, Table 4) (Fukami et al. 2007). However, they were highly divergent compared to those of basal, complex, and corallimorpharians (mean p-distance = 83.8%), whereas the latter three possessed relatively similar HEs (mean p-distance = 43.2%).

Secondary structures of the *cox1* intron for sponges and 7 anthozoans were predicted to confirm its identification in group I based on the consensus primary structure (Fig. 1). All *cox1* introns contained 4 consensus primary structures (P, Q, R, and S) in core structures of group I intron and 9 or 10 paired regions of helices. Paired regions of the secondary structure also reflected the relationship based on primary sequences of introns. For example, I729 of both robust corals and the sponge had lost the P2 helix (Figs. 1A, B). Structures and positions of P1 to P9 were highly similar among I893s of basal and complex

Table 3. Insertion sites of the *cox1* intron in different groups of anthozoans and a sponge. Double arrows indicate insertion sites of the intron

Taxa	Sequence	type
Location	729 876 893	
Zoanthidea		
Palythoa sp.	GCCAT CCGGAGGTTT [149 nt] TGTGTGGGCT→←CACCACATGTTTACAGT - AGGGA	I876
Savalia savaglia	GCCAT CCGGAGGTTT [149 nt] TGTGTGGGCT→←CACCACATGTTTACAGT - AGGGA	I876
Actiniaria		
Metridium senile	GGCAT CCGGAAGTTT [149 nt] TGTGTGGGCA CATCACATGTTTACGGT→←TGGAA	I893
Antipatharia		
Leiopathes glaberrima	GCCAC CCAGAGGTTT [149 nt] TGTGTGGGCT CATCACATGTTCACGGT→←TGGAA	I893
Corallimorpharia		
Ricordea florida	GACAT CCAGAGGTAT [149 nt] TGTGTGGGCA CACCATATGTTTACGGT→←TGGAA	I893
Scleractinia		
Gardineria hawaiiensis	GGCAT CCCGAAGTTT [149 nt] TGGGTGGGCC CATCATATGTTTACGGT→←TGGAA	I893
Siderastrea radians	GGCAT CCAGAAGTTT [149 nt] TGTGTGGGCC CACCATATGTTTACGGT→←TGGGA	I893
Diploastrea heliopora	GGCAT→←CCTGAAGTTT ~	I729
Sponge		
Cinachyrella levantinensis	GGCAT→← CCAGAAGTTT [149 nt] AGTTTGAGCC CATCACATGTTTACAGT TGGAA	I792

corals and corallimorpharians except for the P5 region (Figs. 1C, D). I893s of antipatharians and actiniarians possessed similar secondary structures to corallimorpharians (Fig. 1E), whereas I876 of zoanthiarians had a branched form of the P9.1 paired region representing a unique type of *cox1* intron compared to other anthozoans (Fig. 1F).

Evolutionary rates of the exon and HEG

To evaluate selection forces on the exon and HEG of the intron, we estimated the rate of nonsynonymous substitutions (Ka) versus synonymous substitutions (Ks) in exons and HEGs of corallimorpharians, complex corals, and robust corals. Similar small ratios were observed among *cox1* exons of corallimorpharians and scleractinians, while larger differences were found in comparisons of HEGs among corallimorpharians and scleratinians (Fig. 2, Ka/Ks = 0.03-0.045 on average for *cox1* exons; Ka/Ks = 0.254-0.489 on average for HEGs). Both the *cox1* exon and HEG deviated from neutral variations (Ka/Ks = 1). A lower nonsynonymous ratio of substitutions for the *cox1* exon suggested that more-purified selection of the *cox1* exon was stronger than that of the HEG.

Co-evolution of the *cox1* exon and intron in scleractinians and corallimorpharians

Results of the phylogenetic analyses are summarized in figure 3. Octocorals were used as outgroups for the phylogenetic analyses because of the sister group relationship between hexacorallians and octocorallians. Both the ML and BA analyses strongly supported the monophyly of the Scleractinia composed of “basal”, “complex”, and “robust” clades as proposed by Kitahara et al. (2010) and Stolarski et al. (2011). Corallimorpharian genera were grouped into a monophyletic clade, except for *Corallimorphus profundus* which formed a basal clade next to the Scleractinia, although statistical supports of the MA and BA to this node were not relatively high (86/61).

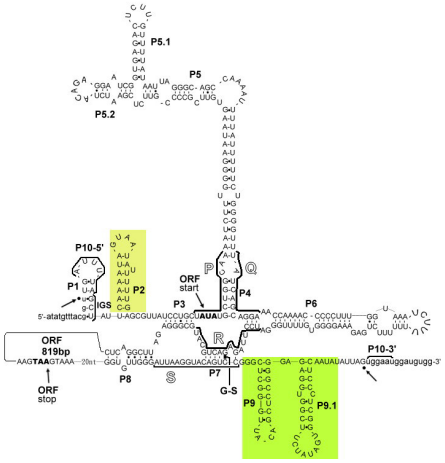
Mapping the occurrence of *cox1* introns onto the phylogenetic tree showed that I893 appeared in all genera of the order Corallimorpharia, the basal scleractinian, *Gardineria hawaiinensis*, and *Fungiacyanthus*, *Porites*, *Goniopora*, *Turbinaria*, *Dendrophyllia*, *Siderastrea*, *Pseudosiderastrea* and *Stephanocoenia* of the complex clade of the Order Scleractinia (herein called complex I) (Fig. 3). I893 was absent from the other lineage, complex II, which contained genera of the families Euphyllidae, Acroporidae, and Agaricidae. I729, in contrast to the distributional pattern of I893 in corallimorpharians, and basal and complex coral clades, had a sporadic but restricted distribution in the robust clade of Pacific scleractinian corals (Fig. 3).

Co-evolution tests of *cox1* exons and introns were separately conducted for corallimorpharians, basal and complex corals (Fig. 4A), and robust corals (Fig. 4B) due to the high divergence of primary DNA sequences between I893 and I729. *cox1* exon and I893 phylogenies were largely congruent in corallimorpharians, and basal and complex corals (Patristic distance correlation = 0.96). The difference was in positions of *Corallimorphus profundus* and *Porites compressa* between these 2 trees (Fig. 4A). In contrast, in the case of robust corals, exon and intron phylogenies substantially differed (Patristic distance correlation = 0.67), suggesting significantly different evolutionary histories for I729 and the *cox1* exons in the robust clade (Fig. 4B, Table 5). To test the co-evolution of the exon and intron, the AU, KH, and SH tests were conducted to examine the congruence of the exon and intron phylogenetic trees. Statistical results of the AU, KH and SH

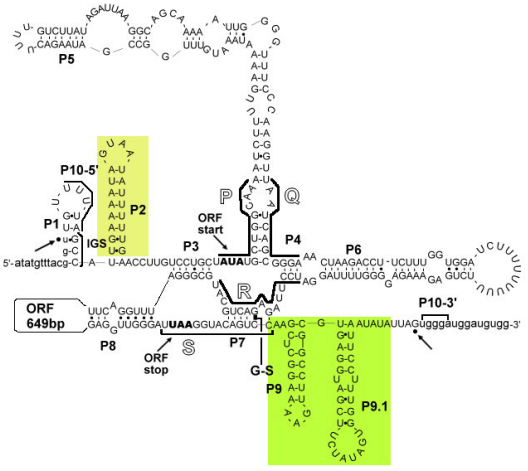
Table 4. Genetic distances of the homing endonuclease gene (HEG) among different organisms. P-distance comparisons of HEG amino acid sequences among different groups of organisms. Distances are listed as percentages (%). nc, not compared

Group	Basal and complex corals	Robust corals	Corallimorpharians	Actiniarians	Sponges
Basal and complex corals	20.6				
Robust corals	82.6	6.9			
Corallimorpharians	43.2	81.5	2.1		
Actiniarians	66.0	82.4	65.1	nc	
Sponges	83.8	22.2	81.2	83.3	19.2

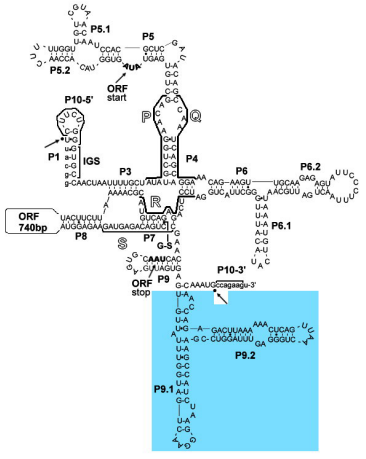
(A)Corallimorpharian



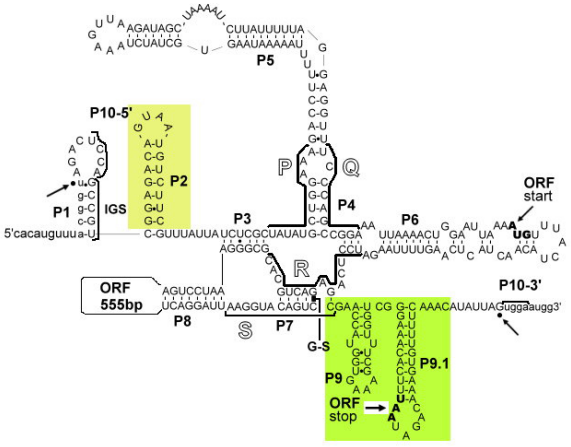
(B)Complex corals



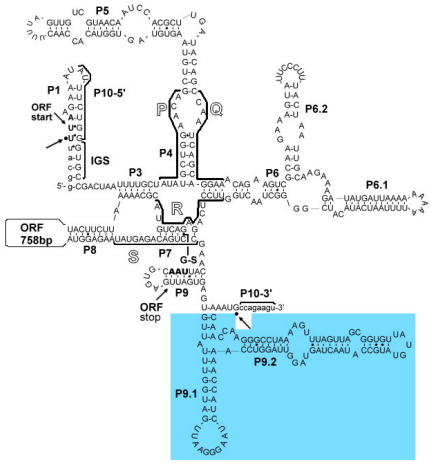
(C)Robust corals



(D)Actiniaria



(E)Porifera



(F)Zoanthids

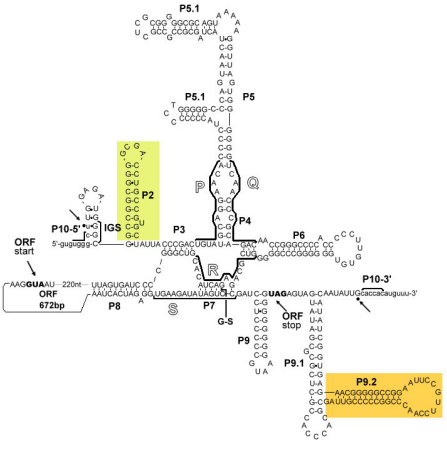
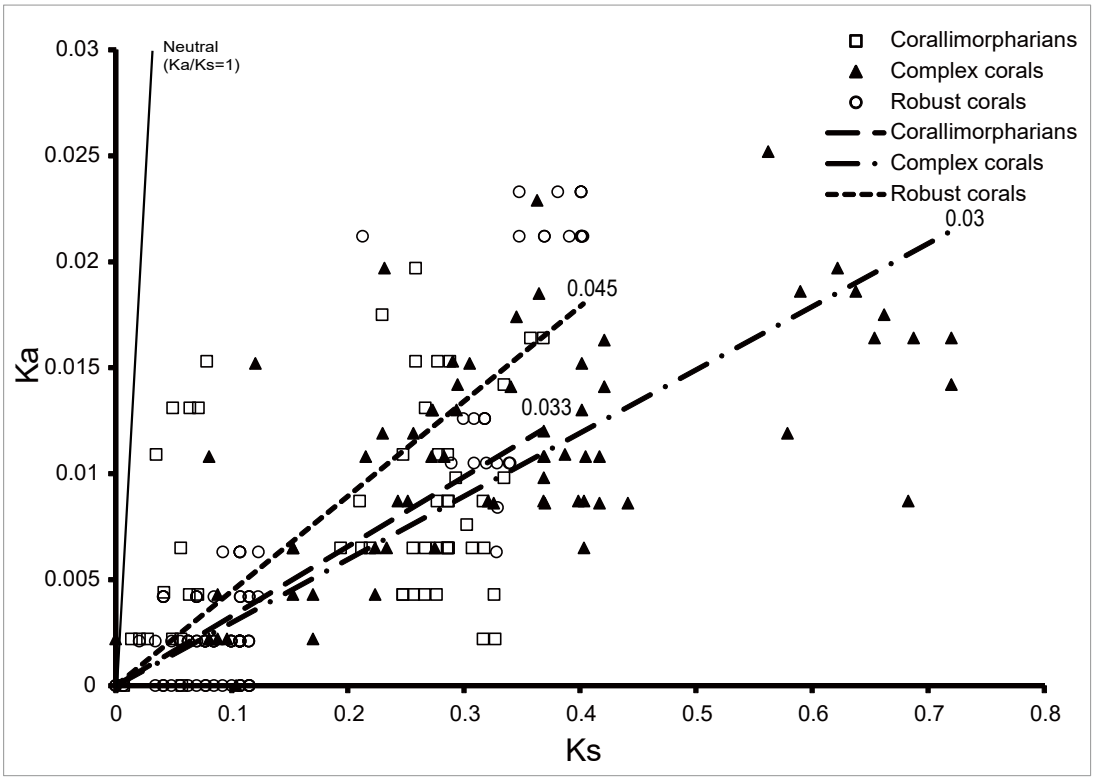


Fig. 1. Secondary structures of representative *cox1* introns in anthozoans. A: Corallimorpharian (*Rhodactis howesii*); B: basal and complex corals (*Gardeneris hawaiinesis*); C: robust corals (*Diploastrea heliopora*); D: actiniarian (*Metridinium senile*); E: poriferian (*Plakortis angulospiculatus*); F: zoantharian (*Savalia savaglia*). Features of the secondary structure indicate the characteristics of group I introns: 10 helical elements P1~P10; consensus primary structures P, Q, R, and S in hollow letters; internal guide sequence, IGS. Initial and terminal sites of the predicted open reading frame are labeled “ORF start” and “ORF stop”, respectively.

(A)*Cox1* Exon



(B)*Cox1* Intron

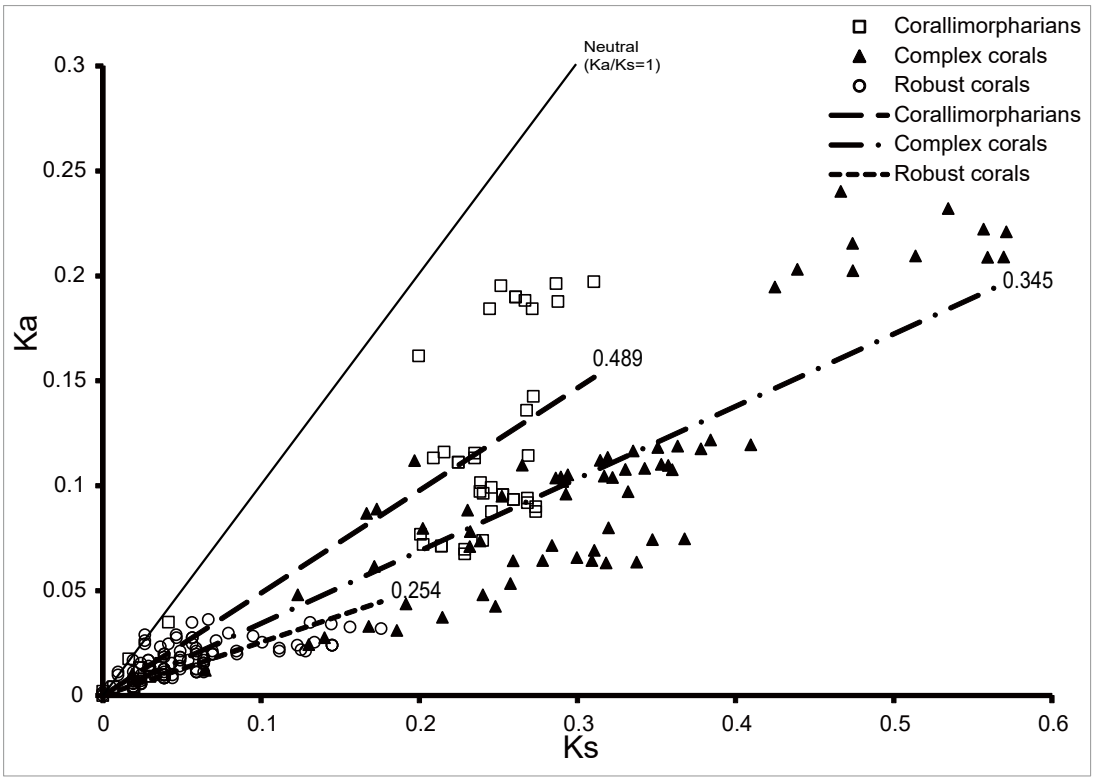


Fig. 2. Ka/Ks comparisons in *cox1* exons and introns among corallimorpharians, complex corals, and robust corals. Values behind the each line show average values in each group, indicating the level of selective forces on each group.

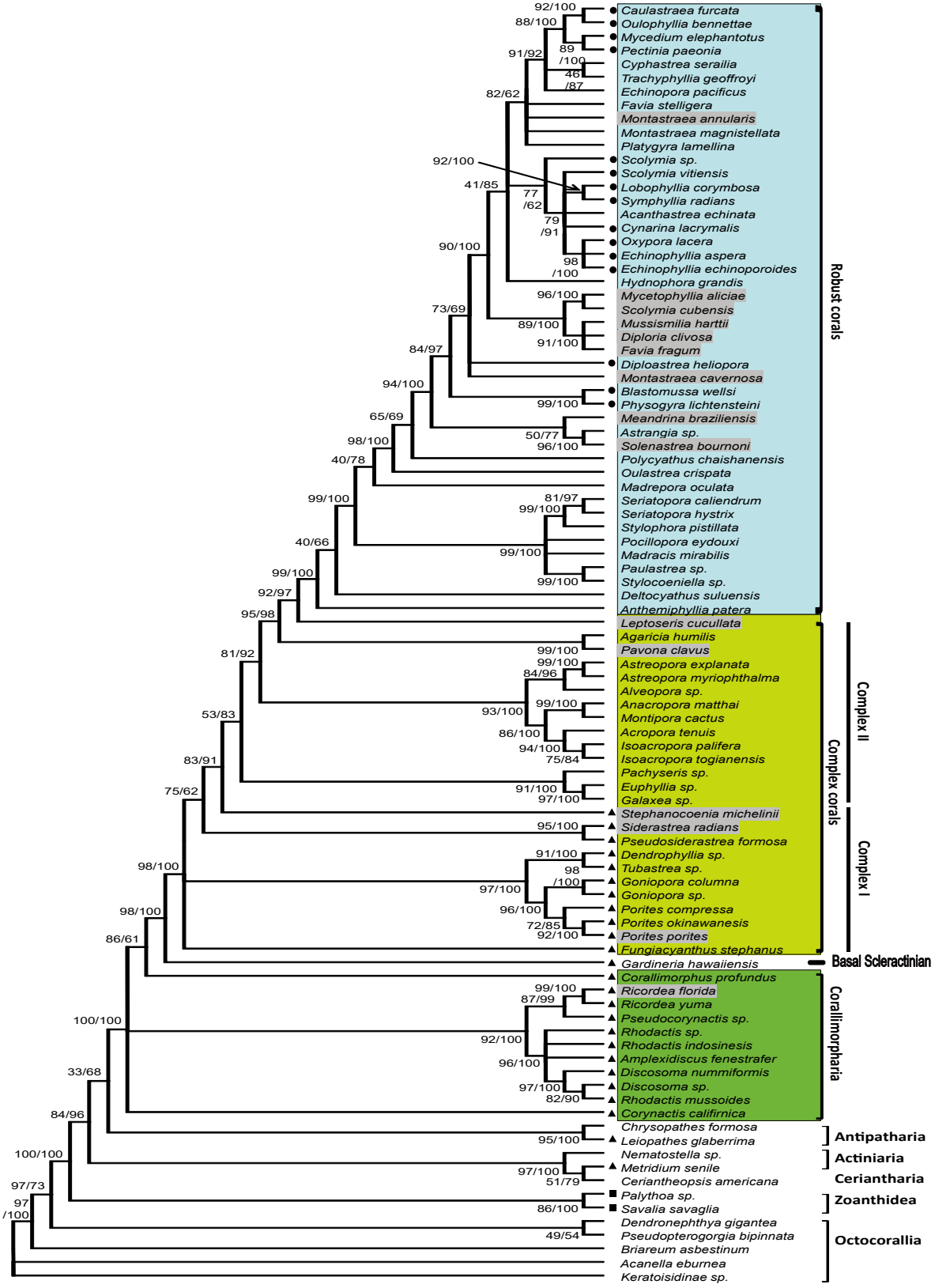


Fig. 3. Phylogeny and characteristics of *cox1* intron traits in hexacorals. The tree topology was constructed with Mrbayes. Numbers labeled on branches are Shimodaira-Hasegawa-like support/posterior probabilities. Species with different types of introns are labeled with symbols: ●, Intron-729 (I729); ▲, Intron-893 (I893); ■, Intron-876 (I876).

tests indicated that tree topologies of the exon and intron significantly differed in robust corals but were consistent in corallimorpharians and complex corals (Table 5).

Molecular clock estimates for intron loss and gain events

Molecular dating based on the *cox1* phylogeny is summarized in figure 5 and table

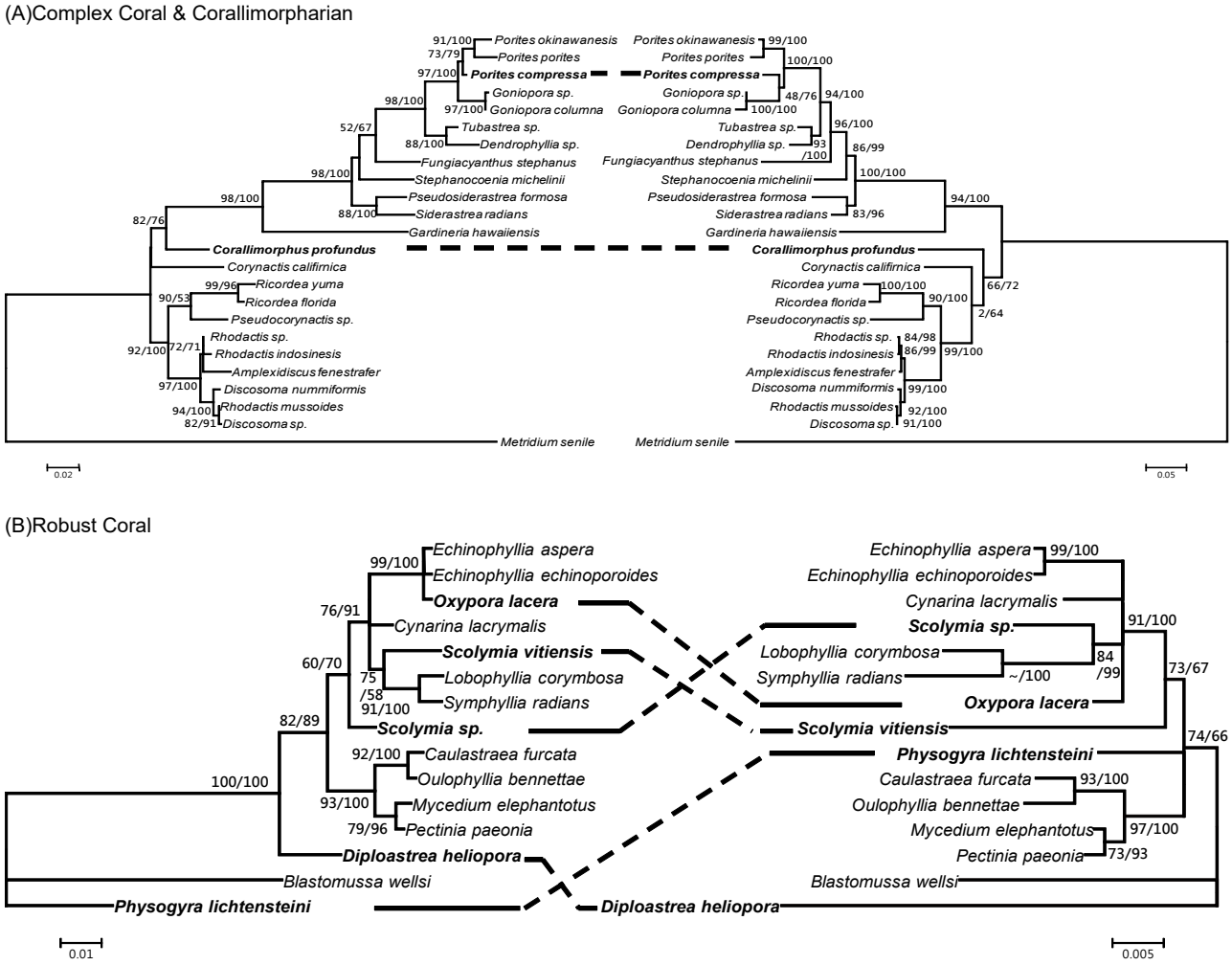


Fig. 4. Comparison of phylogenetic trees between the *cox1* exon (left side) and intron (right side) in complex corals and corallimorpharians (A) and in sponges and robust corals (B). Tree topologies presenting the phylogenetic relationships of exons and introns were consensus trees between the maximum-likelihood analysis and Bayesian algorism. Numbers on branches are Shimedaira-Hasegawa-like/posterior probabilities. Dashed lines are potential changes in phylogenetic positions between the exon and intron trees.

Table 5. Comparisons of *cox1* exon-intron phylogenies. Patristic distance correlations (PDCs) between exon and intron trees were calculated, and the Kishino-Hasegawa (KH), Shimodaira-Hasegawa (SH), and Approximately Unbiased (AU) tests were conducted to estimate the confidence level of topological differences in Mesquite vers. 2.75. ** $p < 0.001$

	Similarity	Statistical test between exon and intron phylogenetic trees		
	PDC	AU	KH	SH
Complex corals and corallimorpharians	0.96	0.243	0.258	0.258
Robust corals	0.67	0.00004**	0.0001**	0.0001**

6. The Corallimorpharia was derived about 552 mya at the end of the Precamrian. The first scleractinian, represented by the "basal" coral, *Gar. howaiinesis*, was derived from a common ancestor of corallimorpharians around 460 mya. "Complex" and "robust" corals were derived from a common ancestor of basal corals around 423 mya during the end of Silurian, whereas robust corals split from complex corals around 358 mya towards the end of the Devonian and beginning of the Carboniferous.

Our results clearly showed that corallimorpharians and scleractinians shared a single origin for the *cox1* intron and subsequently lost and then regained it during evolution by mapping the possession of group I introns onto the Bayesian phylogeny (Fig. 5) and similarities among group I introns (Table 4). I893 already existed in the common ancestor of the corallimorpharian-scleractinian lineage back to 552 mya. All 11 corallimorpharians, ancestors of scleractinians, including both zooxanthellate and azooxanthellate lineages, possess I893. I893 was also observed in the basal coral, *Garderneria howawiinensis*, and complex I corals. Around 213 to 190 mya at the junction between the Triassic and Jurassic, I893 was lost from complex II corals and most robust corals. I729 independently re-invaded several robust corals later at around 130 mya towards the end of the Cretaceous. The inconsistence between exon and intron phylogenies in Robust corals supported the possibility of multiple insertions of I729 (Fig. 4).

DISCUSSION

In this study, *cox1* introns were observed in 12 of 23 species belonging to "basal" and "complex" corals, and 11 species of corallimorpharians. Compared to published *cox1* introns in "robust" corals, these new *cox1* introns suggest that a single origin occurred in the common ancestor of scleractinians and corallimorpharians, and was subsequently lost in complex II corals and most robust corals before re-invasion by new introns from other donor sources, such as sponges or fungi (Fukami et al. 2007).

Evolutionary history of group I introns in scleractinian corals

The "single-origin" scenario of *cox1* introns in scleractinians was supported by several aspects: (i) the insertion site in actiniarians, corallimorpharians, and basal and complex corals was the same, but differed from those of robust corals, which have the same insertion site as sponges (Fukami et al. 2007); (ii) amino acid sequence dissimilarities of HEGs among actiniarians, corallimorpharians, and basal and complex corals were significantly lower (0.43-0.66) than those compared to robust corals and sponges (0.812-0.838), whereas the latter two were highly similar (0.222); (iii) secondary structures of *cox1* introns were similar among actiniarians, corallimorpharians, and basal and complex corals, but differed from those of robust corals and sponges; and (iv) I893 had an ancestral status in the *cox1* gene of all 8 existing corallimorpharian genera and 12 of 14 genera surveyed in actiniarians for over 500 my (Goddard et al. 2006) (Fig. 5). Fukami et al. hypothesized, based on comparisons of intron sequences between the suborder Faviina of robust corals and actiniarians, that both scleractinians and actiniarians independently acquired *cox1* group I introns. However, this hypothesis was not supported because key taxa, such as

Table 6. Results of divergence time estimates. Information includes the mean, 95% highest posterior density interval (HPD lower-upper), effective sample sizes (ESSs), and the posterior probability of each major clade

MRCA	Event	Mean	95% HPD lower-upper	ESS	Posterior probability
A	Origin of corallimorpharians and scleractinians	552.28	473.01-666.97	629	69.24
B	Origin of the *Gardineria* group	460.12	456.14-464.05	4124	98.33
C	Origin of scleractinians	423.04	309.73-463.97	281	1
D	Origin of robust corals	358.31	378.14-461.46	482.53	1
Intron Loss	I893 intron loss	213.02	129.97-290.02	483.26	0.55
Intron Insertion	1st I729 intron insertion	135.38	90.39-186.34	890.99	0.95

MRCA, most-recent common ancestor.

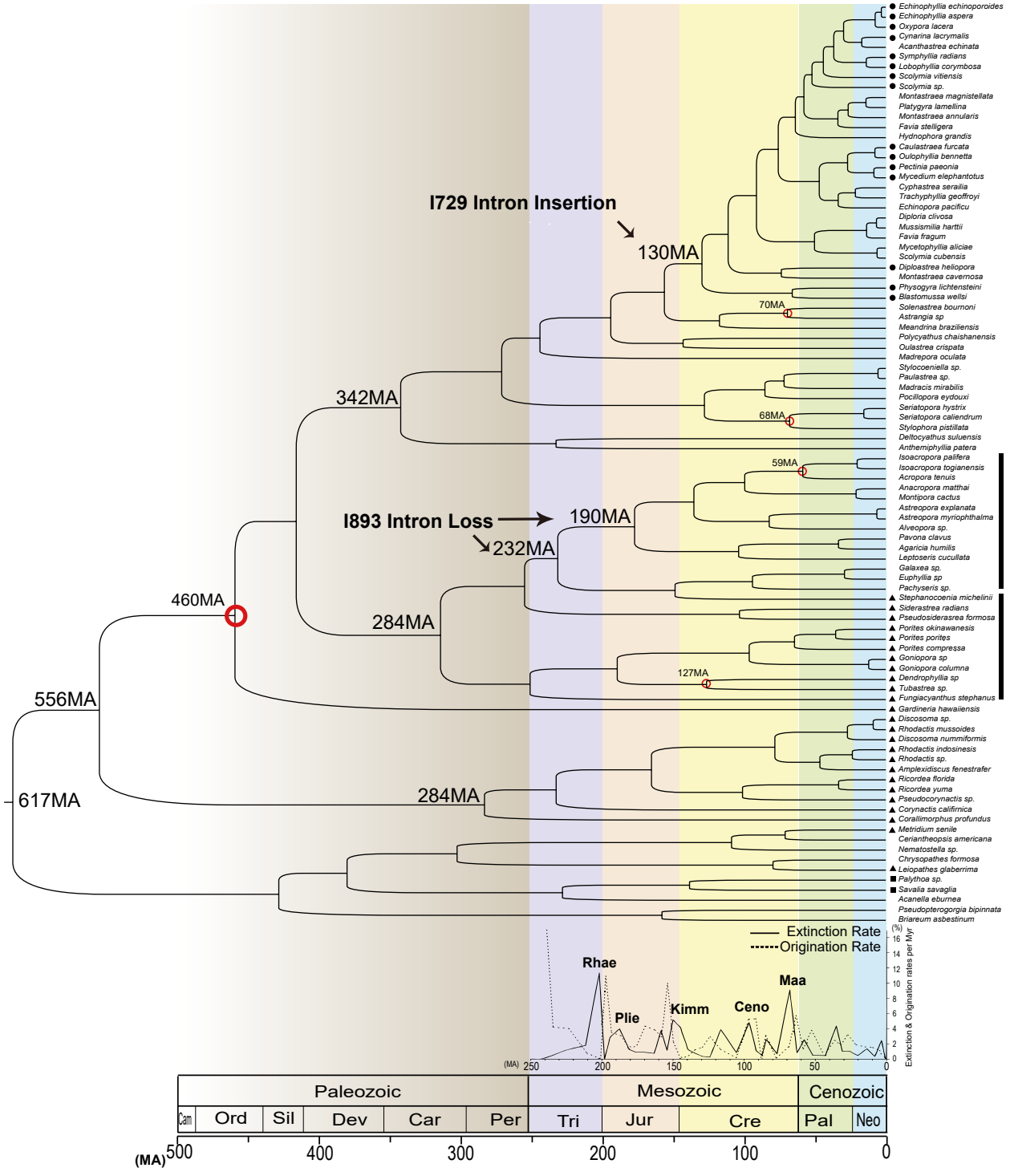


Fig. 5. Bayesian estimates of divergence times in scleractinians. The basal axis is a geologic time scale in units of million years ago (mya). Different time intervals are labeled with abbreviations (Cam, Cambrian; Ord, Ordovician; Sil, Silurian; Dev, Devonian; Car, Carboniferous; Per, Permian; Tri, Triassic; Jur, Jurassic; Cre, Cretaceous; Pal, Paleogene; Neo, Neogene). The chart below the phylogenetic tree gives the extinction rate (solid line) and origination rate (dashed line) in different geological periods, which were modified from Kiessling (2004) with major extinction events labeled with abbreviations (Rhae, Rhaetian; Plie, Pliensbachian; Kimm, Kimmeridgian; Ceno, Cenomanian; Maa, Maastrichtian, KT-extinction). Species with different types of intron are labeled with symbols: ●, Intron-729 (I729); ▲, Intron-893 (I893); ■, Intron-876 (I876).

corallimorpharians and basal and complex corals, were not examined by Fukami et al. (Fukami et al. 2007).

Regardless of the details of the history of introns in *cox1* genes of corals, the sources of coral introns, and anthozoan introns in general, continue to be debated. Sponge (*e.g.*, *Tetilla* sp.) or fungal (*e.g.*, *Smittium culisetae* and *Schizosaccharomyces octosporus*) *cox1* introns were proposed as potential donors to I729 of robust corals based on DNA similarities (Fukami et al. 2007). Whether I729 of robust corals directly invaded from sponges or fungi or indirectly via fungi from sponges or *vice versa* remains unsettled. In contrast, I893 of actiniarians has the highest similarity compared to the fungus, *Neurospora crassa* (Dalgaard et al. 1997; Goddard et al. 2006), suggesting that I893 might have come directly from fungi via homing and horizontal transfer events (Goddard et al. 2006). However, the possibility of other donors (*e.g.*, dinoflagellates) still cannot be excluded because of the complicated symbiotic system in corals (Amend et al. 2012; Baker 2003; Schonberg and Wilkinson 2001).

Our Ka/Ks evolutionary analysis revealed that purifying selection was the predominant force shaping the evolution of *cox1* and HEG of introns (Fig. 2). This observation is consistent with previous observations in the Anthozoa (Emblem et al. 2014) of central roles of most mitochondrial (mt)DNA protein products in the fundamental biological process of the mitochondrial electron transport chain function. However, our analysis also demonstrated that the HEG experienced faster rates of molecular evolution as expressed by Ka/Ks ratios and accelerated raw Ka values. Although inflated relative to other genes (such as *cox1*), calculated HEG Ka/Ks ratios were all < 1, suggesting relaxation of purifying selection rather than positive selection acting on these sequences. Similar findings were also documented in an analysis of actinarian mitochondrial genomes (Emblem et al. 2014).

Results of co-evolutionary comparisons imply that the *cox1* intron was stably persistent in the ancestor of corallimorpharians and basal and complex corals, but not that of robust corals. Previous studies also suggested that group I introns had a low rate of lateral invasion in cnidarians (Fukami et al. 2007; Huchon et al. 2010). It was suggested that HEGs were only able to invade actiniarians because of the slow evolutionary rate of mtDNA, and HEGs did not invade other metazoans possibly either due to a lack of opportunity or because metazoans have faster substitution rates, which means that HEGs degenerate more rapidly (Lin et al. 2011; Medina et al. 2006; Shearer et al. 2002). A similar scenario could be used to explain why HEGs could survive in *cox1* genes of corallimorpharians and basal and complex corals, but were subsequently lost from robust corals. Although global evolutionary rates of mitochondrial genomes in scleractinians and corallimorpharians are slow compared to those of other metazoans (Shearer et al. 2002), the relative evolutionary rate of the mitochondrial genome in robust corals is higher than those of complex corals and corallimorpharians (Chen et al. 2002; Medina et al. 2006), suggesting that HEGs in the *cox1* gene of robust corals will degenerate more rapidly and subsequently be lost compared to those of corallimorpharians and basal and complex corals. Robust corals regained *cox1* introns from other source donors which occurred independently in some of their lineages.

Our analysis implies that there should have been another lateral invasion providing an opportunity for I729 to insert itself into the *cox1* gene of robust corals. Insertion and loss events seem to have occurred more frequently in robust corals than in corallimorpharians and complex corals. It is obvious that insertion sites of I729 in robust corals all lay between the third base of one codon and the first base of the subsequent codon (phase zero, site 729), while I893 lies on the second and third bases of one codon in basal and complex corals and corallimorpharians (phase two, site 893). Roy and Gilbert (Roy and Gilbert 2005) suggested that phase 0 introns were more likely to be lost than other introns, *i.e.*, introns that do not interrupt a codon would have lower selection risks. This suggests that I729 has been inserted and lost more frequently than I893.

Hypothesis: intron loss and gain might be corroborated with major extinction events

Mapping the occurrence of introns on the *cox1* phylogeny of anthozoans suggested that loss and gain of introns in corallimorpharians and scleractinians were corroborated with major extinction events (Fig. 5). I893 was inserted in the common ancestor of hexacorallians some 552 mya, and was maintained in anthipatharians, actiniarians, corallmorpharians, and complex I scleractinians. I893 was lost about 213 mya during the Rhaetian extinction, when about 40% of benthic

taxa disappeared towards the end of the Triassic, although the cause of the Rhaetian mass extinction remains controversial (Galli et al. 2005; Marzoli et al. 2004; Olsen et al. 2002). Kiessling et al. suggested that volcanism causing climate changes led to disturbances in the carbon cycle which could have been the reason for this extinction (Kiessling et al. 2007; Kiessling and Baron-Szabo 2004), including inducing high extinction rates among taxa of inshore habitats and reefs (Kiessling and Baron-Szabo 2004). Although re-invasion of I729 into the *cox1* gene of robust corals occurred around 135 mya, the later Maastrichtian extinction (K-T extinction) which occurred at 65.5 mya might have triggered loss of I729 from some lineages of robust corals. Interestingly, major loss and invasion events of introns also occurred contemporarily with 2 major mass extinction events in the evolutionary history of scleractinians (Kiessling and Baron-Szabo 2004).

These correlations indicate that mass extinctions might have provided *cox1* intronless species with a selective advantage of respiration efficiency allowing them to increase their distribution in harsh environments compared to their counterparts with the *cox1* intron. A previous study indicated that mobile elements would have led hosts to experience greater selective forces unless they were harmless to their host genes (Domart-Coulon et al. 2001). Although the homing process might have provided a successful way to increase the preservation of invaded elements (Edgell et al. 2011; Gagan et al. 2000), it is believed that introns might decrease the efficiency of gene expression because of the prolonged lengths of their transcripts (Chen et al. 2005; Jeffares et al. 2008). The concentration of atmospheric oxygen rapidly declined from approximately 30% to 13% during the beginning of the Triassic to the Rhaetian extinction (Berner 2001, 2009; Berner et al. 2003; Glasspool and Scott 2010). It is believed that non-essential introns would have imposed costs to a gene by prolonging transcription (Lynch 2002). *Cox1* is the catalytic center for reducing oxygen to water in the oxidative phosphorylation of aerobic respiration (Pierron et al. 2012). We speculated that faster transcription efficiency might have provided *cox1*-intronless corals with a selective advantage under a scenario of declining oxygen concentrations.

Acknowledgments: Many thanks go to the New Zealand Institute of Water and Atmosphere Research for providing samples and hosting CAC's visit. We also thank members of the Coral Reef Evolutionary Ecology and Genetics Group (CREEG), Biodiversity Research Center, Academia Sinica (BRCAS) and anonymous referees for their constructive comments. This study was supported by an Academia Sinica Thematic Grant (2005-2010) and a National Science Council, Taiwan grant (2006-2011) awarded to CAC. All coral samples were collected with proper permits.

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