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The Mitochondrial Genome of a Deep-Sea Bamboo Coral (Cnidaria, Anthozoa, Octocorallia, Isididae): Genome Structure and Putative Origins of Replication Are Not Conserved Among Octocorals

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Abstract Octocoral mitochondrial (mt) DNA is subject to an exceptionally low rate of substitution, and it has been suggested that mt genome content and structure are conserved across the subclass, an observation that has been supported for most octocorallian families by phylogenetic analyses using PCR products spanning gene boundaries. However, failure to recover amplification products spanning the *nad4L–msh1* gene junction in species from the family Isididae (bamboo corals) prompted us to sequence the complete mt genome of a deep-sea bamboo coral (undescribed species). Compared to the "typical" octocoral mt genome, which has 12 genes transcribed on one strand and 5 genes on the opposite (*cox2*, *atp8*, *atp6*, *cox3*, *trnM*), in the bamboo coral genome a contiguous string of 5 genes (*msh1*, *rnl*, *nad2*, *nad5*, *nad4*) has undergone an inversion, likely in a single event. Analyses of strand-specific compositional asymmetry suggest that (i) the light-strand origin of replication was also inverted and is adjacent to *nad4*, and (ii) the orientation of the heavy-strand origin of replication (OriH) has reversed relative to that of previously known octocoral mt genomes. Comparative analyses suggest that intramitochondrial recombination and errors in replication at OriH may be responsible for changes in gene order in octocorals and hexacorals, respectively. Using primers flanking the regions at either end of the inverted set of five genes, we examined closely related taxa and determined that the novel gene order is restricted to the deep-sea subfamily Keratoisidinae; however, we found no evidence for strand-specific mutational biases that may influence phylogenetic analyses that include this subfamily of bamboo corals.

Keywords Mitochondrial genome · Origin of replication · Control region · Gene rearrangement · Intramitochondrial recombination · *msh1* · Keratoisidinae · Early metazoan

Mercer R. Brugler and Scott C. France have contributed equally to this work and their names are listed alphabetically.

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Introduction

Anthozoan cnidarians include the familiar and diverse sea anemones, sea fans, soft corals, and stony corals. In general, anthozoan mitochondrial (mt) DNA shows very low levels of variation, and may be uninformative at the interspecific level (Cairns and Bayer 2005; Fukami and Knowlton 2005) and completely void of character state changes at the population level (Shearer et al. 2002; Hellberg 2006; but see Watanabe et al. 2005; Chen et al. 2008). Through February 2008, 28 complete anthozoan mt genomes had been sequenced and made available in GenBank, and an additional 4 mt genome maps had been published without available sequence data (see Beagley et al. 1995; Fukami et al. 2000). These genomes have shown a number of novelties relative to other metazoans, including group I introns, two or fewer tRNAs, and, in the subclass Octocorallia, a putative mismatch repair protein (*msh1*) (Pont-Kingdon et al. 1998; Malik and Henikoff 2000) that has been suggested to be partly responsible for their observed low levels of variation (e.g., France et al.

1996; Shearer et al. 2002; but see Abdelnoor et al. 2006). Comparative studies have shown that *msh1* has a higher substitution rate among octocorals than other mt genes examined (France et al. 1996; France and Hoover 2001, 2002; McFadden et al. 2004; France 2007), and thus it has been widely used for phylogenetic analyses of Octocorallia (e.g., Sanchez et al. 2003; Wirshing et al. 2005; McFadden et al. 2006; France 2007).

However, while analyzing a fragment of mtDNA spanning *nad3–nad4L–msh1* for several octocoral taxa, France and Hoover (2001) encountered difficulties obtaining amplification products for taxa in the family Isididae (bamboo corals) and, more specifically, in the subfamily Keratoisidinae. In subsequent efforts we were able to amplify and sequence a region spanning the 3′-end of *msh1* and the large-subunit rRNA (*rnl*) from several species of bamboo corals (M. C. Barkes and S. C. France, unpublished data), thus demonstrating that the *msh1* gene is present in the family and that it is upstream of *rnl*, as in other octocorals (Fig. 1). PCR amplification products spanning regions upstream and into *msh1* remained elusive and suggested an alternative gene order in the Isididae, in contrast to the prevailing view of "stable gene orders" in octocorallian mt genomes (Kayal and Lavrov 2008). This concept began when shared mt gene arrangements across different taxonomic orders (*Renilla koellikeri* Pfeffer 1886, a sea pansy, order Pennatulacea; *Sarcophyton glaucum* [Quoy and Gaimard 1833], a soft coral, order Alcyonacea) led Beaton et al. (1998) to conclude that gene order among octocorals "appears to be conserved," which was supported by subsequent work by Medina et al. (2006).

To test the hypothesis that bamboo coral mt gene order differs from that of other octocorals, we attempted a series of amplifications using both orthodox (forward-reverse) and unorthodox (forward-forward, reverse-reverse) primer combinations. The results of those amplifications suggested that *msh1* was on the opposite strand relative to *cox1*, and the same strand as *cox2*, unlike all other octocorals sequenced to date (Fig. 1; see Materials and Methods for details). To determine the extent of the genome rearrangement and whether the gene content differed from that of other octocorals, we sequenced the entire mt genome of a deep-sea bamboo coral from the subfamily Keratoisidinae. We also tested species from the remaining isidid subfamilies (Isidinae, Circinisidinae, and Mopseinae), as well as additional deep-sea octocorals belonging to the suborder Calcaxonia, for the presence of this rearrangement.

Study Organism

The study specimen is an undescribed genus/species of bamboo coral (family Isididae) from the subfamily Keratoisidinae that was tentatively assigned to the genus *Isidella* Gray 1857 based on its nodal branching and planar growth form. However, a phylogenetic analysis based on *msh1* sequences places this species intermediate to clades containing *Isidella* and *Keratoisis* Wright 1869 (France 2007), and therefore, to avoid the use of a *nomen nudum*, we reference the specimen simply as BAL208-1 until it is formally described. The family Isididae (Cnidaria: Anthozoa: Octocorallia: Alcyonacea: Calcaxonia) is divided into four subfamilies: Circinisidinae, Isidinae, Keratoisidinae,

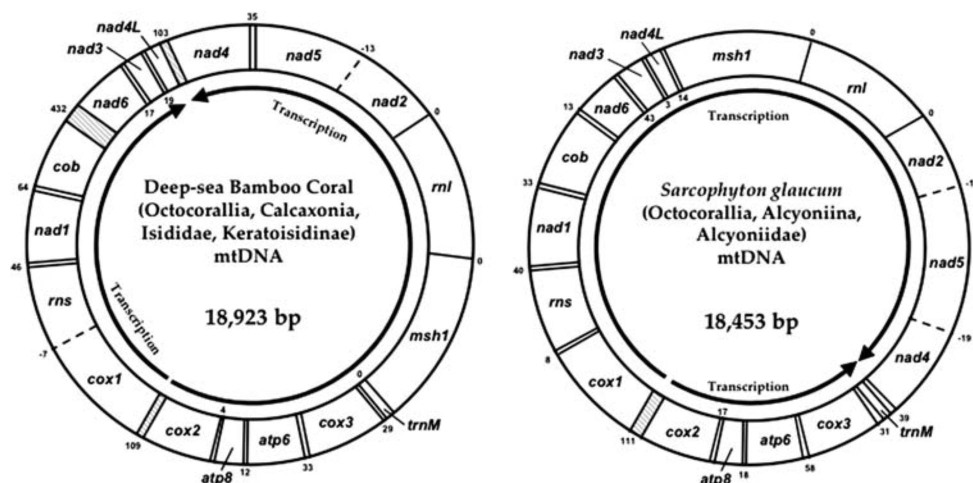

Fig. 1 Mitochondrial (mt) genome maps of the bamboo coral BAL208-1 (left) and a representative octocoral *Sarcophyton glaucum* (right; modified from Beaton et al. 1998). Sizes of intergenic regions (IGR) are presented as nucleotides; a negative value indicates overlapping genes and hatched areas denote IGRs >100 bp. In the bamboo coral mt genome, seven genes (*cox1*, *rns*, *nad1*, *cob*, *nad6*, *nad3*, *nad4L*) are encoded on the light strand (L-strand) while the remaining genes are encoded on the H-strand. We propose the H-strand origin of replication (OriH) to be in the IGR between *cox1* and *cox2* in both genome arrangements, and OriL in the IGR between *nad4L* and *nad4* in the bamboo coral and in the IGR between *trnM* and *nad4* in other octocorals

and Mopseinae (Alderslade 1998). All four subfamilies contain deep-water species, but only the Keratoisidinae are restricted to the deep-sea (>200-m depth). Bamboo corals are so-called because of the jointed appearance of their skeleton, which alternates short proteinaceous nodes with longer calcareous internodes.

Materials and Methods

Collections

BAL208-1 was collected on 1 September 2005 at a depth of 1815 m (3.56°C) on Balanus Seamount (New England Seamounts; western North Atlantic Ocean) using the remotely operated vehicles (ROVs) *Hercules* and *Argus* aboard the research vessel (R/V) *Ronald H. Brown*. Following recovery of the ROVs, several subsamples of the colony were frozen at –80°C, while others were placed in 100% ethanol; a voucher sample was preserved in 70% ethanol and will be deposited in the Smithsonian Institution's National Museum of Natural History following completion of the taxonomic description.

DNA Extractions and PCR Amplification

Total genomic DNA was extracted following protocols described by France et al. (1996), with slight modifications (100 μg proteinase K; single organic [chloroform] extraction). Previous attempts to amplify the mt region spanning *nad4L–msh1* in bamboo corals had failed (France and Hoover 2001; S. C. France, unpublished data), and explanations for the lack of amplification included absence of *msh1* from the mt genome within the family Isididae. However, subsequent work demonstrated that the *msh1* gene is present in the family and that it is upstream of *rnl*, as in other octocorals (M. C. Barkes and S. C. France, unpublished data). Thus, we assumed that the relative positions of *nad4L* and *msh1* had changed. We first paired a reverse bamboo coral-specific primer for the *rnl* gene (16S5Pr; see Table 1 for primer sequences), which we had determined was directly downstream from *msh1*, with a number of general forward primers from genes assumed to be upstream of *nad4L* based on published octocoral mt genome maps (e.g., *cox1*, *rns*, *nad1*, *nad6*, and *nad3*). All reactions failed to yield PCR products for isidids. We hypothesized that one or more genes may have been inverted and, so, paired an internal reverse *msh1* primer (msh3055r) in both orthodox (forward-reverse) and unorthodox (forward-forward, reverse-reverse) primer combinations to allow for the possibility that any one of the tested genes (*cox1*, *cox2*, *cob*, *nad2*, *nad3*, *rns*) could be on the opposite strand. A successful amplification resulted from a pairing of msh3055r with the *cox1* reverse primer HCO2198 (Folmer et al. 1994), generating an ∼3.8-kbp fragment.

Based on this result, we designed new primers optimized for "long amplification" PCR technology (LA PCR; Takara Mirus Bio LA Taq). Pairing a reverse *msh1* primer (mshLA3034R) with a reverse *cox1* (COILA8363R) or a forward *cox2* (CO2LA8092F) primer yielded strong amplification products (∼3.3 and ∼2.8 kbp, respectively) for five different bamboo corals that indicated a change in gene order. We elected to primer-walk the mt genome to determine the extent of the changes. To negate the chance of amplifying mt pseudogenes within the nuclear genome, all subsequent primers were designed to anneal at least 100 bp into the existing sequence. To test the phylogenetic distribution of the novel gene rearrangement in species from other isidid subfamilies, as well as different families from the suborder Calcaxonia, additional primers were designed to cross the following gene boundaries: *nad4L-msh1* and *nad4-cox3* (denoting "typical" octocoral gene order) and *nad4L-nad4* and *msh1-cox3* (indicative of the novel keratoisidin gene order; see Table 1 and Fig. 2).

Table 1 Novel primers used in this study

Primer name	Gene	Primer length (bp)	Primer sequence (5′–3′)
CO1LA8363R	*cox1*	36	CCGTATTAACATACTCGAAGCTGTCCCCGCCATTCC
CO2LA8092F	*cox2*	33	GTGACGCGGCTGAGCCATTTCAACTAGGCTTCC
CO3bam5657F	*cox3*	21	GCTGCTAGTTGGTATTGGCAT
ND4gen4974F	*nad4*	22	TAGGYTTATTTACTCATACWAT
ND4L2475F	*nad4L*	19	TAGTTTTACTGGCCTCTAC
ND42625F	*nad4L*	18	TACGTGGYACAATTGCTG
msh2806R	*msh1*	21	TAACTCAGCTTGAGAGTATGC
msh3055R	*msh1*	20	GGAGAATAAACCTGAGAYAC
mshLA3034R	*msh1*	30	CCTGAGATACTGCGCGTTGTTTAGGCCCCG
16S5Pr	*rnl*	18	TCACGTCCTTACCGATAG

Note: F and R in primer name denote forward and reverse with respect to gene reading frame

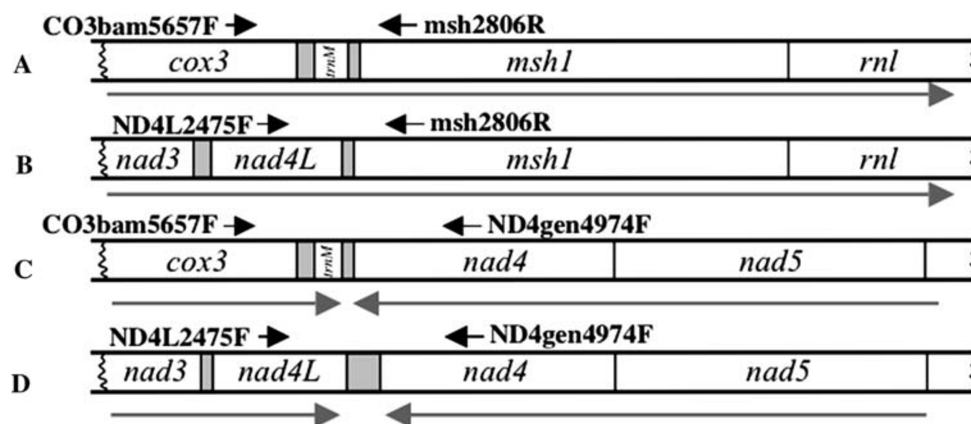

Fig. 2 Linearized gene orders of the regions flanking *msh1* (**A**, **B**) and *nad4* (**C**, **D**) for the two known octocoral mitochondrial genome arrangements. Four primer combinations were tested on species from the subfamily Keratoisidinae, other Isididae, and selected Calcaxonia (see Table 1 for a list of primers) to determine the phylogenetic distribution of the novel gene order. Primers are shown above each gene map, and gray arrows below show the direction of transcription; hatched boxes show the relative sizes of intergenic regions. Species of Keratoisidinae produced amplification fragments of the expected size using primers consistent with gene orders **A** and **D**, but no amplification products were observed for isidids in other subfamilies using the same primer combinations. Circinisdinae, Mopseinae, and Calcaxonia species produced amplification fragments of the expected size using primers consistent with gene orders B and C. See text for further discussion and Supplementary Table S1 for GenBank accession numbers

DNA Sequencing

PCR products were isolated using 1% low-melt agarose SF gels (Amresco, Inc.) followed by overnight agarase digestion (5 U per 100 μl agarose; Sigma-Aldrich Co.). Purified PCR product was used in an ABI PRISM BigDye Terminator v1.1 cycle sequencing reaction following the manufacturer's protocols (except for one-half to one-fourth of the recommended Ready Reaction Premix in 10- to 20-μl total volume reactions). Products were cleaned by ethanol/EDTA precipitation and electrophoresed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems 2002). Sequence traces were edited using Sequencher version 4.6 (Gene Codes Corp.).

Sequence Analysis

Gene identities were determined using the NCBI BLAST program within GenBank (Altschul et al. 1997). Sequences with known gene affinity were then aligned (by eye, based on both nucleotide and amino acid alignments) to other anthozoan sequences using SeqApp version 1.9a169 (Gilbert 1992). Cnidarian start (ATG) and stop (TAA, TAG) codons were used to delineate the beginning and end of all protein-coding genes. Gene boundaries for the two ribosomal genes and single transfer RNA were determined based on alignment similarities alone. Base-pair composition was determined using PAUP* version 4.0b10 (Swofford 2002). Strand-specific compositional asymmetry was measured by implementing the skew formulae of Perna and Kocher (1995) in R (Venables 2005; Charif and Lobry 2007). Specifically, $GC_{skew} = (G - C)/(G+C)$, where G and C are the numbers of those bases on the strand being analyzed, and similarly for AT_{skew}.

Physical mapping of the origins of replication (ORs) (e.g., J. Goddard and Wolstenholme 1980; Clayton 2003) on the H-strand (OriH; also referred to as the control region) and L-strand (OriL) was beyond the scope of this study. We utilized several analytical tools within the software package GraphDNA (Thomas et al. 2007) to identify features and/or sequence similarities generally associated with ORs. Included in the package is Lobry's (1996) DNA-Walk method, which is an inferential technique that generates a graphical representation of cumulative skew across a complete genome by assigning a direction to each consecutive nucleotide in the sequence (C = North, T = East, G = South, and A = West). The method assumes that replication of the mt genome is via the asymmetric strand-displacement model, wherein DNA synthesis begins at OriH and proceeds unidirectionally around the molecule. As the new H-strand is being synthesized using the L-strand as template, the original H-strand is displaced in single-stranded conformation, leaving it only partially shielded by single-stranded mt binding proteins (Reyes et al. 1998) and exposed to free radicals and other mutagens that transverse the highly oxidative environment of the mitochondrion. Only after H-strand replication has progressed approximately 67% around the molecule is OriL exposed and synthesis of the new L-strand initiated in the opposite direction, using the original H-strand as template; synthesis of the new L-strand also proceeds unidirectionally (Clayton 1982, 2003; Saito et al. 2005).

Thus there are significant temporal asymmetries between the strands that cause unequal nucleotide substitution rates, which change along the length of the strand depending on the amount of time spent in single-stranded conformation (Reyes et al. 1998; Tillier and Collins 2000). In most cases, the leading (or H) replicating strand has G > C and T > A, and this bias reverses abruptly at the origin and terminus of replication (Mrazek and Karlin 1998). The graphical output of the DNA-Walk method shows these abrupt changes in base composition bias, although it does not distinguish between OriH and OriL. We also utilized EINVERTED (Rice et al. 2000), Tandem Repeats Finder (Benson 1999), and M-Fold (Zuker 2003) to locate structures characteristic of ORs (i.e., inverted repeats, tandem repeats, and AT-rich hairpins, respectively) both over the entire genome sequence and specifically in the areas showing abrupt reversals in the DNA-Walk method. We analyzed additional anthozoan mt genomes and compared them to BAL208-1 to determine if these structural features are in common locations. For validation purposes, analyses of base composition bias were also performed using the mt genome sequence of the scleractinian coral *Acropora tenuis* (Dana 1846) (GenBank accession no. NC_003522), for which van Oppen et al. (2002) identified a putative control region (OriH) in an intergenic region (IGR) between *rns* and *cox3*. All of the analyses we performed (with the exception of GC-skew) on *A. tenuis* likewise identified reversals in base composition bias in the same IGR.

Results and Discussion

Genome Organization

The circular mt genome of the keratoisidin bamboo coral, BAL208-1, is 18,923 bp in length and contains the typical octocoral complement of 13 energy pathway proteins (*nad1-6*, *nad4L*, *cox1-3*, *atp6*, *atp8*, and *cob*), 2 ribosomal RNA genes (the small [*rns*] and large [*rnl*] ribosomal subunits of RNA), 1 transfer RNA (*trnM*; methionine), and the mismatch repair gene homologue *msh1* (Fig. 1). Compared to other octocoral genomes sequenced to date, BAL208-1 has an unusually large IGR between the *cob* and the *nad6* genes (432 bp), and the entire genome consists of 903 bp (4.77%) of intergenic sequence (nonconsecutive). No novel genes, introns (found within all hexacoral mt genomes sequenced to date; see Fig. 2 of Medina et al. 2006), or motifs characteristic of homing endonucleases (found in the *cox1* intron of several hexacorals [Beagley et al. 1996; M. Goddard et al. 2006; Medina et al. 2006; Fukami et al. 2007; Sinniger et al. 2007; Brugler and France, unpublished data]) were detected in the BAL208-1 mt genome. Seven genes (*cox1-rns-nad1-cob-nad6-nad3-nad4L*) are encoded on the light strand (L-strand; G+T = 9451 bp), while the remaining 10 genes (*cox2-atp8-atp6-cox3-trnM-msh1-rnl-nad2-nad5-nad4*) are on the opposite strand (H-strand; G+T = 9472 bp). In all other octocoral mt genomes sequenced to date, only *cox2-atp8-atp6-cox3-trnM* are encoded on the opposing strand from the remaining genes. Previous phylogenetic analyses do not show Isididae as a basal lineage within Octocorallia (e.g., Berntson et al. 2001; McFadden et al. 2006), and therefore we presume that five genes (*msh1-rnl-nad2-nad5-nad4*) underwent an inversion in the lineage leading to the Keratoisidinae (see below) to produce this derived gene order. Previous studies posit that inverted genes are the result of intramitochondrial recombination (Dowton and Austin 1999; Miller et al. 2004).

Base Composition

Studies of mt genomes typically analyze the H-strand for statistics on base composition. Designation of H- and L-strands depends on the relative proportions of G+T nucleotides, with the higher proportion of G+T being the heavy (H) strand (Clayton 1991); in the "typical" octocoral mt genome, this corresponds to the strand with the greater number of coding genes. The difference in G+T between strands of the BAL208-1 mt genome is minimal ($\Delta_{G+T} = 21$ nucleotides) compared to that in other octocorals (e.g., *Briareum asbestinum* [Pallas 1766]; $\Delta_{G+T} = 1112$). Bamboo coral mt genes are almost evenly distributed between the two strands (59% H:41% L), whereas in other octocorals the distribution is more uneven (71% H:29% L). The difference between genomes is magnified if the number of coding nucleotides, rather than the number of genes, is compared (67% H:33% L vs. 89% H:11% L). As is typical for invertebrates, the base composition of the BAL208-1 mt genome is A+T rich (62.4%) but is on the lower end of the range for octocorals (62.7–64.3%; Table 2). Strand-specific compositional asymmetry (AT- and GC-skew) is shown in Fig. 3 and Table 2. Among anthozoans, hexacorals show greater skews in base composition than octocorals. Several mechanisms have been proposed to explain strand-specific compositional asymmetry, including differences in gene orientation and differential mutational processes, both between transcribed and nontranscribed strands and between leading and lagging strands during replication (Tillier and Collins 2000). The differences between octocorals and hexacorals is likely a reflection of the distribution of genes on the two strands; of the hexacoral mt genomes sequenced to date, all genes are encoded on the same strand. Skew values for the BAL208-1 mt genome deviate from zero less than those of the other three octocorals sequenced to date, corresponding to the almost-even distribution of genes between the two

Table 2 Nucleotide composition of the BAL208-1 mitochondrial genome compared to previously published anthozoan genomes

	# of bp	T (%)	C (%)	A (%)	G (%)	A+T (%)	G+C (%)	AT-skew	GC-skew
Octocorallia									
BAL208-1	18,923	30.9	18.4	31.5	19.2	62.4	37.6	0.01	0.02
P. bipinnata	18,733	33.1	17.2	29.6	20.1	62.7	37.3	−0.06	0.08
S. glaucum	18,453	34.7	16.3	29.6	19.4	64.3	35.7	−0.08	0.09
B. asbestinum	18,632	33.2	17.3	29.7	19.8	62.9	37.1	−0.06	0.07
Hexacorallia									
C. formosa	18,398	33.6	17.9	26.9	21.6	60.5	39.5	−0.11	0.09
M. senile	17,443	34.9	16.9	27.0	21.2	61.9	38.1	−0.13	0.11
A. tenuis	18,338	37.0	13.7	25.1	24.2	62.1	37.9	−0.19	0.28
R. florida	21,376	37.1	13.3	25.0	24.6	62.1	37.9	−0.20	0.30

Note: AT- and GC-skew was measured on the H-strand (see text) and calculated following Perna and Kocher (1995). Octocorallia (all order Alcyonacea): *Pseudopterogorgia bipinnata* (NC_008157), *Sarcophyton glaucum* (AF064823, AF063191), *Briareum asbestinum* (NC_008073); Hexacorallia: *Chrysopathes formosa* (Order Antipatharia; NC_008411), *Metridium senile* (Or. Actiniaria; NC_000933), *Acropora tenuis* (Or. Scleractinia; NC_003522), *Ricordea florida* (Or. Corallimorpharia; NC_008159)

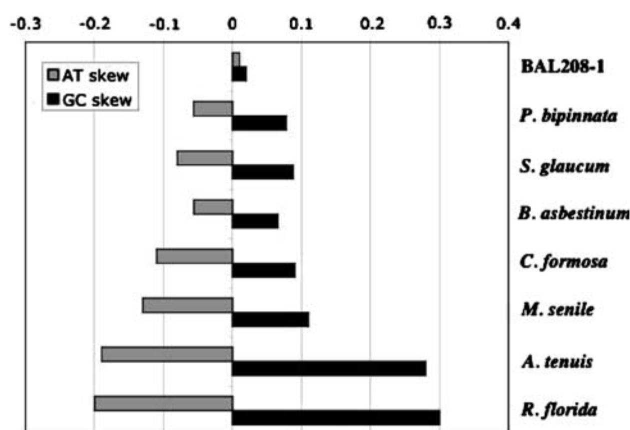

Fig. 3 Strand-specific compositional asymmetry (AT- and GC-skew; calculated following Perna and Kocher 1995) measured on the H-strand of the bamboo coral, BAL208-1, and selected anthozoans (see Table 2 for details)

strands. AT-skew differs in sign between the alternate octocoral genome arrangements: BAL208-1 has 2% more adenine than thymine, whereas the other octocorals have more thymine than adenine on the H-strand (e.g., up to 17% in *Sarcophyton glaucum*).

Table 3 Gene lengths (protein-coding genes, as amino acids; rRNA genes, as base pairs) of BAL208-1 compared to other available octocoral complete mt genome sequences

Gene	Octocorallia			
	Isididae BAL208-1	Alcyoniidae *Sarcophyton glaucum*	Briareidae *Briareum asbestinum*	Gorgoniidae *Pseudopterogorgia bipinnata*
rnl	1961	1962	2224	2211
rns	1031	1021	581[a]	924
cox1	533	532	528	533
cox2	254	254	254	254
cox3	262	262	262	262
nad1	327	326	324	324
nad2	440	458	388	365
nad3	116	118	118	118
nad4	483	496	483	483
nad5	604	606	606	606
nad6	184	186	186	186
atp6	239	238	236	236
atp8	72	73	72	72
nad4L	98	98	98	98
cob	387	387	381	382
msh1	994	983	1022	986

[a] But see Table 4 of Brugler and France (2007)

Protein-Coding and Ribosomal RNA Genes

All 14 protein-coding genes were of similar or exact size to those of *Briareum asbestinum* (NC_008073), *Pseudopterogorgia bipinnata* (Verrill 1864)(NC_008157), and *Sarcophyton glaucum* (AF064823, AF063191) (Table 3), began with an ATG codon triplet, and, with the exception of *cox1*, terminated with either TAG or TAA (Table 4). The nucleotide sequence for *cox1* ends in CTTT, and we surmise that a TAA stop codon is completed by the addition of two 3′ adenines to the mRNA strand, as has been shown in previous studies (Ojala et al. 1981). The 5′- and 3′-ends of the ribosomal RNA genes were deduced by alignment with *rnl* and *rns* rDNA sequences from other octocoral mt genomes. BAL208-1 has the longest *rns* gene among these octocorals, but the shortest *rnl* gene.

Table 4 Organization of the BAL208-1 mitochondrial genome

Gene	Position	Size (bp)	Strand	Start Codon	Stop Codon
igr1	1–109	109		–	–
cox1	110–1706	1597	L	ATG	T(AA)[a]
–	Overlapping	−7		–	–
rns	1700–2730	1031	L	–	–
igr2	2731–2776	46		–	–
nad1	2777–3757	981	L	ATG	TAA
igr3	3758–3821	64		–	–
cob	3822–4982	1161	L	ATG	TAG
igr4	4983–5414	432		–	–
nad6	5415–5966	552	L	ATG	TAG
igr5	5967–5983	17		–	–
nad3	5984–6331	348	L	ATG	TAG
igr6	6332–6350	19		–	–
nad4L	6351–6644	294	L	ATG	TAA
igr7	6645–6747	103		–	–
nad4	6748–8196	1449	H	ATG	TAA
igr8	8197–8231	35		–	–
nad5	8232–10043	1812	H	ATG	TAG
–	overlapping	−13		–	–
nad2	10031–11350	1320	H	ATG	TAG
rnl	11351–13311	1961	H	–	–
msh1	13312–16293	2982	H	ATG	TAA
trnM	16294–16364	71	H	–	–
igr9	16365–16393	29		–	–
cox3	16394–17179	786	H	ATG	TAG
igr10	17180–17212	33		–	–
atp6	17213–17929	717	H	ATG	TAA
igr11	17930–17941	12	–	–	
atp8	17942–18157	216	H	ATG	TAG
igr12	18158–18161	4		–	–
cox2	18162–18923	762	H	ATG	TAG

Note: *cox1-3*, cytochrome *c* oxidase subunits; *atp6* and *atp8*, ATP synthase subunits; *nad1-6* and *nad4L*, NADH dehydrogenase subunits; *cob*, cytochrome b; *rns*, small subunit of ribosomal RNA; *rnl*, large subunit of ribosomal RNA; *trnM*, transfer RNA gene methionine; *igr*, intergenic region. The start of *rns* overlaps by 7 bases with the 3′-end of *cox1*, and the start of *nad2* overlaps by 13 bases with the 3′-end of *nad5*

[a] The *cox1* stop codon may be completed by the addition of two 3′ A residues to the mRNA (see text)

Intergenic Regions and Origins of Replication

The BAL208-1 mt genome consists of 903 bp (4.77%) of intergenic sequence (nonconsecutive). Most of the genes are separated by IGRs that range from 4 to 109 bp in size, although an exceptionally large IGR (*igr4*) was found between *cob* and *nad6* (432 bp). The next largest IGRs known from other octocoral mt genomes are seen in *Renilla koellikeri* (330 bp between *nad5* and *nad4* [Beagley et al. 1995]), and *Briareum asbestinum* (202 bp between *cox1* and *rns*). We are currently analyzing *igr4* across several taxa of the Keratoisidinae to determine its range of variation and utility in species identification, and have observed that it varies in length, from 42 to 605 bp, across the subfamily (J. L. van der Ham and S. C. France, unpublished data). Two pairs of genes (*nad2–nad5* and *rns–cox1*) overlap in the BAL208-1 mt genome, and another three gene boundaries are directly adjacent to one another (*trnM—msh1*, *msh1—rnl*, and *rnl—nad2*). However, analysis of the *trnM-msh1* gene boundary from additional keratoisidin species (see below) reveals an IGR of 11 bp.

Beaton et al. (1998) proposed the *cox1-cox2* IGR to be the location of the origin of replication (OR) within the *Sarcophyton glaucum* mt genome, although they were not able to identify secondary structures typically associated with ORs. As of this writing, ORs have not been reported for any additional octocoral mt genomes. In vertebrates, both H- and L-strands have a distinct OR; however, the location of OriL is less defined in invertebrates (Clary and Wolstenholme 1984). Chen et al. (2008) note that control region (OriH) locations are highly variable among scleractinian corals, and Van Oppen et al. (2002) hypothesized a large degree of heterogeneity in the mechanisms mediating mt replication in hexacorals. Several key regulatory sequences are thought to be important in the initiation of mt replication, including conserved sequence blocks (involved in generating the 3′-ends of RNA primers), A+T-rich regions, and stable stem-loop configurations containing characteristic T-rich loops, within which L-strand synthesis is thought to begin (the hairpin serves as a recognition structure for mtDNA primase [Pearson et al. 1996]).

We used Lobry's (1996) DNA-Walk method to detect abrupt changes in base composition bias associated with ORs (e.g., Picardeau et al. 2000; Touchon et al. 2005). The BAL208-1 mt genome has two abrupt reversals in direction within IGRs (Fig. 4), both of which correspond to the same feature: change in the direction of transcription due to genes being encoded on the opposite strand. There were three other, more gradual, changes in base composition bias, and all mapped within genes (Fig. 4). ORs are typically found in IGRs and not in coding genes. The abrupt changes occur in the regions of *igr1* and *igr7* (Figs. 1 and 4; see Supplementary Fig. 1a for DNA-Walk of *Pseudopterogorgia bipinnata*). Although we found no lengthy inverted or tandem repeats in these regions (Wolstenholme 1992; Saito et al. 2005), an M-Fold analysis of the H-strand *igr1* sequence revealed two stable stem-loop hairpin structures, one of which (positions 60–88) had a T-rich loop characteristic of an mtDNA primase recognition site (see Supplementary Fig. 2a). The second hairpin structure (positions 4–31) has an A-rich loop and is in a region of

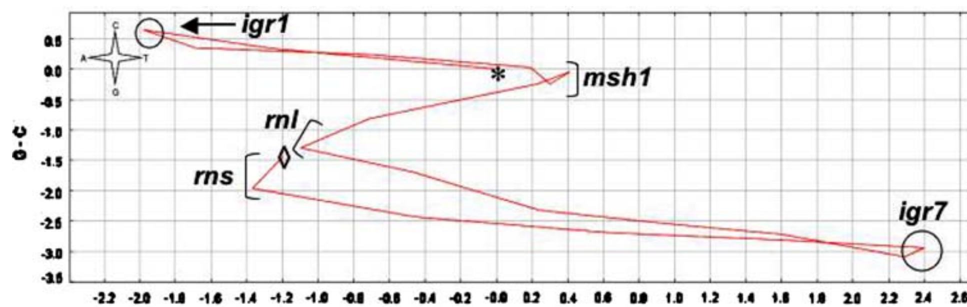

Fig. 4 DNA-Walk result for the bamboo coral, BAL208-1, mitochondrial genome (generated using GraphDNA [Thomas et al. 2007]). The line in the figure is a vectorial representation of a "walk" along the linearized genome, beginning at *cox1* (*) and ending at *rns* (◇). Each step in the walk proceeds in a direction determined by the base composition (indicated by the compass at the upper left). The units on the axes are cumulative skew from the starting point (0, 0), measured as number of nucleotides × 10^2. The line was constructed from 18,923 points, i.e., the length of the genome, but for ease of viewing major changes, a window size of 900 was plotted. Circles highlight intergenic regions of the genome showing abrupt changes in base composition bias that are typically associated with origins of replication. Genic regions displaying changes are also indicated

igr1 sequence that is conserved across at least 20 species representing eight octocoral families (data not shown), including *Briareum asbestinum*. However, in *B. asbestinum* (and other octocorals; see below), it is the opposing strand that is the heavy strand, and therefore this sequence folds into a T-rich loop (Supplementary Fig. 2b). We hypothesize that OriH is located between *cox1* and *cox2* in both mt genome arrangements, but that replication proceeds in opposite directions in each (Fig. 1). We further posit that the location of OriL is in *igr7* in BAL208-1 and between *nad4* and *trnM* in other octocorals (Fig. 1); in both cases these gene regions are approximately two-thirds the distance around the mt genome from OriH, the expected location under the asymmetric strand-displacement model. An M-Fold analysis did not reveal an obvious stable hairpin structure with a T-rich loop within *igr7*; however, such a structure could be derived from the region spanning the *igr7*–*nad4L* junction (data not shown).

Studies on metazoan mt replication have shown that regions near origins of replication, or that include tRNA genes, are hot spots for rearrangement (Moritz and Brown 1987; Boore and Brown 1998; Mueller and Boore 2005; San Mauro et al. 2006), and Macey et al. (1997) indicate that novel vertebrate mt gene orders are statistically linked with movement of OriL. Stanton et al. (1994) report that tRNAs and other genes (rRNA or protein-coding) that are capable of forming energetically stable stem-and-loop structures often occur at the end of rearranged fragments. Hairpin structures formed while mt strands are in a single-stranded state can cause pausing of DNA replication, which may trigger slippage or other replication errors (Bedinger et al. 1989; Viguera et al. 2001). We note that the five inverted genes in the BAL208-1 mt genome are flanked by the putative OriL and *trnM*, and propose another example from the class Anthozoa of an OR-associated mt genome rearrangement: the black coral *Chrysopathes formosa* Opresko 2003 (GenBank accession no. NC_008411). Based on a DNA-Walk analysis and cumulative purine (CT-AG)-, keto (AC-GT)-, and AT-skew estimates, we found two candidate regions for ORs in the *C. formosa* mt genome (Supplementary Fig. 1b). One of these, within an IGR between *rns* and *cob*, corresponds to the OriH identified by van Oppen et al. (2002) in the hexacoral *Acropora tenuis* (see Supplementary Fig. 1b for DNA-Walk of *Acropora tenuis*). This position also marks the sole observed gene rearrangement between *C. formosa* and the sea anemone *Metridium senile* (GenBank accession no. NC_000933), which has three contiguous genes (*cox2*–*nad4*–*nad6*) flanked by *rns* and *cob* (Brugler and France 2007). Although no inverted repeats or significant tandem repeats were noted within this region (data not shown), we did recover a stable stem-and-loop structure at the 5′-end of *cob* (Supplementary Fig. 2c). We reason that these data suggest that errors in replication at ORs may have been responsible for changes in gene order in both hexacorals and octocorals. Interestingly, the second abrupt change in base composition bias in both the *C. formosa* and the *A. tenuis* mt genomes occurred in an IGR adjacent to *trnM* (Supplementary Fig. 1b); a stable stem-loop hairpin with a T-rich loop was identified on the H-strand in this region in *C. formosa*. A *trnM*-associated IGR is also our predicted location for OriL in the "typical" octocoral mt genome, but this region has apparently been part of the inversion in BAL208-1, which has no IGR downstream of *trnM* (Table 4). If this prediction is experimentally confirmed, it suggests that the association of OriL with *trnM* has been conserved across the subclasses Octocorallia and Hexacorallia (but see Flot and Tillier 2007) but is lost in the Keratoisidinae. It is worth noting that *trnM* is the only tRNA encoded in octocoral mt genomes, and one of only two encoded in hexacorallian mt genomes. Maizels and Weiner (1995) hypothesized that tRNAs were originally

used for replication and that their current role in translation is simply an exaptation.

Intramitochondrial Recombination

A number of studies detailing mt gene rearrangements have proposed the mechanism of tandem duplication via slipped-strand mispairing, followed by a random deletion of genes, to explain the rearrangements (e.g., Kumazawa and Nishida 1995; Boore and Brown 1998; Mueller and Boore 2005); however, this mechanism does not explain the inversion of genes from one mt strand to the other as observed in the bamboo coral mt genome and a number of other invertebrate taxa (e.g., the sea star *Pisaster ochraceus* [Smith et al. 1989], honeybee *Apis mellifera* [Crozier and Crozier 1993], sea urchins and sea cucumbers [Smith et al. 1993], phytonematode *Meloidogyne javanica* [Lunt and Hyman 1997], hymenopterans [Dowton and Austin 1999], wallaby louse *Heterodoxus macropus* [Shao et al. 2001], and the freshwater crayfish *Cherax destructor* [Miller et al. 2004]). We propose that the five-gene inversion within the bamboo coral mt genome was brought about via intramitochondrial recombination (IR), which involves breaking and rejoining of double-stranded DNA, thus facilitating gene inversions (see Fig. 7 in Dowton and Austin 1999). Mitochondrial genomes were once thought to lack the ability to recombine (Moritz et al. 1987); however, the mitogenomic research community cannot currently otherwise account for the inversion of genes between strands. Studies by Lunt and Hyman (1997), Kajander et al. (2000), and Dowton and Campbell (2001) have shown that IR occurs at low levels. The five-gene inversion within the bamboo coral mt genome spans 9.5 kb, and may be the longest contiguous inversion yet encountered; Smith et al. (1993) reported a contiguous inversion of 17 genes (3 protein-coding genes and 14 tRNAs), but the total length was only 4.6 kb.

Phylogenetic Distribution of Gene Order Change

To determine the phylogenetic distribution of the gene rearrangement observed in BAL208-1, we developed PCR primers to amplify and sequence mt genome fragments that span the novel *cox3–msh1* (primers CO3bam5657F to msh2806R) and *nad4L–nad4* (ND4L2475F to ND4gen4974F) gene boundaries, and tested them across a range of octocoral taxa (Fig. 2). Amplification products crossing both boundaries were obtained for 12 specimens representing at least five of the eight nominal genera of Keratoisidinae (Supplementary Table S1; see France [2007] for a discussion of taxonomic problems in the subfamily). We attempted amplifications using these primer combinations for species from two additional Isididae subfamilies, the Circinisidinae and Mopseinae, but no PCR products were observed in these reactions. However, the "typical" octocoral gene order was confirmed in these two Isididae subfamilies by pairing primers ND4L2475F and msh2806R (crossing the *nad4L–msh1* gene boundary) and primers CO3bam5657F and ND4gen4974F (crossing the *cox3–nad4* gene boundary). Note that in three of these specimens, no amplification products were observed at the *cox3–nad4* gene boundary (Supplementary Table S1), but we believe that this is an issue with poor-quality DNA extracted from museum specimens and not an indication that the gene order differs. Fragment size was greater across the *cox3–nad4* boundary ($\approx$700 bp) than the *nad4L–msh1* boundary ($\approx$350 bp), and tissues obtained from museum collections have been shown to produce poor results for larger target regions in PCR (e.g., Berntson and France 2001). Confirmation of gene order in these species must await the availability of more recently collected specimens. An amplicon spanning the *nad4L–msh1* gene boundary has also been sequenced for *Isis hippurus* (Linnaeus 1758), representing the remaining isidid subfamily, Isidinae (C. McFadden, Harvey Mudd College, pers. comm.).

We further tested for *cox3–msh1* and *nad4L–nad4* gene boundaries from other families in the suborder Calcaxonia. No amplification products were observed when primers spanning *cox3–msh1* or *nad4L–nad4* were used with species in the families Chrysogorgiidae and Primnoidae. However, expected amplification products characteristic of the "typical" octocoral gene order were observed and sequenced using primers spanning *cox3–nad4* in three species of Chrysogorgiidae and three species of Primnoidae (CO3bam5657F to ND4gen4974F) (additional species were amplified but not sequenced; Supplementary Table S1). An *nad4L–msh1* gene boundary has previously been confirmed for Chrysogorgiidae, Primnoidae, and more than 100 other species across 28 families of Pennatulacea and Alcyonacea (e.g., McFadden et al. 2006). These data suggest that the novel gene order observed in BAL208-1 is restricted within the Isididae to the subfamily Keratoisidinae.

Potential Biases in Phylogenies that Include Keratoisidinae

Recent molecular phylogenetic analyses of Octocorallia have employed the mt gene *msh1* because of its relatively high rate of substitution (Sanchez et al. 2003; Wirshing et al. 2005; McFadden et al. 2006; France 2007). Because *msh1* is one of the five genes encoded on opposite strands among octocorals, there is the possibility that strand-specific compositional asymmetries may blur the true phylogenetic signal (Hassanin 2005) when constructing phylogenies of the Octocorallia that include Keratoisidinae. We thus further analyzed AT- and GC-skew at the five inverted genes, and *msh1* in particular, from available mt

genome sequences to determine if compositional asymmetry differed at these genes between the two octocoral genome arrangements. In all cases except AT-skew at *msh1*, values for BAL208-1 are similar to, or within the range observed for, other octocorals (Supplemental Fig. 3). At *msh1*, the bamboo coral shows a slight negative AT-skew, in contrast to the positive value seen for the other species, although all four taxa have values very close to zero.

We sequenced the entire *msh1* gene from additional species of isidids and other octocorals to determine if this pattern of negative AT-skew was a characteristic of bamboo corals only. Our results show that AT-skew correlates with evolutionary lineage rather than mt genome arrangement when analyzed over both the entire gene (Supplemental Figs. 4a–c) and the 5′-region used for molecular phylogenies (Supplemental Figs. 4d–f). Phylogenetic analyses of the subclass Octocorallia using nuclear (Berntson et al. 2001) and mt (McFadden et al. 2006) sequences resolve most species into two well-supported clades: Calcaxonia/Pennatulacea and Holaxonia/Alcyoniina, and these show, respectively, negative and positive AT-skew in *msh1*. Since our gene boundary analysis (above) and the mt genome map of the pennatulacean *Renilla kollikeri* (Beagley et al. 1995) suggest that only the keratoisidins have the rearranged mt genome (i.e., a potentially different mutational environment), the negative AT-skew at *msh1* is not a result of gene inversion, a conclusion supported by the observation that the other four inverted genes likewise do not show different patterns of skew (Supplemental Fig. 3). A difference in both AT- and GC-skew is observed at apparently neutral sites (third codon position, fourfold degenerate sites) (Supplemental Figs. 4c and f) and suggests the pattern is not driven simply by selection for specific amino acids but rather is a reflection of common ancestry or codon preference. It is of interest to note that the two scleraxonian species included in the analysis (*B. asbestinum* and *Corallium ducale* Bayer 1955) do not share a common AT-skew pattern. In phylogenetic analyses, scleraxonians do not form a monophyletic group and many taxonomists believe that the suborder Scleraxonia is a polyphyletic assemblage (e.g., Fabricus and Alderslade 2001).

If the assumption of the asymmetric strand-displacement model as a mechanism for replication of the mt genome is correct, then the lack of a unique strand-specific mutational bias in the inverted genes of BAL208-1 may be considered further evidence that the origins of replication are not conserved. Based on our hypothesized locations for OriH and OriL (above), the time spent by the *msh1* gene in single-stranded conformation, as estimated by the number of nucleotides between *msh1* and OriL, does not differ markedly between the octocoral genome arrangements; the same can be said for the other contiguous genes involved in the inversion. If the positions of the ORs did not differ between the two genome arrangements, given the different gene orders, we would expect additional compositional asymmetries resulting from differences in time spent in single-stranded conformation.

Conclusion

Octocorals display exceptionally slow nucleotide substitution rates in the mt genome and have been assumed to have a fixed arrangement of genes. We have found a novel arrangement in the mt genome of bamboo corals within the subfamily Keratoisidinae that falsifies this hypothesis. Five genes (*msh1*, *rnl*, *nad2*, *nad5*, *nad4*) have been inverted, and evidence suggests that (i) the light-strand origin of replication was also inverted and is associated with *nad4* in keratoisidins, and (ii) the orientation of the heavy-strand origin of replication has reversed relative to that of previously known octocoral mt genomes. PCR-based analyses spanning gene junctions suggest that the novel mt genome arrangement is a derived state restricted to octocorals in the subfamily Keratoisidinae. Comparisons of strand-specific compositional asymmetries of the five inverted genes made across the Octocorallia appear to reflect evolutionary lineage rather than any specific arrangement of genes. We note that the only other octocoral taxon for which successful amplification of the *nad4L*–*msh1* gene junction has eluded us is the deep-sea genus *Paragorgia* Milne Edwards and Haime 1857 (Scleraxonia, F. Paragorgiidae), and we suggest a mt genome analysis may reveal further novelties. The contribution of this unique mt genome rearrangement that shows evidence for intramitochondrial recombination will add further insight into animal mt evolution (see review by Lavrov 2007).

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