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Evolution of Linear Mitochondrial Genomes in Medusozoan Cnidarians

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Abstract

In nearly all animals, mitochondrial DNA (mtDNA) consists of a single circular molecule that encodes several subunits of the protein complexes involved in oxidative phosphorylation as well as part of the machinery for their expression. By contrast, mtDNA in species belonging to Medusozoa (one of the two major lineages in the phylum Cnidaria) comprises one to several linear molecules. Many questions remain on the ubiquity of linear mtDNA in medusozoans and the mechanisms responsible for its evolution, replication, and transcription. To address some of these questions, we determined the sequences of nearly complete linear mtDNA from 24 species representing all four medusozoan classes: Cubozoa, Hydrozoa, Scyphozoa, and Staurozoa. All newly determined medusozoan mitochondrial genomes harbor the 17 genes typical for cnidarians and map as linear molecules with a high degree of gene order conservation relative to the anthozoans. In addition, two open reading frames (ORFs), polB and ORF314, are identified in cubozoan, schyphozoan, staurozoan, and trachyline hydrozoan mtDNA. polB belongs to the B-type DNA polymerase gene family, while the product of ORF314 may act as a terminal protein that binds telomeres. We posit that these two ORFs are remnants of a linear plasmid that invaded the mitochondrial genomes of the last common ancestor of Medusozoa and are responsible for its linearity. Hydroidolinan hydrozoans have lost the two ORFs and instead have duplicated cox1 at each end of their mitochondrial chromosome(s). Fragmentation of mtDNA occurred independently in Cubozoa and Hydroidae (Hydrozoa, Hydroidolina). Our broad sampling allows us to reconstruct the evolutionary history of linear mtDNA in medusozoans.

Key words: linear mtDNA, medusozoa, cnidaria, ORF314, polB.

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The phylum Cnidaria represents a “hot spot” of animal mitochondrial genomic diversity, with the mtDNA displaying variation in both the gene content and the genome organization. Variation in the gene content is illustrated by the loss of all but one or two tRNA genes, a feature also found in some demosponges and in all chaetognaths (Helfenbein et al. 2004; Papillon et al. 2004; Faure and Casanova 2006; Wang and Lavarov 2008; Miyamoto et al. 2010). In addition, introns (e.g., Beagley et al., 1998; Chen et al. 2008), duplicated genes (Kayal and Lavarov 2008; Voigt et al. 2008), and extra protein genes (mutS in octocorals, polB in Scyphozoa, and unidentified open reading frames (ORFs) in several hexacorals) have been found in several cnidian classes (Pont-Kingdon et al. 1998; Medina et al. 2006; Shao et al. 2006). Furthermore, the structure of the cnidarian mtDNA is also variable: a single circular molecule is found in Anthozoa (hexa- and octocorals), whereas the medusozoan mtDNA consists of one or several exclusively linear molecules (Warrior and Gall 1985; Bridge et al. 1992; Endler and Schiewerwater 2003). Working with linear mtDNA presents unique challenges for both polymerase chain reaction (PCR) amplification and sequencing. As a potential reflection of these challenges, only six medusozoan linear mitochondrial genomes have been determined to date, those of the jellyfishes Aurelia aurita and Chrysaora quinquereirha (Scyphozoa) and the three hydra species Hydra magnipapillata, Hydra oligactis, and Hydra vulgaris (Hydrozoa) (Shao et al. 2006; Kayal and Lavarov 2008; Voigt et al. 2008; Park, Hwang, et al. 2011), while 43 complete circular mitochondrial genomes are available for the anthozoans.

Linear mtDNA is common outside the animals and has been extensively studied in fungi, plants, and several groups of unicellular eukaryotes (Bendich 1993; Burger et al. 2003; Nosek and Tomáška 2003; Hikosaka et al. 2010; Valach et al. 2011). The linear form of these molecules is thought to affect their stability, mechanisms of DNA replication, and expression of the genes they harbor. Unlike circular DNA molecules, linear chromosomes are less stable due to their higher susceptibility to exonucleolytic activity. In addition, replication of linear genomes has to overcome what is known as “the challenge of the ends,” namely the shortening of the linear molecule after each replication cycle (Nosek et al. 1998). In the nucleus, the ends of the linear chromosomes are capped by repetitive sequences known as telomeres that protect the molecule from degradation and allow its replication in a faithful manner (i.e., without the loss of essential sequences). Similarly, linear mtDNAs in algae and fungi have overcome linear instability with an array of telomere structures (Nosek et al. 1998). Recent studies of several yeast species from the genus Candida have suggested the concomitant involvement of the product of 1) a B-type DNA polymerase gene encoded by the mtDNA for 5’ end DNA extension and 2) terminal proteins (TPs) that bind the single stranded end of the linear molecules (Fricova et al. 2010 and reference therein). Thus far, the small number of available linear mitochondrial genomes for medusozoans has hindered attempts to assign any of the available models for the maintenance of chromosome ends proposed for the nonanimal groups (Nosek et al. 1998; Nosek and Tomaska 2002) or to reconstruct the evolutionary history of linear mtDNA in medusozoans.

Here, we present a description of 24 nearly complete mitochondrial genomes from a phylogenetically diverse set of species representing each of the four medusozoan classes, as well as their primary lineages: Cubozoza (Carybdeida and Chirodropida), Hydrozoa (Hydroidolina and Trachylina), Scyphozoa (Coronatae and Discomedusae), and Staurozoa (Cleistocarpida and Eleutheroarcipida). Our study represents the most in-depth exploration of the linear mtDNA in Cnidaria, and includes the first mitochondrial genomes from the classes Cuboza and Staurozoa.

Materials and Methods

Animal Sampling, DNA Extractions, and PCR Amplification

We collected several specimens of Cassiopea andromeda (Scyphozoa) and Millepora platyphylla (Hydrozoa) on the site of Tiahura in Moorea (French Polynesia). We collected specimens of Carybdea xaymacana (Cuboza), Cassiopea frondosa and Chrysaora sp. (Scyphozoa), and Cubaia aphrodite (Hydrozoa) at Bocas del Toro (Panama). The staurozoan Craterolophus convolulus was collected off the island of Helgoland (Germany) and Haliclystus “sanjuanensis” was collected off the coast of Washington state. The cubozoans Carukia barnesi and Chironex fleckeri were collected at Cairns and Weipa, respectively (Queensland, Australia); Alatina moseri was collected in Waikiki (Hawaii), and Chiropsalmus quadramanus was collected on the east coast of the United States, near the border between North and South Carolina. We purchased specimens of Cyanea capillata (Scyphozoa), Clava multicorns, Ectopleura larynx, Laomedea flexuosa, and Pennaria tiarella (Hydrozoa) from the Marine Biological Laboratory at Woods Hole (www.mbl.edu) and Nemopsis bachei from the Gulf Specimen Marine Lab (www.gulfspecimen.org). Tissues from Actostylus mosaicus, Linuche unguiculata, and Rhizostoma pulmo (Scyphozoa) were kindly provided by Dr Michael Dawson, individuals of Obelia longissima (Hydrozoa) by Dr Annette F. Govindarajan, a specimen of Pelagia noctiluca (Scyphozoa) collected at the Station Marine d’Endoume (Marseille, France) by Dr Alexander Ereskovsky, and a DNA aliquot for Lucernaria janetae from the East Pacific Rise by Dr Janet Voight. The identities of hydrozoan species were confirmed morphologically by Dr Peter Schuchert and assessed for all species by comparing the partial cox1 and rnl sequences to the National Center for Biotechnology Information database. We extracted...
We amplified and sequenced parts of cob, cox1, cox2, nad5, ml, and ms using conserved primers for animals (Burger et al. 2007). We used the sequences obtained from these amplifications to design species-specific primers for long PCR amplifications. We amplified nearly complete mitochondrial genomes in a single piece between either cob–cox1 or cob–cox2, or in two parts between ml–nad5 and ms–cox1. We extended our sequences using a modified step-out protocol (Burger et al. 2007) and reamplified the contigs using the standard PCR. We then sheared the long PCR products and processed them for multiplex sequencing (Meyer et al. 2008) using either the 454 high-throughput or Illumina Sequencing platforms, carried out at the Center for Genomics and Bioinformatics of Indiana University and the Office of Biotechnology DNA Facility of Iowa State University, respectively. For C. convolvulus, H. “sanjuanensis,” and L. unguiculata, we constructed random clone libraries from the purified PCR product using the TOPO Shotgun Subcloning Kit from Invitrogen (for details, see Burger et al. 2007) and sequenced at the Office of Biotechnology DNA Facility of the Iowa State University.

We identified mitochondrial contigs from the partial genomic reads of A. moseri generated by the Irdian Genomes project. We used specific primers and step-out protocol on a tissue sample from another individual of A. moseri to extend our sequences toward the end of the mitochondrial chromosomes. Because of the high degree of segmentation of cubozoan mtDNA, we PCR amplified nearly complete coding sequences using the standard PCRs (for ms–nad1 and cox2–cox3) and the step-out protocols (for cob, cox1, nad4, nad2–nad5, ORF314–polB, and ml) and cloned the products into TOPO vectors. Plasmid preparation and sequencing were processed at the Iowa State University Office of Biotechnology DNA facility. We used enzymatic digestion of total DNA with the methylation-sensitive restriction enzyme HpaII to confirm that PCR products amplified in this study are genuine mitochondrial sequences (Kumar and Bendich 2011).

### Genome Assembly, Gene Annotation, and Analysis

We assembled all sequences using the STADEN software suite (Staden 1996). We used both the tRNAscan-SE and ARWEN programs (Lowe and Eddy 1997; Laslett and Canbäck 2008) to identify tRNA genes. We reconstructed the secondary structures of rRNA met (C. multicornis) and TDI (L. unguiculata) by comparing with their homologues in other cnidarian species. Other genes were identified by similar searches in the local databases using the FASTA program (Pearson 1994). We predicted the boundaries of rRNA genes by aligning them to tRNA sequences from the published cnidarians and checking the alignments manually. We also aligned individual protein genes with ClustalW 1.82 (Thompson et al. 1994) using default parameters. We manually verified all alignments based on either amino acid alignments (for protein coding genes) or inferred rRNA or tRNA secondary structures (for RNA-coding genes) to A. aurita and H. oligactis (Shao et al. 2006; Kayal and Lavrov 2008). For the analysis of sequence similarities, we calculated sequence identities using local scripts based on the BioPerl modules (Stajich et al. 2002). We estimated codon usage with the CUSP program in the EMBOSS package (Rice et al. 2000).

### Results

#### The Linear Mitochondrial Genomes of Medusozoa

We amplified and sequenced mitochondrial (mt) genomes of eight species representing the two clades of Scyphozoa (Coronatae: L. unguiculata; Discomedusae: C. andromeda, C. frondosa, C. mosaicus, Chrysaora sp, C. capillata, P. noctiluca, and R. pulmo (partial)), eight species representing the two clades of Hydrozoa (Hydroidolina: C. multicorns, E. larynx, L. flexuosa, M. platyphilla, N. bachei, O. longissima, and P. tiarella; Trachyliena: C. aphrodite), and three species representing both orders of Staurozooa (Cleistocarpia: C. convolvulus; Eleutherothecia: H. “sanjuanensis” and L. janetae). In addition, we obtained substantial mitochondrial sequences from five species belonging to the two major cubozoan clades (Carybdea: A. moseri, C. barnesi, and C. xaymacana; Chiropodica: C. fleckeri and C. quadrumanus).

The mtDNA of all scyphozoan, staurozooan, and hydrozoan species sampled for this study mapped into single linear molecules (see below). Cubozoan mtDNA was composed of eight mitochondrial chromosomes, each encoding one to five mitochondrial genes with the same transcriptional orientation. This is in agreement with earlier DNA hybridization experiments, which suggested that the cubozoan mtDNA was composed of several, up to 4 kb, linear chromosomes (Ender and Schierwater 2003). All cubozoan mitochondrial genes were similar in size to their homologues in other medusozoans and contain neither internal stop codons (TAA or TAG) nor framenshifts. In addition, incubation of the A. moseri total DNA with the methylation-sensitive restriction enzyme HpaII (method described in Kumar and Bendich 2011) inhibited PCR amplifications of the contigs harboring the restriction sites (supplementary fig. S1, Supplementary Material online). Together, this evidence strongly suggested that our cubozoan contigs were genuine mitochondrial sequences, as opposed to nuclear pseudogenes (NUMTs).

All medusozoan mtDNA contained a set of genes for 13 protein subunits conserved in animals and involved in the electron transport chain of oxidative phosphorylation (atp6–8, cob, cox1–3, nad1–6), two ribosomal RNAs (rRNA) (rRNA and rRNA), and two transfer RNAs (tRNA) (tRNA and tRNA). tRNA was missing from the partial mitochondrial genome of the coronate L. unguiculata and no
tRNA genes were found in the partial sequences from cubozoans (see below). We also found some level of class-specific conservation at the ends of the linear mitochondrial chromosomes: duplicated cox1 in most hydrozoans (Hydroidolina), polB and an extra ORF in cubozoans, scyphozoans, staurozoans, and the trachyline hydrozoan C. aphrodite (see below).

Medusozoan mitochondrial genomes displayed a compact organization with few, if any intergenic regions (IGRs), and several pairs of overlapping genes. We found overlapping nad3–nad6 genes in the mtDNA of all hydrozoans, scyphozoans, and staurozoans species (11 ± 3, 35 ± 17, and 9 ± 2 nucleotides respectively). In addition, nad4L–nad3 overlapped by 10 nucleotides in all scyphozoan species, nad1–nad4 overlapped by 10 ± 3 nucleotides in all hydrozoans, atp6–atp8 overlapped by seven nucleotides, and nad2–nad5 by 13 nucleotides in all staurozoans. Similarly, nad2–nad5 overlapped by 26 and 44 nucleotides in the staurozoans H. "sanjuanensis" and L. janetae, respectively. Finally, in the aplanulate E. larynx we found cob–cox1 overlapping by 44 nucleotides. The same two genes overlapped by eight nucleotides in Hydra magnipapillata and H. vulgaris (Voigt et al. 2008), which are also part of the Aplacophora (Collins et al. 2005).

We compared the variation in the mtDNA nucleotide composition among different cnidarian classes (table 1). The linear mtDNA in medusozoans displayed GC-skew values close to zero: cubozoans, hydrozoans, and scyphozoans all shared a small positive GC-skew (on average 0.04 ± 0.21) and similar to those in anthozoans. The A + T content of coding regions varied substantially between different medusozoan classes. Hydrozoan mtDNA had the highest A + T content (74% ± 0.34) between the two strands of the coding sequences, while staurozoans had a slightly negative GC-skew (−0.05 ± 0.02). In comparison, the circular mtDNA in anthozoans displayed a positive GC-skew (0.20 ± 0.09 in hexacorals and 0.09 ± 0.01 in octocorals). AT-skews in all studied medusozoan species were negative for the coding strand (between −0.11 and −0.21) and similar to those in anthozoans. The A + T content of coding regions varied substantially between different medusozoan classes. Hydrozoan mtDNA had the highest A + T content (74% ± 0.34) between the two strands of the coding sequences, while staurozoans had a slightly negative GC-skew (−0.05 ± 0.02). In comparison, the circular mtDNA in anthozoans displayed a positive GC-skew (0.20 ± 0.09 in hexacorals and 0.09 ± 0.01 in octocorals). AT-skews in all studied medusozoan species were negative for the coding strand (between −0.11 and −0.21) and similar to those in anthozoans.

### Table 1

| Gene | Sizea | %ATb | Startc | Endd | Size | %AT | Start | End |
|------|-------|------|--------|------|-------|------|-------|------|
| **Cubozoan** | | | | | | | | |
| atp6 | 708 ± 7 | 63 ± 2 | AG | * | 704 ± 1 | 75 ± 4 | A | * | 704 ± 3 | 69 ± 4 | A | * | 708 ± 0 | 63 ± 1 | A | A |
| atp8 | 210 ± 2 | 64 ± 4 | AG | * | 206 ± 3 | 83 ± 5 | A | * | 208 ± 7 | 73 ± 4 | AG | * | 204 ± 0 | 62 ± 4 | A | A |
| cob | 1149 ± 62 | 72 ± 2 | AG | G | 1148 ± 12 | 73 ± 3 | AG | A | 1146 ± 8 | 66 ± 2 | A | AG | 1068 ± 0 | 60 ± 2 | A | ? |
| cox1 | 1569 ± 58 | 3 ± 3 | A | G | 1569 ± 67 | 67 ± 3 | AG | A | 1580 ± 7 | 64 ± 3 | AG | A | 1578 ± 0 | 61 ± 1 | A | A |
| cox2 | 737 ± 2 | 62 ± 1 | AG | * | 744 ± 10 | 73 ± 4 | AG | A | 746 ± 8 | 67 ± 4 | A | AG | 747 ± 0 | 62 ± 1 | A | AG |
| cox3 | 786 ± 0 | 59 ± 3 | A | G | 786 ± 0 | 72 ± 4 | AG | A | 786 ± 0 | 64 ± 3 | AG | A | 786 ± 0 | 61 ± 1 | A | AG |
| nad1 | 987 ± 8 | 62 ± 3 | AG | AG | 989 ± 4 | 73 ± 4 | AG | A | 972 ± 5 | 66 ± 4 | AG | A | 987 ± 0 | 59 ± 0 | A | A |
| nad2 | 1341 ± 63 | 67 ± 3 | A | G | 1328 ± 32 | 79 ± 5 | AG | A | 1323 ± 13 | 70 ± 5 | AG | A | 1346 ± 2 | 59 ± 4 | A | A |
| nad3 | 351 ± 0 | 62 ± 4 | AG | * | 355 ± 4 | 77 ± 4 | A | * | 357 ± 6 | 69 ± 4 | AG | A | 354 ± 0 | 65 ± 4 | A | A |
| nad4 | 1446 ± 59 | 4 | A | G | 1458 ± 2 | 76 ± 4 | AG | A | 1441 ± 2 | 68 ± 5 | AG | A | 1461 ± 0 | 61 ± 3 | A | AG |
| nad4L | 290 ± 26 | 67 ± 3 | AG | * | 299 ± 2 | 79 ± 4 | A | * | 303 ± 1 | 72 ± 5 | AG | A | 299 ± 2 | 64 ± 1 | A | * |
| nad5 | 1824 ± 62 | 16 ± 3 | AG | G | 1832 ± 2 | 76 ± 4 | AG | A | 1830 ± 19 | 68 ± 5 | AG | A | 1860 ± 13 | 60 ± 2 | A | AG |
| nad6 | 542 ± 24 | 64 ± 3 | AG | G | 556 ± 8 | 79 ± 5 | AG | A | 564 ± 12 | 70 ± 5 | AG | A | 553 ± 2 | 62 ± 2 | A | A |
| ORF3-14 | 6-9 | 5A N A NA | 6-9 | 5A NA | 6-9 | 5A NA | 6-9 | 5A NA | 6-9 | 5A NA | 6-9 | 5A NA | 6-9 | 5A NA | 6-9 | 5A NA |
| polB | 873 ± 85 | 58 ± 0 | G | A | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? |
| mi | 769 ± 57 | 58 ± 0 | NA | NA | 1746 ± 9 | 76 ± 4 | NA | NA | 1818 ± 34 | 69 ± 5 | NA | NA | 1830 ± 57 | 1 ± NA | NA |
| ms | 679 ± 57 | 58 ± 0 | NA | NA | 910 ± 21 | 74 ± 2 | NA | NA | 950 ± 10 | 69 ± 3 | NA | NA | 914 ± 57 | 1 ± NA | NA |
| tmM | — | — | NA | NA | 71 ± 1 | 69 ± 2 | NA | NA | 71 ± 0 | 64 ± 5 | NA | NA | 69 ± 0 | 53 ± 2 | NA | NA |
| tmW | — | — | NA | NA | 70 ± 1 | 65 ± 3 | NA | NA | 70 ± 0 | 64 ± 5 | NA | NA | 71 ± 0 | 52 ± 2 | NA | NA |

**Note.**—The average size in nucleotides (and standard deviation), AT composition (and standard deviation), and putative start and stop codons are reported for each of the Medusozoan subclades. NA, not applicable; ?, data not available.

a Average size in nucleotides, with standard deviation.
b AT composition, with standard deviation.
c Start A and G stand for ATG and GTG start codons, respectively.
d Stop A and G stand for TAA and TAG stop codons, respectively; an asterisk corresponds to an incomplete stop codon (see text).
Staurozoan have relatively higher GC content similar to anthozoans.

codons (glycine, alanine, arginine, and proline [G]) plotted against the percentage of amino acids encoded by G- and C-rich sequences. The total G-C content of mitochondrial protein genes is by very A-T rich genomes and relatively fewer [G] line and correlation coefficient are displayed. Hydrozoa are characterized

**Gene Arrangements in Medusozoan mtDNA**

Analysis of the newly determined mitochondrial sequences revealed a well-conserved mitochondrial gene order within Medusozoa (fig. 2A). Eleven representatives of both discomedusan Scyphozoa and Staurozoa sampled for this study had a completely conserved gene order, identical to that found in A. aurita (Shao et al. 2006). In these groups, the cluster formed by cox1-rnl-ORF314-polB had a different transcriptional orientation to the rest of the genes (fig. 2A). The two clusters were separated by a relatively large noncoding region of about 80 nucleotides, potentially involved in the control of mitochondrial transcription (see below). In the corona L. unguiculata, trnW was not found either in its conserved position between cox2 and atp8 or in the rest of the genome. We were also unable to identify sequences for polB and ORF314 in this species. However, given that the sequence of L. unguiculata is incomplete, it is possible that these genes are present in the mitochondrial genome, downstream of cox7. The gene arrangement was also conserved in all cubozoan species, despite the presence of multiple chromosomes (fig. 2A). Single gene chromosomes harbored cob, cox1, nad4, and rnl genes. Other chromosomes contained gene pairs nad2-nad5 and ORF314-pol8, and gene clusters cox2-atp8-atp6-cox3 and ms-nad6-nad3-nad4L-nad1. When placed together, the gene order across the eight chromosomes in cubozoan mtDNA is nearly identical to that in most scyphozoans and staurozoans except for the absence of tRNA genes.

The mitochondrial genome organization within Hydrozoa was more variable, with at least four different gene orders. The genome organization in the trachyline C. aphrodite was identical to that in discomedusan Scyphozoa and Staurozoa (fig. 2A). All species from the clades Capitata (M. platyphyla and P. tiarella), Filifera III (C. multicorns) and VI (N. bachei), and Leptothecata (L. flexuosa and O. longissima) had a genome organization resembling that of C. aphrodite, with the exception of a duplicated cox1 downstream of cob and the loss of ORF314-polB (fig. 2A; for the description of hydrozoan subclades, see Cartwright et al. 2008). The genome organization in the aplanulate E. larynx was similar to H. oligactis, where rnl was inverted and both tRNA genes (trnM and trnW) moved compared with the other hydrozoans. Interestingly, all nontrachyline hydrozoan species sequenced in this study had two identical copies of cox1, one at each end of the mitochondrial chromosome. In comparison, the three previously published Hydridae mitochondrial genomes (Hydra magnipapillata, H. oligactis, and H. vulgaris) were reported to have only one complete copy of cox1 downstream of cob, with the other copy(ies) being partially degenerated and lacking the 5’ end of the gene (Kayal and Lavrov 2008; Voigt et al. 2008). Furthermore, in H. magnipapillata and H. vulgaris, the mtDNA was split into two nearly equal sized chromosomes, a feature also reported for several other species belonging to the family Hydridae (Warrior and Gall 1985; Bridge et al. 1992; Ender and Schierwater 2003).

Overall, the mitochondrial gene order in medusozoa is well conserved, with only four unique gene orders found so far (ignoring genome fragmentation and cox1 degeneration in Hydridae). We inferred the evolution of mitochondrial genome organization in medusozoa using the currently accepted cnidarian phylogeny (fig. 2B; Collins et al. 2006; Cartwright et al. 2008). Gene order similar to the scyphozoan A. aurita is found in all four of the recognized medusozoa classes. Given the reportedly low rate of genome rearrangement in animals, it is most parsimonious to reconstruct the mitochondrial ancestral medusozoa gene order (AMGO) as identical to the gene order found in A. aurita (fig. 2B).

**Protein Gene Similarity in Medusozoa mtDNA**

Sizes of orthologous protein genes in medusozoa mtDNA showed little variation (0–4%), as compared to anthozoans (0–11%). By contrast, the overall amino acid pairwise identity of protein genes for Medusozoa was highly variable, ranging between 24–96% (average 49% SD = 12) (table 1). Such variability indicates that mtDNA proteins can be good markers for reconstructing the evolutionary history of medusozoa at lower taxonomic levels. Interestingly,
the amino acid pairwise identity of protein genes between coronates and other scyphozoans (50% SD = 2) were similar to the values for all other cnidarian classes (50% and 51% with hexa- and octocorals, 38% with cubozoans, 47% with hydrozoans, and 48% with staurozoans), with a mean of 49% SD = 4 to all cnidarians.

**Fig. 2.**—Evolution of mitochondrial genomes in Medusozoa (Cnidaria) based on phylogenetic relationships according to Collins et al. (2006), Collins et al. (2008), and Cartwright et al. (2008). (A) Comparison of mitochondrial gene orders between various medusozoan clades and the putative mitochondrial AMGO. Breaks in contigs mark the ends of linear mitochondrial chromosomes. Genes are transcribed from left to right unless underlined. *cox1-p* corresponds to the partial copy of *cox1* at the end of the mtDNA molecule(s) in the Hydridae family. *polB* is an inferred member of the B DNA polymerase gene family and *ORF314* is an unidentified ORF located upstream of it. Lightning bolts represent breaks in the mitochondrial chromosomes. Black arrows depict directions of inferred genome rearrangements. (B) Evolution of genome organization of the linear mtDNA in Medusozoa. The phylogeny is based on Collins et al. (2006). Major genomic rearrangements are labeled. 1) Linearization of the mtDNA by insertion of a linear plasmid of unknown source; 2) loss of *polB* and *ORF314*, and duplication of *cox1* at each end of the linear chromosome within Hydrozoa after the divergence of Trachylina; 3) displacement of tRNAs and inversion of *ml* in Aplanulata; 4) genome segmentalization of the mtDNA in several Hydridae species with consequent *trnM* and *cox1* duplication; 5) high level segmentalization of the mtDNA in cubozoan; and 6) displacement of *trnW* in the coronaate *L. unguiculata*. 
The minimally derived genetic code (TGA = tryptophan) was inferred for mitochondrial protein synthesis in all the medusozoan genomes analyzed in this study. Codon usage bias estimated as the effective number of codons \( Nc \) (Wright 1990) was highly correlated with the GC content of the mtDNAs \( (R^2 = 0.97) \), where the AT-rich hydrozoan mtDNA displayed the highest codon usage bias (35 SD = 4), and the lowest values were found in cubozoans and staurazoans (47 SD = 4 and 47 SD = 1 respectively). Within a given codon family, codons ending with A or T were favored over those ending with C or G. CNG codons were nearly absent from the mtDNA of cubozoans, hydrozoans, and scyphozoans, where they represented < 15% of arginine residues with no CGG codon used in hydrozoans. In contrast, CNG codons encoded about 50% of arginine residues in staurazoans.

ATG was inferred as the initiation codon for most mitochondrial coding sequences, yet, we inferred GTG as a start codon for two genes in Hydrozoa (cob in \( L. flexuosa \) and \( O. longissima \), and \( cox1 \) in \( E. larynx \)) and five genes in Scyphozoa (atp8 in \( U. unguliculata \), nad1 in \( P. noctiluca \), nad3 in all but two species, nad4L in \( C. mosaicus \), and nad5 in \( C. aphaedita \)) (table 1). GTG was also the inferred initiation codon in at least 5 of the 13 protein genes in Cubozoza, and mostly present in the order Chirodropida. Stop codons TAA and TAG were found in all medusozoan classes (80% and 20% of the genes respectively, where the former value represents both TAA and incomplete TA stop codons). Incomplete stop codons TA were inferred for several protein genes in medusozoan mtDNA, predominantly in atp6, atp8, nad3, and nad4L (table 1). In the aplanulatan \( E. larynx \) the nearest stop codon for nad5 is located 30 nucleotides past the 5’ end of \( rns \). Interestingly, no stop codon was inferred for nad5 in the scyphozoan \( P. noctiluca \), as the nearest stop codon is found 77 nucleotides within \( rns \).

**RNA Genes in Medusozoan mtDNA**

All complete medusozoan mt-genomes analyzed in this study contained four RNA genes typical for Cnidaria: two genes coding for large and small subunit rRNA (\( ml \) and \( rns \)), and two for methionine and tryptophan tRNAs (\( trnM(cau) \) and \( trnW(au) \)). The genes for the small and large ribosomal RNA subunits were on average 17% smaller in size and displayed less size variation in medusozoans compared to their homologues from anthozoans (table 1). The nucleotide composition of RNA genes was slightly more AT-rich than that of the protein coding regions.

Both medusozoan mt-tRNA genes were more variable in primary sequence and inferred secondary structure than their homologues in anthozoans and demosponges (Wang and Lavrov 2008). The initiator \( trnM^{Met} \) was on average 70 nucleotides long, ranging from 68 in staurazoans to 71 in hydrozoans and scyphozoans. The shorter \( trnM^{Met} \) in Staurazoza lacked two nucleotides at positions 16 and 17 of the D-loop (fig. 3). The anticodon arm in all hydrozoan and staurazoan \( trnM^{Met} \) had an extra base pair typical to type 7 tRNA (Watanabe et al. 1994; Steinberg and Cedergren 1995; Steinberg et al. 1997), resulting in a shorter variable loop (three nucleotides) in the hydrozoans, but a standard four-nucleotides long variable loop in staurazoans. The \( trnM^{Met} \) of all medusozoans but \( C. aphaedita \) and \( H. species \), had two nucleotides (8 and 9) in the connector region between the acceptor and the D-arm, which makes the loss of nucleotide 9 in mitochondrial \( trnM^{Met} \), a synapomorphy for the Hydroidea family. Finally, all hydrozoans but \( C. aphaedita \) and all staurazoans lacked nucleotide 16 in the D-loop of \( trnM^{Met} \). The partial cubozoan mitochondrial sequences determined for this study harbored no RNA genes.

**Intergenic and Putative Control Regions in Medusozoan mtDNA**

We found a single, relatively large (60–94 nucleotides) and AT-rich (> 70%) IGR between \( cox1 \) and \( cox2 \) in all scyphozoan and staurazoan species, and in the trachyline hydrozoan \( C. aphaedita \) mtDNA (fig. 2A). This IGR delimits two sets of genes with opposite transcriptional polarity. A large portion of this region can be folded into a conserved stem-loop motif with up to 13-base pairs in the stem, most of them (23 bases) completely conserved in all scyphozoans sampled in this study and some (12 bases) also conserved in staurazoans and in the hydrozoan \( C. aphaedita \) (fig. 4A). In most other hydrozoan groups (Filifera, Capitata, and Leptotheleata), the largest IGR was located between \( cox2 \) and \( ml \), two genes with opposite transcriptional
polarities (fig. 2A). This IGR also harbored a 20 nucleotide-long sequence that, while displaying low sequence conservation between hydrozoan species, could be folded into a strong stem-loop element (fig. 4A).

In aplanulatan hydrozoans, we found a relatively large (42–52 nt) noncoding region between cox3 and nad2 that displayed up to 70% similarity with trnW sequences. Finally we did not find major IGRs in cubozoan mtDNA.

The “Ends” of Medusozoan mtDNA: Extra ORFs versus Duplicated Genes

All species of Staurozoa, discomedusan Scyphozoa (the sequence of the corresponding region in the coronate scyphozoan *L. unguiculata* remains undetermined), and the trachyline hydrozoan *C. aphrodite* contained two ORFs at one end of the molecule. In Cubozoa, analogous ORFs were found on a separate mitochondrial chromosome. The sequences of the larger ORF were similar with *polB* described in *A. aurita* (Shao et al. 2006) and formed a monophyletic group in a phylogenetic analysis that included *polB* from other organisms (supplementary fig. S2, Supplementary Material online). The sequence of the smaller ORF (*ORF314*) was poorly conserved within Medusozoa, with only two identical amino acid residues between the three classes. This ORF encoded about 100 amino acids and was always located upstream of *polB*. Although preliminary analysis using 3D modeling of the tertiary structure of the putative product of *ORF314* suggests similarities with elongation factors and a potential DNA-binding activity (data not shown), further studies are needed to investigate the role of this gene.

**Fig. 4.**—Putative control region and terminal stem-loop found in medusozoan mtDNA. (A) Consensus sequence of the putative control regions (CR) found in the mtDNA of Hydrozoa, Scyphozoa, and Staurozoa. This CR corresponds to the largest IGR that delimits two sets of genes with opposite transcriptional polarity. (B) Putative stem-loop structure found at the end of scyphozoan mtDNA.
The mtDNA of all hydrozoans but the trachyline C. aphyrodite lacked the two extra ORFs (polB and ORF314) (fig. 2A). Furthermore, we were not able to identify copies of these genes in the nuclear genome of H. magnipapillata (Chapman et al. 2010). Instead, we found a complete copy of cox1 at each end of the mitochondrial chromosome (fig. 2A). The two copies of cox1 had opposite transcriptional polarities and formed part of the inverted terminal repeats (ITRs, fig. 2A). In addition, we found a relatively conserved 116 bp sequence downstream of cox1 in A. aurita and Cassiopea spp. mtDNAs that could be folded into a complex stem-loop structure (fig. 4B). The presence of ITRs is a hallmark for the linear mitochondrial genomes and has been reported in several distantly related organisms (Valach et al. 2011 and references therein).

Discussion

Linear mitochondrial DNA has been well documented in several nonmetazoan groups such as plants and fungi. In yeast, where linear mtDNA has been extensively studied, the distribution of linear and circular mitochondrial genomes has no apparent phylogenetic signal, with closely related species having either linear or circular mtDNA (Nosek et al. 1998; Nosek and Tomaska 2008). In animals, strictly linear mtDNA has been described only in Medusozoa (Warrior and Gall 1985; Bridge et al. 1992), for which only six genomes have been published to date (Shao et al. 2006; Kayal and Lavrov 2008; Voigt et al. 2008; Park, Hwang, et al. 2011). The only other report of linear mtDNA in animals comes from the crustacean isopod, Armadillidium vulgare, where the mtDNA consists of a circular ~28 kb dimer and a linear ~14 kb monomer (Raimond et al. 1999; Marcadé et al. 2007). Our study represents the first thorough survey of the evolution of linear mtDNA in Medusozoa. In order to better understand the evolutionary history of mtDNA linearity in this group, we surveyed species belonging to all medusozoan classes, as well as their primary subclades.

We analyzed medusozoan mitochondrial genomes in a phylogenetic framework and propose the following hypotheses for the evolution of linear mtDNA in this group: 1) a single origin for the linear mtDNA in the lineage leading to Medusozoa, most likely by the integration of a linear plasmid; 2) loss of polB and ORF314, and the duplication of cox1 at each end of the linear mitochondrial chromosome after the divergence of Trachylina from the rest of the hydrozoans (Hydroidolina); 3) gene rearrangement at the stem of Aplanulata; 4) partial loss of cox1 sequence in Hydroidea and split of the mitochondrial chromosome in several species within this clade; 5) high level of mtDNA fragmentation in the branch leading to Cuboza; 6) displacement of tmnW in the coronate L. unguiculata (fig. 28). It is worth noting that the fragmentation of the mitochondrial molecules appears to be an irreversible process as no rearranged mitochondrial genomes have been observed in the taxa where mtDNA is fragmented, as one would expect if different pieces would fuse randomly.

Fukuhara et al. (1993) suggested a positive correlation between the mitochondrial gene order conservation and the linear as opposed to the circular structure of the yeast mtDNA. Our study revealed a similar trend. If we ignore the genome fragmentation and the cox1 duplication, only four unique gene orders can be distinguished in the medusozoan mtDNA: the AMGO is found in all four medusozoan classes, with modified gene orders found in the coronate L. unguiculata, Hydrodolina, and Aplanulata (fig. 24). By contrast, at least nine unique gene orders (more if the presence/the absence of ORFs is considered) have been reported in the circular mtDNA of anthozoans (Uda et al. 2011). Furthermore, most of the differences in the medusozoan mitochondrial gene orders can be explained by a few simple gene rearrangement events, whereas multiple and/or complex rearrangement events are needed to explain differences in the anthozoan gene orders. These observations suggest that the linearization has favored the stabilization of the mitochondrial genome organization in Medusozoa. On the other hand, this result may to some extent reflect the difference in the species richness in Anthozoa versus Medusozoa (about twice as many species in the former; Daly et al. 2007) and/or the number of mitochondrial genomes that have been studied in each group (43 vs. 30).

One other difference between the anthozoan and medusozoan mtDNA is the amount and distribution of noncoding intergenic DNA. Anthozoan mtDNA usually has several relatively large IGRs that sometimes contain repetitive sequences and/or ORFs (Park, Song, et al. 2011). By contrast, medusozoan mitochondrial genomes are compactly arranged with few if any intergenic nucleotides and, sometimes, overlapping genes. Most mitochondrial genomes investigated in this study contained only a single moderately large (~100 bp) IGR separating the two regions of mtDNA with opposite transcriptional polarities. The conservation of sequence and inferred secondary structure of this region suggest that it may be involved in the control of mtDNA transcription and/or replication in Scyphozoa, Staurozoa, and trachyline Hydrozoa. Similarly, the largest IGR in other hydrozoans (situated between trnM and cox1 in Aplanulata and between cox2 and rnl in other hydroidolinans) may be involved in the control of transcription as suggested earlier (Voigt et al. 2008). Compactly arranged mtDNA with a single large IGR involved in the initiation of replication (Desjardins and Morais 1990) and transcription ([L’Abbé et al. 1991]) is also a feature of bilaterian animals. Interestingly, the maturation of a polycistronic pre-mRNA in bilaterian mitochondria involves the excision of tRNA sequences located between most coding sequences in the genome, resulting in monocistronic mRNAs (Ojala et al. 1981). Based on the near absence of IGRs, one can assume that the transcription of medusozoan mtDNA is...
also polycistronic. However, the loss of all but two tRNA genes necessitates a different mechanism for mRNA maturation in medusozoan mitochondria. Finally, the absence of large IGRs and the conservation of unidirectional transcriptional orientation of all genes in cubozoan mtDNA suggest that the initiation of replication and transcription occurs at the end(s) of each of the mitochondrial chromosomes.

We found two distinctive genetic elements at the end of the medusozoan mitochondrial chromosomes: duplicated cox1 in the hydrozoan Liriope pacifica and two extra ORFs (polB and ORF314) in all the other medusozoan classes (Cubozoa, discomedusan Scyphozoa, Staurozoa, and trachyline Hydrozoa). In an earlier study, Shao et al. (2006) showed that polB from the scyphozoan A. aurita mtDNA shares several conserved motifs characteristic of the polymerase domain in family B-DNA polymerases. The authors also attributed the origin of the two extra protein genes to an ancient invasion event by a linear plasmid that resulted in the linearization of the mtDNA in Medusozoa. Similar polB-like sequences are found in the linear mtDNA of several nonanimal groups such as fungi and algae, where they are hypothetically associated with the integration of linear plasmids in the mitochondrial genome (Mouhamadou et al. 2004). The grouping of medusozoan polB sequences into a monophyletic clade suggests a single invasion event early in the evolutionary history of the group that coincided with the origin of its linear mtDNA at the stem of the medusozoan tree. Yet, the low level of sequence conservation in polB-like sequences between medusozoan and nonanimal species hinders our attempts to predict the source of the invading element. Nevertheless, the conservation of both sequence length and position of the two ORFs suggests some level of selection pressure for their maintenance in the mtDNA of most medusozoans. Short ITRs have been previously reported in the mtDNA of A. aurita (Shao et al. 2006) and the hydrozoans H. magnipapillata, H. oligactis, and H. vulgaris (Kayal and Lavrov 2008; Voigt et al. 2008). ITRs are one out of the few telomeric structures found in the linear mtDNAs (Nosek et al. 1998; Nosek and Tomáška 2003) and involved in the maintenance and replication of the linear chromosomes. In yeast, short ITRs are associated with type III linear mtDNA, where a TA covalently binds at the single-stranded 5′ end of the linear molecules (Nosek and Tomáška 2008). The linear molecules generally encode TPs, either as part of a DNA polymerase gene or by a yet unidentified ORF (Fricova et al. 2010 and references therein). In the medusozoan mtDNA, the putative product of ORF314, if shown to have DNA-binding properties, may act as a TP. Consequently, assuming polB and ORF314 are functional genes, we predict that the mtDNA in cubozoans, scyphozoans, staurozoans, and trachyline hydrozoans uses mechanisms of maintenance and replication similar to the type III linear mtDNA as found in yeasts, linear plasmids, and adenoviral DNA. This assertion is further supported by the similarity of polB sequences between the medusozoan and linear plasmids. As described above, both polB and ORF314 are absent from nontrachyline hydrozoan mtDNAs, and no homologues could be found in the completely sequenced nuclear genome of H. magnipapillata (Hydridae, Hydrozoa). Thus, unlike what has been suggested by an earlier study (Voigt et al. 2008), hydrozoans appear to employ an alternative mechanism for the maintenance and replication of their mtDNA. However, the recruitment of a nuclear encoded TP for the maintenance of linear mtDNA in these hydrozoans cannot be ruled out. In addition, the duplication of cox1 at each end of the mitochondrial chromosome(s) is limited to the lineage leading to Hydrozoa, suggesting that a novel mechanism evolved after the divergence of Trachylina and the rest of hydrozoans. Future studies that explore the end conformations of the medusozoan mtDNA will shed light on the maintenance and processing of these linear molecules.

Despite our efforts, we were not able to verify the expression of polB and ORF314 at the RNA level, most likely as a consequence of poor RNA conservation in the preserved tissue samples. We were also unable to investigate the processes involved in transcription and replication of the mtDNA in medusozoans. The analysis of a data set containing ESTs from the scyphozoan C. capillata was unsuccessful, highlighting the difficulties associated in working with the RNA in this group. Interestingly, in expressed sequence tag (EST) assemblies obtained from the staurozoan H. “sanjuanensis,” we found a single mitochondrial hit that spans from the 3′-end of cox1 to the 5′-end of rnl, suggesting that these genes are transcribed in a polycistronic manner. Yet, additional studies are needed to shed light on the role of the two extra ORFs (polB and ORF314) and their putative products in the maintenance of linear mitochondrial chromosomes in medusozoans, and on the mechanisms of mitochondrial gene expression in the group.

**Conclusion**

Medusozoan mtDNA is typically a small compact genome composed of one or several linear chromosomes with a highly conserved gene order, and ITRs at the end of the molecule(s). The linearity of the medusozoan mtDNA is most likely the product of a unique invasion event by a linear plasmid-type element harboring a polB-like and a putative TP gene, with secondary loss of the two extra ORFs within hydrozoans. Medusozoan mitochondrial genomes lack large IGRs, contain several overlapping genes, and harbor shorter protein and ribosomal genes than the representatives from other nonbilaterian clades (Signorovitch et al. 2007; Wang and Lavrov 2008). Besides their strictly linear mitochondrial genomes, a unique feature in animals, the medusozoan mtDNA display characteristics that can be described as intermediary between bilaterian and nonbilaterian animals.
On the one hand, medusozoans use a minimally derived genetic code for mitochondrial protein synthesis and show a relatively low evolution rate of their mitochondrial genes, as found in most nonbilaterian animals. On the other hand, the compactness of their genomes and a relatively stable gene order, both, mirror the evolution of the bilaterian mtDNA. Put into a broader perspective, our results show that the evolution of the animal mitochondrial genomes does not follow a single linear pathway as proposed in an earlier study (Lavrov 2007). This is well illustrated in the cnidarian clade where both bilaterian-like and nonbilaterian-like mitochondrial features are found. Future studies that focus on undersampled animal taxa may further expose the rather unpredictable evolutionary pattern of genome evolution in animal mitochondria, revealing a more complex picture of mtDNA evolution in Metazoa. In addition, we predict that a larger survey of linear mtDNA in medusozoans may uncover original/new solutions to the “challenge of the ends” (Nosek et al. 1998).

Supplementary Material
Supplementary figs. S1 and S2 are available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org).

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