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Extreme mitochondrial evolution in the ctenophore *Mnemiopsis leidyi*: Insight from mtDNA and the nuclear genome

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Abstract

Recent advances in sequencing technology have led to a rapid accumulation of mitochondrial DNA (mtDNA) sequences, which now represent the wide spectrum of animal diversity. However, one animal phylum—Ctenophora—has, to date, remained completely unsampled. Ctenophores, a small group of marine animals, are of interest due to their unusual biology, controversial phylogenetic position, and devastating impact as invasive species. Using data from the *Mnemiopsis leidyi* genome sequencing project, we Polymerase Chain Reaction (PCR) amplified and analyzed its complete mitochondrial (mt-) genome. At just over 10 kb, the mt-genome of *M. leidyi* is the smallest animal mtDNA ever reported and is among the most derived. It has lost at least 25 genes, including *atp6* and all tRNA genes. We show that *atp6* has been relocated to the nuclear genome and has acquired introns and a mitochondrial targeting presequence, while tRNA genes have been genuinely lost, along with nuclear-encoded mt-aminoacyl tRNA synthetases. The mt-genome of *M. leidyi* also displays extremely high rates of sequence evolution, which likely led to the degeneration of both protein and rRNA genes. In particular, encoded rRNA molecules possess little similarity with their homologs in other organisms and have highly reduced secondary structures. At the same time, nuclear encoded mt-ribosomal proteins have undergone expansions, likely to compensate for the reductions in mt-rRNA. The unusual features identified in *M. leidyi* mtDNA make this organism an interesting system for the study of various aspects of mitochondrial biology, particularly protein and tRNA import and mt-ribosome structures, and add to its value as an emerging model species. Furthermore, the fast-evolving *M. leidyi* mtDNA should be a convenient molecular marker for species- and population-level studies.

Keywords: *Ctenophora, comparative genomics, cytonuclear coevolution*

Introduction

Animal mitochondrial DNA (mtDNA) is a small molecule that has been used extensively in population genetic, phylogenetic, and biogeographic studies (Avise 2004). Although originally considered remarkably uniform, recent sampling has uncovered substantial diversity in the organization of animal mitochondrial (mt-) genomes (Lavrov 2011). In particular, each sampled phylum of non-bilaterian animals (e.g. Cnidaria, Placozoa, and Porifera) and even most major lineages within them (e.g. demosponges vs. glass sponges) have revealed distinct modes and tempos of mt-genome evolution (Haen et al. 2007; Signorovitch et al. 2007; Kayal and Lavrov 2008; Wang and Lavrov 2008). Substantial variation has been found in the gene content, rates of sequence evolution, structures of encoded rRNA and tRNA, genetic code used in mitochondrial translation, presence or absence of introns, and percentage of non-coding DNA (reviewed in Lavrov 2011). To date,

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complete mt-genome sequences have been generated for more than 2000 animals, representing the wide spectrum of metazoan diversity, while partial mtDNA sequences (such as *cox1* barcodes) are available for thousands of other species (Ratnasingham and Hebert 2007). Despite this remarkable collection of mtDNA sequences from a multitude of species, one glaring omission remains: no genuine mitochondrial sequence has been reported to date for the phylum Ctenophora.

Ctenophores are a small, well-defined phylum of mostly pelagic, carnivorous, marine animals with a simple body plan and uncertain phylogenetic affinity to other Metazoa (Harbison 1985). They are characterized by biradial symmetry, an oral–aboral axis delimited by a mouth and an apical sensory organ, a locomotor system made of eight rows of laterally reinforced macrocilia or comb plates, and, in most species, a pair of retractable tentacles that contain specialized adhesive cells called colloblasts (Hernandez-Nicaise 1991). All ctenophores have a well-developed gastrovascular system that functions in digestion, circulation, excretion, and reproduction. The rest of the body volume consists primarily of a poorly differentiated gelatinous mesogloea containing several cell types, including muscle cells and neurons (Hernandez-Nicaise 1991). Most ctenophores are extremely fragile and difficult to culture and, as such, we know relatively little about their biology (Pang and Martindale 2008b).

The lack of mtDNA data from the phylum Ctenophora is unfortunate for several reasons. From an evolutionary perspective, ctenophore lineage diverged from other animals very early in metazoan evolution. Fossils with ctenophore-like morphology have been reported from the early Cambrian period (~540 MYA; Chen et al. 2007), and some intriguing morphological connections were suggested for Ctenophora and Ediacaran taxa (Dzik 2002). Because ctenophores evolved independently from the rest of Metazoa for hundreds of millions of years, they may have retained some revealing mitochondrial features from their common ancestor with other animals or evolved some unique features not present anywhere else in the animal kingdom. As an aside, the popular idea that, as an early branching lineage, ctenophores should display mostly ancestral traits is incorrect (*cf.* Crisp and Cook 2005).

From a phylogenetic perspective, the mitochondrial data may help to elucidate the phylogenetic position of Ctenophora, which is still hotly debated, as well as improve our understanding of the relationship within this phylum. Traditionally, two distinct phylogenetic hypotheses have been considered for Ctenophora based on morphological and/or embryological data: (i) grouping them with Cnidaria [the Coelenterata hypothesis (Haeckel 1866; Hyman 1940)], (ii) placing them as a sister group to Bilateria [the Acrosomate hypothesis (Lang 1882; Ax 1996)]. In contrast, early molecular phylogenies tended to place ctenophores as the sister group to Cnidaria + Bilateria, either by themselves [the Planulozoa hypothesis (Wainright et al. 1993; Wallberg et al. 2004)] or with calcareous sponges (Collins 1998). Recent phylogenomic studies utilizing gene supermatrices based largely on Expressed sequence tag (EST) data have yielded conflicting results as to the phylogenetic position of Ctenophora. Two of these studies placed ctenophores as the sister group to all metazoans, including sponges (Dunn et al. 2008; Hejnol et al. 2009). Phylogenomic analyses by a different group of researchers put ctenophores either in a clade with the Cnidaria, supporting the traditional Coelenterata hypothesis (Philippe et al. 2009) or as a sister group to a clade that included Cnidaria, Bilateria, and Placozoa (Pick et al. 2010). Another set of studies utilized whole genome data from all four non-bilaterian phyla, including the ctenophore *Mnemiopsis leidyi*, to examine gene superfamilies (Ryan et al. 2010; Reitzel et al. 2011) and signaling pathway components (Pang et al. 2010) across the Metazoa. Based on the presence or absence of superfamily classes, superfamily subclasses, and pathway components, these authors supported a grouping of Cnidaria, Placozoa, and Bilateria (the ParaHoxozoa) to the exclusion of Porifera and Ctenophora (Ryan et al. 2010). Remarkably, the phylogeny within Ctenophora is also unresolved, mainly because of the surprising conservation of 18S rRNA sequences, the only marker used so far to study it (Podar et al. 2001). Thus, additional markers or new approaches are clearly needed for phylogenetic studies involving ctenophores.

Finally, from structural analyses, ctenophore mitochondria possess an unusual arrangement of tubular cristae (Horridge 1964) unlike most other animals, fungi, and choanozoa that have flat cristae (Cavalier-Smith 1993). Earlier studies placed heavy emphasis on these mitochondrial features in phylogenetic inference (e.g. Cavalier-Smith 1993), although modern investigations have questioned the value of this character for phylogenetic inference (Frey and Mannella 2000). There is little known about the reasons for this difference and whether there is any correlation between mtDNA evolution and mitochondrial morphology.

The ctenophore *M. leidyi* (the warty comb jelly or sea walnut) is an important predator in many pelagic marine ecosystems (Colin et al. 2010). Native to the Atlantic coast of North and South America, it has gained global notoriety for its invasion of several seas of the Mediterranean basin (Shiganova et al. 2001). This ctenophore also has become one of the model species in developmental and evolutionary biology (Pang and Martindale 2008a). The *M. leidyi* genome sequencing project (Pang et al. 2010; Ryan et al. 2010; unpublished) has produced sequences for several candidate mitochondrial genes. We used this information to design primers and to amplify the

complete mt-genome from this species using Polymerase Chain Reaction (PCR). In addition, we used the available nuclear genomic data to obtain deeper insight into the mitochondrial biology of *M. leidyi*. Here, we describe this genome and report its very unusual evolutionary trajectory.

Methods

Specimen collection and genomic DNA preparation

Adult *M. leidyi* were collected from the docks of Woods Hole, MA, during the summers of 2006–2008. Animals were kept in fresh seawater under constant illumination in a large bucket in the laboratory. Adults were induced to spawn by placing them in the dark for 6–7 h, as described in Pang and Martindale (2008a). Fertilized eggs were collected and washed with fresh seawater. The embryos were raised at 17°C for 24–36 h (up to cydippid stage), upon which they were collected and concentrated in 2 ml tubes. These tubes were briefly centrifuged at 2000 *g* to concentrate cydippids (~500–1000). After removing as much excess of seawater as possible, these tubes were placed at − 80°C prior to DNA extractions. Genomic DNA was isolated using DNAzol (Molecular Research Center: Cincinnati, Ohio, USA) as previously described (Pang and Martindale 2008a). DNAzol was added to the tubes of cydippids immediately after they were removed from − 80°C. Following an overnight proteinase K digestion (0.1 mg/ml), tubes were centrifuged at 10,000 *g* to remove the insoluble material. Genomic DNA was then precipitated by adding a half volume of ethanol and incubating at room temperature. Following centrifugation, the DNA pellet was washed with 70% DNAzol (30% ethanol), then 75% ethanol, and then resuspended in sterile water.

mtDNA amplification and sequencing

Mitochondrial *cox1*, *cob*, and *nad5* were identified among the sequences generated by the *M. leidyi* genome sequencing project (Entrez Project ID: 64405) and used to design specific primers: ml-cox1-f1, 5′-CGTCACTTTACATGCTGTTTAC-3′; ml-cox1-r1, 5′-ATACGAGGTAAACACATATCAGC-3′; ml-cob-f1, 5′-ATGGGTCAAATGTCATATTGAGC-3′; ml-cob-r1, 5′-TAAGTAAACTATTAATGACAGGAC-3′; ml-nad5-f1, 5′-TCTGCTGTTTTTCCATTTCATGC-3′; and ml-nad5-r1, 5′-TCCATAGCATAATATAGTCAAGC-3′. The complete mtDNA was amplified in two overlapping fragments using the Long and Accurate PCR kit from TAKARA with primer combinations: (i) cox1-f1 + cob-r1 and cob-f1 + cox1-r1 and (ii) cob-f1 + nad5-r1, nad5-f1 + cob-r1. The total size of the two PCR fragments was identical for both sets of primers. The PCR fragments generated with *cob* and *nad5* primers were used for the shotgun cloning and sequencing as described in our earlier paper (Burger et al. 2007). Briefly, PCR amplifications were combined in equimolar concentration, sheared into pieces 1–2 kb in size, and cloned using the TOPO Shotgun Subcloning Kit from Invitrogen. White colonies containing inserts were collected, grown overnight in 96-well blocks and submitted to the DNA Sequencing and Synthesis Facility of the ISU Office of Biotechnology for high-throughput plasmid preparation and sequencing. The STADEN program suite (Staden 1996) and Phred (Ewing and Green 1998; Ewing et al. 1998) were used to basecall and assemble the sequences. Problematic regions in the assembly (mostly due to mono-T runs) were further investigated by primer walking. The complete mtDNA sequence has been deposited in GenBank (JF760210). Two PCR primers (meta-cox1-f2, 5′-TTKTTYTGRTTYTTYGGNCAYCC-3′ and meta-cox1-r2, 5′-GAKAAKACGTAGTGRAARTGNGC-3′) were designed for *cox1* regions conserved between *M. leidyi* and other animals, and used to amplify a fragment of *cox1* from *Pleurobrachia pileus*, a ctenophore belonging to the order Cydippida (GenBank accession JF760211).

Sequence analysis

We used flip (http://megasun.bch.umontreal.ca/ogmp/ogmpid.html) to predict open reading frames (ORFs) in *M. leidyi* mtDNA and performed similarity searches in local databases with FASTA (Pearson 1994) to identify them. Secondary structure predictions were done using TMHMM for protein coding genes (Krogh et al. 2001). Covariance models for *rns* and *rnl* genes were constructed with Infernal 1.0 (Nawrocki et al. 2009) using manually curated alignments of the corresponding sequences from *Escherichia coli*, *Monosiga brevicollis*, *Oscarella carmela*, *Geodia neptuni*, *Tethya actinia*, *Metridium senile*, *Aurelia aurita*, and *Homo sapiens* with the experimentally determined secondary structures from *E. coli* (Glotz et al. 1981) used as consensus structures. The resulting rRNA secondary structure predictions were visualized using RNAViz (De Rijk et al. 2003). Transfer RNA sequences were searched with tRNAscan (Lowe and Eddy 1997), ARWEN (Laslett and Canback 2008), and manually. The mt-genome map was visualized using CGView (Stothard and Wishart 2005).

Mitochondrial ribosomal protein (MRP) sequences of interest were identified using reciprocal BlastP (Altschul et al. 1997) queries with known MRP sequences from *Saccharomyces cerevisiae* and *H. sapiens* obtained from the Uniprot database (Smits et al. 2007). The identities of aminoacyl-tRNA synthetases and ribosomal proteins were determined based upon

homology to known sequences from *S. cerevisiae* and/or human obtained from the Uniprot database, and mitochondrial targeting presequence predictions were performed using TargetP (Emanuelsson et al. 2007). Intron/exon boundaries in nuclear-encoded *atp6* were annotated using EST sequences from *M. leidyi* publicly available in GenBank.

Protein sequences for *atp9* were obtained from GenBank files, *H. sapiens* NP_001680, *Nematostella vectensis* XP_001634940, *Trichoplax adhaerens* XP_002108967, or by querying the predicted *Amphimedon queenslandica* proteome from the JGI database with the mitochondrial *atp9* sequence from *Amphimedon compressa*. The consensus mitochondrial *atp9* sequence for eukaryotes was obtained from the NCBI Organelle Genome Resources website.

Phylogenetic analysis

Mitochondrial coding sequences for *Cantharellus cibarius* and *Capsaspora owczarzaki* mtDNA were downloaded from http://megasun.bch.umontreal.ca/People/lang/FMGP/proteins.html; other sequences were derived from the GenBank organellar genome database. Translated sequences of *cob*, *cox1–3*, and *nad5* were aligned three times with ClustalW 2 (Larkin et al. 2007) using different combinations of opening/extension gap penalties: 10/0.2 (default), 12/4, and 5/1. The three alignments were compared using SOAP (Löytynoja and Milinkovitch 2001), and only positions that were aligned identically among them were included in phylogenetic analyses.

Phylogenetic inference was done with PhyloBayes (PB) v3.2d (Lartillot and Philippe 2004; Lartillot et al. 2009). We used the CAT + GTR + Γ models for the analysis and ran four chains for 20,000 generation, until convergence (maxdiff = 0.13), sampling every 10th tree.

Results

The smallest animal mt-genome with an extreme nucleotide composition

The sequence of *M. leidyi* mtDNA (Figure 1) was determined to be 10,326 bp in length, making it the smallest animal mt-genome reported to date. We attributed the extremely small size of this genome to the scarcity of intergenic nucleotides, the relocation of *atp6* to the nuclear genome, and the apparent loss of all tRNA genes (see below). The sequence was also extremely AT-rich (%AT = 84.3) and had a strong AT-skew of −0.38 (Perna and Kocher 1995). As a result, we found multiple mono-T runs in the coding strand, 21 of them being greater or equal to 10 and up to 22 nucleotides in size. In some places, the mono-T runs created problems for sequencing this mt-genome.

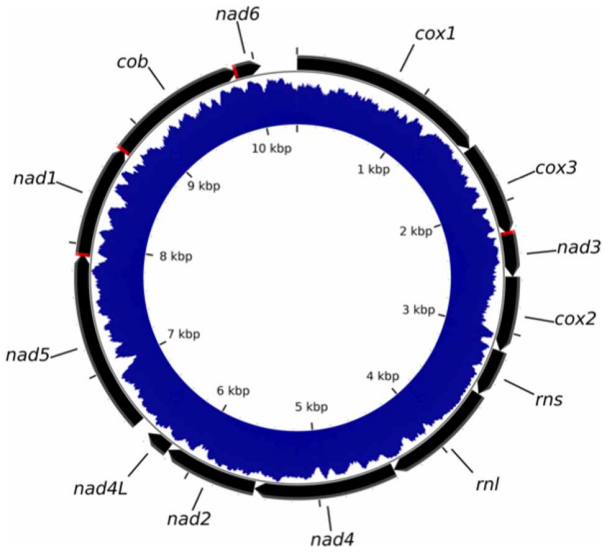

Figure 1. Mitochondrial genome map of *Mnemiopsis leidyi*. Inferred protein and rRNA genes are indicated by black arrows pointing in the direction of their transcriptional orientation; regions of overlap between them are indicated in red. The shaded area between two internal circles indicates AT-richness of the genome, with the inner circle corresponding to 0% AT and the other circle to 100% AT. The genome encodes genes for subunits 1–5 of NADH dehydrogenase (*nad1–5*), subunits 1–3 of cytochrome *c* oxidase (*cox1–3*), *cob*, the large and small subunits of mt-ribosomal RNA (*rnl* and *rns*), and two genes of low homology to other mitochondrial proteins putatively assigned as *nad4L* and *nad6*.

Highly derived coding sequences show extremely low similarity with homologs in other genomes

We located 33 ORFs larger than 50 codons (the usual size of the smallest gene in animal mtDNA) by translating the mtDNA sequence using a minimally modified genetic code (with TGA = Trp), including nine ORFs ≥ 100 codons in length (Figure S1, online). The nine largest ORFs spanned most of the genomes, and their pattern of nucleotide usage (a larger negative AT-skew at the second position and a higher AT content at the third) indicated that these ORFs code for functional transmembrane proteins (Chiusano et al. 2000) (Figure S2, online). Eight of them were identified by sequence similarity as genes for cytochrome *b* (*cob*), cytochrome *c* oxidase subunits 1–3 (*cox1–3*), and Nicotinamide adenine dinucleotide (reduced form) (NADH) dehydrogenase subunits 1,3,4,5 (*nad1,3–5*) (Figure 1). However, their sequence identity with homologous proteins in other organisms was low, ranging from 14% for *nad4* to 41% for *cox1* (Table I). The last ORF—ORF241—was putatively identified as *nad2*, based on the prediction of transmembrane helices in the amino acid sequence it encodes (Krogh et al. 2001; Figure S2, online). We also investigated six ORFs between 50 and 100 codons in length that were located in regions not covered by these genes: four ORFs downstream of the putative *nad2* gene, one downstream of *cob*, and one downstream of *cox2*

Table I. Coding sequences in the *M. leidyi* mt-genome.

Gene	Size	%AT	Start codon	Stop codon	Average similarity of encoded amino acid sequences		
					With non-bilaterian animals[†]	With bilaterian animals[‡]	With outgroups[¶]
cob	1086	79.1	ATT(−27)[§]	TAA(−14)	27.3	27.4	26.9
cox1	1524	75.7	ATT(466)	TAA(0)	40.4	41.0	40.5
cox2	579	79.9	ATT(0)	TAA(0)	17.5	16.8	16.8
cox3	726	84.1	ATT(0)	TAG(−4)	21.4	22.6	22.0
nad1	870	83.7	ATT(−1)	TAA(−27)	21.1	20.3	20.9
nad2	726	92.1	ATT(1)	TAA(0)	5.6	6.1	6.0
nad3	327	90.2	ATA(−4)	TAA(0)	16.6	15.7	14.4
nad4	1095	88.6	ATT(0)	TAA(1)	14.2	14.1	14.0
nad4L	198	90.3	ATG(0)	TAA(108)	15.3	10.8	16.2
nad5	1407	83.9	ATA(108)	TAA(−1)	14.2	14.5	14.7
nad6	204	85.8	ATG(−14)	TAA(466)	3.7	2.6	2.9

[†] Average sequence similarity with homologous proteins in *M. senile*, *Sarcophyton glaucum*, *Ephydatia muelleri*, *O. carmela*, and *T. adhaerens*; [‡] Average sequence similarity with homologous proteins in *Balanoglossus carnosus*, *H. sapiens*, *Katharina tunicata*, *Limulus polyphemus*, and *Xenoturbella blocki*; [¶] Average sequence similarity with homologous proteins in *C. owczarzaki* and *Monosiga brevicolis*; [§] The numbers in parentheses after initiation and termination codons show the number of non-coding nucleotides upstream and downstream of a gene. The negative numbers indicate that the genes are overlapping.

(Figure S1, online). We tentatively identified two of them as *nad4L* and *nad6*, respectively, based on the pattern of nucleotide usage (Figure S2, online), prediction of transmembrane helices (Figure S3, online), and some sequence similarity with corresponding genes in other genomes. While none of these indicators were conclusive (the sequence similarity was low and the proteins encoded by putative *nad4L* and *nad6* were missing some transmembrane domains usually present in their homologs), we note that these genes are typically among the least conserved genes in mtDNA (Wang and Lavrov 2008) and are therefore expected to be difficult to identify. Furthermore, the putative *nad6* is unusually small in size, what contributes to its extremely low sequence identity scores. We were unable to identify genes for any subunits of F_0 Adenosine triphosphate (ATP)-synthase in the mtDNA of *M. leidyi*, but found two of them (*atp6* and *atp9*) in its nuclear genome (see below).

All genes in *M. leidyi* mtDNA had the same transcriptional polarity and several of them overlapped (specifically, *cox3* and *nad3*, *nad5* and *nad1*, *nad1* and *cob*; Table I). There were very few nucleotides outside of the coding sequences, with most of them were present in two regions of the genome: (i) a relatively small region downstream of the putative *nad6* gene, which we interpreted as a non-coding region potentially involved in the initiation of replication and transcription and (ii) a larger region downstream of *cox2*, where we identified genes for the large and small subunit rRNAs.

All subunits of F_0 ATP-synthase are encoded in the nuclear genome

Among the coding sequences identified in the mt-genome of *M. leidyi*, none coded for the subunits of F_0 ATP-synthase. Instead, *atp6* and *atp9* were found in the *nuclear* genome. The nuclear-encoded *atp6* encompassed at least 1742 nt and contained three introns of 544, 137, and 206 bp, respectively. Corresponding cDNA sequences available in GenBank showed the mature *atp6* possessing a 5′-untrans-untranslated region of 9 nt, followed by an ORF of 756 nt and a 3′-untranslated region of 90 nt. The translated ATP6 peptide contained a 19-amino acid presequence at the N-terminus and had an 85% probability of being targeted to the mitochondrion, as predicted by TargetP (Emanuelsson et al. 2007). The amino acid sequence of the ATP6 had been predicted to form four transmembrane domains: the first encoded by exon 2, the following two by exon 3, and the last by exon 4. The fifth transmembrane domain, usually located at the N-terminus of the protein, was not identified and might have been lost.

Similarly, the nuclear-encoded *atp9* gene had acquired an N-terminal extension as well as one intron. Alignments of nuclear-encoded *atp9* sequences from the representatives of several animal phyla revealed a conserved motif just upstream of the core mitochondrial domain that was present in *atp9* presequences from Ctenophora, Cnidaria, Placozoa, and Bilateria, but absent from the demosponge *A. queenslandica* (Figure 2), in which this gene underwent an independent transfer to the nucleus (Erpenbeck et al. 2007).

We were not able to identify *atp8* in either the mitochondrial or the nuclear genome of *M. leidyi*. Because of its small size and poor sequence conservation, this gene may still be present in either of these genomes or might have been lost. Multiple independent losses of *atp8* have been reported in Metazoa (e.g. Hoffmann et al. 1992; Okimoto et al. 1992; Le et al. 2002; Helfenbein et al. 2004; Iannelli et al. 2007) as

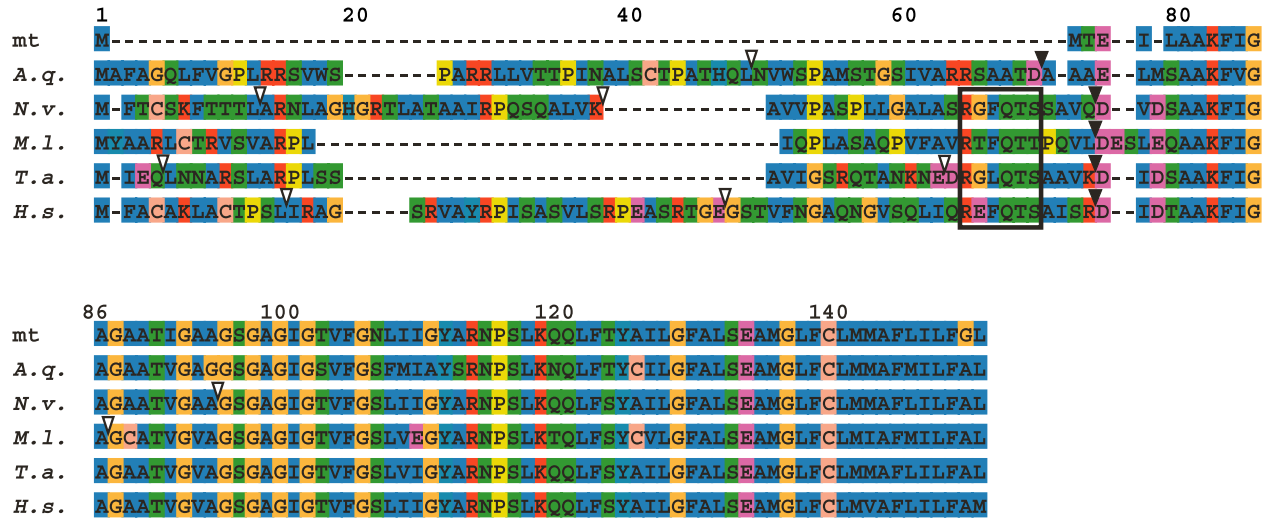

Figure 2. Comparison of *atp9* sequences in *M. leidyi* and other animals. Multiple sequence alignment of *atp9* from *Amphimedon queenslandica* (*A.q.*), *Nematostella vectensis* (*N.v.*), *Mnemiopsis leidyi* (*M.l.*), *Trichoplax adhaerens* (*T.a.*), *Homo sapiens* (*H.s.*), and the consensus sequence from all mt-genomes available on the NCBI Organelle Genome Resources website (mt) were created with MAFFT6 (Katoh and Toh 2008). Presequence cleavage sites as predicted by TargetP are indicated with filled triangles, intron positions are indicated with open triangles, and the conserved motif in the presequence is marked by a rectangle.

well as in other eukaryotes (Denovan-Wright et al. 1998; Burger et al. 2000; Slamovits et al. 2007; Hancock et al. 2010). No nuclear *atp8* has been found to date in these taxa.

An extremely derived mitochondrial ribosome

A 1246 bp region in *M. leidyi* mtDNA downstream of *cox2* contained no large ORFs and is the presumed location of both the large and small subunit ribosomal RNA genes (*rns* and *rnl*, respectively). We found a positive GC-skew in this region, consistent with a nucleotide composition influenced by rRNA secondary structures wherein G–U base pairs are frequently observed. While there was very little similarity with any known sequences, we were able to identify a few potential secondary structures that allowed us to assign the first 368 bp of the region as *rns* and the following 878 as *rnl*.

Two regions within *rns*, the so-called 530 loop (helix 18) and helices 28–30 and 43–45, were partially conserved in sequence and structure (Figure 3A). In particular, we found conservation of the three essential nucleotides (G530 in helix 18 and both A1492 and A1493 in helix 44) used in decoding, a process that discriminates against aminoacyl tRNAs that do not match the codon of mRNA (Ogle et al. 2003). Furthermore, a small (~110 bp) segment at the 3′ end of *rnl* displayed both a high degree of sequence similarity and a similar structure with the peptidyl transferase center (PTC) from domain V of the large subunit rRNA, which constitutes the "catalytic heart of the ribosome" and is highly conserved throughout all of life (Polacek and Mankin 2005; Bokov and Steinberg 2009) (Figure 3B). This conservation was mostly limited to the core of the PTC, while many helices involved in the A-site and P-site of the PTC have been modified or reduced. Among the retained structures were helices 80, 81, and 92, the first of which is responsible for stabilizing the P-site tRNA through base-pairing interactions with its CCA terminus (Polacek and Mankin 2005) and the last being responsible for stabilizing the orientation of the A-site tRNA. The rest of the 1246 bp region did not encode any conserved helices in either srRNA or lrRNA. In particular, the L1-binding domain stalk, present in all other rRNAs, appears to be completely absent from *rnl* in *M. leidyi*. Clearly, the extent of reduction discovered in *M. leidyi* mt-rRNA is unprecedented, far surpassing that in the "minimal RNA" reported in *Leishmania tarentolae* mitochondrial ribosomes (de la Cruz et al. 1985a,b; Sharma et al. 2009).

In mammals (Suzuki et al. 2001; Sharma et al. 2003) and other organisms (Sharma et al. 2009), the truncation of mt-rRNA is compensated by an increase in both the number and size of MRPs. To investigate whether similar changes have occurred in the protein content of the *M. leidyi* mt-ribosome, we surveyed the nuclear genome for the homologs of the 98 known MRPs that could have been present in the most recent common ancestor of opisthokonta (79 from human and 19 from yeast) (Smits et al. 2007). For comparison, we also surveyed the nuclear genome of the demosponge *A. queenslandica* and the cnidarian *N. vectensis*.

We identified 34 putative homologs to human MRPs in the nuclear genome of *M. leidyi* compared to 61 in *A. queenslandica* and 62 in *N. vectensis*. The reduced number of identified proteins in *M. leidyi* is

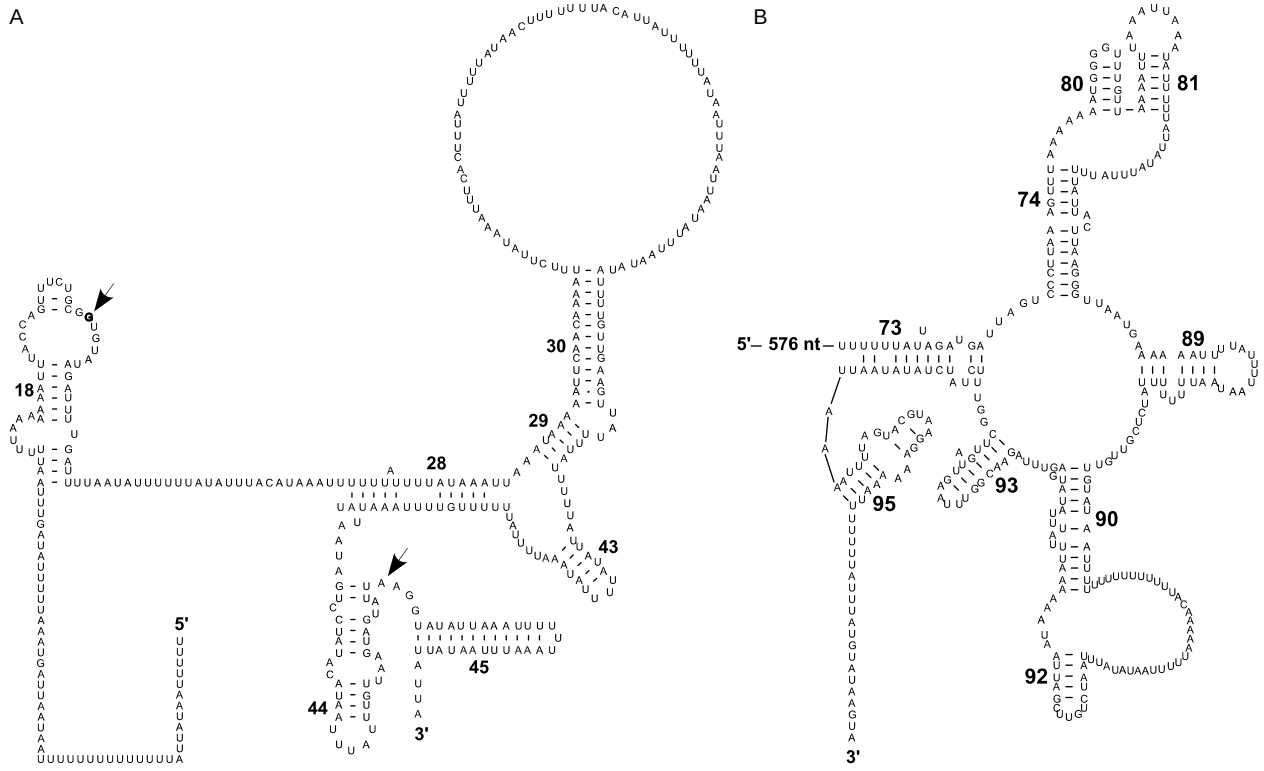

Figure 3. Conserved secondary structures in *M. leidyi* mt-rRNAs. Predicted secondary structures in small (A) and large subunit (B) rRNAs inferred by both manual inspection and Infernal alignments. Helices in srRNA are numbered as in Brimacombe (1995); those in lrRNA as in Leffers et al. (1987). Conserved nucleotide positions referenced in the text are indicated with arrows.

likely a result of their rapid evolution, rather than gene loss, because the observed sequence identity values for these genes were generally lower between human and this ctenophore (mean = 28.0, SD = 6.5%) than between human and either *A. queenslandica* (38.2 ± 6.5%) or *N. vectensis* (41.9 ± 7.4%). In addition, the MRP sequences in *M. leidyi* were on average 35 amino acids longer than their homologs in human and there were more of them showing large expansions (>100 amino acids) than those showing large contractions (6 vs. 2). However, given the drastic reduction in the rRNA content of the *M. leidyi* mt-ribosome, it is likely that novel proteins were also recruited to compensate for the loss of rRNA.

The complete absence of mitochondrial tRNA genes and the loss of nuclear-encoded mitochondrial aminoacyl-tRNA synthetases

No tRNA genes have been found in the mt-genome of *M. leidyi* by using either automated or manual searches. The lack of a mitochondrial gene for $tRNA^{Trp}_{UCA}$ was especially surprising given that tryptophan appears to be specified exclusively by the UGA codon in *M. leidyi* mitochondrial coding sequences (no UGG codons were found), which is an "opal" stop codon in the standard genetic code. A scan of the nuclear genome of *M. leidyi* recovered 478 putative tRNA gene sequences and 708 possible tRNA pseudogenes, with five sequences corresponding to the nuclear *trnW(cca)* but no mitochondrial *trnW(uca)*. This situation is reminiscent of that in *Trypanosoma brucei*, where dual targeting of $tRNA^{Trp}$ requires two different tryptophanyl-tRNA synthetases, one of which recognizes $tRNA^{Trp}$ that has undergone editing from C to U in the first position of the anticodon. This allows decoding of the mitochondrial UGA codons as tryptophan rather than as a stop codon (Alfonzo et al. 1999). Indeed, a search for nuclear genes for mitochondrial aminoacyl-tRNA synthetases (mt-aaRS) recovered a putative homolog to human tryptophanyl mt-aaRS (mt-TrpRS). This was only one of the two mt-aaRS found in the nuclear genome of *M. leidyi*, the second being mt-PheRS.

The lack of interphylum but the presence of intraphylum phylogenetic signal in mitochondrial genes

Given the exceptionally high rate of sequence evolution, one can expect little, if any, phylogenetic signal in the mitochondrial sequences of *M. leidyi* informative for reconstructing the basal animal relationships. Indeed, our phylogenetic analysis based on amino acid sequences from the five best-conserved genes (*cob*, *cox1-3*, and *nad5*) resolved neither the relationships among the four main lineages of animals sampled previously (Bilateria, Cnidaria, Porifera, and Placozoa) nor the phylogenetic position

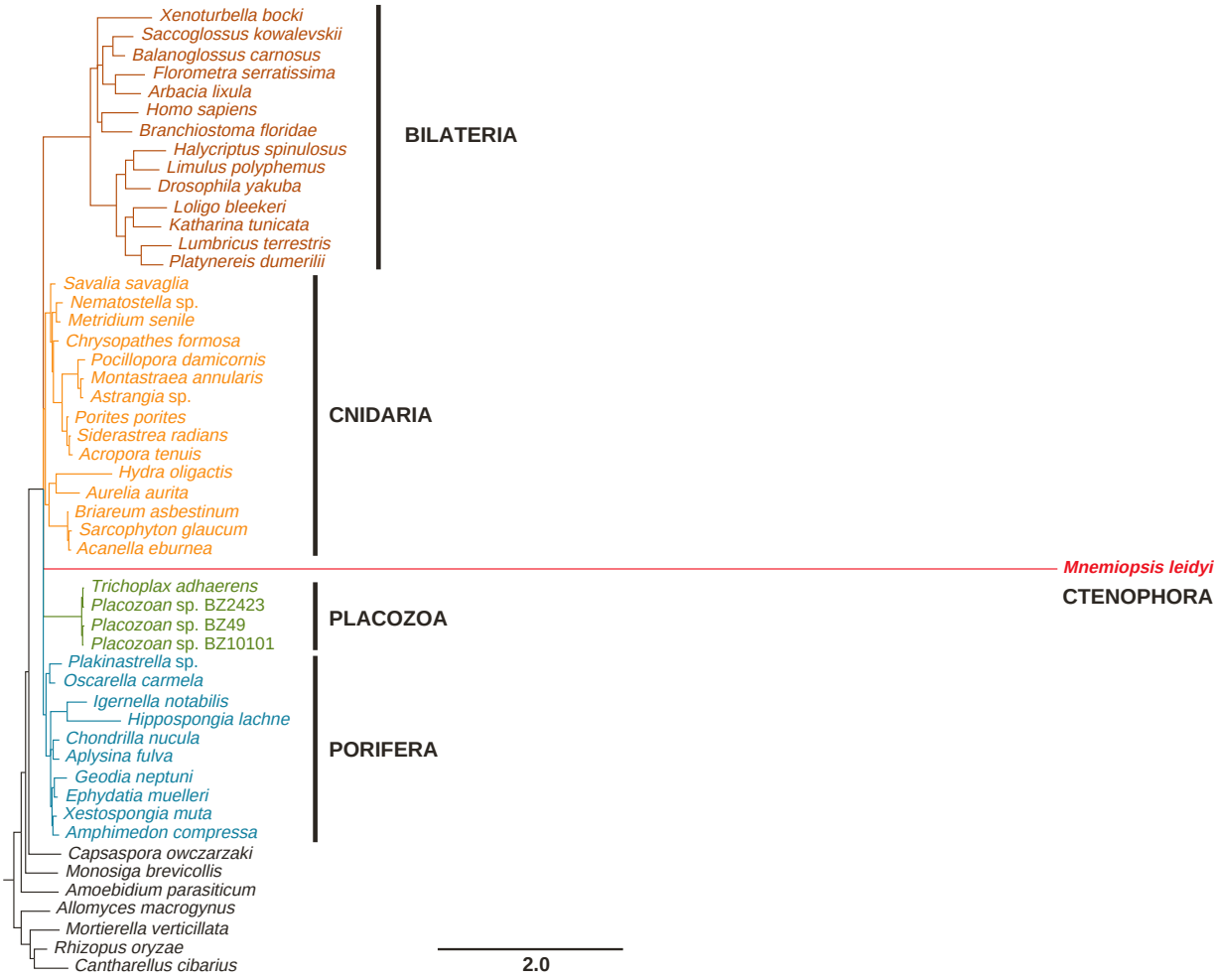

Figure 4. Phylogenetic analysis of animal relationships using mitochondrial sequence data. Posterior majority-rule consensus tree obtained from the analysis of concatenated mitochondrial amino acid sequences inferred from the five genes best conserved in *Mnemiopsis* (*cob*, *cox1–3*, and *nad5*; 1402 aa in total). We used the CAT + F + Γ model in PBs and ran four independent chains for ~20,000 generations sampling every 10th tree after the first 1000 burning cycles. The convergence among the chains was monitored with the maxdiff statistics and the analysis was terminated after maxdiff became <0.15.

of Ctenophora (Figure 4). On the other hand, each of the main lineages was recovered as monophyletic and the relationships within each lineage corresponded closely to recently published phylogenetic studies.

To check whether mtDNA sequences from ctenophores contain any intraphylum phylogenetic signal, we designed two PCR primers for *cox1* regions that were well conserved between *M. leidyi* and other animals and used them to determine the sequence of *cox1* from *Pleurobrachia pileus*, a ctenophore belonging to the order Cydippida. A comparison of *cox1* sequences between *M. leidyi* and *P. pileus* revealed differences at 30% of sites, with $dN = 0.18$, $dS = 40.22$, and $dN/dS = 0.0044$. The large divergence in mtDNA sequences between these two species was in stark contrast to a previous study by Podar et al. (2001), who found very little differences in nuclear 18S sequences among sampled ctenophore species. We also note that the dN/dS ratio for *cox1* sequences is much less than 1, indicating that *cox1* in ctenophores evolves under strong purifying selection and is not a pseudogene.

Discussion

The loss of at least 25 genes explains the extreme size of the genome

Animal mtDNA, although not as highly conserved as previously thought, is an unusually small and economically organized molecule in comparison with other eukaryotic groups (Lang et al. 1999). This compact organization is particularly pronounced in the mtDNA of bilaterian animals, in which intergenic nucleotides are usually few, if any, and genes contain neither introns nor regulatory sequences. The small size of animal mtDNA is also due to reduction in the sizes of genes, which, in the case of structural RNAs, often lack some secondary structures present in homologous molecules in other groups and, in other

cases, are even truncated and completed by post-transcriptional polyadenylation (Yokobori and Pääbo 1997). Given this highly economical organization of animal mtDNA, further reduction in its size is mainly possible through gene loss. Indeed, the mt-genome of *M. leidyi* has lost at least 25 genes: 24 tRNA genes required for translation using the minimally derived genetic code, as well as a gene encoding subunit 6 of the F_0 ATPase. The loss of a mitochondrial-encoded *atp6* gene in animals has previously been reported only in Chaetognatha (Helfenbein et al. 2004). In non-metazoan eukaryotes, there are also examples of *atp6* having been transferred to the nucleus, the chlorophycean alga *Chlamydomonas reinhardtii* being the best studied (Funes et al. 2002). Interestingly, the nuclear encoded *atp6* of *C. reinhardtii* displays a large decrease in the hydrophobicity of the transmembrane region A, corresponding to the missing transmembrane domain in *M. leidyi atp6*, suggesting that the absence of this domain may aid in the import of ATP6 into the mitochondrion. The finding that *atp6* is nuclear encoded in *M. leidyi* makes it another potential model for the study of protein import to mitochondria, which is essential for mitochondrial gene therapy (Manfredi et al. 2002; Figueroa-Martinez et al. 2011).

Although *atp9* was also missing in *M. leidyi* mtDNA, we interpreted its absence as a consequence of a nuclear transfer event that occurred in the lineage leading to all Eumetazoa (all animals minus sponges), rather than an independent transfer. Because *atp9* is mitochondrial encoded in fungi, choanoflagellates, and nearly all poriferans, it was likely present in the mtDNA of the common ancestor to all animals. While parallel losses of organellar genes are common (Martin et al. 1998), the presence of a conserved motif in the presequences of the nuclear-encoded *atp9* gene from Ctenophora, Cnidaria, Placozoa, and Bilateria implies that these presequences share common ancestry and were therefore most likely acquired during a single ancestral nuclear transfer event (Figure 2). Although sequence convergence due to constraints imposed by the presequence cleavage mechanism could also explain the occurrence of this motif (e.g. Liu et al. 2009), we regard it as less likely. For example, no such motif is present in the presequence of *atp9* of the demosponge *A. queenslandica* (Erpenbeck et al. 2007), which transferred to the nucleus independently. Thus, this observation suggests that sponges, rather than ctenophores, form a sister group to the rest of the animals. It also provides further evidence that functional nuclear transfers of mitochondrial genes in addition to numt (Hazkani-Covo 2009) have the potential to be phylogenetically informative as rare genomic changes, even when parallel transfer events are common.

In addition to *atp6*, the mt-genome of *M. leidyi* also lacks all tRNA genes. To date, the complete loss of mt-tRNA genes has been reported only in a few non-metazoans, including apixomplexa (Wilson and Williamson 1997) and trypanosomatids (Schneider 2001), and one species of chaetognaths (Papillon et al. 2004). Furthermore, losses of individual tRNA genes are relatively rare in the mtDNA of bilaterian animals, but are more common in demosponges, homoscleromorphs, cnidarians, and non-metazoan eukaryotes (Glover et al. 2001; Gray et al. 2004; Wang and Lavrov 2008). In fact, all cnidarians and some demosponges lack all but one or two mt-tRNA genes.

In some cases in which both nuclear and mitochondrial data are available, the loss of mt-tRNAs has been shown to be accompanied by the loss of nuclear-encoded mt-aaRS. In particular, the nuclear genome of the cnidarian *N. vectensis* contains genes for only two mt-aaRS (mt-PheRS and mt-TrpRS)—exactly those found also in *M. leidyi* (Haen et al. 2010). In *Nematostella*, the retention of the nuclear-encoded mt-TrpRS and mt-PheRS was attributed to the reassignment of the UGA termination codon to tryptophan in animal mtDNA and the heterotetrameric structure of the cytoplasmic PheRS, respectively (Haen et al. 2010). The analogous situation in *M. leidyi* is consistent with these hypotheses.

Extremely derived ribosomal structures and the trend toward a minimal PTC-like ribosomal RNA

The genes that remain in the mtDNA of *M. leidyi* are characterized by extremely high rates of sequence evolution, which is manifested in extremely low sequence similarity between coding sequences in *M. leidyi* and their homologs in other animals, as well as highly unusual rRNA structures. The mt-genome of *M. leidyi* encodes some of the smallest and most highly derived mt-rRNA sequences ever documented. We show that the reduction in size of mt-rRNA has resulted in the elimination of many secondary structures, with the exception of those involved in decoding in the srRNA and the PTC in the lrRNA. This reduction has also been accompanied by an increase in the evolutionary rate and the overall size of MRPs.

It has been hypothesized that the ribosome may have originally evolved from a free PTC-like RNA molecule capable of catalyzing the formation of peptide bonds. Structural domains were subsequently added in modules throughout the evolution of the ribosome, mostly to stabilize the localization of tRNA molecules near the PTC and to decode mRNA (Bokov and Steinberg 2009). The following accumulation of protein diversity throughout life then led to the reversal of this trend and to their uptake within the ribosomal structure. The degenerative evolution of mitochondrial RNA genes (Lynch and Blanchard 1998) and potentially more efficient and diverse

enzymatics possible with proteins may account for the trend toward the reduction in the size of mt-rRNAs observed in animal mitochondria (Sharma et al. 2003). The rRNA genes in the mt-genome of *Mnemiopsis* represent the most extreme outcome of this reductionary trend ever observed in any organism.

Why is the M. leidyi *mt-genome so derived?*

The primary non-adaptive forces influencing organelle genomic evolution are mutation and random genetic drift [Lynch et al. 2006; but see Gillespie (2001)]. The mitochondrial mutation rate depends on the fidelity of mtDNA polymerase [polymerase γ in most eukaryotes (Graziewicz et al. 2006)] and the presence and efficiency of mitochondrial repair system. The power of random genetic drift, which determines the probability of fixation or the removal of mutant alleles, is defined by the genetic effective size of a population (*Ne*) (Charlesworth 2009). The two evolutionary forces are not independent as the mutation rate influences *Ne*, while a small effective population size can facilitate stochastic fixation of deleterious mutations in genes responsible for DNA replication and repair that can lead to an increased mutation rate.

Two features of ctenophore reproductive biology can negatively affect their *Ne* and hence contribute to their accelerated mitochondrial evolution. First, ctenophores in general and *M. leidyi* in particular are simultaneous hermaphrodites capable of self-fertilization (Baker and Reeve 1974; Pianka 1974). In fact, in *M. leidyi*, eggs are fertilized as soon as they are shed (Pang and Martindale 2008a). Inbreeding caused by self-fertilization is known to have effects on genome evolution as it reduces effective population size, limits the gene flow *via* gamete migration, and reduces the effective recombination rate between polymorphic sites (Charlesworth and Wright 2001). Although some of these effects should have less impact on mitochondrial genes, which are already uniparentally inherited in most organisms, they must influence the nuclear genes that carry out most mitochondrial functions, including replication and repair.

Second, at least some ctenophores are capable of rapid and massive reproduction. *M. leidyi* is an opportunistic omnivore that under optimal conditions can start reproduction at 2 weeks of age and release up to 14,000 eggs/day, creating large blooms (Baker and Reeve 1974; Purcell et al. 2001). Moreover, at least some ctenophores have the ability to reproduce sexually while they are still larvae, a condition known as "dissogeny". In *M. leidyi*, larvae as young as 6 days post-hatching and only 1.8 mm in size were able to produce viable embryos (Martindale 1987). This pattern of early and rapid reproduction followed by massive die-offs could create multiple bottlenecks in the population history of *M. leidyi*, which facilitates the accumulation of deleterious mutations in both mitochondrial and nuclear genes.

It is also possible that the unusually high rate of sequence evolution is limited primarily to mitochondrial components in both nuclear genomes and mt-genomes and is caused by some physiological rather than population biology factors. To this end, we note that the branch leading to ctenophores is not exceptionally long in recent EST-based phylogenomic studies (Hejnol et al. 2009; Pick et al. 2010). Similarly, a study of *M. leidyi* homeobox genes did not find them to be unusually derived (Ryan et al. 2010).

Conclusions

The complete mtDNA sequence of *M. leidyi*—the first from the phylum Ctenophora—is highly unusual in its gene content and the extent of sequence evolution. At just over 10 kb, it has lost at least 25 genes, including *atp6* and all tRNA genes. It also displays an exceptionally high rate of sequence evolution, resulting in highly derived structures of encoded proteins and rRNA. The availability of preliminary *M. leidyi* genome sequence data allowed us to investigate the associated changes in the *M. leidyi* nuclear genome. In particular, we show that *atp6* has been transferred to the nucleus and acquired a targeting pre-sequence, and that the loss of tRNA genes from mtDNA is accompanied by the loss of nuclear encoded mt-aaRS. We also show that the rapid evolution of mt-rRNA is correlated with accelerated evolution of nuclear encoded MRPs. Although the extremely derived genome structure and protein-coding sequences of the *M. leidyi* mt-genome did not allow us to resolve the phylogenetic position of Ctenophora, our results suggest that it should be useful for phylogenetic inference within the phylum and, possibly, as a marker for biogeographic studies. In addition, this work provides a toehold for determining additional ctenophore mt-genomes, which should reveal the full range of mtDNA sequence diversity in this phylum. Finally, several unusual features identified in *M. leidyi* mtDNA make this organism a promising system for the study of various aspects of mitochondrial biology, particularly protein import and mt-ribosome structures, and add to its value as an emerging model species.

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