

Animal Mitochondrial DNA as We Do Not Know It: mt-Genome Organization and Evolution in Nonbilaterian Lineages

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Accepted: August 6, 2016

Abstract

Animal mitochondrial DNA (mtDNA) is commonly described as a small, circular molecule that is conserved in size, gene content, and organization. Data collected in the last decade have challenged this view by revealing considerable diversity in animal mitochondrial genome organization. Much of this diversity has been found in nonbilaterian animals (phyla Cnidaria, Ctenophora, Placozoa, and Porifera), which, from a phylogenetic perspective, form the main branches of the animal tree along with Bilateria. Within these groups, mt-genomes are characterized by varying numbers of both linear and circular chromosomes, extra genes (e.g. *atp9*, *polB*, *tatC*), large variation in the number of encoded mitochondrial transfer RNAs (tRNAs) (0–25), at least seven different genetic codes, presence/absence of introns, tRNA and mRNA editing, fragmented ribosomal RNA genes, translational frameshifting, highly variable substitution rates, and a large range of genome sizes. This newly discovered diversity allows a better understanding of the evolutionary plasticity and conservation of animal mtDNA and provides insights into the molecular and evolutionary mechanisms shaping mitochondrial genomes.

Key words: mitochondrial DNA, Metazoa, Porifera, Cnidaria, Ctenophora, Placozoa.

Introduction

Mitochondria are double membrane-bound organelles found in most eukaryotic cells. Commonly described as cellular “power plants” for their role in the generation of ATP by oxidative phosphorylation, mitochondria perform a variety of additional cellular functions, such as biosynthesis of amino acids and steroids, β -oxidation of fatty acids, FeS metabolism, and initiation of apoptosis (Mcbride et al. 2006; Pagliarini and Rutter 2013; Chandel 2014; Birsoy et al. 2015). However, oxidative phosphorylation appears to be the only function that requires the presence of a mitochondrial genome (mt-genome) (Race et al. 1999), which always codes for some protein subunits of the electron transport chain (Feagin 1992). In addition, nearly all mt-genomes encode ribosomal RNA (rRNA) components of mitochondrial ribosomes, most encode at least some mitochondrial transfer RNA (mt-tRNA), while some encode the ribosomal subunit of RNaseP and proteins involved in mitochondrial transcription, translation, protein import and maturation (Gray et al. 2004).

Extensive biochemical and phylogenetic studies have demonstrated that all mitochondria originated from a single

α -proteobacterial ancestor (Gray et al. 1999) from within the order Rickettsiales (Wang and Wu 2015). Given that the modern representatives of Rickettsiales have small circular genomes (Schulz et al. 2015), such a genome was likely present in the bacterial ancestor of mitochondria. During eukaryotic evolution, this ancestral genome lost most of its genes [retaining as few as 4 (Flegontov et al. 2015) or perhaps even 1 (Petersen et al. 2014) and at most 100 (Burger et al. 2013) genes] and gave rise to a remarkable diversity of architectures, including genomes with varying numbers of linear and circular chromosomes ranging in size from only a few kilobases (kb) to several megabases (Mb) (Gray et al. 2004; Gualberto et al. 2014; Smith and Keeling 2015). In a few lineages, mitochondrial DNA (mtDNA) has been lost altogether (Muller et al. 2012), and in one case mitochondria themselves have been completely lost (Karnkowska et al. 2016). A sample of mtDNA diversity can be seen among close unicellular relatives of animals (defined here as protists that are more closely related to animals than fungi), where both linear and multipartite genomes have evolved (Lavrov and Lang 2014). In contrast, previous reviews of animal mtDNA have emphasized its

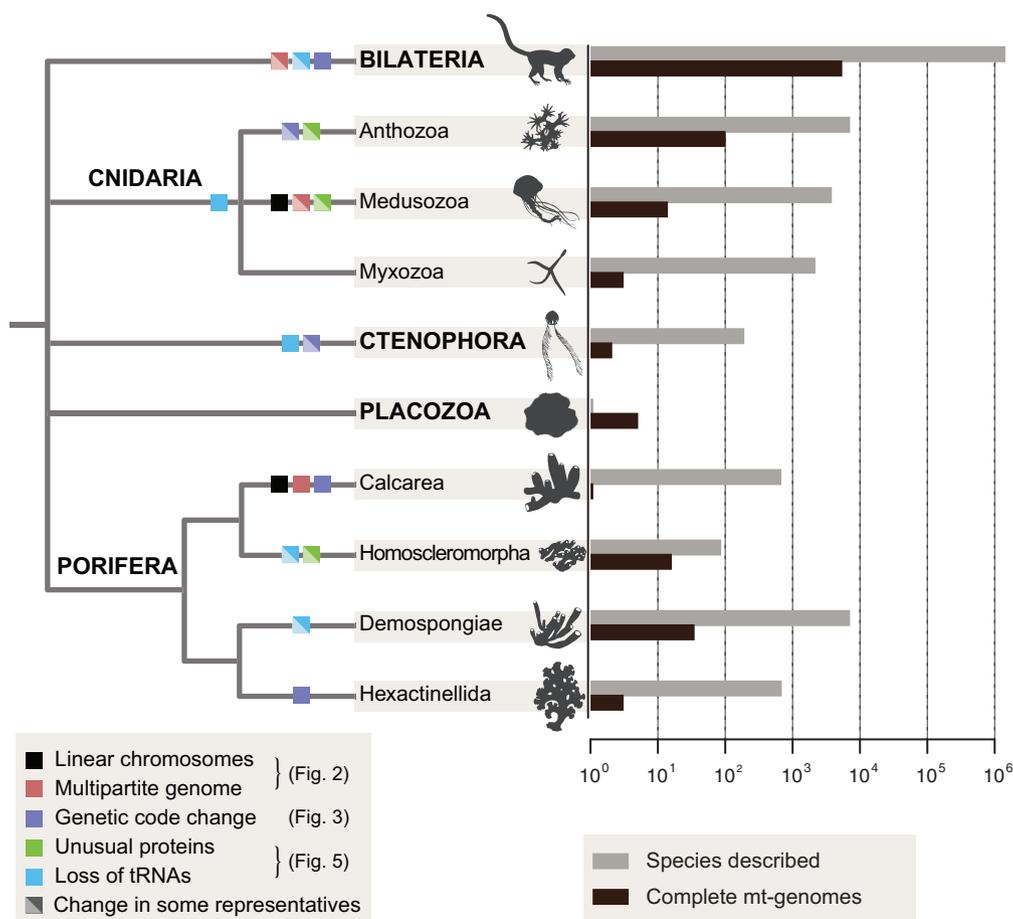


FIG. 1.—Mitochondrial genomes in Metazoa. Consensus view of animal relationships with mapped changes in mt-genome architecture and gene content. Diagonally split boxes indicate changes that occur only in some representatives of the group. Black bars to the right indicate numbers of complete mtDNA sequences in the NCBI RefSeq database as of April 9, 2016 (for data on nuclear genomes, see [Dunn and Ryan 2015]). Gray bars indicate estimated numbers of described species and were obtained from the Global Invertebrate Genomics Alliance (GIGA Community of Scientists 2014). Only the taxa that are mentioned in the text are included. See associated figures for a more detailed depiction of each of these changes. Branch lengths were chosen for illustration purposes only.

remarkable uniformity (Wolstenholme 1992a; Boore 1999; Saccone et al. 2002) but have been focused primarily on bilaterian animals.

Progress in molecular phylogenetics has brought renewed attention to four major groups of nonbilaterian animals (sponges, placozoans, cnidarians, and ctenophores), which have been traditionally categorized as phyla, but which, along with bilaterians, constitute the main branches of the animal tree from a phylogenetic perspective (Ax 1996) (fig. 1). Each of these lineages encompasses hundreds of millions of years of independent evolution (Dos Reis et al. 2015), and their extant representatives demonstrate distinct possibilities in animal mtDNA evolution. Although some aspects of mtDNA diversity in nonbilaterian animals have been reviewed recently by Breton et al. (2014), Bernt et al. (2013), and Osigus et al. (2013), these studies were published before data from all major nonbilaterian lineages became available. In this review,

we put conventional views of animal mtDNA in perspective by providing a comprehensive summary of mt-genome organization in nonbilaterian animals.

Typical Animal Mitochondrial DNA: The Birth of a Myth

The first determined animal mtDNA sequence came from human (Anderson et al. 1981) and that from *Drosophila* followed soon after (Clary and Wolstenholme 1984). Though the gene order was different between mammalian and *Drosophila* mtDNA, resulting in distinct patterns of transcription (Berthier et al. 1986), most of the genetic and genomic features were remarkably similar. Both mt-genomes were small (~16 kb), circular-mapping molecules, containing the same set of 37 genes (13 protein genes encoding subunits of complexes I, III, IV, and V of the oxidative phosphorylation

(OXPHOS) system, 2 rRNA genes, and 22 tRNA genes) with a high degree of synteny. Both genomes encompassed a single large noncoding region, but otherwise contained very few intergenic nucleotides. A modified genetic code was inferred in both species, with AUA and UGA codons specifying methionine and tryptophan, respectively, and AGR codons specifying either serine (in *Drosophila*) or termination (in human). Other unusual genetic and genomic features of bilaterian mtDNA included unorthodox start codons, incomplete stop codons, diminished genes for tRNAs and rRNAs [including a characteristic *trnS(gcu)* that codes for a tRNA with a DHU-arm replacement loop], the absence of introns, and a high rate of sequence evolution (Clary and Wolstenholme 1984; Wolstenholme 1992a).

Given the more than one billion years of combined evolution that separates humans and *Drosophila* (Dos Reis et al. 2015) and their radically different external morphology, the similarity in their mtDNA was astounding. In addition, earlier electron microscopy studies had shown that the sizes of mtDNA in a variety of animal species were similar (Borst and Kroon 1969). Thus, the notion of “typical animal mtDNA” was born, with the assertion that “all metazoa, from Platyhelminthes to mammals possess a mt-genome that consists of a single circular molecule ranging in size from 14.5 to 19.5 kb” and that both gene content and, to some extent, gene order were conserved in all animal mt-genomes (Clary and Wolstenholme 1985). The notion of a conserved, nearly “frozen” (Saccone et al. 2002) genome was reinforced by the observation that most of the mutations in the human mt-genome are associated with disease (Linnane et al. 1989; Wallace 1992).

To date, complete mt-genome sequences from over 6,000 animals have been made available, of which about 97% are from bilaterian animals (<http://www.ncbi.nlm.nih.gov/genome/organelle/>, last accessed April 9, 2016). Although the majority of bilaterian mt-genomes do fit the description presented above, several noticeable exceptions have been found. First, unusual mtDNA organizations have been discovered in several lineages, with multipartite mt-genomes found in the mesozoan genus *Dicyema* (Watanabe et al. 1999) (>3 circles), the rotifer genus *Brachionus* (Suga et al. 2008; Hwang et al. 2014) (2 circles), the nematode genus *Globodera* (Armstrong et al. 2000; Gibson et al. 2007) (6 circles), the booklice genus *Liposcelis* (2 circles) (Wei et al. 2012), the thrips species complex *Scirtothrips dorsalis* (Dickey et al. 2015) (2 circles), and several independent cases in parasitic lice (up to 20 minichromosomes) (Cameron et al. 2011; Jiang et al. 2013; Dong et al. 2014) (fig. 2). In addition, a transient linear genome has been reported in the isopod *Armadillidium vulgare* (Marcadé et al. 2007). Second, the size of bilaterian mtDNA was shown to be more variable than originally thought, ranging from just over 11 kb in chaetognaths (Helfenbein et al. 2004; Papillon et al. 2004; Faure and Casanova 2006; Miyamoto et al. 2010) to over 50 kb in

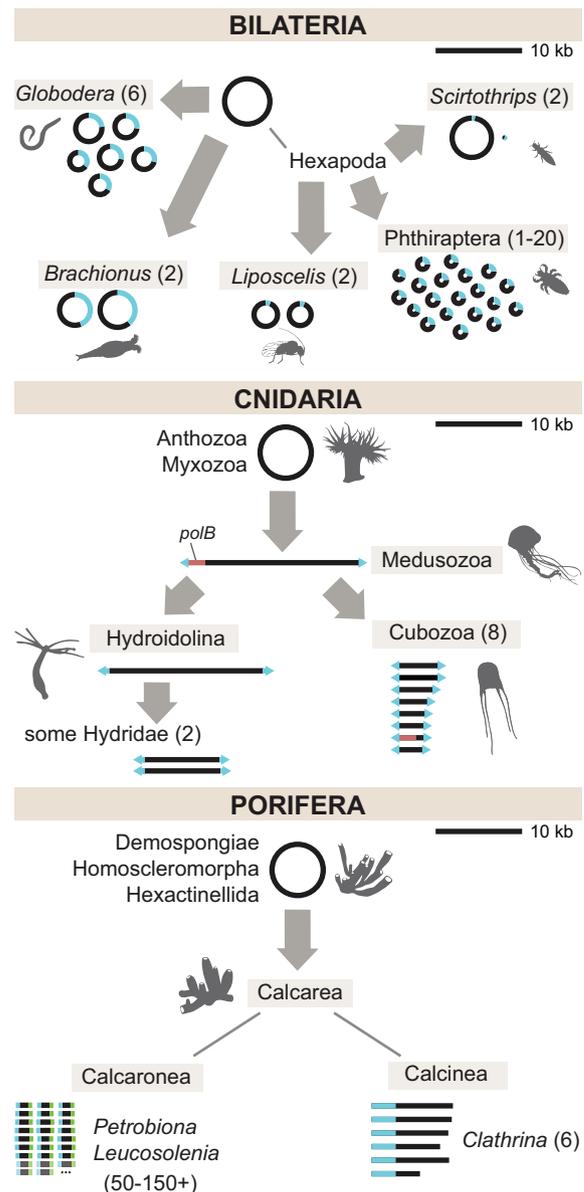


Fig. 2.—Evolution of linear and multipartite mitochondrial DNA in animals. Gray arrows indicate independent reorganization events in the common ancestor of the indicated group. Blue segments indicate regions of homology shared across chromosomes. Numbers in parentheses indicate the range of chromosome numbers (greater than 1) found in the group. Top: Multipartite mtDNA in Bilateria. Not shown: An unknown number of mini-circle chromosomes in *Dicyema* (Watanabe et al. 1999). Middle: Linear and multipartite mtDNA in Cnidaria. The red segment denotes the presence of a *polB* coding sequence in some Medusozoa. Arrowheads at the ends of linear chromosomes signify inverted terminal repeat sequences. Bottom: Multipartite linear mtDNA in Porifera. Faded chromosomes and ellipses indicate the inferred presence of an uncertain number of additional chromosomes. Blue and green segments in *Leucosolenia* and *Petrobiona* show distinct end sequences shared across chromosomes, but which are not homologous between the two genera. Genomes are drawn to scale with respect to the 10 kb scale bar in the upper right part of each panel.

the ark shell *Scapharca broughtonii* (Liu et al. 2013). Third, several changes in gene content have been reported, including losses of *atp8* in most nematodes (Okimoto et al. 1992; Sultana et al. 2013; but see Lavrov and Brown 2001) and flatworms (Le et al. 2000; Von Nickisch-Roseneck et al. 2001; Sola et al. 2015), the loss of *atp6*, *atp8* and most tRNA genes in Chaetognatha (Helfenbein et al. 2004; Papillon et al. 2004; but see Barthelemy and Seligmann 2016), and the gain of a putative novel gene in bivalve molluscs with doubly uniparental inheritance (Breton et al. 2011; Zouros 2013). Furthermore, it has been proposed that additional proteins may be encoded in most if not all animal mt-genomes as (alternative) open-reading frames (ORFs) within standard mitochondrial genes (Guo et al. 2003; Faure et al. 2011; Capt et al. 2015). Fourth, additional genetic codes have been inferred in several groups as shown in figure 3 (Watanabe and Yokobori 2011; Abascal et al. 2012). Fifth, gene order has been found to be much more fluid in some groups (e.g. most tunicates) (Singh et al. 2009; Gissi et al. 2010; Rubinstein et al. 2013) than others. Finally, a highly unusual system was found in bivalve molluscs, which inherit not one but two mt-genomes: one transmitted maternally to all offspring and the second exclusively from father to sons (Zouros et al. 1992; reviewed by Doucet-Beaupré et al. 2010).

With the notable exceptions of those in Chaetognatha and Tunicata, most of the unusual examples of bilaterian mt-genomes have limited taxonomic distribution and have been commonly interpreted as somewhat curious exceptions to the otherwise valid description of “typical animal mtDNA” that has been propagated widely throughout the literature. However, the absence in Cnidaria of almost all of the genetic novelties usually associated with animal mtDNA had already been described by the early 1990s (Wolstenholme 1992b; Beagley et al. 1995). Data from other nonbilaterian phyla (Porifera, Ctenophora, and Placozoa) reinforce the notion that bilaterian mtDNA is not representative of all animals.

Classification and Phylogeny of Nonbilaterian Animals

Nonbilaterian animals display less morphological and species diversity than bilaterian animals and have been traditionally subdivided into four phyla: Porifera, Cnidaria, Ctenophora, and Placozoa, with about 8,500, 11,000, 150, and 1 described species, respectively (Zhang 2011; WoRMS Editorial Board 2015). In addition, Myxozoa, a large (~1,300 species) group of oligo-cellular parasites of fish and annelids with previously unknown phylogenetic affinity, is now placed inside Cnidaria (Nesnidal et al. 2013; Feng et al. 2014; Fook and Siddall 2015; Takeuchi et al. 2015). The relationships among nonbilaterian phyla and bilaterian animals remain contentious (Pisani et al. 2015; Whelan et al. 2015), but are largely irrelevant to the present study. Thus, for the purpose of this review we depict them as a soft polytomy (fig. 1).

Cnidaria. Cnidarians are subdivided into two major lineages: Anthozoa (cnidarians with only a polypoid body form) and Medusozoa (cnidarians with a medusa stage in their life cycle) (Daly et al. 2007; Zapata et al. 2015), but should now also include Myxozoa. Anthozoa is subdivided into Hexacorallia (e.g. sea anemones, hard corals), Octocorallia (soft corals), and Ceriantharia (tube anemones) (Stampar et al. 2014). Within Medusozoa four major clades are recognized: Scyphozoa (jellyfishes), Staurozoa (stalked jellyfishes), Cubozoa (box jellyfishes), and Hydrozoa (hydroids, fire corals, freshwater jellyfishes, siphonophores, etc.) (Collins 2002). Finally, Myxozoa are separated into two classes: Malacosporea and Myxosporea, with the latter containing the majority of species (Fiala et al. 2015).

Ctenophora. Traditionally, ctenophores have been subdivided into two classes based on the presence/absence of their feeding tentacles: Tentaculata (with tentacles) and Nuda (without). Within these classes, 9 orders, 27 families, and about 150 species are currently recognized (Mills 2014). However, the monophyly of both classes and most ctenophore orders is not supported by molecular data and many intraorder relationships remain unresolved (Podar et al. 2001; Moroz et al. 2014; Simion et al. 2015).

Placozoa. Only one placozoan species has been technically described (Schulze, 1883). Nevertheless, molecular studies indicate substantial hidden diversity within this phylum (Eitel et al. 2013). In particular, five substantially different mt-genomes have been published from this phylum (Signorovitch et al. 2007; Miyazawa et al. 2012).

Porifera. Sponges are divided into four major groups (classes): Calcarea (calcareous sponges), Demospongiae (demosponges), Hexactinellida (glass sponges), and Homoscleromorpha (homoscleromorphs) (Gazave et al. 2011). The consensus view on their relationships places demosponges and glass sponges in one group, and homoscleromorphs and calcareous sponges in another (reviewed in Wörheide et al. 2012). Three main lineages are recognized in demosponges: Verongimorpha, Keratosa, and Heteroscleromorpha (Morrow and Cardenas 2015); while calcareous sponges are subdivided into Calcinea and Calcaronea (Manuel 2006; Voigt et al. 2012); glass sponges into Hexasterophora and Amphidiscophora (Dohrmann et al. 2012); and homoscleromorphs into Plakinidae and Oscarellidae (Gazave et al. 2010).

Mitochondrial Genome Architecture in Nonbilaterian Animals

The mt-genomes of most sponges (Wang and Lavrov 2008; Gazave et al. 2010; Haen et al. 2014), anthozoan cnidarians (Brockman and McFadden 2012; Figueroa and Baco 2015), ctenophores (Pett et al. 2011; Kohn et al. 2012), and placozoans (Signorovitch et al. 2007) are mapped as monomeric and circular molecules. This organization is also found in

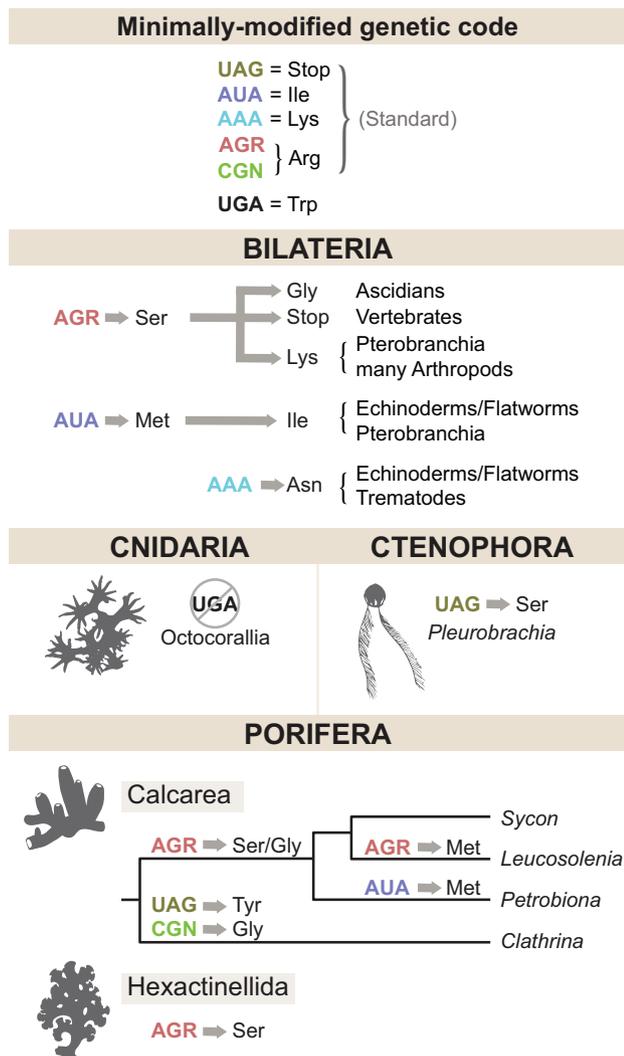


FIG. 3.—Changes in the animal mitochondrial genetic code. Inferred changes in the identities of six codons (listed in the top panel) are shown by arrows. Consecutive changes are shown by a sequence of arrows. Amino-acid identities are designated by the IUPAC three-letter code. A crossed out circle indicates a codon that is no longer used.

choanoflagellates, the closest outgroup to animals, as well as most bilaterian animals and is therefore inferred to be ancestral to Metazoa. However, multipartite and/or linear genomes have evolved in several nonbilaterian lineages, which we will review in more detail below.

Linear Mitochondrial Genomes

Linear mtDNA is found in two major lineages of nonbilaterian animals: Medusozoan cnidarians and calcareous sponges (fig. 2). The occurrence of linear mtDNA in Medusozoa was first inferred based on gel electrophoresis studies (Warrior and Gall 1985; Bridge et al. 1992; Ender and Schierwater 2003).

Subsequently, mt-genome sequences were determined for a variety of medusozoan species revealing two specific features associated with linear mtDNA organization in this group: The presence of identical sequences in inverted orientation at the ends of the chromosome(s) (terminal inverted repeats or TIR) and the existence of two additional ORFs (Shao et al. 2006; Kayal and Lavrov 2008; Voigt et al. 2008; Kayal et al. 2012, 2015; Smith et al. 2012) (fig. 2). One of the ORFs shows significant sequence similarity to B-type DNA polymerase (*polB*), the second is inferred to code for a DNA-binding protein (Shao et al. 2006; Kayal et al. 2012). TIRs are present in all medusozoan mt-genomes and usually include a partial copy of the cytochrome oxidase I gene (*cox1*). The two ORFs are found in cubozoan, schyphozoan, staurozoan, and some hydrozoan mt-genomes but have been lost in hydroidolinan hydrozoans, which instead have longer TIRs that include duplicated *cox1* at each end of their mitochondrial chromosome(s) (Kayal et al. 2012, 2015).

The presence of TIRs and a *polB* homologue in medusozoan mtDNA suggests that their unusual architecture originated through incorporation of a linear plasmid into the ancestral circular genome, while the finding that all medusozoan *polB* sequences form a clade indicates a single origin of linear architecture in this group (Kayal et al. 2012). Linear organellar plasmids with identical but inverted sequences at their termini (known as invertrons: Sakaguchi 1990) are common in organelles of plants and fungi (Hausner 2012) and have been implicated in the evolution of a linear genome organization in some fungi (e.g. Fricova et al. 2010). Interestingly, replication of these plasmids is often initiated from a protein bound to the 5' end of DNA (Klassen and Meinhardt 2007). It has been suggested that the same mode of initiation of replication is retained in mt-genomes that have incorporated such plasmids (Fricova et al. 2010).

All calcareous sponges sampled to date also have multipartite linear mt-genomes (fig. 2), but appear to utilize a different mechanism for the maintenance and repair of their mtDNA, as they do not contain TIRs and do not appear to encode any DNA polymerase-related proteins. The mtDNA of *Clathrina clathrus*, a representative of the subclass Calcinea, consists of six linear chromosomes with a peculiar organization, where about a half of each chromosome is noncoding with hairpin-forming repeat sequences at one end, and with a noncoding region at the other end that is similar across chromosomes (Lavrov et al. 2013). In *Leucosolenia complicata* and *Petrobiona massiliana*, two representatives of the subclass Calcarea, most mitochondrial genes are located on individual chromosomes that have similar but not identical sequences at their ends (Lavrov et al. 2016). The mt-genome organization of *Sycon ciliatum*, another representative of the subclass Calcarea, appears to be more complex and awaits characterization (Lavrov et al. 2016).

Multipartite Mitochondrial Genomes

As is the case with linear genomes, a multipartite organization has evolved several times in animal mtDNA, with both circular (Armstrong et al. 2000; Gibson et al. 2007; Suga et al. 2008; Wei et al. 2012; Jiang et al. 2013) and linear (Voigt et al. 2008; Kayal et al. 2012; Smith et al. 2012; Lavrov et al. 2013, 2016) chromosome architectures (fig. 2). Among nonbilaterian animals, multipartite genomes have evolved at least three times: Two times in Medusozoa (Voigt et al. 2008; Kayal et al. 2012; Smith et al. 2012) and at least one other time in calcareous sponges (Lavrov et al. 2013, 2016). The first report of a multi-chromosomal mt-genome in animals came from the studies of the hydrozoan cnidarians *Hydra attenuata* and *H. littoralis*, in which mtDNA consists of two linear chromosomes, each about 8 kb in size (Warrior and Gall 1985; see also Voigt et al. 2008; Pan et al. 2013). mtDNA from the winged box jellyfish *Alatina moseri* (Smith et al. 2012) and other representatives of class Cubozoa (Kayal et al. 2012) consists of eight linear chromosomes, 2.9–4.6 kb in size (fig. 2).

A remarkable multipartite organization has evolved in calcareous sponges (fig. 2). In both *L. complicata* and *P. massiliana*, all identified coding sequences are located on individual chromosomes. Furthermore, 147 additional mitochondrial chromosomes were identified in *P. massiliana* based on sequence conservation in terminal regions. Some of these chromosomes appear to contain tRNA and rRNA genes, but it is not yet clear whether these sequences are functional. Similarly, at least 50 additional mitochondrial chromosomes were identified in *L. complicata* (Lavrov et al. 2016).

Size Variation and Distribution of Noncoding Nucleotides among Intergenic Regions

The mtDNA of nonbilaterian animals displays at least a 7-fold variation in size (fig. 4). The smallest mt-genomes are found in ctenophores, with those of *Mnemiopsis leidyi* and *Pleurobrachia bachei* measuring 10,326 and 11,016 bp, respectively (Pett et al. 2011; Kohn et al. 2012). The small size of these genomes is due to their reduced gene content, highly abbreviated rRNA structures, and extreme scarcity of intergenic nucleotides. On the other end of the spectrum, the mtDNA of *Trichoplax adhaerens* is 43,079 bp in size (Dellaporta et al. 2006), with several large introns containing intron-associated ORFs. However, the current record in mt-genome size belongs to the multipartite genomes of calcareous sponges, with 6 mitochondrial chromosomes in *C. clathrus* having a total size of about 51 kb (Lavrov et al. 2013), and 150+ mitochondrial chromosomes in *P. massiliana* measuring above 77 kb (the exact sizes of these genomes are unknown because of difficulties associated with sequencing the ends of linear chromosomes and/or identifying all chromosomes) (Lavrov et al. 2016).

Most of the size variation among animal mt-genomes is due to differences in the number of noncoding nucleotides, which are also distributed unevenly among intergenic regions. Bilaterian mtDNA usually contains a single large noncoding segment, also known as the “control” region, or as the “D-loop” region in mammals (Sbisa et al. 1997). In a few species, the control region was shown to contain signals for initiation and termination of replication and transcription (Goddard and Wolstenholme 1980; Clayton 1982). Among nonbilaterian animals, a single large noncoding region is present only in the mtDNA of glass sponges and perhaps ctenophores. In other nonbilaterian taxa, noncoding nucleotides are distributed more evenly among the intergenic regions. The absence of an identifiable control region in many nonbilaterians suggests that their mechanisms of replication and transcription are different from those of bilaterian animals. Furthermore, the “tRNA punctuation” model described in some bilaterian animals for processing of polycistronic mtDNA transcripts (Ojala et al. 1981) is not applicable to many nonbilaterian animals because of the presence of intergenic nucleotides and/or the scarcity or the absence of tRNA genes (see “Loss and gain of mitochondrial tRNA genes”).

More Variation in Protein-Coding Gene Content of Nonbilaterian mtDNA

Mitochondrial protein-coding gene content shows more variation in nonbilaterian compared with bilaterian animals (fig. 5). First, several additional protein-coding genes have been identified. These include *atp9* for subunit 9 (subunit c) of mitochondrial F₀-ATP synthase in all four classes of sponges (Lavrov et al. 2005, 2013; Wang and Lavrov 2008; Haen et al. 2014), *tatC* for the twin-arginine translocase subunit C in the homoscleromorph sponge family Oscarellidae (Wang and Lavrov 2007; Pett and Lavrov 2013), *mutS* for a putative mismatch repair protein in Octocorallia (Pont-Kingdon et al. 1995; Bilewitch and Degnan 2011), *polB* for the DNA dependent DNA polymerase in Medusozoa (Shao et al. 2006; Kayal et al. 2012) and one placozoan (Signorovitch et al. 2007), and intron-associated homing endonucleases, reverse transcriptases, and maturases in several lineages with mitochondrial introns. Among the genes listed above, both *atp9* and *tatC* are also present in the mt-genome of the choanoflagellate *Monosiga brevicollis* (Burger et al. 2003) suggesting that these genes were inherited vertically in sponges, and lost in other animals (Wang and Lavrov 2007; Pett and Lavrov 2013). However, phylogenetic analysis does not rule out the possibility that *tatC* was acquired in Oscarellidae by horizontal transfer from the mtDNA of another eukaryote (Pett and Lavrov 2013). In contrast, cnidarian *mutS* and *polB* were likely acquired by horizontal gene transfer from prokaryotes or viruses (Bilewitch and Degnan 2011).

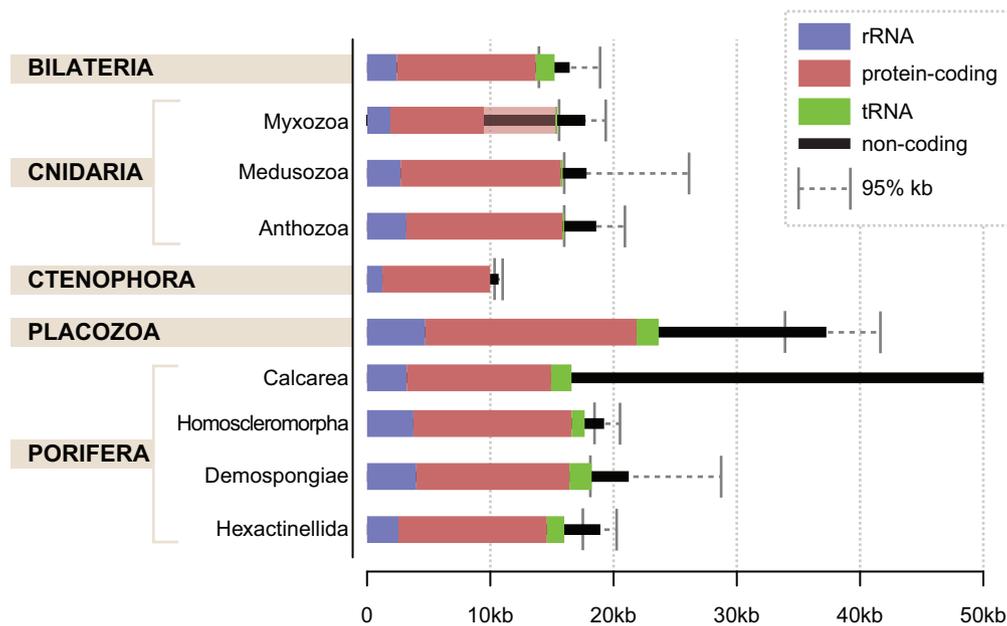


FIG. 4.—Size and coding content of animal mitochondrial DNA. Colored bars indicate the average number of nucleotides coding for ribosomal RNA (blue), protein (red), transfer RNA (green), or noncoding (black) computed using all available complete mt-genome sequences published in the NCBI RefSeq database as of April 9, 2016. Gray error bars indicate the upper and lower boundaries of the 95% interquartile range for genome sizes (2.5th and 97.5th percentiles). The shaded portion of the red bar in Myxozoa indicates additional protein-coding genes likely present in these genomes, but not recognizable due to an extreme rate of sequence evolution.

Second, several protein-coding genes have been lost from some nonbilaterian mt-genomes. In two ctenophores, *atp6* has been transferred to the nucleus, while *atp8* was not identified in either the mitochondrial or the nuclear genomes (Pett et al. 2011; Kohn et al. 2012). Similarly, *atp8* appears to be lost from the mt-genomes of placozoans (Signorovitch et al. 2007; Burger et al. 2009) and calcareous sponges (Lavrov et al. 2013, 2016), while *atp9* has been transferred to the nucleus in several demosponges (Erpenbeck et al. 2007; Lavrov, unpublished data). It has been thought that *atp8* is also absent from mitochondrial genomes of glass sponges (Haen et al. 2007; Rosengarten et al. 2008; Haen et al. 2014). However, a plausible candidate has been recently found in *Oopsacas minuta* (Jourda et al. 2015) with homologous sequences identifiable in other species (unpublished data).

The most unusual mitochondrial gene content has been reported for the myxozoan genus *Kudoa*, where only eight protein-coding genes and some unidentified ORFs were found (Takeuchi et al. 2015). However, because of the extremely high rate of sequence evolution in myxozoan mtDNA, it is probable that some unidentified ORFs represent standard mitochondrial genes that have evolved beyond recognition (fig. 4, shaded red bar). This inference is supported by the observation that only five out of the eight identified genes have retained significant sequence similarity ($E < 0.001$) with their homologues outside of Myxozoa.

Loss and Gain of Mitochondrial tRNA Genes

Besides variation in protein-coding gene content, animal mt-genomes encode a variable number of transfer RNAs (mt-tRNAs) (fig. 5). Most demosponge and placozoan mt-genomes contain 24–25 tRNA genes, including *trnI(CAU)* and *trnR(UCU)*. The latter two genes were lost early in the evolution of bilaterian animals in conjunction with modifications to the mitochondrial genetic code, but their products are needed for mitochondrial protein synthesis in many nonbilaterian animals, which utilize a minimally modified genetic code (see “Changes in the genetic code”). In addition, a second *trnM(CAU)* is present in some sponge mt-genomes, apparently coding for a separate elongator *tRNA^{Met}_{CAU}*.

Representatives of three out of the four main lineages of nonbilaterian animals have experienced significant losses of tRNA genes. First, all mt-tRNA genes have been lost in Ctenophora (Pett et al. 2011) [the two mt-tRNA genes reported for the ctenophore *Pleurobrachia bachei* (Kohn et al. 2012) are annotation artifacts (Pett and Lavrov 2015)]. Second, the mt-genomes of nonmyxozoan cnidarians have lost all but one or two tRNA genes (Kayal et al. 2013), while no mt-tRNA genes have been found in two sampled species in the myxozoan genus *Kudoa* (Takeuchi et al. 2015). Although four mt-tRNAs have been reported from the third representative of this genus, they are also likely annotation artifacts.

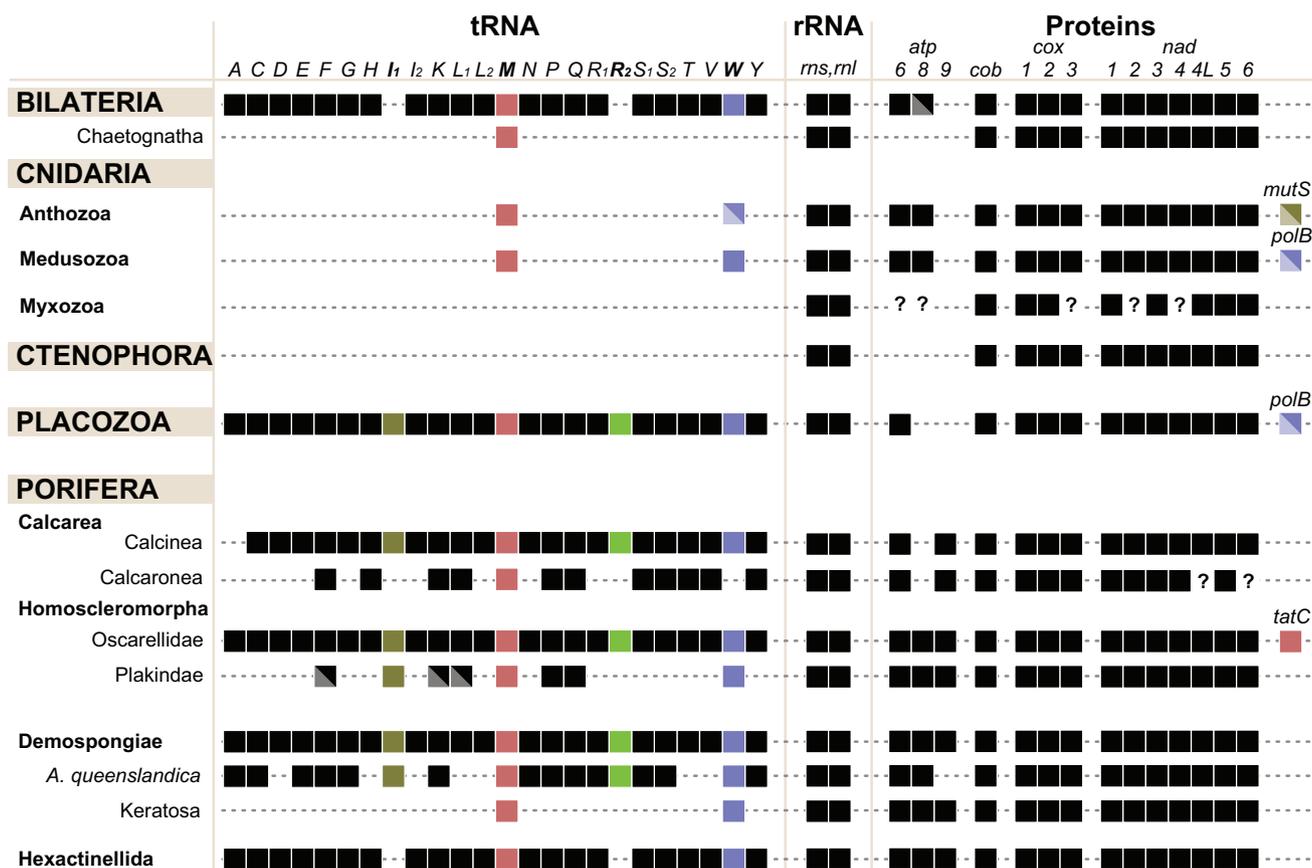


FIG. 5.—Variation in RNA and protein-coding gene content in animal mitochondrial DNA. Left: taxonomic positions of groups with variable gene content. Central: variation in tRNA gene content. Middle: the presence of rRNA genes. Right: the presence/absence of protein-coding genes. Colored boxes highlight the identities of specific genes discussed in the text. Diagonally split boxes indicate genes that are absent in some lineages of the indicated group. Question marks denote genes for which presence or absence has not been confidently determined. Abbreviations: *atp6*, *atp8*–*9*: subunits 6, 8, and 9 of F0 adenosine triphosphatase (ATP) synthase; *cob*: apocytochrome b; *cox1–3*: cytochrome c oxidase subunits 1–3; *nad1–6* and *nad4L*: NADH dehydrogenase subunits 1–6 and 4L; *polB*: DNA polymerase β; *tatC*: twin-arginine translocase component C; *rns* and *rnl*: SSU and LSU rRNAs. The tRNA genes are identified by the one-letter code for their corresponding amino acid; subscripts denote different genes for isoacceptor tRNAs, where *I1* = *trnI(CAU)*, *I2* = *trnI(GAU)*, *L1* = *trnL(UAG)*, *L2* = *trnL(UAA)*, *R1* = *trnR(UCG)*, *R2* = *trnR(UCU)*, *S1* = *trnS(UCN)*, *S2* = *trnS(UGA)*.

Third, mt-tRNA loss has occurred repeatedly in sponges, including several lineages of demosponges, the homoscleromorph family Plakinidae, and possibly some calcaronean sponges. Interestingly, one group of demosponges (Keratosa) has a pattern of mt-tRNA gene loss identical to that in most Cnidaria, encoding only genes for tryptophanyl and methioninyl mt-tRNA (Wang and Lavrov 2008).

The loss of mt-tRNA genes is commonly associated with other changes in the nuclear genome. In particular, the loss of mt-tRNAs may render some components of the mitochondrial translation system redundant, leading to the loss of their genes in the nuclear genome. For example, the maturation of *tRNA^{Leu}_{CAU}* (the product of the *trnI(CAU)* gene) involves a post-transcriptional modification of the cytosine in the anticodon (position 34) to lysidine (2-lysyl-cytidine) performed by *tRNA^{Leu}*-lysidine synthetase (TilS) (Soma et al. 2003), an

enzyme that is not involved in maturation of cytosolic isoleucine tRNAs (Marck and Grosjean 2002). Because of the dispensable nature of this function for cytosolic protein synthesis, the loss of mitochondrial *trnI(CAU)* that occurred in some nonbilaterian groups and all bilaterians, was followed by the loss of the nuclear-encoded TilS gene, rendering a reacquisition of *trnI(CAU)* practically impossible (Pett and Lavrov 2015). Our previous research also uncovered a correlation between the loss of mt-tRNAs and the loss of nuclear-encoded mitochondrial aminoacyl-tRNA synthetases (Haen et al. 2010; Pett et al. 2011), as well as several other RNA-modifying and aminoacyl-tRNA modifying enzymes (Pett and Lavrov 2015).

Finally, some mt-tRNA genes can be gained via a process of “remolding” or “recruitment”, in which a copy of a gene is recruited from one isoaccepting group to another by a point mutation that changes the tRNA’s amino acid identity and its

mRNA coupling capacity. This process has been extensively documented in both bilaterian and nonbilaterian animal mtDNA (Lavrov and Lang 2005; Wang and Lavrov 2011; Sahyoun et al. 2015).

Changes in the Genetic Code

Close unicellular relatives of animals use a nonstandard “minimally modified” genetic code for mitochondrial protein synthesis, where the only deviation from the standard genetic code is the reassignment of the UGA codon from termination to tryptophan (fig. 3). This mitochondrial genetic code was likely the ancestral condition for Metazoa (Knight et al. 2001) and is still used by most nonbilaterian animals. Early in the evolution of bilaterian animals, the meanings of two mitochondrial codons were changed, with AUA reassigned from isoleucine to methionine, and AGR from arginine to serine (Knight et al. 2001), followed by further modifications in some lineages (Watanabe and Yokobori 2011).

Several modifications to the mitochondrial genetic code have also occurred among nonbilaterian animals, some of them in parallel with those in bilaterian animals (fig. 3). Like in bilaterian animals, the identity of AGR codons has been changed from Arginine to Serine in glass sponges (Haen et al. 2007, 2014) and to either Serine or Glycine in the calcarean sponges *Sycon ciliatum*, *S. coactum*, and *P. massiliana* (Lavrov et al. 2016). Furthermore, in *P. massiliana* the AUA codon has been reassigned from Isoleucine to Methionine. More unusual changes have occurred in calcarean sponges *C. clathrus* and *C. aurea*, where UAG has been reassigned from termination to Tyrosine and CGN codons from Arginine to Glycine (Lavrov et al. 2013), and in the calcarean sponge *L. complicata*, where AGR codons have been reassigned to Methionine. In addition, at least some species of octocorals no longer use UAG in their mitochondrial coding sequences (effectively reverting to the standard genetic code), while in some ctenophores this codon has been reassigned to Serine (Pett and Lavrov 2015).

Mitochondrial Introns and Fragmented Genes

Several cases of fragmented mitochondrial genes have been reported in nonbilaterian animals, and the majority of these cases involve mitochondrial introns. Introns in nonbilaterian animal mtDNA were first found in anthozoan cnidarians (Beagley et al. 1996) and subsequently in demosponges (Rot et al. 2006), placozoans (Dellaporta et al. 2006), and homoscleromorph sponges (Wang and Lavrov 2008). It has been suggested that the more common presence of mitochondrial introns in nonbilaterian compared with bilaterian animals [they have been found only in a few annelid species among the latter (Valles et al. 2008; Richter et al. 2015)] may be due either to the lack of early separation between germ and soma,

or to the low mitochondrial substitution rates observed in many of nonbilaterian species (Hausner 2012).

As is the case with organellar introns in other eukaryotes, animal mitochondrial introns are homologous to either group I or group II self-splicing introns found in bacteria and viruses (Martínez-Abarca and Toro 2000; Hausner et al. 2014) rather than to either the spliceosomal introns or tRNA introns present in nuclear genomes (Lang et al. 2007). All hexacoral mt-genomes contain a group I intron inside *nad5* and often another intron inside *cox1* (Beagley et al. 1996; Goddard et al. 2006; Medina et al. 2006; Fukami et al. 2007). The intron within *nad5* is highly unusual because it encompasses at least 2 and as many as 15 standard mitochondrial genes, and sometimes additional ORFs (Emblem et al. 2014). One or two group I introns are also present in *cox1* in some members of the homoscleromorph sponge family Plakinidae (Gazave et al. 2010), and three different group I introns have been described in *cox1* from several groups of demosponges (Rot et al. 2006; Szitenberg et al. 2010; Erpenbeck et al. 2015; Huchon et al. 2015). Finally, all placozoan mt-genomes contain six to seven group I introns (Signorovitch et al. 2007). The structure of *cox1* in these species is most unusual in that it consists of seven to eight exons contained in three segments with different transcriptional polarities, which are joined together by group I intron *trans*-splicing, one of few reports of such a process *in vivo* (Burger et al. 2009). Homing endonucleases of the LAGLIDADG type can often be found encoded in *cox1* group I introns, and the patchy phylogenetic distribution of these proteins within these groups is consistent with the typical life cycle of homing endonucleases, which involves recurrent horizontal transfer among closely related species (Goddard and Burt 1999).

Group II introns are less common in animal mitochondria, but as many as four different forms have been identified in placozoan mt-genomes (Signorovitch et al. 2007; Burger et al. 2009). In addition, two group II introns have recently been found in demosponges (Huchon et al. 2015). Phylogenetic analysis of the reverse transcriptase encoded by the latter introns suggests that they may have originated by horizontal transfer from a red algal donor (Huchon et al. 2015). Surprisingly, group II mitochondrial introns have also been discovered in several annelid species (Valles et al. 2008; Richter et al. 2015), the only example of introns in bilaterian animals.

No introns have been found in glass sponges, calcareous sponges, ctenophores, medusozoan cnidarians, and myxozoans, or most bilaterian animals, groups in which accelerated rates of mt-sequence evolution have been observed. This lack of introns in fast-evolving mtDNA is consistent with observations from other groups of eukaryotes, and can potentially be explained by the mutational hazard hypothesis (Lynch et al. 2006), which asserts that strong mutation pressure, resulting from a high mutation rate, suppresses the proliferation of noncoding elements, including introns. At the same time, no mitochondrial introns have been found in either octocoral

cnidarians or the homoscleromorph sponge family Oscarellidae, two groups with exceptionally low rates of mitochondrial sequence evolution (Gazave et al. 2010; McFadden et al. 2011), indicating that other factors are also at play.

In some cases, not only mitochondrial genes but also their products can be fragmented. For example, in the calcareous sponges *C. clathrus* and *C. aurea*, both rRNA genes are discontinuous and located on different chromosomes (Lavrov et al. 2013, unpublished data), but encode conserved rRNA structures when pieced together. Fragmented rRNA genes have also been found in the oyster genus *Crassostrea* (Milbury and Gaffney 2005; Milbury et al. 2010), and in Placozoa, where large subunit rRNA is encoded in two pieces (Signorovitch et al. 2007). Typically, rRNA fragments assemble as separate molecules, but an example of *trans*-splicing has been recently reported in the Euglenozoan flagellate *Diplonema* (Valach et al. 2014). There is no evidence of rRNA fragment *trans*-splicing in animal mitochondria.

RNA Editing and Translational Frameshifting

Some animal mt-genomes encode aberrant tRNAs and/or mRNAs that are corrected by RNA editing during or after transcription (Gott and Emeson 2000). Mitochondrial RNA editing in bilaterian animals involves primarily tRNAs (Börner et al. 1997). As an extreme example, nearly half of nucleotides in the mt-tRNAs of onychophorans are added post-transcriptionally (Segovia et al. 2011). A few putative cases of mRNA editing have also been reported in bilaterian animal mitochondria, most of which manifest as variation in the length of polythymidine poly(T) tracts (Vanfleteren and Vierstraete 1999; Riepsamen et al. 2008; Deneud et al. 2010; but see Riepsamen et al. 2011). A few RNA-DNA differences have recently been reported in human mtDNA (Bar-Yaacov et al. 2013).

In nonbilaterian animals, both tRNA and mRNA editing has been found. The mtDNA of *C. clathrus* encodes tRNA molecules with multiple mismatches in the aminoacyl acceptor stem, which undergo post-transcriptional RNA editing (Lavrov et al. 2013). In Placozoa, a conserved uracil (U) position in *cox1* is required for proper splicing of a group I intron, but a cytosine (C) is required at the same site for a functional COX protein subunit. This apparent conflict is resolved by converting U to C by an RNA editing mechanism that takes place after splicing is completed (Burger et al. 2009). Recently, widespread and persistent mt-mRNA editing was discovered in calcareous sponges (Lavrov et al. 2016), consisting of single or double U insertions in pre-existing poly(U) tracts. A total of 225, 435, and 451 nucleotides were inserted in mt-mRNAs identified in three analyzed species (Lavrov et al. 2016).

In glass sponges, several single nucleotide insertions in otherwise conserved mitochondrial coding sequences are retained in mRNA and have been interpreted as +1 translational frameshifting sites (Haen et al. 2007; Rosengarten et al. 2008). Most of these insertions occur immediately upstream of conserved glycine GGA codons and result in the formation of in-frame TGG or CGG codons, which are otherwise not used or very rarely used in glass sponge mitochondrial coding sequences (Haen et al. 2014). These unusual sequence features, along with some unusual changes in the corresponding glycine tRNAs, suggest an “out-of-frame pairing” model of translational frameshifting, in which the extra nucleotide is excluded from pairing at the A-site of the ribosome (Buchan and Stansfield 2007). Our recent work revealed that frameshifting sites have evolved repeatedly in glass sponge mtDNA, and can persist for hundreds of millions of years (Haen et al. 2014). Among bilaterian animals, translational frameshifting has been reported in birds and turtles (Harlid et al. 1997; Mindell et al. 1998; Zardoya and Meyer 1998; Parham et al. 2006), *Polyrhachis* ants (Beckenbach et al. 2005) and the eastern oyster (Milbury and Gaffney 2005). Interestingly, AGR codons in human mtDNA, originally inferred to code for termination, have been recently reinterpreted as mitochondrial frameshifting sites (Temperley et al. 2010).

Variable Rates of Sequence Evolution

It was observed early in the study of animal mtDNA that the rate of nucleotide substitutions was an order of magnitude higher in mitochondrial compared with nuclear genes (Brown et al. 1979). Subsequently, a wide range of mitochondrial substitution rates have been estimated within vertebrate lineages, including mammals, where the rates can vary 100-fold and birds, where the rates can vary 30-fold (Nabholz et al. 2008; Nabholz et al. 2009). Analysis of mtDNA sequences from nonbilaterian animals adds another dimension to these observations by showing persistent differences in rates among major animal groups (fig. 6). In particular, demosponges, homoscleromorphs, and most cnidarians have relatively low rates of mitochondrial substitutions, glass sponges, and bilaterian animals have intermediate rates, while ctenophores, calcareous sponges, and myxozoans have high to extremely high rates (fig. 6).

An increased substitution rate may be caused by an elevated mutation rate (which determines the rate of neutral evolution) and/or an increased probability of fixation of non-neutral mutations (Kimura 1993; reviewed in Baer et al. 2007). A high mutation rate in bilaterian mtDNA, first inferred from phylogenetic comparisons, was later corroborated and revised upward by pedigree analyses and mutation accumulation experiments (Parsons et al. 1997; Denver et al. 2000; Haag-Liautard et al. 2008; Xu et al. 2012). The high mutation rate in animal mtDNA is often attributed to damage from reactive

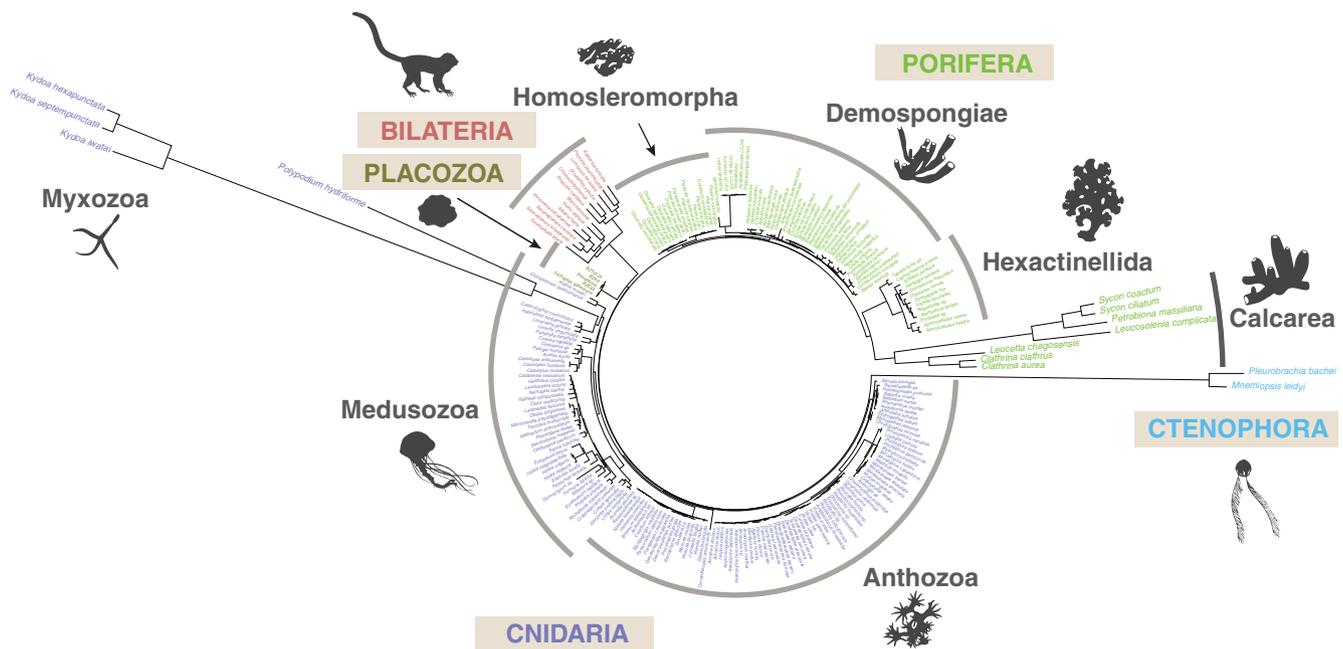


Fig. 6.—mtDNA-based animal phylogeny showing variable rates of mt-sequence evolution. Complete or nearly complete mt-genomes from all available nonbilaterian animals and a few selected bilaterian animals were downloaded from the GenBank. In addition, mitochondrial genomes of *Periphylla periphylla* and *Polypodium hydriforme*, were assembled from high throughput transcriptomic and genomic data (SRX956805 and SRX687102, respectively). Inferred amino acid sequences from nine mitochondrial genes (*cob*, *cox1-3*, *nad1-5*) were aligned with MAFFT v7.215 (Kato and Standley 2013). Conserved blocks within the alignments were selected with Gblocks 0.91 b (Talavera and Castresana 2007) using relaxed parameters (parameters 1 and 2 = ½, parameter 3 = 8, parameter 4 = 5, all gap positions in parameter 5). Cleaned alignments were concatenated in a dataset 2,228 positions in length. Bilateria, Cnidaria, Ctenophora, Placozoa, and Porifera were constrained as monophyletic and the best constrained topology was identified using RAXML with the MTREV + GAMMA + F substitution model and 32 initial tree searches. Subsequently, branch lengths were re-estimated with the CAT + GTR + Γ 4 model in PhyloBayes MPI 1.4e (Lartillot et al. 2013).

oxygen species (ROS) released as byproducts during oxidative phosphorylation (Richter et al. 1988; Yakes and Van Houten 1997) and has been proposed to be a function of the metabolic rate (Martin and Palumbi 1993; Gillooly et al. 2005). However, no support for this “metabolic rate hypothesis” has been found in subsequent studies (Lanfear et al. 2007). Furthermore, recent studies that have utilized ultra-sensitive sequencing of somatic mtDNA revealed that most mutagenic events are not G → T transversions expected as the result of ROS damage but transitions, likely caused by errors made by DNA polymerase and/or spontaneous deamination of cytidine and adenosine during replication (reviewed in Larsson 2010; Ameur et al. 2011; Kennedy et al. 2013; Itsara et al. 2014). Thus, changes in mitochondrial mutation rates are more likely the result of changes in nuclear-encoded proteins responsible for the maintenance and replication of mtDNA rather than different levels of ROS production. Because of the lower mutation load expected for small genomes, natural selection for mtDNA replication fidelity should be relaxed compared with the nuclear genome, resulting in a higher mutation rate (Lynch 2010). It has also been suggested that the elevated mutation rate in animal mtDNA may be driven by positive selection for a greater supply of adaptive mutations (Wallace 2007), but we

view this proposition as highly unlikely. Although alleles causing a high mutation rate (mutator alleles) do exist in natural populations and can become fixed by hitchhiking with linked beneficial mutations (Taddei et al. 1997), this is not expected in mtDNA, which is unlinked from its nuclear-encoded replication machinery (Lynch 2011). Even in asexual bacterial populations, hitchhiker mutator alleles are generally short-lived and/or exist at low frequencies due to the inevitable long-term deleterious mutation load associated with a high mutation rate (Giraud et al. 2001). Experimental increase of mutation rate in mammalian mtDNA lead to aggravated aging, impaired brain development, and reduced lifespan (Ross et al. 2013, 2014).

While the rate of fixation for neutral mutations is equal to the mutation rate, that of non-neutral mutations also depends on their fitness effect (expressed as selection coefficient) and effective population size (Kimura 1993). Both theoretical considerations (Kimura 1968) and analyses of empirical data (Weinreich and Rand 2000; Nielsen and Yang 2003; Popadin et al. 2007; Lartillot and Poujol 2011) suggest that the majority of mtDNA mutations are deleterious and thus should be eliminated by selection. Although positive selection has also been inferred in the mtDNA of some lineages, usually

in association with postulated higher demand for energy production [e.g., in anthropoid primates with an expanded neocortical brain (Grossman et al. 2004), bats (Shen et al. 2010), and mammals at high altitudes (Hassanin et al. 2009)] demonstrating the occurrence of adaptive substitutions in mtDNA evolution can be difficult (Meiklejohn et al. 2007; Nei et al. 2010). Nevertheless, it has been estimated that up to a quarter of nonsynonymous substitutions in various animal species may be fixed by adaptive evolution (James et al. 2016).

The strength of genetic drift, which is inversely proportional to the effective population size, will also influence the probability of fixation of non-neutral mutations, with higher rates of deleterious substitutions in smaller populations, and higher rates of beneficial substitutions in larger populations (Ohta 1973). The effect of genetic drift on mtDNA is amplified by several features of mitochondrial biology, including its uniparental (maternal) inheritance, effective haploidy, and lack (or scarcity) of recombination (Ballard and Whitlock 2004; Aanen et al. 2014). A commonly observed outcome of stronger genetic drift in mtDNA is the more rapid fixation of mildly deleterious mutations (Lynch and Blanchard 1998) as reported for large versus small mammals (Popadin et al. 2007). It has also been inferred that selective sweeps occur predominantly in invertebrate rather than vertebrate animals, due to their larger population sizes (Bazin et al. 2006; Meiklejohn et al. 2007). Finally, genetic drift should influence the mitochondrial mutation rate itself due to accumulation of mildly deleterious mutations in nuclear-encoded mitochondrial replication proteins (Lynch 2010; Sung et al. 2012).

Given the impact that reduced effective population size can have on substitution rates by increasing both the mutation rate and the probability of fixation of slightly deleterious mutations, life history features may help explain the accelerated rates of mitochondrial sequence evolution observed in some nonbilaterian animals. For example, the fastest evolving animal mtDNA belongs to Myxozoa, one of very few examples of parasitic nonbilaterian animals [the others being a related cnidarian endoparasite *Polypodium hydriforme* (Evans et al. 2008), and *Eulampetia pancerina*, a ctenophore with a parasitic larval stage]. As a result of their parasitic lifestyle, there are recurrent bottlenecks in myxozoan populations as their hosts are infected by a small number of spores (Kent et al. 2001). In addition, sexual reproduction usually takes place between descendants of a single founder actinospore, leading to inbreeding and further reduction in the effective population size of mtDNA. Ctenophores, another group with highly accelerated mitochondrial substitution rates, also have an unusual biology, characterized by frequent selfing, hermaphroditism, and large fluctuations in population size (Baker and Reeve 1974; Pianka 1974).

The effect of smaller population size is often most visible in changes (likely deleterious) that accumulate in tRNA and rRNA structures (Lynch 1996). The mt-genome of the ctenophore *M. leidy*, a selfing hermaphrodite (Baker and Reeve 1974;

Pianka 1974), represents one of the most extreme examples, where the sizes of the small and large subunit rRNAs have been reduced to no more than 368 and 878 bp, respectively, with only a few identifiable conserved secondary structures (Pett et al. 2011). Interestingly, in the calcinean sponge *C. clathrus* mt-rRNA structures are relatively well conserved despite high substitution rates in mitochondrial coding sequences.

General Trends in the Evolution of Animal mtDNA

Thirty-five years after the first animal mtDNA sequence was determined (Anderson et al. 1981), we are finally attaining a comprehensive view of animal mt-genome diversity. The emerging picture reveals more variation in animal mtDNA organization and evolution than previously appreciated, much of which is found among nonbilaterian phyla. Conversely, many of the genetic novelties thought to be associated with animal mtDNA (Wolstenholme 1992b) appear to be restricted to Bilateria. The richness of mitochondrial features in nonbilaterian animals is particularly striking in the light of the relatively small number nonbilaterian species with complete mt-genomes (around 200), which is an order of magnitude smaller than the number for Bilateria (over 6000) (fig. 1).

In part, the greater mtDNA diversity in nonbilaterian animals can be explained by the deeper divergences among nonbilaterian comparing to bilaterian phyla (Dos Reis et al. 2015). However, mtDNA diversity does not appear to be simply a function of time, as most major changes map to early branches in animal evolution (fig. 1). This can be seen, for example, in the remarkable uniformity of mtDNA in most bilaterian animals, which suggests that characteristic features of bilaterian mtDNA emerged after the split of Bilateria and Cnidaria in the late Precambrian but prior to the diversification of bilaterian animals in the early Cambrian. Similarly, a distinct organization of glass sponge mtDNA was established prior to the divergence of crown lineages in the Cambrian and has been maintained thereafter (Haen et al. 2014). Comparable patterns of mtDNA evolution are also found within ctenophores and placozoans (Signorovitch et al. 2007; Pett et al. 2011; Kohn et al. 2012), although it is less clear when these patterns were established.

It is not immediately clear why these early patterns should have been so widely conserved, but an intriguing possibility is that early changes in the evolution of certain groups canalized the future course of evolutionary events in mtDNA. For example, early changes in the bilaterian mt-tRNA and/or mt-protein import machinery may have prevented the replacement of mt-tRNAs with their nuclear counterparts, a process that is quite common outside of Bilateria (Schneider 2011; Pett and Lavrov 2015), but very rare among bilaterian animals [the exception of chaetognaths (Faure and Casanova 2006) deserves further investigation (e.g. see Barthelemy and Seligmann 2016)].

As with other biological systems, the evolution of animal mtDNA is also marked by convergence. One of the most striking examples is the parallel evolution of glass sponge and bilaterian mtDNA. Both groups of animals share a similar mtDNA organization with a single large noncoding region, an identical change in the genetic code, a modification in the structure of tRNA(Ser), as well as a particular bias in nucleotide composition (Haen et al. 2007). Other examples of convergent mitochondrial evolution in animals include transitions to a multipartite, linear genome organization in calcareous sponges and the cnidarian class Cubozoa and the loss of the same set of tRNA genes in Cnidaria and the demosponge lineage Keratosa. Interestingly, parallel evolution in mtDNA can precipitate parallel changes in the nuclear genomes, as has been shown by the loss of nuclear encoded mt-aminoacyl-tRNA synthetases in association with the loss of mt-tRNAs. It remains to be seen whether other changes in the content and structure of mt-genomes can lead to convergent evolution in other nuclear genes and molecular pathways.

Future Directions

Faced with the abundance of bilaterian mt-genome sequences, it has been argued that further sampling of animal mt-genomes may be of limited use for studying mtDNA evolution (Smith 2015). This may be true for some groups. However, current mtDNA sampling for nonbilaterian animals remains poor in comparison with their considerable phylogenetic diversity, and further studies in these groups have the potential to significantly expand our understanding of mitochondrial evolution.

In particular, there are several nonbilaterian groups, such as ctenophores, calcareous sponges, and myxozoan cnidarians, where sampling is clearly inadequate. Probably not coincidentally, these are also the groups that have either unusual mt-genome organization or extremely high rates of mt-sequence evolution, or both. For all these groups, mtDNA sequences became available only with the advent of next generation DNA sequencing technologies, which made it possible and cost-effective to sequence total cellular DNA from targeted species and bioinformatically assemble mtDNA. Interestingly, unusual mt-genomes may remain undiscovered in otherwise well-sampled groups of animals because traditional methods have failed to obtain positive results. For example, preliminary data suggest that multipartite mtDNA has evolved in some anthozoan cnidarians (order Ceriantharia) (Brugler and France 2007; Kayal et al. 2013) and demosponges (genus *Mycale*) (unpublished data) but has not been fully described due to sequencing/assembly difficulties. The discoveries of unusual mt-genomes open new questions in the study of animal mtDNA, such as the formation, maintenance, and expression of multipartite genomes, which in the absence of mitosis should represent a formidable task. Studying such genomes could also provide

alternative explanations for unusual experimental results in other supposedly more familiar taxa. Indeed, several recent studies have forced us to rethink mtDNA biology in even the most familiar “model” animal species (e.g. Guo et al. 2003; Temperley et al. 2010; Lewis et al. 2015).

Finally, in many groups our knowledge of mitochondrial biology is limited to the mtDNA sequence itself, while the vast majority of proteins functioning in mitochondria are encoded by nuclear genomes. Recent explosive growth in the quantity of nuclear genomic and transcriptomic data from nonmodel organisms, including many nonbilaterian animals, will provide a fruitful avenue for understanding mitochondrial physiology and evolution in animals.

Acknowledgments

We thank Daniel Sloan, Jonathan Wendel, Kateryna Makova—our associate editor and two anonymous reviewers for their insightful comments on earlier versions of this article, and Paul Veach for his help with illustrations. A large part of the research reviewed here was supported by a grant from the National Science Foundation to DVL (DEB-0829783). W.P. was supported in part by the French National Research Agency (ANR) grant Ancestrome (ANR-10-BINF-01-01).

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Associate editor: Kateryna Makova