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Molluscan Mitochondrial Genomes Break the Rules

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Abstract

The first animal mitochondrial genomes to be sequenced were of several vertebrates and model organisms, and the consistency of genomic features found has led to a “textbook description,” but a more broad phylogenetic sampling of complete animal mitochondrial genomes has found many cases where these features do not exist, and the phylum Mollusca is especially replete with these exceptions. The characterization of full mollusc mitogenomes required considerable effort involving challenging molecular biology, but has created an enormous catalog of surprising deviations from that textbook description, including wide variation in size, radical genome rearrangements, gene duplications and losses, introduction of novel genes, and a complex system of inheritance dubbed “doubly uniparental inheritance”. Here we review the extraordinary variation in architecture, molecular functioning, and intergenerational transmission of molluscan mitochondrial genomes. Such features represent a great potential for the discovery of biological history, processes, and functions that are novel for animal mitochondrial genomes. This provides a model system for studying the evolution and the manifold roles that mitochondria play in organismal physiology, and the many ways that the study of mitochondrial genomes are useful for phylogeny and population biology.

Keywords

mitochondria, mollusc, genome, evolution, doubly uniparental inheritance

Introduction

In the 1980s, as DNA sequencing was becoming common, the fledglings of what we now call “genomics” were diminutive animal mitochondrial genomes. The first reports were of several vertebrates and model organisms, followed quickly by studies of their modes of replication, transcription, RNA processing, and other aspects of molecular biology [see 1]. The consistency of genomic features found and the expectation that these studies were characteristic of all mitochondrial genomes has led to a “textbook description” of mitochondrial genomes that includes a consistent size of about 16 kb, strictly maternal inheritance, a content of 37 genes (encoding 13 proteins, 2 rRNAs, and 22 tRNAs) compactly organized in a nearly invariant arrangement, a single large non-coding “control region” with signals for regulating replication and transcription, and transcription of a single polycistron from each strand that is processed by enzymatic removal of tRNAs into gene-specific (or, in the cases of *nad4L-nad4* and/or *atp8-atp6*, bicistronic) mRNAs. Secondary structures were sometimes inferred for regulatory signals or to compensate for lack of tRNA genes where necessary for enzymatically separating the adjacent gene-specific transcripts.

Clearly, understanding these features is important for interpreting the patterns of evolution of these genomes, but this touches also on many other issues, including interactions with the products of nuclear genes, energy generation, wide-ranging aspects of metabolism and physiology, stress tolerance, susceptibility to oxidative stress, aspects of ecology, patterns of inheritance, and population genomics. A more broad phylogenetic sampling of complete mitochondrial genomes now belies not only these general genomic features, but also makes clear that there is no potential for some of these functional molecular mechanisms.

Among bilaterian animals, the phylum Mollusca is especially replete with such examples. Due to their modest size and considerable phylogenetic information content both in gene sequences and arrangements, molluscan mitogenomes began to be studied in the early 1990s. Then, characterization of full mitogenomes required considerable effort involving challenging molecular biology including physical isolation of mtDNA, restriction enzyme mapping, cloning of large inserts, subcloning into a large number of separate plasmid vectors, and Sanger sequencing by directed primer walking, as evident from the first reports of molluscan mitogenomes from *Mytilus edulis* [Bivalvia: 2], *Katharina tunicata* [Polyplacophora: 3] and several Helicid gastropods [4–6], see Table 1. The revolutions in genome sequencing technology since have greatly accelerated these efforts, and we now have available more than 1000 complete mitochondrial genome sequences from more than 700 species. This, plus a

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3 modest amount of work to understand the biology of these genomes, has created an enormous
4 catalog of surprising deviations from that textbook description, including wide variation in size,
5 radical genome rearrangements, gene duplications and losses, introduction of novel genes, and
6 a complex system of inheritance dubbed “doubly uniparental inheritance” (DUI). This creates
7 great potential for the discovery of biological history, processes, and functions that are novel for
8 animal mitochondrial genomes. Interestingly, expanded non-coding regions, variable repeat
9 content, frequent gene rearrangements, and large numbers of ORFans, while uncommon in
10 other animal lineages, are frequently observed in plants (Mower et al. 2012).
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20 Genome Architecture

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22 The first mollusc mitochondrial genome [2], sequenced nearly three decades ago with Klenow
23 fragment of *E. coli* DNA polymerase on polyacrylamide gels, documented unprecedented
24 genome architectural variation compared to other metazoans and presaged the amazing
25 variation in mollusc mtDNA genome architecture that was soon to be discovered. Several major
26 patterns of molluscan mitochondrial genome biology were largely present, if not fully
27 understood, in that original *Mytilus edulis* mtDNA. This included, to wit, a dramatic departure in
28 gene synteny from other invertebrate mitochondrial genomes, with all genes encoded on one
29 strand, the presence of “doubly uniparental inheritance” (DUI), not recognized until 1994 [7,8],
30 and the seemingly missing ATP Synthase gene *atp8* (and the subsequent question of whether
31 bivalves actually have it [9] or not [10]).
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42 Extensive natural variation

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44 Mollusc mitochondrial genomes vary widely in size. The smallest reported so far belong to the
45 heterobranch gastropods at ~13.6-14.1 kb [see for example: 4,5,11–15] and the scaphopods
46 [16,17]. These are only slightly larger than the smallest animal mitochondrial genomes [18], but
47 still contain all 37 genes typical of metazoan mtDNAs, including 13 protein-coding genes, 22
48 tRNAs, and two rRNAs, as well as a putative control region [11]. Not unexpectedly, these
49 compact mitochondrial genomes feature high levels of overlapping gene boundaries. The
50 largest mtDNAs come from the scallop *Placopecten magellanicus* [up to 42.0 kb, 19] and the
51 Arcidae clams, with *Scapharca broughtonii* ranging [up to ~51.0 kb, 20] and a recent report
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3 claiming that the *S. kagoshimensis* mitochondrial genome is ~56.2 kb in length [21]. The *S.*
4 *broughtonii* mtDNA (and that of *S. kagoshimensis*, if verified) represents the largest animal
5 mitochondrial genome yet recorded out of ~86,900 mtDNAs from >11,600 species present on
6 NCBI. In both scallops and ark shells, the large genome sizes are not primarily a result of
7 duplications or longer intergenic regions, but rather of expansion of the largest non-coding
8 region [20,22], as is commonly the case for size variation in other mollusc mtDNAs (Figure 1).
9 These bivalves are all exceptionally long-lived, especially the Arcidae, raising the question of
10 whether long generation times affect the pace of evolutionary change in mitochondrial genome
11 size, although other long-lived molluscs (e.g., abalone) do not share similar expansions of their
12 mitochondrial genomes [23].
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19 Molluscan mitochondrial genomes have substantial variation in nucleotide composition
20 skew asymmetry [i.e., heavy vs. light strand, 24]. Strand asymmetry occurs when there are
21 more purines (i.e., adenine and guanine) on one DNA strand than there are pyrimidines. The
22 strand with more purines than pyrimidines is heavier and, therefore, moves farther along in
23 cesium chloride density gradient centrifugation when separated than the complementary strand
24 [25] and is therefore termed the heavy or 'H' strand, and the other the light or 'L' strand. This
25 skew is thought to be caused by the bias in types of spontaneous mutations that occur in single-
26 stranded DNA ([i.e., heavy vs. light strand, 24]), a condition that occurs for the displaced strand
27 during transcription or replication (see a characterization in [26], a process known to be
28 unusually slow for mtDNA [27]). The degree of nucleotide skew is particularly large around the
29 control region, as this region is found in single-stranded conformation more commonly than the
30 rest of the molecule. There have been numerous reversals of strand asymmetry in molluscs
31 [28], likely as a result of inversions in the control region, which contains one or both origins of
32 replication [29,30].
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42 Molluscs have experienced many changes in the transcriptional orientations (i.e.,
43 inversions) of genes, placing them variously on strands of differing nucleotide composition
44 skews. For example, some taxa have all genes on one strand, e.g., all marine bivalves [e.g.,
45 scallops, oysters, and clams: 31,32,33] and all protein-coding genes of caenogastropods [34],
46 while others do not, e.g., unionid mussels [35], heterobranchs [14], vetigastropods [36],
47 cephalopods [37,38], scaphopods [16], aplacophorans [39, but see 40, in which all sequenced
48 genes of the *Spathoderm clenchi* mtDNA are on the same strand], monoplacophorans [41], and
49 polyplacophorans [3]. More generally, changes in genome architectures that alter transcriptional
50 patterns across lineages are common and appear to be largely mediated by tRNA transposition
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3 and inversion [14], as the secondary structures are hypothesized to form transcriptional barriers
4 [42] and RNA cleavage signals [43].
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7 Indeed, changes in the gene order are most common for tRNAs. Even families like
8 Haliotidae that exhibit largely conserved synteny of the protein-coding genes exhibit variable
9 tRNA locations [36]. Duplication of tRNAs appears to be a major contributor to mitochondrial
10 genome rearrangement, as expected for the “duplication-random loss model,” with evidence
11 that many molluscs contain extra tRNAs [20,e.g.: 31] beyond the minimal set of the 22 essential
12 for accommodating the “super-wobble” of mitochondrial translation. Interpreting this pattern of
13 tRNA translocations is complicated by cases of remoulding of tRNA anticodons, which occurs
14 sporadically throughout molluscs [44–46] and otherwise [47]. The cases where a single amino
15 acid is specified by two different codon families (serine and leucine) are especially susceptible
16 to this because a switch of anticodons alone would be sufficient since these tRNAs would each
17 have the necessary internal signals for charging with the correct amino acid [48,49].
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25 Still, there has been a large number of rearrangements of the genes encoding proteins
26 or rRNAs, often via tandem duplication [50–52] or large-scale inversions [e.g, vetigastropods:
27 36,vs. caenogastropods: 53]. In contrast to Vertebrata and Arthropoda, in which gene
28 arrangements have remained generally very stable, extensive gene order rearrangements have
29 been documented in every major lineage within Mollusca, including caenogastropods [54],
30 scaphopods [17], cephalopods [37], heterobranchs [55], bivalves [56,57], aplacophorans
31 [39,40], polyplacophorans [58] and monoplacophorans [41]. The extent of this variation has
32 understandably added complexity to inferring ancestral gene order, as until recently many
33 lineages were too lightly sampled to accurately infer evolutionary paths [see for example 59,vs.
34 60,61].
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42 Across animal life, in nearly all lineages, there has been strong selection to maintain the
43 minimal set of 37 genes [but see 62]. With the possible exception of *atp8* in bivalves [see 9,and
44 10], the genes encoding proteins or rRNAs are seldom lost and duplicates are rarely maintained
45 for long periods in molluscs [but see: 44,63,64], and molluscan mtDNAs rarely contain fewer
46 than the necessary minimal set of 22 tRNAs [but see 65]. There has long been speculation
47 about the selection pressures that are responsible for this [66], including suggestions that
48 hydrophobic proteins cannot easily move across membranes, that these proteins may be
49 destructive in the cytoplasm, or that there is value in regulating mitochondrial function with this
50 genome that is a remnant of its prokaryotic ancestor [66–68].
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3 Additions to the mitochondrial genetic repertoire are uncommon but, here too, molluscs
4 provide many of the exceptions. For example, lineage-specific open reading frames have been
5 identified in bivalves that exhibit DUI [69], of which the male version in *R. philippinarum* was
6 proposed to be virally derived [70]. Additionally, there is evidence of nuclear-derived genes
7 inserting into the mitochondrial genome. For example, a novel ORF was discovered with no
8 sequence- or domain-based homology to the rest of the mitochondrial genome of the pearl-lip
9 oyster *Pinctada maxima* but has domain-based homology to the nuclear genome [71]. The
10 mitochondrial genome of the Arcidae clam *Tegillarca granosa* contains 32 novel ORFs, none of
11 which have any homology to the rest of the mitochondrial genome, and eight of which are
12 predicted to have signal peptides, a hallmark of nuclear but not organellar genes [72].
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19 Early studies of transcription and translation in mitochondrial systems showed cases
20 where the adjacent gene pairs *atp8-atp6* and *nad4L-nad4* were not enzymatically separated as
21 mRNAs [see more below and 73] and, instead, were separately translated into proteins by
22 initiation on the ribosome sometimes at the beginning of this bicistron and other times at an
23 internal codon [74–76]. Perhaps this is due to difficulties with translating the very small mRNAs
24 from *atp8* and *nad4L*. Early mitogenome sequencing revealed that these pairs were adjacent
25 even in cases of more highly rearranged genes, suggesting this as a universal molecular
26 process. But some molluscs do not have *atp8-atp6* as adjacent [38,55,77,78] and others do not
27 have *nad4L-nad4* as adjacent [polyplacophorans: 3, heterobranchs: 14, scaphopods: 16,17,
28 unionid mussels: 35,36, cephalopods: 37,38, aplacophorans: 39, monoplacophorans: 41,
29 gastropods: 55], indicating that there must be other modes of translation and regulation.
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37 Not only are gene rearrangements rampant in mollusc mitochondrial genomes, but even
38 individual genes exhibit remarkable architectural variation. Perhaps most prominent among
39 these is the splitting of the large ribosomal rRNA gene (*rnl*) into two distinct genes in
40 *Crassostrea* oysters [79]. The resulting transcripts do not appear to be spliced together into a
41 single RNA, but the ribosome itself appears to be fully functional [80]. The partially duplicated
42 *rnl* and *rns* genes of the vermitid snail *Thylacodes squamigerus* mitochondrial genome bear a
43 superficial resemblance to *Crassostrea*'s split *rnl*, but the fragments appear to be
44 pseudogenes [49].
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50 Evidence for variation in genic architecture also comes from an intriguing case of
51 apparently convergent evolution of the male-specific version of *cox2* in bivalves exhibiting DUI
52 (see more below). In Mytilidae, *cox2* is extended at the 3' end of the transcript [81], but in some
53 Veneridae, *cox2* has a male-specific insertion in the middle of the gene [78]. It is unclear
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3 whether these *cox2* modifications share similar functions, although the former was hypothesized
4 to have a role in reproduction [82]. Finally, tRNAs are commonly found to have truncated D
5 arms, especially in the heterobranchs [83], and there is even a case in which a tRNA has been
6 inserted into *nad5* [84]. These evident departures from the typical mode of intense purifying
7 selection acting on mitochondrial genes likely represent lineage-specific mitochondrial
8 adaptations and more work is required to understand their functional importance.
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13 The largest non-coding region, inferred to perform the functions of the “control region”
14 varies widely in location also; see, for example, its varying positions in *Mytilus* [2] versus
15 scallops [85], squid [86,87], and caenogastropods [88]. And the content and structure of control
16 regions are vastly different across the major molluscan lineages, with high rates of evolutionary
17 turnover by novel tandem duplications, often of previously duplicated regions
18 [19,37,50,72,89,90], transpositions, especially of tRNAs, into this region [20,31,72,91,92], and
19 newly evolved simple sequence repeats such as poly(AT) tracts [93,94]. Together these primary
20 sequence features share the ability to produce secondary structures including stem-loop
21 [33,95], cloverleaf [55,83,96,97], and cruciform [88] structures in the control region, which in
22 other organisms appear to be related to mtDNA replication and transcription [1,55].
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29 Some control regions provide especially valuable insight into biology and evolution of
30 mitochondrial genome architecture. For example, squid control regions harbour relics of
31 tandemly triplicated whole mitochondrial genomes, followed by subsequent loss
32 [60,63,86,87,98]. Heterobranchs have extremely short control regions, reflecting their compact
33 mitochondrial genomes [11], while caenogastropods have control regions of variable length with
34 an inverted repeat interspersed by a simple sequence repeat [53,88]. Control regions of
35 mussels exhibiting DUI have lineage-specific, tripartite control regions consisting of two variable
36 domains interspersed by a conserved domain [92]. Recombination between the F-type and M-
37 type control regions in which an F-type mtDNA acquires an M-type control region appears to
38 coincide with the masculinization of F-type mtDNAs [91,99,100,101; see DUI section below for
39 more details]. Thus, although control regions are often omitted in mitochondrial genome
40 assemblies, generally because of technical difficulties in amplifying or sequencing these
41 regions, those that have been sequenced provide rich sources of information for understanding
42 evolution of mitochondrial genome architecture.
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Moving forward to understand the processes that contribute to variation in mitochondrial genome architecture

This rich phenomenological record described above makes for an ideal system in which to investigate the underlying molecular, genetic, and evolutionary mechanisms contributing to and maintaining variation in genome architecture. Based on this diversity, a few themes have emerged that warrant further investigation. First, tRNA-mediated changes in gene order have been observed across Metazoa [102]. It is hypothesized that at least part of this pattern results from accidental incorporation of tRNAs into the mtDNA when they moonlight as primers for DNA replication [47]. This hypothesis is attractive because it would also help explain why control regions often feature pseudo-tRNAs [e.g., scallops, oysters, and clams: 31,72,92] and other tRNA-like secondary structures [55,83,96,97,103]. Misincorporation of tRNAs might also contribute to the high rates of evolutionary turnover in the control region, as new tRNA incorporation events push older sequences out of the control region. Complicating our understanding of this process is the evolutionary histories of tRNAs, as tRNA remoulding can obscure tRNA evolutionary history (see above). Quantifying the extent of tRNA duplication and remoulding, as well as rates and patterns of control region turnover in molluscan mitochondrial genomes will provide valuable insight into tRNA-mediated genome architectural change.

Second, tandem duplication, which has been implicated in several molluscan genome rearrangements [e.g., 19,20,cephalopods: 37], can happen through a variety of mechanisms [104,105] including slipped-strand mispairing [106], imprecise termination of replication [107,108], dimerization [109], and illegitimate or non-homologous recombination between repeats [110,111]. Support for the role of tandem duplication in shaping mitochondrial genomes is undermined by the scarcity of animal mitochondrial genomes that harbour duplicated copies of protein-coding genes [112]. It may be that duplicates are lost quickly, perhaps responding to selection favouring the maintenance of cytonuclear stoichiometry [113]. Evaluating these various possibilities will require better population-level sampling, especially with the help of long-read sequencing technologies like PacBio or Oxford Nanopore, which can help resolve tandem duplications [114,115].

Third, inversions are perhaps the most commonly retained form of structural rearrangements in molluscan mitochondrial genomes (see above paragraph on changes in transcriptional orientation). Inversions can arise via multiple double-stranded breaks or by inverted repeats [see 116 for description of inverted repeat mechanisms] in which one repeat is deleted, likely via recombination [117]. However, inversions would seem to have immediately

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3 deleterious consequences for transcriptional control of mitochondrial genomes. There has been
4 speculation of an “evolutionary ratchet,” whereby genes rearranging by inversions to be on a
5 single strand would eliminate the selective pressure to maintain transcription of the other strand
6 and, once lost, would make any further inversion of any gene immediately non-functional such
7 that reversion to a state of genes on both strands would be highly unlikely [112]. Investigating
8 mitochondrial transcriptional dynamics in closely related species (or M vs. F-type mtDNAs from
9 the same species) that have inversions relative to one another might prove especially useful in
10 understanding how inversions are able to persist longer than other types of mitochondrial
11 genome rearrangements. How these inversions and subsequent changes in expression affect
12 mitochondrial function and fitness will also be of broad interest to the mitochondrial community.
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19 Fourth is the evident selective pressure for genome streamlining, both in terms of gene
20 content and genome size. One of the more surprising observations of animal mitochondrial
21 genomes is the degree to which genes overlap [95,112,118]. Overlapping mitochondrial ORFs
22 often exhibit alternative reading frames [112], such that elongation of a gene via nonstop
23 mutations may explain variation in the degree of gene overlap. Once genes do overlap, purifying
24 selection is expected to be intense over the region, as mutations occurring in the overlap could
25 have consequences for two separate genes. The greater degree of overlap between *nad4* and
26 *nad6* in the M-type genome of *Solenia carinata* compared to the F-type [95] raises the
27 intriguing question of whether the increased intensity of selection engendered by gene overlap
28 might compensate for the reduced efficacy of selection acting on male vs. female transmitted
29 mtDNAs [119]. Comparing whether mitochondrial genomes with high vs. low N_e (for example, F-
30 type vs. M-type mtDNAs) have lesser degrees of genic overlap and reduced rates of deleterious
31 mutation accumulation [120] would provide a powerful test of the forces contributing to genome
32 streamlining of animal mitochondrial genomes.
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42 Finally, the extent to which gene order can be used as an effective phylogenetic tool for
43 molluscs [46,60,121,122] depends upon low-level taxonomic sampling to infer rates and
44 patterns of structural evolutionary change. The availability of more than 1000 molluscan
45 mitochondrial genomes from over 700 different species as of September 2020 has largely
46 solved that problem, especially for the bivalves (456 mtDNAs from 261 species), gastropods
47 (452 mtDNAs from 358 species), and cephalopods (142 mtDNAs from 60 species). Such gene
48 order analyses should not only take advantage of changes in major gene synteny but also of
49 tRNA movements and inversion events. Together, these five avenues for future research
50 represent central open questions in the evolution of mitochondrial genome architecture and
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3 should provide a framework for understanding how genome architecture contributes to
4 mitochondrial function at molecular, cellular, and organismal levels.
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10 Annotation Challenges

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12 Considerable effort is required for annotation of the genes of molluscan mitogenomes. Most
13 protein-encoding genes are easily identified with orthologs by sequencing similarity, with
14 occasional consideration of hydrophobicity plots for *atp8* and *nad4L*, but there are challenges
15 with inferring the correct start codon in cases where there are multiple, closely-spaced
16 alternatives. An inference must consider the possibility of overlap with the upstream gene and
17 the extent of evolutionary conservation of the open reading frame. This is confounded by the
18 fact that molluscs employ the invertebrate mitochondrial genetic code (NCBI Genetic Code 5)
19 that allows for alternative start codons in addition to ATG, including ATA, ATY, TTG, and GTG
20 (normally encoding for methionine, isoleucine, leucine, and valine, respectively). Each of these
21 would provide a match to the second two nucleotides of the *trnM* anticodon (CAU), which must
22 do double duty in most mitochondrial systems as the tRNA for both methionine and, in the case
23 of protein initiation, formyl-methionine.
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32 Ordinarily, inferring a stop codon for any gene is straightforward but, here too,
33 mitochondrial genomes present a challenge. In many cases, mitochondrial genomes are
34 transcribed as a single polycistronic RNA from each strand [see 123]. The tRNA genes are then
35 removed enzymatically, which liberates gene-specific mRNAs as proposed in the “punctuation
36 model” [43]. In the case of overlapping *atp8-atp6* and of *nad4L-nad4*, these have been shown
37 for yeast [73], fish [124] and mammals [125] to remain as bicistrons that are translated on
38 mitochondrial ribosomes, sometimes from the first codon and sometimes from an internal codon
39 that initiates the second gene. In some other cases of adjacent protein-encoding genes without
40 an intervening tRNA, there are potential secondary structures that have been speculated to
41 serve this function [3, for example]. In many other cases, it remains unknown if these mRNAs
42 are separated or not. The specific challenge for gene annotation from genome sequence is that
43 after enzymatic processing to produce gene-specific messages, some will not have a complete
44 TAG or TAA stop codon, but may terminate on just a TA or T that is completed to a TAA stop
45 codon by polyadenylation of the transcript [126]. Additionally, it is important to consider that
46 some genes are known to overlap, even when on the same strand, further complicating an
47 accurate inference of the correct stop codon from genome data alone.
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3 Of course, there are some cases where these features can be directly observed through
4 the sequencing of expressed sequence tags (ESTs) [13], providing the sequences of the full
5 transcripts from which the genomic boundaries can be reliably determined. This has presented
6 some surprises. For example, ORF analysis had predicted that *nad4* of the gastropod
7 *Biomphalaria glabrata* mitogenome (NC_005439) was unusually long, fully overlapping with
8 *trnT*, in contrast with the reported genes in the gastropods *Cepaea nemoralis* (NC_001816) and
9 *Albinaria caerulea* (NC_001761). Independently determined EST data (AA547758) showed the
10 cDNA for the C-terminus of *nad4* to end before the downstream *trnT* gene, more consistent with
11 those of the other gastropods, and to terminate on a single T nucleotide that was extended by
12 polyadenylation to form a TAA stop codon [13].
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19 Based on genome sequence alone, inferring the exact boundaries of rRNA genes is
20 especially difficult. In fact, in most cases, there is simply the presumption that the rRNA gene
21 extends to the boundary of the flanking genes, with this moderated by the extent of similarity
22 matching to homologous genes of other organisms.
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26 The genes for tRNAs diverge in sequence rapidly and are most commonly found by
27 identifying potential secondary structures with a set of typical features [127–129]. Some
28 lineages are known to have aberrant structures with some of the arms diminished or even
29 missing, complicating this inference.
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33 The rise of next-generation sequencing has been a game-changer for the pace of
34 generating complete mitogenome sequences. These methods generate an enormous number of
35 short sequencing reads, leading to an increased reliance on computational methods for
36 automated genome assembly. Among several alternative software packages that aim to
37 assemble NGS data into large contigs, MITObim was specially designed for the assembly of
38 mitogenomes [129], as well as other tools that were released more recently [130–132]. Using a
39 provided mitochondrial genome or even a short (partial) gene sequence as initial reference to
40 identify sequence data of likely mitogenome origin, this program applies a strategy of BLAST
41 and iterative mapping to select and assemble short reads from a large NGS dataset that
42 provides adequate coverage into a linear representation of a mitogenome. Overlapping,
43 identical sequence termini indicate that the assembly represents the full circular mitogenome. It
44 is worth noting that reliance on computational interpretation of short sequence reads may
45 potentially cause problems in assembling repetitive elements, such as the control region and
46 unsuspected repetitive elements like tandem duplications or repeat regions, that may be
47 resolved only by manual, targeted sequence characterization.
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3 With such relative ease to derive the genome sequences, there is a greater demand for
4 automated annotation. This need was recognized early on by the implementation of semi-
5 automated annotation of genomes of organelles from mitochondria (and plant chloroplasts)
6 through DOGMA (Dual Organellar GenoMe Annotator) that provided predictions of protein- and
7 rRNA-encoding genes through BLAST similarities to previously annotated mitochondrial
8 genomes [133] and provided tools for manually refining the beginning and end of each gene.
9 The identification of tRNA genes employed secondary structure predictions because
10 mitochondrial tRNA sequences share little sequence similarity among animals. Generally,
11 computational predictions were further hindered due to the aberrant structure of several
12 molluscan tRNAs that do not conform to the canonical cloverleaf of animal tRNAs, and typically
13 required manual validation [6]. Current utilities include AGORA [prediction of PCGs in a
14 mitogenome assembly based on BLAST similarities to a reference mitogenome; 134], MitoZ
15 [130–132] and MITOS [135]. The latter software performs *de novo* annotation of protein-
16 encoding genes by sequence similarity and secondary structure predictions of both rRNA and
17 tRNA. MITOS reports annotation results in the standardized format that supports the accepted,
18 consistent nomenclature of mitochondrial genes. Updates (MITOS2 is available at
19 <http://mitos2.bioinf.uni-leipzig.de/index.py>) have improved the prediction accuracy but the
20 results still require manual curation.
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32 Alternative start codons, potential for incomplete stop codons, and molluscan-specific
33 tRNA structures continue to challenge automated annotation. Some possible challenges for
34 annotation are shown in Figure 2, using *atp8* from gastropod mitogenomes as an example. *atp8*
35 is the shortest protein-coding gene in mitogenomes and relatively variable among gastropod
36 species, often not detected by BLAST and thus also not recognized by MITOS. Additionally,
37 *atp8* of several gastropod species employs an alternative start codon, like ATT that normally
38 encodes for an I (isoleucine), serving as start codon (specifying formyl-methionine) only at the
39 initiation of protein translation. Automated gene finding, and inexperienced annotators may fail
40 to recognize ATT as a true start, choosing an upstream M-encoding nucleotide (ATG or ATA),
41 even if part of a different gene as an incorrect start codon. As a consequence, annotation of
42 *atp8* often requires manual inspection and comparison to *atp8* from several species (Figure 2).
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50 A recent paper by Fourdrilis et al. [136] provides a powerful set of criteria to integrate
51 with automated MITOS prediction for correct annotation of gastropod (molluscan) mitogenomes.
52 These criteria include the valid insights into molluscan mitochondrial biology, including the
53 punctuation model, as well as alternative start and stop codons. We summarize these criteria
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3 below: 1) Protein-encoding genes are assumed to begin at the first eligible in-frame start codon
4 in their 5' end, that is, the start codon nearest to the preceding gene without overlapping with it,
5 checking that this start codon is suitable regarding location and gene length by aligning the
6 derived amino acid sequence with that of closely related species; 2) Due to transcription of
7 mtDNA as polycistronic RNA, it is considered physically impossible to have gene overlap
8 between two protein-encoding genes encoded on the same strand and in the same open
9 reading frame, but possible if frames are different; 3) Protein-encoding genes are assumed to
10 end at the first in-frame full stop codon, or an abbreviated stop codon (TA- or T-- in
11 invertebrates) ending immediately before the downstream tRNA. Such an abbreviated codon
12 results from the cleavage of the transcript at the 5' and 3' ends of tRNAs and tRNA-like
13 secondary structures, and is subsequently completed to a TAA stop codon with A residues by
14 polyadenylation; 4) Putatively duplicated genes are evaluated based on quality values provided
15 in the MITOS analysis; 5) The boundaries of tRNA genes are those predicted by MITOS; 6) The
16 boundaries of rRNA genes were those predicted by MITOS and not extended to flanking genes
17 to avoid overestimating rRNA gene length.
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28 Despite these software packages for assistance and the attention of the scientific
29 community, the entries for mitochondrial genomes at NCBI contain a great number of easily
30 recognized annotation errors even in the "Refseq" portion. Despite having this pointed out over
31 a decade ago with specific, simple recommendations for systematically eliminating these and
32 conducting quality control for new entries [26], a recent study identified a great number of errors
33 in a systematic search of complete vertebrate mitochondrial genomes at NCBI [137]. To the
34 best of our knowledge, no such systematic study has been made of annotations for complete
35 mollusc mitogenomes, but there is no reason to suspect that they are immune from similar
36 errors during submission or NCBI review [e.g., 136]. Consistent, accurate, complete annotation
37 of these genomes is critical for comparative and phylogenetic studies. We urge NCBI to
38 implement these simple quality control measures.
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49 Inheritance: DUI in bivalves

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51 Mitochondrial genomes follow a non-Mendelian inheritance pattern of being transmitted
52 uniparentally in most eukaryotes; in animals, mitochondrial inheritance is usually strictly
53 maternal (from now on: strictly maternal inheritance, SMI) [138,139]. Perhaps the most striking
54 feature of mollusc mitochondrial biology is the Doubly Uniparental Inheritance (DUI), a unique
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3 inheritance pattern so far reported in 100+ species of bivalves [140]. In species showing DUI,
4 two sex-linked mitochondrial lineages exist: one is inherited through eggs (F-type) the other
5 through sperm (M-type). Differently from the cases of paternal mtDNA leakage reported in
6 several organisms [141], in DUI the sperm transmission route is stable across evolutionary time,
7 so the F- and M-type coexist as segregated lineages for millions of years accumulating a
8 remarkable sequence divergence. The F-M nucleotide p-distance ranges from 0.08 to 0.449,
9 and the amino acid p-distance of mitochondrial protein-coding genes can reach 0.534 [140].
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14 The dynamics and distribution of F- and M-type in embryos and tissues were firstly
15 investigated in bivalves of the *Mytilus* species complex, in which DUI was observed for the first
16 time [reviewed in 142]. Particularly interesting was the finding that in early embryos (2-8
17 blastomeres) sperm mitochondria stained with MitoTracker Green showed two different
18 distribution patterns: dispersed vs. aggregate. The authors were also able to show a strong link
19 between the pattern and the sex of the progeny: females were associated with the dispersed
20 pattern, males with the aggregated one [143,144]. These observations, together with the results
21 of several molecular works, were used to build a first description of the mitochondrial dynamics
22 in DUI, summarized below. Gametes are homoplasmic for the sex-specific type (F-type in eggs,
23 M-type in spermatozoa), so upon fertilization the zygote is heteroplasmic and the fate of sperm
24 mitochondria is tightly linked with sex. If the embryo develops into a female, the M-type
25 mitochondria are dispersed and actively degraded as happens in some species showing SMI
26 [145], and the animal will be homoplasmic for the F-type. Otherwise, if the embryo develops into
27 a male, sperm mitochondria stay aggregated, as they already are in the midpiece of sperm
28 cells, and are transported into the blastomere 4d, the precursor of the germline, and survive
29 degradation; males are thus heteroplasmic, containing M-type in the germline and F-type in the
30 somatic tissues.
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42 The main points of this model are: 1) homoplasmy of females due to degradation of M-
43 type; 2) heteroplasmy of males with retention of M-type due to the active segregation of sperm
44 mitochondria aggregated in gonad precursors, but not in somatic tissues. A replicative
45 advantage of M-type in males was also hypothesized, to explain its proliferation in
46 spermatogenic tissues [144]. This is still the most commonly used description of the DUI
47 mechanism, but some revisions have become necessary. The existence of the two patterns was
48 confirmed in a distantly related species (divergence time 400+ Mya), the venerid clam
49 *Ruditapes philippinarum* [146], but as new data were gathered and new species analyzed,
50 evidence of deviations from the mechanism as described above started emerging. The
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3 presence of M-type in male somatic tissues is now known to occur in *R. philippinarum* [147],
4 *Venustaconcha ellipsiformis* and *Utterbackia peninsularis* [148], and in *Mytilus galloprovincialis*
5 [149,150].
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9 These works showed also that heteroplasmy is more common than previously thought in
10 both males and females of DUI species, and that the presence, abundance, and distribution of
11 the F- and M-types is quite variable across species, sexes, and tissues. Such differences should
12 be expected when dealing with a quantitative phenomenon like mitochondrial inheritance [138],
13 especially across large evolutionary distances. Recently, immunohistochemistry and microscopy
14 (both confocal and electronic) investigations on *R. philippinarum* showed the presence of
15 heteroplasmy at the organelle level (both types present in the same mitochondrion) in male
16 soma and, quite surprisingly, in undifferentiated germ cells of both sexes, while homoplasmy in
17 both female and male gametes was confirmed [151]. According to these observations, the strict
18 segregation of F-and M-type in gametes would be achieved during gametogenesis—thus much
19 later in development than hypothesized before—and it was suggested that DUI is based on a
20 mechanism of meiotic drive involving selfish genetic elements associated with mitochondria
21 [151,152].
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32 DUI molecular mechanism

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34 Hybrid and triploid DUI mussels have been shown to revert to SMI [153] and the taxonomic
35 distribution of DUI species is scattered across bivalve phylogeny, so DUI must have evolved by
36 the modification of a mechanism of SMI, but which one? There are several different
37 mechanisms by which SMI can be achieved [145,154], but that operating in bivalves is still
38 unknown. Similarly to what happens in mammals, it was hypothesized that ubiquitination could
39 be involved [155] and the results of some investigations seem to be consistent with such
40 supposition [156–159]. A possible approach to understand which molecular mechanism is
41 involved in DUI is to look at the differences between F- and M-type genomes, and numerous
42 works have investigated this issue in the last 25 years. The main findings can be summarized
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51 First, bivalve mtDNA shows an abundance of intergenic regions—or at least regions not
52 containing known genes—and the largest are rich in genetic elements such as repeats, motifs,
53 and DNA/RNA secondary structures which differ between conspecific F and M genomes in DUI
54 species [see for example: 92,160–163]. A strong clue supporting a role of control region
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3 elements in DUI comes from observations in the *Mytilus* complex. Several analyses on F- and
4 M-type mtDNAs in *Mytilus edulis*, *M. galloprovincialis*, and *M. trossulus* revealed the presence in
5 male gonads of genomes having their coding sequences almost identical to those of the F
6 genome (2-3% divergence). It was hypothesized that these genomes originated from F
7 genomes that invaded the male germline and started to be transmitted through sperm, replacing
8 the M-type and accumulating sequence divergence (which is initially reset to zero when the F-
9 type replaces the M-type). This phenomenon was named “role-reversal” or “masculinization”
10 [reviewed thoroughly in 142], and the aberrant F genomes transmitted through sperm have
11 been defined as “masculinized”. Following studies found that the control regions of masculinized
12 genomes contained parts of both the typical F- and M-type mtDNAs, being actually F/M
13 chimaeras. Role-reversal has been observed, so far, only in the *Mytilus* complex. These findings
14 strongly suggest that some elements located in the control region or its proximity have a role in
15 the inheritance mechanism. The identity and the nature of these elements are still unknown and
16 several candidates have been proposed, including DNA and/or RNA secondary structures
17 [161,164], specific sequences/motifs [165], or peptides encoded by open reading frames
18 (ORFs) located near the control region (see second point below).
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29 The second feature that differentiates F and M genomes is the presence of lineage-
30 specific ORFs showing no sequence similarity with known genes, and thus defined “ORFans”
31 [69,70,140,152,164,166–169]. In some cases, a protein product of these ORFans has been
32 detected and localized [70,151,170], but their function remains unknown despite extensive *in*
33 *silico* analyses [70,152,167–169]. Such bioinformatics work has shown that despite high
34 evolutionary rates and large sequence divergences, all the analyzed ORFans have similar
35 predicted structural features, supporting a similar function. The involvement of the ORFans in
36 the DUI mechanism is still a hypothesis and their mechanism of action is an object of
37 speculation, but it is clear that these elements are maintained in bivalve genomes and some
38 surely produce a novel mitochondrial protein. It would be surprising if these elements will turn
39 out to be nonfunctional.
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47 Third, the cytochrome c oxidase subunit 2 gene (*cox2*) shows curious features in
48 bivalves, and in several DUI species, there are important differences between the F-type and M-
49 type *cox2* gene (see also the Genome Architecture section above). The *cox2* gene is duplicated
50 in the F-type of *R. philippinarum* [164] and the M-type of *Musculista senhousia* [160], with
51 paralogous copies showing different length. In some other cases, *cox2* has a different length in
52 the two mtDNAs, due either to 3' coding extensions (550 bp) or big in-frame insertions (up to 3.5
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3 Kb) [140]. It is still not clear if such modifications of *cox2* are linked to DUI for some functional
4 reason, or are a more general feature of bivalve mtDNAs, maybe due to modifications in
5 Complex IV of oxidative phosphorylation.
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8 The fourth and last feature characterizing the differences between the two mitochondrial
9 lineages concerns small non-coding RNAs (sncRNAs). Pozzi et al. [171] sequenced sncRNA
10 libraries from gonads of *R. philippinarum*, and found miRNA-like sequences transcribed by
11 intergenic regions for which a stable hairpin structure was predicted. *In silico* analyses showed
12 that F and M genomes produce different mitochondrial sncRNAs with different nuclear targets.
13 The authors hypothesized that such sncRNAs might affect nuclear gene expression through
14 RNA interference and might influence gonad formation. More recently Passamonti et al. [172]
15 reported *in vivo* clues of the activity of two sncRNAs in *R. philippinarum*. Small mitochondrial
16 RNAs have been so far predicted *in silico* also in several species of amniotes [173], and in
17 *Drosophila melanogaster*, *Danio rerio*, and *Mus musculus* [172].
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27 MtDNA evolutionary patterns in DUI

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29 It is still unclear how DUI emerged and why it has been maintained for hundreds of millions of
30 years. Traits that last so long in evolution are usually maintained by natural selection because
31 they have a function that affects organismal fitness. For this reason, and given the tight link
32 between mitochondrial inheritance pattern and sex in DUI species, it was hypothesized that DUI
33 has a role in sex determination and/or gonad differentiation [142,152,156,158,170,174–177].
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38 Studies on the patterns of molecular evolution of mitochondrial proteins in DUI bivalves
39 clearly show that M-type evolves faster than F-type and both mtDNAs evolve faster than the
40 mitochondrial genomes of other metazoans [142,174]. The reasons behind this pattern are the
41 subject of debate. Relaxed selection is one possible explanation; Stewart et al. [178] suggested
42 that F- and M-type mtDNAs evolve under different degrees of selective constraints as a
43 consequence of different “selective arenas”. Supposing that F-type mtDNA is functional in all
44 somatic tissues and female germline, while M-type functions only in the male germline, F-type
45 would be subject to more stringent constraints, hence the faster sequence evolution of M-type.
46 However, the more recent findings about F- and M-type distribution across tissues (discussed
47 above), and the findings of M-type transcriptional activity in the soma [148,179], may suggest
48 that the above-mentioned arenas of function are not that distinct. Moreover, even if M-type
49 mitochondria are functional only in the male germline, they have a crucial function of providing
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3 energy for sperm swimming. This is a fundamental function, especially in a broadcast spawning
4 animal, and the relaxation of natural selection on such a trait could have long term
5 consequences on DUI species. Many DUI species are quite successful; for example, *Ruditapes*
6 *philippinarum* is highly invasive, and *Arctica islandica* [in which DUI was reported, see 180] is
7 the longest-living non-colonial animal known (maximum reported lifespan ~507 years), so it
8 seems that DUI is not manifestly disadvantageous.
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13 A high-throughput analysis of mtDNA SNPs in F- and M-type of *R. philippinarum* [164]
14 revealed a similar amount of polymorphism in the two genomes, but a different distribution of
15 allele frequencies (probably due to different bottleneck sizes), and the M-type having a lower
16 proportion of SNPs with a predicted deleterious effect. According to these data, the faster
17 evolution of M-type is likely due to the roles of mitochondria in spermatogenesis and sperm
18 motility, the latter being especially important in the intense sperm competition of an animal using
19 broadcast fertilization. Indeed, one interesting feature of DUI is that mtDNA is under selection
20 also for male functions, differently from what happens in all the SMI organisms, in which
21 mitochondria are an evolutionary dead-end in males. This opens a series of interesting
22 consequences and deserves thorough investigations. Recently, two comparative analyses of
23 OXPHOS activity in gametes and somatic tissues of SMI and DUI bivalves reported a metabolic
24 remodeling in M-type mitochondria that suggests an adaptive value of mtDNA variation, and a
25 link between male-energetic adaptation, fertilization success, and the preservation of paternally-
26 inherited mitochondria [181,182].
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36 DUI is generally unknown or considered just a “freak of nature”, but it represents a
37 unique and precious model to study mitochondrial biology and evolution. Thanks to its unusual
38 features, it can be used as a tool to better understand mitochondrial heteroplasmy, inheritance,
39 recombination, and the role of mitochondria in germline formation, meiosis, gametogenesis, and
40 fertilization, in some cases providing the exceptions that address general phenomena in other
41 animal groups. Up to now, DUI has not been found outside bivalves, but, to the best of our
42 knowledge, it has been specifically investigated in just five gastropod species [183].
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The utility and limitations of mitochondrial genomes for phylogeny

During the last three decades, mitochondrial markers, either individually, combined, or as a whole, have been commonly used for phylogenetic reconstruction within Metazoa [97,184–186]. This preference is due to several features that make mitochondrial sequences a well-suited and reliable molecular marker for phylogenetic assessment. Firstly, all Metazoa [except some Loricifera, see 187] possess a mitochondrial genome that can be obtained with relative ease compared with any particular genome region of similar size due to its high abundance and copy numbers within animal cells [97,184]. Secondly, gene orthology, essential for a successful phylogenetic assessment, is expected in the mitogenome, since genes from eventual duplication events, shown to occur in molluscan mtDNA, are rarely retained, and quickly lost or pseudogenized [97,184,186]. Furthermore, uniparental inheritance (see exception in bivalves in the DUI section above) and a general lack of recombination [188] greatly favour the reliable inference of population structure. The variable substitution rates within the different genes/regions of the mitogenome grant a range of phylogenetic signals that might potentially be useful for accessing shallow and deep relationships [97,184,186]. Mitogenomes also possess several structural features that, when thoroughly studied, can be phylogenetically informative, such as genome size, gene arrangement, and content [121], as well as the presence and composition of non-coding regions and repetitive sequences and even RNA secondary structures [184,186].

Despite the overall unarguable utility of mitogenomes for phylogenetic assessments, several limitations may affect their reliability for the same purposes. By being an “independent genetic unity”, that is usually uniparentally inherited with very little recombination, the mitogenome as a whole is itself a single locus that reflects the evolutionary history of the mitochondria, which for several reasons may not be the same as the species evolutionary history [e.g., due to introgression and gender-biased reproductive dispersal: 186]. Furthermore, the presence of non-functional nuclear copies of mitochondrial sequences (numts) may lead to a false interpretation of phylogenetic relationships [186], particularly when single genes are amplified by PCR, and the highly variable substitution rates and base composition between taxa can make direct comparisons difficult [97,186]. Inversions can also complicate phylogenetic analysis using mtDNA gene sequences, as it is likely that genes equilibrate in nucleotide composition to their strand skew, even to the point of having convergent amino acid

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3 substitutions within physio-chemically similar groups that have arisen independently in different
4 lineages [189].
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7 Despite these drawbacks, overall, mitogenomes represent a complete and “isolated”
8 genomic feature, easily available from a wide range of taxa, whose genetic information is
9 comparable and compact enough to be both phylogenetic informative and investigated with low
10 computational effort and therefore a logical choice for a comprehensive phylogenetic study.
11 Consequently, mitochondrial DNA has been used, with a variable range of success, to assess
12 phylogenetic relationships at several taxonomic levels ranging from shallow population-level
13 relationships [e.g., 190], up to phyla [Mollusca: 185, e.g., Annelida: 191, Platyhelminthes:
14 192, Rotifera: 193] and even Metazoa as a whole [97].
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20 Although mitophylogenetics have been successfully used to infer deeper evolutionary
21 relationships within other metazoan taxa, the same success has not been achieved for the
22 Mollusca. The reconstruction of the molluscan deep-level relationships has been extremely
23 challenging, and consistently recovering the monophyly of the Mollusca, or even of the eight
24 molluscan classes, both presumed to be correct based on other data, has not been possible
25 using mitochondrial markers alone [39, 97, Mollusca: 185, 194, 195]. Moreover, only recently and
26 through the application of phylogenomic approaches relying on several nuclear loci, consistent
27 monophyletic Mollusca and monophyletic molluscan classes started to be recovered [196–199].
28 These studies, by contradicting the generally accepted morphocladistic Testaria hypothesis,
29 have resulted in a fundamental reinterpretation of the phylogenetic history of Mollusca. The
30 Testaria hypothesis placed worm-like Aplacophora (Solenogastres and Caudofoveata) as a
31 paraphyletic basal group of the Mollusca and thus postulated a progressive evolution of body
32 complexity, with a true shell occurring only once [199]. Conversely, all the recent phylogenomic
33 studies unambiguously support a basal dichotomy that splits the Mollusca into two major
34 groups, the Aculifera (including the Polyplacophora and the reciprocally monophyletic
35 Aplacophora) and the Conchifera (including the Monoplacophora, Cephalopoda, Scaphopoda,
36 Gastropoda and Bivalvia), thus postulating that the worm-like body plan of Aplacophora was
37 acquired secondarily and has derived from a more complex-bodied ancestor [197, 200].
38 However, the relationships within Conchifera are more controversial, with conflicting results
39 regarding the positioning of Monoplacophora as either basal to all other Conchifera [200] or
40 sister taxa to Cephalopoda [197, 200], as well as the positioning of Scaphopoda as sister to
41 Gastropoda [197, 198, 200] or sister to a clade composed of Gastropoda and Bivalvia
42 [196, 197, 200]. Nevertheless, phylogenomic studies have been fundamental to understanding
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3 early molluscan evolution and although whole genome-scale resources are now easier to
4 obtain, the taxon sampling is still considerably reduced when compared with the mitogenomic
5 data already available [reviewed in 201].
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8 The effectiveness of mtDNA markers to infer deep Molluscan phylogeny has been a
9 thoroughly discussed subject in recent studies [39, Mollusca: 185, 195], describing several
10 factors that may lead to the lack of phylogenetic signal and conflicting tree topologies.
11 Phylogenies often show long-branch attraction artifacts (LBA), with molluscan mitogenomes
12 revealing high differentiation in nucleotide abundance and strand bias. All of these features are
13 a probable consequence of highly frequent gene order rearrangements observed in Molluscan
14 mitogenomes, resulting in heterogeneous substitutions rates and generating systematic
15 analytical errors [see 97, Mollusca: 185, 195, 202 and references within]. Furthermore, ancient
16 (Cambrian) incomplete lineage sorting and uneven taxon sampling may also play a role in the
17 inconsistency of the inferred phylogenetic relationships [195]. These authors also explored the
18 phylogenetic utility of other molluscan specific mitogenome features, such as mitogenome size
19 variation, the highly variable (sometimes absent) protein-coding gene *atp8*, and even the
20 coupling behaviour of particular genes (such as *atp8-atp6* and *nad4L-nad4*) [195]. However, a
21 clear phylogenetic signal is once again hindered, probably by homoplasy of these features.
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31 Within the molluscan classes, deeper relationships based only on mitochondrial markers
32 have also been showing a variable range of success. Recent studies on the Aculifera have
33 expressed promising results using phylomitogenomics, supporting the usefulness of both whole
34 mitogenome sequences and structural features [40, 58, 203]. For instance, new phylogenetic
35 informative mitogenome rearrangements were detected within Polyplacophora, and
36 Caudofoveata, which along with the only Solenogastres published mitogenome, revealed a
37 conserved protein-coding gene order likely consistent to the ancestral molluscan gene order
38 [40, 58, 203]. However, mitogenome availability is still scarce for groups within the Aculifera
39 clade. For example, mitogenome sequences for all the main lineages of the best sampled
40 Aplacophora group, Polyplacophora (n=18), only recently became available [203] (Figure 3).
41 Similarly, Scaphopoda, for which several phylogenetic and systematics doubts persist within its
42 major groups, is very poorly represented regarding mitogenome availability [204]. Furthermore,
43 although phylogenetic analysis using complete mitogenomes revealed promising results for the
44 phylogenetic assessment within the Scaphopoda, using *cox1* alone did not, and therefore, a
45 more comprehensive and intensive whole mitogenome sequencing within the group is urgently
46 needed [204].
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3 Monoplacophoran mitogenomes have been recently sequenced to test their positioning
4 within the Mollusca. However, consistent with the low resolution of mitochondrial markers for
5 deep molluscan classes (see above) the results were inconclusive [41]. Nevertheless, once
6 again unique structural features (e.g., gene arrangement and presence of large intergenic
7 regions) that may be phylogenetically informative were detected and further sampling of the
8 group is needed [41].
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13 Of the three most economically important molluscan classes, Cephalopoda is the best-
14 represented in terms of mitogenome availability, which nonetheless represents only 5.5% of the
15 total species of the group. Unlike in other molluscan classes, mitochondrial markers have shown
16 to be informative regarding the deeper Cephalopoda phylogenetic relationships, revealing their
17 potential to resolve long-lasting phylogenetic questions within the group [60, Mollusca: 185].
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22 As for the two most speciose classes of Mollusca (i.e., Bivalvia and the megadiverse
23 Gastropoda), deep level phylomitogenomics have been constantly inefficient. Both bivalves and
24 gastropods have very unusual mitochondrial evolutionary patterns at both nucleotide and
25 structural level, which render them prone to analytical inconsistencies (e.g., LBA) and hampers
26 a consistent phylogenetic inference [Mollusca: 185, 202, 205]. Inevitably, only through the
27 application of large scale genomic approaches, the interrelationships within both classes are
28 starting to be clarified [205–208].
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33 Contrary to these difficulties in resolution of deeper, older evolutionary relationships,
34 mitochondrial genes and genomes have been much more useful in resolving more recent,
35 intrafamilial phylogenies [209, 210]. Most shallow phylogeny, phylogeographic, and populations
36 genetics studies on molluscs have relied so far on one or two mitochondrial gene fragments
37 sometimes coupled with the same number of nuclear counterparts [211–213]. However, use of
38 these gene fragments alone may lead to biased results and fail to reveal the mitochondrial
39 evolutionary history of species. Furthermore, obtaining a complete mitogenome is not always a
40 possibility, either due to the higher cost of sequencing (when compared with Sanger sequencing
41 of a single gene) or due to logistic limitations (e.g. lack of computational resources). It is
42 therefore important to identify the genes or regions of the mitogenome that better correspond
43 and may be used as surrogates of the whole mitogenome evolutionary history. A study on 41
44 unionid bivalves statistically evaluated the coherence of the individual mitochondrial gene trees
45 and the whole mitogenome tree, indicating that the trees using *nad5* sequences were the most
46 similar to whole mtDNA trees [214]. The results of the gene fragments more widely used in
47 molecular studies within this bivalve taxon, (i.e., *cox1*, *rrnL*, and *nad1*) were less robust). This
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3 study also tested pairs of these widely used gene markers with much higher success, indicating
4 that the trees constructed with the large ribosomal subunit *rrnL* concatenated with *cox1* or *nad1*
5 are highly coherent with the whole mitogenome trees [214]. Another study within the
6 cephalopod Octopodidae family comparing the whole mitogenome with the individual gene tree
7 topologies, also showed that the *nad5* trees best represented the whole mitogenome topologies
8 [215,216]. However, these results were obtained in specific groups of molluscs and should be
9 tested across the Mollusca to evaluate the usefulness of individual and pairs of gene fragments
10 in representing the whole mitochondrial genome phylogenies.
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16 Comparisons of mitochondrial genes have great potential for revealing hidden cryptic
17 diversity aiding in species delimitation and identification [216,217] in understanding molluscan
18 species phylogeographical patterns and population genetic structure, since they have already
19 been used successfully for these purposes in other taxa [218,219]. However, to our knowledge,
20 no comprehensive phylogeographic or population genetics study on mollusc species has used
21 this type of marker.
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26 In summary, studies with phylogenetic analyses of whole mitochondrial sequences and
27 structural features of molluscs, have been increasing steadily over the last decade. These
28 studies have shown limited success in representing deeper evolutionary patterns within the
29 Mollusca and molluscan classes. However, below the family level, robust phylogenies
30 consistent with results of other genomic and morphological studies have been obtained. Given
31 the high potential of whole mitogenomes for barcoding, revealing cryptic diversity, and obtaining
32 robust shallow phylogenetic relationships, it is expected that an increasing number of
33 phylogeographic and population genetics studies using whole mitogenomes will be published
34 shortly.
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45 Summary and Conclusion

46 Despite widespread misunderstanding based on early studies that animal mitochondrial
47 genomes are consistent in structure, function, and inheritance patterns, there is actually
48 enormous diversity among these diminutive genomes across animal life. The phylum Mollusca,
49 in particular, is replete with examples of extraordinary variation in genome architecture,
50 molecular functioning, and intergenerational transmission. This provides a model system for
51 studying the evolution of these features in concert with the diverse and manifold roles of
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3 mitochondria in organismal physiology and the many ways that the study of mitochondrial
4 genomes are useful for phylogeny and population biology.
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41 Figure Legends

42 **Figure 1 – Relationship between the length of (a) non-coding and (b) coding regions on**
43 **total mtDNA length in molluscan classes.** Variation in non-coding length explains a greater
44 proportion of variation in total mtDNA length compared to variation in coding length. Each circle
45 represents a single species. When multiple mtDNAs were available for a single species, the
46 mean across all individual records was taken as the species value. Colors represent different
47 molluscan classes and are indicated by the key in panel (a).
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53 **Figure 2 – In mitogenomes of planobid gastropods, the *atp8* gene is bracketed by *trnN(aac)***
54 **and *trnL2(tta)*.** Shaded boxes: tRNA genes, white boxes: protein coding genes; arrowheads
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3 indicate directionality; *: stop codon. ORF analyses of the mitogenome sequences that ignore
4 the concept of tRNA gene excision from polycistronic mitogenomic transcripts frequently yield
5 incorrect prediction of protein-encoding sequence intervals. Whereas the start codon is correctly
6 indicated, the ORF for *atp8* from *Biomphalaria glabrata* (underlined in both nucleotide and
7 predicted amino acid sequences, NC_005439) falls short, despite an effort to accommodate an
8 incomplete stop codon (T--). Another issue impacts the ORF selected from the *Planorbella duryi*
9 mitogenome (KY514384). It comprises a (correct) start codon and TAA stop codon but overlaps
10 with *trnL2* and yields an unusually long protein sequence. For both snail species, considering
11 the boundaries of the (MITOS predicted) tRNA genes, the ATA is the first possible start codon
12 downstream from *trnN*. At the 3' end, a single T nucleotide remains after excision of *trnL2*,
13 completed by polyadenylation to a TAA (underlined) stop codon. Such peculiarities challenge
14 prediction of multiple genes from molluscan mitochondrial sequences, as is evidenced in
15 several GenBank entries, despite the purported curation of submissions by this NCBI database.
16 Re-evaluation and, if appropriate, updates by contributors of previous GenBank accessions will
17 greatly benefit correct annotation.
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29 **Figure 3** – TOP: Graphic showing the number of complete (dark colours) and partial (Light
30 colours: min. size 10,000 bp) mitogenomes available in GenBank; MIDDLE: mean, minimum
31 and maximum size (bp) of complete mitogenomes per Mollusca class; BOTTOM: graphic
32 showing the percentage of total species with complete mitogenomes published in GenBank.
33 Asterisk superscripts refer to unverified size values, due to assembly challenges, critical
34 evaluation of these publicly available mitogenome sizes and sequence content is highly
35 recommended.
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For Review Only

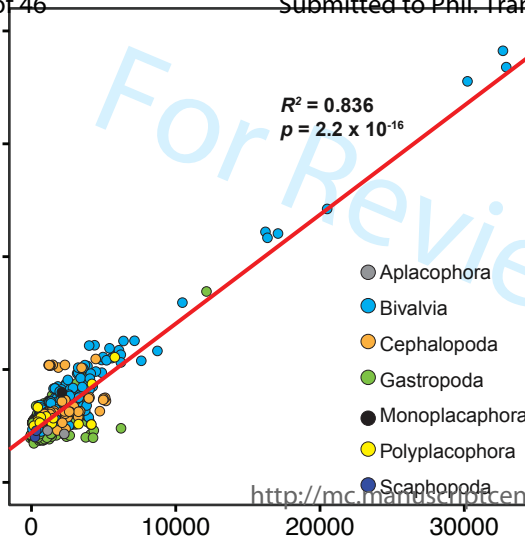
Table 1. Number of molluscan mitogenome sequences in Genbank over time.

The GenBank (GB) search was structured as follows: ("Mollusca"[Organism]) AND (biomol_genomic[PROP] AND mitochondrion[filter] AND ("8000"[SLEN] : "100000"[SLEN]) AND ("1900/01/01"[PDAT] : "1999/12/31"[PDAT])). The term "Mollusca" was replaced for family level searches with "Gastropoda; Bivalvia; Scaphopoda; Cephalopoda; Polyplacophora; Monoplacophora; Aplacophora" and the years were adjusted for specific time intervals. Number in brackets refers to sequences characterized in a time interval prior to deposited in GenBank. RefSeq genomes (restricted to one per species) are described by NCBI as copies of selected assembled genomes available in GenBank, generated by several processes including manual curation.

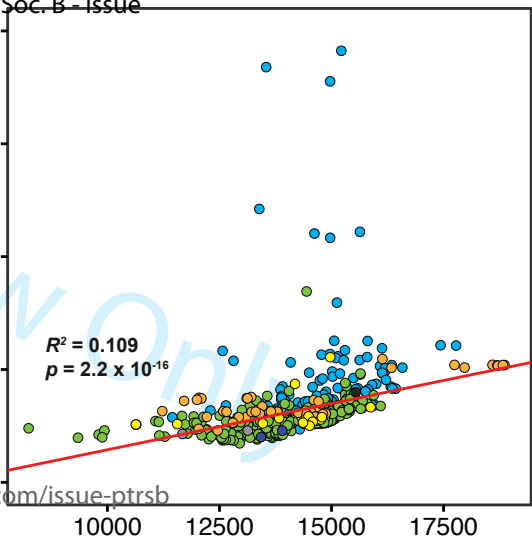
Taxon	GB/RefSeq	-2000	2000-2004	2005-2009	2010-2014	2015-2020
Gastropoda	625/233	1(3)	10	19	104	491
Bivalvia	451/186	0	4	45	130	272
Scaphopoda	3/2	1	1	0	0	1
Cephalopoda	126/50	0	1	7	53	65
Polyplacophora	23/13	1	0	0	2	20
Monoplacophora	3/2	0	0	0	0	3
Aplacophora	8/5	0	0	0	2	6
Mollusca	1239/491	3(3)	16	71	291	858

Total mtDNA Length (bp)

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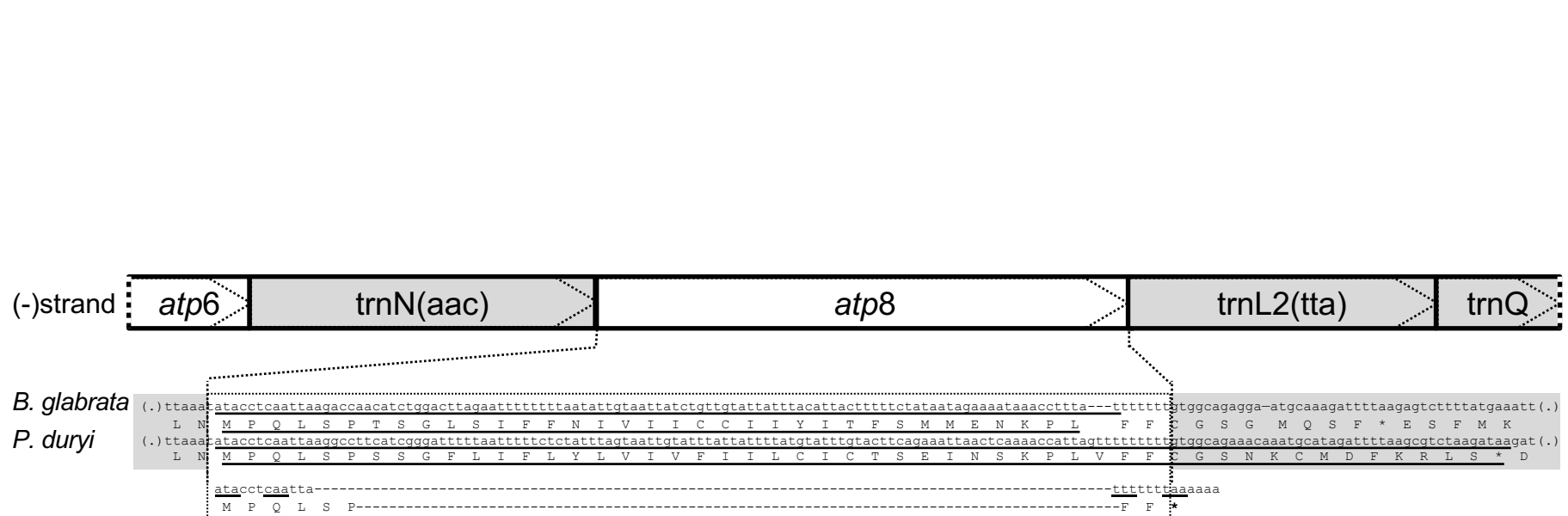


b)



Non-Coding Length (bp)

Coding Length (bp)



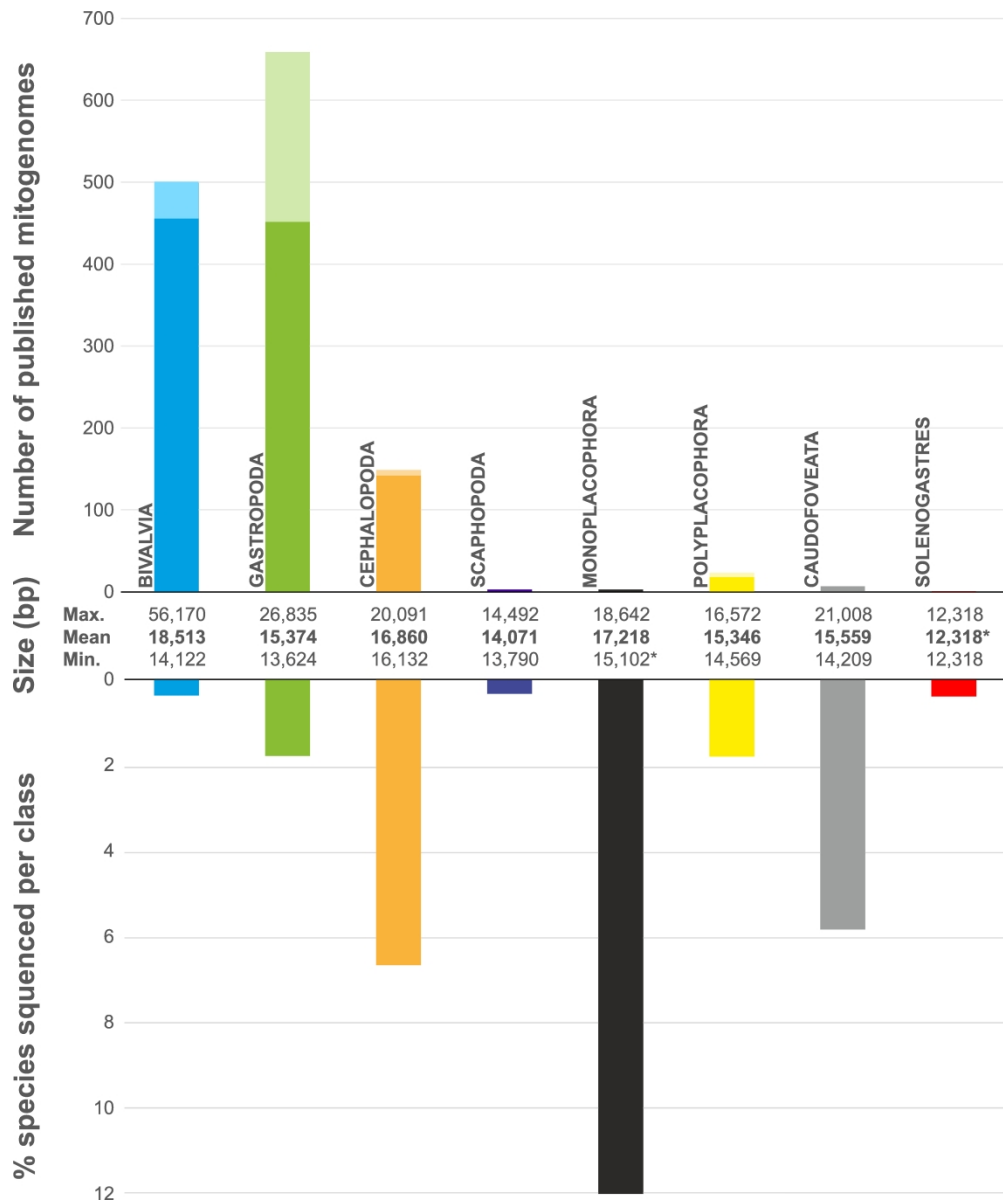


Figure 3 – TOP: Graphic showing the number of complete (dark colours) and partial (Light colours: min. size 10,000 bp) mitogenomes available in GenBank; MIDDLE: mean, minimum and maximum size (bp) of complete mitogenomes per Mollusca class; BOTTOM: graphic showing the percentage of total species with complete mitogenomes published in GenBank. Asterisk superscripts refer to unverified size values, due to assembly challenges, critical evaluation of these publicly available mitogenome sizes and sequence content is highly recommended.