ARPHA Preprints

PENSOFT

PREPRINT

Author-formatted, not peer-reviewed document posted on 01/08/2023

DOI: https://doi.org/10.3897/arphapreprints.e110185

Mitochondrial genomes of *Meghimatium bilineatum* and *Succinea arundinetorum* provide insight into the gene order rearrangement within Stylommatophora (Gastropoda, Panpulmonata)

Gang Wang, Ying Zhu, Chen Yu, Xiaoli Sun, Qiuting Ji, Jun Tang, Bobin Liu, Zijun Sun, Qiuning Liu, Boping Tang, Lianfu Chen, Daizhen Zhang

ARPHA Preprints Author-formatted, not peer-reviewed document posted on 01/08/2023 DOI: https://doi.org/10.3897/arphapreprints.e110185

Mitochondrial genomes of *Meghimatium bilineatum* and *Succinea arundinetorum* provide insight into the gene order rearrangement within Stylommatophora (Gastropoda, Panpulmonata)

Gang Wang[1†], Ying Zhu[1†], Yu Chen[1†], Xiaoli Sun[2†], Qiuting Ji[1], Jun Tang[1], Bobin Liu[1], Zijun Sun[3], Qiuning Liu[1], Boping Tang[1], Lianfu Chen[4*], and Daizheng Zhang[1*]

[1] Jiangsu Key Laboratory for Bioresources of Saline Soils, Yancheng Teachers University, Yancheng 224007, China

[2] College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, Nanjing 211816, China.

[3] School of Stomatology, Xuzhou Medical University, Xuzhou 221004, China

[4] College of Plant Sciences and Technology, Huazhong Agricultural University, Wuhan 430000, China

† These authors contributed equally to this work.

* Correspondence: chenllianfu@foxmail.com, daizhen79wenxin@163.com

Abstract:

In this study, we report the whole mitochondrial genomes of two species, *Meghimatium bilineatum* and *Succinea arundinetorum*, which belong to Stylommatophora, one of the most abundant orders of Gastropoda. The total sizes of *M. bilineatum* and *S. arundinetorum* mitogenomes are 14,352 bp and 15,282 bp, with surprisingly biased proportions of A+T contents that reach to 72.1% and 76.78%, respectively. The protein coding genes (PCGs) in two mitogenomes show negative AT skew values and evolved primarily under purifying selection. Compared with the ancestor of stylommatophora, the mitochondrial genes of *M. bilineatum* exhibited multiple rearrangement events while the mitochondrial genes of *S. arundinetorum* showed only minor differences. Moreover, the order of PCGs were conserved while the tRNA genes showed high frequency of rearrangement among the stylommatophoran species, suggesting that the latter could be one of the major driving forces of mitogenomic evolution in terrestrial molluska species. Our research lays a theoretical foundation for investigating the evolution and divergence of mitochondrial genes and provides valuable resources for studying evolutionary genetics in stylommatophoran species.

Keywords: Gastropoda, Mitogenomes, Stylommatophora, Rearrangement

1. Introduction

Terrestrial gastropods mainly include species of Neritimorpha, Caenogastropoda, and Heterobranchia (Bouchet et al. 2017), which are general name of mollusks living on

land. Terrestrial molluskas are important soil animals and mainly live in humid and humic soil, plant roots, and rock crevices. They are important links along food chains and play important roles in stability of ecosystems (Prezio et al. 1999; Ponder and Winston 2008). Terrestrial gastropods originated in the early Cambrian, and they are not a monophyletic group but evolved from multiple marine lineages (Ejka and Hamerlik 2010). Due to slow movement and limited active dispersal ability, terrestrial molluskas are susceptible to environmental changes and can be used as an indicator species of environmental changes(Lydeard et al. ; Sinos and Moysis 2004). The diversity of terrestrial gastropods is extremely rich, and approximately 30,000 species of terrestrial molluska have been recorded worldwide (Ponder and Winston 2008).

Mitochondria are organelles that are present in most cells with a double membrane, exhibiting rod-like or grainy shapes(Anand and Tikoo 2013). As the power factory of cell, mitochondria are main sites for oxidative phosphorylation and synthesis of adenosine triphosphate (ATP) (Zazueta et al. 2013). As a semiautonomous organelle, mitochondria have a mitogenome independent of nuclear genome, which can encode its own rRNA, tRNA and proteins (Aguileta et al. 2014). The mitogenome is a closed circular double-stranded DNA molecule that consists of one control region and 37 mitochondrial genes, including 22 tRNAs, 13 protein-coding genes (PCGs), and 2 rRNAs. The PCGs include *nad1*, *nad2*, *nad3*, *nad4*, *nad5*, nad4 L and *nad6* genes that control mitochondrial NADH synthesis, and *atp8*, *atp6* that synthesize ATP synthetase, and *cox1*, *cox2*, and *cox3* genes that comprise the core of the cytochrome c oxidase complex("Mam33 promotes cytochrome c oxidase subunit I translation in Saccharomyces cerevisiae mitochondria %J Molecular Biology of the Cell" 2015), and *cob* genes that transferring electrons(Zhao et al. 2012). The mitochondrial PCGs are essential for normal life activities of cells, and the mitochondrial tRNAs enable mitochondria to translate proteins independently (A 2000; Zhao et al. 2012). In addition, the two rRNAs are important components of mitochondrial ribosomes. Mitochondrial genes are distributed on the heavy strand (H strand) and light strand (L strand) that are enriched with guanine (G) and cytosine (C), respectively. Most of the mitochondrial genes are encoded by the H strand. Both the H and L strands can be transcribed into long transcripts, and transcription is initiated by light-strand promoters (LSPs) and heavy-strand promoters (HSPs) (Nicholls and Minczuk 2014).

The control region of mitogenome lies between the L chain of the $tRNA^{Pro}$ gene and the H chain of the $tRNA^{Phe}$ gene. This region consists of promoter, original replication site, and various regulatory elements (D and A 1984; E and A 1985). The control region is also called displacement-loop (D-loop) area because the newly duplicated H chain can replace the original H chain and lead to displacement and formation of a ring structure (Iyengar et al. 2006). According to the previous reports, the D-loop is under weak selective pressure and has a faster rate of evolution

(Davidović et al. 2022). Moreover, the control region can form a triple-stranded structure due to presence of 7S DNA (Walberg and Clayton 1981). Previous studies have suggested that the control region is involved in mitochondrial transcription and replication. Previous studies concerning the control regions in human and mouse mitochondrial DNA have shown that short mitochondrial transcripts originating from TISL are served as primers to initiate the synthesis of nascent H strands (Eva and Nenad 2021). According to the previous reports, variable numbers of tandem repeats (VNTRs) in control region could be the main factor leading to different length of mitogenomes among individuals or species (DAVID et al. 1998; Vives-Bauza et al. 2009; Stoccoro et al. 2021).

Recent improvement of sequencing technology has enabled mitogenome-wide sequencing for stylommatophoran species. To date, the mitochondrial genomes of 60 stylommatophoran species has been published. Previous studies have revealed several rearrangement events in the mitochondrial genomes of stylommatophora species (Groenenberg et al. 2017), such as *Helixpomatia* (Korábek et al. 2019), *Meghimatium bilineatum* (Xie et al. 2019), and *Arion vulgaris* (Özgül et al. 2020). Rearrangement of mitogenome can be served as a useful marker for phylogenetic analysis. However, limitation of the available mitogenomes resources make it difficult to characterize mitogenomic rearrangement events among stylommatophoran species. Therefore, it's urgent need to expand the resource of mitogenomes for Stylommatophora. To this end, we provided the completely high-quality mitogenomes for two stylommatophoran species, *Succinea arundinetorum* and *Meghimatium bilineatum*. Comparative mitogenomic analysis among 23 stylommatophoran species uncovered several mitogenome rearrangement events. Our analysis provides valuable resources and lays theoretical foundation for further studying evolution and divergence of stylommatophoran species.

2. Materials and methods

2.1. Sample Collection, DNA Extraction, and Sequencing

The sample of *M. bilineatum* and *S. arundinetorum* were collected in Yancheng city, Jiangsu, China (120°16′ N, 33°38′ E). Total genomic DNA was extracted from muscle tissue using the Qiagen QIAamp tissue kit according to the manufacturer's protocol and stored at -20 °C before sequencing. The DNA of two species was submitted to Frasergen Bioinformatics Co., Ltd. (Wuhan, China) for Illumina PE library construction and high-throughput sequencing. Two stylommatophorans species sequences were performed on an MGI-SEQ 2000 platform, and each library generated approximately 60 Gb of raw data.

2.2. Gene assembly, annotation, and sequence analysis

The raw reads were subject to quality control by removing low-quality and contaminated reads, and reads with high 'N' ratio, and adapter sequences. After that, the clean reads were *de novo* assembled using SPAdes software (Bankevich et al.). The Circular Consensus Sequencing (HiFi) data were used for error correction. The mitogenomes were annotated with the vertebrate mitochondrial code using MITOS (Donath et al.). The structures of 22 tRNAs were determined using tRNA-scan 2.0 (Chan et al. 2021), and the tRNA structures were mapped by the online web software Forna (Kerpedjiev et al. 2015). Start and stop codons were confirmed using previously published Neritidae mitogenomes as references (Liu et al. 2015; Xie et al. 2019). The circular map of the mitochondrial genome was produced by using OGDRAW v1.3.1 (Stephan et al. 2019). The nucleotide compositions, average nucleotide, amino acid sequence divergences, and the relative synonymous codon usages (RSCU) of PCGs were computed using MEGA v7.0. The strand asymmetries were calculated according to the following formulas: AT-skew = [A-T]/[A+T] and GC-skew = [G-C]/[G+C] (Alexandre et al.). To investigate evolutionary adaptation, rates of nonsynonymous (Ka) and synonymous (Ks) substitutions in the mitogenomes of all Neritidae species were estimated with TBtools (Chen et al. 2018). Repeat sequences were identified using VMatch v2.3.0 (http://www.vmatch.de/) software combined with Python scripts (Wynn and Christensen 2019).

2.3. Phylogenetic analyses and divergence time estimation.

Phylogenetic analyses were performed by using the concatenated alignment of 13 PCGs covering 23 gastropod species from seventeen families of three orders (Stylommatophora, Systellommatophora, and Basommatophora), and *Rhopalocaulis grandidieri* (Systellommatophora) and *Auriculastra duplicate* (Basommatophora) were selected as the outgroup (Suppl. material 1). The CDS and amino acids of PCGs were aligned using MAFFT software (Kazutaka et al. 2013), the conserved blocks were extracted by GBlocks (Dereeper et al. 2010), and the best model of protein evolution was determined by ProtTest (Posada 2011) software. Phylogenetic trees were reconstructed using BI and ML methods.

Estimation of divergence times were based on amino acids (13 PCGs). For divergence time calibration, two calibration points were used as the prior for the corresponding split divergence time. The most recent common ancestor (MRCA) of *Albinaria caerulea* and *Cornu aspersum* commune diverged 269 MYA; the MRCA of *Camaena cicatricosa* and *C. aspersum* diverged 197 MYA. The divergence time of other nodes was calculated by r8s (Sanderson and davis 2004) software with the TN algorithm, PL method, and the smoothing parameter value was set to 1000 through cross-validation.

2.4. Gene rearrangement

Mitogenome organizations and gene rearrangements of stylommatophoran species were analyzed via the CREx web server (Bernt et al. 2007). This method compares two genomes and determines the most parsimonious scenario that has led to the observed rearrangement accounting for duplication reversals, transpositions, or other events. We considered the gene order of Achatina fulica to be the ancestral gene order of stylommatophorans. Based on this method, we conducted pairwise comparisons of mitogenomes to reconstruct genome rearrangement events.

3. Results and discussion

3.1. Genome structural features and organization

Total sizes of *S. arundinetorum* and *M. bilineatum* mitogenomes are 15,282 bp and 14,352 bp, respectively (GenBank accessions OP289102 and OP311642) (Table 1), which are similar with the sizes of other stylommatophoran species that range from 13,798 bp for *Camaena poyuensis* to 16,323 bp for *Achatinella mustelina* [22]. The two mitochondrial genomes show similar structure, including the circular molecules encoding 13 protein-coding genes, 2 rRNAs, and 22 tRNAs, as well as one typical control region characterized by most of the land snails. Furthermore, 9 PCGs (*cob*, *cox1*, *cox2*, *nad1*, *nad2*, *nad4*, *nad4 L*, *nad5*, *nad6*), 12 tRNA genes (*trnA*, *trnC*, *trnD*, *trnF*-*trnL1*, *trnP*, *trnS L*, *trnV*), one rRNA gene (*rrnL*), and the control region are located on the light strand (L-strand). The other mitochondrial genes are located on the H-strand in same orientation, excepting for *trnW* and *trnY* in *S. arundinetorum* that are located on the heavy strand (H-strand) in reversed orientation (Fig. 1).

Similar with other stylommatophoran species, the mitogenomes of *M. bilineatum* and *S. arundinetorum* showed the high A+T contents, which reach to 72.1% and 76.78%, respectively (Suppl. material 3) (Feng et al.). Moreover, the AT skew values are -0.12 (*S. arundinetorum*) and -0.094 (*M. bilineatum*), and GC skew values are 0.047 (*S. arundinetorum*) and 0.019 (*M. bilineatum*). This result indicated the higher ratios of T or G than A and C nucleotides (Table 2 and Suppl. material 3), which is consistent with the previous studies (Özgül et al. 2020). Additionally, 13 genes in *S. arundinetorum* and 18 genes in *M. bilineatum* showed a certain extent of overlaps with nearby genes, with overlapped length ranging from 7 bp to 29 bp and 1 bp to 40 bp, respectively. Totally, we identified 105 bp and 172 bp intergenic sequences in two mitogenomes, with length ranging from 1 bp to 46 bp and 1 bp to 70 bp, respectively (Table 1).

3.2. Protein-Coding Genes, rRNA Genes, and Codon Usage

Among the 13 PCGs, the largest gene is *nad5* (1674 bp in *S. arundinetorum* and 1626 bp in *M. bilineatum*) and the smallest gene is *atp8* (147 bp and 159 bp), which is consistent with the previous studies (Feng et al.). The AT skew values of 13 PCGs were

almost negative, while their GC skew values were almost positive. 'ATG', 'ATA', 'ATT', and 'TTG' were used as the start codons except the *nad4l* in *S. arundinetorum* that displayed incomplete start codon and the *atp8* in *M. bilineatum* that started with 'GTG'. Most of the PCGs stop with TAG or TAA, excepting for the *cox2*, *cox3*, *nad2* in *M. bilineatum* and the *nad2*, *nad4* in *S. arundinetorum* that used a single T-- or TA- as stop codons (Table 1).

Then, we performed Ka/Ks analysis for 12 PCGs to estimate the evolutionary-selection constraints on two species (no value was calculated for the *atp8*). The Ka/Ks ratios of all PCGs are less than 1, indicating that these genes were evolving primarily under purifying selection. The Ka/Ks ratios of the *nad2*, *nad3*, *nad4*, and *nad6* in *M. bilineatum* and the *nad4l* in *S. arundinetorum* are greater than 0.5 (Suppl. material 7, Suppl. material 2), suggesting that these genes received less pressure from purification and evolved relatively more quickly. The *cox1* has the lowest Ka/Ks ratio in both two species and only minor alteration in protein sequence was observed. This gene is also widely used as a molecular marker for species identification and phylogenetic analysis (Astrin et al. 2016). Most of the PCGs (except the *atp8*) in both mitogenomes have negative AT-skew values. As for their GC skew values, seven PCGs (*cox1*, *cox2*, *nad1-nad3*, *nad4 L*, *nad6*) have positive GC skew values and two genes (*atp6* and *atp8*) show negative values.

The lengths of mitochondrial tRNA genes are similar in the two mitogenomes. The nucleotide composition of tRNA genes are biased toward A and T (Suppl. material 8, Table2). Consistent with other gastropodous species, majority of the tRNAs have negative AT skew values and positive GC skew values (Suppl. material 8) (Feng et al. ; Korábek et al. 2019). The highest frequency of amino acids usage by PCGs were Leu2, Phe, Gly, Ala Val, and Ile (Fig. 3) and the lowest frequency of amino acids usage was Arg, which is similar with other gastropodous species (Feng et al. ; Özgül et al. 2020). Moreover, the most frequently used codons were TTA (Leu), ATT (Lie), TTT (Phe), and ATA (Met) that are composed of A/T nucleotides (Fig. 3 and Suppl. material 4).

3.3. The Interspersed Repeat and Control Region

Four types of interspersed repeat, including forward, palindromic, reverse, and complement, were identified in the two mitogenomes. We noticed that the contents of interspersed repeat in *S. arundinetorum* is significantly higher than *M. bilineatum* (Suppl. material 9). The mitochondrial control region (CR) of *S. arundinetorum* and *M. bilineatum* were located between *trnK* (*Lys*) and *trnV* (*Val*), and *trnQ* (*Gln*) and *trnC* (*Cys*), respectively (Fig. 1). The length of CR in *S. arundinetorum* is obviously longer than *M. bilineatum* (1,243 bp versus 411 bp), suggesting that the former has a more complicated mitochondrial CR. Similarly, the nucleotide composition of CR was biased toward A and T in both mitogenomes, with proportions of 78.92% in *S. arundinetorum*

and 76.16% in *M. bilineatum*.

3.4. Phylogenetic Analysis and Divergence Times

To phylogenetically analyze the two systellommatophoran species, we selected 21 additional gastropod species with published mitogenomes for construction of phylogenetic tree. Among them, *Rhopalocaulis grandidieri* (Systellommatophora) and *Auriculastra duplicate* (Basommatophora) were selected as the outgroup. Then, we performed Maximum likelihood (ML) and Bayesian inference (BI) analyses based on the 13 PCGs of these species, which produced almost identical topologies with strong bootstrap and posterior probability values (Fig. 4, Suppl. material 10, and Suppl. material 11). According to the phylogenetic tree, the stylommatophoran species were clustered on the same branch and can be divided into five major subclades. Consistent with previous studies, family Philomycidae, Arionidae, and Oreohelicidae were phylogenetically closed, and the *M. bilineatum* and *Philomycus bilineatus* were clustered together (Tiezhu et al. 2019; Özgül et al. 2020). *S. arundinetorum*, *S. putris,* and *O. wujiaquensis* belonging to Succineidae were grouped together, and *S. arundinetorum* and *O. wujiaquensis* are phylogenetically more closed.

The time-calibrated phylogeny indicated that Stylommatophora diverged from Systellommatophora and Basommatophora approximately 317.17 million years ago (Mya) (Fig. 4), which is complying with the previous study(Guo et al. 2019). Succineidae diverged from other Stylommatophora species about 258.87 Mya ago, and *S. arundinetorum* and *O. wujiaquensis* were differentiated about 13.20 Mya ago. Philomycidae originated approximately 198.08 Mya ago, and *M. bilineatum* and *P. bilineatus* were divergent about 15.39 Mya ago. Based on the time-calibrated phylogenetic tree, Stylommatophora was divergent properly around 200 to 300 Mya ago that match the Triassic Period in the Earth. During this period, the climate was more appropriate and the Earth underwent outbreak of speciation, including cephalopods, crustaceans, fish, amphibians, reptiles, speculations, terrestrial snail and so on (Zhen et al. 2022).

3.5. Mitochondrial gene order and rearrangements

According to the previous report, mitochondrial gene rearrangements were commonly occurred in gastropods species(Kearse et al. 2012) . To study the order of mitochondrial genes in *M. bilineatum* and *S. arundinetorum*, we designated the gene order in *Achatina fulica* mitogenome as the ancestral pattern of Stylommatophora because it displayed same gene order with the outgroups (*Rhopalocaulis grandidieri* and *Auriculastra duplicate*). Overall, we observed 4 unique PCG orders in the mitogenomes of the 23 species analyzed in this study (Fig. 5). Noticeably, the order of mitochondrial PCGs in *M. bilineatum* is identical with *Philomycus bilineatus* while displays great differences

with the other species. We speculated that the mitochondrial PCGs in *M. bilineatum* had experienced four successive inversion arrangements (*nad6-nad5-nad1-nad4L* vs. *cob-cox2-atp8-atp6*, *cox3* vs. *nad4*, *cox2* vs. *atp8-atp6*, and *cox2* vs. *cox3*) (Fig. 6).

When considered all types of genes, we identified 15 types of gene orders in the mitogenomes of the 23 species, indicating the higher frequency of gene rearrangements for the tRNAs. Compared with the ancestral species, three tRNA inversions occurred in *S. arundinetorum* (*trnD-trnC* vs. *trnF*, *trnG-trnH-trnQ-trnl2-atp8-trnN-atp6-trnR-trnE-12S-trnM-nad3-trnS2* vs. *trnY-trnW*, and *trnL1-trnA* vs. *trnP*). Furthermore, we also observed three tRNA inversions in *Punctum randolphii* (*trnS1-nad4* vs. *trnT-cox3*, *trnG-trnH* vs. *trnQ-trnL2-atp8-trnN-atp6-trnR-trnE-12S-trnM-nad3-trnS2-trnT-cox3*, *trnP* vs. *trnA*) and *Arion ater* (*trnL2-atp8-trnN-atp6-trnR* vs. *trnE-12S-trnM*, *trnA* vs. *trnP*, *trnE* vs. *trnQ*) (Fig. 6).

Compared to other stylommatophoran species, *M. bilineatum* displays higher frequency of both PCG and tRNA rearrangements. For example, compared with *A. fulica*, five large-scale gene rearrangement events (*trnP-nad6-nad5-nad1-nad4 L* vs. *cob-trnD-trnC-trnF-cox2-trnY-trnW-trnG-trnH-trnQ-trnL2-atp8-trnN-atp6-trnR-trnE-12S-trnM*, *trnL2-ATP8-trnN-ATP6-trnR-trnE-12S-trnM* vs. *cox2-trnY-trnW-trnG-trnH-trnQ*, *trnS2-trnS1-nad4* vs. *trnT-cox3*, *cox2-trnY-trnW-trnG-trnH-trnQ* vs. *trnP-nad6-nad5-nad1-nad4 L-nad3*, *trnL1* vs. *16S*) were observed (Fig. 6)(Tiezhu et al. 2019; Xie et al. 2019). In addition, some gene clusters, such as *nad6-nad5-nad1-nad4 L*, *trnY-trnW*, *trnG-trnH*, and *trnI-nad2-trnK*, are conserved in these mitochondrial genomes, which can be served as a synapomorphy for the terrestrial molluska species.

4. Conclusion

In this study, we provided and characterized the complete mitogenomes of two stylommatophoran species, *S. arundinetorum* and *M. bilineatum*. The sizes of two mitogenomes are 14,352 bp and 15,282 bp, respectively. The Ka/Ks ratio of mitochondrial genes indicated that the two species were mainly subjected to purifying selection. The longer control region due to the higher interspersed repeat contents in the mitogenome of *S. arundinetorum* than *M. bilineatum* suggested the more complicated gene expression regulation in *S. arundinetorum* mitogenome. The time-calibrated phylogeny analysis revealed the five major clades of stylommatophoran species that were divergent properly around 200 to 300 Mya ago. Comparing with the stylommatophoran ancestor, both the PCGs and tRNAs in the mitogenome of *M. bilineatum* have experienced multiple rounds of gene rearrangements. Moreover, the frequencies of tRNA and rRNA rearrangement are higher than PCGs. Our analysis provides valuable resources and theoretical foundation for investigating the evolution and divergence of stylommatophoran species

Fig. 1. Mitogenome organization of *M. billineatum* and *S. arundinetorum*. Genes transcribed from the J and N strands are shown outside and inside of the circle, respectively. PCGs coding complex I, complex III, and complex IV components are marked with cyan, yellow and green, respectively. rRNA genes are colored red and the ATP synthase is colored yellow, while tRNA genes are colored purple and labeled by the single-letter amino acid code.

Fig. 2. Amino acid frequency in the mitogenomes of *M. billineatum and S. arundinetorum.*

Fig. 3. The relative synonymous codon usage (RSCU) values of *S. arundinetorum* and *M. billineatum* respectively. The outermost cylinder height is the RSCU value, the inner layer is the amino acid, and the innermost three layers are the codons.

Fig. 4 The phylogenetic tree of Gastropoda was inferred from 13 protein genes. Nodes are labeled with numbers referring to divergence time.

Fig. 5. The gene rearrangements in the Gastropoda mitogenomes. Genes encoded by the minor strand are underlined.

Fig. 6. Restriction of mitochondrial gene rearrangement scenarios. The ancestral mitochondrial gene arrangement of *A. fulica*. The tRNA genes are represented by amino acid abbreviations.

Table 1. Summary of gene/element feature of *S. arundinetorum* and *M. billineatum*.

Gene	Position start	Poistion end	Size (bp)	Intervening spacer (bp)*	Start codon	Stop codon	Strand
	Succinea arundinetorum / *Meghimatium billineatum*						
atp6	9831/4205	10487/4855	657/651	-4/-8	ATG/ATG	TAA/TAA	H/H
atp8	9619/4003	9765/4161	147/159	-32/3	ATG/GTG	TAA/TAA	H/H
cob	7483/2717	8577/3799	1095/1083	-20/7	ATA/ATG	TAA/TAA	L/L
cox1	1/1	1530/1527	1530/1527	0/0	TTG/TTG	TAA/TAA	L/L
cox2	8768/11062	9412/11726	645/665	0/-8	TTG/ATA	TAA/TA-	L/L
cox3	13387/5899	14169/6679	783/781	3/0	ATG/ATG	TAA/T--	H/H
nad1	6310/8997	7221/9920	912/924	-25/-7	ATT/ATG	TAA/TAA	L/L
nad2	14247/13416	15231/14295	985/880	-40/3	ATG/ATG	T--/T--	L/L
nad3	11394/10252	11747/10593	354/342	-7/16	ATA/ATG	TAA/TAA	H/H
nad4	12008/12044	13312/13348	1305/1305	0/3	T--/ATG	T--/TAA	L/L
nad4l	7221/9892	7502/10182	282/291	-1/-29	ATG/ATT	TAA/TAG	L/L
nad5	4661/7378	6334/9003	1674/1626	-5/9	ATT/ATT	TAA/TAG	L/L
nad6	4228/6901	4665/7368	438/468	28/21	ATT/ATT	TAA/TAA	L/L
rrnL	2838/1528	4007/2581	1170/1054	0/0			L/L
rrnS	10621/4991	11333/5704	713/714	0/0			H/H
trnA	4133/2647	4199/2709	67/63	-4/2			L/L
trnC	8703/11005	8767/11069	65/65	-5/0			L/L
trnD	8645/3875	8707/3940	63/66	2/3			L/L
trnE	10555/4927	10620/4990	66/64	0/7			L/L
trnF	8579/3806	8642/3871	64/66	1/6			L/L
trnG	9413/11863	9486/11926	74/64	0/10			L/L
trnH	9467/11918	9533/11982	67/65	-20/-9			L/L
trnI	14216/13350	14286/13412	71/63	46/1			L/L
trnK	15232/14296	15282/14352	51/57	0/0			L/L
trnL1	4072/2582	4136/2644	65/63	-3/0			L/L
trnL2	9567/3939	9650/3999	84/61	-28/-2			H/H
trnM	11334/5705	11400/5768	67/64	0/0			H/H
trnN	9770/4163	9834/4212	65/50	4/1			H/H
trnP	4008/6814	4074/6879	67/66	0/1			L/L
trnQ	9535/10175	9594/10235	60/61	1/-8			H/H
trnR	10491/4856	10554/4919	64/64	3/0			H/H
trnS1	11949/11983	12007/12040	59/58	8/0			L/L
trnS2	11752/5769	11811/5827	60/59	4/0			H/H
trnT	13313/5829	13383/5898	71/70	0/1			H/H
trnV	2774/6750	2837/6812	64/63	0/70			L/L
trnW	11871/11796	11940/11852	70/57	5/8			H/L
trnY	11812/11727	11865/11787	54/61	0/0			H/L
D-loop	1531/10594	2773/11004	1243/411	0/0			L/L

Table 2. Composition and skewness of *S. arundinetorum* and *M. bilineatum*.

Gene	A	C	G	T	A+T (%)	G+C (%)	AT-Skew	GC-Skew	Length (bp)
				Succinea arundinetorum / *Meghimatium bilineatum*					
atp6	214/191	91/94	75/86	277/280	0.7473/0.7235	0.2527/0.2765	-0.13/-0.19	-0.1/-0.04	657/651
atp8	59/60	19/23	12/21	57/55	0.7891/0.7233	0.2109/0.2767	0.02/0.04	-0.23/-0.05	147/159
cob	314/288	136/165	151/150	494/480	0.7379/0.7091	0.2621/0.2909	-0.22/-0.25	0.05/-0.05	1095/1083
cox1	401/438	210/219	260/245	659/625	0.6928/0.6961	0.3072/0.3039	-0.24/-0.18	0.11/0.06	1530/1527
cox2	202/207	84/94	100/114	259/250	0.7147/0.6872	0.2853/0.3128	-0.12/-0.09	0.09/0.1	645/665
cox3	233/207	106/119	118/133	326/322	0.7139/0.6773	0.2861/0.3227	-0.17/-0.22	0.05/0.06	783/781
D-loop	436/140	161/50	101/48	545/173	0.7892/0.7616	0.2108/0.2384	-0.11/-0.11	-0.23/-0.02	1243/411
nad1	277/246	90/124	117/158	428/396	0.773/0.6948	0.227/0.3052	-0.21/-0.23	0.13/0.12	912/924
nad2	306/261	82/109	98/123	499/387	0.8173/0.7364	0.1827/0.2636	-0.24/-0.19	0.09/0.06	985/880
nad3	118/100	34/32	36/53	166/157	0.8023/0.7515	0.1977/0.2485	-0.17/-0.22	0.03/0.25	354/342
nad4	412/416	127/180	145/168	621/541	0.7916/0.7333	0.2084/0.2667	-0.2/-0.13	0.07/-0.03	1/1305
nad4l	83/94	17/33	34/34	148/130	0.8191/0.7698	0.1809/0.2302	-0.28/-0.16	0.33/0.01	282/291
nad5	524/457	165/222	192/216	793/731	0.7867/0.7306	0.2133/0.2694	-0.2/-0.23	0.08/-0.01	1674/1626
nad6	151/141	30/56	41/63	216/208	0.8379/0.7457	0.1621/0.2543	-0.18/-0.19	0.15/0.06	438/468
rrnL	461/390	111/125	127/162	471/377	0.7966/0.7277	0.2034/0.2723	-0.01/0.02	0.07/0.13	1170/1054
rrnS	301/272	70/91	86/114	256/237	0.7812/0.7129	0.2188/0.2871	0.08/0.07	0.1/0.11	713/714
trnA	23/25	2009/6	2014/8	21/27	0.6567/0.7879	0.3433/0.2121	0.05/-0.04	0.22/0.14	67/63
trnC	27/14	2005/10	2008/11	25/29	0.8/0.6719	0.2/0.3281	0.04/-0.35	0.23/0.05	65/65
trnD	26/18	2002/5	2004/5	31/22	0.9048/0.8	0.0952/0.2	-0.09/-0.1	0.33/0	63/66
trnE	31/24	2006/6	2003/9	26/22	0.8636/0.7541	0.1364/0.2459	0.09/0.04	-0.33/0.2	66/64
trnF	25/20	2008/10	10/13	21/18	0.7188/0.623	0.2813/0.377	0.09/0.05	0.11/0.13	64/66
trnG	29/20	2010/7	2010/7	25/30	0.7297/0.7813	0.2703/0.2188	0.07/-0.2	0/0	74/64
trnH	24/21	7/13	2010/9	26/21	0.7463/0.6563	0.2537/0.3438	-0.04/0	0.18/-0.18	67/65
trnI	22/22	2012/7	2014/12	23/22	0.6338/0.6984	0.3662/0.3016	-0.02/0	0.08/0.26	71/63
trnK	21/16	2004/7	2004/8	22/26	0.8431/0.7368	0.1569/0.2632	-0.02/-0.24	0/0.07	51/57
trnL1	25/25	2005/8	2012/10	23/23	0.7385/0.7273	0.2615/0.2727	0.04/0.04	0.41/0.11	65/63
trnL2	22/21	2014/10	2017/11	31/28	0.631/0.7	0.369/0.3	-0.17/-0.14	0.1/0.05	84/61
trnM	30/27	2008/7	2006/7	23/24	0.791/0.7846	0.209/0.2154	0.13/0.06	-0.14/0	67/64
trnN	23/25	2008/4	2007/7	27/27	0.7692/0.8254	0.2308/0.1746	-0.08/-0.04	-0.07/0.27	65/50
trnP	26/22	2005/7	2012/9	24/25	0.7463/0.746	0.2537/0.254	0.04/-0.06	0.41/0.13	67/66
trnQ	24/23	2006/6	2010/6	20/24	0.7333/0.7966	0.2667/0.2034	0.09/-0.02	0.25/0	60/61
trnR	24/28	2007/3	2006/10	27/25	0.7969/0.803	0.2031/0.197	-0.06/0.06	-0.08/0.54	64/64
trnS1	16/26	2009/7	2012/9	22/23	0.6441/0.7538	0.3559/0.2462	-0.16/0.06	0.14/0.13	59/58
trnS2	30/23	2005/7	2006/8	19/23	0.8167/0.7541	0.1833/0.2459	0.22/0	0.09/0.07	60/59
trnT	28/19	2004/7	2005/11	34/21	0.8732/0.6897	0.1268/0.3103	-0.1/-0.05	0.11/0.22	71/70
trnV	25/21	2008/8	2006/11	25/23	0.7813/0.6984	0.2188/0.3016	0/-0.05	-0.14/0.16	64/63
trnW	32/22	2009/10	2006/10	23/22	0.7857/0.6875	0.2143/0.3125	0.16/0	-0.2/0	70/57
trnY	18/19	2008/10	2009/6	19/22	0.6852/0.7193	0.3148/0.2807	-0.03/-0.07	0.06/-0.25	54/61

Suppl. material 1. List of gastropoda mitogenomes used in phylogenetic and comparative analyses.

Suppl. material 2. The nonsynonymous/synonymous ratios (ka/ks) in 13 PCGs of *Achatina fulica* with *M. billineatum* and *S. arundinetorum*.

Suppl. material 3. Nucleotide composition of the *S. arundinetorum* and *M. billineatum* mitogenomes.

Suppl. material 4. Amino acid type and percentage of *S. arundinetorum* and *M. billineatum*.

Suppl. material 5. Statistics on the size and number of repetitive sequences in the control region of *S. arundinetorum* and *M. billineatum*.

Suppl. material 6. Statistics of codon preference analysis of *S. arundinetorum* and *M. bilineatum*.

Suppl. material 7. The nonsynonymous/synonymous ratios (Ka/Ks) in 13 mitochondrial PCGs of *M. billineatum* and *S. arundinetorum*.

Suppl. material 8. Composition and skewness of non-coding region in *M. billineatum* and *S. arundinetorum*.

Suppl. material 9. Composition and number of the scattered repeat sequences in *M. billineatum* and *S. arundinetorum*. Red for forwarding repetition, orange for palindromic repetition, blue for reverse repetition, and green for complementary repetition.

Suppl. material 10. The Phylogenetic tree was generated using the Bayesian analyses method from the amino acid composition of complete mitochondrial genomes. Numbers at the nodes are bootstrap values.

Suppl. material 11. The Phylogenetic tree was generated using the Maximum Parsimony method from the amino acid composition of complete mitochondrial genomes. Numbers at the nodes are bootstrap values.

Author Contributions:

Conceptualization, D.-Z.Z., L.-F.C., and G.W.; methodology, X.-L.S.; formal analysis, Y.C.; investigation, B.-B.L.; data curation, J.T., and Z-J.S.; writing-original draft preparation, D.-Z.Z., B.-P.T., and L.-F.C.; writing-review and editing, G.W., Y.Z., and Q.-T.J. All authors have read and agreed to the published version of the manuscript.

Funding:
This study was supported by the Open Foundation of Jiangsu Key Laboratory for Bioresources of Saline Soils [JKLBZ202005], Jiangsu Province industry-university-research cooperation project [BY2021457], and National Natural Science Foundation of China [32070526].

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in National Genomics Data Center (Accession number: CRA008380) https://ngdc.cncb.ac.cn/gsa.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

A Clayton D (2000) Transcription and replication of mitochondrial DNA. Human reproduction (6): 451-465. https://doi.org/10.1093/humrep/15.suppl_2.11

Aguileta G, Vienne D, Ross ON, Hood ME, Giraud T, Petit E, Biology TGJG (2014) High Variability of Mitochondrial Gene Order among Fungi. Genome Biology & Evolution (2):451-465.

Alexandre H, Nelly L, Jean DJSB (2005) Evidence for Multiple Reversals of Asymmetric Mutational Constraints during the Evolution of the Mitochondrial Genome of Metazoa, and Consequences for Phylogenetic Inferences. Systematic Biology (54): 277-298.

Anand SK, Tikoo SKJAiV (2013) Viruses as Modulators of Mitochondrial Functions. Advances in Virology :738-794. http://dx.doi.org/10.1155/2013/738794

Astrin JJ, Hfer H, Spelda J, Holstein J, Muster CJPO (2016) Towards a DNA Barcode Reference Database for Spiders and Harvestmen of Germany. PLoS One 11(9): e0162624. http://dx.doi.org/10.1371/journal.pone.0162624

Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD (2012) SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. Computational Biology and Chemistry (19): 455-477.

Bernt M, Merkle D, Kai R, Fritzsch G, Perseke M, Bernhard D, Schlegel M, Stadler PF, Middendorf MJB (2007) CREx: inferring genomic rearrangements based on common intervals. Bioinformatics 23(21): 2957-2958. https://academic.oup.com/bioinformatics/article/23/21/2957/373406

Bouchet P, Rocroi J-P, Hausdorf B, Kaim A, Kano Y, Nützel A, Parkhaev P, Schrödl M, Strong EE (2017) Revised Classification, Nomenclator and Typification of Gastropod and Monoplacophoran Families. Malacologia (61): 1-526. http://www.xueshufan.com/publication/2781666580

Chan PP, Lin BY, Mak AJ, Lowe TMJNAR (2021) tRNAscan-SE 2.0: improved detection and functional classification of transfer RNA genes. Nucleic Acids Research (49): 9077. http://dx.doi.org/10.1093/nar/gkab688.

Chen C, Rui X, Hao C, He YJCSHL (2018) TBtools, a Toolkit for Biologists integrating various HTS-data handling tools with a user-friendly interface. bioRxiv(8). http://www.biorxiv.org/content/early/2018/03/27/289660

D CD, A CD (1984) Precise identification of individual promoters for transcription of each strand of human mitochondrial DNA. Cell 36(3). http://dx.doi.org/10.1016/0092-8674(84)90343-x

DAVID, LUNT, LAWRENCE, E, WHIPPLE, BRADLEY, C, Ecology HJM (1998) Mitochondrial DNA variable number tandem repeats (VNTRs). utility and problems in molecular ecology.

Davidović S, Marinković S, Kukobat M, Mihajlović M, Tanasić V, Hribšek I, Tanasković M, Stamenković-Radak MJL (2022) CytbGenetic Diversity Analysis of Mitochondrial Gene, Phylogeny and Phylogeography of Protected Griffon Vulture (Gyps fulvus) from Serbia. Life (Basel)12(2):164.

Dereeper A, Audic S, Claverie J, Blanc GJBeb (2010) BLAST-EXPLORER helps you building datasets for phylogenetic analysis. BMC Evolutionary Biology (10):8. http://dx.doi.org/10.1186/1471-2148-10-8

Donath, Jühling, Externbrink, Middendorf, Pütz, Florentz, Stadler, Fritzsch, Bernt MITOS (2013) Improved de novo metazoan mitochondrial genome annotation. Molecular Phylogenetics and Evolution (69): 313-319.

E HJ, A CD (1985) Initiation of transcription from each of the two human mitochondrial promoters requires unique nucleotides at the transcriptional start sites. Proceedings of the National Academy of Sciences of the United States of America (82): 2660-2664. http://dx.doi.org/10.1073/pnas.82.9.2660

Ejka T, Hamerlik LJPJoE (2010) Land snails as indicators of soil humidity in Danubian woodland (SW Slovakia). Polish Journal of Ecology (57): 741-747. http://yadda.icm.edu.pl/yadda/element/bwmeta1.element.baztech-article-BGPK-2859-1187

Eva K, Nenad B (2021) Mechanisms and regulation of protein synthesis in mitochondria. Nature Reviews Molecular Cell Biology 22(5). http://dx.doi.org/10.7554/eLife.59264

Feng JT, Xia LP, Yan CR, Miao J, Reports ZLJS (2021) Characterization of four mitochondrial genomes of family Neritidae (Gastropoda: Neritimorpha) and insight into its phylogenetic relationships. Scientific reports (11): 11748. http://www.xueshufan.com/publication/3164272016

Groenenberg DSJ, Harl J, Duijm E, Gittenberger EJH (2017) The complete mitogenome of Orcula dolium (Draparnaud, 1801); ultra-deep sequencing from a single long-range PCR using the Ion-Torrent PGM. Hereditas 54(1). http://dx.doi.org/10.1186/s41065-017-0028-2

Guo Y, Zhang Y, Liu Q, Huang Y, Xiao NJG (2019) A chromosomal-level genome assembly for the giant African snail Achatina fulica. Gigascience 8(10). http://dx.doi.org/10.1093/gigascience/giz124

Iyengar A, Diniz FM, Gilbert T, Woodfine T, Maclean NJMP (2006) Structure and evolution of the mitochondrial control region in oryx. Molecular Phylogenetics and Evolution 40(1): 305-314. http://dx.doi.org/10.1016/j.ympev.2006.02.015

Kazutaka, Katoh, Daron, Biology SJM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Molecular Biology and Evolution (30): 772-780.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran CJB (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics (28): 1647-1649. http://dx.doi.org/10.1093/bioinformatics/bts199

Kerpedjiev P, Hammer S, Hofacker ILJB (2015) Forna (force-directed RNA): Simple and effective online RNA secondary structure diagrams. Bioinformatics 31(20): 3374-3376. http://dx.doi.org/10.1093/bioinformatics/btv372

Korábek O, Petrusek A, Rovatsos MJZ (2019) The complete mitogenome of Helix pomatia and the basal phylogeny of Helicinae (Gastropoda, Stylommatophora, Helicidae). Hereditas (154): 1-10.

Liu C, Wu X, Shen HDJMD (2015) Complete mitochondrial genome of Vaginulus alte and Homoiodoris japonica. Mitochondrial DNA Part A 27(5): 1-4. http://dx.doi.org/10.3109/19401736.2015.1066345

Lydeard, Charles, Cowie, Robert H, Ponder, Winston F, Bogan, Arthur E, Bouchet (2004) The Global Decline of Nonmarine Mollusks. BioScience (54): 321-330.

Roloff GA, Henry MF (2015) Mam33 promotes cytochrome c oxidase subunit I translation in Saccharomyces cerevisiae mitochondria. Molecular Biology and Evolution 26(16): 2885-2894. http://dx.doi.org/10.1091/mbc.E15-04-0222

Nicholls TJ, Minczuk MJEG (2014) In D-loop: 40 years of mitochondrial 7S DNA. Experimental Gerontology (56): 175–181. http://dx.doi.org/10.1016/j.exger.2014.03.027

Özgül D, Michael S, Zeyuan C (2020) The complete mitogenome of Arion vulgaris Moquin-Tandon, 1855 (Gastropoda: Stylommatophora): mitochondrial genome architecture, evolution and phylogenetic considerations within Stylommatophora. PeerJ (8): e8603.

Ponder, Winston (2008) Phylogeny and Evolution of the Mollusca. Molluscan Evolutionary Development : 435-437. http://www.onacademic.com/detail/journal_1000039429161510_62df.html

Posada DJB (2011) ProtTest 3: fast selection of best-fit models of protein evolution. Bioinformatics 27(8): 1164-1169. https://academic.oup.com/bioinformatics/article/27/8/1164/227935

Prezio, John, R., Lankester, Murray, W., Lautenschlager, R. A, Bell, F. WJRCDRFer (1999) Effects of alternative conifer release treatments on terrestrial gastropods in regenerating spruce plantations. Canadian Journal of Forest Research.

Sanderson MJJsoe, davis e (2004) r8s, Version 1.70 User's Manual. Section of Evolution & Ecology Davis,USA. http://dx.doi.org/10.1139/cjfr-29-7-1141

Sinos G, Moysis MJJoMS (2004) Dispersal patterns and population structure of the land snail Albinaria coerulea (Pulmonata: Clausiliidae). Journal of Molluscan Studie (2): 107-116. https://academic.oup.com/mollus/article/70/2/107/991437

Stephan G, Pascal L, Ralph BJNAR (2019) OrganellarGenomeDRAW (OGDRAW) version 1.3.1: expanded toolkit for the graphical visualization of organellar genomes. Nucleic Acids Res (1): 59-64. http://dx.doi.org/10.1093/nar/gkz238

Stoccoro A, Smith A, Baldacci F, Coppede FJMA (2021) Mitochondrial D-Loop Region Methylation and Copy Number in Peripheral Blood DNA of Parkinson's Disease Patients. Genes (12): 750.

Tiezhu, Yang, Guolyu, Xu, Bingning, Gu, Yanmei, Shi, Hellen, Genes LJ (2019) The Complete Mitochondrial Genome Sequences of the Philomycus bilineatus (Stylommatophora: Philomycidae) and Phylogenetic Analysis. Genes (10): 198.

Vives-Bauza C, Vries RLAD, Tocilescu MA, Przedborski SJPRD (2009) Is there a pathogenic role for mitochondria in Parkinson's disease? Parkinsonism & related disorders 15 Suppl 3: 241-244. http://dx.doi.org/10.1016/S1353-8020(09)70823-5

Walberg MW, Clayton DAJNAR (1981) Sequence and properties of the human KB cell and mouse L cell D-loop regions of mitochondrial DNA. Nucleic Acids Res 9(20): 5411-5421. http://dx.doi.org/10.1093/nar/9.20.5411

Wynn EL, Christensen ACJGG, Genomes, Genetics (2018) Repeats of Unusual Size in Plant Mitochondrial Genomes: Identification, Incidence and Evolution. G3 (Bethesda) 9(2):549-559. http://www.biorxiv.org/content/early/2018/07/24/376020?rss=1

Xie GL, Köhler F, Huang XC, Wu RW, Zhou CH, Ouyang S, Wu XPJMP, Evolution (2019) A novel gene arrangement among the Stylommatophora by the complete mitochondrial genome of the terrestrial slug Meghimatium bilineatum (Gastropoda, Arionoidea).

Zazueta C, Correa F, Buelna-Chontal M, Hernandez-Resendiz SJCDT (2013) Targeting Mitochondria for Cardiac Protection. Molecular Phylogenetics and Evolution (135): 177-184. http://www.ingentaconnect.com/content/ben/cdt/2013/00000014/00000005/art00008

Zhao L, Mi T, Zhen Y, Yu Z (2012) Cloning, characterization, and expression of Cytochrome b (Cytb)—a key mitochondrial gene from Prorocentrum donghaiense. Chinese Journal of Oceanology and Limnology 30(3).

Zhen X, Jason H, Jianxin Y, B. WP, Hongfu Y, Qing X, Weiju R, Hui L, Jun S, Fansong M (2022) End Permian to Middle Triassic plant species richness and abundance patterns in South China: Coevolution of plants and the environment through the Permian–Triassic transition. Earth-Science Reviews.8.