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Received: 15 September 2021 | Revised: 8 December 2021 | Accepted: 11 December 2021

DOI: 10.1111/zsc.12524

Zoologica Scripta | WILEY

ORIGINAL ARTICLE

Mitogenomics reveals phylogenetic relationships of Patellogastropoda (Mollusca, Gastropoda) and dynamic gene rearrangements

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Funding information
National Natural Science Foundation of China, Grant/Award Number: 31772414; Fundamental Research Funds for the Central Universities, Grant/Award Number: 201964001

Abstract

Patellogastropoda has been recognized as the most 'primitive' group of living Gastropoda and plays important role in littoral marine ecosystems. Both morphological and molecular works have attempted to resolve the phylogenetic framework of Patellogastropoda, but evolutionary relationships among major lineages remain controversial. In addition, a few mitogenomes sequenced of Patellogastropoda exhibit extensive rearrangements; however, it was unclear that which one can represent the ancestral gene arrangement. In this regard, we sequenced 10 new mitochondrial genomes and analysed them with previously published nine mitogenomes and six transcriptomic data which represent six families of Patellogastropoda. A well-supported phylogeny was reconstructed based on the amino acid sequences of 13 protein-coding genes, recovered Lottioidea as non-monophyletic with two well-supported lineages. Comprehensive taxon sampling in Lottiidae allows us to recover *Lottia digitalis* sister to *Lottia cassis*. Comparing gene order among families, genera and species showed that Nacellidae has a conserved gene arrangement which is similar to that of hypothetical ancestral Gastropoda, whereas Lottiidae exhibits extensive rearrangements even without considering the changes of tRNA genes. A chronogram dating mayor cladogenetic events within the group was also reconstructed. Our results provide new insights into the phylogenetic relationships of Patellogastropoda and detect dramatic gene rearrangements within the group.

KEYWORDS

gene rearrangement, mitogenome, Patellogastropoda, phylogeny

1 | INTRODUCTION

The Patellogastropoda (Lindberg, 1986) or true limpets are characterized by cap-shaped shells with the apex typically situated at the centre of the shell or slightly anterior (Lindberg, 2008). They distribute worldwide and can be found in diverse habitats from deep-sea hydrothermal vents to the highest reaches of the intertidal and brackish water (Branch, 1985a, 1985b; Lindberg, 1990; Nakano & Sasaki, 2011; Sasaki et al., 2005). The species diversity of Patellogastropoda is highest in intertidal rocky shores and plays important roles in littoral marine ecosystems (Branch, 1985b; Nakano & Ozawa, 2007). Studies on Patellogastropoda have focused on diverse aspects of their biology including ecology, physiology, behaviour, reproduction, population genetics, historical biogeography

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(Underwood, 1979; Hodgson & Bernard, 1988; Healy, 1988; Sasaki & Okutani, 1994; Koufopanou et al., 1999; Fraser et al., 2002; Nakano & Ozawa, 2004; Nakano et al., 2009; González-Wevar et al., 2010; Ponder & Lindberg, 2020) and perhaps most prominently evolutionary biology due to its distinctive morphology (e. g. secondarily uncoiled shells, two pairs of outer lateral radular teeth, subpallial sensory streaks, the displacement of both kidneys, shell microstructure including foliated and conical crossed-lamellar layers, the presence of pallial gills, rotation of the pericardium, the ultrastructure of the cephalic tentacle epithelium, the osphradium and the kidney) (Andrews, 1985; Haszprunar, 1985; Künz & Haszprunar, 2001; Lindberg, 1998) and plesiomorphic characters (e.g. homogeneous jaw with the dorsal lateral extensions, dorsolateral cartilages, asymmetric and specialised kidney) (Sasaki, 1998; Ponder & Lindberg, 2020).

The current classification of Patellogastropoda was largely established by Nakano and Ozawa (2007) and modified slightly by Nakano and Sasaki (2011) on the basis of systematically summarizing predecessors' work. Mostly following Nakano's classification, Patellogastropoda was further revised by Bouchet et al. (2017), who elevated the order Patellogastropoda to the rank of subclass and assigned a single-order Patellida with three superfamilies (with constituent families), namely Lottioidea (Nacellidae, Lepetidae, Acmaeidae, Lottiidae, Pectinodontidae and Neolepetopsidae), Eoacmaeoidea (Eoacmaeidae) and Patelloidea (Patellidae).

More recently, attempts have been made to reconstruct the phylogeny of Patellogastropoda using molecular data, and some of them also combined with microstructure and mineralogy in the fossil limpet shell to estimate approximate divergence time of patellogastropods (Cunha & Giribet, 2019; Gaitan-Espitia et al., 2019; Harasewych & McArthur, 2000; Nakano & Ozawa, 2004; Nakano & Ozawa, 2007; Nakano & Warén, 2008; Uribe et al., 2019; Yoon & Kim, 2007). Although these studies have improved our understanding of evolutionary relationships within Patellogastropoda, the phylogenetic position of Nacellidae, Patellidae and Eoacmaeidae remains contentious. Nacellidae was a paraphyletic group at the base of patellogastropods in the studies based on partial COI + histone H3, partial 18S rRNA and compete 18S rRNA (Harasewych & McArthur, 2000; Nakano & Warén, 2008; Yoon & Kim, 2007). However, in the studies based on partial 12S rRNA + 16S rRNA + COI, Nacellidae is a monophyletic clade and only sister to the deep-sea clade (Nakano & Ozawa, 2007). Although Eoacmaeidae appears as the sister group to all other patellogastropods in the studies based on partial 12S rRNA + 16S rRNA + COI, study based on partial COI + histone H3 have placed Eoacmaeidae only related to Patellidae (Nakano & Ozawa, 2007; Nakano & Warén, 2008). In addition, there are also many controversies on whether Patellidae and Lottiidae were paraphyletic or monophyletic groups and even their positions in phylogenetic trees (Harasewych & McArthur, 2000; Nakano & Ozawa, 2004, 2007; Nakano & Warén, 2008).

Mitochondrial genomes have been used with success to reconstruct phylogenetic relationships in different metazoan groups (e.g. Li et al., 2015; Stöger & Schrödl, 2013). Compared to gene fragments, it contains more molecular sequence information, richer gene structure information and has proven useful in enhancing resolution and statistical confidence of inferred phylogenetic trees (Li et al., 2012; Mueller, 2006; Russo et al., 1996; Zardoya & Meyer, 1996). Within Patellogastropoda, phylogenetic inference based on mitogenomes has shown less efficiency due to long-branch attraction (LBA) biases caused by *Lottia digitalis*, but also in some instances associated to limited taxon sampling (Gaitan-Espitia et al., 2019; Uribe et al., 2019). In these studies, *L. digitalis* was sister to Bivalvia and Heterobranchia (Gaitan-Espitia et al., 2019) or the first divergent lineage of Patellogastropoda (Uribe et al., 2019). These results were contradicted each other and the true position of *L. digitalis* within Patellogastropoda is still unclear.

In addition, mitogenome gene order rearrangements can provide an independent dataset to resolve the evolutionary relationships because shared mitogenome gene order rearrangement patterns in different taxonomic groups most likely provide evidence of common ancestry rather than being the product of convergent evolution (Basso et al., 2017; Tan et al., 2018). Prior to this study, only nine complete or nearly complete mitochondrial genomes of Patellogastropods were available. These published sequence data represented three families: Lottidae, Patellidae and Nacellidae, which show, respectively, high, intermediate and low levels of gene rearrangement compared to the hypothetical ancestral mitochondrial gene order of Gastropoda (Gaitan-Espitia et al., 2019; Uribe et al., 2019). Patellogastropoda is a diverse group encompassing up to eight families, 36 genera, it is worthy to explore the gene order rearrangements of other patellogastropods and figure out which one can represent the ancestral gene arrangement of Patellogastropoda.

In this study, we newly sequenced 10 complete or nearly complete Patellogastropoda mitochondrial genomes and analysed together with mitogenome and transcriptomic data available from NCBI, which represent six lineages (Nacellidae, Lottiidae, Pectinodontidae, Neolepetopsidae, Eoacmaeidae and Patellidae) of Patellogastropoda. Our aims were (a) to alleviate the LBA of *L. digitalis* and elucidate the phylogenetic relationship of Patellogastropoda based on six families, 25 species in mitogenome level; (b)

to determine the ancestral gene order of Patellogastropoda and compare Patellogastropoda mitochondrial gene arrangement to the hypothetical ancestral Gastropoda; and (c) to estimate the divergence time of major cladogenetic events within the comprehensive phylogenetic context of Patellogastropoda.

2 | MATERIALS AND METHODS

2.1 | Specimen collection and mitochondrial genome sequencing

The limpet samples were collected from the coast of China from August 2009 to September 2020 (except *Cellana nigrolineata*, which collected from Jeju Island, South Korea). The collection data of each specimen are listed in Table 1. After collection, specimens were immediately preserved in 95% ethanol. The total genomic DNA was extracted from 5 to 10 mg of foot tissue (one specimen per species was used for DNA extraction) using TIANamp Marine Animals DNA Kit (TIANGEN Biotech Beijing Co. Ltd.) following manufacture's protocols. Genomic DNA was submitted to Beijing Novogene Technology Co., Ltd, for library construction and high-throughput sequencing. Sequencing libraries with average insert sizes of approximately 300 bp were prepared and then sequenced as 150 bp paired-end runs on the Illumina NovaSeq 6000 platform.

2.2 | Mitochondrial genome assemblies and annotation

Raw sequence data for all 16 samples (including six transcriptomic data and 10 new sequenced genomic data) were trimmed using Trimmomatic 0.36 (Bolger et al., 2014) with the parameters "ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 LEADING:3TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36." Resulting clean reads were assembled de novo using NOVOPlasty 4.1 (Dierckxsens et al., 2017) and MitoZ v. 2.3 (Meng et al., 2019).

Annotation of the protein-coding genes (PCGs) was defined by using Open Reading Frame Finder (https://www.ncbi.nlm.nih.gov/orffinder/) and corroborated using the MITOS web server (Bernt, Donath, et al., 2013) with default settings and the invertebrate genetic code for mitochondria and followed by manual genome annotation in Artemis (Rutherford et al., 2000). Gene boundaries were examined and subsequently adjusted manually by comparison with the previously published patellogastropod mitochondrial genome. The tRNA genes were identified using ARWEN (Laslett & Canbäck, 2008) and tRNA scan-SE 1.21 (Lowe & Eddy, 1997). The ribosomal RNA (rRNA) genes were identified by comparing with the mitochondrial genomes of other limpets, and their boundaries were assumed to be between the adjacent genes (Boore et al., 2005).

Transcriptomic data (Cunha & Giribet, 2019) for *Patella ulyssiponensis*, *Patelloida saccharina*, *Testudinalia testudinalis*, *Lottia* cf. *fenestrata*, *Eoacmaea pustulata* and *Paralepetopsis* sp. were download from NCBI SRA database, and raw reads were assembled using Trinity v. 3.2.1 (Grabherr et al., 2011) with the "trimmomatic" flag. Mitochondrial protein-coding genes were identified by TBLASTX (Camacho et al., 2009) using the published nucleotide sequences of 13 protein-coding genes of the closest relatives as queries. The full-length sequences of all 16 newly sequenced mitochondrial genomes can be accessed through GenBank (Table 1). Mitochondrial protein-coding genes derived from transcriptomic data acquired from NCBI SRA Database were deposited to figshare (https://figshare.com/articles/dataset/Patellogastropoda_mt_genome/16436880).

To verify the reliability of this method and the data obtained, the transcriptomic data of *Nacella magellanica* (SRR8318359), which had a publicly available mitochondrial genome (KT990125), were downloaded, assembled, annotated and deposited as described above. The number of different positions and percentages of similarity between the mitochondrial genome sequences assembled from the transcriptomes and obtained in GenBank were examined (Abalde et al., 2019).

2.3 | Concatenated alignments

A total of 31 taxa were used for the phylogenetic analysis, of which three species from Cephalopoda and three species from Vetigastropoda were selected as outgroup based on current understanding of gastropod evolutionary history (Cunha & Giribet, 2019; Kocot et al., 2011; Uribe et al., 2019; Zapata et al., 2014) (Table 1). Amino acid sequences of the 13 mitochondrial protein-coding genes (PCGs) used in this study were aligned separately using MAFFT (Katoh & Standley, 2013). Ambiguously aligned positions were removed using Gblocks (Talavera & Castresana, 2007) with default parameters. Finally, resulting alignments were concatenated into final supermatrix using FASconCAT (Kueck & Meusemann, 2010) for downstream phylogenetic analysis.

2.4 | Phylogenetic analysis

Phylogenetic analyses were inferred using maximum-likelihood (ML) method and Bayesian inference (BI).

TABLE 1 List of species used in this study

Species	Length (bp)	GenBank Acc. No.	Locality	Reference
Order Patellogastropoda				
Famliy Nacellidae				
Nacella clypeater[a]	16,742	KT990124		Gaitan-Espitia et al. (2019)
Nacella magellanica[a]	16,663	KT990125		Gaitan-Espitia et al. (2019)
Nacella magellanica		SRR8318359		Cunha and Giribet (2019)
Nacella concinna[a]	16,761	KT990126		Gaitan-Espitia et al. (2019)
Cellana radiata[a]	16,194	MH916651		Uribe et al. (2019)
Cellana toreuma[a]	16,260	MW716504	Yangjiang, Guangdong, China	This study
Cellana nigrolineata[a]	16,148	MW727704	Jeju Island, South Korea	This study
Cellana grata[a]	16,181	MW722939	Ningde, Fujian, China	This study
Famly Patellidae				
Patella vulgata	14,808	MH916653		Uribe et al. (2019)
Patella ferruginea	14,460	MH916654		Uribe et al. (2019)
Patella ulyssiponensis		SRR8318349		Cunha and Giribet (2019)
Scutellastra flexuosa	15,233	MW735839	Sansha, Hainan, China	This study
Family Lottiidae				
Lottia digitalis[a]	26,835	NC_007782		Simison et al. (2006)
Nipponacmea fuscoviridis[a]	18,720	MK820636		Feng et al. (2020)
Nipponacmea radula[a]	19,231	MZ901174	Weihai, Shandong, China	This study
Patelloida ryukyuensis	16,319	MZ130310	Weihai, Shandong, China	This study
Patelloida saccharina lanx	19,427	MW735838	Wenchang, Hainan, China	This study
Patelloida saccharina		SRR8318348		Cunha and Giribet (2019)
Patelloida conulus[a]	17,030	MZ048283	Weihai, Shandong, China	This study
Lottia cassis	18,797	MZ130258	Weihai, Shandong, China	This study
Lottia goshimai	17,849	MZ766128	Qingdao, Shandong, China	This study
Lottia cf. *fenestrata*		SRR8318352		Cunha and Giribet (2019)
Testudinalia testudinalis		SRR8318355		Cunha and Giribet (2019)
Family Pectinodontidae				
Bathyacmaea nipponica	16,792	MF095859		Sun et al. (2019)
Family Neolepetopsidae				
Paralepetopsis sp.		SRR8318345		Cunha and Giribet (2019)
Family Eoacmaeidae				
Eoacmaea pustulata		SRR8318360		Cunha and Giribet (2019)
Order Vetigastropoda				
Tegula lividomaculata[a]	17,375	NC_029367		Uribe et al. (2016)
Cittarium pica[a]	17,949	KY212109		Uribe et al. (2017)
Lunella aff. *Cinerea*[a]	17,670	KF700096		Williams et al. (2014)
Class Cephalopoda				
Octopus vulgaris[a]	15,744	NC_006353		Yokobori et al. (2004)
Octopus minor[a]	15,974	NC_015896		Cheng et al. (2012)
Vampyroteuthis infernalis[a]	15,617	NC_009689		Yokobori et al. (2007)

[a]Complete mt genome.

ML analyses were carried out using software IQ-TREE 1.6.1 (Nguyen et al., 2015) with 1000 replicates of ultrafast bootstrapping (-bb 1000). BI analyses were performed with MrBayes v. 3.1.2 (Ronquist et al., 2012), running four simultaneous Monte Carlo Markov chains (MCMC) for 10,000,000 generations, sampling every 1000 generations and discarding the first 25% generations as burn-in on the CIPRES Science Gateway V. 3.3 (Miller et al., 2010). Parameter convergence was achieved within 10 million generations, and the standard deviation of split frequencies was <0.01. All parameters were checked with Tracer v. 1.7 (Rambaut et al., 2018), and the effective sample size (ESS) was more than 200. The resulting phylogenetic trees were visualized in FigTree v. 1.4.4 (Rambaut, 2014). The best partition schemes and best-fit models of substitution for the data sets were identified using Partition Finder Protein 2 (Lanfear et al., 2017) with the Bayesian information criterion (Schwarz, 1978). The partitions tested were all genes combined; all genes separated (except *atp6-atp8* and *nad4-nad4L*); and genes grouped by subunits (*atp*, *cob*, *cox* and *nad*).

In order to check whether the presence of long branches generate biases in the phylogenetic reconstruction, BI analyses using the site-heterogeneous mixture model (Lartillot & Philippe, 2004) were also performed in PhyloBayes MPI v.1.8c (Lartillot et al., 2013) using the CAT-GTR model and discarding constant sites ("-dc" option). These methods have shown well performance avoiding phylogenetic biases in gastropod phylogeny (Uribe et al., 2016, 2019). Two independent MCMC chains were running until convergence (checked a posteriori using the tools implemented in PhyloBayes; maxdiff <0.1, maximum discrepancy <0.1 and effective sample size >100). Consensus trees were obtained after discarding the first 10% cycles as burn-in.

2.5 | Gene order analyses

Pairwise comparisons of the gene order within all available patellogastropod mitochondrial genomes were performed with CREx (Bernt et al., 2007) using the common intervals parameter, which considers events of transpositions, inverse transpositions, inversions and tandem duplication-random loss to infer which one could represent the most ancestral architecture for this order. Given tRNA replication and extensive rearrangement, Lottiidae were not considered into this comparison. Besides, we also compared the gene order between true limpets and hypothetical ancestral Gastropoda (Osca et al., 2014). Since we could not find the protein-coding genes of *nad3* and *nad4L* in *Nipponacmaea radula*, it was not included in comparative analysis of gene arrangement.

2.6 | Divergence time estimation

Divergence times within Patellogastropoda were estimated on the amino acid dataset using the uncorrelated, lognormal relaxed clock model, random starting trees and Yule speciation model in BEAST v. 1.7 (Drummond et al., 2012). The final Markov chain was run twice for 200 million generations, sampling every 20,000 generations. The first 10% of samples were discarded as burn-in, according to the convergence of chains checked with Tracer v. 1.7 (Rambaut et al., 2018), and the effective sample size of all the parameters was above 200.

The first calibration point was set at the root of the tree. A lognormal distribution was applied, with the minimum of 488.3 Mya and a 95% upper limit of 501 Mya (mean: 3.3; SD: 1.8) based on the oldest known Cephalopoda fossil, *Plectronoceras cambria* (Cope, 1997; Nishiguchi & Mapes, 2008). The second calibration point was set at the split between *Patelloida* and *Nipponacmea* + *Lottia*. The most ancient fossil is *Patelloida* recovered from the Late Cretaceous (Campanian) of California (Lindberg, 1983; MacClintock, 1967). The 95% lower and upper limits were set to 100.5 and 113.0 Mya (lognormal distribution, mean: 3.3; SD: 1.9). The third calibration point was set for the divergence of *Cellana* and *Nacella*, since the oldest fossil of *Cellana* from Mexico has been recognized in the Ypresian (Squires & Demetrion, 1992). We set the 95% lower and upper limits to 47.8 and 56.0 Ma (lognormal distribution, mean: 2.1; SD: 1.7). For the fourth calibration point, the earliest fossil of *Lunella*, *Lunella miyarensis* (MacNeil, 1964), was reported from the Eocene Miyara group, which has been accurately aged using foraminiferans and can be placed in the Priabonian stage (Saito et al., 1984). The 95% lower and upper limits were set to 34.2 and 37 Mya to represent the most recent common ancestor of this clade (lognormal distribution, mean: 0.9; SD: 1.0). The maximum clade credibility tree was determined and annotated in TreeAnnotator v. 2.4.1 after removal of 10% of the trees as burn-in.

3 | RESULTS

3.1 | Mitochondrial genome organizations

Within Patellogastropoda, the mitochondrial genomes that were determined complete are indicated with the letter a in Table 1. The size of 10 newly sequenced mitochondrial genomes ranged from 15,233 bp (*Scutellastra flexuosa*) to 19,427 bp (*P. saccharina lanx*). It is worth noting that the mitogenomes of lottiids are larger in length (~ 2kb) than patellids and nacellids. The AT contents ranged

from 53.07% in *P. saccharina lanx* to 68.36% in *Cellana toreuma*. Lottiids showed lower AT contents than other species (Table S1). The newly determined mitogenomes, except for lottiids, encode for 13 PCGs, two rRNA and 22 tRNA genes. At least one tRNA gene (trnM, trnW, trnK, trnN or trnY) duplication was found in six newly sequenced mitochondrial genomes of lottiids (Table S2). We cannot find the protein-coding genes of *nad3* and *nad4L* in *Nipponacmea radula*. The intergenic nucleotides of lottiids were larger than other species, but the length of PCGs and rRNAs did not change significantly between species (Tables S1 and S2). The same phenomenon was also observed in previously sequenced Lottiidae species (*L. digitalis* and *Nipponacmaea fuscoviridis*). Six different start codons were observed (Table S2) but most protein-coding genes (85.94%) start with conventional initiation codon ATG or ATA. Similar results were also observed in mitochondrial genomes of other patellogastropods (Feng et al., 2020; Gaitan-Espitia et al., 2019; Uribe et al., 2019).

For *Nacella magellanica*, we identify the 12 protein-coding genes except ATP8, and the percentage of sequence similarity was above 97.44% for all genes (see Table S3). Although there are slight sequence differences due to different geographic origins, individual variability or sequencing errors (Abalde et al., 2019), it is clear that our methods and data are reliable.

3.2 | Phylogenetic relationship

The concatenated alignment of 13 amino acid sequences from 31 taxa had a total length of 3,150 positions. The best partition scheme was the one combining genes by subunits, while the best substitution models were MTART+G for *atp*, MTART+I+G for *cob*, LG+I+G+F for *cox* and MTART+I+G+F for *nad* (Table S4). The ML (Figure 1) and BI analyses arrived at almost identical topologies and most nodes received high support values, except one internal node within *Patella*. Although both ML and BI analyses suggest that *Patella* was monophyletic, the phylogenetic tree indicated that *Patella ferruginea* was sister to *Patella vulgata* + *P. ulyssiponensis* in ML analyses and BI analyses with site-heterogeneous mixture model (Figure S1) while *P. vulgata* was sister to *P. ferruginea* + *P. ulyssiponensis* in BI analysis with the best substitution models.

All families and genera are monophyletic groups except genus *Lottia*. Eoacmaeidae and Lottiidae forms a well-supported clade (PP = 1; BS = 100%). Patellidae was the sister group of a clade containing Nacellidae + Pectionodontidae and Neolepetopsidae (PP = 1; BS = 90%). In Lottiidae, *Patelloida* sister to a clade formed by *Lottia* and *Nipponacmea*. The genus *Lottia* was found to be polyphyletic if take *T. testudinalis* into account. *Testudinalia testudinalis*, the only representative of *Testudinalia*, was nested in the genus *Lottia* and was clustered with *L.* cf. *fenestrata*, which indicate that either *T. testudinalis* or genus *Testudinalia* should be transferred to genus *Lottia*.

3.3 | Mitochondrial gene order and rearrangements

Among the 18 species with known gene arrangement, all limpets belong to family Nacellidae have the same

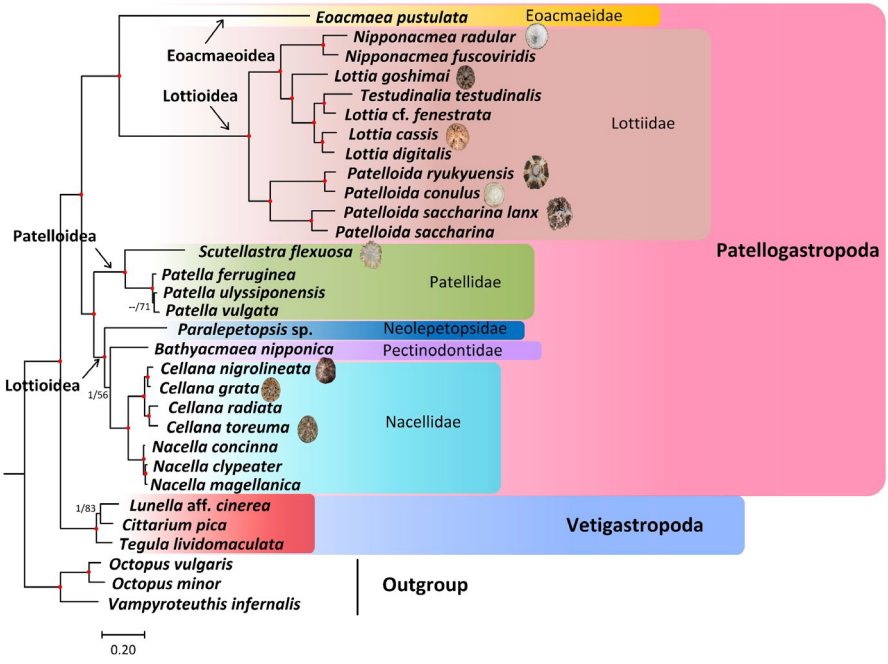

FIGURE 1 Phylogenetic relationships of Patellogastropoda based on concatenated amino acids of 13 mitochondrial protein-coding genes. The ML phylograms are shown. Numbers at nodes are statistical support values for BI (posterior probabilities)/ML (bootstrap proportions in percentage). Solid red circles represent nodes with posterior probabilities ≥0.95 and bootstrap proportions ≥90

gene arrangement (Figure 2). The CREx analysis of patellogastropod mitogenomes indicated that the gene order of nacellid limpets are the most primitive condition of Patellogastropoda, because every gene order can be obtained with a minimum number of events only when start from nacellid mitogenomes (Table 2). When compare to gastropod ancestor, the rearrangements of nacellids were only restricted to tRNA genes (with 10 translocations and two inversions), the most mobile genes in animal mitochondrial genomes (Boore, 1999; Dowton et al., 2009). *Bathyacmaea nipponica* have the similar gene organization with nacellids. Compared with gene arrangement of nacellids, there are only three tRNA translocations and one reverse translocation in *B. nipponica*. In Patellidae, there was only one translocation of *cox2*/*cob* between *Patella* and *Scutellastra* when not considering tRNAs. When compared to the ancestral order of Gastropoda, *Scutellastra* retain a relatively large cluster (*cox2-nad4L-nad4-nad5-atp6-atp8*, with the *nad4L* to *atp8* fragment inverted). In Lottiidae, due to extensive rearrangements of PCGs, rRNA and tRNA, our pairwise comparison was only based on 13 PCGs and two rRNA genes. However, the gene arrangements within the family and genus still shows high level of uncertainty, except *Patelloida conulus* and *Patelloida ryukyenis*, which have same gene order not only 13 PCGs and two rRNA genes, but also tRNA genes. Whether including tRNAs or not, each lottiid species shows a high level of rearrangement when compared with hypothetical ancestral Gastropoda.

3.4 | Divergence times

The origin of the stem lineage leading to order Patellogastropoda was dated 341.8 Mya, although with a relatively large credible interval (95% highest posterior density: 232.3–441.7 Mya; Figure 3). The first event of diversification within Patellogastropoda was estimated at a mean of 283.4 (180.0–371.8) Mya, separating *E. pustulata* and lottiid limpets. The split between two clades of Lottiidae were dated to 170.9 (100.5–231.5) Mya. The split time of the clade comprising Patellidae, *Paralepetopsis* sp. and *B. nipponica* + Nacellidae was estimated around 222.8 (130.8–329.2) Mya. The splitting of *Paralepetopsis* sp. was estimated to occur around 152.9 (83.3–243.3) Mya. The split time of *B. nipponica* from nacellid species was inferred as 119.2 (61.5–200.7) Mya. The radiation of the analysed congeneric species (*Nipponacmaea*, *Lottia*, *Patelloida*, *Cellana*, *Nacella*, *Patella*) is estimated to have occurred from the late Eocene to the Pliocene (3–43 Mya; Figure 3).

4 | DISCUSSION

4.1 | Patellogastropoda phylogeny

Patellogastropoda was recovered as a monophyletic group, while incongruent with the previous study of Nakano and Ozawa (2007), Lottioidea was recovered as non-monophyletic with two well-supported lineages. Lottiidae formed one clade and the second clade included *Paralepetopsis* sp. from Neolepetopsidae, *B. nipponica* from Pectinodontidae and monophyletic Nacellidae. *Eoacmaea pustulata*, the only representative of Eoacmaeidae, was recovered as the sister taxon to the clade Lottiidae with high nodal support, consistent with (Nakano & Warén 2008); Cunha and Giribet (2019). In previous studies based on mitogenomes, Lottiidae (only represented by *L. digitalis*) was recovered as the sister taxon of *Eoacmaea*, *Cellana* and *Patella* (Uribe et al., 2019), or formed a clade with bivalves (Gaitan-Espitia et al., 2019), which is most likely an artifact caused by long-branch attraction (LBA) and limited taxon sampling. In this study, we select more extensive taxa and 13 amino acid sequences to alleviate LBA and further checked by using the site-heterogeneous mixture model, allow us to obtain reliable phylogenetic position of *L. digitalis*.

Previous molecular phylogenies recovered Neolepetopsidae as a sister group to Acmaeidae or Lottiidae + Eoacmaeidae (Cunha & Giribet, 2019; Harasewych & McArthur, 2000; Nakano & Warén, 2008). In our study, however, Neolepetopsidae, represent by *Paralepetopsis* sp., was recovered as the sister taxon of a clade included *B. nipponica* (belongs to Pectinodontidae) and nacellids. The affinity between Pectinodontidae and Nacellidae was consistent with Nakano and Ozawa (2007). Both ML and BI analysis support Patellidae as the sister group to the clade included *Paralepetopsis* sp. from Neolepetopsidae, *B. nipponica* from Pectinodontidae and monophyletic Nacellidae, which raising the necessity of taxonomy revise to Lottioidea in the light of our results.

4.2 | Mitochondrial gene arrangements

Gene rearrangements in true limpets are much more common than previously thought. Our analysis inferred that the gene order of Lottiidae showed high levels of rearrangement even without considering tRNAs. The gene order of the newly sequenced mitogenome of *L. cassis* differs substantially with the sister taxon *L. digitalis*. Mitochondrial gene rearrangements have often been associated with increased evolutionary rates (Bernt, Bleidorn, et al., 2013). Moreover, previous studies have found a positive significant correlation in mitochondrial genomes between gene

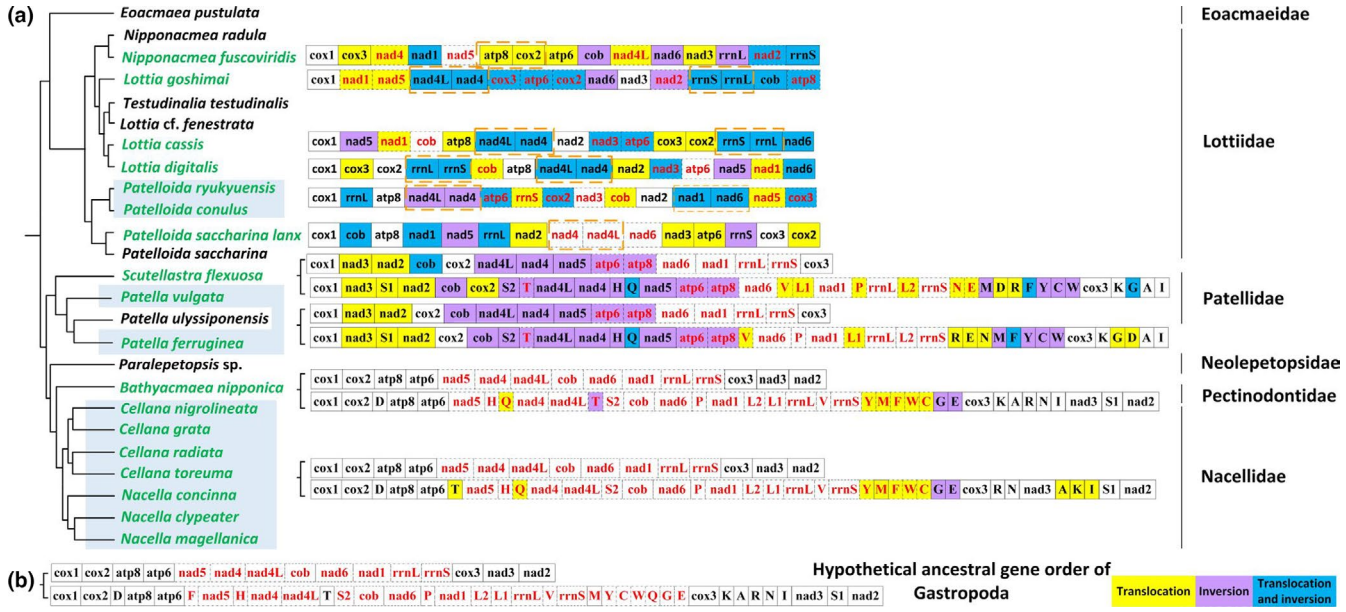

FIGURE 2 Mitochondrial gene order within Patellogastropoda and hypothetical ancestral gastropoda. (a) Phylogenetic relationships were recovered by Bayesian inference utilizing alignment. The tRNA genes are not shown in Lottiidae, which shown tRNA duplication and extensive gene rearrangements. The species with known gene arrangement were coloured in green. The species included in the light blue box indicate the same gene arrangement. Genes in the light strand (reverse) are represented in red and dashed boxes. The gene in orange dashed boxes indicates they preserves the cluster when compared to the ancestral mitochondrial gene order of Gastropoda. (b) Hypothetical ground pattern of gastropod mitochondrial genome

order rearrangement rates and faster evolutionary rates (Xu et al., 2006). Perhaps the high levels of gene rearrangement within Lottiidae were due to a high rate of sequence evolution, which confirmed by the long branches of *L. digitalis* in mitochondrial genome phylogenies (Gaitan-Espitia et al., 2019; Uribe et al., 2019). Interestingly, compared to other species in Patellogastropoda, lottiid limpets showed remarkable variation in mitochondrial genome size, from 16,319 bp (*Patelloida ryukyuensis*) to 26,835 bp (*L. digitalis*), and several species have large mitochondrial genome size (>18 bp). We propose that the variable mitochondrial genome size in these limpets may be correlated with their high levels of gene rearrangement. Further studies are needed to test this association in more Mollusca group.

In Patellidae, the newly sequenced mitogenomes of *Scutellastra flexuous* shown medium and low levels of gene rearrangement when compared to the gene organization of gastropod ancestor and *Patella*, respectively. Hence, intermediate levels of gene rearrangement, at the family level, characterize the mitochondrial genomes of Patellidae. However, the gene rearrangement of genera within Patellidae is relatively conservative. Our new sequenced mitogenomes of three species belongs to *Cellana* share the same gene organization with other nacellids, which agreed with the idea that mitochondrial genome organization of Nacellidae was conserved (Gaitan-Espitia et al., 2019; Uribe et al., 2019). Besides, *B. nipponica* and nacellids not only share the similar gene organization, but also sisters to each other in our phylogenetic tree.

4.3 | Divergence time estimation

The reconstructed time tree dated the origin of Patellogastropoda in the early Carboniferous (about 341.8 Mya) and shown that the principal clades and antitropical distribution pattern of the Patellogastropoda established during the Mesozoic and Cenozoic, which is consistent with previous studies (Nakano & Ozawa, 2004, 2007). The emergency of Patellogastropoda might have triggered by the greenhouse interval, which occurs between the earlier Visean and the later Serpukhovian–Pennsylvanian icehouse times (Pfefferkorn et al., 2014).

According to our time trees, the early divergence within Patellogastropoda occurred in the early Permian. The earth warmed rapidly and glaciers retreated to high latitudes from the late Sakmarian period, and reached the warmest period of the entire Early Permian in the late Artinskian–Kungurian time. A suitable climate might have triggered the splitting of *E. pustulata* (Chumakov & Zharkov, 2002). The origin of Lottiidae was dated at the Middle Jurassic. During the period, the sea temperature in the mid-latitude area suddenly dropped 10°C, which might lead to the emergency of Lottiidae (Korte et al., 2015).

Our molecular clock analyses inferred that the origin of Patellidae in the Upper Triassic. This period was characterized by hot and arid, but was interrupted during the Mid- to Late Carnian by increased rainfall (Simms & Ruffell, 1990). These climate change might have triggered these diversification events. The

TABLE 2 CREx analysis of the most ancestral gene order in Patellogastropoda

Events									
From	**To**	**Common intervals**	**Breakpoints**	**Reversal distance**	**Transp.**	**Rev.**	**Rev. transp.**	**TDRL**	**Total events**
Bathyacmaea nipponica	Nacellidae	724	9	7	1	0	1	1	3
	Patella	126	26	24	7	3	0	2	12
	Scutellastra flexuosa	84	30	25	5	6	1	3	15
Nacellidae	*Bathyacmaea nipponica*	724	9	7	3	0	1	0	4
	Patella	70	29	27	6	2	0	3	11
	Scutellastra flexuosa	44	33	28	5	5	1	3	14
Patella	*Scutellastra flexuosa*	476	15	13	4	5	0	0	9
	Bathyacmaea nipponica	126	26	24	6	3	0	2	11
	Nacellidae	70	29	27	6	2	0	3	11
Scutellastra flexuosa	*Bathyacmaea nipponica*	84	30	25	3	6	1	4	14
	Nacellidae	44	33	28	4	5	1	4	14
	Patella	476	15	13	4	5	0	0	9
Hypothetical ancestral Gastropoda	*Bathyacmaea nipponica*	456	13	11	3	3	0	1	7
	Nacellidae	252	19	17	4	2	0	2	8
	Patella	78	25	24	3	6	0	3	12
	Scutellastra flexuosa	52	29	24	4	8	1	3	16

Note: The arrangement of all PCGs, tRNAs and rRNAs was considered. When more species had the same order, only one of them was used as a representative for that arrangement. The gene order of Nacellidae represents all Nacellid limpets and *Patella* represent *Patella vulgate* + *Patella ferruginea*. Due to tRNA replication and extensive rearrangements in Lottiidae, they were not included in this analysis. The gene rearrangement events are abbreviated as follows: Transp., transposition; Rev., reversal; Rev. transp., reverse transposition; TDRL, tandem duplication-random loss.

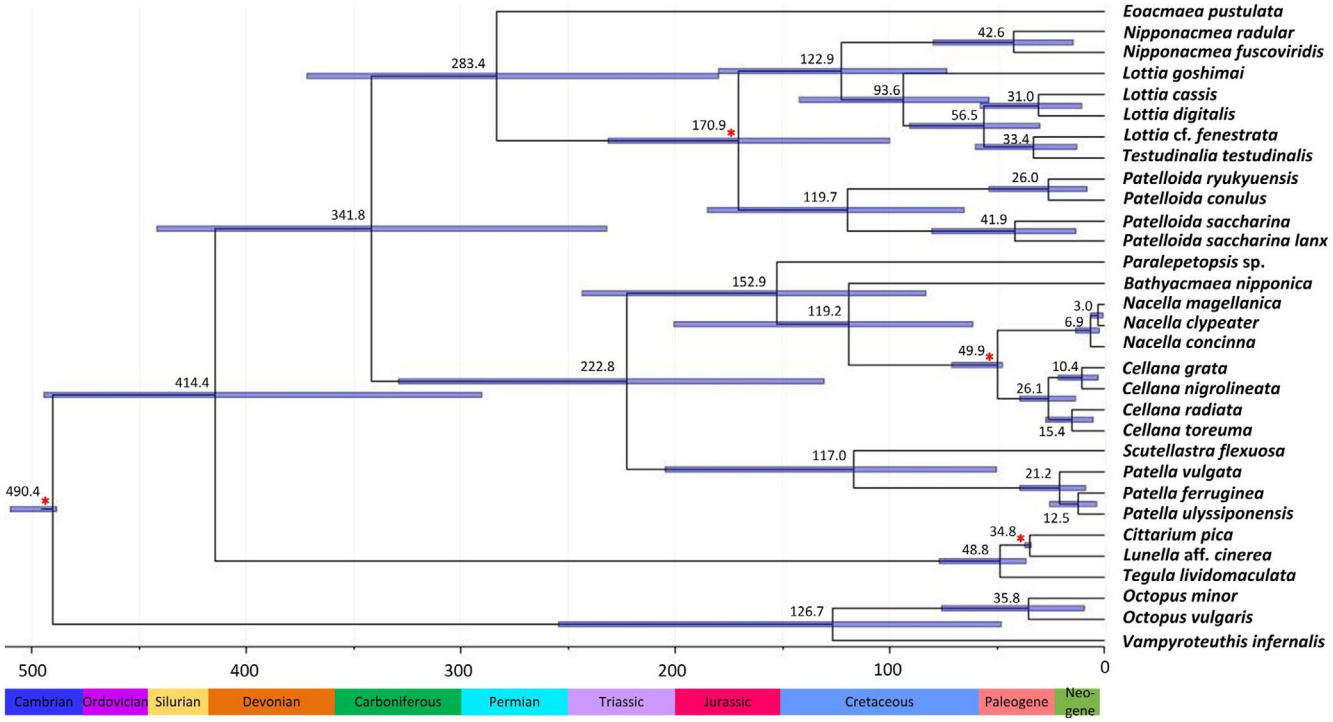

FIGURE 3 Chronogram of the Patellogastropoda based on the amino acid sequences of the concatenated mitochondrial protein-coding genes, and using Bayesian relaxed dating methods (BEAST). Blue bars indicate 95% highest posterior density intervals (HPD), and calibration constraints are indicated with an asterisk on the corresponding nodes. Dates (and credible intervals) are in millions of years

divergence of *Paralepetopsis* sp. from the *B. nipponica* and Nacellidae were dated at the Tithonian (Jurassic) period, which is the greenhouse phase in the Middle Jurassic–Early Cretaceous 'cool' mode (Chumakov & Zharkov, 2002). Warm climate in the period might lead to the splitting of *Paralepetopsis* sp. The origin of *B. nipponica* was dated at the Middle Cretaceous. After a prolonged cooling, the climate warmed significantly across the Aptian–Albian boundary, which might have triggered the emergence of *B. nipponica* (Bottini et al., 2015). The origin of Nacellidae was dated at Upper Eocene. The sea temperature increased gradually from the middle Paleocene to the early Eocene, but marine waters experienced a progressive cooling began from the middle Eocene, which might lead to the emergency of Nacellidae (Kennett & Stott, 1990; Miller, 1992; Miller et al., 1987; Zachos et al., 1993).

5 | CONCLUSIONS

The reconstructed mitogenomic tree alleviated the LBA of *L. digitalis* and provided high resolution of Patellogastropoda phylogenetic relationships. Given its distribution, this result calls for taxonomy revise of superfamily Lottioidea and either *T. testudinalis* or genus *Testudinalia* should be transferred to genus *Lottia*. By comparing the gene order of all sequenced patellogastropod species to the hypothetical ancestral mitochondrial gene order of Gastropoda, Lottiidae, *S. flexuosa* and Nacellidae shown high, intermediate and low levels of gene rearrangement. Within family, the gene order of Lottiids and Nacellids is irregular and conservative, respectively. And the mitochondrial gene order of Nacellidae appears to represent the ancestral state of Patellogastropoda, with 10 translocations and two inversions of tRNAs from the putative ancestral gastropod arrangement. Our results indicate that improve taxon sampling and reconstruct phylogenetic tree based on the amino acids sequences of 13 PCGs can effectively alleviate the LBA of *L. digitalis* and improve our understanding of mitogenomic evolution and phylogenetic relationships in Patellogastropoda.

ACKNOWLEDGEMENT

This work was supported by the National Natural Science Foundation of China under Grant 31772414; Fundamental Research Funds for the Central Universities under Grant 201964001.

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How to cite this article: Xu, T., Qi, L., Kong, L., & Li, Q. (2021). Mitogenomics reveals phylogenetic relationships of Patellogastropoda (Mollusca, Gastropoda) and dynamic gene rearrangements. *Zoologica Scripta*, 00, 1–14. https://doi.org/10.1111/zsc.12524

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