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EDITED BY

Khor Waiho,
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REVIEWED BY

Yingying Ye,
Zhejiang Ocean University, China
Leila Belén Guzmán,
Instituto de Biología Subtropical, Argentina
Jun Liu,
Institute of Deep-Sea Science and
Engineering, Chinese Academy of Sciences
(CAS), China

*CORRESPONDENCE

Joong-Ki Park

✉ jkpark@ewha.ac.kr

†These authors have contributed equally to
this work

SPECIALTY SECTION

This article was submitted to
Marine Fisheries, Aquaculture and Living
Resources,
a section of the journal
Frontiers in Marine Science

RECEIVED 31 December 2022

ACCEPTED 20 March 2023

PUBLISHED 26 April 2023

CITATION

Putri ET, Lee D, Kwak H, Kim Y, Nakano T
and Park J-K (2023) Complete
mitochondrial genomes of the
“Acmaeidae” limpets provide new insights
into the internal phylogeny of the
Patellogastropoda (Mollusca: Gastropoda).
Front. Mar. Sci. 10:1134991.
doi: 10.3389/fmars.2023.1134991

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Complete mitochondrial genomes of the “Acmaeidae” limpets provide new insights into the internal phylogeny of the Patellogastropoda (Mollusca: Gastropoda)

Eggy Triana Putri^{1†}, Damin Lee^{1†}, Haena Kwak¹, Yukyung Kim¹,
Tomoyuki Nakano² and Joong-Ki Park^{1,3*}

¹Division of EcoScience, Ewha Womans University, Seoul, Republic of Korea, ²Seto Marine Biological
Laboratory, Kyoto University, Nishimuro, Wakayama, Japan, ³Natural History Museum, Ewha Womans
University, Seoul, Republic of Korea

The subclass Patellogastropoda (called “true limpets”) is one of the most primitive groups of the Gastropoda and contains approximately 350 species worldwide. Within this subclass, internal phylogeny among family members, including relationships of the “Acmaeidae” with other patellogastropod families, remains incompletely clarified. Here, we newly determined two complete mitochondrial genome sequences of “Acmaeidae” (*Acmaea mitra* and *Niveotectura pallida*) and one sequence from Lottiidae species (*Discurria insessa*) and combined them with mitochondrial genome sequences of 20 other published limpet species for phylogenetic analysis of the sequence dataset (nucleotides and amino acids) of 13 protein-coding genes using maximum likelihood and Bayesian inference methods. The resulting phylogenetic trees showed monophyly of Patellogastropoda species that were subsequently subdivided into two clades [clade I (Nacellidae, Pectinodontidae, Acmaeidae, and Patellidae) and clade II (Eoacmaeidae and Lottiidae)]. The sister relationship between the Acmaeidae and Pectinodontidae species revealed by phylogenetic analysis was also supported by sharing their similar gene arrangement patterns, which differ substantially from those of clade II members including the Lottiidae species. The polyphyletic relationship between Acmaeidae (grouped with Pectinodontidae as a sister taxon in clade I) and Lottiidae species (grouped with Eoacmaeidae in clade II) corroborates that they are phylogenetically distinct from each other. This mitochondrial genome phylogeny contradicts previous morphology-based hypotheses, yet highlights that Acmaeidae and Pectinodontidae are the most closely related. Further in-depth analysis of the complete mitochondrial genome sequences based on a broad range of samples including those from relatively unstudied and/or underrepresented taxa is required to fully understand the mitochondrial genome evolution and a more comprehensive phylogeny among the major groups of the Patellogastropoda.

KEYWORDS

mitochondrial genome, Acmaeidae, Pectinodontidae, Lottiidae, Patellogastropoda
phylogeny

1 Introduction

Acmaeidae is a family of Patellogastropoda (called “true limpets”) that represents one of the most primitive groups of the Gastropoda and contains approximately 350 species worldwide. They are found in lower intertidal to subtidal zones and constitute macrobenthic communities on coastal rocky shores, playing an important role in littoral ecosystems (Branch, 1985). This family is characterized by having a foliated shell with a fibrillar prismatic shell microstructure, one feather-shaped ctenidium, and a single pair of tricuspid radular teeth (Lindberg, 1998). Over the last century, external shell morphology has been the feature most widely used for both species-level taxonomy and investigating the internal phylogeny of the Patellogastropoda. However, morphological traits of limpet species are highly variable with different morphs from the ontogenic or ecophenotypic origin, resulting in substantial taxonomic confusion and disagreement on their phylogenetic relationships (Nakano and Ozawa, 2007). Soft body anatomy including radula characters has also been considered phylogenetically informative and widely used to investigate the relationships among the Patellogastropoda families in the past decades (Lindberg, 1998; Sasaki, 1998). Nevertheless, the taxonomic confusion associated with the absence of key characters for defining the family boundaries, and convergent evolution and ecophenotypic plasticity in their morphological features has resulted in different classification systems of the Patellogastropoda over the last decades (Sasaki et al., 2006; Nakano and Sasaki, 2011).

Against this background, phylogenetic relationships including the taxonomic status of the “Acmaeidae” with the Patellogastropoda have remained unresolved from previous studies that employed different sources of taxonomic information, such as shell characters, internal morphology, and molecular sequence data [Dall, 1876; Lindberg, 1998; Sasaki, 1998 (morphology); Harasewych and McArthur, 2000; Nakano and Ozawa, 2007 (molecular data)]. The Acmaeidae was traditionally divided into two subfamilies, Acameinae and Pectinodontinae, based on morphological perspectives (Lindberg, 1998), but these two subfamilies (*sensu* Lindberg, 1998) were not found as a monophyletic group by subsequent molecular analysis of three mitochondrial genes (mtDNA 12S rDNA, 16S rDNA, and *cox1*; Nakano and Ozawa, 2007). Moreover, despite this molecular study reporting that the Acmaeidae species (*Acmaea mitra*, *Niveotectura pallida*, and *Erginus sybaritica*) were grouped with Lottiidae species, their relationships were not concordant with other studies using the morphology or using different molecular markers, i.e., nuclear vs. mitochondrial DNA: The Acmaeidae species were included as a subgroup of Lottiidae based on their morphological similarity with lottiid species (e.g., shell microstructure, radula characters, and presence of a ctenidium) (Lindberg, 1998), but they were revived as a separate family (Nakano and Sasaki, 2011) from the Lottiidae. These phylogenetic uncertainties of the “Acmaeidae” necessitate further study using different molecular evidence to elucidate their relationships with other Patellogastropoda families.

During recent decades, comparative analysis of the complete mitochondrial genome information (i.e., nucleotide sequence and

gene order data) has been widely used for investigating the phylogenetic relationships in many molluscan groups including Patellogastropoda groups where interrelationships were not fully resolved (Uribe et al., 2019; Feng et al., 2020; Xu et al., 2022). In this study, we aimed to (1) resolve phylogenetic uncertainty of the Acmaeidae and (2) extend our knowledge of mitochondrial genome diversity among major groups of the Patellogastropoda. For this, we determined two complete genome sequences of “Acmaeidae” (*Acmaea mitra* and *Niveotectura pallida*), the first representatives from the Acmaeidae, and one complete genome sequence of Lottiidae (*Discurria insessa*), combined them with the mitochondrial genome sequences of 20 other published limpet species plus 12 non-patellogastropod species (including 2 chiton species), and performed phylogenetic analysis based on nucleotide sequence information and gene order data.

2 Materials and methods

2.1 DNA extraction

Genomic DNA from three limpet species (*A. mitra*, *N. pallida*, and *D. insessa*) was extracted from muscle tissues of an ethanol-fixed single individual using E.Z.N.A. mollusk DNA kit (Omega Biotek, Norcross, GA, USA), in accordance with the manufacturer’s instructions, and subjected to next-generation sequencing (NGS) library construction and sequencing.

2.2 Library preparation and next-generation sequencing

NGS sequencing libraries were prepared using Illumina TruSeq DNA library preparation kit and sequenced with a 151 bp paired-end run of the Illumina HiSeq X sequencing platform (Illumina, San Diego, CA, USA).

2.3 Mitochondrial genome assembly and gene annotation

Low-quality reads and adaptor sequences were trimmed using Trimmomatic v0.39 (Bolger et al., 2014). The clean reads of *A. mitra* (clean reads: 76,270,083), *D. insessa* (clean reads: 73,627,056), and *N. pallida* (clean reads: 14,140,656) were *de novo* assembled into circular DNAs using NOVOPlasty V.4.2 (Dierckxsens et al., 2017) and their genes were annotated on the MITOS webserver (Bernt et al., 2013). The protein-coding genes (PCGs) and ribosomal RNA (rRNA) genes were also confirmed by comparing them with those of other Patellogastropoda species using Geneious® v.11.0.11. The tRNA genes for each mtDNA sequence were identified using the tRNAscan-SE program (Lowe and Eddy, 1997) or by manually inspecting potential secondary structures and anticodon sequences.

2.4 Phylogenetic analyses

We performed phylogenetic analyses for 29 mitochondrial genome sequences from 23 Patellogastropoda species (including three newly sequenced in this study) and 4 non-Patellogastropoda (Heterobranchia and Vetigastropoda) species, as well as 2 chiton species as outgroups (Table 1). Nucleotide sequences of 13 protein-coding genes were individually translated to amino acid sequences and multiple-aligned by 'reverse translation' of the aligned protein sequences using RevTrans 1.4 webserver (Wernersson and Pedersen, 2003), and concatenated using MEGA v10.2.6 (Kumar et al., 2018). The best-fit substitution models for nucleotide (NT) and amino acid (AA) sequences for each of the 13 PCGs were identified (see Table 2) using jModelTest (NT; Darriba et al., 2012) and ProtTest 3.4.2 (AA; Darriba et al., 2011) on the CIPRES webserver (Miller et al., 2010). Phylogenetic relationships were reconstructed using Bayesian inference (BI) with MrBayes 3.2.6 (Ronquist et al., 2012) and maximum likelihood (ML) with RAxML V.8.2.9 (Stamatakis, 2014) on the CIPRES portal (Miller et al., 2010). A Markov chain Monte Carlo (MCMC) analysis was performed using two independent runs of 1×10^6 generations with four chains, sampling every 100 generations for the BI tree. Bayesian posterior probability (BPP) values were estimated after discarding the initial 2×10^3 trees that reached a stationary stage of the parameters as burn-in. The bootstrap analysis was also performed with 1,000 replicates for the resulting

2.5 Phylogenetic analysis using gene order information

Pairwise comparisons of the gene order among Patellogastropoda species were conducted to infer the parsimonious rearrangement scenarios (e.g., transpositions, reverse transpositions, reversals and tandem-duplication-random-loss events) using CREx (Bernt et al., 2007) based on common intervals. The MLGO web server was used to determine the phylogeny among Patellogastropoda species based on gene order information (PCGs, rRNAs, and tRNAs) (Hu et al., 2014).

2.6 Divergence time estimation

Divergence times among Patellogastropoda species were estimated based on nucleotide dataset of 13 protein-coding genes using an uncorrected lognormal relaxed clock model with a Yule speciation process as a tree prior in BEAST v2.6.7 (Bouckaert et al., 2019). A calibrated Yule speciation model was used as a tree prior, and the best partition schemes and the best evolution models were estimated in Partition Finder 2 based on BIC. We set the time calibration on three points based on Nacellidae (47.8–56 MYA, million years ago) from Nakano & Ozawa (2007), Patellogastropoda (336–339.4 MYA), and Neoloricata (460–466 MYA) data from the

Paleobiology Database (<https://paleobiodb.org/>). We ran Markov chain for three times, each running for 10×10^7 generations, and sampling every 1,000 generations. The convergence of chains were confirmed with Tracer v1.7.2 (Rambaut et al., 2018) and the first 10% of samples were discarded as burn-in. The results were combined with Log combiner and Tree annotator of the BEAST package. The maximum clade credibility (MCC) tree was visualized in FigTree 1.4.4 (Rambaut, 2018).

3 Results

3.1 Characteristics of mitochondrial genomes of the Acmaeidae

The mitochondrial genomes of the two Acmaeidae species are 17,903 bp (*A. mitra*), 18,670 bp (*N. pallida*) and *D. insessa* mtDNA is 18,225 bp in size, each containing 37 genes (13 PCGs, 22 tRNA genes, and 2 rRNA genes). The *D. insessa* mtDNA has a duplicated copy of *trnM*, a universal feature of Lottiidae species that were determined thus far. All tRNA genes from these three newly determined mitochondrial genomes have a typical clover-leaf secondary structure, with the exception of one tRNA in *N. pallida* (*trnS1*) and three tRNAs in *D. insessa* (*trnS1*, *trnS2*, and *trnE*). Both *trnS1* and *trnS2* lack a DHU stem, whereas the T Ψ C arm is absent in *trnE*. This result is consistent with an earlier study that DHU stems are missing in *trnS1* and *trnS2* in *Lottia goshimai* and *Nipponacmea fuscoviridis* (Feng et al., 2020). Information on the genome organization, overall nucleotide composition, start/termination codons, and codon usage of 13 PCGs for the three species is presented in the supplementary data (Supplementary Tables 1–9). Nucleotide compositions of the three newly sequenced species were 26% A, 36.9% T, 24.2% G, and 12.9% C for *A. mitra*; 28% A, 37.8% T, 22.3% G, and 11.91% C for *N. pallida*; and 30.1% A, 30.3% T, 17.5% G, and 22.1% C for *D. insessa* (see Supplementary Tables 2, 5, 8). In their entire genome sequences, there is a strong bias toward A and T, with the A + T content ranging from 60.4% (*D. insessa*) to 65.8% (*N. pallida*), which is similar to previously reported findings in other Patellogastropoda species (Feng et al., 2020).

3.2 Phylogenetic relationships of the Acmaeidae within the Patellogastropoda

Phylogenetic analyses of nucleotide (NT) and amino acid (AA) sequences using ML and BI methods found an identical tree topology (Figure 1): The resulting trees show that the Patellogastropoda species formed a monophyletic grouping that was strongly supported [76% BP in ML and 1.0 BPP in BI (NT dataset) and 84% BP in ML and 0.99 BPP in BI (AA dataset)], relative to other gastropod groups (e.g., Heterobranchia,

TABLE 1 Complete mitochondrial genomes used for phylogenetic analysis in this study.

Family	Species	Length (bp)	GenBank accession no.	Reference
Acmaeidae	<i>Acmaea mitra</i> *	17,903	OQ133456	This study
	<i>Niveotectura pallida</i> *	18,670	OQ133458	This study
Eoacmaeidae	<i>Eoacmaea</i> sp.	5,801 (partial sequence)	MH916652.1	Uribe et al., 2019
Lottiidae	<i>Discurria insessa</i> *	18,225	OQ133457	This study
	<i>Lottia cassis</i>	18,797	MZ130258	Xu et al., 2022
	<i>Lottia digitalis</i>	26,835	DQ238599.1	Simison et al., 2006
	<i>Lottia goshimai</i>	17,849	MZ766128	Xu et al., 2022
	<i>Nipponacmea fuscoviridis</i>	18,720	MK395167.1	Feng et al., 2020
	<i>Patelloida conulus</i>	17,030	MZ048283	Xu et al., 2022
	<i>Patelloida ryukyuensis</i>	16,319	MZ130310	Xu et al., 2022
	<i>Patelloida saccharina lanx</i>	19,427	MW735838	Xu et al., 2022
Nacellidae	<i>Cellana grata</i>	16,181	MW722939	Xu et al., 2022
	<i>Cellana nigrolineata</i>	16,148	MW727704	Xu et al., 2022
	<i>Cellana radiata</i>	16,194	MH916651.1	Uribe et al., 2019
	<i>Cellana toreuma</i>	16,260	MW716504	Xu et al., 2022
	<i>Nacella clypeater</i>	16,742	KT990124.1	Gaitán-Espitia et al., 2019
	<i>Nacella concinna</i>	16,761	KT990126.1	Gaitán-Espitia et al., 2019
	<i>Nacella magellanica</i>	16,663	KT990125.1	Gaitán-Espitia et al., 2019
Patellidae	<i>Patella ferruginea</i>	14,400	MH916654.1	Uribe et al., 2019
	<i>Patella vulgata</i>	14,808	MH916653.1	Uribe et al., 2019
	<i>Scutellastra flexuosa</i>	15,233	MW735839	Xu et al., 2022
Pectinodontidae	<i>Bathyacmaea lactea</i>	18,446	MW309841.1	Liu et al., 2021
	<i>Bathyacmaea nipponica</i>	16,792	MF095859.1	Sun et al., 2019
Aplysiidae	<i>Aplysia californica</i>	14,117	AY569552.1	Knudsen et al., 2006
Fissurellidae	<i>Diodora graeca</i>	17,209	KT207825.2	Uribe et al., 2016a
	<i>Fissurella volcano</i>	17,575	JN790612.1	Unpublished data
Hydrocenidae	<i>Georissa banguyensis</i>	15,267	KU342664.1	Uribe et al., 2016b
Neritidae	<i>Nerita melanotragus</i>	15,261	GU810158.1	Castro and Colgan, 2010
Onchidiidae	<i>Onchidella celtica</i>	14,150	AY345048.2	Grande et al., 2004
Peltospiridae	<i>Chrysomallon squamiferum</i>	15,388	AP013032.1	Nakagawa et al., 2014
	<i>Gigantopelta aegis</i>	16,097	MW442948.1	Lan et al., 2021
Pomatiopsidae	<i>Tricula hortensis</i>	15,179	EU440735.1	Unpublished data
Vermetidae	<i>Ceraesignum maximum</i>	15,578	HM174253.1	Rawlings et al., 2010
Acanthochitonidae	<i>Cryptochiton stelleri</i>	15,082	KJ569363.1	Irisarri et al., 2014
Ischnochitonidae	<i>Lepidozona coreanica</i>	16,572	NC_046935.1	Unpublished data

*: newly determined in this study.

TABLE 2 The best-fit models estimated from each of the 13 protein-coding genes of 23 patellogastropod and 6 other gastropod species used for phylogenetic analysis.

Gene	Nucleotide	Amino acid
<i>atp6</i>	GTR+I+G	LG+I+G+F
<i>atp8</i>	TIM3+I+G	HIVb+I+G+F
<i>cytb</i>	TVM+I+G	CpREV+I+G+F
<i>cox1</i>	GTR+I+G	LG+I+G+F
<i>cox2</i>	GTR+I+G	LG+I+G+F
<i>cox3</i>	GTR+I+G	CpREV+G+F
<i>nad1</i>	GTR+I+G	MtArt+I+G+F
<i>nad2</i>	GTR+I+G	LG+I+G+F
<i>nad3</i>	GTR+I+G	MtArt+I+G+F
<i>nad4</i>	TVM+I+G	MtREV+I+G+F
<i>nad4L</i>	TVM+I+G	MtArt+I+G+F
<i>nad5</i>	TVM+I+G	JTT+I+G+F
<i>nad6</i>	GTR+I+G	MtREV+G+F

Vetigastropoda), and were subsequently subdivided into two groups (clades I and II) with high branch support. Inclusion of other gastropod groups (e.g., Neritimorpha, Caenogastropoda, and Neomphalina) in the analysis did not change the internal relationships among Patellogastropoda species (Supplementary Figure 1). Within the Patellogastropoda, clade I is represented by four monophyletic limpet families (Nacellidae, Pectinodontidae, Acmaeidae, and Patellidae), whereas clade II comprises the two remaining families: Eoacmaeidae and Lottiidae. Compared with clade II, clade I was more strongly supported [92% BP in ML and 1.00 BPP in BI (NT dataset) and 97% BP in ML and 1.00 BPP in BI (AA dataset)]. Within clade I, the Patellidae was positioned basal to the group of families Acmaeidae, Pectinodontidae, and Nacellidae, in which the former two families formed a robust sister relationship. The two Acmaeidae species (*A. mitra* and *N. pallida*) were grouped with Pectinodontidae species (*Bathyacmaea lactea* and *B. nipponica*) receiving high supporting values from the NT dataset (93% BP in ML and 1.00 BPP in BI; Figure 1), but relatively moderate support in ML analysis for the AA dataset (72% BP in ML; see high branch support of 1.00 BPP in BI). Within clade II, a partial sequence of *Eoacmaea* sp. representing the family Eoacmaeidae formed a sister relationship with the strongly supported monophyletic Lottiidae including *D. insessa* (newly sequenced) [70% BP in ML and 1.00 BPP in BI (NT dataset); 80% BP in ML and 0.99 BPP in BI (AA dataset)]. Within Lottiidae, the monophyletic *Patelloida* species was sister to the group of *Lottia*, *Discurria*, and *Nipponacmea* species with high branch support (100% BP in ML and 1.00 BP in BI). Phylogenetic relationships among the Patellogastropoda species inferred from inclusion of other gastropod groups in phylogenetic analysis (e.g., Neritimorpha, Caenogastropoda, and Neomphalina; Supplementary Figures 1, 2) were identical to those depicted in Figure 1.

3.3 Gene arrangement patterns among major Patellogastropoda groups

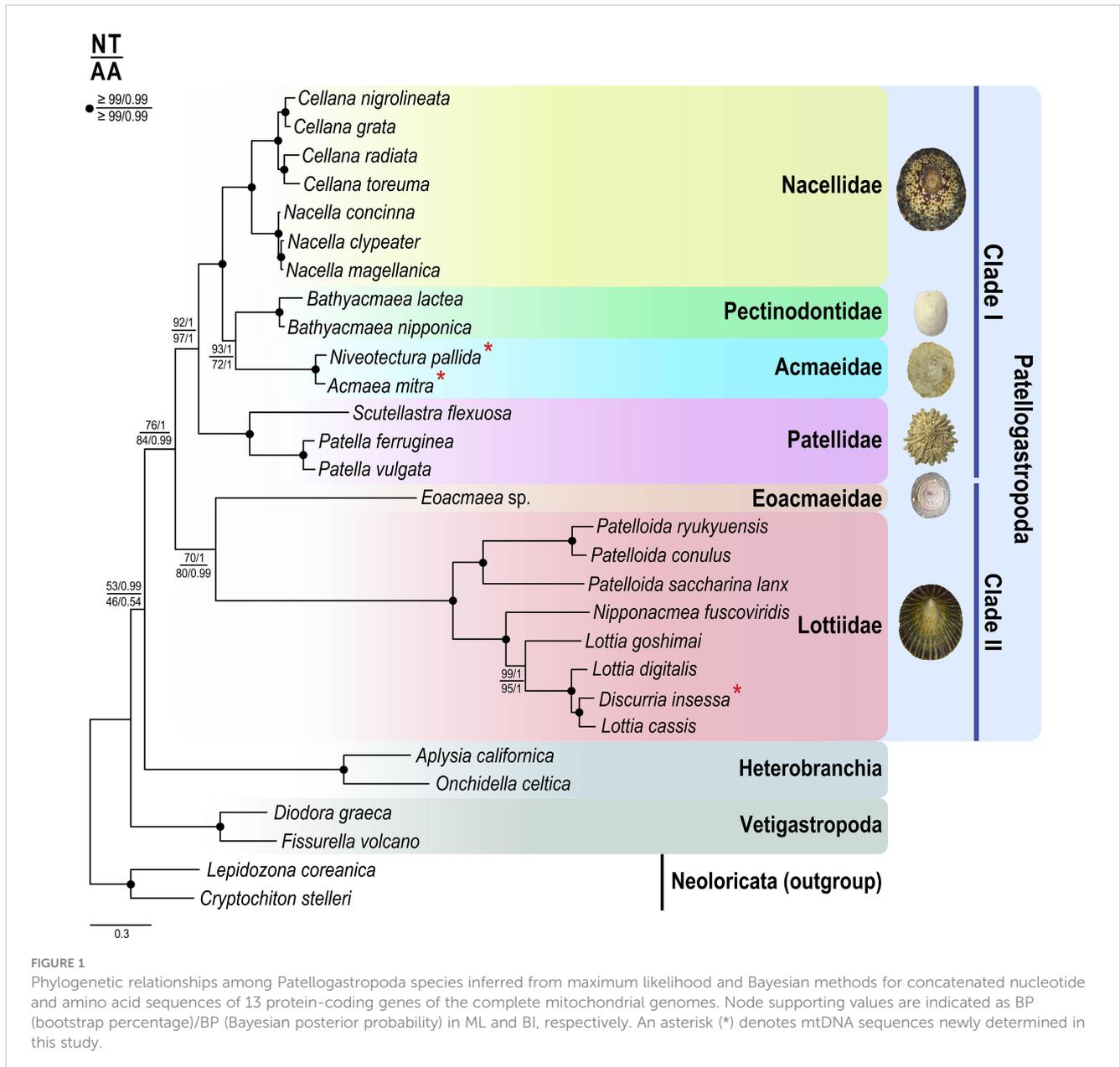
Comparison of gene order information among Patellogastropoda species indicates that the gene arrangement patterns among clade I members are relatively conserved, compared to the clade II members in which gene rearrangements are very substantial (Figure 2). Excluding tRNA genes that are highly variable in their position even between closely related species, the gene arrangement pattern of 13 PCGs and two rRNA genes among the group of three families Nacellidae, Pectinodontidae, and Acmaeidae is identical, but radically differs from that of their sister group Patellidae. By contrast, an extensive gene order rearrangement was observed among clade II members, even between congeneric species belonging to the genera *Patelloida* and *Lottia*. Phylogenetic tree from parsimony analysis based on gene order data (Supplementary Figure 3) is generally consistent with relationships among the family members inferred from phylogenetic analysis of the protein-coding gene sequence data (see Discussion section for the details of their relationships).

3.4 Estimated divergence times among major Patellogastropoda groups

Divergence time estimation using mitochondrial 13 PCGs inferred the origin of Patellogastropoda was dated 337.20 MYA (95% HPD interval 332.60–341.81 MYA) in the middle Carboniferous period (Figure 3). Subsequent divergence among major family groups belonging to clade I and clade II was estimated to begin in the Permian-Triassic boundary (clade I; 257.67 MYA; 185.20–325.34) and in the early Permian period (clade II; 295.84 MYA; 95% HPD interval 255.72–329.43 MYA), respectively. Within clade I, the family Patellidae started branching off first from the common ancestor of the remaining families (Nacellidae, Pectinodontidae, and Acmaeidae) in the Permian-Triassic boundary, followed by lineage splitting of the Acmaeidae from the Pectinodontidae in the Jurassic-Cretaceous boundary (132.62 MYA; 95% HPD interval 65.84–206.46 MYA).

4 Discussion

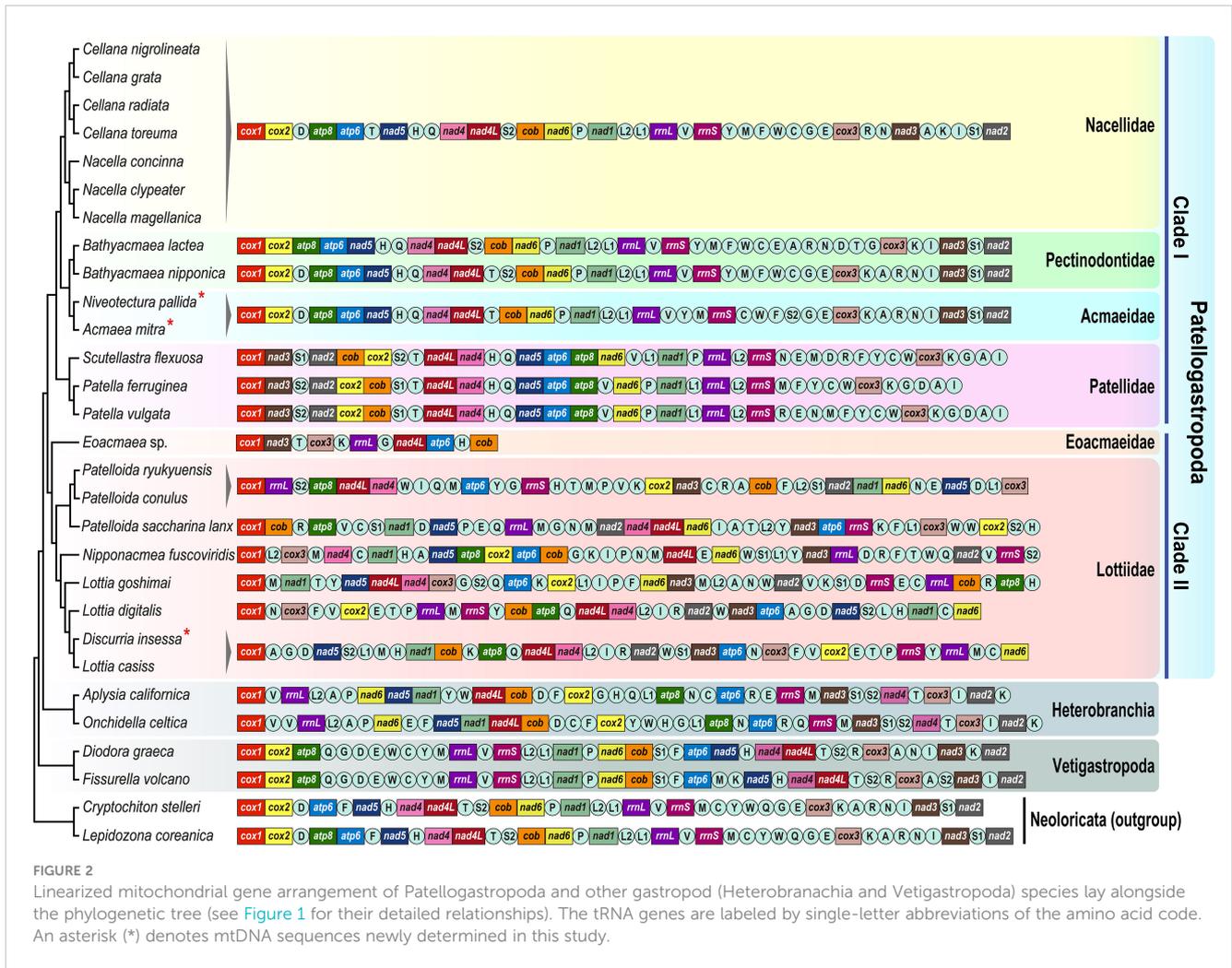
The Acmaeidae is one of the most taxonomically debated Patellogastropoda families, particularly in terms of their taxonomic status and phylogenetic relationships with other groups. Nonetheless, to date there has been only minimal study of this group using molecular data (Nakano and Sasaki, 2011). The relationships inferred in many previous studies did not agree with each other, according to the data sources used in the analysis, such as morphology vs. molecular data, or even between molecular markers (i.e., nuclear vs. mitochondrial DNA). Members of the family Acmaeidae were originally assigned to many lottiid genera and species (e.g., Pilsbry, 1891). However, this scheme was substantially revised based on the shell structure (Lindberg, 1986). After this revision, the genus *Acmaea* was restricted to the type



species *Acmaea mitra*, and the rest of the species formerly belonging to Acmaeidae were transferred to the Lottiidae (Lindberg, 1986). The mitochondrial genome phylogeny reconstructed in this study provides new and invaluable insights into the internal phylogeny of the Patellogastropoda. The monophyletic grouping of Acmaeidae + Pectinodontidae species contradicts the previous analysis of partial gene fragments of mtDNA (Nakano and Ozawa, 2007), in which the “Acmaeinae” was nested within the Lottiidae, whereas the “Pectinodontinae” was clustered with Lepetidae species. These inferred relationships provided a basis of taxonomic treatment in which the “Pectinodontinae” was elevated to the familial rank (Nakano and Ozawa, 2007). Nakano and Sasaki (2011) later corrected their earlier erroneous interpretation derived from sequence contamination of *Acmaea mitra* by placing it as a junior synonym of Lottiidae. The Pectinodontidae still retained its familial status thereafter, awaiting taxonomic clarification from further

analysis. In this regard, our mitochondrial genome tree highlights that Acmaeidae and Pectinodontidae are the most closely related, and their close affinity lends strong support to the previous morphology-based classification in which the pectinodontid species were treated as a subfamily of the family Acmaeidae (Lindberg, 1998). Nevertheless, further extensive studies based on re-evaluation of their morphological characters including shell and soft body anatomy will be necessary to clarify the taxonomic status of the pectinodontid species as a subgroup or confirm them as a taxonomically valid, separate family from the Acmaeidae.

Historically, phylogenetic relationships of the Acmaeidae with other groups have not yet clearly defined (Nakano and Sasaki, 2011). Based on morphological similarity [e.g., shell microstructure, radula characters (Lindberg, 1998), and presence of a left ctenidium (Sasaki, 1998)] and molecular analysis [12S and 16S mtDNA sequence data (Nakano and Ozawa, 2004)], Acmaeidae was once



considered more closely related to Lottiidae than to Patellidae, Nacellidae, and Lepetidae, and was included as a subgroup of Lottiidae. In contrast, later analysis of mtDNA *cox1* and histone H3 sequence data revealed that they were distinct from Lottiidae and were subsequently revived as a separate family from Lottiidae (Nakano and Sasaki, 2011). Polyphyletic relationships between Acmaeidae and Lottiidae species discovered from this mitochondrial genome phylogeny provide strong taxonomic justification of their independent family status. Moreover, the clade I monophyly (Patellidae, Nacellidae, Pectinodontidae + Acmaeidae) that is separated from clade II (Eoacmaeidae + Lottiidae) indicates that Acmaeidae is a member of the superfamily Patelloidea along with other clade I family members, not belongs to the superfamily Lottiioidea. Note that the time-calibrated phylogenetic tree (Figure 3) indicates that the divergence of the Acmaeidae from the Pectinodontidae is estimated to begin in the Jurassic-Cretaceous boundary (132.62 MYA), being much more recent event than the clade I/clade II separation (in the middle Carboniferous; 337.20 MYA) and the Lottiidae/Eoacmaeidae splitting (in the Carboniferous-Permian boundary; 295.84 MYA).

In many metazoan groups including molluscan species, comparison of the gene arrangement of the complete

mitochondrial genome has been widely used and sharing similar synteny pattern observed among certain taxonomic groups can often be interpreted as additional evidence of their phylogenetic affinity (Lee et al., 2019). Likewise, gene order comparison across the limpet species provides additional insights into the phylogenetic relationships and mitochondrial genome evolution among the family members of the Patellogastropoda. The gene arrangement patterns of the two Acmaeidae species (*A. mitra* and *N. pallida*) showed the same gene order. Excluding the tRNAs that are often highly mobile even between closely related species, the arrangement patterns of PCGs and rRNA genes of the clade I members are highly conserved showing very similar synteny blocks within each family member (Figure 2). Notably, the three families Acmaeidae, Pectinodontidae, and Nacellidae display the identical gene arrangement in PCGs and rRNA genes, lending additional support to their phylogenetic relatedness. By contrast, the gene order of the Lottiidae species belonging to clade II is highly variable according to the genus and even between congeneric species (see *Lottia* species), as previously reported (Xu et al., 2022). Moreover, the gene order of Lottiidae species shows substantially different patterns from those of clade I members (see Figure 2 for more details). Inferred relationships among Patellogastropoda species

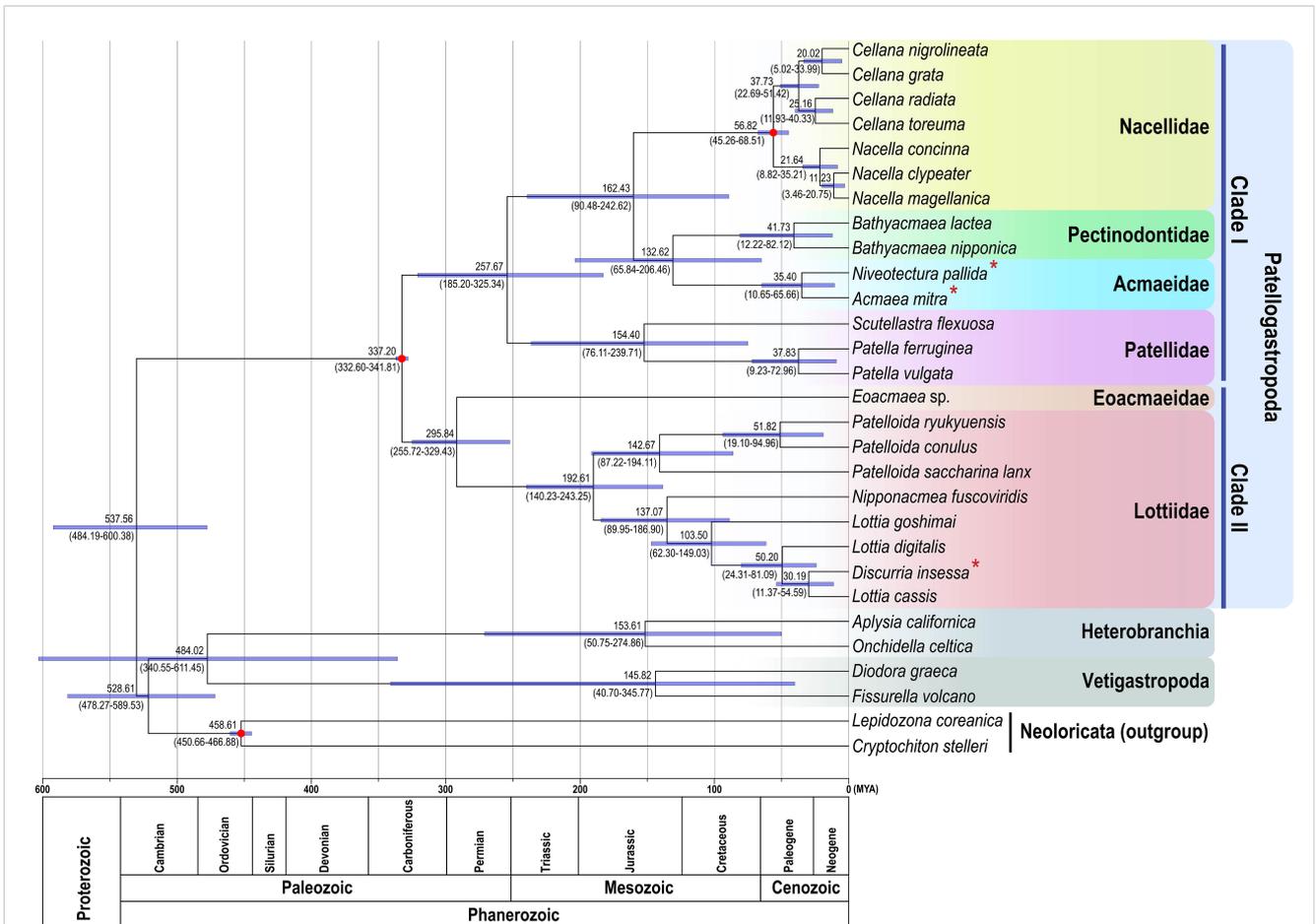


FIGURE 3

Time-calibrated phylogenetic chronogram showing the mean age estimates (with 95% HPD intervals) based on 13 protein coding genes of patellogastropod mitochondrial genomes. Newly determined mtDNA sequences in this study are marked with an asterisk (*), and calibration constraints are represented by red dots on the corresponding nodes.

from gene order analysis (Supplementary Figure 3) agreed with tree topology from the sequence analysis (Figure 1 and Supplementary Figures 1, 2) in that Patellogastropoda species were subdivided into two clades (clade I and clade II) and inferred relationships among clade I family members. One exception is that the two Pectinodontidae species (*B. lactea* and *B. nipponica*) were not grouped as monophyletic in their gene order tree: the former was clustered with Nacellidae species, whereas the latter was grouped with Acmaeidae species (Supplementary Figure 3). This discrepancy results from *B. lactea* species shows an intermediate pattern between the Nacellidae and Acmaeidae in some tRNA synteny blocks. Within clade II, most of branches leading to the Lottiidae species were unresolved and showed extremely long branches that are ascribed to their extensive gene rearrangement events between the Lottiidae species. In addition, it is also interesting to note that the Acmaeidae and one Pectinodontidae species (*B. nipponica*) retain the same consecutive tRNA block of “tRNA KARNI” (*trnK*, *trnA*, *trnR*, *trnN*, and *trnI*), which has long been considered an ancestral state across the major clades of the Gastropoda including other subclasses (Uribe et al., 2016b; Gaitán-Espitia et al., 2019) and outgroup (chiton) species (Irisarri et al., 2014). At present, we are unable to precisely address the

evolutionary history of gene arrangement patterns across the patellogastropods. To this end, in-depth analysis of complete mitochondrial genome sequences based on a full breadth of taxon coverage from relatively unstudied and/or underrepresented taxa is required to fully understand the mitochondrial genome evolution and a more comprehensive phylogeny among the major groups of the Patellogastropoda.

Data availability statement

The data presented in the study are deposited on GenBank (accession numbers: OQ133456, OQ133457 and OQ133458).

Author contributions

J-KP designed and led the study. EP, DL, HK, YK collected samples, conducted the laboratory work, and performed phylogenetic analysis of the sequence data. EP, TN, and J-KP wrote the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the management of Marine Fishery Bio-resources Center (2023) funded by the National Marine Biodiversity Institute of Korea (MABIK) and the Basic Science Research Program of the National Research Foundation of Korea (NRF) funded by the Ministry of Education (grant no. 2020R1A2C2005393).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2023.1134991/full#supplementary-material>

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