Sequence comparison of the mitochondrial genomes of five brackish water species of the family Neritidae: Phylogenetic implications and divergence time estimation

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Abstract
Neritids are ancient gastropod species which can live in marine, brackish water, and freshwater environments. In this study, we sequenced and annotated the mitochondrial genomes of five brackish water neritids (i.e., Clithon corona, Clithon lentiginosum, Clithon squarrosum, Neritina iris, and Septaria lineata). The mitogenomes ranged from 15,618 to 15,975 bp, and all contain 13 protein-coding genes (PCGs), 22 tRNA genes, and two rRNA genes, with a closed ring structure. We calculated the Ka/Ks values of all 13 PCGs of Neritidae species, all ratios are less than 1, under purification selection. Phylogenetic analysis of the 13 PCGs showed that Neritimorpha is a sister group with Vetigastropoda and Caenogastropoda, genus Clithon is a sister group with Neritina and Septaria. Estimation of divergence time for all species of Neritidae showed that the main differentiation of Neritidae occurred in Cenozoic period (65 Mya), C. corona and C. lentiginosum were differentiated in the Cenozoic Neogene, the other three species diverged in the Cenozoic Paleogene. These results will help to better understand the evolutionary position of Neritidae and provide reference for further phylogenetic research on Neritidae species.

Keywords
divergence time, mitogenome, Neritid, phylogenetic

Taxonomy Classification
Genomics; Phylogenetics
1 | INTRODUCTION

Neritidae (Gastropoda: Neritimorpha: Cycloneritida) is one of the most diverse taxa in the Neritimorpha (Rafinesque, 1815). At present, there are 16 genera, comprising around 280 species (Hamish et al., 2007), with about 40 species having been found on the southeast coast of China before 2008 (Zhang, 2008). The fossil record of neritids dates back to the late Cretaceous (Kano, 2002), showing ecological radiation and extreme diversity in form. Neritids occur mainly in intertidal zone (Sasaki et al., 2002). They are euryhaline, and can live in marine, brackish water, and freshwater ecosystems. Nerita species are almost exclusively found in marine environments, Clithon and Neritina animals are mostly found in freshwater and brackish water environments (Tan & Clements, 2008). There have been at least five or six evolutionary transitions from hypersaline environments to freshwater in the evolutionary history of Neritidae (Frey, 2010; Holthuis, 1995). However, most freshwater lineages retain a dispersed planktonic marine larval stage, in which adults develop, reproduce in rivers, hatch larvae enter the sea, grow into adults, and return to freshwater in a cycle (Abdou et al., 2015).

The metazoan mitochondrial genome (mitogenome) is a double-stranded molecular structure in the form of a closed ring. It usually has 37 coding genes, including 13 protein-coding genes (PCGs), two ribosomal RNA genes (rRNA), 22 transfer ribonucleic acid (tRNA) genes, and a noncoding control region (CR) (Fernández-Silva et al., 2003; Wolstenholme & David, 1992). The mitogenome is characterized by high conservation, lack of extensive recombination, maternal inheritance, and a high mutation rate (Curole & Kocher, 1999; William et al., 2004). Compared with some gene fragments, such as COI (cytochrome c oxidase subunit 1) and 16S rRNA, mitogenome sequences can better elucidate evolutionary relationships between species, it has been widely used in phylogenetic researches (Zardoya & Meyer, 1996).

Next-generation sequencing (NGS) have been widely used in phylogenetic analysis, and the study of Neritidae classification has been ongoing for a long period. However, there are insufficient studies on the mitochondrial data and divergence time of neritids. In this study, we chose five neritid species: Clithon corona, Clithon lentiginosum, Clithon squarrosum, Neritina iris, and Septoria lineata, which can live in both fresh and brackish water environments. After sequencing, assembly, annotation, and analysis of the complete mitogenome, we analyzed their basic characteristics in the five species, calculated the average nonsynonymous to synonymous substitution ratio (Ka/Ks) of 19 Neritidae species, constructed the phylogenetic tree of mitogenomes of Gastropoda to analyze the phylogenetic position and relationship in Neritidae, and speculated the differentiation time of neritids.

2 | MATERIALS AND METHODS

2.1 | Sample collection and DNA extraction

Five species of Neritidae C. corona, N. iris, S. lineata, C. squarrosum, and C. lentiginosum were collected from the coastal area of Huizhou, Guangdong Province, China (Table 1). The preliminary morphological identification of these samples was carried out by consulting the taxonomic experts of the Marine Biological Museum of Zhejiang Ocean University. Store samples in absolute ethanol, take a small piece of fresh foot tissue to extract total DNA by salting-out method (Aljanabi & Martinez, 1997), and store at −20°C.

2.2 | Mitogenome sequencing, assembly, and annotation

The complete mitogenomes of five species were sequenced on the Illumina Hiseq X Ten platform by Originging Bio-pharm Technology Co., Ltd. (Shanghai, China). The Covaris M220 physical method (ultrasonic) was used to fragment the DNA, and the length of the fragments was 300–500 bp. Then, the DNA fragments were purified to construct a sequencing library. The Illumina HiSeq platform was used for sequencing after library quality inspection, and a 10 Gb data volume was used for sequencing. Data quality control was performed by Trimmomatic v0.39 (http://www.usadellab.org/cms/index.php?page=trimmomatic) (Bolger et al., 2014), filter out low-quality reads, duplicated reads, sequences with an “N” rate greater than 10%, and sequencing linker sequences. Clean data with high quality was obtained and the reads of the five species were de novo assembled using NOVOPlasty assembly software (https://github.com/ndierckx/NOVOPlasty) (Dierckxsens et al., 2017). The stack cluster was compared with reference genome in the GenBank database, and majority of the mitogenome sequence information was obtained. Then, the online software MITOS (http://mitos.bio-inf.uni-leipzig.de/index.py) was used for structural and functional annotation and manual correction (Bernt et al., 2013), the complete mitogenome was finally obtained. Sequenced mitogenomes were uploaded to GenBank database at the National Center for Biotechnology Information (NCBI).

2.3 | Sequence analysis

Circular genome visualization of five species was generated using the online CGView server (http://cgview.ca/) (Stothard & Wishart,
2.4 | Phylogenetic analyses

The phylogenetic analyses of the five species were performed using the sequences of complete mitogenomes from 81 species (Table 2). A total of 74 Gastropoda species from Neritimorpha, Vetigastropoda, Caenogastropoda, Patello gastropoda, and Heterobranchia were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) for phylogenetic analysis. Two Veneridae species Dosinia tro nd (NC_037917) and Dosinia japonica (NC_038063) were used as outgroups. The sequence of the 13 PCGs of each species were identified using DAMBE 7 (Xia, 2018), the PCGs of each sample were concatenated together in the same order, the tree building sequence set was obtained by combining them in a unified sequence. The PCGs sequences of these 81 species were aligned using ClustalW of MEGA-X. Nucleotide substitution saturation was analyzed using DAMBE 7 to evaluate whether these sequences were suitable for phylogenetic tree construction.

The Bayesian inference (BI) method of the program MrBayes 3.2.7a (Ronquist et al., 2012) and the maximum likelihood (ML) method of IQ-tree 2.1.3 (Minh et al., 2020) were used to analyze the phylogenetic relationships. The Bayesian method model measurement firstly used PAUP 4 (Swofford, 2002) software for format conversion, and then used MRMTGUI (Nhuin, 2005) software to associate PAUP 4, ModelTest 3.7 (Posada, 2005) and MRModelTest 2.3 (Nylander et al., 2004) programs to determine the best alternative model under the Akaike information criterion (AIC) as GTR + I + G. BI analysis was performed using two Markov chain Monte Carlo (MCMC) run with 2 million generations, and sampling was performed once every 10000 generations. The first 25% of trees were discarded as burn-in, and convergence for independent operation was evaluated using the mean standard deviation of the splitting frequency (≤0.01).

The ML tree best fit replacement model (GTR + F + I + G4) selected by Bayesian information criterion (BIC) using ModelFinder (Kalyaanamoorthy et al., 2017), setting the boot copy number with 1000 ultra-fast bootstraps in order to reconstruct the consensus tree. Finally, the phylogenetic tree was viewed, edited, and visualized using the Figtree 1.4.4 (Rambaut, 2018) software.

3 | RESULTS AND DISCUSSION

3.1 | Genome structure, composition, and skewness

The complete mitogenome sequences of the five Nerita species consist of 15,975 bp (C. corona), 15,885 bp (C. lentiginosum), 15,905 bp (C. squarrosus), 15,618 bp (N. iris), and 15,697 bp (S. lineata), the smallest being for N. iris and the largest for C. corona. The GenBank accession numbers are MZ189741, MZ152905, MZ297477, MZ189742, and MZ315041, respectively (Figure 1). They are all closed, circular, double-stranded DNA molecules, containing 37 typical coding genes, including 13 PCGs, 22 tRNA genes, two rRNA genes (12S rRNA and 16S rRNA), and a control region (CR). Among them, 15 genes (seven PCGs and eight tRNA genes) are located on the heavy chain, while the others were located on the light chain (Figure 1). The longest gene was ND5, with a length of 1702 to 1717 bp, and the shortest was the ATP8 gene, with a consistent length of only 165 bp (Table 3).

In the five mitogenomes at present study, the average AT content was higher than CG, with a bias of 64.90%. The average AT-skew was ~0.0545, and GC-skew was 0.1486 (Table 4). The base content of As was lower than that of Ts, and the base content of Gs was higher than that of Cs. In general, the average content of each species in...
| Subclass             | Family                  | Species                  | Size (bp) | Accession no. |
|---------------------|-------------------------|--------------------------|-----------|---------------|
| Heterobranchia      | Placobranchidae         | Elysia cornigera         | 14,118    | NC_035489     |
|                     | Plakobranchus ocellatus | 14,173                   | AP014544  |               |
| Aplysiidae          | Aplysia dactylo molea  | 14,128                   | DQ991927  |               |
|                     | Aplysia kurodai         | 14,131                   | KF148053  |               |
| Onchidiidae         | Peronia peroni          | 13,968                   | JN619346  |               |
|                     | Platevindex mortoni     | 13,991                   | NC_013934 |               |
| Ellobiidae          | Myosotella myosotis     | 14,246                   | NC_012434 |               |
|                     | Auriculinella bidentata | 14,135                   | JN606066  |               |
|                     | Ellobium chinense       | 13,979                   | NC_034292 |               |
|                     | Carychi um tridentatum  | 13,908                   | KT696545  |               |
|                     | Ovatella vulc oni       | 14,274                   | JN615139  |               |
| Volvatellidae       | Ascobulla fragilis      | 14,745                   | AY345022  |               |
| Siphonariidae       | Siphonaria gigas        | 14,514                   | NC_016188 |               |
|                     | Siphonaria pectinata    | 14,065                   | NC_012383 |               |
| Polyceridae         | Nembrotha kubaryana     | 14,395                   | NC_034920 |               |
|                     | Roboaistra europaea     | 14,472                   | NC_004321 |               |
|                     | Notodoris gardineri     | 14,424                   | NC_015111 |               |
| Patellogastropoda   | Nacellidae              | Nacella clypeater        | 16,742    | KT990124      |
|                     |                         | Nacella magellanica      | 16,663    | KT990125      |
|                     |                         | Nacella concinna         | 16,761    | KT990126      |
|                     |                         | Cellana grata           | 16,181    | MW722939      |
|                     |                         | Cellana nigrolineata    | 16,153    | LC600801      |
|                     |                         | Cellana radiata         | 16,194    | MH916651      |
|                     | Patella ferruginea      | 14,400                   | MH916654  |               |
|                     | Patella pellucida       | 14,949                   | OU795045.1|               |
|                     | Patella vulgata         | 14,808                   | MH916653  |               |
| Pectinodontidae     | Bathycmaela lactea     | 18,446                   | MW309841  |               |
|                     | Bathycmaela nipponica   | 16,792                   | MF095859  |               |
| Caenogastropoda     | Muricidae               | Ceratostoma burnettii    | 15,334    | NC_046569     |
|                     |                         | Ceratostoma roriflum     | 15,338    | MK411750      |
|                     |                         | Ocinebrellus falcatus    | 15,326    | NC_046052     |
|                     |                         | Boreotrophon candelabrum| 15,265    | NC_046505     |
|                     | Conidae                 | Conus betulinus          | 16,240    | NC_039922     |
|                     |                         | Conus tulipa             | 15,756    | KR006970      |
|                     | Naticidae               | Euspira silva            | 15,315    | NC_046593     |
|                     |                         | Euspira pila             | 15,244    | NC_046703     |
|                     |                         | Mammilla kurodai        | 15,309    | NC_046596     |
| Pomatiopsidae       | Oncomelania quadrasi    | 15,184                   | LC276227  |               |
| Muricidae            | Chicoreus torrefactus   | 15,359                   | NC_039164 |               |
|                     | Indothis lacer          | 15,272                   | NC_037221 |               |
|                     | Rapana venosa           | 15,272                   | NC_011193 |               |
|                     | Menathais tuberosa      | 15,294                   | NC_031405 |               |
| Clavatulidae         | Turricula nelliae spuria| 16,453                   | MK251986  |               |
|                     | Turritella bacillum     | 15,868                   | NC_029717 |               |
the complete mitogenome was T > A > G > C (Table 3), which is consistent with the reported complete neritids mitogenomes (Arquez et al., 2014; Feng et al., 2020, 2021).

### 3.2 | Protein-coding genes and codon usage

The mitogenome of the Neritidae in this study contains 13 PCGs, including a cytochrome b (Cyt b), two ATPases (ATP6 and ATP8), three cytochrome oxidases (COI–III), and seven NADH dehydrogenases (ND1–6 and ND4L). The length of the PCGs in these five species is between 11,054 and 11,140 bp (Table 3). The base composition of these species also showed a high AT bias, with the highest AT content being seen in S. lineata, at 65.75%. The AT bias values of each species were negative, in addition to N. iris at −0.07, the values of the other four species are −0.05, with the T base content being higher than that of the A base. In these five neritid species, the start codon was ATN, almost all genes initiated with ATG, and only a few genes initiated with ATA (Table 5). The majority of the 13 PCGs terminated with TAG or TAA as stop codons, and some of the PCGs terminated with T as an incomplete codon, which was often found in ND2 and ND5. This incomplete stop codon was usually

### Table 2 (Continued)

| Subclass            | Family       | Species                | Size (bp) | Accession no. |
|---------------------|--------------|------------------------|-----------|---------------|
| Vetigastropoda      | Turbinidae   | Angaria neglecta       | 19,470    | NC_028707     |
|                     |              | Astratium haematragum  | 16,310    | NC_031858     |
|                     |              | Bolma rugosa           | 17,432    | NC_029366     |
|                     |              | Lunella granulate      | 17,190    | NC_031857     |
| Tegulidae           | Chlorostoma argyrostromum | 17,780  | KX298892   |
|                     | Omphalius nigerinus       | 17,755    | NC_031862   |
|                     | Tegula brunnea          | 17,690    | NC_016954   |
|                     | Tegula lividomaculata   | 17,375    | NC_029367   |
| Haliotiidae         | Haliotis iris          | 17,131    | NC_031361   |
| Trochidae           | Gibbula umbilicalis     | 16,277    | NC_035682   |
|                     | Monodonta labio         | 16,440    | MK240320    |
|                     | Stomatella planulata    | 17,151    | NC_031861   |
|                     | Umbonium thomasi        | 15,998    | MH729882    |
| Peltospiridae       | Chrysomallon squamiferum| 15,388    | AP013032    |
|                     | Gigantopelta aegis      | 15,176    | MT312227    |
| Phasianellidae      | Phasianella solida      | 16,698    | NC_028709   |
| Neritimorpha        | Neritidae              | Clithon corona*       | 15,975    | MZ189741     |
|                     |                        | Clithon lentiginosum*  | 15,885    | MZ152905     |
|                     |                        | Clithon squarrosus*    | 15,905    | MZ297477     |
|                     |                        | Clithon ovalaniense    | 15,705    | MT568501     |
|                     |                        | Clithon retroptictus   | 15,802    | NC_037238    |
|                     |                        | Clithon sowerbianum    | 15,919    | MT230542     |
|                     |                        | Nerita iris*           | 15,678    | MZ189742     |
|                     |                        | Neritina violacea      | 15,710    | KY021066     |
|                     |                        | Septaria lineata*      | 15,697    | MZ315041     |
|                     |                        | Nerita albigilla       | 15,314    | MK516738     |
|                     |                        | Nerita balteata        | 15,571    | MN477253     |
|                     |                        | Nerita chamaeleon      | 15,716    | MT161611     |
|                     |                        | Nerita undata          | 15,583    | MN477254     |
|                     |                        | Nerita versicolor      | 15,866    | KF728890     |
|                     |                        | Nerita fulgurans       | 15,343    | KF728888     |
|                     |                        | Nerita tessellata      | 15,741    | KF728889     |
|                     |                        | Nerita japonica        | 15,875    | MN747116     |
|                     |                        | Nerita melanotragus    | 15,261    | GU810158     |
|                     |                        | Nerita yoldii          | 15,719    | MK395169     |
supplemented during transcription to obtain a complete stop codon T(AA) (Ojala et al., 1981).

The amino acid composition used in PCGs was relatively similar in all five species (Figure 2). The use of Leu, Lys, Ser, Phe, and Val were relatively frequent, and His and Arg were the least common amino acids. Comparing the relative synonymous codon usage (RSCU) of five species, the result showed that the average frequency of GCU (Ala), CCU (Pro), UUA (Leu2), and ACU (Thr) codons were higher than others. The amino acid content and codon usage of the 13 PCGs in these five species are similar.

3.3 | Transfer RNAs, ribosomal RNAs, and CR

Like other complete neritids mitogenomes, there are 22 tRNA genes in these five species, including two larger regions: MYCWQGE (tRNA-Met, Tyr, Cys, Trp, Gln, Gly, Glu) and KARNI (tRNA-Lys, Ala, Arg, Asn, Ile) between 12S rRNA and ND3, and separated by COIII gene. The other ten tRNAs are scattered between PCGs and rRNAs (Figure 1, Table 6). The average total length of the tRNAs is 1467 bp, ranging from 56 to 72 bp (Tables 4 and 7). All of the tRNAs show significant AT base bias, with an AT content of 63.23%. The AT-skew and GC-skew are -0.0187 and 0.1725, respectively, showing a slight bias toward the use of T and a large bias toward C (Table 4).

The average length of the rRNAs is 2198 bp, with the shortest lengths of 16sRNA and 12sRNA being 1328 and 864 bp, respectively (Tables 4 and 7). These also show an AT base bias, with an AT content of 67.16%. Both the AT-skew (0.0841) and GC-skew (0.0405) are positive, indicating a bias toward A and G.

In the complete mitogenome of the Neritidae, the control region (CR) is the largest noncoding region, and the mitochondrial CR of all neritid species in this study was located between tRNA-Glu and COI, with a length of 527–891 bp (Table 6). This area usually presents a high AT bias, being an A + T rich area. This is an essential element involved in mitogenome replication and transcription initiation (Fernández-Silva et al., 2003).

3.4 | Ka/Ks

Ka/Ks has been used as an effective way to understand the dynamic evolution of protein-coding genes. Therefore, the Ka/Ks ratios of the 13 PCGs were calculated using the 19 sequenced Neritidae species in order to study the relationship between evolution and selection pressure (Figure 3). The results showed that the Ka/Ks ratios of the PCGs range from 0.053 for COI to 0.712 for ND6. COI has the lowest Ka/Ks value, suggesting that COI is under the lowest selective pressure to conserve the protein sequence. It is therefore widely used as a potential molecular marker in species identification and phylogenetic studies (Astrin et al., 2016).

In general, a gene is considered to be positively selected only when the Ka/Ks ratio is greater than 1. The majority of the 13 PCGs genes of the species involved in this study had relatively lower Ka/
| Table 3: Composition and skewness in the mitogenomes of five neritid species |
|-------------------------------------------------|
| **Mitogenome** | **Size(bp)** | **A(%)** | **T(%)** | **G(%)** | **C(%)** | **A+T(%)** | **G+C(%)** | **A-T skew** | **T-A skew** |
|-----------------|--------------|----------|----------|----------|----------|------------|------------|-------------|-------------|
| **Mitogenome**  | 15,975       | 30.66    | 34.06    | 20.24    | 15.04    | 64.72      | 35.28      | 0.05         | 0.29         |
| **COI**         | 15,975       | 30.66    | 34.06    | 20.24    | 15.04    | 64.72      | 35.28      | 0.05         | 0.29         |
| **COII**        | 15,975       | 30.66    | 34.06    | 20.24    | 15.04    | 64.72      | 35.28      | 0.05         | 0.29         |
| **ATP8**        | 15,975       | 30.66    | 34.06    | 20.24    | 15.04    | 64.72      | 35.28      | 0.05         | 0.29         |
| **ATP6**        | 15,975       | 30.66    | 34.06    | 20.24    | 15.04    | 64.72      | 35.28      | 0.05         | 0.29         |
| **COIII**       | 15,975       | 30.66    | 34.06    | 20.24    | 15.04    | 64.72      | 35.28      | 0.05         | 0.29         |
| **ND3**         | 15,975       | 30.66    | 34.06    | 20.24    | 15.04    | 64.72      | 35.28      | 0.05         | 0.29         |
| **ND1**         | 15,975       | 30.66    | 34.06    | 20.24    | 15.04    | 64.72      | 35.28      | 0.05         | 0.29         |
| **ND5**         | 15,975       | 30.66    | 34.06    | 20.24    | 15.04    | 64.72      | 35.28      | 0.05         | 0.29         |
| **ND4**         | 15,975       | 30.66    | 34.06    | 20.24    | 15.04    | 64.72      | 35.28      | 0.05         | 0.29         |
| **ND4L**        | 15,975       | 30.66    | 34.06    | 20.24    | 15.04    | 64.72      | 35.28      | 0.05         | 0.29         |
| **ND6**         | 15,975       | 30.66    | 34.06    | 20.24    | 15.04    | 64.72      | 35.28      | 0.05         | 0.29         |
| **Cytb**        | 15,975       | 30.66    | 34.06    | 20.24    | 15.04    | 64.72      | 35.28      | 0.05         | 0.29         |
| **ND2**         | 15,975       | 30.66    | 34.06    | 20.24    | 15.04    | 64.72      | 35.28      | 0.05         | 0.29         |

(Continues)
| S1               | Size(bp) | A(%)  | T(%)  | G(%)  | C(%)  | A+T(%) | AT-skew | Size(bp) | A(%)  | T(%)  | G(%)  | C(%)  | A+T(%) | AT-skew |
|------------------|----------|-------|-------|-------|-------|--------|---------|----------|-------|-------|-------|-------|--------|---------|
| tRNAs            | 1471     | 30.80 | 31.61 | 22.16 | 15.43 | 62.41  | -0.01   | 1417     | 31.12 | 32.67 | 21.38 | 14.82 | 63.79  | -0.02   |
| rRNAs            | 2204     | 35.53 | 30.54 | 17.60 | 16.33 | 66.07  | 0.08    | 2197     | 36.55 | 30.45 | 17.25 | 15.75 | 67.00  | 0.09    |
| PCGs             | 11,140   | 25.89 | 37.35 | 18.23 | 18.53 | 63.24  | -0.18   | 11,078   | 25.46 | 38.02 | 18.51 | 18.01 | 63.48  | -0.20   |
| Mitogenome       | 15,697   |       |       |       |       |        |         |          |       |       |       |       |        |         |
| COI              | 1548     |       |       |       |       |        |         |          |       |       |       |       |        |         |
| COII             | 690      |       |       |       |       |        |         |          |       |       |       |       |        |         |
| ATP8             | 165      |       |       |       |       |        |         |          |       |       |       |       |        |         |
| ATP6             | 702      |       |       |       |       |        |         |          |       |       |       |       |        |         |
| COIII            | 780      |       |       |       |       |        |         |          |       |       |       |       |        |         |
| ND3              | 354      |       |       |       |       |        |         |          |       |       |       |       |        |         |
| ND1              | 933      |       |       |       |       |        |         |          |       |       |       |       |        |         |
| ND5              | 1716     |       |       |       |       |        |         |          |       |       |       |       |        |         |
| ND4              | 1323     |       |       |       |       |        |         |          |       |       |       |       |        |         |
| ND4L             | 294      |       |       |       |       |        |         |          |       |       |       |       |        |         |
| ND6              | 495      |       |       |       |       |        |         |          |       |       |       |       |        |         |
| Cytb             | 1137     |       |       |       |       |        |         |          |       |       |       |       |        |         |
| ND2              | 1003     |       |       |       |       |        |         |          |       |       |       |       |        |         |
| tRNAs            | 1478     |       |       |       |       |        |         |          |       |       |       |       |        |         |
| rRNAs            | 2204     |       |       |       |       |        |         |          |       |       |       |       |        |         |
| PCGs             | 11,140   |       |       |       |       |        |         |          |       |       |       |       |        |         |
Ks ratios, ratio is less than 1. Therefore, we suggest that these PCGs may be under the influence of purification selection.

### 3.5 Phylogenetic relationships

The 13 PCGs of the mitogenome of 79 species from five subclasses of Gastropoda (Vetigastropoda, Caenogramopoda, Neritimorpha, Patellogramopoda, and Heterobranchia) and other two species as outgroups were used to construct phylogenetic trees (Figure 4, Table 2). The result showed that the ML tree and BI tree have a consistent topological structure, therefore, only the topology of BI tree was displayed, with strong bootstrapping for the ML tree and posterior probability values.

Our phylogenetic analysis showed that Neritimorpha is closely related to Caenogastropoda and Patellogastropoda, five subclasses within the Gastropoda show the following relationship: (Vetigastropoda + Caenogastropoda + Neritimorpha + Patellogastropoda + Heterobranchia), which was consistent with Feng et al. (2020, 2021). Kocot et al. (2011) analyze the phylogenetic relationships of Gastropoda species showing that Caenogastropoda and Heterobranchia were sister groups, and Neritimorpha is closely related to them. Patellogastropoda is on the outermost side of the phylogenetic tree. Osca et al. (2014) constructed a
FIGURE 2  The frequency of mitochondrial PCG amino acids and relative synonymous codon usage (RSCU) of five newly sequenced neritid mitogenomes.
phylogenetic tree, finding a different result, Neritimorpha is closely related to Caenogastropoda, and then closely with Vetigastropoda. Subsequently, Uribe, Colgan, et al. (2016) added a subclass, Neomphalina, based on the research of Osca. This subclass is between Heterobranchia and Vetigastropoda in terms of evolutionary time. Zapata et al. (2014) assessed the various hypotheses that have been put forward about the inner branches of gastropod evolutionary trees in recent decades, concluding that Neritimorpha appeared on the outermost branch only once.

The phylogenetic tree of the Neritidae showed that the genus *Neritina* and *Septaria* clustered together, as a sister group with *Clithon*, the genus *Nerita* is independently distributed in Neritimorpha. According to their living habits, *Nerita* species were the only organisms widely distributed in the marine environment. Species from the genus *Neritina*, *Septaria*, and *Clithon* were common in fresh and brackish water, so they had relatively closed evolutionary relationships. Phylogenetic relationships analysis showed that all of Neritidae species were grouped together, all the posterior probability values

### Table 6: Intergenic nucleotides of five neritid species

| Intergenic | Cc | Cl | Cs | Ni | Sl | Summary |
|------------|----|----|----|----|----|---------|
| COI        | 11 | 11 | 11 | 11 | 11 | 11      |
| COII       | −5 | 2  | 1  | 1  | 1  | −5 to 2 |
| tRNAAsp    | 0  | 0  | 0  | 0  | 0  | 0       |
| ATP8       | 6  | 6  | 6  | 6  | 6  | 6       |
| ATP6       | 31 | 22 | 28 | 22 | 22 | 22–31   |
| tRNAAsp    | 0  | 0  | 0  | 0  | 0  | 0       |
| ND5        | 0  | 0  | 0  | 0  | 0  | 0       |
| tRNAAsp    | 0  | 0  | 0  | 0  | 0  | 0       |
| ND4        | 2  | 2  | 2  | 2  | 2  | 2       |
| ND4L       | 4  | 4  | 4  | 4  | 4  | 4       |
| tRNAThr    | 8  | 8  | 9  | 8  | 3  | 3–9     |
| tRNAAsp(CUN) | 5  | 5  | 5  | 5  | 5  | 5       |
| Cytb       | 10 | 10 | 11 | 10 | 19 | 10–19   |
| ND6        | 7  | 7  | 7  | 7  | 7  | 7/13    |
| tRNAPro    | 1  | 1  | 1  | 1  | 1  | 1       |
| ND1        | 0  | 0  | 0  | 0  | 0  | 0       |
| tRNAAsp(UUR)| 0  | 0  | 0  | 14 | 4  | 0       |
| tRNAAsp(CUN)| −25 | −25 | −25 | −22 | −22/−25 |
| 16S rRNA   | −4 | −4 | −8 | −10| −10| −4 (−10)|
| tRNAVal    | −1 | −1 | −1 | −1 | −1 | −1      |
| 12S rRNA   | −1 | −1 | −1 | −1 | −1 | −1      |
| tRNAMet    | 4  | 4  | 4  | 5  | 5  | 4–5     |
| tRNAThr    | 4  | 4  | 4  | 5  | 6  | 4–6     |
| tRNAPro    | 0  | 0  | 0  | 0  | 0  | 0       |
| tRNAThr    | 0  | 0  | 0  | 0  | 0  | 0       |
| tRNAAsn    | 10 | 11 | 10 | 10 | 13 | 10–13   |
| tRNAThr    | 1  | 1  | 1  | 1  | 0  | 0/1     |
| ND3        | 3  | 3  | 3  | 4  | 4  | 3/4     |
| tRNAAsp(AGY)| 9  | 9  | 9  | 9  | 9  | 9       |
| ND2        | 99 | 99 | 99 | 99 | 99 | 99      |
were 1, and the bootstraps values were greater than 78. Using COI and 16s rRNA to conduct a phylogenetic tree, the results of Bunje and Lindberg (2007) show the genus Neritina and Septaria as sister groups, Nerita is a separate branch in the Neritidae. Chee and Mohd (2014) constructed a NJ tree using DNA barcoding of 12 species in the Neritidae, finding that Neritina and Clithon had a closed phylogenetic relationship, as sister groups with Nerita, this result was also consistent with recent research. Such branching results correspond to their living environment, species in Neritidae were distinguished by the difference in the salt content of the living environment.

| gene       | Cc | Cs | Cl | Ni | Sl | Summary   |
|------------|----|----|----|----|----|-----------|
| tRNA<sup>Asp</sup> | 66 | 67 | 66 | 66 | 67 | 66/67     |
| tRNA<sup>Phe</sup> | 66 | 66 | 66 | 68 | 68 | 66/68     |
| tRNA<sup>His</sup> | 66 | 66 | 66 | 66 | 66 | 66        |
| tRNA<sup>Thr</sup> | 68 | 68 | 68 | 68 | 68 | 68        |
| tRNA<sup>Met</sup> | 68 | 67 | 68 | 67 | 67 | 67/68     |
| tRNA<sup>Tyr</sup> | 68 | 68 | 68 | 68 | 69 | 68/69     |
| tRNA<sup>Cys</sup> | 64 | 64 | 64 | 65 | 65 | 64/65     |
| tRNA<sup>Pro</sup> | 66 | 66 | 66 | 67 | 66 | 66/67     |
| tRNA<sup>Ala</sup> | 67 | 67 | 67 | 67 | 67 | 67/68     |
| tRNA<sup>Thr</sup> | 68 | 67 | 68 | 68 | 69 | 68/69     |
| tRNA<sup>Met</sup> | 68 | 67 | 68 | 68 | 69 | 69        |
| tRNA<sup>Tyr</sup> | 68 | 68 | 68 | 68 | 68 | 68        |
| tRNA<sup>Cys</sup> | 64 | 64 | 64 | 65 | 65 | 64/65     |
| tRNA<sup>Pro</sup> | 66 | 66 | 66 | 67 | 66 | 66/67     |
| tRNA<sup>Ala</sup> | 67 | 67 | 67 | 67 | 67 | 67        |
| tRNA<sup>Thr</sup> | 68 | 67 | 68 | 68 | 69 | 68/69     |
| tRNA<sup>Met</sup> | 68 | 67 | 68 | 68 | 69 | 69        |
| tRNA<sup>Tyr</sup> | 68 | 68 | 68 | 68 | 68 | 68        |
| tRNA<sup>Cys</sup> | 64 | 64 | 64 | 65 | 65 | 64/65     |
| tRNA<sup>Pro</sup> | 66 | 66 | 66 | 67 | 66 | 66/67     |
| tRNA<sup>Ala</sup> | 67 | 67 | 67 | 67 | 67 | 67        |
| tRNA<sup>Thr</sup> | 68 | 67 | 68 | 68 | 69 | 68/69     |
| tRNA<sup>Met</sup> | 68 | 67 | 68 | 68 | 69 | 69        |
| tRNA<sup>Tyr</sup> | 68 | 68 | 68 | 68 | 68 | 68        |
| tRNA<sup>Cys</sup> | 64 | 64 | 64 | 65 | 65 | 64/65     |
| tRNA<sup>Pro</sup> | 66 | 66 | 66 | 67 | 66 | 66/67     |
| tRNA<sup>Ala</sup> | 67 | 67 | 67 | 67 | 67 | 67        |
| tRNA<sup>Thr</sup> | 68 | 67 | 68 | 68 | 69 | 68/69     |
| tRNA<sup>Met</sup> | 68 | 67 | 68 | 68 | 69 | 69        |
| tRNA<sup>Tyr</sup> | 68 | 68 | 68 | 68 | 68 | 68        |
| tRNA<sup>Cys</sup> | 64 | 64 | 64 | 65 | 65 | 64/65     |
| tRNA<sup>Pro</sup> | 66 | 66 | 66 | 67 | 66 | 66/67     |
| tRNA<sup>Ala</sup> | 67 | 67 | 67 | 67 | 67 | 67        |
| tRNA<sup>Thr</sup> | 68 | 67 | 68 | 68 | 69 | 68/69     |
| tRNA<sup>Met</sup> | 68 | 67 | 68 | 68 | 69 | 69        |
| tRNA<sup>Tyr</sup> | 68 | 68 | 68 | 68 | 68 | 68        |

**FIGURE 3** The average nonsynonymous to synonymous substitution ratio (Ka/Ks) of all 13 PCGs of 19 Neritidae species
3.6 | Divergence times

Our results showed that Neritimorpha originated from about 216.53 Mya (95% highest posterior density (HPD) = 206.56–226.37 Mya) (Figure 5), which is close to previous studies (Feng et al., 2020, 2021). The first divergence of the Neritimorpha was in the Triassic period, the first period of Mesozoic, which was the transition period involving the disappearance of Paleozoic biota and the formation of post-modern biota. During this period, the marine invertebrate fauna underwent great changes (Uribe, Kano, et al., 2016). In the Neritidae, the differentiation of the four genera occurred about 102.74 Mya, the results obtained from this analysis were slightly older than the age of the origin of the Spadonidae estimated in previous reports (Feng et al., 2020, 2021). This may be due to differences...
between results from the fossil record and different evolutionary classification methods, which are limited by their different areas of experience and expertise. Further revision of the fossil record of the genus is needed to address the attribution of the different genera.

The genus Nerita was differentiated in 70.94 Mya, and other three genera were differentiated in 71.35 Mya. These five species differentiated in the Paleogene and Neogene of Cenozoic (23.03–65.50 Mya), the period of the emergence and evolution of modern organisms. The most striking effect of the Early Tertiary was the Himalayan movement: this was the period when the Qinghai-Tibet Plateau began to rise. At this time, the continental transgression of China decreased rapidly and marine sediments appeared in the marginal areas. This crustal movement might have contributed to the rapid differentiation of the neritids during this period.

4 | CONCLUSIONS

We sequenced the complete mitogenomes of five species in Neritidae, and analyzed basic characteristics of gene sequences, found the genome size, gene order, and nucleotide composition were similar with previous findings. The Ka/Ks ratios of 13 PCGs in 19 Neritidae species showing that these genes were under purifica-
tion selection. Phylogenetic analyses indicated genus Neritina and Septaria were sister groups, and clustered with Clithon, genus Nerita was a separate branch in Neritidae. According to the estimation of divergence times, five species differentiated in the Cenozoic. This result provides a reference for the study of phylogenetic analysis and evolution research. In this study, three of five species belong to genus Clithon, data from genus Neritina and Septaria are limited, further studies are needed to follow up these findings and explore the evolutionary processes of neritids.

AUTHOR CONTRIBUTION

Jing Miao: Data curation (equal); Writing – original draft (equal). Jiantong Feng: Data curation (equal); Writing – original draft (equal). Xiaojuan Liu: Methodology (equal); Resources (equal). Chengrui Yan: Methodology (equal); Resources (equal). Yingying Ye: Funding acquisition (lead); Supervision (lead); Writing – review & editing (lead). Jiji Li: Funding acquisition (lead); Supervision (lead); Writing – review & editing (lead). Kaida Xu: Data curation (supporting); Writing – original draft (supporting). Baoying Guo: Data curation (supporting); Writing – original draft (supporting). Zhenming Lü: Data curation (supporting); Writing – original draft (supporting).
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CONFLICT OF INTEREST
All the authors declared no potential interest.

DATA AVAILABILITY STATEMENT
The following information was supplied regarding the availability of DNA sequences: The complete mitogenomes of Clithon corona, Clithon lentiginosum, Clithon squarrosum, Nerita iris, and Septaria lineata are deposited in GenBank of NCBI under accession number MZ189741, MZ152905, MZ229747, MZ189742, and MZ315041, respectively.

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