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MITOGENOME REPORT

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The mitochondrial genome of *Neoperla bimaculata* (Li et al. 2021) (plecoptera: perlidae) from Tibet of southwest China and its phylogenetic analysis

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ABSTRACT

The complete mitochondrial genome (mitogenome) of *Neoperla bimaculata* was sequenced and annotated in this study. We found that the mitogenome of *N. bimaculata* is 15,774 bp in length with an $A + T$ content of 64.3%. It exhibits the classic structure of a mitogenome. Most protein-coding genes (PCGs) of the mitogenome initiate with the standard start codon ATN. Ten PCGs use the standard stop codon TAA/TAG, while the *COI*, *COII*, and *ND5* genes terminate with a single T nucleotide. Phylogenetic analyses suggested that *N. bimaculata*, along with two unpublished *Neoperla* species, formed a cluster within the phylogenetic tree. Our results indicated that the genus Neoperla and Neoperlops were sister groups. Meanwhile, the monophyly of Perlinae and Acroneuriinae was supported in the mitochondrial phylogeny.

Introduction

Perlidae is a large family of Plecoptera (stoneflies), encompassing more than 1,100 species (DeWalt and Ower 2019; DeWalt et al. 2023). Within this family, the genus *Neoperla* comprises over 280 species distributed worldwide in Eastern North America, Tropical and Temperate Asia, and central Africa (Sivec et al. 1988; Li et al. 2021; DeWalt et al. 2023). Despite its extensive taxonomic diversity, current information regarding the mitochondrial genome (mitogenome) of Perlidae is very limited. To date, no mitogenome of *Neoperla* has been published, and only one complete and one partial mitogenome are available in GenBank (http://www.ncbi.nlm.nih.gov/genbank/, accessed on 8 October 2023). As a result, our understanding of the mitogenome information of *Neoperla* is limited, hindering our ability to establish a phylogenetic relationship for this genus.

Neoperla bimaculata Li et al. 2021 is a newly described species confined to Motuo County in Tibet, China (Li et al. 2021). The nymphs prefer to inhabit unpolluted or minimally polluted mountain streams, often being found beneath shallow rocks. As adults, they possess a soft body and are terrestrial, typically residing in dead trees, stones, or crevices on embankments. They are distinguishable by their brown bodies with black patterns, predominantly black heads, yellow bases of the antennae, and black tips. Additionally, two large triangular black stripes are present on the sides of the pronotum, with a light-colored area in the middle resembling

a sandglass. Their legs are dark brown with a light color, and their cerci are pale yellow, with slightly darker coloring at the apical segments (Figure 1). In our current investigation, we successfully sequenced the complete mitogenome of *N. bimaculata* and analyzed its general features. The outcomes of this research will aid in the future reconstruction of higher-level phylogenetic relationships within Perlidae or Perloidea based on mitogenomic data.

Materials and methods

The male adult specimens of *N. bimaculata* (Figure 1) used for this study were captured by Jianyun Wang at Motuo County, Tibet, China (coordinate as follows: N29°14′45″ E95°10′16″) on August 8, 2015. The specimens are unregulated invertebrates, and as such, no approval from the relevant institutional ethics committee was required. The freshly collected specimens were preserved immediately in absolute ethanol. The specimens were identified by Weihai Li (Department of Plant Protection, Henan Institute of Science and Technology, China). The voucher specimen was deposited at the Entomological Museum of the Henan Institute of Science and Technology, Henan Province, China (contact person: Jinjun Cao, email: cjj1986108@163.com) under the voucher number VHL-0062.

Total genomic DNA was extracted from the thorax muscles using the QIAamp DNA Blood Mini Kit (Qiagen,

Hilden, Germany) and stored at −20 °C until needed. The mitogenome of *N. bimaculata* was generated by amplification of overlapping PCR fragments and 11 pairs of primers were designed (Table S1) by using Primer Premier 6.0 (Lalitha 2000). The PCR products were gel purified before Sanger

Figure 1. The male habitus of *neoperla bimaculata* in lateral view. The picture was taken by Weihai Li.

sequencing. Finally, sequences were assembled using BioEdit version 7.0.5.3 (Hall 1999). The complete mitogenome of *N. bimaculata* was annotated by the MITOS Web Server (Bernt et al. 2013) and the Protein-coding genes (PCGs) and ribosome RNAs (rRNAs) were identified by comparison with sequences from other published stoneflies. The mitogenome map was completed by CGView Web server (https://cgview. ca/). Nucleotide composition was calculated with MEGA version 5.1 software (Tamura et al. 2011). Composition skew analysis was calculated according to formulas: AT-skew = [A-T]/[A + T] and GC-skew = [G-C]/[G + C] (Perna and Kocher 1995).

To obtain the phylogenetic position of *N. bimaculate*, phylogenetic relationships were analyzed using the concatenated nucleotide sequences of PCG12 (including the first and second codon positions) from other 17 perlid stoneflies and two species (*Pteronarcella badia* and *Styloperla spinicercia*) from Styloperlidae as the outgroups. All sequences of the 13 PCGs were individually aligned in MEGA version 5.1 software. Maximum Likelihood (ML) phylogenetic tree was estimated using the IQ-TREE Web Server in W-IQ-TREE (Trifinopoulos et al. 2016) with 10,000 replicates of ultrafast likelihood bootstrap. MrBayes 3.2.6 (Ronquist et al. 2012) was used to carry

Figure 2. Gene arrangement in the mitogenome of *neoperla bimaculata*. GC content is represented by black, negative GCskew is represented by green, positive GCskew is represented by deep purple and other colors represent different gene types.
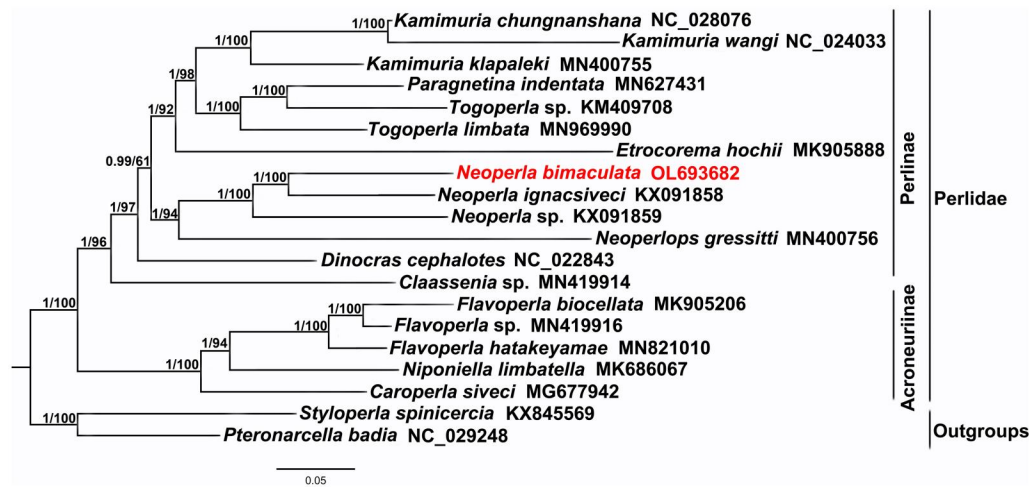
Figure 3. Phylogenetic tree based on the concatenated nucleotide sequences of PCG12 (including the first and second codon positions) by bayesian inference (BI) and maximum-likelihood (ML) methods from the complete mitogenomes of *N. bimaculata* and 17 other stoneflies. The mitogenomes of *pteronarcella badia* and *styloperla spinicercia* were selected as outgroups.

out BI analysis under the following conditions: 10 million generations with sampling every 100 generations and the first 25% discarded as burn–in.

Results

The mitogenome of *N. bimaculata* is a typical circular DNA molecule, spanning 15,774 bp in length (GenBank: OL693682). It comprises 13 PCGs, 2 rRNA genes, 22 tRNA genes, and an A + T-rich region (also known as the control region) with a length of 862 bp (Figure 2). The nucleotide composition (A: 34.0%, T: 30.3%, C: 23.4%, G: 12.3%) of the *N. bimaculata* mitogenome shows a marked A + T bias (64.3%). The AT skew and GC skew of the entire mitogenome were calculated as 0.058 and −0.309, respectively, indicating a higher occurrence of As and Cs compared to Ts and Gs. Most PCGs used ATG as the start codon, while *COII* and *ND1* started with TTG, *ND3* and *ND6* started with ATC, and *ATP8* started with ATT, respectively. Eleven PCGs employed the standard stop codon TAA/TAG, while the *COII* and *ND5* genes terminated with a single T nucleotide. The length of all 22 tRNA genes ranged from 65 to 72 bp. The predicted secondary structures were typically cloverleaf-shaped, except for $tRNA^{Ser(AGN)}$, in which the dihydrouridine (DHU) stem was replaced by a 7-bp simple loop (data not shown). Similar characteristic is also observed in most other insects (Cameron 2014). In the *N. bimaculata* mitogenome, the two rRNA genes (large and small subunit ribosomal RNA: *lrRNA* and *srRNA*) were positioned between $tRNA^{Leu(CUN)}$ and $tRNA^{Val}$, and between $tRNA^{Val}$ and the A + T-rich region, respectively. The length of *lrRNA* and *srRNA* were 1,368 and 830 bp, respectively, falling within the range observed in completely sequenced Perlidae insects. The 802 bp control region of *N. bimaculata* was located between *srRNA* and $tRNA^{Ile}$, exhibiting the highest A + T content of 73.7%.

Both ML and BI phylogenetic analyses indicated that *N. bimaculata* is positioned within the subfamily of Perlinae. Furthermore, *N. bimaculata* exhibited the closest genetic relationship with two other species, namely *N. ignacsiveci* and

Neoperla sp., all belonging to the same genus. These three *Neoperla* species formed a well-supported cluster, which in turn constituted a sister group to *Neoperlops gressitti* (Figure 3). The phylogenetic results also supported the monophyly of Perlinae and Acroneuriinae (Bayesian posterior probabilities (PP) = 1.00, bootstrap probabilities (BP) = 100).

Discussion and conclusions

In this study, the complete mitogenome of *N. bimaculata* was successfully assembled and annotated for the first time, representing the genus *Neoperla*, with only one complete and one partial unpublished mitogenome available before our study. The gene size and arrangement of the newly sequenced mitogenome of *N. bimaculata* are consistent with those of other insects in the family Perlidae (Huang et al. 2015; Wang et al. 2016; Li et al. 2019; Hao et al. 2020; Shen and Du 2020).

Currently, the classification system of Perlidae comprises two subfamilies based on morphological characters: the Perlinae and Acroneuriinae (Zwick 2000). In this study, the monophyly of Perlinae and Acroneuriinae was supported in the mitochondrial phylogeny (PP = 1, BP = 100). However, in other molecular studies, Acroneuriinae was considered a paraphyletic group (Terry and Whiting 2003; Shen and Du 2020). The relationships within the subfamily Acroneuriinae were found to be complex. In future studies, dense sampling can help us understand the phylogenetic relationships within this subfamily.

Ethical approval

Authors' contributions

Conceptualization: CY Guo, Y Wang and JJ Cao; data curation and analysis: CY Guo, and Y Wang; investigation: CY Guo and WH Li; writing—original

Data availability statement

The data that support the findings of this study are openly available in GenBank of NCBI at https://www.ncbi.nlm.nih.gov, reference number OL693682.

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