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# Integrative taxonomy uncovers hidden species diversity in the rheophilic genus *Potamometra* (Hemiptera: Gerridae)

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**Abstract**

The genus *Potamometra* Bianchi, 1896 represents big rheophilic semi-aquatic bugs that typically inhabit middle-altitude mountainous streams. Here, we integrated molecular and morphological data for delimiting species boundaries and understanding the evolutionary history of the genus *Potamometra*. Twenty-seven complete mitochondrial genomes of *Potamometra* were sequenced, with samples representing most of the known geographically distributed locations around the Sichuan Basin. The results of different species delimitation methods (ABGD, bPTP, GMYC and BPP) based on the monolocus or multilocus data strongly supported the existence of two cryptic new species (*Potamometra anderseni* Zheng, Ye & Bu, sp. nov. and *Potamometra zhengi* Zheng, Ye & Bu, sp. nov.) although more entities were found in the tree-based delimitation methods. The two new species were successfully validated using morphological characters within a detailed taxonomic framework. Phylogenetic analyses supported the reciprocal monophyly of the seven highly node-supported clades, which were matched with the five known species and two new taxa. A novel gene arrangement pattern that two trnF (trnF1 and trnF2) genes separated by an intergenic spacer (IGS) were found in all the species except the sister group of *Potamometra berezowskii* Bianchi, 1896 and *Potamometra linnavuorii* Chen, Nieser & Bu, 2016. This gene rearrangement event could be explained by the tandem duplication and random loss (TDRL) model. Our study emphasized that the combination of molecular sequence data, morphological characters and mitochondrial structural information could improve the accuracy of species delimitation.

KEYWORDS

gene rearrangement, integrative taxonomy, mitochondrial genome, phylogenetics, *Potamometra*, species delimitation

## 1 | INTRODUCTION

Species are the fundamental biological units, and species delimitation is a central issue in systematics and evolutionary biology (Fujita, Leaché, Burbrink, McGuire, & Moritz, 2012; May, 1988; Wiens, 2007). In recent years, the employment of molecular-based delimitation methods has made great contributions to the defining of species boundaries, especially in morphologically conserved groups (Bluemel et al., 2014; Chan et al., 2017; Devitt, Wright, Cannatella, & Hillis, 2019; Fujisawa & Barraclough, 2013; Yang, 2015). Molecular species delimitation can provide preliminary taxonomic hypotheses and valuable complements to morphological taxonomy

(Huang et al., 2019; Luo, Ling, Ho, & Zhu, 2018). However, defining a new species when relying solely on molecular information is inadequate. Empirical studies have shown that using molecular methods alone sometimes over-split populations that might not represent distinct species, leading to taxonomic inflation (Bauer et al., 2011; Pinacho-Pinacho, García-Varela, Sereno-Uribe, & Pérez-Ponce, 2018; Villamil et al., 2019; Zhang, Kapli, Pavlidis, & Stamatakis, 2013). The uncertainties in delimiting species boundaries based on the molecular data ideally need to be tested and carefully verified using other sources of information (e.g. morphological data) within an integrative framework (Lee & Palci, 2015; Noguerales, Cordero, & Ortego, 2018) that could provide guiding significance in studying the morphologically conserved groups (Barberousse & Samadi, 2010; Grummer, Bryson, & Reeder, 2014; Pedraza-Marrón et al., 2019).

Mitochondrial genomes are considered powerful markers for resolving different levels of phylogenetics that include deep divergent lineages (Condamine, Nabholz, Clamens, Dupuis, & Sperling, 2018; Tang et al., 2018) or recently radiated groups (Jacobsen et al., 2012; Stokkan et al., 2018), due to their maternal inheritance, relatively high evolutionary rate and rare recombination (Barr, Neiman, & Taylor, 2005; Boore, 1999; Cameron, 2014; Curole & Kocher, 1999). In addition to the information on nucleotide sequences, the significance of mitochondrial gene rearrangement to support relationships of taxa that are inferred from the phylogenetic context have been proposed since the mid-1980s (Brown, 1985) and have been elucidated by some previous studies (Cameron, 2014; Galaska, Li, Kocot, Mahon, & Halanych, 2018; Rokas & Holland, 2000). Studies indicated that gene rearrangements were not only restricted to clades at higher taxonomic categories but also could be found at lower taxonomic levels in some invertebrate groups (Cameron, 2014; Yan et al., 2014). Therefore, mitochondrial genomes can provide robust phylogenetic information, especially when phylogenetic inferences based on the gene sequences and arrangement patterns arrive at a congruent topology (Cameron, 2014; Galaska et al., 2018).

The genus *Potamometra* Bianchi, 1896 (Insecta: Hemiptera: Heteroptera: Gerridae: Ptilomerinae) is mainly distributed around the Sichuan Basin of China restricting to a series of mountain ranges and inhabits in secluded environment of quiet side of fast flowing mountainous streams with dense forest cover at elevations of 390–1650 m (Chen, Nieser, & Bu, 2016; Drake & Hoberlandt, 1965). The history of taxonomic studies of *Potamometra* is uncomplicated compared with many other semi-aquatic bugs because only a few publications exist on this genus. Initially, Bianchi (1896) placed the genus in the subfamily Halobatinae because of its shortened abdomen. However, according to Bergroth (1908), this genus did not belong to Halobatinae. Esaki (1927) erected the subfamily Ptilomerinae within the family Gerridae and assigned *Potamometra* to this subfamily based on its morphology. Drake and Hoberlandt (1965) revised the genus including the four species, that is, *Potamometra berezowskii* Bianchi, 1896; *Potamometra macrokosos* Drake & Hoberlandt, 1965; *Potamometra montandoni* (Kirkaldy, 1899) and *Potamometra tibetensis* Esaki, 1927. More than 40 years later, one new species, *Potamometra linnavuorii* Chen et al., 2016, was described by Chen et al. (2016), and therefore, the genus currently comprises five nominal species. Although this genus has undergone a relatively long history of taxonomic studies based on morphology, since the description of the first nominal species in the 19th Century, it has never been studied outside the context of taxonomic work, and, as a result, little is known of its hidden species diversity and phylogenetic relationships between the putative species.

Here, we integrate molecular and morphological data for delimiting species boundaries and understanding the evolutionary history of the genus *Potamometra*. Our sampling of *Potamometra* includes most of the known distribution locations from literature and new localities collected in the last 5 years around the Sichuan Basin. Preliminary taxonomic work reveals subtle morphological variation in some newly discovered and geographically isolated populations, leading us to hypothesize that several cryptic lineages and even new species might exist in this genus. Therefore, we apply a two-step approach for species delimitation with a wide range of common single-locus- or multilocus-based methods using mitochondrial genomes to form preliminary hypotheses of species boundaries. We then test these putative boundaries within a taxonomic framework and describe two new species. Furthermore, features of the mitochondrial genomes are compared, and a phylogeny is constructed for the first time based on mitochondrial sequence data and morphological characters. We also discover a unique gene arrangement pattern and further discuss the evolutionary mechanisms of this gene rearrangement and its beneficial effect in a phylogenetic context.

## 2 | METHODS AND MATERIALS

### 2.1 | Samples, DNA extraction and sequencing

We selected 27 individuals of *Potamometra* from 24 locations, comprising the five known species and two undescribed taxa and covering all of the known distribution range of this genus (Table S1). All specimens were preserved in 100% ethanol and stored in a freezer at −20°C in the College of Life Sciences at Nankai University (Tianjin, China). Total genomic DNA was extracted from thorax tissue using the CTAB method (Reineke, Karlovsky, & Zebitz, 1998). For each sample, the whole mitochondrial genome was obtained using the Illumina HiSeq 2000 platform (Illumina) with a 350-bp insert size and a paired-end 150-bp sequencing strategy at Novogene. Traditional PCR amplification and Sanger sequencing were used to verify the region of gene rearrangement. The primers used for amplification and sequencing are listed in Table S2. The thermal cycling program for the PCR amplification was set as follows: an initial denaturation at 94°C for 2 min; denaturation for 35 cycles at 94°C for 30 s; annealing at 47–60°C for 30 s; elongation at 72°C for 40–60 s, depending on the size of the products; and a final elongation at 72°C for 10 min.

## 2.2 | Gene annotation and sequence analyses

The entire mitogenome was obtained by a combination of de novo assembly using SOAPdenovo-Trans in Shenzhen Genomics Institute and MITObim following the tutorial I (https://github.com/chrishah/MITObim). All tRNA genes and their secondary structures were identified on the MITOS web server (http://mitos.bioinf.uni-leipzig.de/index.py/) (Bernt et al., 2013) using the invertebrate mitochondrial genetic code. Two rRNA genes were determined by alignment with homologous sequences of published mitochondrial genomes of *Ptilomera tigrina* Uhler, 1860 (Gerridae: Ptilomerinae) and *Aquarius paludum* Fabricius, 1794 (Gerridae: Gerrinae) in GenBank, and the corresponding GenBank accession numbers are listed in Table S1. Protein coding genes (PCGs) were initially identified using the Open Reading Frame Finder (ORF Finder) implemented at the NCBI website (https://www.ncbi.nlm.nih.gov/orffinder/) with the invertebrate mitochondrial genetic code and then compared with published mitochondrial sequences of Insect using the program BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

We used CG View server V 1.0 (Grant & Stothard, 2008) to draw the mitochondrial genome map. Nucleotide composition and codon usage of the PCGs were calculated by Mega 7.0 (Kumar, Stecher, & Tamura, 2016). AT-skew $[(A - T)/(A + T)]$ and GC-skew $[(G - C)/(G + C)]$ were used to measure the bias of the nucleotide composition. We used DnaSP 5.0 (Librado & Rozas, 2009) to analyse the non-synonymous substitution rate (Ka) and synonymous substitution rate (Ks) of all PCGs, and the evolution rate of each PCG was represented by Ka/Ks.

## 2.3 | Molecular phylogenetic analysis

A phylogenetic tree of *Potamometra* was reconstructed. The taxa *Ptilomera tigrina* Uhler, 1860 (Gerridae: Ptilomerinae) and *Aquarius paludum* Fabricius, 1794 (Gerridae: Gerrinae) were selected as outgroups (Table S1). The sequences of 13PCGs, two rRNAs and 22 typical tRNAs from the mitochondrial genome were used in the phylogenetic analyses. Sequences of rRNAs and tRNAs were aligned using MAFFT 7.402 (Katoh & Standley, 2013) under the G-INS-i strategy, and alignments of 13 PCGs were performed based on amino acid sequences using Muscle implemented in Mega 7.0 (Kumar et al., 2016). Individual genes were then concatenated to generate various data sets: PCG123RT (all three codon positions of the 13 PCGs, two rRNAs and 22 tRNAs), PCG123 (all three codon positions of the 13 PCGs), PCG12 (the first and second codon positions of the 13 PCGs) and AA (amino acid sequences of 13 PCGs). We used PartitionFinder 2.0 (Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2017) to test the various partitioning schemes and optimal models for each data set. The Bayesian information criterion (BIC) and the 'greedy' algorithm with branch lengths estimated as 'unlinked' were selected to search for the best-fit scheme (Table S3). All data sets were analysed by Bayesian inference (BI), maximum likelihood (ML) and maximum parsimony (MP). BI analyses were conducted using MrBayes 3.2.6 (Ronquist et al., 2011) with partitioned models (Table S3). Two simultaneous runs of 10,000,000 generations were conducted for each matrix. The trees were sampled in every 1,000 generations. Convergence of runs was assessed using Tracer 1.7 (Rambaut, Drummond, Xie, Baele, & Suchard, 2018). With the first 25% of trees discarded as burn-in, Bayesian posterior probabilities were calculated for a 50% majority-rule consensus tree of the remaining trees. ML analyses were performed with 1,000 bootstrap replicates under the program RAxML 8.0.12 (Stamatakis, 2006) using the GTRGAMMAI model. MP analyses were carried out with the program TNT 1.1 (Goloboff, Farris, & Nixon, 2008). All characters were equally weighted, and gaps were treated as missing characters. New technology searches consisting of 10,000 random addition sequence replicates were applied. Analyses were performed with the default options. Clade robustness was evaluated with jackknife resampling and Poisson bootstrap reweighting values.

## 2.4 | Molecular species delimitation

We implemented the following four widely used single-locus- or multilocus-based methods: Automatic Barcode Gap Discovery (ABGD) (Puillandre, Lambert, Brouillet, & Achaz, 2012), Bayesian Poisson Tree Processes (bPTP) (Zhang et al., 2013), Generalized Mixed Yule Coalescent model (GMYC) (Fujisawa & Barraclough, 2013; Pons et al., 2006) and Bayesian coalescent method in the software Bayesian Phylogenetics and Phylogeography (BPP) (Yang, 2015). ABGD, bPTP and GMYC are suitable for analysing single-locus data sets, whereas the Bayesian method in BPP is designed to analyse multilocus data. We used the COI segment and 13 PCGs of the mitogenome for single-locus and multi-locus analyses, respectively. ABGD analysis was performed at the web server (http://wwwabi.snv.jussieu.fr/public/abgd/) using the following parameters: prior intraspecific divergence values (Pmin = 0.001 and Pmax = 0.1), relative gap width ($X = 1.5$) and the Kimura 2-P (K80) distance model. For the bPTP analysis, we first built a non-ultrametric phylogenetic tree under the program RAxML 8.0.12 (Stamatakis, 2006) using the same model and outgroups as above. Then, the output from the RAxML was used as the input for the bPTP analysis, which was conducted on the species delimitation web server for bPTP (http://species.h-its.org/ptp/). The analysis was run for 200,000 generations with a thinning of 100 and burn-in of 10%. The GMYC method integrates both Yule and coalescent modes to identify species boundaries by referring to the relative node times in an ultrametric tree. We inferred the ultrametric tree as the input for GMYC analysis using BEAST 2.4.8 (Bouckaert et al., 2014) without outgroups. Models of nucleotide substitution were evaluated with PartitionFinder 2.0 (Lanfear et al., 2017). The best substitution models for 1st + 2nd and 3rd codon positions of COI were TRN + I and TRN + G, respectively. A Yule model tree prior and strict clock were used. MCMC chains were run for 1 million generations and sampled every 5,000 generations. Convergence of runs was assessed by ESS values using Tracer 1.7 (Rambaut et al., 2018). Consensus trees were obtained in TreeAnnotator 2.4.8 (Bouckaert et al., 2014), with a burn-in percentage at 10%. The ultrametric tree generated by BEAST was used to run GMYC with a single-threshold method in the species delimitation web server (http://species.h-its.org/gmyc/). BPP runs under the multispecies coalescent model (MSC), which account for incomplete lineage sorting and gene tree conflicts (Rannala & Yang, 2013; Yang & Rannala, 2010, 2014). We used the A10 analysis in the program BPP 3.3 (Yang, 2015) to validate the delimitation results generated by the above methods. The species tree was estimated by BEAST 2.4.8 (Bouckaert et al., 2014) using 13 PCGs of mitogenome. We assumed that the 13 PCGs shared the same gene tree owing to their linkage in the mitochondrial genome. The best substitution model was TVM + I + G, which was evaluated by jModeltest 2.1.7 (Posada, 2008). A Yule model tree prior and strict clock were selected. The MCMC chains were run for 1 million generations and sampled every 5,000 generations. The convergence of the runs was assessed by ESS values using Tracer 1.7 (Rambaut et al., 2018). The final species trees were obtained using TreeAnnotator 2.4.8 (Bouckaert et al., 2014) with a burn-in percentage of 10%. We conducted three BPP analyses using different combinations of the ancestral population size ($\theta$) and the root age ($\tau_0$) as proposed by Leaché and Fujita (2010): (a) large population sizes $\theta \sim G\ (1, 10)$ and deep divergences $\tau_0 \sim G(1, 10)$; (b) small population sizes $\theta \sim G\ (2, 2000)$ and shallow divergences $\tau_0 \sim G\ (2, 2000)$; and (c) large population sizes $\theta \sim G\ (1, 10)$ and relatively shallow divergences $\tau_0 \sim G\ (2, 2000)$. Each analysis was run for 250,000 generations with a burn-in of 10%.

## 2.5 | Morphological delimitation and cladistic analysis

The specimens used for morphological analyses were from material collected in the last 5 years. All measurements are presented in millimetres (mm). Photographs of apterous images were made using a Nikon SMZ 1000 microscope equipped with a computer-controlled SPOT RT digital camera and Helicon software. Male genital segments were treated with warm 10% KOH to study its structure. Photographs of male parameres were acquired using an OLYMPUS BX53 microscope equipped with a computer-controlled Canon OLYMPUS DP72 digital camera and Cell sens Standard software.

Fifteen morphological characters were selected for cladistic analysis among species of *Potamometra* (Tables 1 and 2). All morphological characters were given equal weight in the analyses and unordered. Some morphological structures between *Potamometra* and other closely related genera are so different that the homologies of these structures are difficult to assess. For this reason, we used an artificial outgroup (all zeros) for rooting the trees. MP analysis was performed using TNT v1.1 (Goloboff et al., 2008) using the same parameters as above. Abbreviations:

**TABLE 1** Character state matrix used in the cladistic analysis

| Taxon | Character | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Potamometra berezowskii* | 0 | 1 | 2 | 1 | 2 | 0 | 2 | 0 | 0 | 1 | 1 | 0 | 2 | 0 | 1 |
| *Potamometra linnavuorii* | 0 | 1 | 1 | 0 | 2 | 0 | 2 | 0 | 0 | 1 | 1 | 0 | 2 | 0 | 1 |
| *Potamometra tibetensis* | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 2 | 0 | 1 |
| *Potamometra macrokosos* | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 |
| *Potamometra zhengi* sp. nov. | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 |
| *Potamometra anderseni* sp. nov. | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 |
| *Potamometra montandoni* | 1 | 0 | 2 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 |
| Outgroup | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

NKUM, Institute of Entomology, College of Life Sciences, Nankai University, Tianjin, China; m (or mm), Male of *Potamometra* species; f (or ff): Female of *Potamometra* species.

## 3 | RESULTS

### 3.1 | Mitogenome features and gene rearrangement of *Potamometra* species

The length of the mitogenomes of *Potamometra* ranged from 15,339 bp to 15,588 bp (Appendix S1). *Potamometra berezowskii* and *P. linnavuorii* contained 37 typical mitochondrial genes (13 PCGs, two rRNAs, and 22 tRNAs) and the putative CR. The remaining species included 38 genes (i.e. the typical 37 genes and an extra trnF) (Figure S1, Appendix S1). These mitogenomes exhibited high A + T bias (74.3%–74.9%) as well as a similar AT-skew and GC-skew (Table S4). All PCGs were initiated with the standard start codon ATN (N represents one of four nucleotides, A, T, C and G), except for ND5, which began with TTG. Seven PCGs had complete termination codons (TAA or TAG), while the remaining six PCGs (ATPase6, COII, COIII, ND3, ND4 and ND6) had incomplete termination codons (T-) that probably were completed by posttranscriptional polyadenylation (Appendix S1, Ojala, Montoya, & Attardi, 1981). A total of 3,690 codons were present in each *Potamometra* mitogenome other than the stop codons. The most frequent codon families were Leu, Ile, Phe and Met (>300), whereas the least common codon family was Cys (<50) (Figure S2). The low Ka/Ks value ($\omega < 0.3$) for each PCG implied that all PCGs were under strong purifying selection. The animal DNA barcoding gene COI had the lowest $\omega$ value (Figure S3). All predicted tRNAs could be folded into the typical clover-leaf secondary structure (Figures S4–S10). The secondary structure of trnS1 (GCU) contained a complete dihydrouridine (DHU) arm instead of a simple loop, which was also found in *Corythucha ciliate* (Yang, Yu, & Du, 2013).

We identified two trnF genes (trnF1 & trnF2) separated by an intergenic spacer (IGS) in *P. macrokosos*, *P. montandoni*, *P. tibetensis* and the two putative new species (Figure 1). The two trnF genes were absolutely identical except for one base difference in samples of the SCLS and SCYB (Figure S11). The results suggested that the two trnF might be functional genes, since both of them could form a standard clover-leaf secondary structure and the same 5′-GAA-3′ anticodon, which was the normal anticodon of trnF. The results of the sequence alignment showed that IGS had high similarity (71.4%–76.1%), with the homologous sequences of ND5 (Figure S12). We speculated that IGS was the pseudo gene of the homologous sequences in ND5 due to the location of IGS and the high sequence similarity between IGS and the homologous sequences in ND5.

**TABLE 2** Characters and states used in the cladistic analysis

| Number | Characters and states |
|---|---|
| 1 | Median yellow stripe on pronotum: slightly widened behind the middle (0), distinctly widened in posterior half (1) |
| 2 | Median yellow stripe on metanotum: broad (0), narrow (1) |
| 3 | Hind coxal outgrowth of female: absent (0), poorly developed, triangular (1), extremely long, subcylindrical, extended backward, about twice the length of coxal segment itself (2) |
| 4 | Inner side of the hind coxa in male: without projections or not obvious (0), with short but distinct projections (1) |
| 5 | Posterior margin of abdominal tergite I of female: without projection (0), with a median, short projection (1), with a median, long projection (2) |
| 6 | Hairs in the middle of posterior margin of abdominal tergite I in female: short (0), long (1) |
| 7 | Abdominal sternite 5: relatively short, not reaching the hind coxa (0), relatively longer, extending to the rear, surpassing the hind coxa (1), long, bend to the dorsal side (2) |
| 8 | Posterior margin abdominal sternite 5: without long hairs on both sides of median notch (0), with long hairs on both sides of median notch (1) |
| 9 | Sternite VII: a narrow band (0), with long outgrowth at top margin, subcylindrical, along lateral and posterior margin with dense hairs (1) |
| 10 | Size of genital segments: relatively small (0), relatively large (1) |
| 11 | Parameres of male: relatively shorter, scarcely reaching as far as hind margin of pygophore (0), extremely long, protruding distinctly beyond apex of pygophore (1) |
| 12 | Parameres of male: apically widened into a caudal knob (1), without such caudal knob (0) |
| 13 | Apical margin of pygophore in male: narrow, rounded (0), broad (1), with laterocaudal projection each side (2) |
| 14 | Proctiger of male: maximum width beyond its median length (0), maximum width slightly shorter than its median length (1) |
| 15 | Proctiger of male: with several long hairs at anterior margin (0), with long hairs along lateral margins (1) |

## 3.2 | Molecular phylogenetic analyses

The phylogenetic trees showed highly compatible topological structure in the three analytical methods (i.e. BI, ML and MP) based on the four data sets (PCG123RT, PCG123, PCG12 and AA, Appendix S2), supporting the reciprocal monophyly of seven highly-supported clades (corresponding to *P. berezowskii*, *P. linnavuorii*, *P. macrokosos*, *P. montandoni*, *P. tibetensis* and two undescribed taxa) (Figure 2a). The three species (*P. montandoni*, *P. zhengi* sp. nov. and *P. tibetensis*) with gene rearrangement events appeared as successively branched lineages in the basal clades. The remainders formed two distinct clades: one of the clades, comprising *P. macrokosos* + *Potamometra anderseni* sp. nov., had a gene rearrangement, whereas its strongly supported sister group (*P. berezowskii* + *P. linnavuorii*) did not have this rearrangement (Figure 2a,c).

## 3.3 | Molecular species delimitation

For single-locus species delimitation, we used the COI data set for the ABGD, bPTP and GMYC analyses. The distance-based method ABGD retrieved seven molecular operational taxonomic units (MOTUs) when the prior intraspecific divergence (*p* value) was .00129 (Figures 2b and S14). Details of individuals in each MOTU are shown in Table S5. The bPTP method provided two results: the maximum likelihood solution result and the highest Bayesian supported solution result, which were identical in this study. The bPTP retrieved eight MOTUs without outgroups and split *P. linnavuorii* into two MOTUs (Figure 2b and Table S6). The single threshold GMYC analysis, with a confidence interval ranged from 1 to 26, yielded ten MOTUs; *P. berezowskii* and *P. linnavuorii* were separated into multiple MOTUs (Figure 2b and S14). BPP fully supported the seven putative species derived from the result of the ABGD. All nodes had perfect speciation probabilities for all three analyses for the different combinations of the ancestral population size ($\theta$) and the root age ($\tau_0$) (Figure 2b, S15, Table S7).

## 3.4 | Morphological delimitation and cladistic analysis

Two new species, *P. anderseni* Zheng, Ye & Bu, sp. nov. and *P. zhengi* Zheng, Ye & Bu, sp. nov., are described in this study, and a key to the males and females of all the species of *Potamometra* was made to facilitate identifications (see details of revision of the rheophilic genus *Potamometra* (Hemiptera: Gerridae) in supporting information and Figures 3, 4, S16–S19).

Maximum parsimony analysis of the morphological data set under equal weights yielded one most parsimonious tree with a length of 121,050 steps (Figure S20). The most parsimonious tree with Bremer support and bootstrap values is shown and is largely congruent with the result from the molecular data. *Potamometra montandoni* was sister to the remaining clades in the genus (Bremer support/bootstrap values = 10/100). All other clades formed a strongly supported monophyletic group, which was further supported by four synapomorphic characters (posterior margin of abdominal tergite I in female with long hairs; sternite VII with long outgrowth at top margin, subcylindrical, along lateral and posterior margin with dense hairs; small genital segments; and maximum width of male proctiger beyond its median length). *Potamometra berezowskii* and *P. linnavuorii* formed a clade, and their relationship was supported by four synapomorphies (median yellow stripe on pronotum slightly widened behind the middle; median

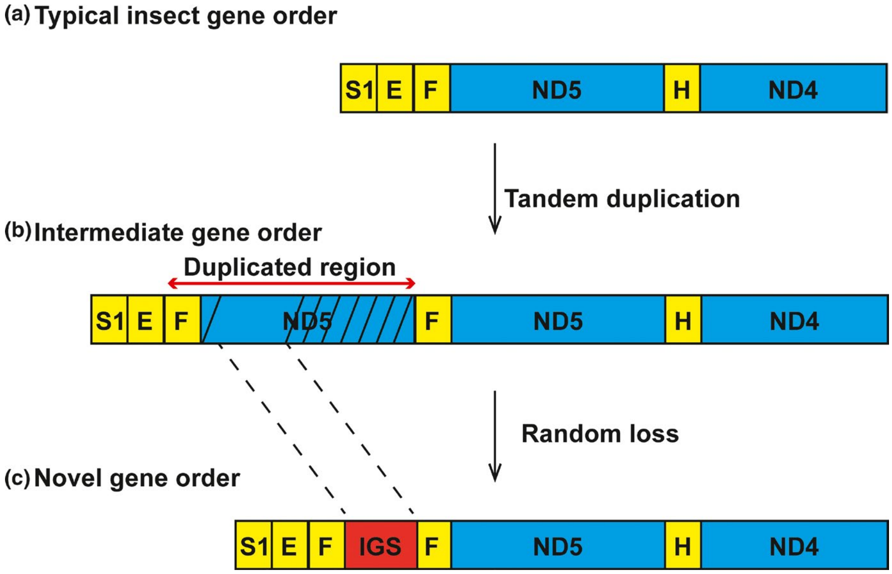


**FIGURE 1** The mechanism proposed for mitochondrial gene rearrangement in partial species of *Potamometra* in tandem duplication and random loss (TDRL) model. A double-arrow dash in red colour indicates the region of gene duplication. The regions covered by oblique lines in ND5 indicate sequences of random loss. IGS is the abbreviation for intergenic spacer and the names of PCGs are indicated by standard abbreviations, while names of tRNAs are represented by a single letter abbreviation [Colour figure can be viewed at wileyonlinelibrary.com]

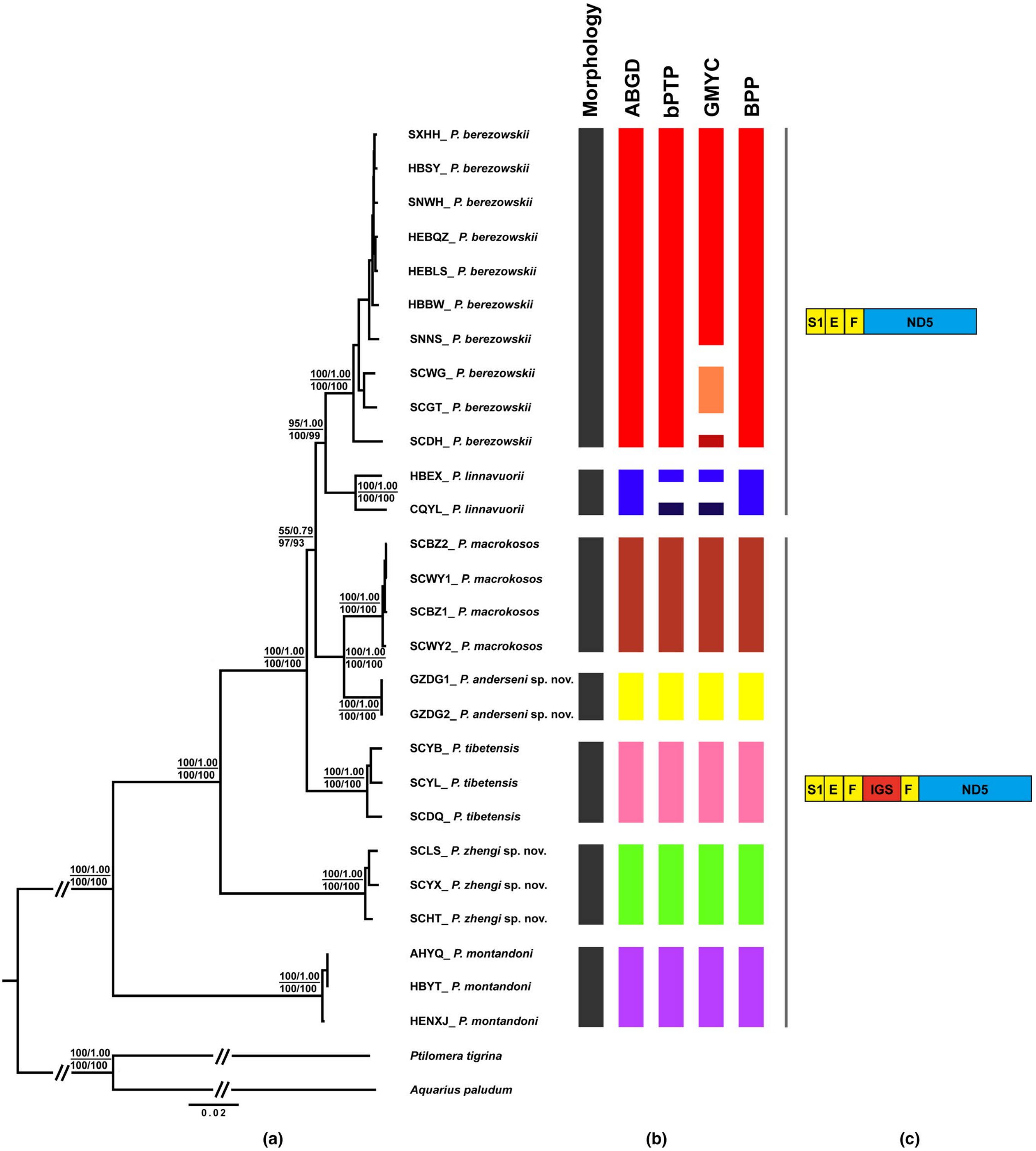
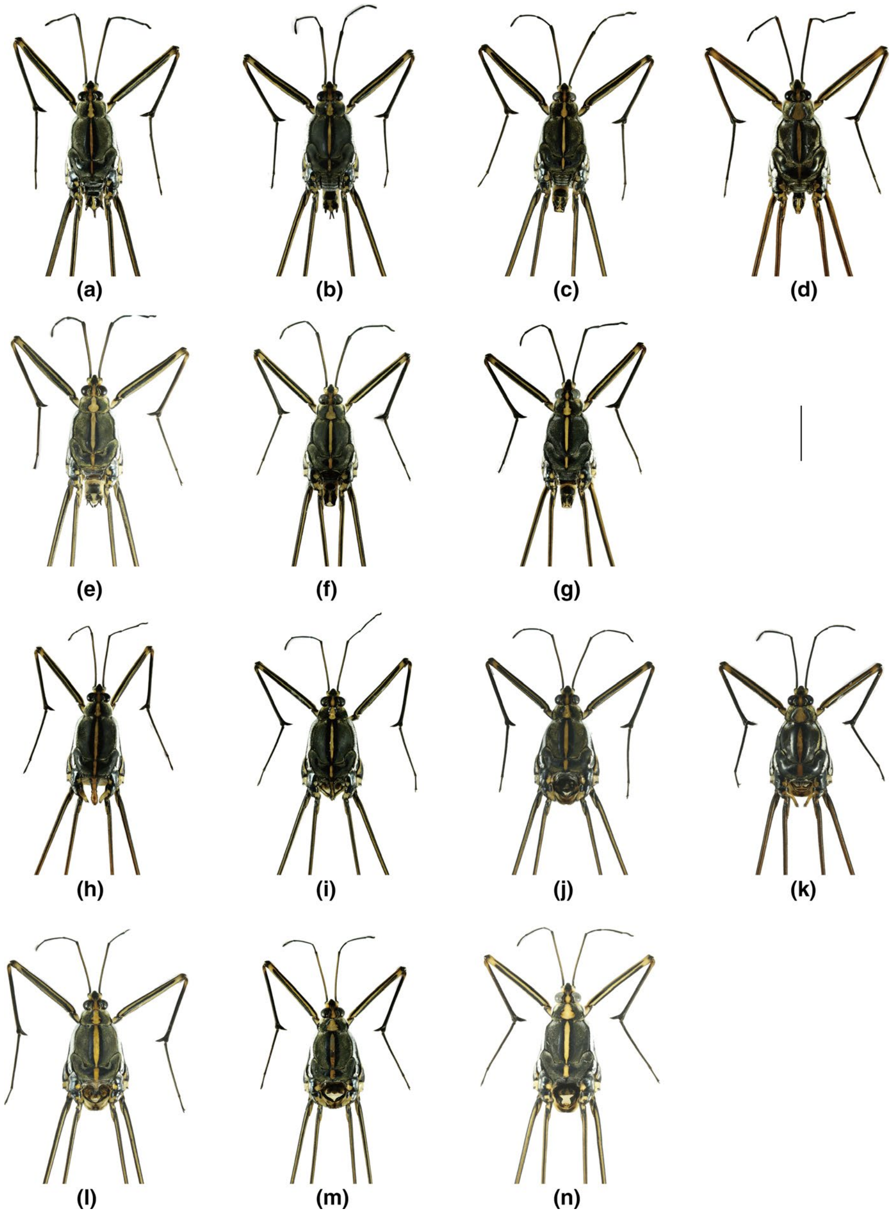


**FIGURE 2** *Potamometra* phylogenetic relationships and species delimitation. (a) Phylogenetic tree of *Potamometra* species based on PCG123RT data set. Numbers associated with each node are, from left to right, ML bootstrap values (upper), BI posterior probabilities (upper), MP jackknife values (lower) and MP poisson bootstrap values (lower). The lengths of the branches follow the phylogram of the ML tree. (b) Summary of species delimitation results. Columns are taxonomic identification based on morphology and the results of four molecular delimitation methods: ABGD, bPTP, GMYC and BPP. The putative species inferred by molecular delimitation methods are represented in different colours. (c) Comparison of mitogenome structure between trnS1 and ND5 among *Potamometra* species. The ND5 gene, tRNAs and IGS (Intergenic spacer) are shown with blue, yellow, and red respectively [Colour figure can be viewed at wileyonlinelibrary.com]

**FIGURE 3** Photographs of *Potamometra* species in dorsal view, middle and hind legs are partly removed. (a)–(g), apterous male: (a) *P. berezowskii*; (b) *P. linnavuorii*; (c) *P. macrokosos*; (d) *P. montandoni*; (e) *P. tibetensis*; (f) *P. anderseni* sp. nov.; (g) *P. zhengi* sp. nov. (h)–(n), apterous female: (h) *P. berezowskii*; (i) *P. linnavuorii*; (j) *P. macrokosos*; (k) *P. montandoni*; (l) *P. tibetensis*; (m) *P. anderseni* sp. nov.; and (n) *P. zhengi* sp. nov. Scale 5.0 mm [Colour figure can be viewed at wileyonlinelibrary.com]



yellow stripe on metanotum narrow; posterior margin of abdominal tergite I of female with a median, long projection; and abdominal sternite 5 long, bend to the dorsal side). *Potamometra macrokosos* and *P. anderseni* sp. nov. formed a clade, which was supported by two characters (inner side of the hind coxa in male with short but distinct projections and posterior margin abdominal sternite 5 with long hairs on both sides of median notch). *Potamometra tibetensis* was clustered with the *P. macrokosos* + *P. anderseni* sp. nov. clade, which was supported by three synapomorphies (male parameres extremely long, protruding distinctly beyond apex of pygophore; apical margin of male pygophore with laterocaudal projection each side; and male proctiger with long hairs along lateral margins). However, relationships among the species (*P. zhengi* sp. nov.), the clade (*P. anderseni* sp. nov., *P. macrokosos*) and the clade (*P. tibetensis*, *P. berezowskii*, *P. linnavuorii*) remain unresolved (Figure S20, Tables 1 and 2).

## 4 | DISCUSSION

### 4.1 | Phylogeny within *Potamometra*

Previous studies of *Potamometra* focused only on morphological classification (Chen et al., 2016; Drake & Hoberlandt, 1965), and the interspecific phylogenetic relationships of the genus have never been explored. In this study, we reconstructed the phylogenetic relationships among *Potamometra* using BI, ML and MP methods based on different data matrixes of mitochondrial genomes. Nucleotide and amino acid sequences of PCGs were used

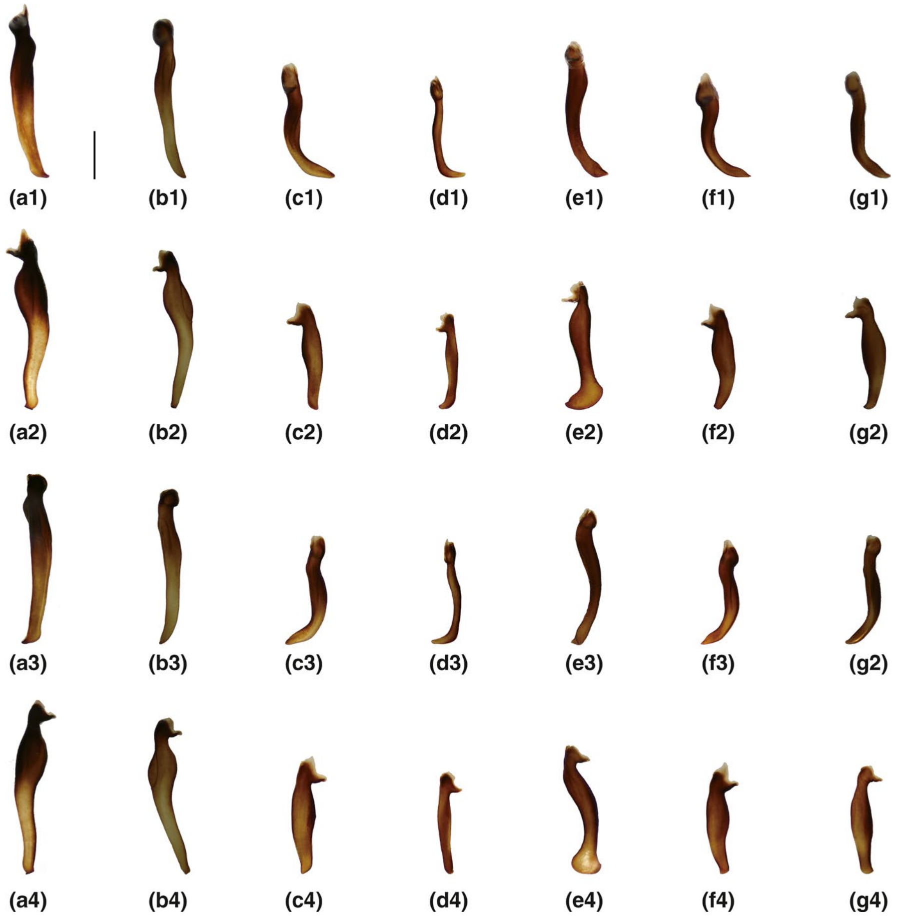


**FIGURE 4** Parameres of *Potamometra* species in various views. The numbers (1–4) represent various views, 1. dorsolateral view; 2. dorsal view; 3. ventrolateral view; 4. ventral view. The letters (a–g) represent different species. (a) *P. berezowskii*; (b) *P. linnavuorii*; (c) *P. macrokosos*; (d) *P. montandoni*; (e) *P. tibetensis*; (f) *P. anderseni* sp. nov.; and (g) *P. zhengi* sp. nov. Scale 0.5 mm [Colour figure can be viewed at wileyonlinelibrary.com]

in our phylogenetic study. Nucleotide sequences performed better than amino acid sequences according to the nodal supports. This could be caused by the amino acid sequences eliminating some valuable phylogenetic signals for the lower-level phylogenetic analyses (Cameron, 2014; Li et al., 2016).

Both molecular and morphological analyses supported the monophyly of the genus *Potamometra*. The synapomorphic character of the genus is the lack of ventroapical depression in the fourth antennal segment (Chen et al., 2016). The main difference between the phylogenetic topology based on the mitochondrial and morphological data was the position of the clade (*P. macrokosos* + *P. anderseni* sp. nov.), which acted as a sister group to the *P. berezowskii* + *P. linnavuorii* clade in the mt tree but exhibited unresolved status in the morphological tree, which could potentially be due to the limited phylogenetic information of morphological characters.

## 4.2 | Gene rearrangement and its phylogenetic implications

In the true bugs, the phenomenon of gene rearrangements has been found in some groups, such as the gene order rearranged from CR-trnI-trnQ-trnM-ND2 to CR-trnQ-trnI2-trnI1-trnM-ND2 in an assassin bug (Heteroptera: Reduviidae) (Jiang et al., 2016), and trnQ-trnI switched positions in flat bugs (Heteroptera: Aradidae) (Song et al., 2016). However, instances of tRNA duplication have rarely been observed in Heteroptera. Prior to this study, the tRNA duplication had not been reported in water striders (Heteroptera: Gerridae). We discovered two trnF genes separated by an intergenic spacer (IGS) in *P. macrokosos*, *P. montandoni*, *P. tibetensis* and two putative new species. The presence of extra tRNAs is usually accompanied by the existence of pseudogenes (Jiang et al., 2016), which was helpful for us as we speculated on the mechanisms of these rearrangements. Three models were generally used to explain the gene rearrangements: the tandem duplication and random loss (TDRL) model (Boore, 2000), the tandem duplication and non-random loss (TDNL) model (Lavrov, Boore, & Brown, 2002) and recombination (Lunt & Hyman, 1997). Based on the results of sequence alignment and the position of IGS and trnF2, we speculated that this gene rearrangement could probably be explained by the TDRL model (Figure 1): the gene tandem duplication first occurred in the trnF-ND5 region and generated two sets of the same gene region (Figure 1b); the ND5 was then randomly eliminated and became a pseudogene, which shaped the IGS during subsequent evolutionary events (Figure 1c).

The gene arrangement pattern of the block from trnF to ND5 was matched to the molecular phylogenetic tree. *Potamometra* species could be separated into two major groups according to the gene arrangement pattern. The two species (*P. berezowskii* and *P. linnavuorii*) without the gene rearrangement formed a strongly supported derived clade, whereas the gene rearrangement event was shared by the remaining species, which suggested that the presence or absence of the unique gene arrangement pattern could provide useful information for understanding the evolutionary relationships among *Potamometra* species.

## 4.3 | Species delimitation

Here, we applied conservative criteria to establish the number of species within an integrative taxonomic framework since DNA-based approaches have the tendency to overestimate species (Luo et al., 2018; Pentinsaari, Vos, & Mutanen, 2016; Will, Mishler, & Wheeler, 2005; Zhang et al., 2013). Searching for powerful diagnostic characters is in fact one of the essential tasks of taxonomy (Zhang et al., 2018). We highlighted the importance of male genital structure and the projections on the inner side of the hind coxa in defining species boundaries among the apterous males of *Potamometra*, which allowed the 27 individuals to be divided into seven clusters, including five known species and two new species (*P. anderseni* sp. nov. and *P. zhengi* sp. nov.).

The distance-based method ABGD retrieved seven MOTUs that corresponded well with the morphological taxa. The perfect performance of ABGD might be due to the low speciation rate of *Potamometra* (Dellicour & Flot, 2015). Although more than seven MOTUs were discovered by the bPTP model, two MOTUs representing the two new species were still defined as independent MOTUs. The bPTP method divided *P. linnavuorii* into two putative species that were inconsistent with morphological delimitation. The discordance between the bPTP and the morphological methods might be because the bPTP tends to overestimate the number of species when using multiple sequences per population (Zhang et al., 2013). Similarly, the single-threshold GMYC method also yielded a high number of MOTUs, which is not surprising since GMYC usually over-splits species, mainly due to low genetic divergence between lineages and overlap of inter- and intraspecific divergences, or due to lack of reciprocal monophyly between sister clades (Pentinsaari et al., 2016; Stokkan et al., 2018; Talavera, Dincă, & Vila, 2013). In addition, sampling bias, differences in population size and speciation rates might also be the reason for the overestimate with GMYC (Esselstyn, Evans, Sedlock, Khan, & Heaney, 2012; Pentinsaari et al., 2016; Talavera et al., 2013).

The Bayesian coalescent method of BPP is designed to analyse multiple loci and has proven to be efficient in species delimitation (Leaché et al., 2017; Yang, 2015; Yang & Rannala, 2010) and may better reflect observations of morphological divergences (Hurtabo-Burillo, May-Itzá, Quezada-Eúan, Rúa, & Ruiz, 2016; Lin, Stur, & Ekrem, 2018; Yang & Rannala, 2017). In this study, the BPP method fully supported the seven species, and all the nodes had perfect posterior probabilities for all three combinations of the ancestral population size ($\theta$) and the root age ($\tau_0$), which indicated that BPP could yield high posterior probabilities for correct species delimitations when appropriate priors were chosen (Luo et al., 2018; Yang, 2015; Yang & Rannala, 2017).

# 5 | CONCLUSION

In the present study, species boundaries of the genus *Potamometra* were explored within an integrative taxonomic framework based on the mitochondrial genomes and morphological data. Although different species delimitation methods failed to produce an identical number of species, most species delimitation approaches indicated seven distinct species, including two new species (*P. anderseni* sp. nov. and *P. zhengi* sp. nov.). Features of the mitochondrial genomes of the *Potamometra* species were analysed. Gene rearrangement occurred in some species of the genus, and the mechanism could be explained by the TDRL model. The presence or absence of the unique gene arrangement pattern could provide useful information for understanding the evolutionary relationships among *Potamometra* species. This study conducted integrative taxonomy to uncover hidden species diversity of the genus *Potamometra* and provides a framework for further study of the biogeography and evolution of this genus.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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