Phylogenetic discordance is due to incomplete lineage sorting and pre-speciation introgression in the erosion-mediated radiation of Asian warty newts

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ABSTRACT

The genus *Paramesotriton*, comprising 15 species classified into two groups, exhibits the broadest geographical distribution among modern Asian newts, spanning southern China from west to east. The group represents a successful example of adaptive radiation. The intrageneric phylogenetic relationships among the species, however, remain unresolved, particularly within the *P. caudopunctatus* group (PCSG). In this study, we employed restriction-site associated DNA sequencing across five representative PCSG species and integrated data from previously published mitochondrial genomes to conduct a comprehensive phylogenomic analysis. Our results provide robust support for resolving interspecific relationships within the PCSG. Incomplete lineage sorting was identified as the primary cause of gene tree discordance, supplemented by gene flow events predating speciation (e.g., ASTRAL, HyDe, Dsuite, and PhyloNet). We also found evidence of hybridization between *P. longliensis* and an unidentified *Paramesotriton* lineage, suggesting that *P. zhijinensis* may be of hybrid origin. Our findings further suggest that erosional isolation caused by the exposure of carbonate sedimentary rocks has facilitated allopatric divergence among the PCSG species. Moreover, our results integrate the origin and diversification of the PCSG with Miocene paleoclimatic conditions and the erosion of carbonate sedimentary rocks, providing crucial insights into the speciation mechanisms of Asian warty newts. We propose an erosion-driven speciation model, wherein repeated episodes of allopatric diversification were promoted by the dynamic geomorphological processes in karst mountain ecosystems during both tectonically active and quiescent periods.

Keywords: Phylogenomics, incomplete lineage sorting, hybridization, radiation, rock erosion

INTRODUCTION

Evolutionary biologists have long been skeptical of sympatric speciation, as geographic or reproductive isolation is traditionally viewed as the prerequisite for lineage divergence. Increasing evidence, however, suggests that speciation can proceed without such isolation (Morjan and Rieseberg, 2004; Ravinet et al., 2017; Ronco et al., 2021; Sousa and Hey, 2013; Sun et al., 2022; Wang and Liu, 2025). This paradigm shift has renewed interest in the role of interspecific gene flow, particularly in how introgression may persist during lineage divergence. Several studies have shown that post-speciation introgression among closely related species is fairly common, shifting the focus toward the evolutionary consequences of gene flow (Lescroart et al., 2023; Meleshko et al., 2021; Wang et al., 2024; Zhang et al., 2023; Zou et al., 2022). Compared with other driving factors such as paleoclimatic changes, gene flow has been shown to promote adaptive radiation in several taxa (Du et al., 2024; Meleshko et al., 2021; Qian et al., 2023). As the extent of gene flow can vary across different stages of divergence (Feder et al., 2012), its evolutionary impact likely depends on whether it occurs before or after speciation (Choin et al., 2021; Meleshko et al., 2021; Wang et al., 2024). The presence of gene flow, however, complicates phylogenetic reconstruction by introducing conflicting signals that may obscure the true evolutionary relationships (Alexander et al., 2017). Therefore, accounting for the dynamics of introgression is crucial when inferring evolutionary histories (Santos et al., 2025; Zhang et al., 2021). In this context, introgression signals have been successfully detected across multiple taxa (Du et al., 2024; Lescroart et al., 2023; Meleshko et al., 2021; Rancilhac et al., 2021) using tools such as PhyloNet (Than et al., 2008) and Dsuite (Malinsky et al., 2021).

Incomplete lineage sorting (ILS) is another major source of gene tree discordance. When ILS occurs during speciation, detecting gene flow becomes particularly difficult, as both processes can leave similar genomic signatures (Pinho and Hey, 2010; Ravinet et al., 2017). ILS refers to the phenomenon where lineages fail to coalesce during speciation events due to the stochasticity of the coalescent process, a pattern commonly observed for rapid speciation events (Avise and Robinson, 2008; Degnan and Rosenberg, 2009; Maddison and Knowles, 2006; Zwickl et al., 2014). ILS reflects the retention of ancestral polymorphism that may become fixed in descendant lineages (Suh et al., 2015), leading to phylogenetic discordance among loci even after divergence (Szöllősi et al., 2015). Consequently, the stochastic nature of ILS produces discordant phylogenetic signals among genomic loci when inferring species relationships (Avise and Robinson, 2008; Rivas-González et al., 2023; Suh et al., 2015; Tan et al., 2023). Distinguishing ILS from introgression remains challenging, as ILS is more prevalent during rapid radiations (Feng et al., 2022; Maddison and Knowles, 2006), whereas gene flow may persist over longer evolutionary timescales. Although methods such as QuIBL (Edelman et al., 2019) and Phytop (Shang et al., 2025) have been developed, they require well-resolved phylogenies. Despite these methodological advances, inferring species relationships and quantifying introgression remain difficult when both ILS and gene flow are present (Kapli et al., 2020). Applying complementary phylogenomic approaches across sequential nodes, however, has proven effective in various taxa (Meleshko et al., 2021; Tan et al., 2023; Wang et al., 2024; Zou et al., 2022). Genome-wide data concerning gene flow and ILS in amphibians remain limited, although genomic sequencing efforts are increasing. Recent studies, however, have documented introgression and ILS during speciation in the eastern red-backed salamander (Waldron et al.,

2025), microhylid frogs (Alexander et al., 2017), banded newts (Van Riemsdijk et al., 2022), and Old World salamanders (Rancilhac et al., 2021). Notably, transcriptomic data from Old World salamanders revealed widespread ancient hybridization across genera within the Salamandridae family, leading to cytonuclear conflict (Rancilhac et al., 2021).

The Asian warty newt genus *Paramesotriton* (Caudata: Salamandridae) is distributed from southern China to northern Vietnam (Figure 1A). The genus represents a successful example of adaptive radiation among modern Asian salamanders (Luo et al., 2022; Yuan et al., 2022). Currently, 15 species are recognized in the genus, and these can be subdivided into two species groups based on morphology and genetics: the *P. caudopunctatus* group (PCSG) and the *P. chinensis* group (see Figure S1 for the phylogeny) (Fei and Ye, 2016; Gu et al., 2012a; Gu et al., 2012b; Luo et al., 2021; Luo et al., 2022; Wang et al., 2013; Wu et al., 2010; Yuan et al., 2014), or into three subgenera (Fei and Ye, 2016). The interspecific relationships within these two species groups, however, remain controversial, particularly within the PCSG (see Figure S2 for the morphology). Previous phylogenetic analyses have primarily relied on mitochondrial ND2, mitogenomes, and a limited number of nuclear gene markers, leading to conflicting phylogenetic topologies (Gu et al., 2012a; Gu et al., 2012b; Luo et al., 2021; Luo et al., 2022; Wang et al., 2013; Wu et al., 2010; Yuan et al., 2014). ILS may be the primary driver of phylogenetic discordance within the PCSG, given the rapid speciation inferred from the estimated divergence times (Luo et al., 2021; Luo et al., 2022; Yuan et al., 2022); however, the relative contributions of ILS and introgression remain unclear. Importantly, several PCSG species are classified as threatened in the China's Red List of Biodiversity (Jiang and Xie, 2021), with *P. longliensis* (Endangered), *P. zhijinensis* (Endangered), *P. wulingensis* (Near Threatened), and *P. caudopunctatus* (Vulnerable), all of which have experienced significant population declines (Jiang and Xie, 2021; Zhao et al., 2012). This underscores the urgent need for both conservation interventions and a robust understanding of their taxonomy, genetic diversity, and evolutionary history—particularly as species delimitation is fundamental to effective conservation policies and elucidating ecological and evolutionary processes.

Guizhou Province is the headwater region of several major river systems, including the Wujiang, Hongshui, Qingshuijiang, and Wuyang Rivers. The province's geological framework is dominated by highly erodible carbonate sedimentary rocks interspersed with mixed sedimentary formations (Figure S2) (Guizhou Provincial Bureau of Geology and Mineral Resources, 1987; Sayre et al., 2014). Notably, species within the PCSG are found almost exclusively in areas composed of carbonate sedimentary rocks (Figure S2). Consequently, populations residing in different tributaries are geographically isolated by river systems that flow through carbonate substrates and are further separated by intervening barriers of mixed sedimentary rocks (Figure S2). The spatial distribution of surface bedrock, however, is not static. Erosional processes acting on non-vertical stratigraphic layers drive the lateral migration of lithological boundaries across the landscape. In the karst mountains of Guizhou, ongoing erosion at rates of 44–76 m/Ma on hillslopes and 280–1138 m/Ma along river channels (Chen, 1985;2019; Yang, 1985) has progressively expanded the surface exposure of carbonate rocks while also increasing the outcrop area of mixed sedimentary rocks (Sayre et al., 2014). These dynamics suggest that suitable habitats for PCSG species were likely more extensive in the past. Over geological timescales, such erosional forces may have

gradually fragmented the ancestral populations across tributary systems bounded by carbonate substrates, thereby facilitating allopatric lineage diversification.

In this study, we traced the complex and likely reticulate evolutionary history of the PCSG. Forty-six individuals of five representative species (*P. caudopunctatus*, *P. zhijinensis*, *Paramesotriton* sp., *P. longliensis*, and *P. maolanensis*) and an outgroup (*Pachytriton inexpectatus*) were sequenced using restriction-site associated DNA sequencing (RAD-seq). The sequencing data were processed through the STACKS pipeline for de novo identification of single-nucleotide polymorphisms (SNPs) in the absence of a reference genome, a similar approach having been applied to *Paramesotriton deloustali* (Van Tran et al., 2025). These data, combined with the eight published mitochondrial genomes of PCSG salamanders, were used in a comprehensive genomic analysis of the five species representing the PCSG. The phylogeny of PCSG salamanders was reconstructed, ILS and gene flow among the species were investigated, and their evolutionary history was traced.

MATERIALS AND METHODS

Taxon sampling

A total of 46 samples were obtained through field surveys and museum collections (Figure 1B). The samples included nine *P. caudopunctatus*, ten *P. longliensis*, two *P. maolanensis*, ten *P. zhijinensis*, eight *Paramesotriton* sp. (Table S1 presents morphological materials), and seven outgroup individuals, providing a comprehensive representation for subsequent analyses (Table S2). All of the muscle samples were collected from the tail tips and preserved in 95% anhydrous ethanol at the Animal Ecology Laboratory, Guizhou Normal University, Guiyang, Guizhou Province, China. Despite extensive multi-seasonal fieldwork conducted in the Wuling Mountains between 2018 and 2024, fresh tissue samples of *P. wulingensis* could not be obtained due to the species' rarity. Consequently, *P. wulingensis* was excluded from this study.

Laboratory protocols, sequencing, and bioinformatic methods

A cetyltrimethylammonium bromide protocol was used to extract genomic DNA from ethanol-preserved tissues (n = 46) (Hanania et al., 2004). Following digestion with EcoRI (GAATTC), RAD libraries were prepared (Baird et al., 2008) and size-selected (350–550 bp) for paired-end sequencing on an MGISEQ-2000 platform (MGI Tech, Shenzhen City, China). The raw sequencing data are available under NCBI BioProject PRJNA1016311.

We processed raw reads using fastp v0.23.4 (Chen, 2023) for quality filtering, followed by STACKS v2.61 (Rochette et al., 2019) for RAD-seq data analysis. The pipeline comprised (1) cleaning and filtering the raw data using process_radtags and clone_filter; (2) building loci (ustacks), creating a catalog of loci (cstacks), matching samples back against the catalog (sstacks), transposing the data (tsv2bam), adding paired-end reads to the analysis, and calling genotypes; and (3) de novo SNP calling with denovo_map.pl. Key assembly parameters were optimized following the established r80 method (Gundappa et al., 2022; Paris et al., 2017). We systematically evaluated the maximum number of allele mismatches allowed between stacks (M = 3–8) and coverage depth requirements (m = 4–14), with the number of mismatches allowed between sample loci (n) set equal to M. The final parameters selected were M = 6, m = 10, and n =

6, ensuring robust locus recovery while maintaining data quality. In total, the STACKS program identified 15,581,647 biallelic SNPs across 46 individuals.

We implemented a rigorous SNP filtering pipeline: (1) initial variant filtering using VCFtools v0.1.16 (MAF > 0.01, missing data < 50%, biallelic sites) (Danecek et al., 2011); (2) sample quality control with PLINK v1.90 (Purcell et al., 2007) (excluding samples with greater than 50% missing genotypes); (3) genotype imputation via Beagle v5.0 (Browning and Browning, 2016); and (4) linkage disequilibrium (LD)-based SNP pruning using bigsnpr v1.9.11 (Privé et al., 2018).

Phylogenetic reconstruction based on mitochondrial genomes and SNP data

We constructed a maximum likelihood (ML) tree using IQ-TREE v.1.6.12 (Nguyen et al., 2015) based on concatenated loci under the GTR + ASC model. Node support was evaluated with 2000 ultrafast bootstrap replicates (Hoang et al., 2018).

We used two approaches for reconstructing the species tree. First, we estimated the species tree using SVDquartets (Chifman and Kubatko, 2014) implemented in PAUP* 4.0a (Wilgenbusch and Swofford, 2003), an algorithm that evaluates all of the possible taxon quartets under the coalescent model. Node support was assessed with 100 bootstrap replicates. This approach additionally helped identify cases of ILS or introgression where nodal support was low. Second, 1,475,246 SNPs were partitioned into non-overlapping windows (2-kb, 5-kb, 10-kb, 20-kb, 30-kb, and 50-kb) using raxml_sliding_windows.py (https://github.com/simonhmartin/genomics_general, accessed on July 12, 2024), each serving as an independent SNP subset. These subsets were used to infer gene trees under the GTRCAT model in RAxML. The species tree was subsequently inferred using ASTRAL-III (Zhang et al., 2018) by summarizing topological information from the reconstructed gene trees.

The 18 mitochondrial genomes used for phylogenetic reconstruction were primarily obtained from our previously published study (Table S3) (Luo et al., 2022). Multiple sequence alignment employed MUSCLE (Edgar, 2004) within MEGA v.7.0 (Kumar et al., 2016). The best-fit partitioning schemes and nucleotide substitution models were selected based on the Bayesian Information Criterion using PartitionFinder v.2.1.1 (Lanfear et al., 2017). Both ML and Bayesian inference (BI) methods were used to reconstruct the phylogenetic trees. The ML analysis was run in IQ-TREE v.2.0.4 (Nguyen et al., 2015) under the optimal model (Table S4), with 20,000 ultrafast bootstrap (UFBoot) replicates. BI analysis was performed in MrBayes v.3.2.1 (Ronquist et al., 2012), with two independent runs of five million generations each, sampling every 1,000 generations and discarding the first 25% as a burn-in. Nodes were considered well-supported if the Bayesian posterior probability (BPP) exceeded 0.95 and the UFBoot values exceeded 95%.

Genetic cluster analysis, genetic diversity, and population dynamics

We performed LD-based pruning to reduce the correlations among SNPs using PLINK v.1.9 (Purcell et al., 2007) with the following parameters: --indep-pairwise 50 10 0.5. The R package bigsnpr v.1.9.11 was used to conduct principal component analysis (PCA) (Privé et al., 2018), and scatter plots of the first two principal components (PC1 and PC2) were generated for visualization. Population genetic structure was inferred using ADMIXTURE v.1.3.0 (Alexander et al., 2009) under the default settings, with the number of ancestral clusters (K) ranging from 2 to 5. The optimal K value was determined based on the lowest

cross-validation (CV) error. VCFtools v0.1.16 was used to calculate nucleotide diversity (π) and pairwise genetic differentiation (Fst) for each species (Danecek et al., 2011). Stairway Plot v2.1.1 was used to reconstruct demographic history (Liu and Fu, 2015), based on the site frequency spectrum generated with easySFS (Gutenkunst et al., 2009). A generation time of three years (Funk et al., 1999) and a per-site, per-generation mutation rate of 2.2×10^{-9} (Reilly, 2009) were used in the model.

Detection of gene flow and incomplete lineage sorting

Currently, it remains unclear whether the observed phylogenetic discordance in the PCSG (Luo et al., 2021; Luo et al., 2022) was caused by gene flow, ILS, or a combination of both. To address this issue, we used multiple approaches to detect potential introgression and ILS within the PCSG. First, we used the Treemix method (Pickrell and Pritchard, 2012) to detect the presence and direction of gene flow among the five species. The analysis was independently run 10 times using the following parameters: -root -m 1~10 -k 1000 -se -bootstrap 1000 -global -noss. The optimal number of migration events was determined by comparing the observed changes in the likelihood value using the R package OptM (Fitak, 2021). Second, we used the Dtrios program implemented in Dsuite (Malinsky et al., 2021) to assess gene flow among species of the PCSG based on the species relationships revealed by the phylogenetic trees. We used three methods: *D*-statistics (Durand et al., 2011), *f*4-ratio (Patterson et al., 2012), and *f*-branch methods (Malinsky et al., 2018). For *D*-statistics and the *f*4-ratio, we ran "Dsuite Dtrios" by inputting a VCF file, a species tree file, and a sample file. For the *f*-branch analysis, we performed "Dsuite Fbranch" using the species tree and utilized the output of the "Dsuite Dtrios" analysis to map gene flow intensities to the phylogenetic tree topology.

We conducted a hybridization detection analysis using HyDe to further validate the ancient introgression signals indicated by the D-statistics (Blischak et al., 2018). HyDe is a site-pattern probabilistic method for detecting hybrid speciation events, identifying putative parental populations, and estimating the genetic parameter γ to quantify the genomic contribution of each parental population to the hybridization event (Blischak et al., 2018). A rooted, four-taxon hybridization test was performed for all of the possible triplet combinations, i.e., an outgroup (O), two putative parental populations (P1 and P2), and a hybrid population (Hyb) comprising a mixture of P1 and P2, i.e., ((P1, (Hyb), P2), O). Values of γ around 0.5 and *p*-values < 0.05 indicate a 50:50 hybridization origin with the two parental lineages contributing, while very low or very high values indicate no hybridization events (Blischak et al., 2018). In this test, the hybridizing population is either the sister population of P1 with probability $1 - \gamma$ or the sister population of P2 with probability γ (Meng and Kubatko, 2009). We performed hybridization tests using a three-step analytical pipeline. Using the "run_hyde_mp.py" script, we evaluated hybridization events between all triplet combinations of the five species, with *Pachytriton inexpectatus* as the outgroup, and filtered the results based on whether there was significant evidence of hybridization (*P*-value < 0.05). Next, we tested each individual in the putative hybridization population using the "individual_hyde.py" script. Finally, we used the "bootstrap_hyde.py" script for bootstrap resampling (500 replicates) of individuals in the putative hybrid lineage for each of the specified triplets.

Based on the geographic distribution (Figure 1B) and previous phylogenetic results (Luo et al., 2021; Luo et al., 2022), we hypothesized that *P. zhijinensis* may have originated from an ancient hybridization

event. To test this hypothesis, we used PhyloNet-MPL and HyDe to quantify the genomic contributions of the two parental lineages (*P. longliensis* and *Paramesotriton* sp.) to the hybrid species *P. zhijinensis*. We used the tree-based maximum pseudo-likelihood method InferNetwork_MPL in PhyloNet v3.8.2 to infer species networks in introgression and ILS scenarios (Than et al., 2008). The 295 gene trees from ASTRAL were used as input. Ten parallel network searches were performed, allowing 0–5 reticulation events. The number of runs (-x) of the search was set to 100, and the starting tree was the species tree from ASTRAL to produce the five networks with the highest likelihood scores. The branch lengths and inheritance probabilities of the returned species networks were optimized under full likelihood conditions by specifying the "-po" option. Four independent runs were performed, with the other parameters set as the default values. To estimate the optimal network among models with different numbers of reticulation events, the inferred network with the highest likelihood score was chosen, and the optimal network was visualized in Dendroscope v3.8.10 (Huson and Scornavacca, 2012).

We used Phytop (Shang et al., 2025) to identify and visualize signals of ILS and introgression/hybridization. Based on the output of ASTRAL (Zhang et al., 2018) under the parameter setting "-t 2," Phytop quantifies the degree of ILS and introgression/hybridization by analyzing the proportions of internal node topologies in the species tree (Shang et al., 2025).

Divergence time estimation, biogeography, and analysis of diversification dynamics

We used SNAPPER v1.1.2 to estimate divergence times and reconstruct the species tree (Bryant et al., 2012; Stoltz et al., 2021); SNAPPER v1.1.2 implements a strict molecular clock model coupled with the Wright-Fisher diffusion model (Brown and Yang, 2011; Stange et al., 2018). SNAPPER analysis was implemented in BEAST v2.7.6 (Bouckaert et al., 2019) with input datasets prepared through random SNP selection using the snapp_prep.rb script (https://github.com/mmatschiner/snapp_prep, accessed on July 12, 2024) (parameters: ruby snapp_prep.rb -a SNAPPER -v -t -c -s -m 3860 -l 1000000 -x -o) to generate the required XML input files. Calibration for the timing of the split between *Paramesotriton* and *Pachytriton* was constrained to 25.42 Ma (a lognormal distribution, standard deviation = 0.0859) (Luo et al., 2022). Three independent runs were performed in BEAST v2.7.6 (Bouckaert et al., 2019), each with one million generations and sampling every 500 generations. Convergence of the runs was assessed using Tracer v1.7.2 (Rambaut et al., 2018) by verifying that the effective sample sizes exceeded 200. DensiTree v2.0 (Bouckaert et al., 2019) and TreeAnnotator v2.7.6 (Bouckaert et al., 2019), with a burn-in of the first 10% of the MCMC chains, were used to generate maximum-credibility trees with median heights.

The ancestral ranges of the PCSG were reconstructed using BioGeoBEARS (Matzke, 2013) following established protocols (Luo et al., 2021). The current distribution was classified into six distinct biogeographic regions based on mountain ranges, the river network structure, and the geological structure: (A) the Leigong Mountains, (B) the Northern Wumeng Mountains-Wujiang River, (C) the Southern Wumeng Mountains-Wujiang River, (D) the Lianjiang River Basin, (E) the Libo Karst Region, and (F) the Wuling Mountains. Based on comprehensive field investigations and a literature review, the maximum ancestral range size was constrained to two adjacent regions to ensure biologically meaningful reconstructions.

We followed the methods outlined in a previous phylogenetic study (Li et al., 2024) to infer the diversification dynamics of the PCSG. The BEAST results were summarized to obtain time intervals with 95% confidence intervals for lineage divergence events. Lineage divergence events were defined as the maximal number of observed lineage divergence events per 0.5 million years (MDE) and were used to illustrate trends over time.

RESULTS

Genome sequencing, single-nucleotide polymorphism calling, and the mitogenome dataset

A total of 46 samples generated approximately 2948.5 Gb of raw data and 1,004 million paired-end reads, with an average of 64.09 Gb of data and 21.8 million paired-end reads per sample (Table S1). Of these, 92.80% of the bases had quality scores greater than or equal to 30, and the guanine-cytosine content was 43.91% (Table S1). A total of 15,581,647 biallelic SNPs were generated from the STACKS-cleaned reads. After filtering for low-quality individuals using PLINK, 1,475,246 SNPs remained. A total of 438,504 SNPs were retained after linkage disequilibrium (LD)-based SNP filtering. In total, three SNP datasets were generated: Dataset 1 (1,475,246 SNPs) for phylogenetic reconstruction, Dataset 2 (438,504 SNPs) for PCA and genetic clustering, and Dataset 3 (3,860 SNPs) for estimation of divergence times. The mitogenomes of 18 species (15,466 bp in length) contained 4,334 variable and 2,852 parsimony-informative sites. The best-fit substitution models were TVM + I + G (12S/16S rRNA), HKY + I + G (tRNAs), GTR + G (most protein-coding genes), and HKY + G (ND6).

Phylogenetic relationships between the species

Phylogenetic analysis based on the mitochondrial genome revealed minor mitochondrial divergence among species within the PCSG. The genetic distance of mitochondrial genes ranged from 0.53% to 7.80% (Table S5) and indicated division into two clades. *Paramesotriton zhijinensis* was positioned at the base of Clade II, followed in sequence by *P. maolanensis*, *P. longliensis*, and *Paramesotriton* sp. The phylogenetic relationship between the latter two remained unresolved, as demonstrated by the low nodal support values (BPP = 0.47, UFB = 42) (Figures 2A and S4). This supported our suggestion that mtDNA alone is insufficient to resolve species-level relationships within this rapidly diverging group. In contrast, the ML tree inferred from the SNPs dataset provided a highly resolved phylogeny with strong support (UFBoot = 100) (Figure 2B). In this ML tree, *Paramesotriton* sp., however, was positioned at the base of Clade II, followed by *P. zhijinensis*, *P. maolanensis*, and *P. longliensis*. The species tree inferred using ASTRAL exhibited a topology consistent with the SNP-based ML tree (Figures 2C and S5), whereas the SVDQuartets species tree supported *P. zhijinensis* as the basal lineage of Clade II (Figure 2D).

Contributions of ILS, genetic introgression, and hybridization to gene tree heterogeneity

Evidence of gene flow among species within the PCSG was provided by the Treemix, *D*-statistics, *f*4-ratio, and *f*-branch analyses. The TreeMix analysis detected significant gene flow from the ancestor of Clade II to *P. maolanensis*, from *P. caudopunctatus* to the common ancestor of *P. longliensis* and *P. maolanensis*, and from *P. zhijinensis* to *P. caudopunctatus* (Figure 3A), consistent with the results of the *D*-statistics and f_4-ratio tests (Figure 3B–D). The *D*-statistics (Figure 3B), f_4-ratio test (Figure 3C), and *f*-branch method (Figure 4D) provided further evidence of historical gene flow among the species (D = 0.01–0.14, |*Z*-

score| > 3, $P < 0.001$) (Figure 3B and Table S6), particularly between *P. zhijinensis* and *P. longliensis*, as well as between *P. zhijinensis* and *Paramesotriton* sp. (Figure 3D).

The PhyloNet-MPL analysis supported hybridization among the three species, suggesting that *P. zhijinensis* is likely of hybrid origin, with 45% of its genome inherited from *P. longliensis* and 55% from *Paramesotriton* sp. (Figures 3E and S6). The PhyloNet-MPL results were further corroborated by the HyDe analysis. The HyDe results showed that only 2 of 10 significant hybridization events produced γ-values close to 0.5 (Table S6), suggesting that *P. zhijinensis* is likely of hybrid origin (Figure 3F). Next, we further validated the authenticity of these two hybridization events at the individual level. The γ-values for *P. longliensis* and *Paramesotriton* sp. individuals ranged from 0.35 to 0.46 and from 0.54 to 0.65, respectively (Table S8). This supported *P. zhijinensis* as a hybrid species, with genomic contributions of approximately 62.1% from *Paramesotriton* sp. and 37.9% from *P. longliensis* (Figure 3F; Table S7). Finally, bootstrap resampling (500 samples) was performed on individuals within the hybrid population to obtain the distribution of γ-values for assessing the heterogeneity in levels of genetic introgression (Figure 3F; Table S9). The results revealed nearly uniform genetic admixture in *P. zhijinensis* at both the species and individual levels (Figure 3E; Tables S7–S9). However, because our data were not genome-wide, whether *P. zhijinensis* represents a true hybrid requires cautious interpretation.

Additional phylogenomic analyses using Phytop on the genome data indicated that ILS accounted for 11.0–42.7% of the conflicts within Clade II, while introgression explained the remaining discordance, corroborated by introgression events between *P. zhijinensis* and *P. longliensis*, as well as between *P. zhijinensis* and *Paramesotriton* sp. (Figure 3G). Thus, ILS primarily contributed to the conflicting relationships within the PCSG, and this was complemented by introgression.

Population genetic structure, diversity, and demographic history of the PCSG

Population genetic analyses were employed to infer the genetic structure and evolutionary history of the PCSG. When K = 4, the population structure analysis identified four distinct genetic clusters within the PCSG (Figures 4A and S7), a finding that contrasted with the five clusters determined by PCA (Figure 4B). The genetic structure revealed admixture among *P. longliensis*, *P. maolanensis*, *P. zhijinensis*, and *Paramesotriton* sp., with particularly strong signals between the first two species (Figure 4A). In addition, the estimates of population differentiation (Fst) between *P. maolanensis* and *P. longliensis*/*P. caudopunctatus* were 0.06 and 0.03, respectively, suggesting limited genetic divergence (Figure 4C). These values, however, should be interpreted with caution due to the extremely small sample size (n = 2) for *P. maolanensis* (Weir and Hill, 2002). In contrast, the remaining species exhibited moderate genetic differentiation, with Fst values ranging from 0.11 to 0.16 (Hartl and Clark, 1997).

Genetic diversity assessed using π revealed that *P. longliensis* and *P. zhijinensis* exhibited comparable π values, both higher than that of *Paramesotriton* sp. (Figure 4D). Notably, *P. caudopunctatus* exhibited the highest level of genetic diversity, indicating a relatively large effective population size, consistent with field observations.

Population demographic analyses indicated that a bottleneck event occurred between 1 Ma and 130 ka, characterized by a rapid decline in the effective population sizes (*Ne*) of *P. caudopunctatus* (~800 ka), *P. longliensis* (~200 ka), *P. zhijinensis* (~130 ka), and *Paramesotriton* sp. (~130 ka), followed by a

subsequent rapid recovery (Figure 4E). Thereafter, *Ne* remained relatively stable from 100 ka to 8 ka before entering a declining phase. From 8 ka to the present, *Ne* has undergone a continuous decrease (Figure 4E).

Divergence time, biogeography, and diversification dynamics

The species tree inferred by SNAPPER showed topological congruence with both the SNP-based ML tree and the ASTRAL species tree (Figure 5A). The time to the most recent common ancestor of the PCSG was estimated at ~16.90 Ma (95% highest posterior density (HPD): 20.09–13.80 Ma). Subsequent speciation events within this group occurred between 13.89 Ma and 8.15 Ma (Figure 5B).

The ancestral range reconstruction suggested that the ancestors of the PCSG were most likely distributed in the Wumeng Mountains-Wujiang River (Figures 5B and C, S8, and S9; Table S10). A vicariance event at ~16.90 Ma divided the common ancestors into eastern and western groups (Figure 5B). The biogeographic results also suggested that the eastern group may have originated from the dispersal of the western group, while the ancestors of the southern species likely dispersed stepwise from the Wumeng Mountains through the central area during the Miocene (Figure 5B and C).

MDE analyses showed that lineage divergence within the PCSG occurred ~18 Ma, with a rapid increase at approximately 17 Ma and 10 Ma. Two distinct peaks in divergence occurred: the first at ~15 Ma and the second at ~8.5 Ma, with a valley at ~11 Ma (Figure 5E).

DISCUSSION

Phylogeny under incomplete lineage sorting and ancient introgression

Our analyses have resolved long-standing phylogenetic uncertainties within the PCSG. While morphological studies have proposed either a two-group (Fei et al., 2006) or three-subgenus classification (Fei and Ye, 2016), the molecular data consistently supported a division into two major clades (Gu et al., 2012a; Gu et al., 2012b; Luo et al., 2021; Luo et al., 2022; Wang et al., 2013; Yuan et al., 2014; Yuan et al., 2022). The relationships among *P. zhijinensis*, *P. longliensis*, *P. maolanensis*, and *Paramesotriton* sp., however, remain unresolved, with the mitochondrial data suggesting two conflicting topologies (Figure S10) (Luo et al., 2021; Luo et al., 2022). Our coalescent- and concatenation-based analyses strongly support *P. longliensis* as the sister species of *P. maolanensis* (Figure 2). Phylogenetic discordance analyses further indicated that ILS was the main source of cytonuclear conflict, with introgression playing a secondary role (Figure 3).

The phylogenetic analyses indicated that ILS was the primary cause of observed conflicts, although distinguishing ILS from introgression remains difficult, as both generate similar signals (Fontaine et al., 2015). There was an absence of recent large-scale admixture signals in the population structure and DensiTree analyses (except in *P. maolanensis*). A plausible explanation is that while current interspecific gene flow is limited by geographic isolation, historical introgression during the early phase of speciation may have contributed to the rapid diversification of the PCSG into distinct ecological niches (Luo et al., 2021). This scenario is consistent with recent geological changes in the region and the relatively recent divergence times of these species (Luo et al., 2021; Zhou and Chen, 1993). Notably, ancient admixture involving ancestral variation has been shown to drive rapid radiation in other taxa (Marques et al., 2019), a pattern supported by our tests for introgression and ILS. Further analysis of discordance sources revealed

that ILS consistently contributed more than introgression across internal nodes (Figure 3G), reinforcing the view that the phylogenetic conflicts were primarily due to ILS (Edelman et al., 2019; Meleshko et al., 2021). Importantly, these ILS signals likely reflect episodes of rapid lineage diversification within the PCSG. Similar patterns of introgression-associated discordance have been reported between *P. deloustali* and *P. guangxiensis* (Van Tran et al., 2025). Nevertheless, we acknowledge the potential for underestimating recent gene flow due to several factors. First, introgression from unsampled "ghost" lineages or interspecific differences in effective population size may bias *D*-statistics (Zheng and Janke, 2018), introducing unexplained error. Second, our SNP dataset lacks whole-genome coverage, and missing loci may have affected the estimates of gene flow. These limitations underscore the need for future studies that incorporate de novo genome assemblies (e.g., HiFi sequencing with Hi-C scaffolding) and assess the functional relevance of introgressed regions.

Conservation assessments have revealed alarming trends. Based on the IUCN Red List criteria, *P. zhijinensis* is classified as Endangered (B1ab(iii)), *P. caudopunctatus* as Near Threatened (A2cd), and *P. longliensis* as Vulnerable (B1ab(iii,v)). The effective population size of the PCSG has declined by more than 96% since the Last Glacial Maximum (Figure 4E), and in combination with low nucleotide diversity (Figure 4D) and the continued reduction of potential suitable habitat area (Yan, 2023), these indicators collectively meet the thresholds for classification as Critically Endangered. Similar demographic declines have been reported in music frogs (Lyu et al., 2024). Moreover, due to only two sequenced samples, the genetic diversity of *P. maolanensis* remains unassessed. Since its discovery, the species has only been recorded by the type specimens (Gu et al., 2012a), with no subsequent findings, suggesting an extremely small population and a potentially underestimated risk of extinction. Although all of the *Paramesotriton* species are listed as Class II protected wildlife in China, only four are currently covered by the protected area network in Guizhou (Figure S11). We therefore recommend prioritizing conservation efforts for species outside these protected areas.

Paramesotriton wulingensis was not included in this study due to constraints such as the small population size and legal restrictions. Previous studies have identified *P. wulingensis* along with *P. caudopunctatus* as part of the basal clade of the PCSG (Gu et al., 2012a; Gu et al., 2012b; Luo et al., 2021; Luo et al., 2022; Yuan et al., 2022). Both biogeographic reconstruction and gene flow analyses, however, require a complete phylogenetic framework. The absence of *P. wulingensis* thus introduces some uncertainty, although its impact may be limited. Future inclusion of this species will be essential for a more comprehensive understanding of introgression and the biogeographic history of the PCSG.

Possible ancient hybrid origin of *P. zhijinensis* is supported by multiple lines of evidence

Multiple lines of evidence suggest that *P. zhijinensis* may have originated through hybridization. Although the population structure inferred by ADMIXTURE indicated that *P. zhijinensis* harbors only a small proportion of genetic ancestry from *Paramesotriton* sp. and *P. longliensis*, both PhyloNet-MPL and HyDe analyses inferred nearly equal (~50%) genomic contributions from these two parental lineages (Figure 3E and F). In addition, the *D*-statistics (Figure 3B), the f_4-ratio test (Figure 3C), and the *f*-branch method detected strong signals of gene introgression. This discrepancy is likely attributable to differences in the sensitivity and assumptions of the various analytical methods. ADMIXTURE, which employs a model-

based approach using allele frequency data, is optimized for detecting recent population structure (Alexander et al., 2009). The method may be less effective in identifying ancient or complex hybridization events, especially when the admixture signals have been eroded by subsequent recombination, backcrossing, or strong purifying selection following the ancestral introgression. In contrast, HyDe and PhyloNet-MPL are specifically designed to infer hybridization under a phylogenetic framework based on invariants and network likelihood (Blischak et al., 2018; Than et al., 2008), making them more powerful tools for detecting deep or ancient hybrid origins. Therefore, despite the very weak admixture signals observed in the ADMIXTURE results, the results from HyDe and PhyloNet-MPL provide strong support for an ancient hybrid origin of *P. zhijinensis* from *P. longliensis* and *Paramesotriton* sp. Similar hybrid origins supported by HyDe and PhyloNet-MPL have been reported in other taxa, including macaques (Zhang et al., 2023), Asiatic black bears (Zou et al., 2022), South American fur seals (Lopes et al., 2023), and swordtail fishes (Du et al., 2024). Another possible explanation for the discrepancy is that RAD-seq data may be affected by biases in the distribution of restriction enzyme cut-sites (Andrews et al., 2016). If the sampled loci are located outside of regions under neutral selection or active introgression, or fail to capture parental lineage-specific divergent regions, ADMIXTURE may be unable to detect the admixture signals, potentially misclassifying *P. zhijinensis* as a distinct cluster. In contrast, RAD-seq typically yields thousands to tens of thousands of SNPs, providing sufficient resolution for HyDe, PhyloNet-MPL, and the *f*-branch method to recover congruent signals of ancient hybridization.

Additionally, although we hypothesized that *P. zhijinensis* may have originated through hybridization between *Paramesotriton* sp. and *P. longliensis*, with its intermediate body size providing morphological support (Table S11), the limitations of our data analysis underscore the need for further studies incorporating multiple lines of evidence. These may include comparative genomics using high-quality genomes, karyotype analysis, and reproductive studies.

Effects of ancestral introgression on the inference of divergence time

Molecular clock models can significantly influence estimates of divergence time. Relaxed molecular clock models accommodate rate variation across lineages and may better reflect natural evolutionary processes (Chen et al., 2021), while nucleotide substitution rates among closely related species often show minimal variation (Brown and Yang, 2011; Ho and Duchêne, 2014). In such cases, strict molecular clock models are frequently preferred and demonstrate superior performance (Brown and Yang, 2011; Yang and Rannala, 2006). Similar to SNAPP (Stange et al., 2018), SNAPPER enables highly precise estimation of divergence time using SNP data through the Wright-Fisher diffusion model that approximates the multi-species coalescent model (Stange et al., 2018; Stoltz et al., 2021). Owing to these advantages, both SNAPP and SNAPPER have been successfully applied to various animal taxa (Oury et al., 2023; Sánchez et al., 2023; Tunström et al., 2023), lending support to the reliability of our results.

Historical introgression may inflate estimates of species divergence times. Molecular dating revealed that the genus *Paramesotriton* originated in the late Oligocene, ~25.29 Ma, and the MRCA of the PCSG originated in the early Miocene, ~16.90 Ma, consistent with previous nuclear DNA-based estimates (Luo et al., 2022). Our study estimated significantly earlier interspecific divergence times for the PCSG than previous mtDNA-based results (Luo et al., 2021; Shen et al., 2024; Yuan et al., 2022), with an approximate

5 Ma discrepancy. The divergence times near the phylogenetic root, however, remained consistent. While mtDNA is conventionally considered a rapidly evolving marker that typically yields relatively ancient divergence time estimates (Zheng et al., 2011), our findings presented the opposite pattern. We propose that interspecific gene flow during speciation events likely caused mtDNA homogenization and reduced the level of genetic variation among lineages. This process systematically biases divergence time estimates toward more recent dates (Joly et al., 2009; Rosenzweig et al., 2016), particularly at the tips of branches. Therefore, when using mtDNA for molecular dating, especially in rapidly radiating lineages, it is crucial to evaluate potential gene flow to avoid systematic underestimation of divergence times.

Evolution of biodiversity is driven by the erosion of heterogeneous rock

Diversification of PCSG has been driven by orogeny and the monsoon through the erosion of heterogeneous rocks. The geological and topographic features of the karst mountains in Guizhou provide an additional test for the hypothesized link between geology and biological diversification. Currently, the PCSG species in different tributaries are restricted to exposed carbonate sedimentary rock and are isolated by mixed sedimentary rock (Figure S3). How, then, did their ancestors disperse throughout the river network? Current geological data indicate that the present-day landscape of Guizhou initially formed during the late Tertiary period due to a west-to-east tilting uplift (Guizhou Provincial Bureau of Geology and Mineral Resources, 1987; Zhang, 2012; Zhou and Chen, 1993), with carbonate sedimentary rock overlying the uppermost strata (Sayre et al., 2014). As surface erosion exposed the contact zone between carbonate sedimentary and mixed sedimentary rocks, fluvial erosion and paleoclimatic conditions drove the upstream movement of this contact zone, resulting in a terrain characterized by higher elevations in the north and lower elevations in the south (Li, 2001; Zhou and Chen, 1993). Although all extant PCSG species are currently confined to carbonate sedimentary rocks, the forces of orogeny, paleoclimatic changes, and progressive erosion have collectively shaped distinct river networks (Chen, 2019; Guizhou Provincial Bureau of Geology and Mineral Resources, 1987; Yang, 1985). Together with the exposure of mixed sedimentary rocks, these factors may have acted as barriers to dispersal, promoting the geographic isolation and divergence of populations across different tributaries (Figures 5C and S3). We hypothesized that the erosion-induced migration of the geological contact zone led to geographic isolation among drainage basins and thereby served as a key mechanism for allopatric differentiation among PCSG species.

To test this hypothesis, we conducted a biogeographic analysis that integrated both distributional patterns and divergence time estimates. Our findings indicated that the origin, dispersal, and diversification of PCSG were closely synchronized with major Miocene paleogeoclimatic events. These include the rapid uplift of the Qinghai-Tibetan Plateau (25–15 Ma) (Ding et al., 2022), the accelerated left-lateral extrusion of Indochina (35–16 Ma) (Gilley et al., 2003; Leloup et al., 2001), and the intensified East Asian monsoon rainfall (23–13 Ma) (Farnsworth et al., 2019; Wan et al., 2025). The combined effects of vigorous orogenic activity, warm climatic conditions, and heavy precipitation contributed to the development of the region's characteristic topographic gradients—namely, higher elevations in the north and west relative to the south and east (Zhou and Chen, 1993). This geomorphological evolution likely facilitated the southward dispersal of PCSG populations, followed by geographic isolation and subsequent diversification of species. Our robust phylogenetic evidence provides strong support for this evolutionary scenario.

In addition, our analysis suggested that the maximal number of observed lineage divergence events per 0.5 million years (MDE) occurred during the early Miocene, with the origin of this peak dating to approximately 18.2 Ma (95% HPD: 19.91–11.62 Ma). A rapid increase in lineage divergence began around 17 Ma, reaching an initial peak at ~15 Ma (Figure 5E). Afterward, a decline began at ~14.5 Ma and reached the valley at ~12.5 Ma, coinciding with the left-lateral extrusion of Indochina that began slowing at ~16 Ma, transitioned to right-lateral extrusion by ~13 Ma, and underwent continuous Miocene cooling (15–12 Ma) (Leloup et al., 2001; Leloup et al., 1995; Li et al., 2024; Wan et al., 2025; Wang et al., 2020). Finally, during the period of the right-lateral extrusion of Indochina (~11–5 Ma) (Li et al., 2024), the Ailao Shan orogenic activity (8.4–6.8 Ma) (Fyhn and Phach, 2015; Zhang et al., 2009), the rapid uplift of the Hengduan Mountains (~11–2.5 Ma) (Favre et al., 2015; Su et al., 2019; Sun et al., 2011; Wang et al., 2012), and the intensification of the Asian monsoon (~10–4 Ma) (Farnsworth et al., 2019), the MDE rose sharply again at ~11 Ma and reached a second peak at ~8 Ma. Under the influence of these paleogeological and climatic events, the rapid diversification and radiation of the PCSG may have led to the retention of ancestral polymorphisms across lineages (Suh et al., 2015; Szöllősi et al., 2015), resulting in ILS signals underlying the observed phylogenetic conflicts at key nodes (Figure 3G). The temporal span between the earliest and most recent divergence events was approximately 13.5 Ma. Based on estimated erosion rates of ~34–76 m/Ma (Chen, 1985;2019; Yang, 1985), this duration would theoretically result in an elevation difference of 459–1026 m. This erosion-based elevation range partially overlaps with the present-day elevational variation in the study region (160–2400 m) (Li, 2001; Zhang, 2012). This discrepancy, however, could be reconciled by considering additional factors that may have accelerated erosion rates, including glacial erosion, geological disturbances, and intense precipitation events (Ji, 1992; Yang, 1985; Zhang, 2012; Zhou and Chen, 1993). Despite these uncertainties, the broad temporal congruence between estimated divergence times and landscape evolution, when considered in the context of karst geomorphological development, is consistent with the phylogenetic evidence. These findings collectively support a scenario in which PCSG diversification was at least partially mediated by long-term topographic erosion processes.

Isolation and diversification can be caused by rock erosion. Bedrock type can drive heterogeneity in biodiversity across landscapes by promoting the evolution of local endemics (Rahbek et al., 2019; Zhao et al., 2024), and erosion of heterogeneous substrates has been shown to drive diversification in the freshwater fishes and of North America (Kim et al., 2023; Stokes et al., 2023) and the flora of Madagascar (Liu et al., 2024). Our findings suggest that the erosion of carbonate sedimentary rock facilitated allopatric lineage divergence—and even speciation—in mountain stream salamanders, representing a diversification mechanism distinct from orogeny-driven speciation, although the erosional processes themselves may have been modulated by long-term climatic forces (Chen, 2019; Wan et al., 2025). The specific effects of lithology on karst species, however, likely vary by taxonomic group and geological context (Zhao et al., 2024). For example, the near-continuous carbonate formations spanning northern to southern Guizhou have resulted in minimal genetic differentiation between northern and southern populations of the *Odorrana schmackeri* complex (Jiang et al., 2022). Hydrological features, including river morphology and substrate composition, are likely critical for PCSG survival, as these species predominantly inhabit slow-

flowing streams or pools with minimal disturbance (Fei and Ye, 2016; Zhao et al., 2012). The fluvial characteristics of mixed sedimentary terrain, i.e., steep gradients, turbulent currents, and reduced sediment accumulation, do not support such habitats. Therefore, even where physical distances appear small, ecological unsuitability restricts dispersal and gene flow, reinforcing the barrier effect. Notably, PCSG species are scarce in mixed sedimentary rock terrain (Figure 5C), where deeply incised gorges typically foster turbulent flow and minimal accumulation of sediment. Nevertheless, the precise ecological linkage between carbonate erosion and PCSG diversification remains unresolved. The current restricted distribution and habitat specificity could alternatively reflect post-dispersal adaptive processes under erosion-mediated isolation (Waters et al., 2020). For example, the progressive erosion of carbonate deposits (Wan et al., 2025), being reshaped by Miocene climatic and tectonic forces into the north-high-south-low topography, may have initially permitted the southward expansion and divergence of the ancestors of Clade II. Subsequent intensification of erosion and drainage network development, coupled with the emergence of mixed sedimentary rock barriers, likely imposed biogeographic constraints on the PCSG, mirroring the abiotic segregation patterns described here. Moreover, subterranean rivers may serve as corridors for gene flow, resulting in moderate genetic differentiation (Figure 4C) and possible introgression (Figure 4B–G) among species from different surface drainages. For example, based on river erosion rates of 280–1138 m/Ma (Yang, 1985), the onset of tributary isolation is estimated to have occurred ~1.5–4.6 Ma (Table S12), coinciding with the mid-Pliocene (~3.6 Ma) uplift of the Qinghai-Tibetan Plateau (Chen et al., 2020) and the intensified west-to-east tilting and erosion of Guizhou during the Pleistocene (Zhou and Chen, 1993). Notably, this time frame aligns with previous divergence estimates derived from mitochondrial fragments (Luo et al., 2021; Yuan et al., 2022), suggesting that recent mitochondrial introgression may have occurred among these species during the Pliocene–Pleistocene transition. Given that genetic introgression may have occurred prior to speciation, the impact of geographic isolation is likely to have outweighed the effects of subsurface connectivity in shaping current patterns of divergence. Regardless of the underlying mechanisms, persistent carbonate erosion has provided the essential preconditions for the protracted allopatric diversification observed in the PCSG. This study highlights the potential of integrating ecological, genomic, and geological datasets to fully understand how geophysical processes influence freshwater biodiversity.

CONCLUSIONS

This study has presented a comprehensive phylogenomic framework for the PCSG, revealing that ILS, introgression, and hybridization have jointly contributed to its complex evolutionary history. By integrating topographic and phylogenetic data, we conclude that heterogeneous bedrock erosion may have driven the diversification of the PCSG. Our findings have direct significance for conservation. Demographic analyses have indicated a continuous decline in effective population size and low current genetic diversity since the LGM, consistent with IUCN threat assessments. Despite their ecological and evolutionary importance, most species remain outside the established protected area network, highlighting a significant conservation gap. This underscores the urgent need for genome-informed conservation strategies, particularly for microendemic and cave-restricted species such as *P. maolanensis*, to ensure

their long-term survival. Overall, our results demonstrate the value of an integrative eco-evolutionary framework in disentangling complex phylogenetic histories and informing effective conservation planning.

DATA AVAILABILITY

The datasets containing the accession numbers for *Paramesotriton caudopunctatus* group generated in this study are available as supplemental information. Original sequence reads of genome data are available at the National Center for Biotechnology Information (Accession number SAMN37379588–SAMN37379633).

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHORS' CONTRIBUTIONS

J.Z. and T.L. conceived and designed the research; T.L., J.J.W., and M.L. conducted field surveys and collected samples; T.L., J.J.W., and M.Y.X. analyzed molecular and environmental data; and T.L., H.Q.D., N.X., and J.Z. wrote, discussed, and revised the manuscript. All authors read and approved the final version of the manuscript. J.Z. provided funding support.

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Figures and Tables

Figure 1. Map of study area. (A) Geographic distribution of *Paramesotriton* species in southern China and northern Vietnam. (B) Topographic map of drainage basins in Guizhou, showing the river network (colored lines) and recorded occurrences of the PCSG (circles). Gray circle indicate species not sampled in this study.

Figure 2. Phylogeny and cytonuclear discordance. (A) ML tree reconstructed from mitogenomes. Complete phylogenetic relationships illustrated in Figure S4. (B) ML tree inferred from concatenated SNPs. (C) Species trees constructed using ASTRAL and SVDquartets. The two analyses produced conflicting topologies, each with strong support (100%).

Figure 3. Gene flow and ILS contribute to gene tree heterogeneity. (A) Population tree inferred by TreeMix assuming 3 migration events. Arrows indicate migration edges, with color intensity reflecting migration weight (estimated proportion of ancestry from the source population in the admixed population). (B–D) The gene flow analysis results for PCSG, using the *D*-statistic (B), f_4-ratio test (C), and the *f*-branch method (D). (E) Phylogenetic network inferred from PhyloNet. The blue line indicates reticulation and red numerical values indicate inheritance probabilities. (F) The introgression schematic diagram detected by HyDe on the species levels within PCSG. Density plot of estimated γ values across 500 bootstrap replicates in HyDe analyses. γ represents the estimated probability of inheritance from the ancestor of the PL, whereas 1-γ representsthe probability of inheritance from the PS. (G) Proportion of introgression (IH) and ILS detected at nodes N3, N4, and N5 by Phytop. P is the *p*-value of χ2 test to check whether the number of topologies q2 and q3 are equal, ILS-i and IH-i represent the calculated ILS index and IH index respectively, and ILS-e and IH-e represent the proportion of gene tree topological incongruence that can be explained by the ILS and IH, respectively. Abbreviations: PC, *P. caudopunctatus*; PL, *P. longliensis*; PM, *P. maolanensis*; PS, *Paramesotriton* sp.; PZ, *P. zhijinensis*.

Figure 4. Population genetic structure, diversity, and demographic history. (A) Admixture proportions of the genetic clusters of each individual of the PCSG. Scenarios with K = 2 to 5 are shown. According to CV analysis, the best value is K=4. The number below each cluster indicates the sample number. (B) PCA plot based on the first two principal components. (C) Genetic differentiation among species and corresponding Fst values. (D) Genetic diversity and endangered status in the China's Red List of Biodiversity (Jiang and Xie, 2021). (E) Demographic history reconstructed for four PCSG species. Abbreviations: N_e, effective population size; LIG, Last Interglacial (140–120 ka); LGM, Last Glacial Maximum (~21 ka); MHO, Mid-Holocene (8.3–4.2 ka), LHO: Late-Holocene (4.2–0.3 ka). PC, *P. caudopunctatus*; PL, *P. longliensis*; PM, *P. maolanensis*; PS, *Paramesotriton* sp.; PZ, *P. zhijinensis*. EN, Endangered; NT, Near Threatened; VU, Vulnerable; NE, Not Evaluated.

Figure 5. Divergence time, reconstructed biogeography, and divergence dynamics. (A) The chronogram inferred using SNAPPER. (B) Ancestral area reconstruction based on the DEC + J model. The blue bars at each node indicate the 95% CIs for divergence time, and the numbers above and below the nodes indicate the Bayesian posterior probability of divergence time and the species tree. Circled nodes indicate the most probable ancestral region, and arrows indicate the dispersal direction. (C) Possible dispersal routes of the PCSG in Guizhou and adjacent areas. (D) Global temperature inferred from deep-sea oxygen isotope records ($\delta^{18}O$) in benthic foraminifera (Zachos et al., 2008), used to model mean annual precipitation at idealized CO_2 levels (Farnsworth et al., 2019). (E) Divergence dynamics of the PCSG lineages based on the maximum number of observed lineage divergence events (MDE) per Ma. OIHMs: orogeny intensification of the Hengduan Mountains; RUQTP: rapid uplift of the Qinghai-Tibetan Plateau.

Supporting materials

Figure S1. Phylogenetic tree reconstructed using BI and ML methods based on concatenated mitochondrial genome and 32 nuclear genes datasets for the genus *Paramesotriton* and outgroups. This figure was modified from Luo et al. (2022).

Figure S2. Dorsal and ventral views of five species of the genus *Paramesotriton* distributed in Guizhou. (A) *Paramesotriton* sp.; (B) *P. zhijinensis*; (C) *P. longlisis*; (D) *P. maolanensis* (modified from Gu et al. 2012); (E) *P. caudopunctatus*; and (F) *P. wulingensis*.

Figure S3. Geological map of Guizhou and the distribution of its PCSG.

Figure S4. Phylogenetic tree reconstructed using BI and ML methods based on concatenated mitochondrial genome.

Figure S5. Summary of topologies among multi-dataset. The species trees inferred by multi-species coalescent approach (ASTRAL) from fixed-window local trees with different lengths, 50 kb, 30 kb, 20 kb, 10 kb, 5 kb, and 2 kb.

Figure S6. The reticulation history of PCSG inferred from PhyloNet. The best-supported networks were derived from analyses of 295 concatenated SNP gene trees using PhyloNet in four replicated runs. The inheritance probabilities are indicated next to the reticulation edges.

Figure S7. The CV error values corresponding to K-values evaluated by Admixture analysis. The optimal K-value is determined by the lowest CV error.

Figure S8. Raw details of the ancestral area of PCSG using BioGeoBEARS (DEC+J). Alphabetical abbreviations for tips and nodes: (A) Leigong Mountains, (B) North of Wumeng Mountains–Wujiang River, (C) South of Wumeng Mountains–Wujiang River, (D) Lianjiang River Basin, (E) Libo Karst Area, and (E) Wuling Mountains.

Figure S9. Raw details of the ancestral area of the PCSG using BioGeoBEARS (BAYAREALIKE+J). Alphabetical abbreviations for tips and nodes: (A) Leigong Mountains, (B) North of Wumeng Mountains–Wujiang River, (C) South of Wumeng Mountains–Wujiang River, (D) Lianjiang River Basin, (E) Libo Karst Area, and (E) Wuling Mountains.

Figure S10. A summary of the phylogenetic relationship conflicts of species within PCSG.

Figure S11. Distribution of PCSG within nature reserves in Guizhou.

Table S1 The diagnostic characters selected for the newly described species in this study and the six species of the *P. caudopunctatus* group. Grey shading indicates clear difference in characteristics compared to that of *Paramesotriton* sp.

Table S2 Sample collection distribution and sequencing information in this study.

Table S3 The mitochondrial genomic sequences used in this study

Table S4 Best-fit partitioning and evolutionary models used for mitochondrial genome phylogenetic analysis.

Table S5 Uncorrected *p*-distance (%) between six species of the PCSG based on mitochondrial genes.

Table S6 Gene introgression among species based on *D*-statistics analysis. Abbreviations: PZ, *P. zhijinensis*; PC, *P. caudopunctatus*; PS, *Paramesotriton* sp.; PL, *P. longliensis*; PM, *P. maolanensis*; OG, *Pachytriton inexpectatus.*

*** Significant at |*Z*-score| >= 3.**

**** Significant at |*Z*-score| >= 5.**

Table S7 HyDe results of hybridization detection analyses using the run_hyde.py script.

Note: P1 and P2 correspond to two putative parental populations respectively, and Hybrid was the potential hybrid population.

[a] P value less than 0.05 indicates a significant signal of hybridization event.

[b] γ parameter is the probability that Hybrid is sister to P2. In the hybrid speciation model, it represents the relative proportion of the genetic composition derived from P2.

Table S8 HyDe results of hybridization detection at the individual-level analysis using the individual_hyde.py script.

Table S9 HyDe results of hybridization detection at the species-level (500 replicates) analysis using the bootstrap_hyde.py script.

Table S10 Statistical results from BioGeoBEARS. The best model is indicated by boldface. Abbreviations: LnL=log-likelihood; *d*=dispersal rate per million years along branches; *e*=extinction rate per million year along branches; *j* = likelihood of founder-event speciation at cladogenesis; AIC= Akaike information criterion.

Table S11 Potential morphological evidence supporting the hybrid origin of *P. zhijinensis.*

Table S12 Estimated timing of river isolation in Guizhou based on erosion rates.

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