

# Diversification outbreaks and dynamics of Asian leaf-litter frogs, genus *Leptobrachella* (Anura, Megophryidae), with the description of a new species from Guizhou Province, China

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## Abstract

The uplift of the Qinghai-Tibet Plateau and the Indochina extrusion are two of the most prominent consequences of the India–Asia collision. These two geologic events greatly altered topography and drainage patterns that, in turn, affected the regional climate, the landscape and the evolution of biodiversity. Despite this, little is known about how orogeny and climate affect the evolution of biodiversity, especially the dynamics of diversification, including origins, peaks and endings. Here, we performed phylogenetic and biogeographic analyses of *Leptobrachella* distributed in Southeast Asia and southern China, based on mitochondrial 16S ribosomal RNA. The results revealed that *Leptobrachella* may be roughly divided into Clade I from south of the Indo-Burma and Clade II from central and northern Indo-Burma and southern China. We then investigated the diversification of *Leptobrachella* over time through biogeographic meta-analyses. We showed that the speciation of *Leptobrachella* was dominated by *in situ* diversification that was most likely associated with the uplift of the Qinghai–Tibet Plateau, the Indochina extrusion and the intensification of the Asian monsoon and that diversification may have been less influenced by temperature. *In situ* diversification experienced three small accelerated phases and one decelerated phase initiated at ~ 32 Ma, with a sharp increase at ~ 15 Ma, a peak at ~ 8.7 Ma and a gradual decline after ~ 6 Ma and the peaks of diversification were asynchronous in Southeast Asia and southern China. Our results suggest a three-phase scenario for the diversification of *Leptobrachella*, with periods of acceleration and deceleration at every stage, a pattern consistent with the Indochina extrusion, the uplift of the Qinghai–Tibet Plateau and the intensification of the Asian monsoon since the Oligocene. This study highlights how biogeographic meta-analyses can be utilised to estimate diversification history in taxa lacking sufficient molecular markers to quantify the impact of orogeny and climatic shifts on diversification processes. In addition, we also identified four undescribed species and described one new species, *Leptobrachella xishuiensis* sp. nov., from Xishui County, Guizhou Province, China.

## Key Words

Biogeography, diversification dynamics, *Leptobrachella*, mitochondrial DNA, nuclear gene, phylogeny

\* Authors contributed equally to this work.

## Introduction

The Indochina and southern China are well-known biodiversity hotspots (Myers et al. 2000). Such high biodiversity has prompted researchers to propose hypotheses concerning the coupling of species diversification and paleogeoclimatic events (Che et al. 2010; Xu et al. 2021). These hypotheses are related to the uplift of the Qinghai–Tibetan Plateau (QTP), the Indochina extrusion and the intensification of the Asian monsoon climate since the Oligocene (Chen et al. 2018; Ding et al. 2020; Feijó et al. 2022; Li et al. 2022; Luo et al. 2022a, 2023). However, less is known about the dynamics of species dispersal and diversification between the two regions, i.e., when this diversification was initiated, when it peaked and if and when it ended.

Asian leaf-litter frogs, genus *Leptobranchella* Smith, 1925 are a group of small frogs that inhabit the forests of the Indochina, Borneo, north-eastern India, and southern China (Frost 2024). Species within this genus were initially assigned to multiple genera, including *Nesobia* Van Kampen, 1923, *Paramegophrys* Liu, 1964, *Carpophrys* Sichuan Biological Research Institute, 1977, *Lep-tolalax* Dubois, 1980, *Lalax* Delorme, Dubois, Grosjean & Ohler, 2006, and *Lalos* Dubois, Grosjean, Ohler, Adler & Zhao, 2010. Despite prior controversies regarding the taxonomic associations of these genera, a recent large-scale phylogenetic study has shown that the above genera are synonymous with *Leptobranchella* (Chen et al. 2018). As of 13 August 2024, 104 species have been recorded in the genus, with nearly half of the species described in the last 10 years (Suppl. material 5). The genus has the highest species diversity within the family Megophryidae (Frost 2024). The most recent common ancestor (MRCA) of *Leptobranchella* originated in the early Oligocene (~31.55 Ma), with an “upstream” colonisation pattern from south to north and its diversification may be related to the warm and humid climate during the Miocene (Chen et al. 2018). Nevertheless, the dispersal history of *Leptobranchella* between the Indochina Peninsula and southern China remains unknown. Additionally, based on recent phylogenetic analyses (Chen et al. 2024), *Leptobranchella* may have undergone radiations in major clades that have not been detected. In the last 6 years, multiple new species have been described (41 species), especially in southern China (32 species) (Suppl. material 5), suggesting that southern China is a biodiversity hotspot for *Leptobranchella* and that species diversity may be severely underestimated (Chen et al. 2018).

Guizhou Province in south-western China has a high diversity of amphibian species (Zhou et al. 2023). Currently, 14 species of the genus *Leptobranchella* are recorded in Guizhou, representing 33.33% of the total number of *Leptobranchella* in China (42 species; AmphibiaChina (2024)), i.e., *L. bashaensis* Lyu, Dai, Wei, He, Yuan, Shi, Zhou, Ran, Kuang, Guo, Wei & Yuan, 2020, *L. bijie* Wang, Li, Li, Chen & Wang, 2019, *L. chishuiensis* Li, Liu, Wei & Wang, 2020,

*L. dong* Liu, Shi, Li, Zhang, Xiang, Wei & Wang, 2023, *L. dorsospina* Wang, Lyu, Qi & Wang, 2020, *L. dushanensis* Li, Li, Cheng, Liu, Wei, Wang, 2024, *L. jinshaensis* Cheng, Shi, Li, Liu, Li & Wang, 2021, *L. liui* (Fei & Ye, 1990), *L. oshanensis* (Liu, 1950), *L. pelodytoides* (Boulenger, 1893), *L. purpuraventra* Wang, Li, Li, Chen & Wang, 2019, *L. suiyangensis* Luo, Xiao, Gao & Zhou, 2020, *L. ventripunctata* (Fei, Ye & Li, 1990), and *L. wulingensis* Qian, Xiao, Cao, Xiao & Yang, 2020 (Wu et al. 1986; Wang et al. 2019; Luo et al. 2020; Lyu et al. 2020; Li et al. 2020a; Wang et al. 2020a; Cheng et al. 2021; Liu et al. 2021; Liu et al. 2023; Li et al. 2024a). Ten of these species have been described in the last four years, suggesting that the diversity of the genus in Guizhou may be underestimated.

Here, we build on genetic samples obtained from field surveys and sequencing integrated with published genetic data. Our objectives were to: (1) assess the phylogeny of *Leptobranchella*, based on the most comprehensive genetic data available; (2) identify and characterise potentially cryptic species; and (3) test for the presence of rapid radiation and assess the dynamics of diversification in *Leptobranchella*.

## Materials and methods

### Sampling, DNA extraction, PCR and sequencing

There was a total of 43 specimens that were collected throughout our biodiversity surveys in Guizhou, Guangxi, Yunnan and Chongqing, China, from 2017 to 2024. Six specimens were identified as *L. yunyangensis* from Qiyaoshan Nature Reserve and Lianhua Village, Renhe Town, Yunyang County, Chongqing; six were *L. alpina* from Wulian Mountain, Jingdong County, Yunnan; one was *L. ventripunctata* from Jianguin Village, Lingyun County, Guangxi; eight were *L. suiyangensis* from Huoqiuba Nature Reserve, Suiyang County, Guizhou; 12 were *Leptobranchella* sp1 and *Leptobranchella* sp2 from Wuliang Mountain, Jindong County, Yunnan; three were *Leptobranchella* sp3 from Shibing County, Guizhou; and seven were an undescribed species from Xianyuan Town, Xishui County, Guizhou. All of the specimens used for morphological studies were fixed in a 10% formalin buffer and then transferred to 75% ethanol and stored at the Animal Ecology Laboratory of Guizhou Normal University (GZNU), Guiyang City, Guizhou Province, China.

The catalogue and distribution of *Leptobranchella* were reorganised through field surveys, literature reviews and database searches using Amphibian Species of the World (<https://amphibiansoftheworld.amnh.org/>) (Frost 2024) and GBIF (<https://www.gbif.org/>). Both databases were accessed on 13 August 2024. After that, the distribution sites for every species were rasterised to a  $0.5^\circ \times 0.5^\circ$  resolution (~111 × 111 km<sup>2</sup>) grid system using ArcGIS v.10.4 and species richness was obtained by summing the total number of species occurring in the grid cells.

Genomic DNA was extracted from muscle tissues using a DNA extraction kit (Tiangen Biotech Co., Ltd., Beijing, China). We referred to prior studies (Chen et al. 2018; Luo et al. 2022b) that amplified and sequenced the mitochondrial 16S ribosomal RNA gene (16S rRNA) and six nuclear gene fragments, i.e., brain-derived neurotrophic factor (BDNF), sodium/calcium exchanger 1 (NCX1), neurotrophin 3 (NTF3), recombination activating gene 1 (RAG1), rhodopsin (RHOD) and solute carrier family 8 member 3 (SLC8A3) (Suppl. material 6). PCR amplifications were performed in a 20  $\mu$ l reaction volume with the following cycling conditions: an initial denaturing step at 95 °C for 5 min, 35 cycles of denaturing at 95 °C for 1 min, annealing at 50–57 °C for 1 min and extending at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The products were sequenced on an ABI Prism 3730 automated DNA sequencer at Chengdu TSING KE Biological Technology Co. Ltd (Chengdu, China). All of the newly-obtained sequences have been submitted to GenBank (Suppl. material 7).

### Phylogenetic construction and divergence time estimation

We used a total of 296 sequences and constructed two sequence matrices for phylogenetic analyses, i.e. mitochondrial 16S rRNA (dataset 1) and the combined sequences of six nuclear genes (dataset 2). Multiple sequence alignment was performed using MAFFT v.7.4 (Kato and Standley 2013) within PhyloSuite v.1.2.3 (Zhang et al. 2020) and checked using MEGA v.7.0 (Kumar et al. 2016) to rule out possible errors. Partitionfinder v.2.1.1 (Lanfear et al. 2017) was used to select the best-fit partitioning and nucleotide substitution model for the two datasets, based on the Bayesian information criterion. In dataset 2, every gene fragment was pre-set as an independent partition. We referred to Chen et al. (2018) to select *Leptobranchium boringii*, *Leptobranchium huashen*, and *Megophrys glandulosa* as outgroups (Suppl. material 7).

Phylogenetic trees were reconstructed using Bayesian Inference (BI) and Maximum Likelihood (ML) methods, based on best-fit partitioning and nucleotide substitution models. The BI analysis was performed using MrBayes v.3.2.1 (Ronquist et al. 2012). Each BI analysis was run independently using four Markov Chain Monte Carlo chains (three heated chains and one cold chain) starting with a random tree; each chain was run for  $2 \times 10^7$  generations and sampled every 1000 generations. Convergence of the data runs was confirmed when the average standard deviation of split frequencies was less than 0.01. The ML analysis was performed using IQ-tree v.2.0.4 (Nguyen et al. 2015), based on the best-fit model with 10000 ultrafast bootstrap (UFB) replicates (Hoang et al. 2018). The ML analysis was performed until a correlation coefficient of at least 0.99 was achieved. Nodes were considered to be highly supported when the Bayesian Posterior Probabil-

ity (BPP) value for the BI analysis was  $> 0.95$  (Ronquist et al. 2012) and the UFB value for the ML analysis was  $> 95\%$  (Hoang et al. 2018). Genetic distances were calculated based on dataset 1 using the uncorrected  $p$ -distance model and 1000 bootstrap replications in MEGA v.7.0 (Kumar et al. 2016).

The sampling of species, molecular dating and phylogenetic reconstruction were performed in BEAST v.1.8.2 (Drummond et al. 2012) using dataset 1. Two calibration nodes were employed for the estimation of divergence times with reference to recent studies: (1) the crown age for the family Megophryidae was ca. 51.5–57.7 million years ago (Ma) (Mahony et al. 2017); the most recent common ancestor of the genus *Leptobranchella* occurred at 31.55 Ma (95% highest posterior density (HPD): 22.74–43.65 Ma) (Chen et al. 2018). The BEAST analysis used an uncorrelated lognormal relaxation clock and a Yule tree prior. The BEAST analysis was run for 200 million generations and sampled every 1000 generations. All of the calibrations used a normal prior, monophyly and standard deviation values of 2.2. Convergence of the run parameters was checked using Tracer v.1.7.1 (Rambaut et al. 2018) to ensure that the effective sample size of all of the parameters was greater than 200. Three runs were made in total and the trees were finally merged using LogCombiner v.2.4.7. A maximum clade credibility tree was generated using TreeAnnotator v.2.4.1 (implemented in BEAST) by applying a burn-in of 25%.

### Biogeographic meta-analyses and diversification dynamic analysis

Due to the limitations of our molecular markers, dispersal pathways were not the focus of this study; instead, we were more interested in whether or not lineages had radiated and diversified. Therefore, we assessed the dispersal and *in situ* diversification history of *Leptobranchella* referring to the biogeographical meta-analyses utilised in prior studies (Klaus et al. 2016; Jiang et al. 2019; Xu et al. 2021; Li et al. 2022), i.e., the maximum number of observed dispersal events (MDisE) per million years and the maximum number of *in situ* diversification events (MDivE) per million years.

The ancestral ranges of *Leptobranchella* were estimated using the R package BioGeoBEARS (Matzke 2013). We divided the distribution ranges into five major regions: (A) Borneo, (B) Southern Thai-Malay Peninsula, (C) Northern Thai-Malay Peninsula, (D) Indo-Burma and (E) Southern China (Chen et al. 2018). Three models, i.e., dispersal-extinction-cladogenesis (DEC) (Ree and Smith 2008), dispersal-vicariance analysis (DIVALIKE) (Ronquist 1997) and the Bayesian biogeographical inference model (BAYAREALIKE) (Landis et al. 2013), as well as the +J model (founder-event), were employed in the analyses. The maximum range was set to two, based on actual surveys and the consensus (Chen et al. 2018) in literature that current *Leptobranchella* are narrowly distributed.

To infer the patterns of speciation in *Leptobranchella* based on the results of the biogeographic analyses, we referred to the criteria of Xu et al. (2021) concerning the phylogenetic tree to categorise these patterns into two types: dispersal and *in situ* diversification. We divided the observed dispersal events and *in situ* diversification events defined as the maximum number of dispersal events per 0.1 million years (MDisE) (Klaus et al. 2016) and the maximum number of *in situ* diversification events per 0.1 million years (MDivE) (Xu et al. 2021), respectively, to show the trends of these two types of speciation events over time. The MDisE/MDivE rates were calculated by summing potential dispersal or *in situ* diversification events over time in 0.1-Mya slices, based on the obtained confidence intervals for divergence times. In this analysis, we used the *cpt.mean* function (method = "AMOC," Q = 0.1) of the R package *changept* (Killick and Eckley 2014) to identify significant change points in the MDisE/MDivE curve.

Another concern was whether rapid radiation had occurred in *Leptobranchella*. Lineage-Through-Time (LTT) plots and Bayesian Analysis of Macroevolution Mixtures (BAMM) were used to estimate the rates of speciation and the accumulation of lineages over time. The LTT plot was drawn from the total set of tree files from the BEAST analysis using Tracer v.1.7.2. BAMM v.2.5.0 (Rabosky et al. 2013) and BAMMtools v.2.1.10 (Rabosky et al. 2014) were used to estimate the diversification rates of *Leptobranchella* over time. The R package BAMMtools v.2.1.10 (Rabosky et al. 2014) evaluated the a priori parameters of BAMM. Time trees generated using BEAST were used for species-extinction analysis. Due to the small number of endpoints in the time tree (< 500 tips), we assigned an a priori value of 1.0 to the variable "expectedNumberOfShifts." One million generations were run and the trees were sampled every 1,000 generations; the first 10% of these were burn-in within BAMMtools v.2.1.10 (Rabosky et al. 2014). We also performed a macroevolutionary cohort analysis using BAMMtools to summarise the probability of species sharing common macroevolutionary rate dynamics (Rabosky et al. 2014).

## Morphological measurements and statistical analysis

Morphological data for the putative new species followed Fei et al. (2009) and Rowley et al. (2013). Morphological data were measured for description and comparison and measured to the nearest 0.1 mm utilising digital calipers. The measurements were as follows: **SVL** = snout-vent length (from tip of snout to vent); **HDL** = head length (from tip of snout to rear of jaws); **HDW** = head width (head width at the commissure of the jaws); **SNT** = snout length (from tip of snout to the anterior corner of the eye); **EYE** = eye diameter (diameter of the exposed portion of the eyeballs); **IOD** = interorbital distance (minimum distance between upper eyelids); **IND** = internasal distance (distance between nares); **UEW** = upper eyelid width

(measured as the greatest width of the upper eyelid); **NEL** = nostril-eyelid length (distance from nostril to eyelid); **TMP** = tympanum diameter (horizontal diameter of the tympanum); **TEY** = tympanum-eye distance (distance from anterior edge of the tympanum to posterior corner of the eye); **HND** = hand length (distance from distal end of radioulna to tip of phalanx of finger III); **LAHL** = length of the lower arm and hand (distance from tip of the third finger to elbow); **LW** = lower arm width (maximum width of the lower arm); **HLL** = hindlimb length (distance from tip of fourth toe to vent); **THL** = thigh length (distance from vent to knee); **TIB** = tibia length (distance from knee to heel); **TW** = maximal tibia width; **TFL** = length of foot and tarsus (distance from the tibiotarsal articulation to the distal end of toe IV) and **FOT** = foot length (from proximal edge of the inner metatarsal tubercle to the tip of the fourth toe) (Suppl. material 8). Comparative morphological data for species of the genus *Leptobranchella* were obtained from literature (Suppl. material 9). Due to the high likelihood of undiagnosed diversity within the genus (Rowley et al. 2016; Yang et al. 2016), where available, we relied on examination of topotype material and original species descriptions. Sex was determined by the presence of internal vocal sac openings and the presence of eggs in the abdomen through external inspection.

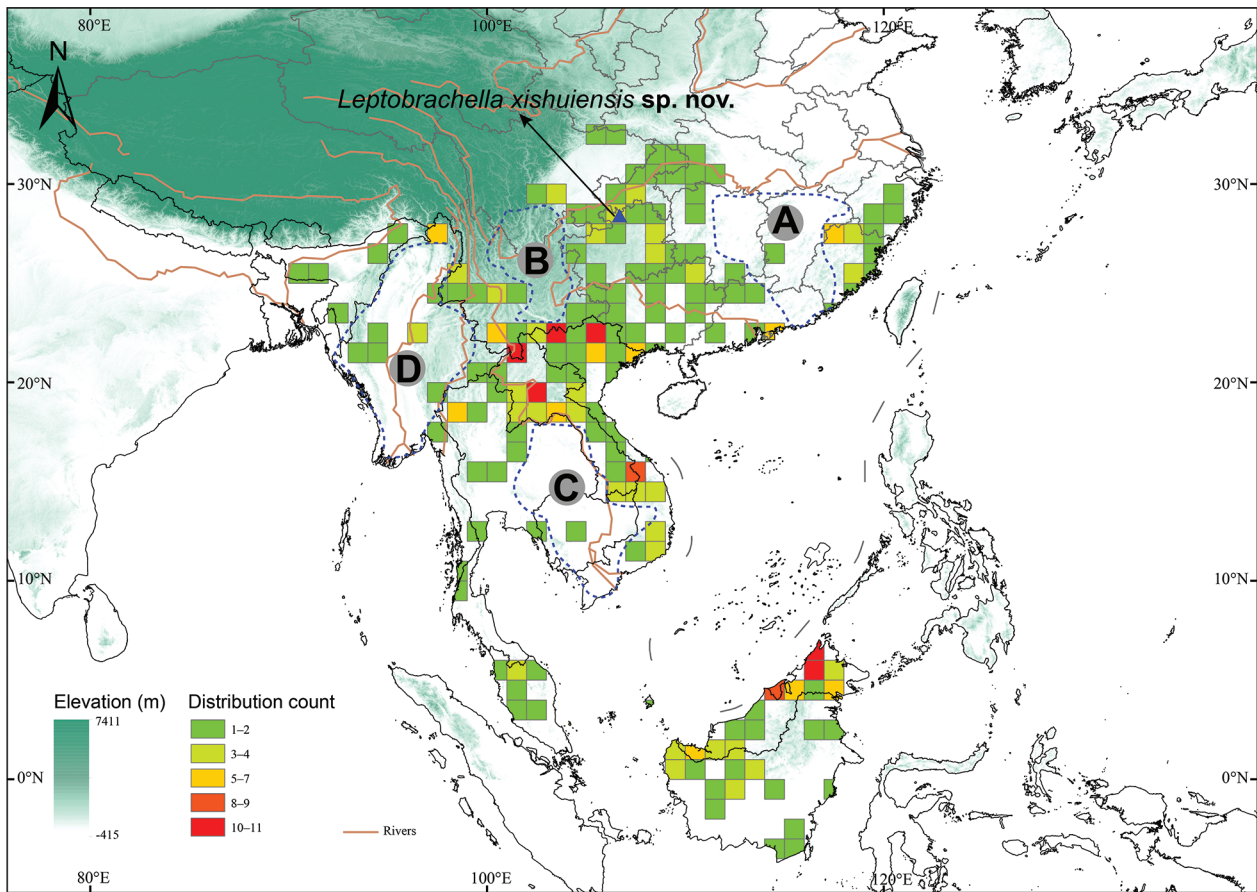
We performed a statistical analysis of the morphometric data for both species because of the morphological similarity between the new species and similar species. A size-corrected value defined as the ratio of every character to SVL was calculated and, then, the data were log-transformed for subsequent morphometric analyses in order to reduce the impact of allometry. We performed a principal components (PCs) analysis of the morphology values of these measurements, based on eigenvalues greater than one. The maximum variance method and simple bivariate scatter plots were used to explore and characterise the morphometric differences between the new species and closely-related species. One-way analysis of variance (ANOVA) was conducted to determine the significance of differences in morphometric characters between the new species and the aforementioned similar species. All of the statistical analyses were performed using SPSS 21.0 (SPSS, Inc., Chicago, IL, USA) and differences were considered statistically significant at a *p*-value < 0.05.

## Results

### Species distributions and related hotspots

We collected a total of 388 distribution sites for 104 species using field surveys and published data (Suppl. material 10). The distribution of these species was mapped in 1° × 1° (approximately 111 × 111 km<sup>2</sup>) grid cells. The most abundant cells were located in Borneo, Indo-Burma and southern China, in that order. For Indo-Burma, the hotspot cells were concentrated in central and northern Vietnam-southern Yunnan, China and northern Laos. For





**Figure 1.** Distribution of *Leptobranchella* hotspots and collection sites for the new species. **A–D.** Denote four record-gap regions: **A.** Eastern China, comprising the Luoxiao Mountains, the Lianhua Mountains, the Xuefeng Mountains and the Wuyi Mountains; **B.** The Hengduan Mountains; **C.** Myanmar **D.** Cambodia and southern Vietnam. The base maps are from the Standard Map Service website (<http://bzdt.ch.mnr.gov.cn/index.html>; Map Approval No. GS(2020)4619). This illustration was created and provided by Tao Luo.

southern China, the hotspot cells were concentrated in the Wuyi Mountains in the east and the Nanling and Wuling Mountains in the west (Fig. 1).

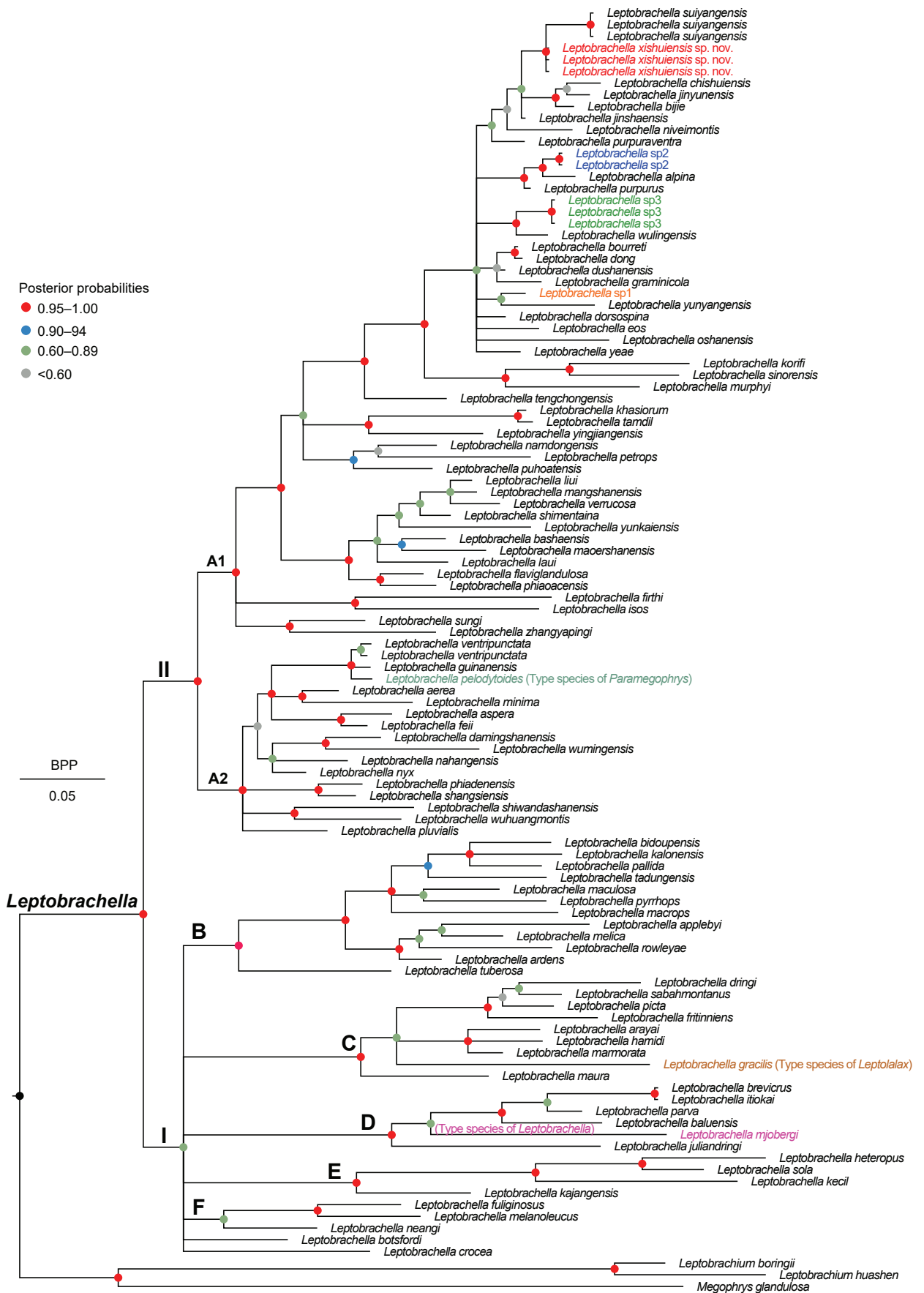
### Phylogeny, haplotype network, and genetic distance

The BI phylogenetic tree was based on the mitochondrial 16S rRNA gene with a length of 532 base pairs (bp). This tree had poorly resolved phylogenetic relationships between species associated with short marker lengths; however, the analysis was still able to delineate two large clades. Following Chen et al. (2018), clades I and II may be further divided into five and two subclades, respectively, where subclades A1 and A2 were highly resolved (Fig. 2). Three samples from Xishui, Guizhou, were clustered together as the sister clade of *L. suiyangensis*. Three putative undescribed species, namely, *Leptobranchella* sp1, *Leptobranchella* sp2, and *Leptobranchella* sp3, from Yunnan and Guizhou, China, were also identified (Fig. 2).

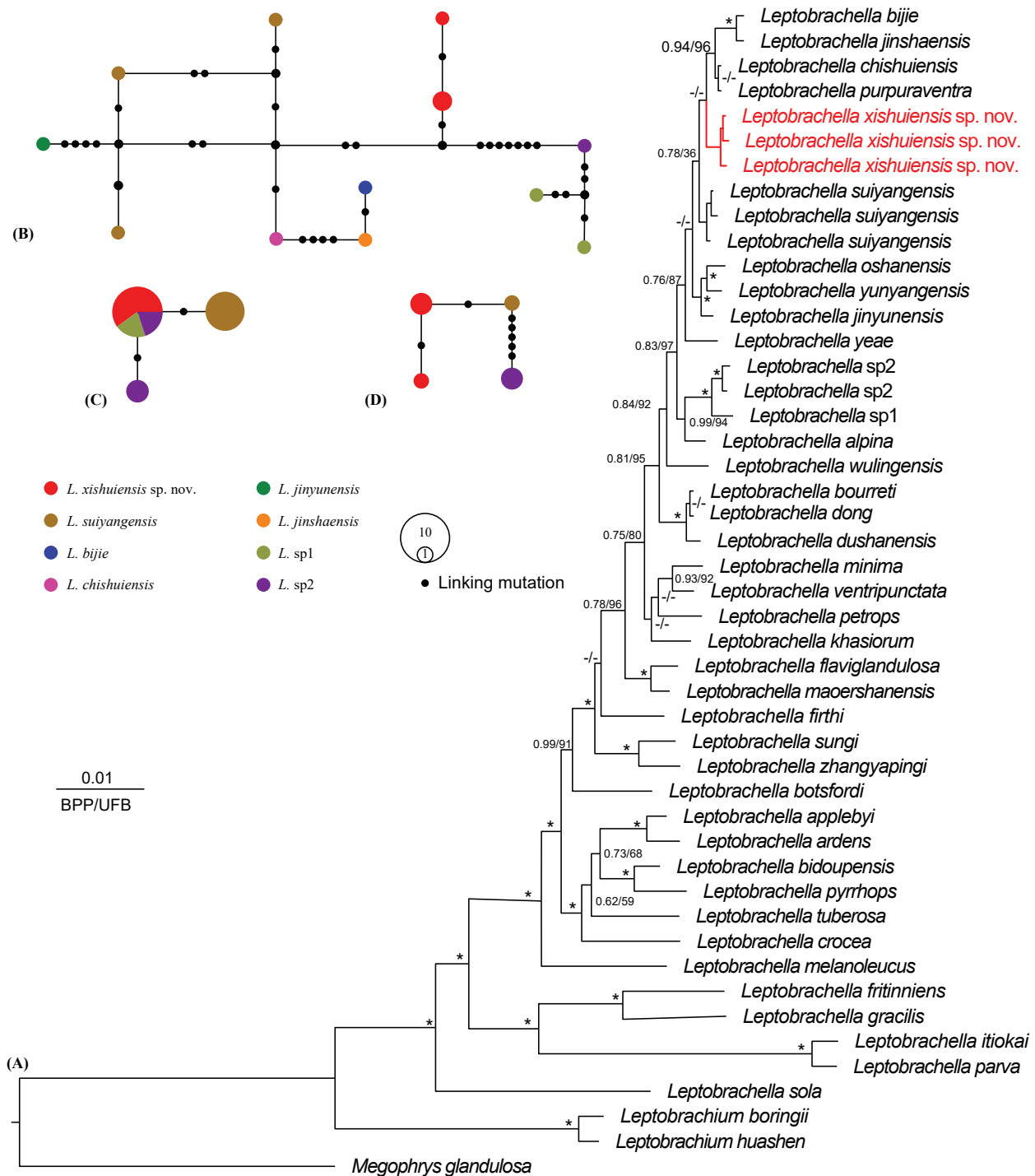
The combined sequence data from six nuclear genes (3594 bp in total length) also failed to resolve the phylogeny of *Leptobranchella* (Fig. 3A). This was possibly due to

the lack of a large number of species being sampled, generating a topology inconsistent with the mitochondrial DNA data; however, the three samples from Xishui, Guizhou formed an independent lineage as a basal clade of ((*L. bijie* + *L. jinshaensis*) + (*L. chishuiensis* + *L. purpuravenetra*)) (Fig. 3A). Haplotype networks, based on RAG1 and NTF3, showed that unique, non-shared haplotypes were present in the Xishui samples and multiple linked mutations occurred within closely-related species (Fig. 3B, D); however, in the nuclear gene NCX1, the Xishui samples shared haplotypes with two undescribed species, *Leptobranchella* sp1 and *Leptobranchella* sp2 (Fig. 3C).

The smallest pairwise genetic divergence between the Xishui samples and 96 species of the genus *Leptobranchella* was 1.7% (vs. *L. suiyangensis*) to 17.9% (vs. *L. gracilis*). These levels were similar to or higher than the divergence levels amongst recognised sister species; for example, 1.2% between *L. bijie* and *L. jinyunensis*, 0.2% between *L. dong* and *L. bourreti* and 1.9% between *L. liuig* and *L. mangshanensis* (Suppl. material 11). In conclusion, the genetic divergence, matrilineal tree and nuclear gene tree were all highly supportive of the *Leptobranchella* populations in Xianyuan Town, Xishui County, Guizhou, as an independent phylogenetic species.



**Figure 2.** Bayesian Inference (BI) tree based on mitochondrial 16S rRNA (532 bp). The scale bar represents 0.05 nucleotide substitutions per site. This illustration was created and provided by Tao Luo.

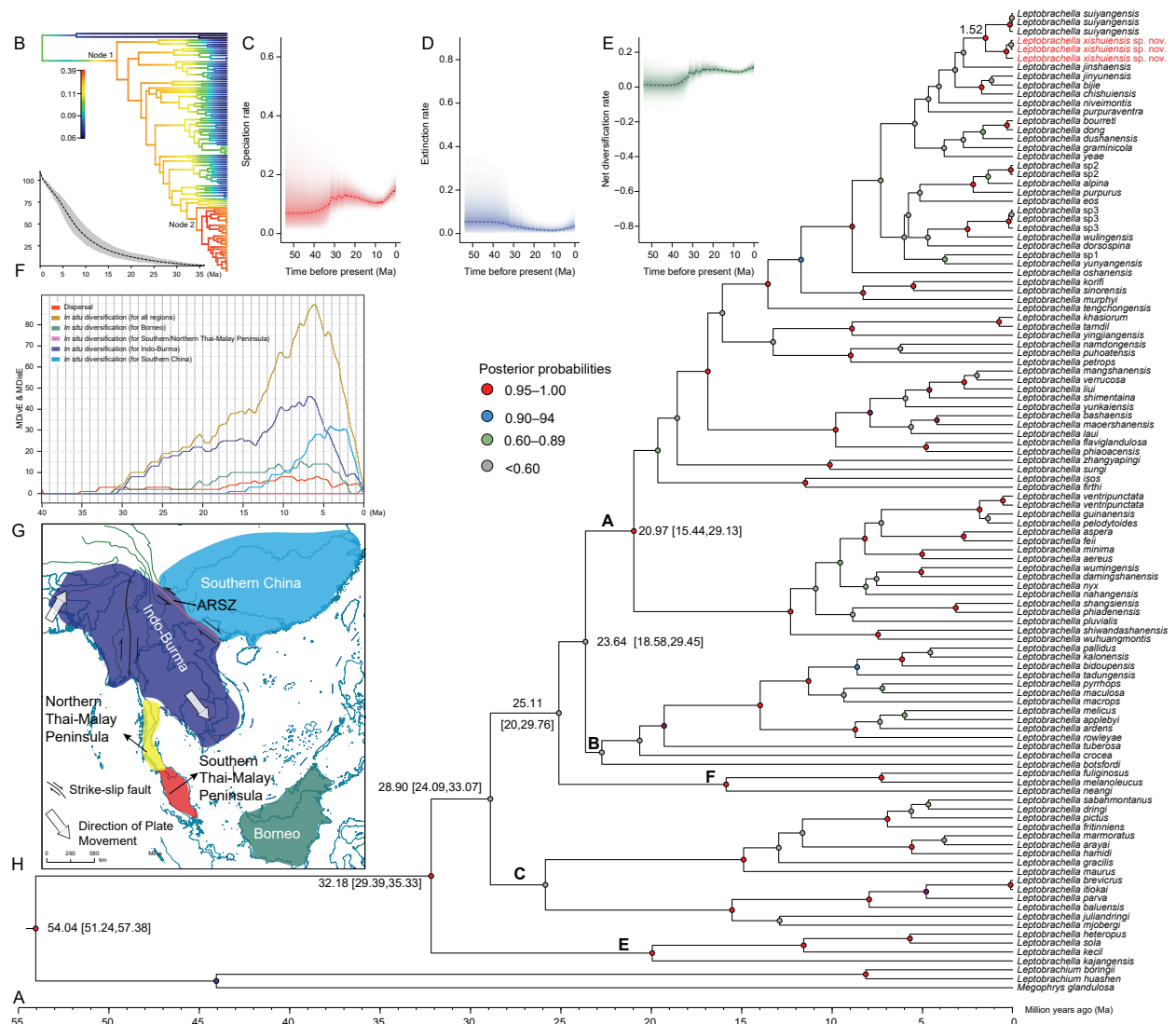


**Figure 3.** Bayesian Inference (BI) tree based on six nuclear genes (A) and nuclear haplotypes (B–D). BPP from BI analyses/UFB from ML analyses are listed next to the nodes. Asterisks and “-” indicate support values of 1.00/100 and below 0.60/60. The scale bar represents 0.01 nucleotide substitutions per site. **B.** RAG1; **C.** NCX1; **D.** NTF3. This illustration was created and provided by Tao Luo.

### Historical diversification revealed by biogeographic meta-analyses

The time tree, estimated using BEAST, was inconsistent with the BI tree obtained using the 16S rRNA (Fig. 4A and Suppl. material 1). The most recent common ancestor of the genus *Leptobranchella* was estimated as occur-

ring in the Early Oligocene, ca. 32.18 Ma (95% HPD = 29.39–35.33 Ma). Subsequently, divergence between sister clades for Clade F was estimated to have occurred in the Oligocene to Early Miocene, ca. 20.97–28.90 Ma. The Xishui samples diverged from *L. suiyangensis* at 1.52 Ma (95% HPD = 0.93–2.79 Ma). According to the best model DEC+J (Suppl. material 12), the ancestors of



**Figure 4.** Mitochondrial 16S rRNA-based time tree and diversification dynamics. **A.** Speciation rates along the phylogeny (the rates are reflected by the branch colours; cold colours denote slower rates and warm colours denote faster rates); **B–E.** Speciation rates through time from the analysis of five datasets. Lines denote means and lighter shadows denote the 95% highest posterior density; **F.** LTT plot showing mean lineage accumulation over time in millions of years (Ma); **G.** Diversification dynamics of the genus *Leptobrachella* based on the maximum number of dispersal events (MDiE) and the maximum number of *in situ* diversification events (MDivE) per Ma; **H.** Geographical distribution and tectonic zone range, modified from Chen et al. (2018). ARSZ: Ailao Shan–Red River shear zone. This illustration was created and provided by Tao Luo.

*Leptobrachella* inhabited Borneo and Indo-Burma (Suppl. material 1). Based on this time tree, Indo-Burma was identified as the source of outward dispersal, but there was also the reverse, i.e. southern China to Indo-Burma (Suppl. material 2).

The phylorate plot generated from the BAMM analysis showed a distinct increase in the speciation rate of *Leptobrachella* in the basal and apical branches at around 32.18 Ma and 8.89 Ma (Fig. 4B, nodes 1 and 2). In the LTT plot, acceleration of the lineage accumulation rate occurred around 10 Ma, 5 Ma, and 1 Ma (Fig. 4F). Plots of the speciation rate through time for the whole 16S rRNA dataset showed that the speciation rate and net diversification rate of *Leptobrachella* increased from 30

to 25 Ma, decreased from 25 to 10 Ma and continued to increase after 10 Ma (Fig. 4C, F). Extinction rates were somewhat similar (Fig. 4D). The differences in speciation rates amongst *Leptobrachella* may also be observed in the macro-evolutionary cohort display (Suppl. material 3). Taken together, the results suggest that *Leptobrachella* has historically undergone several rapid radiations.

We summarised the events associated with *Leptobrachella* diversification, including 178 *in situ* diversification events and 21 dispersal events (Suppl. material 13). *In situ* diversification was the primary pattern of speciation. Biogeographic meta-analyses showed similar trends for *in situ* diversification and dispersal over time. Both dispersal and *in situ* diversification events began in the



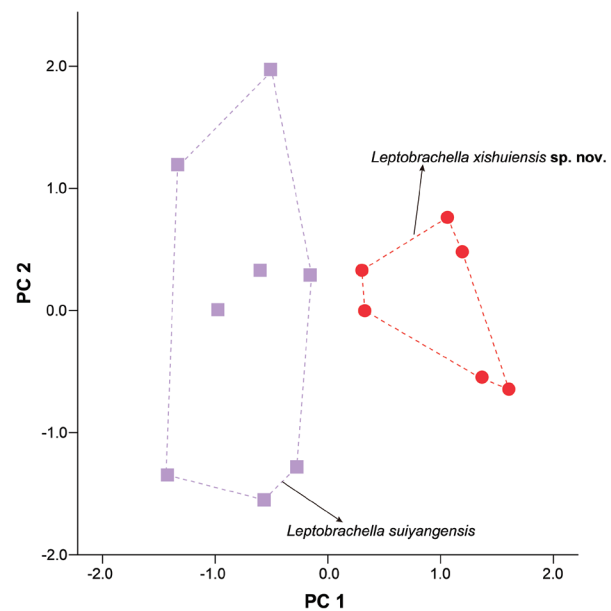
**Table 1.** Peak and inflection times for maximum number of dispersal events per 0.1 million years (MDisE) and the maximum number of *in situ* diversification events per 0.1 million years (MDivE) diversification dynamics. NA – denotes data not applicable.

| Features of the diversification dynamics | Observations (99% confidence interval) (Ma) | Comment        |
|--|---|----------------|
| Peak of MDivE                            | 8.70 (7.78–9.57)                            | All regions    |
|  | 9.85 (7.67–12.25)                           | Borneo         |
|  | 10.75 (9.46–12.02)                          | Indo-Burma     |
|  | 4.74 (4.06–5.44)                            | Southern China |
| Peak of MDisE                            | 10.52 (6.56–14.42)                          | NA             |
| Change points of MDivE                   | 2.557 (1.80–3.32)                           | All regions    |
|  | 15.44 (13.59–17.29)                         |                |
|  | 2.11 (1.26–2.96)                            | Borneo         |
|  | 7.07 (4.89–9.25)                            |                |
|  | 3.20 (1.88–4.53)                            | Indo-Burma     |
|  | 17.02 (15.55–18.50)                         |                |
|  | 1.30 (0.55–2.05)                            | Southern China |
|  | 11.95 (9.31–12.90)                          |                |
| Change points of MDisE                   | 4.53 (1.94–7.11)                            | NA             |

Early Oligocene (~ 30 Ma). The initial speciation rates were slow, then increased rapidly at around 15.0 Ma and peaked around 10.52 Ma [95% HPD = 6.56–14.42] and 8.70 Ma [95% HPD = 7.78–9.57] (Table 1). These events were followed by rapid declines at 4 Ma and 12 Ma. For Borneo, Indo-Burma and southern China, the inflection points of the rapid increase in the *in situ* diversification rates occurred at 7.07 Ma, 17.02 Ma, and 11.95 Ma, peaked at 9.85 Ma, 10.75 Ma, and 4.74 Ma, and decreased significantly at 2.11 Ma, 3.20 Ma, and 1.30 Ma (Table 1). Additionally, the *in situ* diversification peaks for Indo-Burma and southern China were staggered, suggesting that diversification was not synchronised. Taken together, the results suggest that the Miocene (5–15 Ma) was a key period for the diversification of *Leptobranchella*.

### Morphological analyses

Principal component analysis applied to the Xishui samples and *L. suiyangensis* using 15 measured characters showed that a total of five principal components were extracted. The first three components explained 63.42% of the total variance, with PC1 accounting for 28.31%, PC2 for 19.73% and PC3 for 15.38% (Suppl. material 14). In the scatterplot of PC1 versus PC2, Xishui samples and *L. suiyangensis* formed distinct clusters and were separated on the PC1 axis (Fig. 5). HDW, EYE, IOD, UEW, HND, LW, and HLL were the mainly loaded PC1 (Suppl. material 14). The results of the ANOVA tests indicated that the Xishui samples differed significantly from *L. suiyangensis*, based on several morphometric characters, including EYE, IOD, HND, LW, and HLL (all *p*-values < 0.05; Table 2). Combining morphological and genetic differences, we describe the *Leptobranchella* population from Xianyuan Town, Xishui County, Guizhou, China, as the new species *Leptobranchella xishuiensis* sp. nov.



**Figure 5.** Scatter plots of the first and third principal components for *Leptobranchella xishuiensis* sp. nov. and *L. suiyangensis*. This illustration was created and provided by Tao Luo.

### Taxonomic accounts

***Leptobranchella xishuiensis* Luo, Deng & Zhou, sp. nov.**

<https://zoobank.org/9A971D18-BE09-493A-BE76-CAAA3ABE78DC>

Table 2; Suppl. material 7; Figs 6, 7

**Material examined. Holotype.** • GZNU20240726001, adult male collected by Tao Luo on 26 July 2018 from the Xianyuan Town, Xishui County, Guizhou, China (28.30094224°N, 106.71286583°E, 1601 m a.s.l.; Fig. 1).

**Paratypes.** • GZNU20240726002–006, four adult males were collected from the type locality on 6 June 2018, by Tao Luo, Zi-Fa Zhao, Chang-Ting Lan, and Zhong-Lian Wang.

**Table 2.** Morphometric statistics and results of ANOVA from *Leptobranchella xishuiensis* sp. nov. (LX) and *L. suiyangensis* (LS). The significance level was set at  $P < 0.05$ . Morphometric characters are explained in the Materials and Methods section. Bold black indicates a significant difference. NA – denotes data not applicable.

| Measurements | <i>L. xishuiensis</i> sp. nov. |            | <i>L. suiyangensis</i> |            | P-value from ANOVA<br>LX vs. LS |
|--------------|--------------------------------|------------|------------------------|------------|---------------------------------|
|              | Male (n = 6)                   |            | Male (n = 8)           |            |                                 |
|              | Range                          | Mean ± SD  | Range                  | Mean ± SD  |                                 |
| SVL          | 28.1–36.2                      | 31.0 ± 2.8 | 28.7–29.7              | 29.2 ± 0.4 | 0.177                           |
| HDL          | 10.4–13.6                      | 12.0 ± 1.1 | 9.9–12.1               | 11.2 ± 0.9 | 0.192                           |
| HDW          | 9.8–12.6                       | 10.9 ± 1.1 | 9.3–10.4               | 10.0 ± 0.4 | 0.118                           |
| SNT          | 4.2–5.2                        | 4.6 ± 0.4  | 4.0–5.0                | 4.4 ± 0.4  | 0.414                           |
| EYE          | 3.8–4.5                        | 4.2 ± 0.2  | 2.4–3.9                | 3.3 ± 0.6  | <b>0.010</b>                    |
| IOD          | 3.8–4.3                        | 4.1 ± 0.2  | 2.8–3.4                | 3.1 ± 0.2  | <b>0.000</b>                    |
| IND          | 2.7–3.7                        | 3.1 ± 0.3  | 2.8–3.7                | 3.1 ± 0.3  | 0.972                           |
| UEW          | 2.3–3.6                        | 2.8 ± 0.4  | 1.6–3.1                | 2.3 ± 0.6  | 0.121                           |
| NEL          | 2.2–2.4                        | 2.3 ± 0.1  | 2.3–2.8                | 2.5 ± 0.2  | 0.600                           |
| TMP          | 1.9–2.3                        | 2.2 ± 0.1  | 1.2–2.3                | 1.9 ± 0.4  | 0.139                           |
| TEY          | 1.3–2                          | 1.6 ± 0.3  | 1.1–1.9                | 1.4 ± 0.3  | 0.396                           |
| HND          | 7.3–8.9                        | 7.9 ± 0.6  | 6.5–7.4                | 7.0 ± 0.3  | <b>0.017</b>                    |
| LAHL         | 13.6–16.1                      | 14.3 ± 1.0 | 13.2–13.4              | 13.3 ± 0.1 | 0.058                           |
| LW           | 1.8–2.4                        | 2.2 ± 0.2  | 1.8–2.0                | 1.9 ± 0.1  | <b>0.014</b>                    |
| HLL          | 48.8–59.1                      | 51.8 ± 3.8 | 41.8–44.4              | 43.1 ± 0.9 | <b>0.001</b>                    |
| TIB          | 13.5–17.3                      | 14.9 ± 1.4 | 13.1–13.8              | 13.5 ± 0.3 | 0.055                           |
| TW           | 3.5–4.3                        | 3.8 ± 0.3  | 3.8–4.2                | 4.0 ± 0.2  | 0.174                           |
| FOT          | 12.1–15.7                      | 13.7 ± 1.3 | 12.3–12.9              | 12.6 ± 0.3 | 0.100                           |
| TFL          | 19.5–24.1                      | 21.1 ± 1.7 | NA                     | NA         | NA                              |
| THL          | 13.8–17.7                      | 14.6 ± 1.5 | NA                     | NA         | NA                              |

**Etymology.** The specific epithet “*xishuiensis*” refers to the name of the type locality, Xishui County, Guizhou, China. We suggest the English name “Xishui Leaf-litter Toad” and the Chinese name “Xí Shuǐ Zhǎng Tū Chán (习水掌突蟾)”.

**Diagnosis.** *Leptobranchella xishuiensis* sp. nov. can be distinguished from its congeners by a combination of the following characters: (1) SVL 28.1–36.2 mm in males; (2) toes rudimentary webbed, lateral fringes on toes absent; (3) dorsal surface shagreened with small, raised tubercles and longitudinal ridges; (4) ventral surface greyish-white without dark brown spots; (5) throat, chest and ventral surfaces of limbs purplish-grey; (6) iris bicoloured, upper 1/3 copper, transitioning to silver in lower 2/3; (7) distinct black spots present on flanks; and (8) prominent inner palmar tubercle separated from the small outer palmar tubercle; (9) tibia-tarsal articulation reaching the tip of snout.

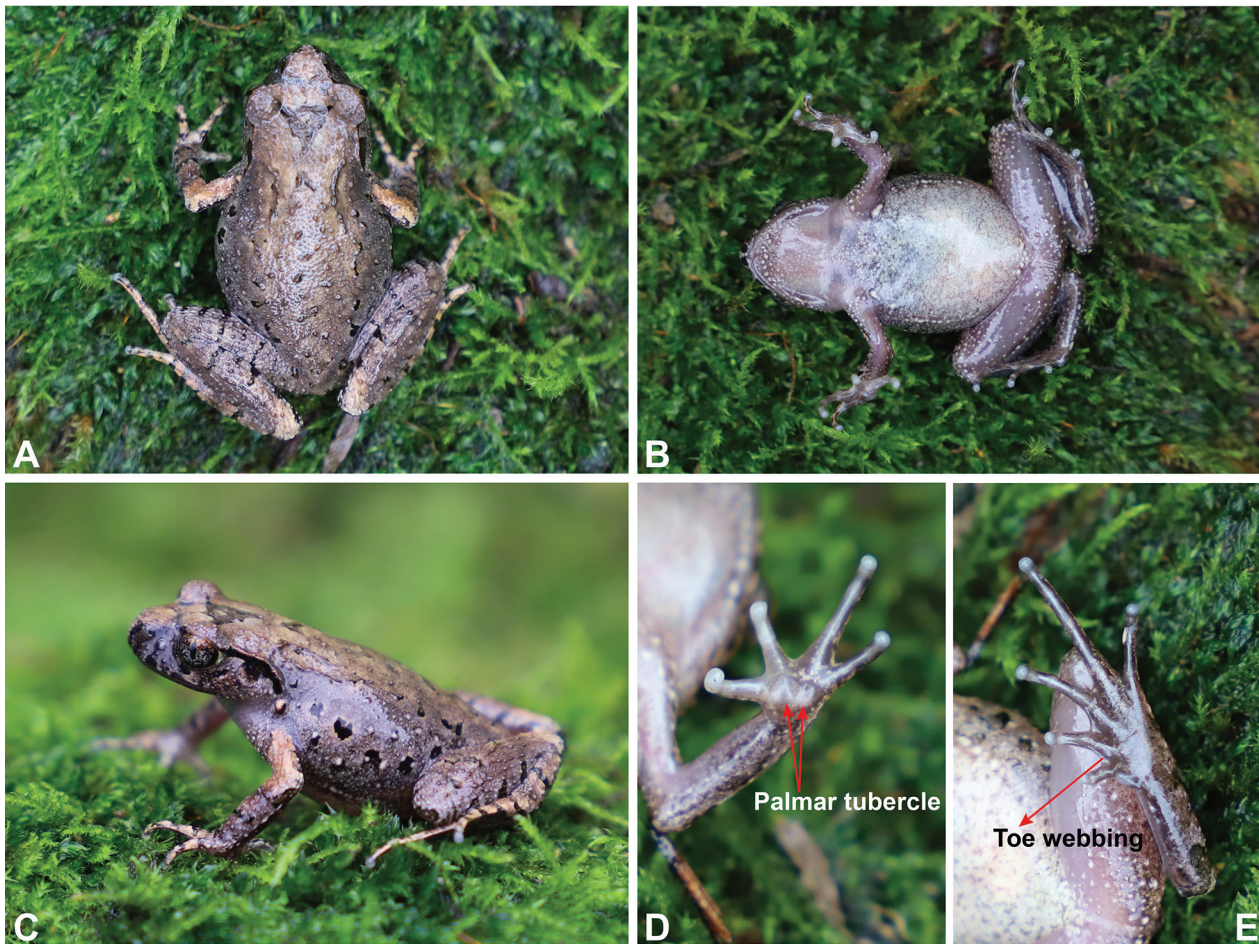
**Description of the holotype.** Adult male, body size moderate, SVL 36.2 mm. Head length slightly wider than head width; snout short, rounded, protruding, projecting over the lower jaw, longer than eye diameter; nostril oval, closer to the tip of snout than eye; canthus rostralis distinct; loreal region sloping and slightly concave; interorbital region flat, slightly longer than internarial distance; pupil vertical; eyes large, diameter near equal to snout length (EYE/SNT = 0.9); tympanum distinct, rounded and slightly concave and its diameter conspicuously less than eye diameter (TD/EYE = 0.6); supra-tympanic fold distinct, raised from corner of eye to supra-axillary gland;

vocal sac openings located laterally on the floor of mouth; tongue cordiform, shallow notch at the posterior tip.

Fore-limbs slender and short, length of lower arm and hand 45.1% of SVL; tips of fingers rounded and slightly swollen; relative finger lengths I < II < IV < III; nuptial pad absent; subarticular tubercles absent; prominent inner palmar tubercle, separated from the small outer palmar tubercle; finger webbing and dermal fringes absent. Hind-limbs slender, tibia slightly nearly equal to thigh length (THL/TIB = 0.9); TIB/SVL = 0.5; tips of toes rounded, slightly swollen; relative toe length I < II < V < III < IV; subarticular tubercles absent, replaced by distinct dermal ridges; pronounced large, oval inner metatarsal tubercle; outer metatarsal tubercle absent; 1/3 toe webbing; lateral fringes absent; tibia-tarsal articulation reaching the tip of snout; heels overlapping when thighs are appressed at right angles to body.

Dorsal surface shagreened with small, raised tubercles and longitudinal ridges; ventral and chest skin smooth without tubercles; anterior throat with several tubercles; pectoral glands oval; femoral glands oval, located on the posteroventral surface of thighs, closer to the knee than to the vent; supra-axillary glands distinct and rounded; a pair of glands under the vent; and ventrolateral glands distinctly visible and raised, forming an incomplete line.

**Colour of the holotype in life.** Dorsal surface greyish-brown, an inverted triangle marking between eyes, irregular markings on shoulder and rear of the back; flanks with greyish-white tubercles and irregular black spots;



**Figure 6.** Holotype of *Leptobranchella xishuiensis* sp. nov. **A.** Dorsal view; **B.** Ventral view; **C.** Lateral view; **D.** Palmar; **E.** Plantar. This illustration was created and provided by Tao Luo.

tympaanum bicoloured, with the upper half of the tympanum pale brown and the lower half light greyish-brown; supratympanic line black from posterior corner of the eye to supra-axillary glands; light brown between the posterior corner of the eyes and the tympanum; wide brown bars on the upper lip; brown transverse bars distinct on dorsal surface of fore-limbs and hind-limbs; upper arm surfaces light orange; ventral surface greyish-white without dark brown spots; throat immaculate purplish-grey and its margin concentrated white tubercles; chest purplish-grey; ventral surfaces of limbs purplish-grey, scattered with white tubercles; pectoral and femoral glands and a pair of creamy-white glands under the vent, supra-axillary glands white; pupil black; iris bicoloured, upper one-third copper, transitioning to silver in lower two-thirds (Fig. 7A4).

**Colour of the holotype in preservation.** In a 10% formalin preservation solution, dorsum and limb surfaces faded to a uniform dark brown; brown, inverted triangle markings distinctly visible between the eyes; irregular black spots distinct on flanks; dorsolateral markings and longitudinal skin ridges and spots on dorsal becoming more distinct; throat, chest and belly creamy-white; pectoral, femoral, supra-axillary and ventrolateral glands creamy-white; dark transverse bars on limbs, fingers and toes remained distinct; upper arm and tibiotarsus faded to greyish-white.

**Variation.** Measurements of the type series are shown in Suppl. material 7. All of the male paratypes matched the overall characters of the holotype. The black spots and tubercles on the flanks exhibited variation between individuals. Certain individuals possess lesser tubercles and continued longitudinal ridges on their dorsum and hind-limb surfaces (Fig. 7C1), while others exhibit a light brown colouration on their dorsum (Fig. 7C1).

**Ecology and distribution.** *Leptobranchella xishuiensis* sp. nov. was found only in bamboo forests at 1600 m elev. All of the individuals were inhabiting bamboo leaves about 1.2 m from the stream and did not call. Juveniles that came ashore were also found during the survey. Based on this, the breeding season may be around May to June. Frogs discovered in the area included *Megophrys qianbeiensis* and *Quasipaa boulengeri*.

**Comparisons.** Suppl. material 9 presents a concise overview of the diagnostic morphological characters of species found north of the Isthmus of Kra. From the 26 congeners of the genus *Leptobranchella* occurring south of the Kra Isthmus, by the presence of supra-axillary and ventrolateral glands, new species can easily be distinguished from *L. arayai*, *L. dringi*, *L. fritinniensis*, *L. gracilis*, *L. hamidi*, *L. heteropus*, *L. kajangensis*, *L. kecil*, *L. marmorata*, *L. melano-leuca*, *L. maura*, *L. picta*, *L. platycephala*, *L. sabahmontana*,





**Figure 7.** Morphological characters compared between *Leptobranchella xishuiensis* sp. nov. (A, C) and *L. suiyangensis* (B). (1) dorsal view, (2) lateral view, (3) ventral view, (4) bicoloured iris, (5) palmar and (6) plantar. The species photos were provided by Tao Luo.



and *L. sola* (vs. absent in the latter); by having a moderate body size (SVL 28.1–36.2 mm in males), the new species differs from *L. baluensis*, *L. brevicrus*, *L. bondangensis*, *L. fusca*, *L. itiokai*, *L. juliandringi*, *L. mjobergi*, *L. natunae*, *L. parva*, *L. palmata*, and *L. serasanae* (vs. less than 18 mm in the latter males or females).

By the moderate body size of the male (SVL 28.1–36.2 mm), the new species differs from the smaller males *L. alpina*, *L. applebyi*, *L. ardens*, *L. aspera*, *L. bashaensis*, *L. bidoupensis*, *L. crocea*, *L. feii*, *L. flaviglandulosa*, *L. graminicola*, *L. khasiorum*, *L. laui*, *L. maculosa*, *L. mangshanensis*, *L. melica*, *L. murphyi*, *L. pallida*, *L. petrops*, *L. tengchongensis*, *L. verrucosa*, *L. wumingensis*, and *L. yingjiangensis* (vs. < 28 mm in the latter); and from the larger *L. nahangensis*, *L. sungi*, and *L. zhangyapingi* (vs. larger than 40 mm in the latter).

By the presence of black spots on flanks, the new species differs from *L. aerea*, *L. botsfordi*, *L. eos*, *L. firthi*, *L. isos*, and *L. tuberosa* (vs. absent). From the rudimentary webbing on the toes, the new species differs from *L. jinshaensis*, *L. jinshaensis*, *L. kalonensis*, *L. oshanensis*, *L. rowleyae*, *L. shiwandashanensis*, and *L. tadungensis* (vs. absent); from *L. pelodytoides* and *L. tamdil* (vs. wide); and from *L. guinanensis* (vs. 1/3 toe webbing). By the absence of lateral fringes on toes, the new species differs from *L. bijie*, *L. bourreti*, *L. chishuiensis*, *L. damingshanensis*, *L. dong*, *L. dorsospina*, *L. dushanensis*, *L. fuliginosa*, *L. jinyunensis*, *L. korifi*, *L. liui*, *L. maoershanensis*, *L. niveimontis*, *L. phiadenensis*, *L. phiaoacensis*, *L. puhoatensis*, *L. purpurus*, *L. purpuraventra*, *L. shangsiensis*, *L. shimentaina*, *L. sinorensis*, *L. wuhuangmontis*, *L. wulingensis*, *L. yae*, *L. yunkaiensis*, and *L. yunyangensis* (vs. present in the latter).

By the tibia-tarsal articulation reaching the tip of the snout when the leg is stretched forward, the new species differs from *L. macrops* (vs. reaching the eye), *L. neangi* (vs. reaching just past the anterior edge of the eye), *L. nokrekensis* (vs. reaching the posterior corner of eye), *L. pluvialis* (vs. reaching the nostril), and *L. ventripunctata* (vs. reaching between the eye and the tympanum). By having dorsal surface shagreened with small, raised tubercles and longitudinal ridges, ventral surface greyish-white without dark brown spots and throat and chest purplish-grey, the new species differs from *L. lateralis* (vs. dorsum with roughly granular and belly creamy-white), *L. minima* (vs. dorsum smooth and ventral surface creamy-white), *L. namdongensis* (vs. dorsum with finely tuberculate and belly creamy-white with brown dusting on the margins), *L. nyx* (vs. dorsum with rounded tubercles and belly creamy-white with brown margins), *L. pingbianensis* (vs. dorsum smooth and chest and belly with dark brown spots), and *L. pyrrophops* (vs. dorsum slightly shagreened and belly reddish-brown with white speckling).

The new species can clearly be distinguished from its phylogenetically close congener *L. suiyangensis*. *L. xishuiensis* sp. nov. differs from *L. suiyangensis* as follows: lateral fringes on the toes absent (vs. present), dorsal surface shagreened with small, raised tubercles and longitudinal ridges (vs. dorsum slightly smooth, with light

reddish-brown tubercles), ventral surface greyish-white without dark brown spots (vs. yellowish creamy-white with marble texture chest and belly or with irregular light brown speckling), throat and chest purplish-grey (vs. throat is grey-white and chest yellowish-creamy-white), tibia-tarsal articulation reaches the tip of snout (vs. reaching the anterior eye), prominent inner palmar tubercle, separated from the small outer palmar tubercle (vs. palmar tubercle not distinct), and iris bicoloured, upper one-third copper, transitioning to silver in lower two-thirds (vs. coppery-orange on the upper half and silver grey on the lower half) (Fig. 7A4, B4, C4). For morphs EYE, IOD, HND, LW and HLL, the new species is significantly larger than *L. suiyangensis* (Table 2) and can be distinguished on the PC1 axis (Fig. 5; Suppl. material 14).

## Discussion

### Phylogeny, taxonomy and diversity

The classification of Asian leaf-litter frogs has been an enigma and several taxonomic schemes have been proposed. The genus *Leptolalax* Dubois, 1980 was widely accepted and assigned two subgenera, *Lalos* Dubois, Grosjean, Ohler, Adler & Zhao, 2010 and *Leptolalax* Dubois, 1980 (Dubois 1980; Delorme et al. 2006; Dubois et al. 2010). Despite phylogenetic support for both subgenera (Poyarkov et al. 2015; Yuan et al. 2017), controversy remains (Ohler et al. 2011; Matsui et al. 2017). Based on large-scale sampling, Chen et al. (2018) revealed that *Leptobranchella* nested within *Leptolalax*, thus placing it in the synonymy of *Leptobranchella* and they rejected the hypothesis that two subgenera were contained within *Leptolalax*, ending a long-standing controversy. Several genera have also been proposed by Chinese taxonomists, for example, *Paramegophrys* and *Carpophrys*. Thus, *Paramegophrys* was also utilised as a valid genus name for Asian leaf-litter frogs in China for some time (Jiang et al. 2008; Mo et al. 2008; Fei et al. 2009). The genus *Paramegophrys* was rejected by Dubois et al. (2010), based on Article 9.9.9 of the International Code of Zoological Nomenclature (4<sup>th</sup> ed.); however, considering the morphological differences, *Leptobranchella* within China was placed in *Paramegophrys* by Fei and Ye (2016). For *Carpophrys*, the genus name is invalid as a nomen nudum, based on Article 14 of the International Code of Nomenclature (4<sup>th</sup> ed.), as the type species was not specified (Dubois 1981; Fei and Ye 1992; Dubois et al. 2010). As it is a taxon with many new species descriptions in recent years, the complete phylogeny of this genus remains to be constructed.

Through amplification and analysis of published data, we have provided additional insights into the phylogeny of *Leptobranchella*. Our results clearly show that *Leptobranchella* is divided into two major clades (BPP = 1.00), Clade I and Clade II, that are highly resolved in the BI tree for 16S rRNA. This result differs from those of prior studies (Chen et al. 2018; Luo et al. 2022b; Chen et al. 2024). We support

*Leptolalax* as a synonym of *Leptobranchella* (Chen et al. 2018) and we also propose three hypotheses for the classification of the genus: (1) place all of the species in *Leptobranchella* (the single genus option) comprising Clades I and II; (2) retain either *Leptobranchella* or *Leptolalax* in clade I only, assigning either *Leptolalax* or *Leptobranchella* to Clade II (a two-genera option); (3) retain *Leptobranchella*, Clade I only, with *Leptolalax* as a synonym of *Leptobranchella* and create a new genus for Clade II that may restore the validity of *Paramegophrys* (a two-genera option). Geographically and morphologically, the majority of Clade I is from south of Indo-Burma, where the clade is characterised by the absence of supra-axillary and ventrolateral glands and small body size; Clade II is largely from central and northern Indo-Burma and southern China and it is characterised by the presence of supra-axillary and ventrolateral glands and medium to large body size (Suppl. material 4). While the two clades differ morphologically and geographically to some extent, a detailed morphological examination of Clade I is needed for possible revisions. In addition, the phylogeny, based on multiple nuclear gene loci, was more consistent with the results of Chen et al. (2018). Therefore, we prefer the most conservative “single-genus option” until additional data are available.

Based on field surveys and published data, we identified three hotspots in the distribution of *Leptobranchella*. Only the Borneo and Indo-Burma regions corresponded to prior global biodiversity hotspots (Myers et al. 2000). The remaining area is located in the mountainous regions of southern China, including the Wuyi, Nanling and Wuling Mountains and is the habitat of 55.6% of *Leptobranchella* species in China. This overlaps with new hotspots for amphibians recently identified in China (Xu et al. 2024). Southern China has experienced rapid economic growth and severe loss of protected areas in recent years (Xu et al. 2017; Ma et al. 2019, and the fauna has experienced pressure from human activities (Xu et al. 2024). Therefore, these three mountainous regions need urgent protection. We note that there are four recorded gap areas and that additional surveys of these areas are necessary.

### Bursts of diversification stimulated by interacting geological and climatic processes

To estimate the origin and dynamic history of diversification in *Leptobranchella*, we assessed the divergence times and MDEs of 101 species using mitochondrial 16S rRNA sequences. Although the topology was not well resolved, utilising 95% HPD overcomes the effects of this uncertainty to some extent and this approach is widely utilised (Klaus et al. 2016; Jiang et al. 2019; Xu et al. 2021; Li et al. 2022). Therefore, the results of the MDivE analysis are utilised in the discussion. The distribution of *Leptobranchella* spans nearly 30 degrees of latitude and the diversity hotspots are concentrated near orogenic zones, i.e. the QTP and the Ailao Shan–Red River shear zone. Thus, the diversification of *Leptobranchella* was most likely

driven by geology and the Asian monsoon climate and less by temperature (Luo et al. 2023).

Molecular dating and MDivE analyses revealed that *Leptobranchella* originated at ~ 32 Ma. The MDivE has two major (35–14 Ma, 13–6 Ma) or three minor acceleration phases (35–20 Ma, 19–14 Ma and 13–6 Ma) and a deceleration phase (6 Ma–present), increasing rapidly at ~ 15 Ma, with the highest peak occurring at ~ 8.7 Ma (Fig. 4G, Table 2). The origin of the genus is consistent with geological and phylogenetic evidence hypothesising that 35 Ma was the initiation of the left-lateral extrusion in Indochina (Schärer et al. 1994; Leloup et al. 2001; Gilley et al. 2003; Li et al. 2020b) or was in the midst of an accelerated process (Li et al. 2024b), but predates the rapid QTP uplift (25–15 Ma; Ding et al. (2017); Ding et al. (2022)). The first small peak of the MDivE is at ~ 20.5 Ma, which is consistent with the dramatic change in the India-Asia convergence rate and angle (Li et al. 2024b). The MDivE first decreased at ~ 20 Ma, implying a slowing of the Indochina left-lateral extrusion and coinciding with the end of the geological left-lateral offset (Chen et al. 1992; Leloup et al. 1995; Leloup et al. 2001; Wang et al. 2020b). However, the rapid uplift of the QTP at 20 Ma (Ding et al. 2017) may have led to the end of a short-lived slowdown in diversification in the MDivE.

From 19 to 16 Ma, the MDivE continued to increase, likely driven by the continued sharp uplift of the QTP (Ding et al. 2017), although the left-lateral extrusion in Indochina may have been slowing (Li et al. 2024b). A second decrease in the MDivE occurred at ~ 15 Ma, a period that is often regarded as the end of the left-lateral offset (Leloup et al. 1995, 2001; Wang et al. 2020b). The MDivE reached a second valley at 13.5 Ma (Fig. 4G) and the Red River Fault began to be dislocated at the same time (~ 13–12 Ma) (Zhang et al. 2006; Zhang et al. 2009). Phylogenetic evidence supports the hypothesis that the left-lateral extrusion in Indochina may also have continued until this time (Li et al. 2024b); that is, the shift in the left-to-right lateral movements of the ARSZ (~ 13 Ma) and the slowing of the rapid uplift of the QTP (~ 15 Ma) may have occurred in the mid-Miocene (Ding et al. 2017, 2022; Li et al. 2024b).

From 13 to 6 Ma, the MDivE underwent a final dramatic increase, peaking at 8.70 Ma (95% CI: 9.57–7.78 Ma; Table 2), suggesting that more intense orogenic and climatic shifts occurred during this period. Geology and phylogeny have shown that a second, more rapid outward extrusion of Indochina occurred at this time and that the rate and angle of Indo-Asian convergence again changed rapidly (Li et al. 2024b). Geological studies also suggest that the right-lateral extrusion of Indochina occurred during the late Miocene (11–5 Ma) (Zhang et al. 2009; Fyhn and Phach 2015). The most distinct peak of the MDivE occurred at 8.70 Ma, consistent with the Ailao Shan thermochronologic age (8.4–6.8 Ma) (Zhang et al. 2009) and the age of the Asian monsoon intensification (Farnsworth et al. 2019). From 6 Ma to the present, MDivE has continued to decline as orogeny and monsoon climate intensification slowed.

A similar three-stage diversification pattern was also reflected in the speciation rate-through-time plots (Fig. 4D). The dynamic patterns of MDivE suggest that the Indochina extrusion (left and right) and QTP uplift went through multiple phases. Global temperatures continued to decline over the same period (Westerhold et al. 2020), while the MDivE continued to rise and, thus, the contribution of temperature to diversification may have been limited.

## Conclusions

This study identified three biodiversity hotspots and four record-vacant regions as priority areas for conservation research in the future. We revealed the dynamics and patterns of diversification in *Leptobranchella* by integrating molecular dating and ancestral range reconstructions of multiple species, explaining the paleogeoclimatic events that influenced its diversification. This unique approach will help overcome the difficulty of obtaining the correct topology for inferring evolutionary dynamics due to too few molecular markers. Our estimates of the diversification dynamics of *Leptobranchella* augment studies of orogeny and the Asian monsoon climate and provide new data for understanding species diversification patterns in Southeast Asia and southern China. We also describe a new species of *Leptobranchella* and identify three cryptic species, suggesting that the diversity of the genus needs further exploration.

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## Authors' contributions

Tao Luo and Jiang Zhou conceived and designed the research; Tao Luo, Zi-Fa Zhao, Zhong-Lian Wang, Chang-Ting Lan and Ming-Yuan Xiao conducted field surveys and collected samples; Tao Luo, Zi-Fa Zhao and Zhong-Lian Wang measured specimens and analysed morphological and genetic data; Tao Luo, Huai-Qing Deng, Ning Xiao, and Jiang Zhou wrote and revised the manuscript. All authors have read and approved the final version of the manuscript.

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## Supplementary material 1

### Evolutionary timescales for *Leptobranchella*

Authors: Tao Luo, Zi-Fa Zhao, Zhong-Lian Wang, Chang-Ting Lan, Ming-Yuan Xiao, Huai-Qing Deng, Ning Xiao, Jiang Zhou

Data type: png

Explanation note: Evolutionary timescales for *Leptobranchella* were estimated in BEAST v.1.8.2 using mitochondrial 16S rRNA. This photo was created and provided by Tao Luo.

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Link: <https://doi.org/10.3897/zse.101.137392.suppl1>

## Supplementary material 2

### Raw details of the ancestral range of *Leptobranchella* estimated at the species level using BioGeoBEARS

Authors: Tao Luo, Zi-Fa Zhao, Zhong-Lian Wang, Chang-Ting Lan, Ming-Yuan Xiao, Huai-Qing Deng, Ning Xiao, Jiang Zhou

Data type: png

Explanation note: This photo was created and provided by Tao Luo.

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Link: <https://doi.org/10.3897/zse.101.137392.suppl2>

### Supplementary material 3

#### Macroevolutionary cohort speciation matrix

Authors: Tao Luo, Zi-Fa Zhao, Zhong-Lian Wang, Chang-Ting Lan, Ming-Yuan Xiao, Huai-Qing Deng, Ning Xiao, Jiang Zhou

Data type: png

Explanation note: Every cell in the matrix is coded by a colour denoting the pairwise probability that two species share a common macroevolutionary rate regime. The species tree is shown for reference on the left and upper margins of every cohort matrix. This photo was created and provided by Tao Luo.

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Link: <https://doi.org/10.3897/zse.101.137392.suppl3>

### Supplementary material 4

#### Distribution of species in Clades I and II in Southeast Asia and southern China

Authors: Tao Luo, Zi-Fa Zhao, Zhong-Lian Wang, Chang-Ting Lan, Ming-Yuan Xiao, Huai-Qing Deng, Ning Xiao, Jiang Zhou

Data type: png

Explanation note: This photo was created and provided by Tao Luo.

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Link: <https://doi.org/10.3897/zse.101.137392.suppl4>

### Supplementary material 5

#### References for morphological characters for congeners of the genus *Leptobrachella*

Authors: Tao Luo, Zi-Fa Zhao, Zhong-Lian Wang, Chang-Ting Lan, Ming-Yuan Xiao, Huai-Qing Deng, Ning Xiao, Jiang Zhou

Data type: docx

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Link: <https://doi.org/10.3897/zse.101.137392.suppl5>

### Supplementary material 6

#### Primers used in PCR and sequencing in this study

Authors: Tao Luo, Zi-Fa Zhao, Zhong-Lian Wang, Chang-Ting Lan, Ming-Yuan Xiao, Huai-Qing Deng, Ning Xiao, Jiang Zhou

Data type: docx

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Link: <https://doi.org/10.3897/zse.101.137392.suppl6>

## Supplementary material 7

### Localities, voucher information and GenBank numbers for all of the samples used in this study

Authors: Tao Luo, Zi-Fa Zhao, Zhong-Lian Wang, Chang-Ting Lan, Ming-Yuan Xiao, Huai-Qing Deng, Ning Xiao, Jiang Zhou

Data type: docx

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Link: <https://doi.org/10.3897/zse.101.137392.suppl7>

## Supplementary material 8

### Measurements of the specimens of *Leptobrachella xishuiensis* sp. nov. and *L. suiyangensis*

Authors: Tao Luo, Zi-Fa Zhao, Zhong-Lian Wang, Chang-Ting Lan, Ming-Yuan Xiao, Huai-Qing Deng, Ning Xiao, Jiang Zhou

Data type: docx

Explanation note: All of the units are in mm. See the abbreviations for the morphological characters in the Materials and Methods section. \*For the holotype.

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Link: <https://doi.org/10.3897/zse.101.137392.suppl8>

## Supplementary material 9

### Selected diagnostic characters for the new species described herein and for the species in the genus *Leptobrachella* occurring north of the Isthmus of Kra

Authors: Tao Luo, Zi-Fa Zhao, Zhong-Lian Wang, Chang-Ting Lan, Ming-Yuan Xiao, Huai-Qing Deng, Ning Xiao, Jiang Zhou

Data type: xlsx

Explanation note: Grey shading indicates non-overlapping characters compared to *Leptobrachella xishuiensis* sp. nov.

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Link: <https://doi.org/10.3897/zse.101.137392.suppl9>

## Supplementary material 10

### Distribution site of *Leptobrachella* in this study

Authors: Tao Luo, Zi-Fa Zhao, Zhong-Lian Wang, Chang-Ting Lan, Ming-Yuan Xiao, Huai-Qing Deng, Ning Xiao, Jiang Zhou

Data type: xlsx

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Link: <https://doi.org/10.3897/zse.101.137392.suppl10>



## Supplementary material 11

### Uncorrected *p*-distance between *Leptobrachella* species for the 16S rRNA gene sequences

Authors: Tao Luo, Zi-Fa Zhao, Zhong-Lian Wang, Chang-Ting Lan, Ming-Yuan Xiao, Huai-Qing Deng, Ning Xiao, Jiang Zhou

Data type: docx

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Link: <https://doi.org/10.3897/zse.101.137392.suppl11>

## Supplementary material 12

### Evaluation results of *Leptobrachella* for ancestral areas using the R package BioGeoBEARS

Authors: Tao Luo, Zi-Fa Zhao, Zhong-Lian Wang, Chang-Ting Lan, Ming-Yuan Xiao, Huai-Qing Deng, Ning Xiao, Jiang Zhou

Data type: docx

Explanation note: The best model is indicated in bold. Abbreviations: LnL = log-likelihood; *d* = dispersal rate per million years along branches; *e* = extinction rate per million years along branches; *j* = likelihood of founder-event speciation at cladogenesis; AIC = Akaike Information Criterion.

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Link: <https://doi.org/10.3897/zse.101.137392.suppl12>

## Supplementary material 13

### Inferred *in situ* diversification events and dispersal events related to *Leptobrachella* summarised from Suppl. material 1

Authors: Tao Luo, Zi-Fa Zhao, Zhong-Lian Wang, Chang-Ting Lan, Ming-Yuan Xiao, Huai-Qing Deng, Ning Xiao, Jiang Zhou

Data type: docx

Explanation note: The age (upper and lower bounds of the 95% intervals) of the respective clades are drawn from the time tree constructed using mitochondrial 16S rRNA.

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Link: <https://doi.org/10.3897/zse.101.137392.suppl13>

## Supplementary material 14

### Variable loadings for principal components with eigenvalues greater than one from morphometric characters corrected by SVL

Authors: Tao Luo, Zi-Fa Zhao, Zhong-Lian Wang, Chang-Ting Lan, Ming-Yuan Xiao, Huai-Qing Deng, Ning Xiao, Jiang Zhou

Data type: docx

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