Rediscovery of *Protohynobius puxiongensis* (Caudata: Hynobiidae) and its phylogenetic position based on complete mitochondrial genomes

Rui Peng a,b,1, Peng Zhang c,1, Jian-Li Xiong d, Hai-Jun Gu e, Xiao-Mao Zeng b,* Fang-Dong Zou a,*

a Sichuan Key Laboratory of Conservation Biology on Endangered Wildlife, College of Life Sciences, Sichuan University, Chengdu 610064, PR China
b Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu 610041, PR China
c College of Animal Science and Technology, Henan University of Science and Technology, Luoyang 471003, PR China
d School of Life Sciences, Sun Yat-sen University, Guangzhou 510275, PR China
e Wildlife Resource Investigation and Conservation Station of Sichuan Forestry Department, Chengdu 610082, PR China

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**ABSTRACT**

The mysterious Asian hynobiid salamander, *Protohynobius puxiongensis*, was described based on a single specimen collected in 1965 and never found again since then. Because the specimen had an internasal bone, *Pr. puxiongensis* was thought to retain a primitive character lost by a common ancestor of all other hynobiid salamanders, and it was thus considered to be not only a new genus, but also a new subfamily. This conclusion bothered herpetologists for decades because it was based on only one specimen and one character without other living specimens being rediscovered. After years of field effort, we rediscovered living individuals of *Pr. puxiongensis* at its type locality. All characters observed in rediscovered specimens are identical to the original description of the holotype except the internasal bone, implying that the internasal bone observed in the holotype may be just an individual variation. To examine the phylogenetic position of *Pr. puxiongensis*, we sequenced complete mitochondrial genomes for this species, together with two *Pseudohynobius* species. By combining 18 published hynobiid mitochondrial genomes and our new sequences, we reconstructed a comprehensive phylogenetic relationship of Hynobiidae at the genus level. Our results indicate that *Pr. puxiongensis* is deeply nested within the hynobiid phylogeny. It is the sister group of the *Pseudohynobius* species, and the validity of subfamily Protohynobiinae is not supported.

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**1. Introduction**

*Protohynobius puxiongensis*, one of the most enigmatic species of Asian hynobiid salamanders, was accidentally discovered in a potato cellar in a remote area of Hengduan Mountains, western China in 1965. Despite many attempts to rediscover this species, no one succeeded in collecting a second individual prior to this report. Remarkably, *Pr. puxiongensis* is the only five-toed hynobiid salamander discovered in the area of Hengduan Mountains, where four-toed *Batrachuperus* were widely recorded for a long time (Liu, 1950; Zhao and Adler, 1993). In addition, *Pr. puxiongensis* possesses many distinct characteristics that distinguish it from *Batrachuperus* species, such as no labial fold and distinct costal folds. Therefore, after being described by Fei and Ye (2000), the species was then widely accepted (AmphibiaWeb, 2009; Frost, 2009), although it was based on only a single specimen.

Interestingly, an internasal bone was found between the nasal bones on the skull of the type specimen of *Protohynobius puxiongensis* (Fei and Ye, 2000). This character, however, has not been observed in any living hynobiid salamanders or even any living tailed amphibians. The internasal (or median rostral) bone is present in some fossil tetrapods, such as *Ichthyostega*, *Loxomma*, and *Tersomius texensis* (Carroll, 1964; Duellman and Trueb, 1994; Heatwole and Carroll, 2000; Huttenlocker et al., 2007; Schoch and Rubidge, 2005), thus considered a primitive character by Fei and Ye (2000). Based on this unusual characteristic, Fei and his colleagues proposed that *Pr. puxiongensis* was the most primitive group within hynobiid salamanders and belonged to a new hynobiid subfamily—Protohynobiinae (Fei et al., 2005, 2006; Fei and Ye, 2000). On the other hand, previous molecular study (Zhang et al., 2006) suggests that relationships among living hynobiids are shaped primarily by geography. Therefore, the overlapping distribution ranges of *Pr. puxiongensis* and other *Batrachuperus* species (a young hynobiid group) indicate a close relationship between these hynobiid lineages, which would disprove the hypothesis that *Pr. puxiongensis*...
is phylogenetically outside a clade comprising all other hynobiid salamanders.

Through years of field work and help from local people, we have rediscovered living adults, larvae, and egg sacs of *Protohynobius puxiongensis* from its type locality. This finding provides us an opportunity to study the phylogenetic position of *Pr. puxiongensis* by DNA analysis. The complete mitochondrial genomes have been successfully used to study phylogenetic questions for different animal groups because they have become more readily available and are expected to produce more reliable phylogenetic inference than small gene fragments (Cummings et al., 1995; Zardoya and Meyer, 1996; Zhang et al., 2008). In addition, a phylogenetic study including 8 of 10 genera of Hynobiidae has been done based on complete mitochondrial genomes by Zhang et al. (2006). In this study, we sequenced the complete mitochondrial genomes of *Pr. puxiongensis*, and two representatives of the genus *Pseudohynobius*: *Ps. flavomaculatus* and *Ps. shuichengensis*, to cover the two missing hynobiid genera not been reported before. These new sequences, together with published hynobid mitochondrial genomes, are used to address the phylogenetic position of *Pr. puxiongensis* among living hynobids and to reconstruct phylogenetic relationships of hynobids more comprehensively.

## 2. Materials and methods

### 2.1. Sample collection and identification

The specimens of *Protohynobius puxiongensis* (CIB-XM3126, 28°38.168’N, 102°30.485’E, altitude 2900 m), *Pseudohynobius flavomaculatus* (CIB-XM2084, 30°31.468’N, E109°05.680’E, altitude 1900 m) and *Ps. shuichengensis* (CIB-XM2855, 26°34.366’N, 104°48.455’E, altitude 1944 m), were collected from Puxiong, Yuexi County of Sichuan province, Hanchi, Lichuan County of Hubei province, Shangshilong, Shuicheng County of Guizhou province of China, respectively (Fig. 1).

The holotype specimen was unexpectedly found in May 1965 from an abandoned potato cellar used by local people, Yi nationality, for food storage. The potato cellar is obviously not the breeding habitat where hynobiid salamanders are usually collected, which may explain why the rediscovery took such a long time. In 2007, we changed our searching strategy to educate local people, giving detailed lectures and having personal conversations with local people one by one to make them pay attention to this rare animal. Finally, the first news came in the summer by a local shepherd boy who caught a larva in a brook 2 km away from the type locality. By autumn, our group had rediscovered more larvae with external gills in nearby field spots. In spring of 2008–2009, we successfully re-collected adults together with egg sacs. The new localities of *Protohynobius puxiongensis* are less than three kilometers away from its type locality.

All characters observed in the rediscovered adult specimens of *Protohynobius puxiongensis* match the original description of the holotype except the internasal bone and premaxillary fontanelle (Table 1). The holotype of *Pr. puxiongensis* lacks the premaxillary fontanelle, whereas we observed a very small one in the specimen (CIB-XM3323) that we examined (Fig. 2). Although the premaxillary fontanelle is thought to be an important character for hynobiid identification, its size varies greatly in different individuals of the same species, sometimes being difficult to see (Jian-Li Xiong, Ph.D. Thesis, Sichuan University, China). Therefore, the difference on the premaxillary fontanelle between the holotype and the rediscovered specimens may result from individual variation. As to the internasal bone (present in the holotype but absent in our rediscovered specimens; Fig. 2), we speculate that the internasal bone observed in the holotype may be introduced by an individual variation (see Section 4). Based on the nearly identical collecting locality and the overall morphological similarity, we consider the hynobiid specimen that we collected to be the missing hynobiid species—*Protohynobius puxiongensis*.

### 2.2. Laboratory protocols

Total genomic DNA was extracted from muscle tissues using the standard Proteinase K method (Sambrook and Russell, 2001). A suite of 28 primers (see Supplementary material Table 1 for details) was used to amplify 14 contiguous and overlapping fragments that covered the entire mt genome. PCR reactions were performed with a PTC-200 thermal cycler (BioRad, USA) in a volume of 25 µl containing 1 × Ex PCR buffer, 0.1 mM dNTPs, 1.5 mM MgCl$_2$, 1 µM each primer, 1.5 U Ex Taq polymerase (TaKaRa Bio, Dalian). Cycling conditions were 95°C for 4 min, followed by 35 cycles of 30 s at 95°C, 45 s annealing at 50–55°C, and a 1–3 min extension at 72°C, with a final extension at 72°C for 10 min. PCR products were purified from 1% TAE agarose gels with the DNA Agarose Gel Extraction Kit (Omega, USA) and sequenced directly with the corresponding PCR primers and some specific walking primers on an ABI 3730 sequencer.

### 2.3. Sequence data and phylogenetic analyses

DNA sequences were analyzed using the software DNAMAN version 3.0 (Lynnon Biosoft, Quebec, Canada). Protein-coding genes were identified using the ORF-Finder tool of NCBI (http://www.ncbi.nlm.nih.gov/projects/gorf/). The locations of tRNA genes were determined by comparison with known sequences from other hynobiids. The tRNA genes were identified using the tRNA-Scan-SE v.1.21 (Lowe and Eddy, 1997) or by the proposed secondary structures (Kumazawa and Nishida, 1993) and anti-codons. The mitochondrial genome sequences of *Protohynobius puxiongensis*, *Pseudohynobius flavomaculatus* and *Ps. shuichengensis*, were deposited in GenBank under Accession No. FJ532058 – FJ532060.

Another 18 hynobid and four outgroup (three cryptobranchids, one salamandrid) mitochondrial genomes were downloaded from GenBank. The details for these sequences are given in the Supplementary material Table 2. All sequences from the L-strand-encoded genes (ND6 and eight tRNA genes) were converted into complementary-strand sequences. Thirteen protein-coding, 22 tRNA and two RNA gene sequences were aligned using Clustal W (Thompson et al., 1997) at default settings. All 22 tRNA alignments were then combined to generate a concatenated alignment. To avoid artificial bias in refining alignments, we used Gblocks (Castresana, 2000) to extract regions of defined sequence conservation from the two RNAs, concatenated tRNAs, and 13 protein-coding gene alignments at default settings. Finally, a DNA dataset combining all 16 Gblock-refined alignments was generated. This DNA dataset was divided into 42 partitions according to genes and codon positions (tRNAs, 2 tRNAs, every codon position for 13 protein genes). Model selection for each partition was done according to the Akaike information criterion (AIC) as implemented in MrModelTest 2.2 (http://www.abc.uu.se/systzoo/staff/nylander.html). The best fitting model for each partition was used in subsequent Bayesian phylogenetic analyses. In addition to the DNA alignment, we made an amino acid alignment of the deduced amino acid sequences of all 13 mt protein-coding genes using a similar methodology. This protein data set was divided into 13 partitions according to genes. MP analyses were performed using heuristic searches (TBR branch swapping; MULPERS option in effect) with 100 random-addition sequences by PAUP* 4.0b10 (Swofford, 2002). All sites were treated equally. ML analyses were conducted using PHYML version 2.4.4 (Guindon and Gascuel, 2003) with GTR+Γ4 (for DNA) or MTMAM+Γ4 (for protein) substitution models. Branch
Fig. 1. The localities of specimens used in this study. The collection locality of *Protohynobius puxiongensis* is marked by triangle, *Pseudohynobius shuichengensis* by square and *Pseudohynobius flavomaculatus* by circle. Solid lines of BlueGreen, red and blue indicate the distribution ranges of three western hynobiid genera *Batrachuperus*, *Liua* and *Pseudohynobius*, respectively.

Table 1
A morphological character comparison among four hynobiid genera, *Protohynobius*, *Pseudohynobius*, *Batrachuperus*, and *Liua*.

<table>
<thead>
<tr>
<th>Character</th>
<th><em>Protohynobius</em></th>
<th><em>Protohynobius</em></th>
<th><em>Pseudohynobius</em></th>
<th><em>Batrachuperus</em></th>
<th><em>Liua</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Shape of body</td>
<td>Slender</td>
<td>Slender</td>
<td>Slender</td>
<td>Stout</td>
<td>Stout</td>
</tr>
<tr>
<td>Shape of vomerine teeth</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Internasal bone</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Premaxillary fontanelle</td>
<td>Absent</td>
<td>Present (small)</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Lacrimals reach to external nares</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Labial fold</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Obvious</td>
<td>Obvious</td>
</tr>
<tr>
<td>Fingers</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Toes</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Horny cover on the palms and soles</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent or present</td>
<td>Present</td>
</tr>
<tr>
<td>Tail fin fold</td>
<td>Not obvious</td>
<td>Not obvious</td>
<td>Not obvious</td>
<td>Not obvious</td>
<td>Obvious</td>
</tr>
<tr>
<td>Life history</td>
<td>Mainly terrestrial</td>
<td>Mainly terrestrial</td>
<td>Mainly terrestrial</td>
<td>Lifetime aquatic</td>
<td>Lifetime aquatic</td>
</tr>
<tr>
<td>Karyotype</td>
<td>2n = 52</td>
<td>2n = 52</td>
<td>2n = 52</td>
<td>2n = 66</td>
<td>2n = 66</td>
</tr>
<tr>
<td>Egg sacs</td>
<td>Spiral</td>
<td>Spiral</td>
<td>Spiral</td>
<td>Sprial or &quot;C&quot;-shaped</td>
<td>C-shaped</td>
</tr>
<tr>
<td>The arrange mode of eggs in the egg sacs</td>
<td>Single line or interlaced</td>
<td>Single line or interlaced</td>
<td>Single line or interlaced</td>
<td>Single line</td>
<td>Single line</td>
</tr>
<tr>
<td>Habitat</td>
<td>Unkown</td>
<td>Mountain brooks</td>
<td>Mountain brooks</td>
<td>Streams</td>
<td>Streams</td>
</tr>
</tbody>
</table>

* Data of *Protohynobius puxiongensis* are from the Holotype (CIB-65II0220) (Fei and Ye, 2000).
* Data of *Protohynobius puxiongensis* are from the specimens recently collected from the type locality (CIB-XM3332, CIB-XM3364, CIB-XM3365 and CIB-XM3198).
* Data of *Pseudohynobius*, *Batrachuperus* and *Liua* are from Fei et al. (2006).
* Unpublished data from Xiong and Zeng.
* The Holotype (CIB-65II0220) was collected in a potato cellar near a brook, but its habitat was unclear.
support for MP and ML analyses was evaluated with non-parametric bootstrap analysis (1000 replicates). Partitioned Bayesian inferences were conducted with MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003) with one cold and three heated chains (temperature set to 0.1) for 20 million generations and sampled every 5000 generations. Due to computation cost, the BIs for the protein data were run for five million generations and sampled every 500 generations. The burn-in parameter was empirically estimated by plotting -ln L against the generation number using Tracer version 1.4 (http://evolve.zoo.ox.ac.uk/beast/help/Tracer), and the trees corresponding to the first 15–50% generations were discarded. To ensure that our analyses were not trapped in local optima, four independent MrBayes runs were performed. Topologies and posterior clade probabilities from different runs were compared for congruence.

3. Results

3.1. General features of new mitochondrial genomes

The complete mitochondrial genomes of Protohynobius puxiongensis, Pseudohynobius flavomaculatus and Ps. shuichengensis, are 16,398, 16,389, and 16,394 bp in length, respectively. As in most of the published higher vertebrate sequences, all three newly sequenced hynobiid mitochondrial genomes encode two rRNAs, 22 tRNAs, and 13 proteins in a typical vertebrate order. The long non-coding region between tRNA-Thr and tRNA-Pro genes, which is observed in all published salamander mtDNAs, is also present in the mitochondrial genomes of Pr. puxiongensis (125 bp), Ps. flavomaculatus (125 bp) and Ps. shuichengensis (127 bp).

3.2. Phylogenetic analyses

The mitogenomic DNA data set combining two rRNAs, the concatenated tRNAs, and 13 protein-coding gene alignments contains 15,007 characters (7320 constant, 1578 parsimony-uninformative, and 6109 parsimony-informative). The protein data set derived from the deduced amino acid sequences of all 13 mitochondrial protein-coding genes contains 3718 characters. Of these, 2150 are constant, 512 are parsimony-uninformative, and 1056 are parsimony-informative.

DNA maximum parsimony, partitioned DNA Bayesian and partitioned protein Bayesian analyses produced nearly identical tree topologies except for some trivial differences within the genus Batrachuperus (node h, Fig. 3). The other three methods gave largely congruent results but with some difference: protein MP tree put Salamandrella as the sister group of Hynobius (node e, Fig. 3), but the bootstrap support for this result is below 50%; DNA and protein ML trees placed the Ranodon–Paradactylodon clade as the sister taxon to all other hynobiids except Onychodactylus, but the bootstrap support for this result is weak (~50%; node c, Fig. 3). In all tree-building analyses, Protohynobius puxiongensis is strongly supported as the sister group of genus Pseudohynobius species by Bayesian posterior probabilities (BPP) of 1.0, and MP or ML bootstrap values over 95% (Fig. 3). Liua is the sister group of the Protohynobius–Pseudohynobius clade but with low support (bootstrap 50–60%, BPP 0.8–0.9; node g, Fig. 3). The closest relative of Protohynobius, Pseudohynobius, and Liua is Batrachuperus, and these four genera form a well supported clade in most tree-building analyses (node f, Fig. 3). The successively more distant outgroups to the Protohynobius–Pseudohynobius–Liua–Batrachuperus clade are Salamandrella, Hynobius, a sister-pairing of Ranodon and Paradactylodon, and Pachyhynobius, respectively. The genus Onychodactylus is strongly supported as the sister taxon of all other hynobiids (BPP = 1.0, bootstrap = 100%; node b, Fig. 3). The new mitogenomic phylogenetic tree of living hynobiids is largely in congruence with the previous study using overlapping sampling and similar methods (Zhang et al., 2006), except for the placement of Pachyhynobius. Here, Pachyhynobius is recovered as the sister taxon to all other hynobiids except Onychodactylus (node c, Fig. 3), whereas Zhang et al. (2006) recovered the genus as the sister taxon to a clade comprising all other hynobiids except Onychodactylus and the sister taxa Ranodon and Paradactylodon.

4. Discussion

4.1. Validity of the genus Protohynobius and the subfamily Protohynobiinae

The holotype of Protohynobius puxiongensis possesses an internasal bone between two nasal bones on the skull. Based on the unusual characteristic, Fei and his colleagues considered Pr. pux-
Phylogenetically outside a clade comprising all other hynobiid salamanders, and placed it in a new hynobiid subfamily—Protohynobiinae (Fei and Ye, 2000). We checked four specimens (three male adult specimens and one larva) recently collected from the type locality and found that all specimens examined have no internasal bone between the nasal bones while the remaining morphological characteristics of our specimens correspond with the original description of the holotype specimen. Actually, the internasal bone is not a very stable character and can be occasionally introduced by individual mutation. Dalquest and Scheffer (1994) reported a mutant phylotype of pocket gophers with an internasal bone. A small, narrow, internasal bone situated medially in the frontonasal suture was observed in two populations of spiny rats, whereas the presence of this bone is not stable (Martin, 1970). More importantly, we have observed a mutant individual with an internasal bone situated medially between two nasal bones in another hynobiid species, *Hynobius maoershanensis*, whereas most individuals of this species lack this feature (unpublished data). Based on the above examples and our observation, we speculate that the internasal bone in the specimen of *Pr. puxiongensis* collected in 1965 may be just an individual variation. Therefore, morphologically, using the internasal bone as a key characteristic to establish the subfamily Protohynobiinae is questionable.

The complete mitochondrial genome sequences of *Protohynobius puxiongensis*, *Pseudohynobius flavomaculatus* and *Ps. shuichengensis* reported here, together with other published sequences of *Pseudohynobius shiuchengensis* and *Pr. puxiongensis* within the tree is bold.

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**Fig. 3.** Phylogenetic relationships of living hynobiids inferred from analyses of mitochondrial genomic data (DNA level and protein level). Tree topology and branch lengths are taken from the partitioned Bayesian analysis on mitochondrial genomic sequences. Branches with letters have branch support values given below the tree for maximum parsimony bootstrapping (MP), maximum-likelihood bootstrapping (ML) and Bayesian posterior probabilities (BA). Branches with bootstrap support >95% and Bayesian posterior probability >0.99 are indicated as stars. Hyphens indicate nodes that are not supported in the corresponding analyses. Note that the branch leading to *Protohynobius puxiongensis* within the tree is bold.
hynobiids, represent all 10 recognized genera of Hynobiidae. Based on substantial molecular data, all tree-building methods indicate that Onychodactylus, not Protohynobius, is the sister taxon of all other living hynobiids, and Pr. puxiongensis is deeply nested within the hynobid phylogeny (Fig. 3). Pseudohynobius is strongly supported as the sister group of Protohynobius. Liua and Batrachuperus are successively more distant outgroups to the Protohynobius + Pseudohynobius clade (Fig. 3). Therefore, on a molecular level, our analyses again disproved the validity of the subfamily Protohynobiniace.

Protohynobius puxiongensis is no doubt a valid species because it is genetically distinct from other hynobiid species in our molecular phylogenetic analyses (Fig. 3). However, there is no strong evidence to support the validity of the genus Protohynobius according to our investigation. Firstly, Pr. puxiongensis is morphologically very close to Pseudohynobius species; most characters used to identify Pseudohynobius, such as cylindrical trunk, vomerine teeth in two arched series like ‘ ‘ ‘ ‘ shape, four fingers and five toes without claws, lunged, no labial fold (see Table 1 for details) can also be observed in Protohynobius. More importantly, we observed a premaxillary fontanelle between premaxillae and nasal bones in the adult specimen of Pr. puxiongensis (Fig. 2). This feature is an important character to identify the genus Pseudohynobius. In addition, our field survey shows that Pr. puxiongensis has similar living and breeding habits to Pseudohynobius species. Adults of both genera are terrestrial in high mountain areas; breeding occurs in gently moving cold brooks, and egg sacs commonly attach to walls of spring holes or undersurface of stones. Secondly, Protohynobius and Pseudohynobius form an apparent clade, and the genetic distance between the two genera is not large compared with the distances between other hynobid genera (Fig. 3). The genetic difference between Ranodon and Paradiclytodon is somewhat like the Protohynobius–Pseudohynobius situation (Fig. 3), but morphologically Ranodon (five toes) and Paradactylodon (four toes) are more distinct. Considering that there are no distinct morphological or genetic differences between Protohynobius and Pseudohynobius, and the two genera compose a clade in our phylogenetic analyses, we suggest that Protohynobius puxiongensis could be merged into the genus Pseudohynobius as Pseudohynobius puxiongensis.

4.2. Phylogeny and biogeography of hynobiids

In this study, we added three new hynobid mitochondrial genomes to the substantial database that exists for Hynobiidae. These new data represent the two hynobid genera (Pseudohynobius and Protohynobius) missing from the previous mitogenomic study for Hynobiidae (Zhang et al., 2006), and allow us to investigate the interrelationships among the 10 hynobiid genera for the first time. Compared to Zhang et al.’s results (2006) there are two other topological differences. (1) The three new hynobids (Pseudohynobius and Protohynobius) represent a previously unstudied clade that is the sister group of Liua (node f, Fig. 3). (2) The second branch within the hynobid phylogeny is recovered as Pachyhynobius in this study (node c, Fig. 3), not the Ranodon–Paradiclytodon clade as in Zhang et al. (2006). This incongruence may be due to the use of more outgroup species (presently 4 and previously 2) and the use of protein data for phylogenetic analyses. It is worth to point out that in both studies, not all tree-building methods produced identical results and some support for the placement of Pachyhynobius is not conclusive (e.g., Bayesian posterior probabilities <0.95). These results imply that the split between Pachyhynobius and the Ranodon–Paradiclytodon clade may take place within a small window of time, the mitochondrial genome alone is unable to resolve this tritomy, and more data (especially nuclear genes) are needed.

Zhang et al. (2006) suggested that the relationships among living hynobiids have been shaped primarily by geography, and that West China is the secondary diversification center for mountain-type hynobiids. Our analyses support this hypothesis because the two new hynobid genera (Pseudohynobius and Protohynobius), which are distributed in West China, are the closest relatives to sympatric Liua and Batrachuperus (node f, Fig. 3). Furthermore, Zhang et al. (2006) also suggested that the split between Batrachuperus and other mountain-type hynobiids distributed in West China outside the Tibetan Plateau (e.g., Liua, Pseudohynobius), may have been the outcome of active orogenic movements on the eastern edge of the Tibetan Plateau. However, their study lacked two essential hynobid taxa, Pseudohynobius and Protohynobius, to support their hypothesis. Only when all mountain-type hynobiids distributed in West China outside the Tibetan Plateau (Liua, Pseudohynobius, or even Protohynobius) form a monophyletic group, the hypothesis seems rational. In this study, the sister–taxon relationship between the Pseudohynobius–Protohynobius clade and Liua (node g, Fig. 3) is favored by all analyses although the support for this node is not strong. If this grouping is correct, it will indirectly support Zhang et al.’s hypothesis.

If we accept Zhang et al.’s hypothesis, we could speculate that non-Batrachuperus mountain-type hynobiids originated in the mountain areas east to the Sichuan Basin, after the eastern edge of the Tibetan Plateau elevated quickly. Considering the current distribution between Liua and Pseudohynobius is apparently separated by the Yangtze River (Fig. 1), it is rational that the forming of the Yangtze River divided the mountain-type hynobid ancestors into two groups: the north group gave rise to Liua and the south group gave rise to Pseudohynobius. Remarkably, Pseudohynobius puxiongensis is the only five-toed hynobid salamander distributed in the Hengduan Mountains of the Tibetan Plateau. Taking into account that Pseudohynobius most likely originated outside the Tibetan Plateau, we infer that Ps. puxiongensis is a young species rather than a primitive one as been thought before: it reached its current distribution area by dispersal rather than relic restriction.

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Appendix A. Supplementary data


References


