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Cryptic species and systematics of the hynobiid salamanders of the *Liua–Pseudohynobius* complex: Molecular and phylogenetic perspectives

Xiaomao Zeng [a,b], Jinzhong Fu [b,*], Liqiao Chen [c], Yingzhou Tian [d], Xiaohong Chen [e]

[a] *Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu 610041, Sichuan, China*
[b] *Department of Integrative Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada*
[c] *Department of Biology, East China Normal University, Shanghai 200062, China*
[d] *Department of Biology, Liupanshui Teachers' College, Shuicheng 553004, Guizhou, China*
[e] *College of Life Science, Henan Normal University, Xinxiang 453002, China*

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Abstract

Using mitochondrial DNA sequencing and allozyme electrophoresis, we examined 18 populations of the *Liua–Pseudohynobius* complex, endemic to China. Based on their phylogenetic affiliation and exhibited fixed allelic differences, the complex comprises at least six species, two of which are previously unknown cryptic species. The complex is clearly divided into two groups, genus *Liua* including *Liua shihi* and *Liua tsinpaensis*, and genus *Pseudohynobius* including *Pseudohynobius flavomaculatus*, *Pseudohynobius shuichengensis* and the two new species. The previously often used genus name *Ranodon* is inappropriate, because the type species of the genus, *Ranodon sibricus*, is distantly related to this complex. The species diversity among Chinese hynobiid salamanders are far from being recognized and further effort should be directed at extensive field collection in central and western China.

Keywords: DNA sequencing; Allozyme; Cryptic species; *Liua*; *Pseudohynobius*; *Ranodon*; Hynobiid salamanders; China

1. Introduction

Discovering and describing species diversity is a fundamental part of evolution and biodiversity studies, and even among the best studied groups, such as the vertebrates, this task is far from complete. This is certainly the case for hynobiid salamanders in the *Liua–Pseudohynobius* complex of central and western China. The complex includes four described species, *Liua shihi*, *Ranodon tsinpaensis*, *Pseudohynobius flavomaculatus* and *Pseudohynobius shuichengensis* (Zhao and Adler, 1993; Tian et al., 1998; Fei, 1999), and despite the small number of species, its taxonomy is still quite controversial. The validity of *P. flavomaculatus* has been in dispute, as were the generic placements of

* Corresponding author. Fax: +1 519 767 1656.
E-mail address: jfu@uoguelph.ca (J. Fu).

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other species in the complex. For example, Zhao and Adler (1993) considered *P. flavomaculatus* a synonym of *R. tsinpaensis*, and placed *shihi* in the monotypic genus *Liua*. On the other hand, Fei (1999) considered *P. flavomaculatus* to be valid, and placed *flavomaculatus* and *tsinpaensis* in the genus *Pseudohynobius*, and *shihi* in the genus *Ranodon*. Recently, Kuzmin and Thiesmeier (2001) and Frost (2004) synonymized *Liua* and *Pseudohynobius* with *Ranodon*, and placed all three species in the genus *Ranodon*. While *P. shuichengensis* was published in 1998, no other major works ever listed it as a valid species. A fifth species, *Ranodon sibricus*, was often placed in this complex in one way or another (Zhao and Adler, 1993; Fei, 1999). Recent molecular works have begun to clarify these confusions. Li et al. (2004) confirmed the validity of *P. flavomaculatus* using DNA sequencing data, and concluded that *P. flavomaculatus*, *L. shihi* and *R. tsinpaensis* are more closely related to the genus *Batrachuperus* than to *R. sibricus*, the type species of the genus *Ranodon*. These results were confirmed by Weisrock et al. (1999).

Several factors have contributed to our poor understanding of this complex. First, we know very little about the general biology of this group due to their secretive lifestyle. These salamanders are highly terrestrial (except *L. shihi*) and uncommonly sighted, only appearing near bodies of water during the breeding season. Consequently, these species are known from only a few specimens from a limited number of localities. Second, much of the taxonomic discussions are based on only a few morphological characters from a small number of museum specimens (e.g. Zhao and Wu, 1995), so the full extent of character variation is unknown. In addition, the conserved body plan of this group provides few variable morphological characters, which consequently have limited power in delineating species.

In the midst of these taxonomic difficulties, a more fundamental question has largely been ignored – how many species are there? The recent discovery of two new hynobiid species in central China (Chen et al., 2001; Sheng et al., 2004) suggests that more collecting, particularly outside of the conventional collecting season (spring and summer), will reveal more hynobiid species. Many hynobiids are winter breeders, and the mountain ranges in central and western China have rarely been surveyed in winter for amphibians. Furthermore, salamanders of the *Liua–Pseudohynobius* complex have never been exposed to comprehensive molecular investigation. Molecular data have repeatedly revealed a surprisingly high amount of genetic divergence in morphologically conservative amphibian species that might otherwise be indistinguishable. These include hynobiid salamanders such as *Hynobius* and *Batrachuperus*, where allozyme and mitochondrial DNA sequence data have revealed several cryptic species (Matsui, 1987; Matsui et al., 2000).

The objective of this study is to reveal cryptic species and clarify the taxonomy of the *Liua–Pseudohynobius* complex. We sampled a large range of the distribution area, including all known locations of *Liua–Pseudohynobius* complex as well as several other mountain ranges with no previous records. Furthermore, we employed molecular techniques, including allozyme electrophoresis and mitochondrial DNA sequencing, and a phylogenetic approach to delineate species boundaries and to determine the taxonomic grouping of the species.

2. Materials and methods

2.1. Sample collection

A total of 76 salamanders of the *Liua–Pseudohynobius* complex from 18 locations were collected during 1999 to 2002 (Appendix I, Fig. 1). For adult or subadult specimens, liver and skeletal muscle tissues were removed and immediately frozen in liquid nitrogen. The frozen tissues were stored at −80 °C until DNA extraction or protein electrophoresis. Larval specimens were preserved in 95% ethanol and stored at −20 °C until DNA extraction. Voucher specimens are deposited in Chengdu Institute of Biology (Chengdu) and Museum of Vertebrate Zoology, University of California (Berkeley).

For phylogenetic analysis, *Pachyhynobius shangchengensis*, *Hynobius leechii*, *Hynobius amjiensis* and *Batrachuperus pinchonii* were used as outgroups. *R. sibricus* was also included in the analysis to test the relationships among *Ranodon*, *Batrachuperus* and the *Liua–Pseudohynobius* complex.

2.2. DNA sequencing and phylogenetic analysis

Two specimens from most populations were sequenced. Four specimens from populations 15 and 17 were sequenced and only one specimen from locations 4, 14, and 16 was available. Genomic DNA was isolated from liver or muscle

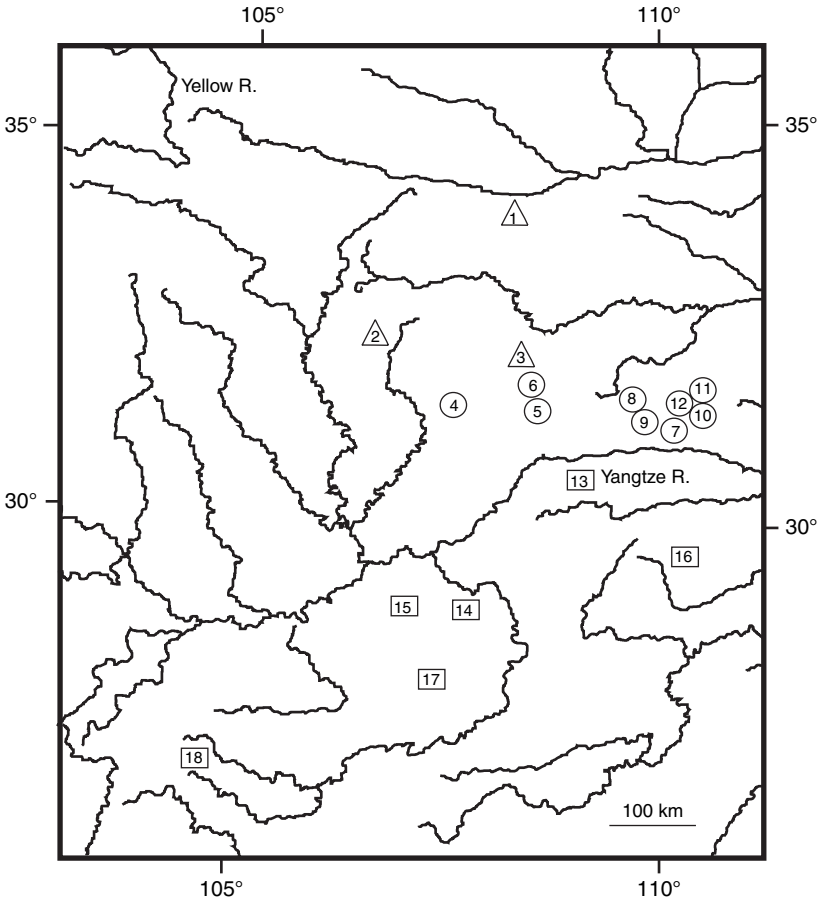

Fig. 1. Map of central and western China with sample locations. Circles are *Liua shihi*, triangles are *L. tsinpaensis*, and squares are *Pseudohynobius*.

tissues using the Wizard genomic DNA isolation protocol (Promega). A fragment of the cytochrome *b* gene from the mitochondrial genome was chosen for sequencing. Polymerase chain reactions (PCR) and sequencing were conducted with primers MVZ15 and MVZ16 (Moritz et al., 1992). PCR products were purified with Qiaquick protocols (Qiagen), and the DNA sequencing was performed using an ABI 377 with BigDye terminator sequencing chemistry (Perkin Elmer).

Sequences were edited and aligned using Sequencher (version 3.1.1). Maximum parsimony (MP) and Bayesian inference (BI) were employed to generate a phylogenetic hypothesis. Tree search and calculation were performed using PAUP (version 4.0b10; Swofford, 2002) and MrBayes (version 3.1; Ronquist and Huelsenbeck, 2003). Data set editing was accomplished using MacClade (version 4.01; Maddison and Maddison, 2000).

For the parsimony analysis, each nucleotide site was treated as a character and all characters were treated equally and unordered. A heuristic search via TBR branch swapping with 1000 random step addition replicates was conducted. Confidence limits were estimated by the bootstrap analysis (Felsenstein, 1985) with 1000 replicates. For the Bayesian analysis, the GTR + I + G model was used, as chosen by Modeltest (version 3.06; Posada and Crandall, 1998). Four Markov chains were used and the data set was run for four million generations to allow for adequate time of convergence. The default ''prior'' settings were used. Trees were sampled every 100 generations and we designated the first 30,000 sample trees as ''burn in'' and used the last 10,000 sample trees to estimate the consensus tree and the Bayesian posterior probabilities. Two separate runs, which include a total of four independent tree searches, were conducted and the resulting trees were compared and pooled. The pairwise differences (uncorrected *p* distance) were used to represent the magnitude of genetic divergence.

2.3. Allozyme electrophoresis and analysis

Horizontal starch gel (11%) electrophoresis was used to separate the allozymes. Homogenates of a combination of liver and muscle tissues were used. All protocols, and enzyme and allelic nomenclature follow Murphy et al. (1996). The buffer systems used are as in Fu et al. (2003).

Data were analysed with GDA (version 1.1; Lewis and Zaykin, 2001). Genetic divergence was evaluated using the percentage of polymorphic loci (P), the mean number of alleles per locus (A), and the mean heterozygosity by direct count (H). Population substructure was tested using Wright's hierarchical F-statistics. Nei's distance was also employed to represent genetic differences between populations.

3. Results

3.1. DNA sequences and gene tree

A total of 35 individuals from 19 populations, including one outgroup member, were sequenced. Seven sequences, including four outgroup and three ingroup members, were obtained from GenBank (Fu et al., 2001, 2003; Zhang et al., 2003). Together, a total of 32 haplotypes were detected and analysed. The sequences had a total length of 755 after alignment. No insertions or deletions were found. All sequences could be translated into amino acids with vertebrate genetic code, suggesting these sequences represent genuine cytochrome *b* gene. All sequences are deposited in GenBank (accession numbers DQ335713–DQ335747).

The data set produced 321 variable sites and 280 parsimony-informative characters. For the ingroup members, there were 265 variable sites and 233 parsimony-informative characters. The 50% majority consensus tree from the Bayesian analysis is presented in Fig. 2. The maximum likelihood value stabilized after 30,000 generations and the overall best log value was −4472.82. Maximum parsimony (MP) analysis resulted in 16 equally most parsimonious trees, with 764 steps, a CI of 0.5294, and an RI of 0.8112. The strict consensus tree was nearly identical to and completely compatible with the Bayesian tree, with only a few differences between the tip nodes in the *tsinpaensis* clade and the *shihi* clade. For example, the Huaeshan population formed a polytomy with the Houzhenzi and Zhongba populations at the base of the *tsinpaensis* clade on the MP tree while it was the sistergroup of the Houzhenzi population on the Bayesian tree. The bootstrap proportions (BSPs) from the parsimony analysis and the Bayesian posterior probabilities (BPPs) were mapped on the tree (Fig. 2). There were several prominent features of this phylogenetic hypothesis. First, all ingroup members were grouped into two well-supported major clades, the *Pseudohynobius* group and the *Liua* group (BSP = 100, BPP = 100; Fig. 2). The latter further divided into two well-supported clades, the *tsinpaensis* clade and the *shihi* clade. Second, most haplotypes from the same population were grouped together, with three exceptions in the *shihi* clade, although haplotypes from same population were always grouped in the same major clade. Third, the *Pseudohynobius* group showed relatively long branch lengths, while the *shihi* clade and the *tsinpaensis* clade had short branch lengths.

Pairwise differences (uncorrected *p* distances) among the populations are presented in Table 1. All populations of *L. shihi* were lumped together because of low intraspecific genetic variation. The pairwise differences within the major clades varied dramatically: the *shihi* clade had a maximum of 1.6% difference, while the *Pseudohynobius* group had up to 15.4% difference (Kuankuoshui vs. Baimashan). The genetic divergences between these major lineages were high, ranging from 10.8% to 21.1%.

3.2. Allozymes data and the F-statistics

Sixty specimens from 10 populations were examined for allozyme variation. Within the 24 resolved loci, 12 were monomorphic: sAAT-A, sACOH-A, CBP-1, EST-D, βGLUR-A, GTDHP-A, G6PDH-A, sIDH-A, LDH-A, LDH-B, αMAN-A, and mMDH-A. The genotypic frequencies of polymorphic loci are listed in Table 2.

All loci conformed to Hardy–Weinberg predictions without significant deviation. Wright's F_{st} was calculated for *L. shihi* and *Liua tsinpaensis* (=*R. tsinpaensis*, see Section 4). An F_{st} of 0.054 suggested little genetic differentiation among populations of *L. shihi*, which were near panmictic (Table 3). Conversely, an F_{st} of 0.885 indicated great genetic differentiation among populations of *L. tsinpaensis*. Pairwise Nei's genetic distances revealed the same patterns (Table 4).

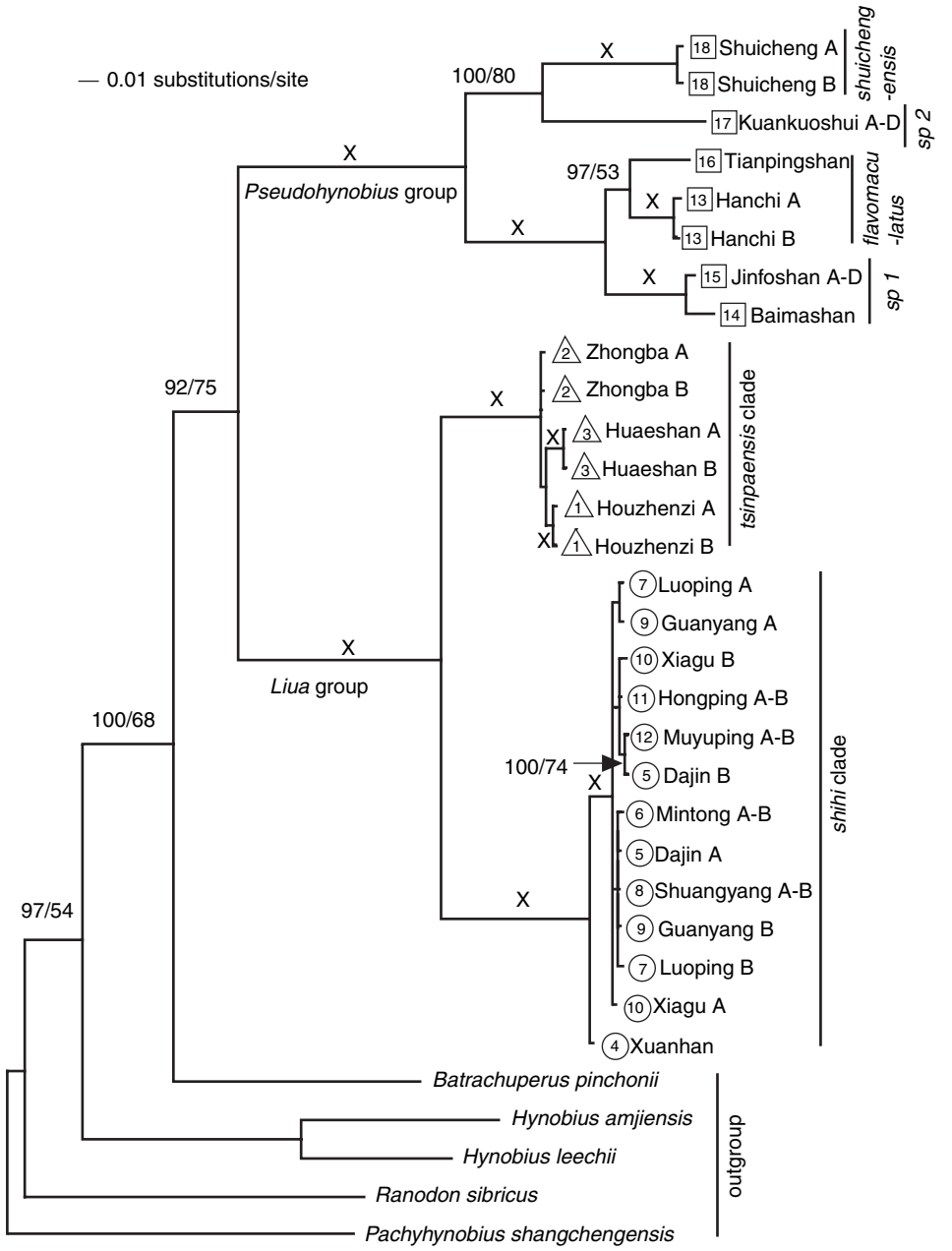

Fig. 2. The phylogenetic hypothesis derived from the Bayesian analysis of the cytochrome *b* sequence data. Numbers above branches are Bayesian posterior probabilities (BPPs)/bootstrap proportions (BSPs), and only nodes with greater than 95 BPPs are mapped on the tree. A "X" represents a 100 BPP and a greater than 95 BSP. The taxon names are location names. Numbers in front of location names are location numbers in Fig. 1.

Most remarkably, several clades and populations revealed fixed or nearly fixed allelic differences from other populations. The *Pseudohynobius* group had fixed allelic difference at loci PK-A(*c*) and sMDHP-A(*c*), and the *tsinpaensis* clade had nearly fixed allelic difference at loci ADH-A(*a*) and sMDH-A(*a*). Surprisingly, the *shihi* clade did not display any fixed differences, although morphologically it is very distinctive from all other species in the complex and is the only species in the group that lives in an aquatic environment through the entire year. Within the major clades, the Jinfoshan population showed fixed allelic differences at locus PGDH-A (*b*), and the Hanchi population had fixed allelic differences at loci sCAT-A(*b*) and mCAT-A(*b*). The Houzhenzi population had fixed allelic difference at locus sMDHP-A(*a*), while the Huaeshan population was fixed at locus mCAT-A(*f*).

Table 1
The average pairwise differences of the cytochrome *b* sequences among lineages of the *Liua–Pseudohynobius* complex and other hynobiid salamanders

	1	2	3	4	5	6	7	8	9	10	11	12	13
1 Shiusheng (18)	**0.5**												
2 Kuankuoshui (17)	11.4	**0.0**											
3 Tianpingshan (16)	14.6	14.7	–										
4 Hanchi (13)	13.6	14.2	4.7	**0.4**									
5 Jinfoshan (15)/Baimashan (14)	14.5	15.0	7.1	7.5	**1.9**								
6 Zhongba (2)	18.4	20.0	19.3	18.2	19.4	**0.1**							
7 Huaeshan (3)	18.5	19.3	18.7	17.4	19.4	1.3	**0.1**						
8 Houzhenzi (1)	18.7	20.3	19.5	18.3	19.8	0.8	1.7	**0.1**					
9 *shihi* (4–12)	19.1	20.7	21.1	20.2	21.1	10.8	11.3	11.4	**1.6**				
10 *Batrachuperus*	18.9	18.1	18.1	17.0	18.4	18.1	18.2	18.6	17.8	–			
11 *Hynobius*	20.8	20.2	21.0	20.2	21.4	19.1	19.6	19.4	20.7	18.1	**12.8**		
12 *Ranodon sibricus*	19.6	19.8	19.1	18.7	20.2	19.1	19.9	19.3	19.5	17.6	19.6	–	
13 *Pachyhynobius*	20.7	21.3	21.9	20.9	22.6	20.1	21.0	20.3	19.9	17.7	18.3	17.9	–

Numbers on diagonal are the maximum difference within each lineage.

4. Discussion

4.1. Define genus: Liua, Pseudohynobius or Ranodon?

As shown in previous molecular studies (e.g. Li et al., 2004), none of the species in the *Liua–Pseudohynobius* complex were found to be closely related to *R. sibricus*, the type species of the genus *Ranodon* (Fig. 2). Therefore, *Ranodon* is an inappropriate genus name for the complex. *Liua* and *Pseudohynobius* are available genus names, which have been previously used for these species. We recommend using *Liua* to encompass species *shihi* and *tsinpaensis*, and *Pseudohynobius* to encompass *flavomaculatus*, *shuichengensis* and the two undescribed species (Fig. 2). Despite the small number of species in this complex, we would like to keep them divided between two genera for two reasons. First, both names have been in use for more than two decades and should be maintained to provide stability. Second, the two genera may not form a monophyletic group, since some of the Bayesian trees suggest that *Liua* may be more closely related to *Batrachuperus* than *Pseudohynobius*. As in previous studies (Weisrock et al., 1999; Li et al., 2004), *Liua* and *Pseudohynobius* were found to be most closely related to the genus *Batrachuperus* based on our parsimony analysis. In the Bayesian analysis, however, while 92% of the trees placed the genera *Liua* and *Pseudohynobius* as sistergroups, 8% of the trees placed *Batrachuperus* in the ingroup as the sistergroup to *Liua* (Fig. 2). Several morphological studies also suggested the close association between *L. shihi* and *Batrachuperus* (Fei and Ye, 1984; Zhao and Hu, 1984). Our data have limited power of resolving the relationships among the genera; other ongoing projects (e.g. the AmphibiaTree Project) will certainly shed more light on this issue.

Cytological evidence also supports the two-genus classification. *L. shihi* from Guanyan (9) and *L. tsinpaensis* from Houzhenzi (1) have an almost identical karyotype of $2n = 66$, including 8 large biarmed, 2 medium biarmed, and 23 medium and small acrocentric chromosome pairs (Ikebe, 1993; Ikebe et al., 2000). On the other hand, *P. flavomaculatus* from Hanchi (13) has a karyotype of $2n = 52$ with 7 large biarmed, 6 medium biarmed, and 13 small acrocentric chromosome pairs, while *P. shuichengensis* from Shuicheng (18) has less one small acrocentric chromosome pair (Tian et al., 1998; Ikebe et al., 2000). Geographically, the genera are separated by the Yangtze River, with *Liua* found to the north and *Pseudohynobius* found to the south (Fig. 1). Although amphibians cannot survive without water, a major river such the Yangtze can form an effective physical barrier to gene flow and eventually lead to speciation.

4.2. Species delineation in the genus Pseudohynobius

Pseudohynobius exhibited great genetic diversity. Within the genus, cytochrome *b* showed pairwise difference up to 15.39% (Table 1), a relatively high value compared to other salamanders (e.g. 4% in *Hynobius yiwuensis*, Fu et al., 2003; 12.1% in *Ensatina*, Moritz et al., 1992; 14.7% in bolitoglossine salamanders, Jackman et al., 1997). All populations in the genus, except that from Shuicheng, were previously identified as *P. flavomaculatus*. We have found,

Table 2
Genotypic frequencies of the polymorphic loci in the *Liua–Pseudohynobius* complex

Locus	*Liua shihi*					*L. tsinpaensis*			*P. flav.*	*P.* sp. 1
	Luoping ($n=13$)	Dajin ($n=6$)	Hongping ($n=3$)	Muyuping ($n=6$)	Xiagu ($n=15$)	Houzhenzi ($n=5$)	Zhongba ($n=4$)	Huaeshan ($n=3$)	Hanchi ($n=2$)	Jinfoshan ($n=3$)
ADH-A	bb	bb	bb	bb	bb	aa	aa	aa(2) ab(1)	bb	bb
EST-1	bb(1) cc(4) bc(8)	bb(1) cc(4) bc(1)	cc	bb(3) bc(2) ?(1)	bb(6) cc(4) bc(5)	aa	aa	bb(2) cc(1)	cc	cc(1) bc(1) ?(1)
sMDH-A	bb	bb	bb	bb	bb	aa	aa	aa	ab(1) bb(1)	bb
PEP-A	ab(1) bb(12)	bb	bb	bb	bb	bb	bb	bb	bb	aa(2) ab(1)
PGM-A	aa(12) ab(1)	aa(5) ab(1)	aa	aa	aa(14) ab(1)	aa(3) ?(2)	bb	bb	cc	bb(1) cc(2)
PGDH-A	aa(1) cc(12)	cc	cc(2) cd(1)	cc(5) dd(1)	cc(14) cd(1)	cc	cc	cc	cc	bb
PK-A	bb	bb	bb	aa(1) bb(5)	bb	bb(1) ?(4)	bb	bb	cc	cc(1) ?(2)
sCAT-A	aa(1) cc(12)	cc	cc	cc	cc	cc	cc	cc	bb	cc
mCAT-A	aa(1) cc(4) dd(4) cd(4)	cc(2) dd(2) cd(2)	dd	cd(1) dd(5)	cc(2) dd(12) ee(1)	dd	dd	ff	bb	dd
sMDHP-A	bb	bb	bb(2) bd(1)	bb(5) bd(1)	bb(13) bd(2)	aa	bb	bb	cc	cc
mMDHP-A	bb	bb	bb	bb	bb	bb	bb	bb	aa	aa(1) bb(2)
GPI-A	aa	aa	aa	aa	aa	aa	aa(3) bb(1)	aa	aa	aa
P	28.0	12.0	8.0	24.0	20.0	0.0	4.0	8.3	4.0	17.39
A	1.24	1.12	1.08	1.24	1.24	1.00	1.04	1.08	1.04	1.17
H	0.046	0.027	0.027	0.029	0.024	0.000	0.000	0.014	0.020	0.036

P = percentage of polymorphic loci (0.95 criterion); A = mean number of alleles per locus; H = mean heterozygosity. *P. flav.* = *Pseudohynobius flavomaculatus*.

however, that there are four species within the genus, which clearly correspond to *P. flavomaculatus*, *P. shuichengensis*, and two previously unrecognized species.

The Jinfoshan population, together with Baimashan population, constitute a separate species, most closely related to *P. flavomaculatus* (Fig. 2). The Jinfoshan population has fixed allelic differences from its sistergroup, the Hanchi population (=*P. flavomaculatus*), at loci PGDH-A, sCAT-A, and mCAT-A (Table 2), suggesting established

Table 3
F_{st} for *Liua shihi* and *L. tsinpaensis*

	shihi	*tsinpaensis*
Overall	0.054	0.885
Upper bound	0.127	1.000
Lower bound	−0.019	0.0769

The upper and lower bound were calculated from 10,000 replicates of bootstrapping over loci at 95% confidence interval.

Table 4
Nei's genetic distance for all populations of *Liua–Pseudohynobius* complex

Populations	1	2	3	4	5	6	7	8	9	10
1 Dajin	–									
2 Luoping	0.057	–								
3 Xiagu	0.098	0.068	–							
4 Hongping	0.165	0.152	0.173	–						
5 Muyuping	0.213	0.150	0.015	0.246	–					
6 Houzhenzi	0.929	0.825	0.863	0.961	0.821	–				
7 Zhongba	0.882	0.778	0.821	0.896	0.798	0.825	–			
8 Huaeshan	0.792	0.734	0.794	0.857	0.742	0.932	0.839	–		
9 Hanchi	0.916	0.861	0.900	0.941	0.862	0.991	0.959	0.925	–	
10 Jinfoshan	0.866	0.831	0.867	0.856	0.786	0.925	0.898	0.867	0.792	–

reproductive isolation. Therefore, the population should be recognized as a valid species (the biological species concept; Mayr, 1942). The high level DNA sequence divergence between the two sister species (7.5%) lends further support to this conclusion. We, therefore, tentatively refer to this taxon as *P*. sp. 1. The placement of the Baimashan population in this taxon was based on the phylogenetic relationships since we do not have allozyme data for the population. Similarly, we tentatively place the Tianpingshan population in *P. flavomaculatus* based on its phylogenetic affiliation and lack of allozyme data (Fig. 2), although it displayed a high degree of divergence from the Hanchi population (4.7%).

The Kuankuoshui population morphologically resembles *P. flavomaculatus* (Wu et al., 1986), but phylogenetically it is more closely related to *P. shuichengensis*. Tian and Gu (2001) compared LDH between the Kuankuoshui and Shuicheng populations, and a re-interpretation of the results suggests that there was fixed allelic difference at the locus LDH-A. The Kuankuoshui population has 11.4% sequence divergence with *P. shuichengensis*, and a 14.2% divergence with the Hanchi population of *P. flavomaculatus* (Table 1). Considering the phylogenetic relationships, the morphological differences, and the magnitude of genetic divergence, we suggest that this is also a separate species, tentatively named here as *P*. sp. 2.

Geographically, the four species occupy different mountain ranges (Fig. 1). *P. shuichengensis* occurs to the west in the Wumeng Mts. (18), and both *P*. sp. 1 and *P*. sp. 2 occur in the Dalou Mt. area (14, 15 and 17). *P. flavomaculatus* occurs in the Wuling Mt. region to the east (13 and 16).

4.3. Species delineation in the genus Liua

The genus is clearly divided into only two species, *L. shihi* and *L. tsinpaensis*, which are morphologically and ecologically distinct. *L. shihi* is an aquatic species and lives in water through the entire year while *L. tsinpaensis* is only found in water during the breeding season. Several characters associated with aquatic lifestyle are present in *L. shihi*, such as labial fold and laterally compressed tail. Such characters are absent in *L. tsinpaensis*.

L. shihi is a genetically cohesive group maintaining a low degree of intraspecific divergence. Within species pairwise differences of cytochrome *b* gene were less than 1.6%, and the Nei's distances among its populations were small, ranging from 0.057 to 0.246. Furthermore, a large amount of gene flow occurs between the populations, as indicated by the *F*-statistics ($F_{st} = 0.054$) and the separated clade locations of haplotypes from the same populations. The species is genetically distinct, exhibiting substantial differences from its phylogenetic sistergroup (e.g. 10.8–11.4% from *L. tsinpaensis*) and its geographically neighbouring populations [e.g. 11.3% from Huaeshan (3)]. *L. shihi* occurs in Daba Mts., along both the south and north sides.

The status of *L. tsinpaensis* is more complex than that of *L. shihi*. While DNA sequence data suggest a genetically cohesive unit with an average intraspecific divergence of 1.7%, almost the same as that for *L. shihi*, there were several fixed allelic differences among the three populations, suggesting a lack of gene flow between the three populations. The Huaeshan population, for example, has a fixed allele (*f*) at locus mCAT-A (Table 2). Correspondingly, the Nei's distances among the three populations were high (0.825–0.932; Table 4). Geographically, the three populations are located in three different mountain ranges. Houzhenzi (1) is located at the northern slope of the Tsinling Mt. and is part of the Yellow river drainage. Zhongba (2) is located at south side of Micangshan and Huaeshan (3) is located at south

side of Daba Mts.; both are part of the Yangtze River drainage. Considering this evidence, we suggest that the three populations be considered as species candidates, pending further investigation. Our hesitation rested mainly on the small sample sizes.

4.4. Taxonomic recommendation and further research

We recommend separating the *Liua–Pseudohynobus* complex into two genera, *Liua* which includes *L. shihi* and *L. tsinpaensis*, and *Pseudohynobius* which includes *P. shuichengensis*, *P. flavomaculatus* and the two undescribed species. We did not formally describe these two new species due to the lack of adult specimens. Currently, we are working on collecting more specimens, particularly adult specimens. The diversity of the hynobiid salamanders in China is far from being fully recognized, which is particularly true for the terrestrial species, such as *Pseudohynobius* and *Hynobius*. Further effort should be directed to field collection in central and western China.

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Appendix I. Specimens examined

Numbers in parentheses are location numbers in Fig. 1. MVZ = Museum of Vertebrate Zoology, University of California (Berkeley), CIB = Chengdu Institute of Biology (Chengdu). Specimens with DNA sequencing data are underlined; specimens with allozyme data are in italics. Sequence data of MVZ231141, MVZ231150, and MVZ231151 are obtained from GenBank (AY028775, AY028773, and AY028774, respectively).

Liua shihi, n = 50

MVZ231141, *MVZ231142*, *MVZ231143*, *MVZ231144*, *MVZ231145*, *CIB-XM1218*, *CIB-XM1219*, *CIB-XM1220*, *CIB-XM1221*, *CIB-XM1222*, *CIB-XM1223*, *CIB-XM1224*, *CIB-XM1225*, Luoping (7), Wushan Co., Chongqing Municipality, 31.17 °N 110.14 °E, elevation 1250 m.

CIB-XM1155, CIB-XM1156, Guanyang (9), Wushan Co., Chongqing Municipality, 31.33 °N 109.91 °E, elevation 1450 m.

CIB-XM1152, CIB-XM1153, Mintong (6), Chengkou Co., Chongqing Municipality, 31.78 °N 108.58 °E, elevation 1900 m.

CIB-XM1226, *CIB-XM1227*, *CIB-XM1228*, *CIB-XM1229*, *CIB-XM1230*, *CIB-XM1231*, Dajin (5), Kaixian Co., Chongqing Municipality, 31.52 °N 108.45 °E, elevation 1400 m.

CIB-XM1161, CIB-XM1162, Shuangyang (8), Wuxi Co., Chongqing Municipality, 31.48 °N 109.80 °E, elevation 1200 m.

CIB-XM285, Xuanhan Co. (4), Sichuan Province, 31.5 °N 107.6 °E.

CIB-zyc1008, *CIB-zyc1009*, *CIB-zyc1011*, Hongping (11), Shennongjia Nature Reserve, Hubei Province, 31.41 °N 110.25 °E, elevation 1700 m.

CIB-zyc1031, *CIB-zyc1032*, *CIB-zyc1033*, *CIB-zyc1034*, *CIB-zyc1035*, *CIB-zyc1038*, Muyuping (12), Shennongjia Nature Reserve, Hubei Province, 31.43 °N 110.28 °E, elevation 1740 m.

CIB-XM858, *CIB-XM859*, *CIB-XM860*, *CIB-XM861*, *CIB-XM865*, *CIB-XM866*, *CIB-XM867*, *CIB-XM868*, *CIB-XM870*, *CIB-XM872*, *CIB-XM896*, *CIB-XM897*, *CIB-XM898*, *CIB-XM899*, *CIB-zyc1047*, Xiagu (10), Shennongjia Nature Reserve, Hubei Province, 31.43 °N 110.27 °E, elevation 2000 m.

Ranodon tsinpaensis, n = 12

MVZ231153, *MVZ231154*, *MVZ231155*, *MVZ231156*, *MVZ231157*, Houzhenzi (1), Zhouzhi Co., Shaanxi Province, 33.85°N 107.72°E, elevation 1850 m.

CIB-XM825, *CIB-XM826*, *CIB-XM839*, *CIB-XM840*, Zhongba (2), Nanjiang Co., Sichuan Province, 32.34°N 106.42°E, elevation 1780 m.

CIB-zyc914, *CIB-zyc915*, *CIB-zyc920*, Huaeshan (3), Wanyuan Co., Sichuan Province, 32.06°N 108.08°E, elevation 1790 m.

Pseudohynobius flavomaculatus, n = 3

MVZ231150, *MVZ231151*, Hanchi (13), Lichuan Co., Hubei Province, 30.31°N 109.05°E, elevation 1900 m.

CIB-200087, Tianpingshan (16), Sangzhi Co., Hunan Province, elevation 1400 m.

Pseudohynobius shuichengensis, n = 2

CIB-XM1078, CIB-XM1079, Shuicheng (18), Guizhou Province, 26.34°N 104.51°E, elevation 1820 m.

Pseudohynobius sp. 1, n = 5

CIB-zyc825, *CIB-zyc826*, *CIB-zyc827*, *CIB-zyc828*, Jinfoshan (15), Nanchuan Co., Chongqing Municipality, 29.00°N 107.11°E, elevation 2100 m.

CIB-bms20010715020, Baimashan (14), Wulong Co., Chongqing Municipality, elevation 1500 m.

Pseudohynobius sp. 2, n = 4

CIB-XM1071, CIB-XM1072, CIB-XM1073, CIB-XM1074, Kuankuoshui (17), Suiyang Co., Guizhou Province, 28.12°N 107.10°E, elevation 1350 m.

Pachyhynobius shangchengensis, n = 1

CIB-N105N, Shangcheng, Henan Province.

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