Genetics and Molecular Research

Phylogenetic relationships of *Pseudohynobius* (Urodela, Hynobiidae) inferred from DNA barcoding analysis

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Genet. Mol. Res. 15 (2): gmr.15028155
Received August 8, 2015
Accepted November 26, 2015
Published July 15, 2016
DOI http://dx.doi.org/10.4238/gmr.15028155

ABSTRACT. As a proven tool, DNA barcoding can identify species rapidly and unambiguously. In this study, we used mtDNA cyt b, COI, and 16s rRNA sequences of six species of *Pseudohynobius*, *Protohynobius puxiongensis*, *Liua shihi*, *Ranodon sibiricus*, and *Pachyhynobius shangchengensis*, to reconstruct the phylogenetic relationships using Bayesian inference and maximum likelihood methods. Approximate lineage divergence times were also estimated, the divergence between them was calculated to have taken place mainly in Miocene. Our results showed that: 1) *Ps. guizhouensis* is an independent and valid species that is a sister species to *Ps. kuankuoshuiensis*; 2) five *Pseudohynobius* species formed a monophyletic group; 3) *Ps. tsinpaensis* is different from *L. shihi*, and should be classified as belonging to the *Liua* genus; and 4) *Pr. puxiongensis* is the sister lineage to all *Pseudohynobius*

species, and should therefore be named *Pseudohynobius puxiongensis*.

Key words: *Pseudohynobius*; *Protohynobius puxiongensis*; mtDNA COI; mtDNA cyt b; mtDNA 16s rRNA

INTRODUCTION

Molecular genetic approaches are widely used to study taxonomy, population genetics, and phylogenetic relationships of many organisms (Moritz and Hillis, 1996). The mitochondrion has some unique features, for example, maternal mode of inheritance, a high rate of evolution, and lack of recombination (Moritz et al., 1987). As a result, the mitochondrion is frequently used in comparative studies among closely related species and populations (Moritz et al., 1987). Molecular approaches, particularly those using mtDNA sequences, are often used for species delimitation (Knowlton, 2000). Although different genes in the mitochondrial genome have different rates of evolution, some more conserved gene regions within the genome have been used to study the deeper branches in amphibian evolution (Munasinghe et al., 2003). For DNA barcoding, mitochondrial cytochrome oxidase subunit I (COI), for example, has been shown to delimitate species rapidly and unambiguously. This region has also been used, for example, in genealogical reconstructions, forensics, and biodiversity surveys. Recently, a partial fragment of the mitochondrial COI gene has been used to barcode amphibian species in many studies (Che et al., 2012).

Barcoding of amphibians is essential in part because many species are now endangered (Che et al., 2012). Compared with other mitochondrial genes, COI is more frequently used in studies determining amphibian phylogenies or phylogeography. Therefore, other genes should be identified that could complement COI (Smith et al., 2008). In this study, we investigated the mtDNA cytochrome b (cyt b) and 16s rRNA genes as alternative molecular markers.

Pseudohynobius is an important group within the Hynobiidae. It was discovered and named by Fei and Ye (1982). Six species have been recorded in this genus so far; *Ps. flavomaculatus* (Fei and Ye, 1982), *Ps. guizhouensis* (Li et al., 2010), *Ps. jinfo* (Wei et al., 2009), *Ps. kuankuoshuiensis* (Xu et al., 2007), *Ps. shuichengensis* (Tian et al., 2006), and *Ps. tsinpaensis* (Hu et al., 1966). In addition to the above mentioned species, other species have been identified and named mainly based on morphological characters. cyt b was used by Li et al. (2010) to study five species of *Pseudohynobius* system development. Zeng et al. (2006) suggested that *Ps. tsinpaensis* should be called *Liua tsinpaensis*, based on a phylogenetic study of the *Liua-Pseudohynobius* complex using cyt b only.

Protohynobius puxiongensis, the only known five-toed hynobiid salamander, was discovered in the Hengduan mountain area (Liu, 1950; Zhao and Adler, 1993). It was described based on a single specimen and has never been found again, since its discovery in 1965. It was considered to belong to a new genus as well as a new subfamily. Because it had an internasal bone, a primitive character, the specimen was thought to represent a common ancestor of all other hynobiid salamanders. This conclusion bothered herpetologists for decades. In order to resolve the problem, based on living individuals of *Pr. puxiongensis* that were rediscovered at its type locality, Peng et al. (2010) reexamined the phylogenetic position of this species in relation to two *Pseudohynobius* species. They concluded that *Pr. puxiongensis* is, in fact, a sister group of the *Pseudohynobius* species. They also suggested that *Pr. puxiongensis*

should be reclassified as a *Pseudohynobius* species and named *Pseudohynobius puxiongensis* (Peng et al., 2010). However, in their study, they only compared *Pr. puxiongensis* with two *Pseudohynobius* species.

In order to further examine the validity of the *Pseudohynobius* species, we recreated the phylogeny of six *Pseudohynobius* species. In addition, our aim was to determine whether *Pr. puxiongensis* is indeed the sister group of all the known *Pseudohynobius* species or whether it was nested within the *Pseudohynobius*. This was done based on partial sequences of mtDNA COI, mtDNA cyt b, and 16s rRNA genes.

MATERIAL AND METHODS

Sequence data

We downloaded mtDNA cyt b, COI, and 16s rRNA sequences of *Pr. puxiongensis*, *L. shihi*, *Ranodon sibiricus*, *Pachyhynobius shangchengensis*, and the six *Pseudohynobius* species from GenBank (Table 1). *Pa. shangchengensis* and *R. sibiricus* were included as the outgroup.

Table 1. Accession numbers of all species included in this study that were downloaded from GenBank.

Species	Accession numbers		
	mtDNA cyt b	mtDNA COI	mtDNA 16s rRNA
Pseudohynobius guizhouensis	JX867277.1	JN165828.1	JN165944.1
Pseudohynobius kuankuoshuiensis	JX867278.1	JN165830.1	JN165946.1
Pseudohynobius shuichengensis	FJ532060.1	FJ532060.1	FJ532060.1
Pseudohynobius flavomaculatus	FJ532059.1	FJ532059.1	FJ532059.1
Pseudohynobius jinfo	DQ335723.1	JN165827.1	JN165943.1
Pseudohynobius tsinpaensis	DQ333813.1	DQ333813.1	DQ333813.1
Protohynobius puxiongensis	FJ532058.1	FJ532058.1	JN165950.1
Liua shihi	107991059: 14180-15321	107991059:5352 -6893	107991059:1074-2671
Ranodon sibiricus	21450026: 14182-15322	21450026:5355 -6905	21450026:1077-2676
Pachyhynobius shangchengensis	107991129: 14169-15310	107991129:5330-6880	107991129:1074-2652

The sequences were aligned with ClustalX v. 1.83 (Chenna et al., 2003). The aligned sequences were edited using BioEdit v. 7.0.9.0 (Hall, 1999). The total number of sites (excluding sites with gaps/missing data) was 1802, of which 583 bp were sequenced for the COI gene, 754 bp for the cyt b gene, and 465 bp for the 16s rRNA gene.

Phylogenetic analyses

The phylogenetic relationships among the species were estimated using maximum likelihood (ML) analyses in PHYML v. 3.0 (Guindon et al., 2010), as well as Bayesian ML analyses with 3,000,000 generations in MrBayes v. 3.2 (Ronquist et al., 2012). To find the best-fitting substitution model we used MODELTEST (Posada and Crandall, 1998) and MrModeltest v2 (Nylander, 2004). A GTR+I+G model was adopted in both the ML and Bayesian analyses. Branch support for the ML analyses was evaluated with a non-parametric bootstrap analysis (1000 replicates).

Divergence time estimate

The partial mtDNA sequences were used to estimate the approximate divergence

times of the species included in this study using BEAST v. 1.4.7 (Drummond and Rambaut, 2007). All sequences including the outgroups and a Bayesian Markov chain Monte Carlo approach with an uncorrelated log-normal relaxed molecular clock were used in the analysis. Two independent runs, each composed of 120 million generations, were performed. The results were visualized in Tracer V1.6 (Rambaut and Drummond, 2013), to test for stationarity. We used LogCombiner 1.4.7 (Drummond and Rambaut, 2007) to combine both runs, TreeAnnotator 1.4.7 (Drummond and Rambaut, 2007) to annotate tree information, and FigTree 1.4.2 (Rambaut, 2014) to visualize the final tree information.

Weisrock et al. (2001) proposed a molecular evolutionary rate of the mitochondrial genome of hynobiids (0.64% per myr per lineage). Therefore, 0.64% per Myr per lineage was used to estimate the divergence between the species.

RESULTS

Phylogenetic analyses

The tree topologies of the ML and Bayesian analyses were identical (Figures 1 and 2). With respect to the outgroup, the *Pseudohynobius* species formed a monophyletic group. *Ps. tsinpaensis* and *L. shihi* formed a separate branch. Based on these data, we were able to conclude that: 1) *Ps. guizhouensis* is an independent and valid species, which is a sister species to *Ps. kuankuoshuiensis*; 2) five species of *Pseudohynobius* formed a monophyletic group; 3) *Ps. tsinpaensis* is different from and a sister species to *L. shihi*; 4) *Pr. puxiongensis* is sister lineage to all the *Pseudohynobius* species.

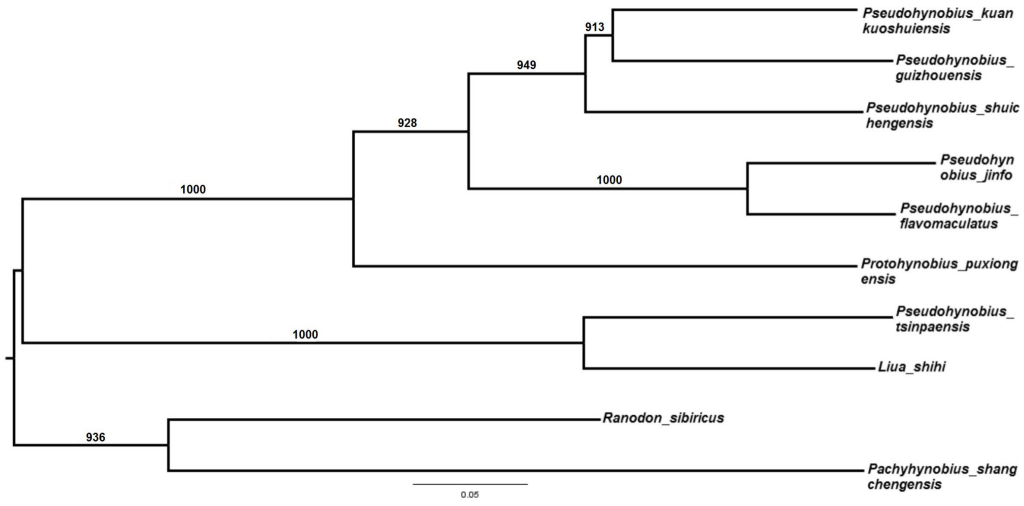

Figure 1. Maximum likelihood tree based on mtDNA sequences of six species of *Pseudohynobius*, *Protohynobius puxiongensis*, and *Liua shihi*. *Ranodon sibiricus* and *Pachyhynobius shangchengensis* are included as outgroup. Numbers above the branches represent the bootstrap values. The scale bar represents the number of substitutions per site.

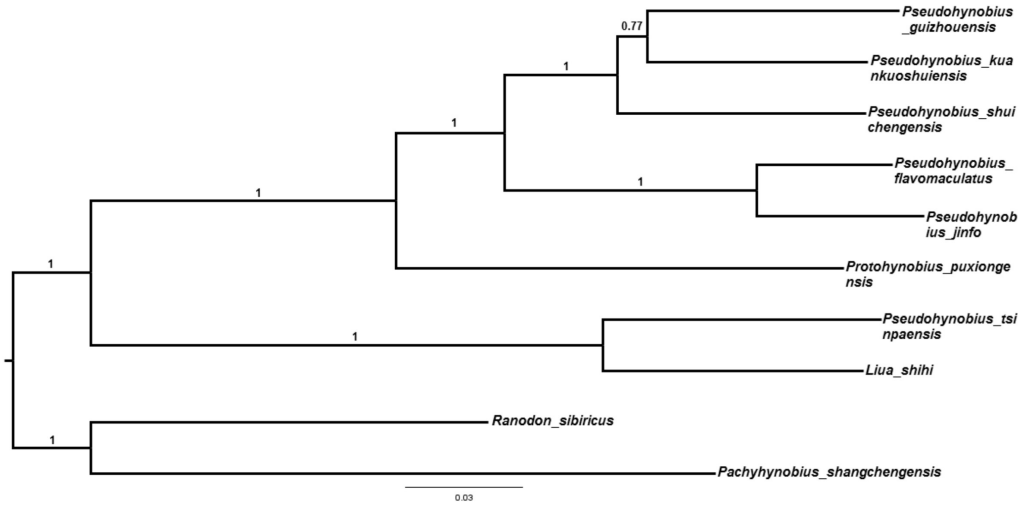

Figure 2. Bayesian tree based on mtDNA sequences of six species of *Pseudohynobius*, *Protohynobius puxiongensis*, and *Liua shihi* from China. The tree is rooted with sequences from *Ranodon sibiricus* and *Pachyhynobius shangchengensis*. Values above the branches indicate Bayesian posterior probabilities. The scale bar represents the number of substitutions per site.

Divergence time estimate

When in geological time the divergences happened, and the results of the BEAST analysis showed that the divergence times among *Ps. flavomaculatus*, *Ps. guizhouensis*, *Ps. jinfo*, *Ps. kuankuoshuiensis*, and *Ps. shuichengensis* all occurred at the end of Miocene (Figure 3).

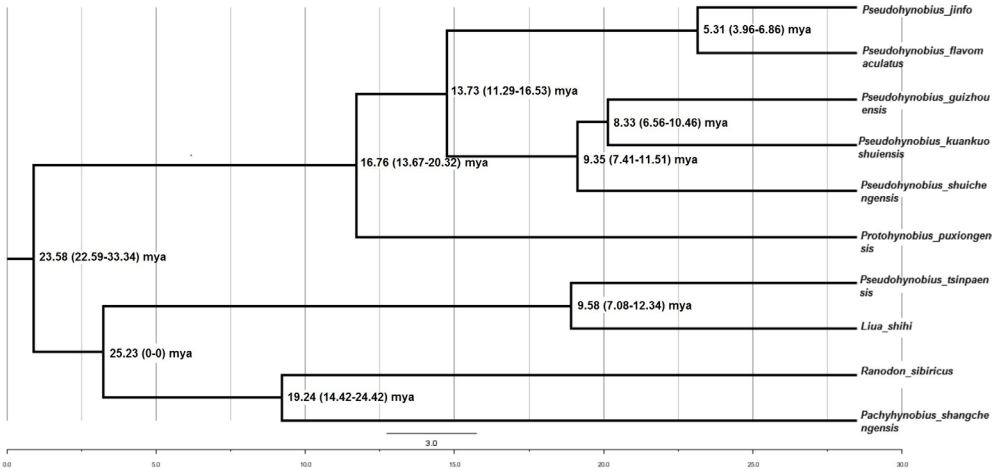

Figure 3. Phylogenetic neighbor joining tree based on mtDNA sequences of six species of *Pseudohynobius*, *Protohynobius puxiongensis*, and *Liua shihi* in China, rooted with two sequences from *Ranodon sibiricus* and *Pachyhynobius shangchengensis*. Numbers above the branches represent the bootstrap values and the values at the nodes are the exact divergence times with the 95% highest posterior density interval as estimated by BEAST. The scale bar represents the number of substitutions per site.

DISCUSSION

Validity of the *Pseudohynobius* species

The divergence times between each species pair was large, so we believe that these five *Pseudohynobius* species are valid. Based on the phylogenetic analyses, we found that *Ps. tsinpaensis* and *L. shihi* formed a branch separate from the other five *Pseudohynobius* species and *Pr. puxiongensis*. The divergence time between these species occurred at the end of Oligocene 25.23 mya (Figure 3). This indicates that *Ps. tsinpaensis* and *L. shihi* are closely related and that *Ps. tsinpaensis* should be classified into the *Liua* genus, as suggested previously (Zeng et al., 2006; Zhang et al., 2006). Hence, the *Liua* genus should contain two species: *L. shihi* and *L. tsinpaensis*.

Validity of *Protohynobius puxiongensis*

The phylogenetic analyses showed that *Pr. puxiongensis* and the five remaining *Pseudohynobius* species form a single branch, indicating a close relationship between them (Figures 1 and 2). The divergence time between *Pr. puxiongensis* and the five *Pseudohynobius* species was calculated to have taken place in the middle of Miocene and Pliocene, at around 16.76 mya (Figure 3). Hence, it can be concluded that *Pr. puxiongensis* is a sister lineage to all *Pseudohynobius* species. *Protohynobius puxiongensis* should therefore be named *Pseudohynobius puxiongensis* (Figures 1 and 2), as suggested by Peng et al. (2010).

Conservation of *Pseudohynobius* species

All *Pseudohynobius* species have very specific living condition requirements (Xiong et al., 2010) and the distribution range of each of the *Pseudohynobius* species is narrow. For example, *Ps. kuankuoshuiensis* is only found in the Kuankuoshui Reserve of the Guizhou Province (Xu et al., 2007), *Ps. shuichengensis* is only distributed within the Shuicheng county of the Guizhou Province (Tian et al., 2006), and *Ps. jinfo* is found only in the Jinfo mountains outside of Chongqing city (Wei et al., 2009). Many factors have been found to cause a drastic decline in the number of *Pseudohynobius* species, including climate change, environmental destruction, and intensified human activity (Xiong et al., 2010). Therefore, these species are in need of urgent protection and our best efforts are needed to protect the *Pseudohynobius* genus from extinction.

ACKNOWLEDGMENTS

Research supported by the Doctoral Fund of Zunyi Medical University (#F-702).

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