

# Identification of conservation units of the hynobiid salamander *Pachyhynobius shangchengensis*

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Genet. Mol. Res. 14 (3): 9772-9778 (2015) Received December 28, 2014 Accepted May 8, 2015 Published August 19, 2015 DOI http://dx.doi.org/10.4238/2015.August.19.10

**ABSTRACT.** The evolutionary significant units (ESUs) of the salamander *Pachyhynobius shangchengensis* (Hynobiidae) in the Dabieshan mountains, southeastern China, were identified based on mitochondrial DNA data. We used methods for detecting cryptic species, such as the minimum spanning tree, the automatic barcode gap discovery, and the generalized mixed Yule-coalescent model; geographical partitioning was also used to identify the ESUs. A total of four ESUs were identified.

**Key words:** Conservation unit; Cytochrome c oxidase subunit I; Cytochrome b; *Pachyhynobius shangchengensis*; China

## **INTRODUCTION**

Ryder (1986) described conservation units as "evolutionarily significant units, ESUs", and Moritz (1994) defined management units (MUs) as "significant divergence of allele frequencies at nuclear or mitochondrial loci, regardless of the phylogenetic distinctiveness of the alleles". Genetic divergence is one of the most important criteria for the identification of ESUs (Ryder, 1986; Dizon et al., 1992; Moritz, 1994; De Guia and Saitoh, 2007). Another criterion for identifying ESUs is geographic partitioning (Dizon et al., 1992).

The salamander *Pachyhynobius shangchengensis* (Hynobiidae) was first described by Fei et al. (1985) in Henan Province, China, and it is endemic to the Dabieshan mountains, southeastern China. It is a rare and endangered species (Wu et al., 1994; Cai, 2001; Fei et al., 2006; Wang et al., 2009; Xiong et al., 2009; Duan et al., 2010). Based on phylogenetic studies, four MUs (Zhao et al., 2013) or three ESUs (Pan et al., 2014) have been identified. The four MUs recognized by Zhao et al. (2013) are actually four ESUs, since they were identified based on the criterion of reciprocal monophyly. The genetic criterion for the identification of an ESU is that "ESUs should be reciprocally monophyletic for mtDNA alleles, and exhibit significant divergence of allele frequencies at nuclear loci" (Moritz, 1994). ESUs are characterized by significant genetic distances (Dizon et al., 1992). Methods for detecting cryptic species, such as the minimum spanning tree constructed using TCS 1.21 (Clement et al., 2000), the automatic barcode gap discovery (ABGD) (Puillandre et al., 2012), and the generalized mixed Yule-coalescent (GMYC) model developed by Pons et al. (2006), could be used to identify ESUs. These methods may be effective at identifying ESU boundaries.

We investigated four natural populations of this species: the Jingangtai (JGT) population, the Huangbaishan (HBSH) population, the Tiangtangzhai (TTZH) population, and the Yingshan-Yuexi-Huoshan (YSHX-YXX-HSHX) population. All of the populations were geographically separated from each other (Figure 1). The present study aimed to identify the ESUs of *P. shangchengensis* using methods that can detect cryptic species, and compare the results with the reported descriptions of the ESUs for this species (Zhao et al., 2013; Pan et al., 2014). Mitochondrial cytochrome b (mtDNAcyt b) and cytochrome c oxidase subunit I (mtDNA COI) were used as molecular markers in the analysis.

#### **MATERIAL AND METHODS**

### Sampling and DNA sequence data

A total of 79 *P. shangchengensis* individuals were collected from six locations in the Dabieshan mountains, southeastern China (Figure 1). The mtDNAcyt b (942 bp) and mtDNA COI (1011 bp) data were the same as used by Zhao et al. (2013).

#### Data analysis

A minimum spanning tree was built using TCS 1.21 (Clement et al., 2000) based on combined mtDNA cyt b and mtDNA COI data. The cryptic species hypothesis was tested us-

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Figure 1. Geographical distribution of the four evolutionary significant units (ESUs) of *Pachyhynobius shangchengensis*. These four ESUs include HBSH, JGT, YSHX-YXX-HSHX, and TTZH and are represented by circles in different colors.

ing ABGD based on combined mtDNA cyt b and mtDNA COI data. The ABGD web-interface, as well as a command-line program, is available at http://wwwabi.snv.jussieu.fr/public/abgd/ abgdweb.html (Puillandre et al., 2012). The prior intraspecific divergence was set to between 0.01 and 0.03 (Puillandre et al., 2012).

A GMYC model developed by Pons et al. (2006) and Monaghan et al. (2009) was used to detect the ESUs. This analysis required a rooted ultrametric tree, which was constructed in BEAST v. 1.6.1 (Drummond and Rambaut, 2007). The GMYC model has been successfully used to detect cryptic species (Fontaneto et al., 2009, 2011); therefore, we analyzed our data with the GMYC model using the R package's "splits" (species' limits by threshold statistics) (r-forge.rproject.org/projects/splits/, accessed 2011; R Development Core Team, 2008) to check for cryptic species within *P. shangchengensis*. We used the single-threshold method since it outperforms the multiple-threshold method (Fujisawa and Barraclough, 2013). Splits between cryptic species and major intraspecific lineages were estimated at a substitution rate of 0.64% per million years per branch (lineage), as suggested by Weisrock et al. (2001) for hynobiid salamander mitochondrial DNA. This evolutionary rate has been used previously for hynobiid mitochondrial DNA data (Matsui et al., 2007, 2008; Yoshikawa et al., 2008; Malyarchuk et al., 2010). The GMYC analysis of COI and cyt b was performed consecutively, since the GMYC procedure is based on single-locus sequence data. The geographical distances between the ESUs were calculated online at http://www.gpsvisualizer.com/calculators.

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## RESULTS

The maximum parsimony network yielded four unconnected subnetworks (Figure 2). Subnetwork HBSH included all of the haplotypes from HBSH, subnetwork JGT included all of the haplotypes from JGT, subnetwork TTZH included all of the haplotypes from TTZH, and subnetwork YSHX-YXX-HSHX included all of the haplotypes from YSHX-YXX-HSHX. The ABGD analysis detected four distinct groups (Figure 3). Group 1 included all of the haplotypes from HBSH, group 2 included all of the haplotypes from HSHX, YSHX, and YXX, group 3 included all of the haplotypes from TTZH.

The GMYC analysis (based on cyt b) detected four independent evolutionary entities in *P. shangchengensis* (Figure 4); there was a significant difference between this and the null hypothesis of one single species (P = 0.007). Entity 1 included all of the haplotypes from HBSH, entity 2 included all of the haplotypes from JGT, entity 3 included all of the haplotypes from HSHX, YSHX, and YXX, and entity 4 included all of the haplotypes from TTZH (Figure 4). The GMYC analysis based on COI failed to give satisfactory results.

The minimum geographical distance between the ESUs was 20.5 km (Figure 5); therefore, the four ESUs were completely geographically isolated.



Figure 2. Minimum spanning tree of haplotypes based on the combined data. Each haplotype is represented by an ellipse or square (ancestor), with the area proportional to its frequency.

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![](_page_4_Figure_0.jpeg)

Figure 3. Analysis results of ABGD (automatic barcode gap discovery) based on the combined data.

![](_page_4_Figure_2.jpeg)

**Figure 4.** GMYC model analysis based on cyt b fragment. Number of entities =  $4 (P = 0.007^{**})$ . Rectangles indicate groups identified by the GMYC model as independent evolutionary entities (populations).

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![](_page_5_Figure_1.jpeg)

Figure 5. Geographical distances between the evolutionary significant units.

### **DISCUSSION**

The maximum parsimony network yielded four unconnected subnetworks suggesting that there are four cryptic species in *P. shangchengensis*. The ABGD and GMYC analyses confirmed the existence of four cryptic species. The same lineage (subnetwork, group, or entity) in different analyses (maximum parsimony network, ABGD, and the GMYC model) was composed of the haplotypes that came from the same locality. Different cryptic species-detection analyses yielded the same results, so it can be concluded that there are four ESUs (HBSH, JGT, TTZH, and YSHX-YXX-HSHX) for *P. shangchengensis* (Figure 1). These four ESUs are geographically isolated from each other, which decreases the level of gene flow between them. Significant geographical partitioning is one of the most important criteria for ESU identification (Dizon et al., 1992).

Four ESUs were identified, which is consistent with the results of Zhao et al. (2013) but differs from the results of Pan et al. (2014). There are at least four criteria for the identification of ESUs: significant genetic differentiation, geographical isolation, ecological traits, and morphometric data (De Guia and Saitoh, 2007), and the absence of any of these criteria will yield only partial ESUs (De Guia and Saitoh, 2007). For the identification of *P. shangchengensis* ESUs, we could only satisfy the significant genetic differentiation and geographical isolation criteria, since no morphological or ecological differences between the ESUs were detected.

## ACKNOWLEDGMENTS

Research supported by Project #31071888 of the National Natural Science Foundation of China and the Science and Technology Program of Shaanxi Academy of Science (2014 K-33, 2008K-02).

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