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Hynobiidae origin in middle Cretaceous corroborated by the new mitochondrial genome of *Hynobius chinensis*

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ABSTRACT

Hynobius chinensis was first described by Günther in the nineteenth century. At present, the origins of the extinct *Hynobius chinensis* on the Zhoushan Island (*Hynobius chinensis*-ZI) remain a mystery. It is the only species of family Hynobiidae on the Zhoushan Island. However, there is very little empirical evidence regarding *Hynobius chinensis*-ZI phylogenetic relationship, and when or how did its ancestors colonized the island. Here, we used mitochondrial genome data to recover the phylogeny of family Hynobiidae. Results suggested that the origin of Hynobiidae was most likely in Middle Cretaceous (~112.9 Mya), and some *Hynobius* species of Taiwan and Japan diverged earlier than that of the mainland of China. *Hynobius chinensis*-ZI diverged from its closest living relative (*Hynobius yiwuensis*) around 6.5 Mya, and *Hynobius chinensis*-ZI was isolated on Zhoushan Island since the postglacial transgression in Holocene period.

1. Introduction

The most primitive family of terrestrial salamanders, the Hynobiidae (hynobiid salamanders), comprises 59 species belonging to 10 genera (MacLeod et al., 1997; Robertson et al., 2004) Now, most species native to East Asia are found to be extinct, endangered or vulnerable (AmphibiaWeb, http://amphibiaweb.org/). Of these, *Hynobius chinensis* (Chinese salamander) is endangered (assigned by IUCN Red List of Threatened Species, http://www.iucnredlist.org/ and China Red Data Book of Endangered Animals (Zhao, 1998)). *Hynobius chinensis* was first described by Günther (1889) from two type specimens in Yichang (Hubei Province, China), and re-described by Adler and Zhao in 1990 (Adler and Zhao, 1990). However, this type locality has been doubted by morphologists, because there is no living individuals had been found for more than a century. Recently, some morphologists have reported living individuals in Yichang (Wang et al., 2007) and revealed the *Hynobius* populations in the Chong'an and Wenling area become extirpated (Fu et al., 2003). At present, the populations occurred in southeastern China (Fig. 1), including Yichang (Hubei Province) (Adler and Zhao, 1990), Chong'an (Fujian Province) (Pope, 1931), Wenling (Zhejiang Province) (Boring and Chang, 1933; Chang, 1933) and Zhoushan (Zhejiang Province) (Ma and Gu, 1999) were described under the same name – *Hynobius chinensis*. In addition, the samples from Qiyang (Hunan Province, China) were also assigned to *Hynobius chinensis*, because this locality is geographically close to Yichang (Zhang et al., 2006).

Zhoushan, also known as the fourth largest island of Zhejiang Provincei, is located in East China Sea (N 30°, E 122°). This island is about 20 km away from Mainland China, leading to a geographical isolation for many animals. Currently, *Hynobius chinensis* is the only terrestrial salamanders on the island (*Hynobius chinensis*-ZI), and as many as 4000 living individuals of this geographical population in the lentic ponds. *Hynobius chinensis*-ZI was thought morphologically different from other mainland species (Ma and Gu, 1999). Recently, research suggested that *Hynobius chinensis*-ZI was also genetically different from the other species in southeastern China (Fu et al., 2003). Here, we reported the new mitochondrial genome of *Hynobius chinensis* from Zhoushan Island to discuss its main features, and combined with published mitochondrial DNA to investigate the phylogenetic relationship of Hynobiidae, because few Hynobiidae phylogeny has collected *Hynobius chinensis* DNA samples from Zhoushan Island (Weisrock et al., 2013). It is important to note at the outset that some geographic barriers appear to have a significant influence on speciation and diversification, so we are trying to find new perspectives to help in understanding the Hynobiidae origin by collecting paleogeographic information for past 150 million years (from Colorado Plateau Geosystems, http://jan.ucc.nau.edu/~rcb7/, and Goal of the PALEOMAP Project, http://www.scotese.com/). A geochronologic time point related to the formation of the arc-shaped Japanese archipelago was used as the internal calibration point in divergence estimating. We hope that the

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Localities	References	Assigned name
Yichang	Günther (1889)	*H. chinensis*
Chong'an	Pope (1931)	*H. chinensis*
Wenling	Chang (1933), Boring et al. (1933)	*H. chinensis*
Wenling, Yiwu, Zhenhai	Cai (1985)	*H. yiwuensis*
Anji	Gu (1992)	*H. amjiensis*
Zhoushan	Ma and Gu (1999)	*H. chinensis*-ZI
Qiyang	Zhang et al. (2006)	*H. chinensis*-Q

Fig. 1. The distribution of *Hynobius* populations in southeastern China. Circles indicated localities in Hubei, Hunan, Fujian and Zhejiang, colored according to the assigned name (yellow: *H. amjiensis*; red: *H. chinensis*; green: *H. yiwuensis*; blue: *H. guabangshanensis*). Cai (1985) assigned all hynobiids in Zhejiang Province to *H. yiwuensis*. Specimens for the studies of Zhang et al. (2006) were collected from Qiyang, Hunan Province, where is the habitat of *H. guabangshanensis*. Recent investigations of the Wenling and Chong'an area did not reveal any trace of *Hynobius* species.

phylogenetic reconstruction and molecular timescale described in this study could be beneficial in the explore and advance of hynobiid salamanders evolution.

2. Methods

2.1. Sampling, sequencing and annotating the mitochondrial genome

The material used for sequencing was collected from Zhoushan Island, Zhejiang Province. Total genomic DNA isolation was conducted as detailed in standard phenol-chloroform extraction method (Sambrook and Rusell, 2001). PCR reactions were performed on the PTC-200 (MJ) and eleven pairs of primers were designed for amplification (Supplemental Table S1). The mixture included 0.2 μM of primers, 0.2 mM of dNTPS, 1 μl of DNA template, 2 unit of *Taq* Plus DNA polymerase, and 5.0 μl of 10× *Taq* Plus polymerase buffer. The quality of fragments was assessed by agarose gel electrophoresis and then purified using the Gel Extraction Kit (Takara). After conversion of clones into plasmids, we selected clones and sequenced them. The clones were sequenced using a ABI 3730 automated sequencer with M13 forward and/or M13 reverse primers. All determined sequence were compared with all sequences available in DDBJ/EMBL/GenBank on the basis of BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi/). tRNAs were annotated by first screening by using tRNAscan-SE (Lowe and Eddy, 1997) and then manual identification of missing tRNA genes by comparing with that of other related organism. Nucleotide composition was calculated using the EditSeq program of DNAStar package (DNASTAR Inc., USA).

2.2. Taxonomic coverage and sequence alignment

In current update of AmphibiaWeb (AW, http://amphibiaweb.org/), there are ten genera (59 species) in Hynobiidae. *Pseudohynobius* and *Protohynobius* were recognized as two separate genera (Frost, 2011), but in Amphibian Species of the World (ASW, http://www.amnh.org/), *Protohynobius* was abolished. Total 27 mitochondrial sequences of family Hynobiidae are available from GenBank searches (http://www.ncbi.nlm.nih.gov/taxonomy). Sequences used in this study were given in Table 1, and Cryptobranchidae was used as the out group. The multiple alignments of mitogenome sequences were subjected to G-INS-I, FFT-NS-1 or FFT-NS-2 strategies in MAFFT software (Katoh et al., 2005). The ambiguously aligned or highly diverged alignment of data set were excluded to make the phylogenetic analyses be more reliable (Gatesy et al., 1993). The gaps retained in aligned sequences are recognized as missing data (Nei and Kumar, 2000). The final data consisted of 11,370 bp from 13 protein-coding genes, 2537 bp from two rRNA genes, 1534 bp from 22 tRNA genes, and 786 bp from the control region. Two species, *Hynobius tokyoensis* and *Hynobius retardatus* are lack of 12S rRNA genes in the published genomes, and the control region of former was incomplete.

2.3. Phylogenetic reconstruction

Phylogenetic relationships were reconstructed by the Minimum Evolution (ME), Maximum Likelihood (ML), Maximum Parsimony (MP), and Neighbor Joining (NJ) using MEGA 5 and RAxML ver. 7.2.8 (Stamatakis, 2006; Tamura et al., 2011). In NJ and ME analyses, phylogenies were performed under the Maximum Composite Likelihood method and node reliability was estimated with 1000 bootstraps. Both MP and ML analyses were derived by using heuristic searches with 500 bootstraps, and all characters were treated as being equally-weighted in MP searches. Best-fit evolutionary models were selected by jModeltest 3.7 using Bayesian Information Criteria strategy (Posada, 2008).

Partitioned Bayesian analysis was constructed with MrBayes 3.2 (Ronquist and Huelsenbeck, 2003). We partitioned the combined dataset into mixed models based on gene fragment types (protein-coding genes, rRNAs, tRNAs, and codon positions), shown in Supplementary Table S2 and S3. Random starting tree was used, and the Markov Chain Monte Carlo run over 1,000,000 generations sampling every 100 generations, finally 'burn-in' the first 2500 trees. Selection of best partition strategy was based on Bayes Factors calculations, using the harmonic mean approximation of the marginal model likelihood. The harmonic mean approximation of the marginal model likelihood was used for calculating Bayes factors (Kass and Raferty, 1995; Nylander et al., 2004).

2.4. Tracing ancestral divergence time

The Bayesian molecular dating was performed with MCMCtree program in PAML 4.6 software (Yang and Rannala, 2006). The inferred ML tree was used as the required topology. An independent rates model (IR model) was used to specify the rates among internal nodes (Zhong et al., 2009). A conservative minimum bound of the