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# Toward understanding the distribution of Laurasian frogs: A test of Savage's biogeographical hypothesis using the genus *Bombina*

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ABSTRACT

Several anuran groups of Laurasian origin are each co-distributed in four isolated regions of the Northern Hemisphere: central/southern Europe and adjacent areas, Korean Peninsula and adjacent areas, Indo-Malaya, and southern North America. Similar distribution patterns have been observed in diverse animal and plant groups. Savage [Savage, J.M., 1973. The geographic distribution of frogs: patterns and predictions. In: Vial, J.L. (Ed.), Evolutionary Biology of the Anurans. University of Missouri Press, Columbia, pp. 351–445] hypothesized that the Miocene global cooling and increasing aridities in interiors of Eurasia and North America caused a southward displacement and range contraction of Laurasian frogs (and other groups). We use the frog genus *Bombina* to test Savage's biogeographical hypothesis. A phylogeny of *Bombina* is reconstructed based on three mitochondrial and two nuclear gene fragments. The genus is divided into three major clades: an Indo-Malaya clade includes *B. fortinuptialis*, *B. lichuanensis*, *B. maxima*, and *B. microdeladigitora*; a European clade includes *B. bombina*, *B. pachypus*, and *B. variegata*; and a Korean clade contains *B. orientalis*. The European and Korean clades form sister-group relationship. Molecular dating of the phylogenetic tree using the penalized likelihood and Bayesian analyses suggests that the divergence between the Indo-Malaya clade and other *Bombina* species occurred 5.9–28.6 million years ago. The split time between the European clade and the Korean clade is estimated at 5.1–20.9 million years ago. The divergence times of these clades are not significantly later than the timing of Miocene cooling and drying, and therefore can not reject Savage's hypothesis. Some other aspects of biogeography of *Bombina* also are discussed. The Korean Peninsula and the Shandong Peninsula might have supplied distinct southern refugia for *B. orientalis* during the Pleistocene glacial maxima. In the Indo-Malaya clade, the uplift of the Tibetan Plateau might have promoted the split between *B. maxima* and the other species.



## 1. Introduction

Many animal and plant groups share a similar disjunct distribution pattern, of which members of a group occur in all or part of the following four isolated regions of the Northern Hemisphere: central/southern Europe and adjacent areas, Korean Peninsula and adjacent areas, Indo-Malaya, and southern North America (e.g., Gould and Donoghue, 2000; Soltis et al., 2001; Choi, 2007; Wang et al., 2008). Anuran groups of Laurasian origin, including *Ascaphus*, Alytidae, Bombinatoridae, Pelobatoidea, and Rhinophrynidae, typically display this pattern (Duellman and Trueb, 1994; AmphibiaWeb, 2009). Savage (1973) proposed a hypothesis that explains this common distribution pattern among anurans. In early Cenozoic, many groups of Laurasian origin had widespread northern distribution and some probably even had a circumpolar distribution. In Miocene (23.8–5.3 million years ago), the global cooling trend and the increasing aridities in interiors of Eurasia and North America caused a southward displacement and range contraction that led to the current disjunct distribution of these frogs (Savage, 1973). Consequently, as the result of geographic isolation, diverged Laurasian frogs lineages formed within each aforementioned isolated region after these climatic change events. Although this hypothesis initially was proposed for anurans, it can be applied to other organisms as well. So far, the hypothesis has not been rigorously tested.

The divergence time is the key to test Savage's hypothesis, which predicts that divergence between Laurasian frog lineages from different isolated regions occurred no later than the Miocene climatic changes. If one such divergence time is significantly later than the time of the corresponding climatic change, the hypothesis must be rejected. Diversification of some Laurasian anurans almost certainly existed prior to the climatic change in Miocene (Roelants et al., 2007). For example, breaking and connecting of two land bridges between North America and Eurasia might cause divergence between these frogs in North America and their sister group

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in Eurasia. The Greenland Land Bridge broke at the end of the Eocene; the Bering Land Bridge connected North America and Eurasia since the early Oligocene (Cox and Moore, 2005). Both events occurred much earlier than the cooling and drying events employed in Savage's hypothesis (e.g., Zachos et al., 2001; An et al., 2006), and likely had caused divergence in frogs, which have limited dispersal ability (e.g., Berven and Grudzien, 1990; Nyström et al., 2002). Therefore, dating of these divergence events provides no evidence of rejecting Savage's hypothesis. For this reason, recently diverged Laurasian frog groups would be the best study organisms to test Savage's hypothesis, because split dates estimated between lineages from different isolated regions would have high possibility to be later than Miocene.

The Laurasian frog genus *Bombina* (Bombinatoridae) is perhaps the best model system for testing Savage's (1973) hypothesis. Presently the genus *Bombina* includes eight species (AmphibiaWeb, 2009; but see Yu et al., 2007), and its fragmented distribution includes western Eurasia, Korean Peninsula and adjacent areas, and southern China and adjacent Vietnam. The western Eurasian group includes three species, *B. bombina*, *B. pachypus*, and *B. variegata*, which are distributed in Europe, Turkey, and western Russia. Hofman et al. (2007) recovered five major mitochondrial clades within this group. Like other temperate and boreal species, the intraspecific diversifications of these European *Bombina* species were notably linked to the Pleistocene climatic oscillations (e.g., Avise, 2000; Hewitt, 2004; Canestrelli et al., 2006; Hofman et al., 2007). The Korean Peninsular group includes another temperate and boreal species, *B. orientalis*. It is distributed in two isolated areas—one is the Korean Peninsula and adjacent northeastern China and southern Russian Far East, and the other is the Shandong Peninsula of China. The Indo-Malaya group harbors the other four species, *B. fortinuptialis*, *B. lichuanensis*, *B. maxima*, and *B. microdeladigitora*. Their distribution includes southern China and adjacent northern Vietnam. The uplift of the Tibetan Plateau may have promoted speciation in this group (Tian and Hu, 1985; Liu and Yang, 1994). Previous studies suggest that major split events between extant *Bombina* species did not occur much earlier than Miocene (e.g., Fromhage et al., 2004; Roelants et al., 2007). However, *Bombina* only occurs in three of the four regions (minus North America), and therefore will only permits a test of Savage's hypothesis for a subset of the areas that Savage originally addressed. Other anuran groups of Laurasian origin are either very old and therefore include many divergence that occurred earlier than Miocene (i.e. Pelobatoidea), or are limited to only one region possibly due to extinction events (e.g., Duellman and Trueb, 1994) (i.e. *Ascaphus*, Alytidae, and Rhinophrynidae).

We used a phylogenetic with molecular clock approach to test the Savage (1973) hypothesis. We formulated one simple prediction and tested it with a new molecular dataset derived from all species of the Laurasian genus *Bombina*. We predicted that divergence dates between lineages from different regions would be not later than the hypothesized periods of Miocene cooling and drying. If the estimated dates are statistically later than those periods, we would consider it as a rejection of the Savage's (1973) hypothesis.

## 2. Materials and methods

### 2.1. Taxon sampling

All eight described species of *Bombina*, a total of 88 specimens, were sampled, of which 85 specimens were from 40 collecting sites and the other three individuals did not have locality data. Sampling effort was particularly concentrated in the Asian species. Sampling localities for the five Asian species, *B. fortinuptialis*, *B. lichuanensis*, *B. maxima*, *B. microdeladigitora*, and *B. orientalis*, are shown in Fig. 1. For the European species, representatives of each of the five major mitochondrial clades (Hofman et al., 2007) were included.

Six species from the genera *Alytes* and *Discoglossus*, a total of six specimens, were selected as outgroups based on the current understanding of their phylogenetic relationships (e.g., San Mauro et al., 2004; Roelants and Bossuyt, 2005; Frost et al., 2006). All specimen information is presented in Appendix A.

### 2.2. Laboratory protocols

For phylogenetic reconstruction, three fragments from the mitochondrial genome and two fragments from the nuclear genome were selected for sequencing. The first mitochondrial fragment is part of the COI gene, and was 1066 base pairs in length. The second mitochondrial fragment is part of the *cyt b* gene, and was 966 base pairs in length. The third mitochondrial fragment includes part of the 12S and 16S genes and the $tRNA^{Val}$ gene between them, and was approximately 1950 base pairs before alignment. The first nuclear fragment is part of the RAG-2 gene, and was 668 base pairs in length. The second nuclear fragment includes part of Exons 1 and 2 of the Rhodopsin gene and the Intron 1 between them, and, in the ingroup, was approximately 1000–1360 base pairs before alignment. For the 12S-16S, RAG-2, and Rhodopsin fragments, only parts of samples were sequenced as representatives. The Rhodopsin fragment is mainly composed of intron sequence, because its exon regions contained only 151 base pairs. Although this fragment has not been used (to the best of our knowledge) for phylogenetic reconstruction in anurans, nuclear introns are commonly used as phylogenetic markers (e.g., Creer et al., 2006; Di Candia and Routman, 2007). The other four fragments have been used previously for phylogenetic reconstruction in anurans (e.g., Hoegg et al., 2004; Frost et al., 2006; Hofman et al., 2007).

The COI and *cyt b* fragments were initially sequenced for all specimens. Then the 12S-16S fragment was sequenced for a subset of samples that represent the major lineages identified from the phylogenetic reconstruction of the COI and *cyt b* data. The data from the 12S-16S fragment were employed to estimate deeper divergences than those revealed by COI and *cyt b* fragments. For slowly evolving nuclear genes, only a few individuals from each species were selected for sequencing.

DNA was extracted from tissues preserved in 95% ethanol using the genomic DNA extraction protocols of the Qiagen QIAamp DNA Mini Kit. A standard polymerase chain reaction (PCR) was used to amplify the genomic DNA. For the mitochondrial and RAG-2 genes, PCR products were purified and directly sequenced with BigDye-labeled terminator sequencing protocols in conjunction with an ABI 3700 automatic sequencer (Applied Biosystems). For the Rhodopsin gene, in the ingroup, PCR products were cloned and multiple clones were sequenced using universal M13 forward and reverse primers. For the Rhodopsin gene of the outgroup specimen, PCR products were directly sequenced. All primers, except the universal ones, used in PCR and sequencing are listed in Appendix B.

Sequence editing was conducted with BioEdit (Version 7.0.5; Hall, 1998). Alignment was conducted with ClustalX (Version 1.83; Thompson et al., 1997) and checked by eye. The rRNA secondary structures of *Xenopus laevis* (Cannone et al., 2002) and the amino acid sequences for coding regions were used for checking.

### 2.3. Phylogenetic analysis

The mitochondrial data set, the RAG-2 data set, and the Rhodopsin data set were analyzed independently and in combination. Two mitochondrial data sets were analyzed separately. The first one included the COI and *cyt b* fragments of all the ingroup samples ex-