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Complete mitochondrial genomes resolve phylogenetic relationships within *Bombina* (Anura: Bombinatoridae)

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ABSTRACT

A highly resolved and time-calibrated phylogeny based on nucleotide variation in 18 complete mitochondrial genomes is presented for all extant species and major lineages of fire-bellied toads of the genus *Bombina* (*Bombinatoridae*). Two sets of divergence time estimates are inferred by applying alternative fossil constraints as minima. Divergence time estimates from both analyses differed for the two oldest nodes. The earliest phylogenetic split occurred between small- and large-bodied *Bombina* (subgenera *Bombina* and *Grobina*, respectively) either in the Middle Oligocene or the Early Miocene. East Asian *B. orientalis* and European *B. bombina* + *B. variegata* diverged in the early or Middle Miocene. Divergence times inferred using the alternative fossil calibration strategies converged for the younger nodes, with broadly overlapping HPD intervals. The split between *Bombina bombina* and *B. variegata* occurred in the Late Miocene of Europe and somewhat preceded another deep mtDNA division between the Balkan *B. v. scabra* and *B. v. variegata* inhabiting the Carpathian Mts. Concurrently, the genetically distinct *B. maxima* diverged from other *Grobina* in southeast Asia in the Late Miocene or Pliocene. Our mtDNA phylogeny and a new species-tree analysis of published data (nuclear and mtDNA) suggest that *B. fortinuptialis*, *B. lichuanensis* and *B. microdeladigitora* may be conspecific geographic forms that separated due to Pleistocene climatic fluctuations in southeastern Asia. In the western Palearctic, the Late Pliocene to Pleistocene climatic vagaries most probably induced vicariant events in the evolutionary history of *B. variegata* that led to the formation of the two Balkan *B. v. scabra* lineages and the allopatric *B. v. pachypus* in the Apennine Peninsula. Divergence among *B. bombina* mtDNA lineages is low, with an Anatolian Turkey lineage as the sister group to the European mtDNA clades. In sum, Miocene diversification in the genus *Bombina* established six allopatrically distributed major mtDNA lineages that diversified during the Pliocene and Pleistocene and have survived until the present. The narrow habitat requirements of fire-bellied toads and extensive environmental changes throughout the Palearctic in the Neogene may have contributed to a putatively high extinction rate in these anurans resulting in the current east/west disjunction of their ranges.

1. Introduction

Extant discoglossoid anurans are confined to the western (*Alytes*, *Bombina*, *Discoglossus*) and eastern Palearctic (*Barbourula*, *Bombina*). Each of these genera are clearly distinct and monophyletic (Fromhage et al., 2004; San Mauro et al., 2004; Roelants and Bossuyt, 2005; Clarke, 2007; Blackburn et al., 2010). Crown group discoglossoid radiations are relatively recent, originating after the Middle Miocene (Fromhage et al., 2004; Martínez-Solano et al., 2004; Zangari et al., 2006; Hofman et al., 2007; Blackburn et al., 2010; Pabijan et al., 2012). The most speciose extant discoglossoid lineage is *Bombina* (variously referred to as fire-bellied, yellow-bellied or bell toads) with only eight species, all of which are morphologically similar with a warty, cryptically colored dorsum and bright, aposematic belly patterns involved in the warning posture or Unkenreflex (Bajger, 1980).

The western Palearctic is inhabited by the wide ranging parapatric sister species *B. bombina* (Linnaeus, 1761) and *B. variegata* (Linnaeus, 1758) that meet in a narrow hybrid zone (Szymura, 1993). The former is composed of two clades confined to northern and southern Europe, with shallow mtDNA divergence both between and within clades (Hofman et al., 2007; Fijarczyk et al., 2011). On the other hand, mtDNA variation across the range of *B. variegata* is extensive (Hofman et al., 2007; Fijarczyk et al., 2011).

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Three subspecies are traditionally recognized: *B. v. variegata* (Linnaeus, 1758) inhabiting western and central Europe including the Carpathians; the Balkan yellow-bellied toad *B. v. scabra* (Küster, 1843), and the Apennine endemic *B. v. pachypus* (Bonaparte, 1838), sometimes viewed as a legitimate species (e.g. Lanza and Vanni, 1991; Canestrelli et al., 2006). The Korean peninsula and parts of northeastern China are home to *B. orientalis* (Boulenger, 1890) which together with the western Palearctic lineages form a monophyletic clade of small-bodied species referred to the subgenus *Bombina*. Body size, chromosome number, some morphological traits and a suite of osteological traits distinguish the subgenus *Bombina* from *Grobina* (Tian and Hu, 1985; Szymura and Passakas, 1988; Liu and Yang, 1993; Clarke, 2007). The subgenus *Grobina* (formerly *Glandula*, see Dubois, 1987) encompasses four large-bodied, southeast Asian taxa with putatively allopatric geographic distributions: *B. maxima* (Boulenger, 1905), *B. microdeladigitora* (Liu et al., 1960), *B. fortinuptialis* (Tian and Wu, 1981), and *B. lichuanensis* (Ye and Fei, 1993). However, the degree to which the southeast Asian taxa are differentiated remains poorly known. Although morphological (Tian and Hu, 1985; Vukov et al., 2006), biochemical (Szymura, 1993) and DNA sequence analyses (Fromhage et al., 2004; Yu et al., 2007; Zheng et al., 2009) have elucidated the general patterns of decent within *Bombina*, the phylogenetic relationships within small-bodied (subgenus *Bombina*) and large-bodied (subgenus *Grobina*) are still contentious, particularly the affinities of the closely related east Asian species (Zheng et al., 2009) and the European *B. variegata* which is known to include strongly differentiated lineages that could represent distinct species intergrading in a broad cline (Spolsky et al., 2006).

Sequences of entire mitochondrial genomes offer an exceptional wealth of informative nucleotide and amino acid sites at different taxonomic levels. For example, complete mitogenomes have been used to unveil the phylogenetic relationships among major orders of vertebrates (e.g. Arnason et al., 2002; Zhang et al., 2005; Pacheco et al., 2011). At a finer scale, previously intractable polytomies resulting from low levels of sequence divergence or rapid radiations have also been unraveled in this way (Morin et al., 2010; Wielstra and Arntzen, 2011). Entire mitochondrial genomes are known for four species of *Bombina* and have been used to place a molecular timescale on the origin of modern lissamphibians (Zhang et al., 2005) and for the reconstruction of affinities among discoglossoid genera (San Mauro et al., 2004). Moreover, mitochondrial gene organization and patterns of nucleotide variation within *Bombina* have been described in detail (Pabijan et al., 2008) with an emphasis on the considerable divergence between the mitogenomes of *B. bombina* and *B. variegata.* These two species are morphologically, ecologically and genetically distinct but form hybrid zones in areas where their parapatric meet in central Europe (Szymura and Barton, 1991; Szymura, 1993; Yanchukov et al., 2006).

Several biogeographic scenarios have been invoked for the origins and current distributions of extant *Bombina* species. A Middle Miocene desiccation event across Laurasia is thought to have formed the east – west disjunction between *B. orientalis* and European *Bombina* (Savage, 1973; Zheng et al., 2009). The uplift of the Tibetan plateau and its surroundings has been proposed for initiating differentiation in *Grobina* (Liu and Yang, 1994; Zheng et al., 2009). The origin of *B. bombina* and *B. variegata* has been attributed to isolation in separate glacial refugia in the Pliocene or Pleistocene (Mertens, 1928; Arntzen, 1978; Szymura, 1983) or late Miocene/Pliocene (Szymura, 1993; Fromhage et al., 2004; Hofman et al., 2007). The Apennine, Balkan and Carpathian lineages of *B. variegata* are thought to have originated in separate Pleistocene refugia (Szymura, 1993; Hofman et al., 2007). Nearly all of these hypotheses were based on the propagation of standard rates of protein or DNA evolution and thus ignore error associated with the primary fossil calibration, rate variation among lineages and loci, and other sources of uncertainty (e.g. Graur and Martin, 2004; Ho, 2007).

In this paper, we extend our mitochondrial genome sampling to representatives of all *Bombina* species and major intraspecific clades. We capitalize on the resolving power of 12 newly obtained and 6 previously published mitogenomes representing all *Bombina* taxa and establish the phylogenetic relationships within eastern and western Palearctic *Bombina* and among distinct mitochondrial lineages within species. Using 3 fossil calibrations, we estimate divergence times for major nodes within the *Bombina* mitochondrial phylogeny and test earlier hypotheses on the origins of major *Bombina* lineages. Moreover, preliminary results encouraged us to re-analyze a previous multigene dataset with extensive sampling from eastern Palearctic species (Zheng et al., 2009). Our reanalysis of this dataset involved a coalescent-based species tree method which is better suited for studies of closely related taxa because, unlike concatenation, it allows unlinked loci to have independent histories (Degnan and Rosenberg, 2009; Heled and Drummond, 2010). We thus provide a phylogenetic framework and fossil-calibrated timescale for understanding the evolution of both eastern and western Palearctic *Bombina* and discuss the taxonomic implications of our findings.

2. Material and methods

2.1. Samples and molecular methods

The analysis included 12 new and 6 previously published *Bombina* mtDNA genomes for which species designation, sampling localities, GenBank accession numbers, references and abbreviations used herein are given in Table 1. Our sampling includes all known species and major *cytb* haplogroups (Canestrelli et al., 2006; Vörös et al., 2006; Hofman et al., 2007; Fijarczyk et al., 2011) within both western and eastern Palearctic *Bombina* (Table 1, Fig. 1). For most of these clades, we sequenced the mitogenomes of two or three individuals from geographically distant localities; the mtDNA haplotypes of each of these individuals coalesce within each major haplogroup, excluding the potential confounding effect of incomplete lineage sorting within our mtDNA phylogeny. The complete mtDNA genome of *Discoglossus galganoi* (AY585339, San Mauro et al., 2004), was used as an outgroup.

Purified mtDNA (Szymura et al., 1985, 2000) was available for BbomA, BbomM, BvarB1, BvarB2, BvarP1 and Bmax1. Genomic DNA was isolated by phenol/chloroform extraction from ethanol preserved tissues for BvarB3, BvarC2, BvarC3, BvarP2, Blich, and Bmic. The *Bombina* mtDNA genomes were obtained through PCR amplification of several overlapping mtDNA fragments spanning the entire mtDNA molecule according to the PCR/sequencing strategy of Pabijan et al. (2008). Species-specific PCR and sequencing primers were developed for Blich, Bmax and Bmic where required (Supplementary Tables S1–S2). Amplicons were sequenced using both PCR and sequencing primers with the BigDye Terminator v3.1 Cycle Sequencing Kit and run on a 3100 Applied Biosystems Genetic Analyzer. The length variable control regions, (*cf.* Fig. 2 in Pabijan et al., 2008) that could not be unambiguously sequenced due to heteroplasmy caused by indels and a variable number of tandem repeats were first amplified and then cloned using the Promega pGEM®-TEasy Vector System (Promega), following the manufacturer's instructions. Positive, white colonies were used for colony PCR using T7 and SP6 primers and then sequenced with specific primers. Chromatograms were edited, where necessary, and assembled using SeqMan v. 5.0.5 (DNASTAR, Inc.) and Bioedit v.7.05 (Hall, 1999). Both strands of the mtDNAs were sequenced, therefore a minimum of two reads covered each mtDNA genome. However, because of the overlap of amplicons, many sites received