Molecular Phylogenetics and Evolution 63 (2012) 113–130

Contents lists available at SciVerse ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Molecular phylogenetics and historical biogeography of the west-palearctic common toads (*Bufo bufo* species complex)

J. Garcia-Porta [a], S.N. Litvinchuk [b], P.A. Crochet [c], A. Romano [d], P.H. Geniez [c], M. Lo-Valvo [e], P. Lymberakis [f], S. Carranza [a,*]

[a] *Institute of Evolutionary Biology (CSIC-UPF), Passeig Marítim de la Barceloneta, 37-49, 08003 Barcelona, Spain*
[b] *Institute of Cytology, Russian Academy of Sciences, Tikhoretsky pr. 4, 194064 St. Petersburg, Russia*
[c] *CNRS – UMR 5175, Centre d'Ecologie Fonctionnelle et Evolutive, 1919 Route de Mende, F-34293 Montpellier Cedex 5, France*
[d] *Dipartimento di Biologia, Università di Roma "Tor Vergata", Via della Ricerca Scientifica, I-00133 Rome, Italy*
[e] *Dipartimento di Biologia ambientale e biodiversità, Via Archirafi 18, I-90123 Palermo, Italy*
[f] *Natural History Museum of Crete, University of Crete, Knosou Av., PO Box 2208, 71409 Irakleio, Greece*

ARTICLE INFO

Article history:
Received 27 May 2011
Revised 18 December 2011
Accepted 20 December 2011
Available online 30 December 2011

Keywords:
Amphibian
Phylogeny
Biogeography
Deserts
Diversification
Pleistocene glaciations

ABSTRACT

In most pan-Eurasiatic species complexes, two phenomena have been traditionally considered key processes of their cladogenesis and biogeography. First, it is hypothesized that the origin and development of the Central Asian Deserts generated a biogeographic barrier that fragmented past continuous distributions in Eastern and Western domains. Second, Pleistocene glaciations have been proposed as the main process driving the regional diversification within each of these domains. The European common toad and its closest relatives provide an interesting opportunity to examine the relative contributions of these paleogeographic and paleoclimatic events to the phylogeny and biogeography of a widespread Eurasiatic group. We investigate this issue by applying a multiproxy approach combining information from molecular phylogenies, a multiple correspondence analysis of allozyme data and species distribution models. Our study includes 304 specimens from 164 populations, covering most of the distributional range of the *Bufo bufo* species complex in the Western Palearctic. The phylogenies (ML and Bayesian analyses) were based on a total of 1988 bp of mitochondrial DNA encompassing three genes (*tRNAval*, *16S* and *ND1*). A dataset with 173 species of the family Bufonidae was assembled to estimate the separation of the two pan-Eurasiatic species complexes of *Bufo* and to date the main biogeographic events within the *Bufo bufo* species complex. The allozyme study included sixteen protein systems, corresponding to 21 presumptive loci. Finally, the distribution models were based on maximum entropy. Our distribution models show that Eastern and Western species complexes are greatly isolated by the Central Asian Deserts, and our dating estimates place this divergence during the Middle Miocene, a moment in which different sources of evidence document a major upturn of the aridification rate of Central Asia. This climate-driven process likely separated the Eastern and Western species. At the level of the Western Palearctic, our dating estimates place most of the deepest phylogenetic structure before the Pleistocene, indicating that Pleistocene glaciations did not have a major role in splitting the major lineages. At a shallow level, the glacial dynamics contributed unevenly to the genetic structuring of populations, with a strong influence in the European–Caucasian populations, and a more relaxed effect in the Iberian populations.

1. Introduction

Throughout the Neogene (23–2.6 Ma), the Palearctic region has experienced several climatic and physiographic changes that have modulated the diversification of its biotas and shaped their distributions. This is particularly true for pan-Eurasiatic groups with distributions extending from the Western to the Eastern Palearctic; several paleoclimatic or paleogeographic events ranging from a regional to a global scale (Blondel and Aronson, 1999; Azanza et al., 2000; Fortelius et al., 2002; Melville et al., 2009) have likely structured these populations. Singularly, the rise of the Himalayas is one of the most important landmarks for understanding the distribution patterns in the Palearctic. This process, initiated 45–55 Ma, is considered the continents' largest perturbation to atmospheric circulation, ultimately originating the Central Asian Deserts and the monsoon-like climate in Eastern Asia (Molnar et al., 2010). Several cases of sister-species complexes at both sides of the deserts have led to the hypothesis that the origin of the Central Asian Deserts separated many Eastern and Western species complexes by

---

* Corresponding author. Fax: +34 93 230 95 55.
E-mail address: salvador.carranza@ibe.upf-csic.es (S. Carranza).

1055-7903/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2011.12.019

vicariance (Savage, 1973; Borkin, 1984; Voelker, 1999). For amphibians, Savage (1973) and Borkin (1984) hypothesized that the progressive aridification of Central Asia coupled with global cooling trends during the Miocene (23–5.3 Ma) forced the amphibian faunas to retract their ranges to the South, forming isolates at both sides of the great Central Asian Deserts.

After the above-mentioned splits between Eastern and Western domains, each lineage diversified regionally throughout the rest of the Neogene. However, the major causes of these cladogenetic events in most cases are debated. In the Western Palearctic, the classic "glacial refugia" theory attempts to explain most of these cladogenetic events as a consequence of shifts in the distributional ranges towards the South during the glacial maxima, leading to subsequent allopatric isolation and genetic differentiation in the Mediterranean Peninsulas (Hewitt, 2000). The existence of species or subspecies broadly dividing into Eastern and Western groups backed this theory (e. g. *Pelobates cultripes*/*Pelobates fuscus*), suggesting that both groups were derived from refugia located in different Mediterranean Peninsulas (mainly Iberian Peninsula, Italian Peninsula and the Balkans) (Llorente et al., 1995). However, dating estimates revealed that although some of the splits were associated with the glacial cycles, this was not a general rule and many splits could be firmly placed in Pre-Pleistocene times (Seddon et al., 2001; Babik et al., 2007). Therefore, the role of Pleistocene glacial cycles shifted from being one of the most important processes for explaining the current diversity of species in the Palearctic to a more labile process with different degrees of relevance depending on the particular organism and the temporal scale considered (Klicka and Zink, 1997; Soria-Carrasco and Castresana, 2011). A more modern view is that the phylogeographic structure of most Paleartic groups is actually a combination of deep splits during the Miocene or Pliocene, followed by a re-structuring caused by fluctuations in population sizes experienced during the Quaternary (e.g. Paulo et al., 2001; Mattoccia et al., 2005; Nascetti et al., 2005; Ursenbacher et al., 2008). Nevertheless, in most cases the historical causes of these deep splits usually remain elusive.

Amphibians constitute a very good model to explore the historical aspects of species distributions due to their low dispersal capacity and retention of a strong phylogeographic signal. Moreover, they are very sensitive to climatic changes, which make them optimal organisms for discriminating the effects of glacial cycles and other environmental changes upon their genetic structure and biogeographic patterns (Zeisset and Beebee, 2008). The European common toad belongs to the genus *Bufo* (*sensu stricto*), a pan-Eurasiatic group comprising two species complexes. Eastern Eurasia contains the greatest species richness of the genus, with 13–14 recognized species distributed across Central and Eastern China, Northern Vietnam, Korea, far Eastern Russia, and Japan (here and after the *Bufo gargarizans* species complex) (Frost, 2011; see also Zhan and Fu, 2011).

The second complex occurs in the Western Palearctic, and only two or three valid species are currently recognized (here and after the *Bufo bufo* species complex) (Litvinchuk et al., 2008; Frost, 2011): the Eichwald toad (*Bufo eichwaldi* Litvinchuk et al., 2008), restricted to the Talysh mountains of the Southeastern Caucasus; the Caucasian toad (*Bufo verrucosissimus* (Pallas, 1814), not recognized by e.g. Crochet and Dubois, 2004), which inhabits the Caucasus and Anatolia; and the European common toad (*Bufo bufo* (Linnaeus, 1758)), the Palearctic anuran with the largest distributional range, spanning from North Africa to the Polar circle and from the Western Iberian Peninsula to the Baikal Lake in Siberia (Lizana, 2002). Despite this huge distributional range, according to Mertens and Wermuth (1960), the European common toad is a single species with three subspecies: (1) the nominate subspecies *Bufo bufo bufo*, the Eurosiberian form, distributed across Northern and Central Europe, Western Siberia, the British Islands and the Eurosiberian enclaves of the Mediterranean peninsulas, (2) *Bufo bufo spinosus* Mertens, 1925, considered the Mediterranean counterpart of the nominal subspecies, occupying the Mediterranean margins of Europe, North Africa and most parts of Western and Central France (Geniez and Cheylan, 2005, in press), and (3) *Bufo bufo gredosicola* Müller & Hellmich, 1925, with a very limited distributional range restricted to the highest prairies and lakes of the Sierra de Gredos, in Central Iberian Peninsula.

Since the European common toad and its closest relatives present a disjunct distribution across Eurasia (Lizana, 2002), and also show regional structure in the Western Palearctic, they provide an interesting opportunity to examine the importance of the Central Asian Deserts as the vicariant event that separated Eastern and Western species complexes, and secondly to assess the relative contribution of both glacial and preglacial events in the regional structure of the Western Palearctic.

The aim of the present study is to combine data from molecular phylogenies, multiple correspondence analyses of allozyme data and species distribution models, to unravel the historical processes that have contributed to shaping the biogeography and cladogenesis of the most abundant and widely distributed amphibian genus in the Palearctic.

2. Methods

2.1. Taxon sampling, DNA extraction, amplification and sequencing

A total of 151 specimens were included in the mitochondrial DNA study, covering the entire distribution range of the species complex in the Western Palearctic (Table 1 and Fig. 1). Of these, 147 are members of the *Bufo bufo* species complex, with four specimens obtained from GenBank (Benson et al., 2008). The remaining four specimens belong to the *Bufo gargarizans* species complex and were used as outgroups (all obtained from GenBank). A list of all the samples used in the present work with their extraction codes, voucher references, corresponding localities and GenBank accession numbers can be found in Table 1. Genomic DNA was extracted from ethanol-preserved tissue samples using the Qiagen DNeasy Blood & Tissue Kit. A total of 1988 bp of mitochondrial DNA were sequenced for most of the specimens (5.8% of missing data), encompassing fragments of three genes: *tRNAval* (48 bp), *16SrRNA* (1386 bp) and *ND1* (554 bp). Already published primers for the amplification and sequencing of the mitochondrial gene fragments included in the present study as well as PCR conditions used are given in detail in Biju and Bossuyt (2003) and Roelants and Bossuyt (2005). All amplified fragments were sequenced for both strands. Contigs were assembled in Geneious v. 5.3.6 (Biomatters Ltd.).

2.2. Phylogenetic analyses of mitochondrial DNA

The sequences obtained were aligned using the online version of MAFFT 6.240 (Katoh et al., 2002) (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/), following a FFT-NS-i strategy (slow, iterative refinement method) with the rest of the settings left by default (scoring matrix 200PAM ($k = 2$), gap opening penalty = 1.53). The gaps generated by the process of alignment were considered missing data in all the following analyses.

Two methods of phylogenetic analysis, namely maximum likelihood (ML) and Bayesian analysis (BI), were employed and their results compared. The ML analysis was performed using RaxML 7.0.4 (Stamatakis, 2006) with the dataset split in two partitions: one partition including the RNA-coding genes and the other including the protein-coding gene (*ND1*). JModeltest (Posada, 2008) was used to select the most appropriate model of sequence evolution