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Nuclear and mitochondrial phylogeography of the European fire-bellied toads *Bombina bombina* and *Bombina variegata* supports their independent histories

ANNA FIJARCZYK,* KRYSTYNA NADACHOWSKA,† SEBASTIAN HOFMAN,* SPARTAK N. LITVINCHUK,‡ WIESŁAW BABIK,† MICHAŁ STUGLIK,† GÜNTER GOLLMANN,§ LUKÁŠ CHOLEVA,¶ DAN COGĂLNICEANU,** TANJA VUKOV,†† GEORGE DŽUKIĆ†† and JACEK M. SZYMURA*

**Department of Comparative Anatomy, Institute of Zoology, Jagiellonian University, Gronostajowa 9, 30-387 Kraków, Poland, †Institute of Environmental Sciences, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland, ‡Russian Academy Sciences, St Petersburg 199034, Russia, §Department of Evolutionary Biology, University of Vienna, Althanstraße 14, 1090 Wien, Austria, ¶Institute of Animal Physiology & Genetics of the Academy of Sciences the Czech Republik, Rumburská 89, 277 21 Liběchov, Czech Republic, **Ovidius University Constanta, Constanta 900470, Romania, ††Institute for Biological Research 'Siniša Stanković', University of Belgrade, Belgrade, Serbia*

Abstract

Exact location and number of glacial refugia still remain unclear for many European cold-blooded terrestrial vertebrates. We performed a fine-scaled multilocus phylogeographic analysis of two *Bombina* species combining mitochondrial variation of 950 toads from 385 sites and nuclear genes (*Rag-1, Ncx-1*) from a subset of samples to reconstruct their colonization and contemporary variation patterns. We identified the lowlands northwest of the Black Sea and the Carpathians to be important refugial areas for *B. bombina* and *B. variegata*, respectively. This result emphasizes the importance of Central European refugia for ectothermic terrestrial species, far north of the Mediterranean areas regarded as exclusive glacial refugia for the animals. Additional refugia for *B. variegata* have been located in the southern Apennines and Balkans. In contrast, no evidence for the importance of other east European plains as refugial regions has been found. The distribution of mtDNA and *Ncx-1* variation suggests the presence of local refugia near the Black Sea for *B. bombina*; however, coalescent simulations did not allow to distinguish whether one or two refugia were present in the region. Strong genetic drift apparently accompanied postglacial expansions reducing diversity in the colonization areas. Extended sampling, coupled with the multilocus isolation with migration analysis, revealed a limited and geographically restricted gene flow from the Balkan to Carpathian populations of *B. variegata*. However, despite proximity of inferred *B. bombina* and *B. variegata* refugia, gene exchange between them was not detected.

Keywords: *Bombina*, isolation with migration, nuclear and mitochondrial markers, phylogeography, Pleistocene refugia, postglacial expansion

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Introduction

Climate oscillations during the Pleistocene played a significant role in shaping the genetic structure of boreal and temperate species. Quaternary temperature changes resulted in periodic expansions and contractions of ranges, as species followed suitable habitats determined by climatic factors. Populations that survived multiple glacial cycles in areas with favourable conditions often conserved high genetic diversity, whereas the populations that occupy recently deglaciated areas have

Correspondence: Jacek M. Szymura, Fax: +48 12 634 3716; E-mail: jacek.m.szymura@uj.edu.pl

commonly lost genetic variation because of repeated bottlenecks during colonization (Hewitt 1999, 2004; Varga 2010). Analysis of present distributions of species inhabiting western Eurasia, together with paleoclimatic and fossil (e.g., pollen) data, has led to the identification of main refugial areas in the Mediterranean peninsulas and unveiled several patterns of postglacial colonization (Hewitt 2004; Schmitt 2007; Varga 2010). The advances in laboratory and analytical methods, a combination of molecular and paleontological approaches, and broad taxon sampling revealed additional areas, well north of the typically postulated classical refugia, where animals and plants could have survived colder periods (Stewart & Lister 2001; Schmitt 2007; Provan & Bennett 2008). A 'continental' group of species, according to a recent classification proposed by Schmitt (2007), had multiple extra-Mediterranean glacial centres of expansion such as the perialpine region, Carpathian Basin and various parts of the Balkan Peninsula. The importance of some of these northern refugia has been confirmed for multiple species (Schmitt & Seitz 2001; Willis & van Andel 2004, Ursenbacher *et al.* 2006; Ronikier *et al.* 2008; Pauls *et al.* 2009), including the *Bombina* toads (Hofman *et al.* 2007).

An increasing but still limited number of studies on terrestrial organisms with broad Eurasiatic distributions attempted to reconstruct the history of Eastern Europe and Northern Asia colonization (Jaarola & Searle 2002; Brunhoff *et al.* 2003; Babik *et al.* 2004; Deffontaine *et al.* 2005; Durka *et al.* 2005; Ursenbacher *et al.* 2006; Marmi *et al.* 2006; Fedorov *et al.* 2008; Tougard *et al.* 2008; Tollefsrud *et al.* 2008, Korsten *et al.* 2009). Most of these areas were colonized either from the above-mentioned refugia in Europe or from locations farther to the east (Jaarola & Searle 2002; Babik *et al.* 2004; Deffontaine *et al.* 2005; Marmi *et al.* 2006; Fedorov *et al.* 2008; Tougard *et al.* 2008; Tollefsrud *et al.* 2008; Korsten *et al.* 2009). While the Ponto-Caspian region was an essential refugium for aquatic fauna (Culling *et al.* 2006; Kotlík *et al.* 2008), its importance as a major glacial refugium for terrestrial or semi-aquatic organisms is still unclear. This region was suggested as a refugium for *B. bombina* (Hofman *et al.* 2007), but limited sampling did not allow for reliable inference of postglacial migration routes of this species in Eastern Europe. Thus, detailed phylogeographic analyses of the species inhabiting Eastern Europe and particularly the Ponto-Caspian region are needed.

The fire-bellied toad (*B. bombina*) and its sister species, the yellow-bellied toad (*B. variegata*), are anurans that are widespread throughout Central and Eastern Europe. These toads have parapatric distributions, which reflect their distinct habitat preferences. *B. bombina* occupies larger, more permanent ponds of the European lowlands, while *B. variegata* prefers small, ephemeral pools and puddles in the mountainous areas (Szymura 1993). The species hybridize wherever their ranges meet, forming narrow hybrid zones (Szymura & Barton 1991; Szymura 1993). The zones differ in genetic structure depending on their location (Szymura 1993; Vines *et al.* 2003; Yanchukov *et al.* 2006), but no introgression has been detected outside the hybrid zone (Hofman & Szymura 2007; Hofman *et al.* 2007; but see Vörös *et al.* 2006).

The two species differ in their morphology, physiology, behaviour, ecology and life history. In addition, three subspecies are currently recognized within *B. variegata*: *B. v. variegata* in Western Europe and the Carpathians, *B. v. scabra* in the Balkans and *B. v. pachypus* in the Apennines. The latter form is sometimes regarded as a separate species (Canestrelli *et al.* 2006). Distinctness of species and subspecies was confirmed by the patterns of allozyme variation (Szymura 1993).

A recent phylogeographic study based on nucleotide variation of mitochondrial cytochrome *b* gene (*cyt b*) (Hofman *et al.* 2007) provided further insights into the genetic differentiation and history of both species. This study revealed a complex pattern in *B. variegata*, partially at odds with earlier research. Yellow-bellied toads from Western Europe display a strong affinity to the populations from the Balkans and Apennines, whereas the Carpathian mtDNA clade is very distinct, suggesting an ancient divergence and long-term survival of toads in Carpathian refugia (Szymura *et al.* 2000; Spolsky *et al.* 2006; Vörös *et al.* 2006; Hofman *et al.* 2007). The study confirmed a shallow genetic structure in *B. bombina* already suggested by allozymes (Szymura 1993). It was concluded that two recently diverged *B. bombina* clades expanded postglacially from putative refugia situated at the western and/or northwestern shore of the regressed Black Sea (Hofman *et al.* 2007). However, inference of refugial areas and subsequent migration routes of *B. bombina* were solely based on a limited data set, which was restricted to a minor part of the species range. Therefore, a more detailed study to reconstruct the initial colonization pattern and to identify glacial refugia is needed to present robust conclusions. Mitochondrial DNA (mtDNA) markers have long been regarded as useful tools for deciphering species histories because of such features as fast pace of evolution and substantial intraspecific polymorphism (Avise 2000). Nevertheless, conclusions based solely on mitochondrial inferences have been continuously questioned (Ballard & Whitlock 2004; Bazin *et al.* 2006, Galtier *et al.* 2009). An increasing number of studies reveal apparent discrepancies between nuclear and mitochondrial gene genealogies (Renoult *et al.* 2009; Wahlberg *et al.* 2009). Such conflicts may result from either incomplete lineage

sorting or introgression events. Hence, to develop a more comprehensive phylogeographic reconstruction, an application of several unlinked loci is essential. Nuclear markers become helpful in reconstructing demographic processes such as population growth and gene flow (Zink & Barrowclough 2008; Wahlberg *et al.* 2009). Given the advantages of a multilocus approach, it appears to be a desirable step in the phylogeographical study of the *Bombina* toads.

We applied a multilocus approach and coalescent analysis on an extensive data set to identify historical glacial refugia and to reconstruct colonization pathways in the European *Bombina*, as well as to draw general conclusions about the important geographic regions for semi-terrestrial ectothermic species. Specifically, we aimed to (i) comprehensively describe the distribution of mtDNA lineages, with an emphasis on previously unsampled regions of Eastern Europe; (ii) analyse the genetic variation and sequence divergence of two nuclear gene fragments, *Rag-1* and *Ncx-1*, across the ranges in relation to postulated refugia; (iii) compare patterns of mitochondrial and nuclear DNA sequence variation; and (iv) test for the historical and contemporary gene exchange between the Carpathian and Balkan *B. variegata* populations.

Methods

Sampling, amplification and sequencing

Genomic DNA was extracted from tissue fragments, mostly toe-clips stored in 96% ethanol, by using the phenol–chloroform method. We obtained 586 new samples from 229 new localities complementing the collection of mtDNA used by Hofman *et al.* (2007) and covering entire distribution of two species (Table S1, Fig. S1a, Data S1, Supporting information). A few individuals of *B. variegata* were found in the range of *B. bombina* (22, 272 and 309 in Table S1, Fig. S1a, Supporting information), although not far away from the boundaries of their own species range (Yanchukov *et al.* 2006); hence, they were also included in the analyses. Procedures of amplification and sequencing of *cyt b* (1069 bp) are described in Hofman & Szymura (2007). We sampled also 80 individuals from 73 localities for *Ncx-1* gene and 111 individuals from 68 localities for *Rag-1* gene. Nuclear sequences were sampled randomly from the whole DNA collection and four additional localities in Poland and Romania (Mogielica, Cărăşeu, Ghilvaci and Hinova; sites 389, 386–388, respectively; Fig. S1b,c, Table S1, Supporting information), but more extensively in the regions of putative refugia. Fragments of nuclear genes were 1061 bp (*Rag-1*) and 702 bp (*Ncx-1*) long. Fragments of the *Rag-1* (~1100 bp) and *Ncx-1* gene (~740 bp) were, respectively, amplified with primers Rag1L (5′-GAGGAAAGCCCTATACTTCTGG-3′) and Rag1H (5′-CTCCATGATGGCCTCCAAT-3′), and NcxF (5′-TCATCCGCTCCTGAAATTCT-3′) and NcxR (5′-CACAGTCCCACAGTTTTCCA-3′), designed on the basis of the *Bombina* sequences available from GenBank (AY523705 and AY523715). Amplification of both nuclear markers was performed with Fermentas *Taq* DNA polymerase and other reagents. The *Rag-1* reaction mixture contained 2 μL of DNA, 2.5 μL of 10× PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.12 μM of each primer, 1.25 U of *Taq* polymerase, 0.5 μL of 10× bovine serum albumin (BSA) and 17.15 μL of molecular biology grade water. Thermal conditions of the reaction were as follows: denaturation at 94 °C for 3 min, annealing at 54 °C for 45 s and elongation at 72 °C for 2 min, followed by 34 cycles with denaturation at 94 °C for 30 s, annealing at 57 °C for 45 s and elongation at 72 °C for 1 min and a final elongation at 72 °C for 10 min. For amplification of *Ncx-1*, we used 2 μL of DNA, 1.8 μL of 10 × PCR buffer, 2.5 mM of $MgCl_2$, 0.28 mM of dNTPs and each primer, 1 U of *Taq* polymerase, 0.7 μL of 10× BSA and 10 μL of Sigma water per sample. We applied cycling scheme similar to that used for *Rag-1* amplification, with the following exceptions: an initial denaturation step at 94 °C for 2 min, 35 cycles with denaturation at 94 °C for 30 s, an annealing temperature of 55 °C for 45 s, and elongation at 72 °C for 1 min. Amplicons were purified with Clean-up columns (A & A Biotechnology) or *ExoI-SAP* digestion. The sequencing reaction was conducted with BigDye-Terminator reagents with the same primers (the central part of *Rag-1* PCR products was sequenced with an additional primer: 5′-AAACACATTGCAAGAAGATCC-3′), and the products of the sequencing reactions were resolved on an ABI Prism 3100 Avant Genetic Analyzer.

Haplotypes in sequences with heterozygous nucleotide positions were resolved by using two methods. To determine the haplotypes of *Rag-1* sequences, we designed primers with 3′ end matching first or last ambiguous position in heterozygous sequences and specifically amplified only one allele to be sequenced. In *Ncx-1* gene, we disentangled the ambiguities using Phase (Stephens *et al.* 2001; Stephens & Donnelly 2003). We used *B. orientalis* from East Asia as the outgroup.

Population genetic analyses

We distinguished several geographic groups in each species (Fig. 1) and compared the amounts of variation, measured as nucleotide diversity (π), haplotype diversity (H_d) and the number of segregating sites (S), present in these groups. The parameters were calculated with DnaSP software (Librado & Rozas 2009). We also

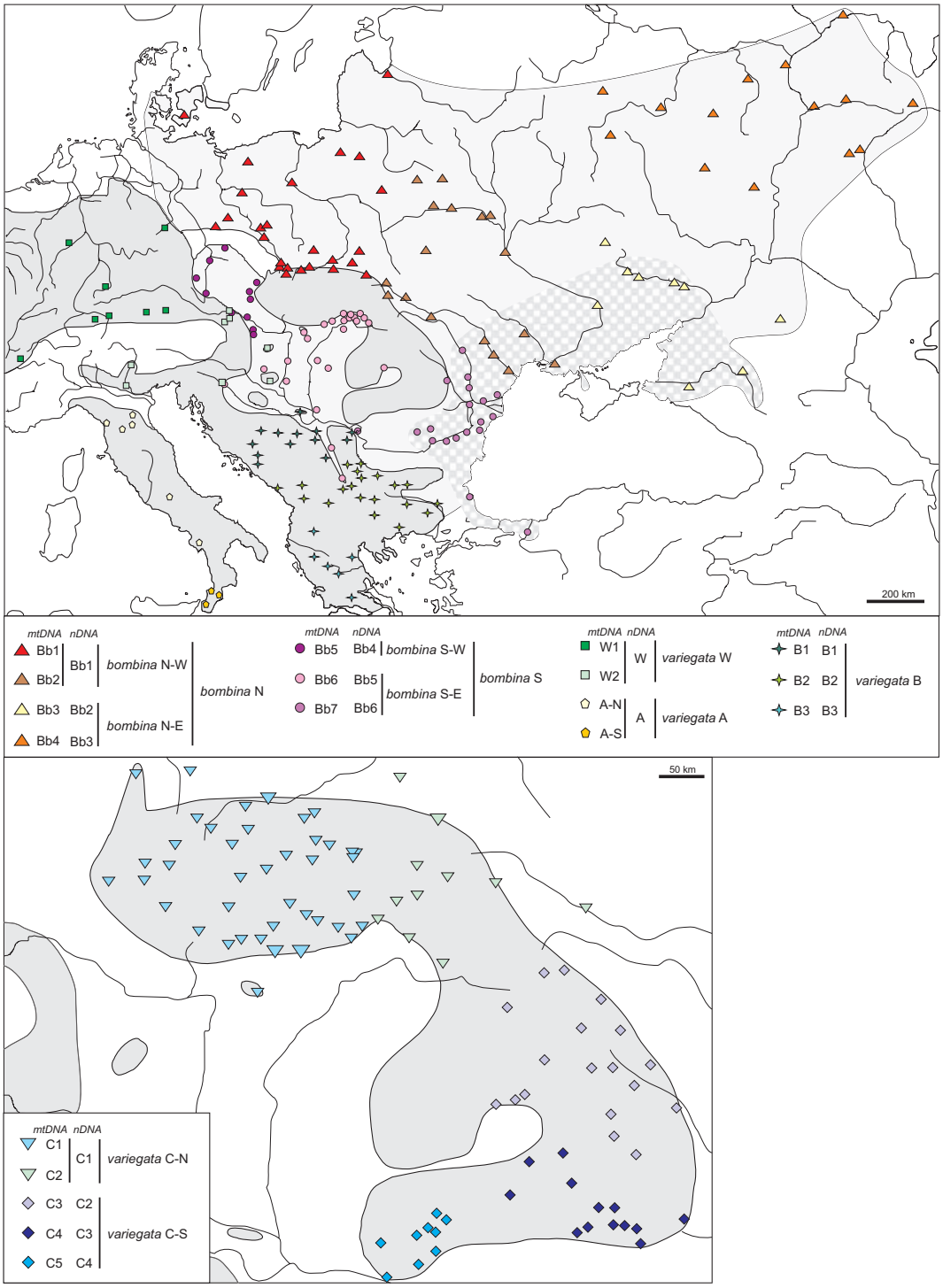

Fig. 1 Partitioning of all sampling sites into geographical regions within the main clades of *Bombina bombina* (light grey) and *Bombina variegata* (dark grey). The Black Sea region is checked. Carpathian sites are shown on the lower map.

computed the statistics commonly used to test for demographic expansions (Tajima's D, Fu's F_S) using Arlequin version 3.5 (Excoffier & Lischer 2010). To infer haplotype networks of nuclear markers, we used a median-joining calculation implemented in NETWORK 4 (Bandelt *et al.* 1999). To investigate whether the relationships among mtDNA haplotypes within major mtDNA clades have not changed in comparison with the previous study (Hofman *et al.* 2007), we also inferred networks for *cyt b* using a maximum

parsimony calculation in the same programme. The clades were named after the Hofman *et al.* (2007) study to retain continuity.

Analysis of molecular variance (AMOVA) and pairwise F_{ST} was conducted by using Arlequin version 3.5 (Excoffier & Lischer 2010). Because the three main mtDNA clades exhibited almost nonoverlapping geographic distributions, we performed a separate analysis of genetic structure for each clade. Within each clade, we distinguished broad geographical regions, and smaller geographically homogenous areas within them were designated as populations composed of sampled sites (Fig. 1). To evaluate whether the increase in the level of within-population variation was not an artefact of pooling geographically proximal localities into populations, we also performed AMOVA on a subset of localities, for which at least three sequences were available; here, regions were delineated as in the first AMOVA analysis, and localities were designated as populations.

To detect maximally differentiated groups of populations, we ran spatial analysis of molecular variance in the SAMOVA 1.0 (Dupanloup *et al.* 2002) program with 100 simulated annealing processes. We explored values of K (number of groups) from 2 to 15. In the analysis, we only included sites with three or more individuals. We selected a K value for which F_{CT} (corresponding to among-groups variation) reached a plateau (i.e. did not increase considerably with increasing K beyond that value).

Genetic landscape shapes were generated with Alleles in Space (AIS) software (Miller 2005) to visualize the 'genetic boundaries' marking the abrupt transition between populations and groups of populations characterized by divergent mtDNA. The program calculates genetic distance between sampling sites, which are connected into a network based on the Delaunay Triangulation. The values of genetic distance, referred to as 'surface heights', are set in the midpoints of each connection in the network. Raw genetic distances acquired from the program were interpolated afterwards. The 'height' values, with their respective latitude and longitude coordinates, were then imported into the ArcGIS program. We used spatial analyst extension to produce genetic diversity surface image with the inverse distance weighted (IDW) algorithm, which were plotted onto a map of Europe, where altitude reflects the genetic distance between population pairs.

To discriminate between processes of demographic expansion and purifying selection, which both result in an excess of rare alleles, we applied a heterogeneity test presented by Hahn *et al.* (2002). It utilizes neutrality tests such as Tajima's D and Fu and Li's D and compares the values of these statistics performed on synonymous and nonsynonymous mutations. The program is available on the website: http://sites.bio.indiana.edu/~hahnlab/Software.html. We also applied McDonald & Kreitman's (1991) test, which compares the ratio of synonymous and nonsynonymous changes in fixed and polymorphic mutations.

Isolation with migration analysis

The isolation with migration (IM) model of population divergence was used to evaluate the level of gene flow between the Carpathian (C) and the Balkan (B) populations. This model assumes two descendent population split from a single ancestral population t generations ago, and since then, the populations may, or may not, have been subject to gene exchange (Hey & Nielsen 2004). The IMa2 program (Hey 2010) uses information from multilocus sequence data and performs Markov chain Monte Carlo (MCMC) sampling of gene genealogies to estimate six parameters: effective ancestral population size ($\theta_A = 4N_e\mu$), effective population sizes of descendent populations (θ_1 and θ_2), time of divergence (t) and migration rates between descendent populations in either direction (m_1 and m_2). We analysed all *Rag-1* ($n = 126$) and *Ncx-1* ($n = 61$) sequences and randomly sampled 60 (30 per group) sequences from *cyt b*. The sampling procedure was repeated five times, and we created five data sets comprising the three loci. Because within locus recombination can violate parameter estimations, we analysed only maximally informative blocks of nonrecombining sequences for individuals obtained in the IMgc program (Woerner *et al.* 2007). We ran MCMC under HKY model of sequence evolution, using a geometric heating scheme, 100 heated chains and burn-in of 100 000 steps. The upper bounds of priors were defined experimentally during shorter initial runs. To ensure that the MCMC converged to its stationary distribution, we monitored effective sample size (ESS) values of parameter t, trend-line plots of the parameter and swapping rates between chains. We analysed each of the data sets at least twice in two independent runs (up to 7.5 million steps). We applied likelihood ratio test to verify significance of the estimated migration rates.

Coalescent simulations

We applied coalescent simulations in an attempt to distinguish between two hypotheses regarding the number of *B. bombina* glacial refugia: (1) two populations of *B. bombina* (reflecting two genetically separate regions revealed by AMOVA) diverged around 120 000 years ago, survived the last glacial maximum (LGM) in separate refugia and colonized Europe, with one population following the Danube and its northern tributary the Prut

River (southern population) (Arntzen 1978) and the other expanding along the Dniester, Southern Bug, Dnieper and Don River (northern population) and (2) all populations persisted the LGM in one refugium at the Black Sea coast and diverged into the southern and northern populations after the LGM around 12 000 years ago.

To test the two competing hypotheses, we used the program SIMCOAL 2.1.2 (Laval & Excoffier 2004) and performed coalescent simulations for *cyt b* and *Ncx-1*. *Rag-1* was not included because it did not show substantial variation. The program generates a coalescent history for a set of sequences and simulates genetic diversity under different demographic parameters such as deme size N (where $\theta = 2N_e u$, and N_e is an effective haploid population size), time of split (t) and migration (m) between demes. For each model, we applied a wide range of parameter values for ancestral population size (N_A), severity of the bottleneck (N_B), duration of the bottleneck (t_B) and mutation rate (u). We calculated θ in Arlequin and estimated the effective population size for southern (N_S) and northern (N_N) populations for each marker. Time of divergence was set to 4000 generations in the model with one refugium and 40 000 generations in the model with two refugia, assuming the generation time for *Bombina* is 3 years (Szymura 1998; Gollmann & Gollmann 2002). We also assumed a bottleneck event preceding the expansion from the refugia at about 4000 generations ago. To discriminate between the two scenarios of population divergence, we ran several simulations under each model with 100 iterations exploring a wide range of parameter estimates, including ancestral population size (N_A), population sizes during bottleneck (N_B), duration of the bottleneck (t_B) and mutation rate (u). We checked different values of N_A, N_B and t_d ranging from 10 000 to 1 000 000, from around 100 to no bottleneck and from 50 to 500 generations, respectively. The mutation rate for *cyt b* in Anura ranged from 0.7 to 1.8×10^{-8} per site per year, according to the literature (Babik *et al.* 2004; Jang-Liaw *et al.* 2008). In simulations, we applied the minimum, maximum and universal mtDNA mutation rate calibration of 1×10^{-8}. For *Ncx-1*, we used the mutation rate estimated for about 1×10^{-9} by Roelants & Bossuyt (2005). To evaluate how the simulated sequences correspond with the observed data, we sampled 10 sequences from each population and compared the distribution of the F_{ST} statistic under two scenarios of one or two refugia with the actual values. Statistical significance was based on 1000 simulations.

Results

In this study, we obtained 586 new *cyt b* sequences, which, together with those reported by Hofman *et al.* (2007), increased the number of available *B. bombina* and *B. variegata* sequences to 950 (Fig. S1a, Supporting information). We sequenced 160 copies of *Ncx-1* fragment and 222 copies of *Rag-1* fragment as well (Figs. S1b,c, Supporting information). Detailed information about sequence variation can be found in Table S2 (Supporting information). For new *cyt b*, *Ncx-1* and *Rag-1* haplotypes, see GenBank accession nos. JF898320–JF898442, JF898443–JF898463 and JF898464–JF898491, respectively.

mtDNA

Division into main mtDNA clades is shown in Fig. 2, and networks of mtDNA haplotypes are presented in Fig. 3. Some of the newly acquired mtDNA sequences fell beyond the previously delineated clades yet did not form distinct clades.

In *B. bombina*, only clade B3-2 was present over vast expanses of the Eastern European lowlands. The B3-1 clade had a more southern distribution and occurred along the Danube in the Carpathian Basin and on the western and northwestern shores of the Black Sea. It extended farther to the north along the Dnieper to the western part of Belarus and to the south along Morava

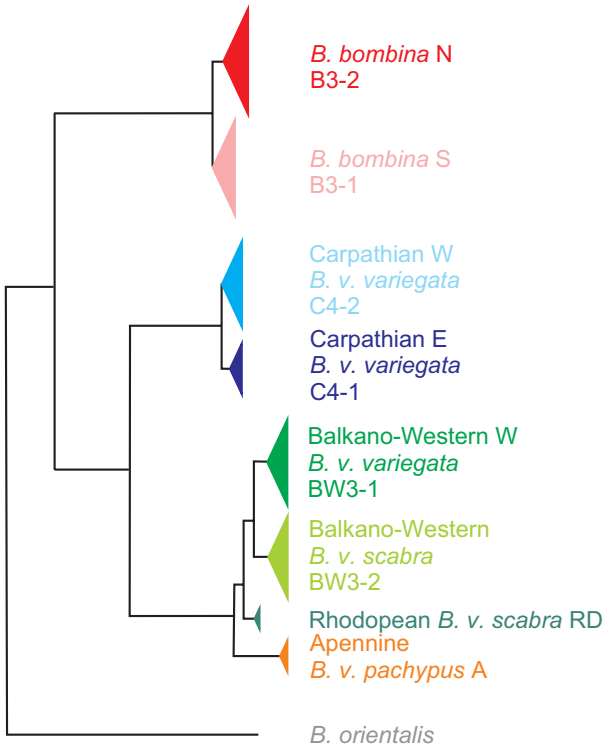

Fig. 2 Two major mtDNA clades distinguished for haplotypes of *Bombina variegata* and one for *Bombina bombina*. Topology and branch lengths are based on maximum-likelihood analysis. The tree was rooted by using the East Asian *B. orientalis*.

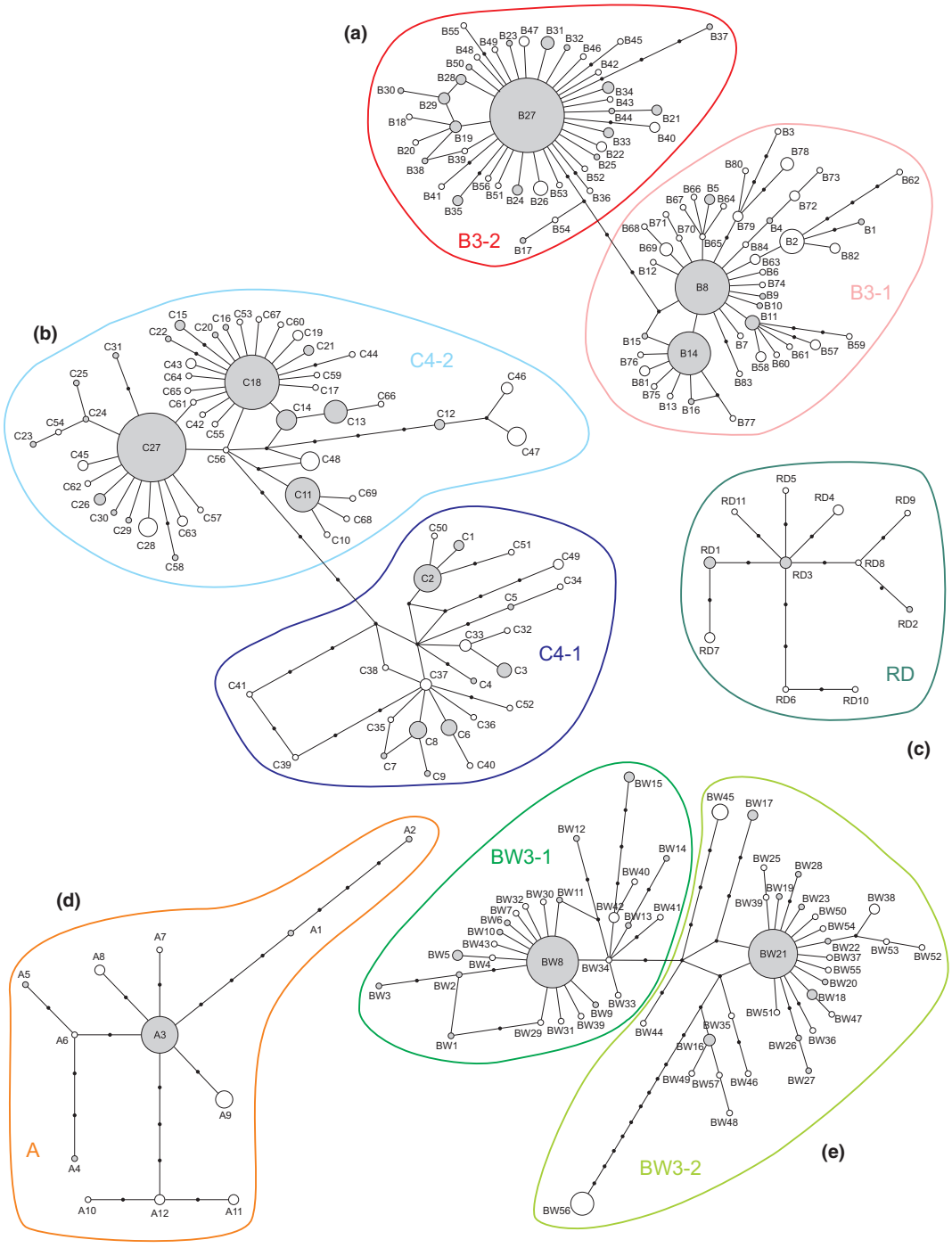

Fig. 3 Maximum parsimony network of mtDNA haplotypes for the *Bombina bombina* clade (a), Carpathian *Bombina variegata* clade (b), Rhodopean *B. variegata* clade (c), Apennine *B. variegata* clade (d) and Balkano-Western *B. variegata* clade (e). Circle size indicates haplotype abundance, and straight lines and dots reflect mutations and median vectors, respectively. Grey circles refer to the haplotypes discovered by Hofman *et al.* (2007), and empty circles represent the new haplotypes found in this study. Coloured lines delimit larger mtDNA clades. Colours match Fig. 2.

River where it was surrounded by *B. variegata* populations. Although the two clades were separated in their western extreme by the Sudety Mountains, a few B3-2 haplotypes penetrated into northern Bohemia along the Elbe River and perhaps meet the southern B3-1 clade (Fig. S1a, Table S1, Supporting information) in accordance with one of the hypotheses suggested by Arntzen (1978). Haplotypes from the B3-2 clade were detected in

a previously unsampled area at the northwest Black Sea coast, postulated as a location for two *B. bombina* glacial refugia (Hofman *et al.* 2007).

The major Carpathian and Balkano-Western clades in *B. variegata* were previously thought to have nonoverlapping distributions. Yet, populations in their geographic proximity share both types of mtDNA. These populations (Caraşova, Poiana Muşuroane, and Şvinita; sites 177, 178, and 253, respectively; Fig. S1a, Table S1, Supporting information) are located north of the Danube in the southwest Carpathians.

New data revealed that the Rhodopean clade (belonging to the Balkano-Western lineage, Fig. 2), previously known to be geographically restricted to mountainous regions of southeastern Bulgaria, reached the northeastern parts of Greece.

The southern margins of the *B. v. scabra* range in Greece possessed distinct haplotypes clustering with the major Balkano-Western clade. *B. bombina* had the highest nucleotide and haplotype diversities in the region of the Black Sea, and *B. variegata* showed the highest DNA variability in the putative refugial areas of the southern Carpathians and Balkan Peninsula (Table 1). Significantly low Tajima's *D* values were found in the populations north of the putative refugia (Table 1). Low genetic variation was detected in Western Europe, the northern Carpathians and the eastern part of the *B. bombina* distribution. Surprisingly, there was no clear sign of variation loss in *B. bombina* within the Carpathian Basin, the southwestern *bombina* population, which penetrated the narrows of the Iron Gate up along the Danube River valley. The southernmost Apennine populations of *B. v. pachypus* were characterized by high haplotype but low nucleotide diversity.

The map of genetic landscape shapes for *B. bombina* showed an increase in genetic diversity around the Black Sea region (Fig. 4a). A few places situated north of the Black Sea were characterized by substantially higher variation, which may be interpreted as an overlap of two distinct haplogroups, B3-1 and B3-2. The southern lineage (B3-1) apparently extended westward, following the Danube River and its tributaries (the southern Morava). The ridge of differentiation between the haplogroups ran from the southern to western Carpathians and then instead of continuing along the Sudety Mountains, turned south across the Bohemian Uplands, separating three *B. bombina* sites with northern clade affinities. The areas located at the western and eastern limits of the *B. bombina* distribution were represented as 'plains'. In the map of genetic landscape shapes for *B. variegata*, the highest 'mountain range' separated the two most distinct clades: the Carpathian and Balkano-Western clades (Fig. 4b). Two other highly divergent belts separated the Apennine Peninsula and Rhodope Mountains, each with distinct mtDNA lineages (A and RD, respectively; Fig. 2). Separate maps focusing on either the Balkan Peninsula and Western Europe (Fig. 4c) or the Carpathians (Fig. 4d) showed that the highest divergence was found in their southern regions.

Nuclear DNA

Overall nucleotide variation given by molecular analyses and haplotype network reconstruction in the *Ncx-1*

Table 1 Estimates of *cyt b* genetic diversity: number of segregating sites (*S*), percentage of nucleotide diversity (% π) and haplotype diversity (H_d) with the estimated standard deviation (SD) and tests for demographic expansion accounted for *n* individuals in geographic groups

Geographic group	*n*	*S*	% π ± %SD	H_d ± SD	Tajima's *D*	Fu's F_s
bombina N-nBS	150	46	0.12 ± 0.20	0.564 ± 0.050	−2.55*	−28.61*
bombina N-BS	21	12	0.29 ± 0.06	0.705 ± 0.095	−0.14	0.32
bombina N-W	141	47	0.20 ± 0.03	0.618 ± 0.049	−2.28*	−27.12*
bombina N-E	40	14	0.08 ± 0.02	0.479 ± 0.099	−2.35*	−10.61*
bombina S-nBS	120	26	0.15 ± 0.02	0.756 ± 0.026	−1.92*	−13.11*
bombina S-BS	54	30	0.22 ± 0.03	0.828 ± 0.051	−2.06*	−18.48*
bombina S-W	104	14	0.12 ± 0.01	0.712 ± 0.028	−1.38	−7.91*
bombina S-E	61	30	0.22 ± 0.03	0.813 ± 0.050	−2.03*	−19.54*
variegata W	46	8	0.04 ± 0.01	0.356 ± 0.091	−2.06*	−8.63*
variegata B	143	64	0.52 ± 0.04	0.842 ± 0.025	−1.57*	−24.72*
variegata C-N	157	29	0.14 ± 0.01	0.644 ± 0.036	−2.03*	−19.34*
variegata C-S	170	53	0.54 ± 0.02	0.933 ± 0.009	−1.09	−18.30*
variegata A-N	43	13	0.10 ± 0.03	0.533 ± 0.087	−1.99*	−4.14*
variegata A-S	5	2	0.09 ± 0.02	0.800 ± 0.164	0.24	−0.48

N, North; S, South; W, West; E, East; B, Balkans; C, Carpathians; A, Apennines; BS, regions in the vicinity of the Black Sea; nBS, regions distant from the Black Sea. * $P < 0.05$.

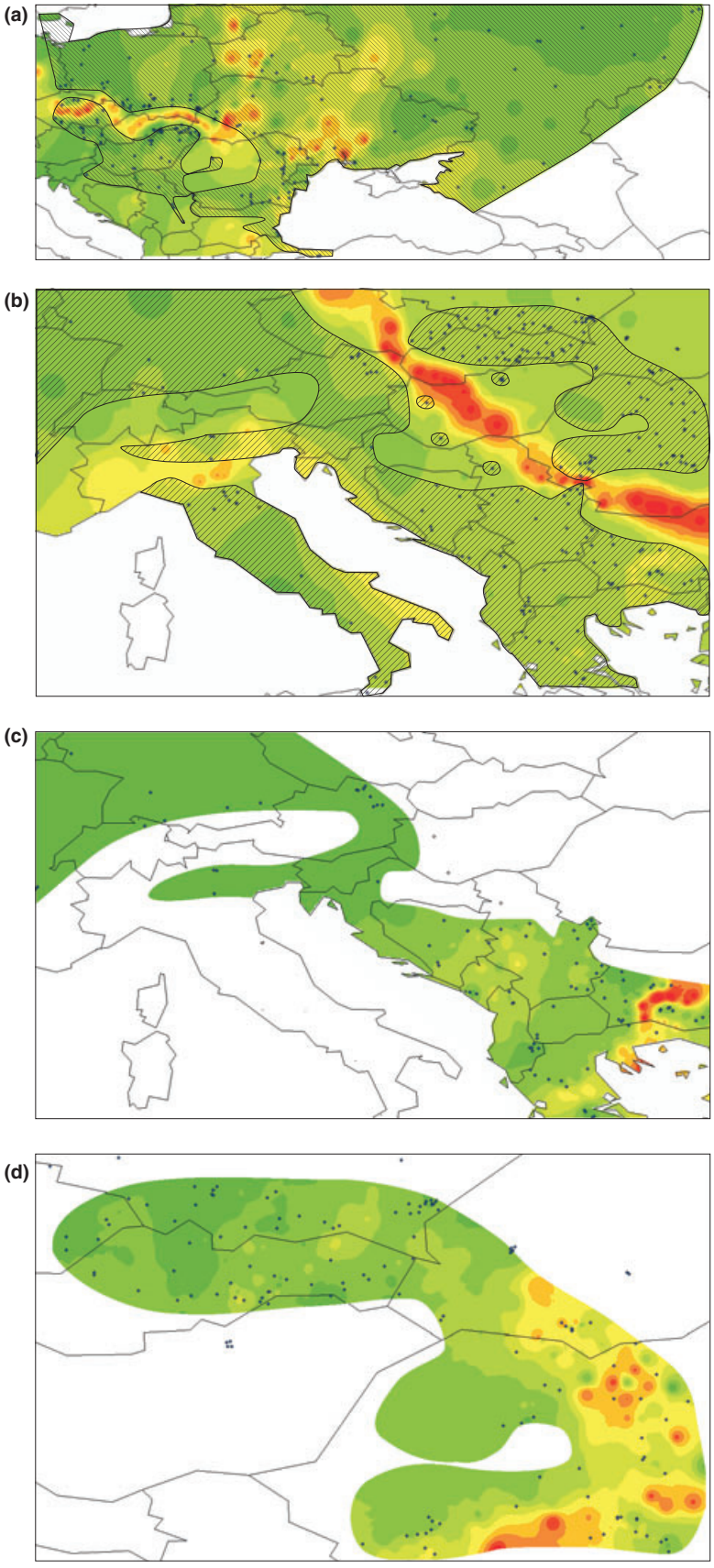

Fig. 4 Genetic landscape maps based on mtDNA overlaid onto a map of Europe. Maps were generated for *Bombina bombina* (a), *Bombina variegata* (b), *B. variegata* in the Balkans and Western Europe (c) and *B. variegata* in the Carpathians (d). Higher altitudes in the map (red colour) reflect high genetic distance between neighbouring localities, and lower altitudes (green colour) correspond to areas of decreased genetic diversity. Dashed line areas (a, b) show the distribution of *Bombina* species in the study area.

and *Rag-1* markers was low. Substantial genetic diversity was found only in the southern Carpathians and Balkans (Table 2 and Fig. 5). In contrast, northern Italy and Western Europe were each occupied by lineages with a single haplotype per locus. Reconstruction of haplotype networks distinguished three *B. variegata* groups at both markers. The group inhabiting the area of the Apennine Peninsula, Balkans and Carpathians was represented by the R11–R19 haplotypes of *Rag-1* and the N4–N10 haplotypes of *Ncx-1*. Alleles R1–R10 and N1–N3 were restricted to the Balkans with an exception (R5), whereas alleles R21–R24 and N13–N15 were mainly found in the Carpathians and Western Europe.

Bombina bombina had substantially lower genetic variability than *B. variegata* in both markers. We found no variation in *Rag-1*, except for one rare variant found in the east. *Ncx-1* was represented by three closely related variants, N17–N19. Haplotype N18 was widespread, mainly in northeastern Europe, whereas N17 and N19 were constrained to the Danube valley and lowlands west of the Black Sea. The latter variant followed the course of the Dnieper River in a manner similar to the B3-1 mtDNA clade.

Genetic structure of populations

We assessed genetic structure using two approaches: (i) by measuring the distribution of genetic variation within and among predefined populations and population groups within the AMOVA framework and (ii) by using SAMOVA to identify homogenous and maximally differentiated groups of localities. Grouping of localities into regions and regional populations is shown in Fig. 1, and the results of AMOVAs are shown in Table 3.

In *B. bombina* and Balkano-Western *B. variegata*, most of the mtDNA variation was partitioned between geographic regions; the variation among populations within regions explained only a small fraction of the variation observed, and a substantial portion of the variation was distributed within populations (Table 3). In contrast, no significant differentiation was detected on a regional scale in the Carpathians, where most of the variation was distributed within populations (Table 3). Analyses in which individual localities were treated as populations gave essentially identical results for *B. bombina* and the Carpathian *B. variegata* (Table 3), indicating that substantial within-population variation was not an artefact of pooling localities into populations, but accurately reflected the high level of haplotype diversity within single sampling sites. In Balkano-Western *B. variegata*, the variation among populations was more pronounced than that within populations.

Additional insights were gained from the inspection of the matrix of pairwise F_{ST} between populations (Table S3a–c, Supporting information). In all three clades, differentiation among most populations in the northern regions was nonsignificant, whereas relatively high and significant F_{ST} values were detected among the southern populations.

The results of SAMOVA for each group are presented in Table 4 and Fig. S2 (Supporting information). In

Table 2 Estimates of *Ncx-1 and Rag-1* genetic diversity: number of segregating sites (*S*), percentage of nucleotide diversity (% π) and haplotype diversity (H_d) with the estimated standard deviation (SD) accounted for *n* individuals in geographic groups

	Ncx-1				*Rag-1*			
Geographic group	*n*	*S*	% π ± %SD	H_d ± SD	*n*	*S*	% π ± %SD	H_d ± SD
bombina N-BS	30	1	0.05 ± 0.01	0.370 ± 0.084	22	3	0.03 ± 0.02	0.091 ± 0.081
bombina N-BS	14	2	0.06 ± 0.02	0.385 ± 0.149	10	3	0.16 ± 0.02	0.556 ± 0.075
bombina N-W	22	2	0.08 ± 0.01	0.558 ± 0.057	12	0	0.00	0.000
bombina N-E	22	0	0.00	0.000	20	3	0.12 ± 0.02	0.442 ± 0.087
bombina SnBS	23	1	0.06 ± 0.01	0.403 ± 0.091	16	0	0.00	0.000
bombina S-BS	12	2	0.15 ± 0.02	0.530 ± 0.076	14	0	0.00	0.000
bombina S-W	17	1	0.05 ± 0.02	0.382 ± 0.113	12	0	0.00	0.000
bombina S-E	18	2	0.13 ± 0.02	0.569 ± 0.096	18	0	0.00	0.000
variegata W	8	0	0.00	0.000	20	0	0.00	0.000
variegata B	28	12	0.60 ± 0.03	0.796 ± 0.053	52	21	0.49 ± 0.02	0.918 ± 0.021
variegata C-N	11	5	0.34 ± 0.04	0.636 ± 0.089	16	6	0.13 ± 0.07	0.233 ± 0.126
variegata C-S	26	7	0.36 ± 0.06	0.594 ± 0.095	62	14	0.28 ± 0.03	0.665 ± 0.038
variegata A-N	4	0	0.00	0.000	8	0	0.00	0.000
variegata A-S	4	1	0.07 ± 0.04	0.500 ± 0.265	2	1	0.09 ± 0.05	1.000 ± 0.500

N, North; S, South; W, West; E, East; B, Balkans; C, Carpathians; A, Apennines; BS, regions in the vicinity of the Black Sea; nBS, regions distant from the Black Sea.

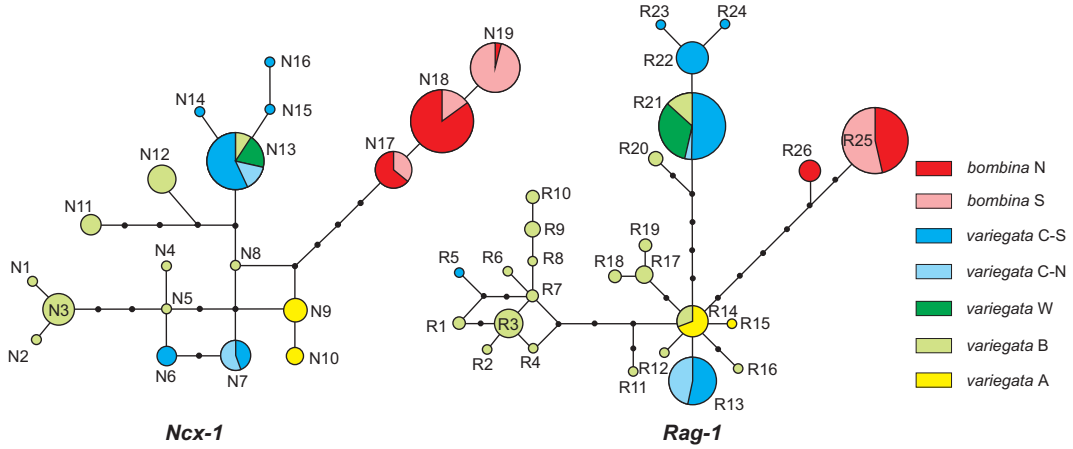

Fig. 5 Median-joining haplotype networks for *Ncx-1* and *Rag-1* in *Bombina bombina* and *Bombina variegata*. Under the assumption that homoplasy reflects recombination, loops in the network indicate where different DNA segments had different phylogenetic histories. Circle size represents haplotype abundance. Black dot represents one mutation step. The colour assigns haplotypes to a geographical location.

Table 3 AMOVA—percentage of variation in three mtDNA clades: *Bombina bombina* (Bb), *Bombina variegata* Carpathian (C) and Balkano-Western (BW), estimated among groups, among populations within groups and within populations

	Bb†	Bb‡	C†	C‡	BW†	BW‡
Among groups	65.47*	69.37***	7.14	13.69***	76.74*	78.96***
Among populations within groups	6.68***	12.00***	19.04***	19.47***	6.60***	18.28***
Within populations	27.85***	18.62***	73.82***	66.84***	16.65***	2.76***

†Geographical regions within groups designated as populations.
‡Single locations comprising three or more individuals designated as populations.
Statistical significance *$P < 0.05$, ***$P < 0.001$.

B. bombina, F_{CT} equalled 0.73 when number of groups, $K = 3$, and it stabilized for higher values of K. Two of these groups were located in the northern and southern part of the *B. bombina* distribution, respectively, whereas the third group constituted a single allopatric site in Turkey with four unique haplotypes. In the Carpathian *B. variegata*, F_{CT} reached 0.42 when $K = 5$. One major group prevailed over the whole Carpathian area, whereas the four other groups were smaller and restricted to the peripheries in the southeast and southwest of the Carpathians. In the Balkano-Western *B. variegata*, the F_{CT} values plateaued at 0.95 when $K = 5$. Three widespread groups were distributed in the Apennines, Western Europe and most of the Balkan Peninsula, whereas two smaller local groups inhabited the Rhodope Mountains in the east and the Parnassus in the south.

AMOVA failed to reveal well-defined geographical structuring of nuclear sequences (Table S4, Supporting information). We computed pairwise F_{ST} for *Rag-1* and *Ncx-1* between regional populations delineated as for mtDNA (Fig. 1). Results are presented in Table S5a–c (Supporting information). Comparison of *B. bombina* populations for *Ncx-1* (*Rag-1* was not considered here as it shows only minimal genetic variation) revealed high F_{ST} values between *B. bombina* populations 2 and 3 in the north, and these were significantly different from *B. bombina* populations 4 and 5 in the south. The Carpathian populations of *B. variegata* were not significantly different from each other, except for the most geographically distant groups of the *Rag-1* gene. Significant F_{ST} values were found among almost all of the populations from three regions: Western Europe, the Balkans and the Apennine Peninsula. Only the northern part of the Balkan Peninsula was genetically indistinct from the Western European toads.

To rule out the possibility of selection on the three loci, we conducted heterogeneity tests on three groups: *B. bombina*, Carpathian and Balkano-Western (Table 5). The test was significant for *cyt b* in the Balkano-Western group, suggesting a weak purifying selection on slightly deleterious alleles. It was also significant in the

Table 4 SAMOVA—percentage of variation in three mtDNA clades: *Bombina bombina* (Bb), and *Bombina variegata* Carpathian (C) and Balkano-Western (BW), estimated among groups, among populations within groups and within populations calculated for *cyt b*, where *K* is a number of groups

	Bb ($K = 3$)	C ($K = 5$)	BW ($K = 5$)
Among groups	72.81**	41.65**	95.02**
Among populations within groups	7.42**	– 4.12*	2.68**
Within populations	19.76**	62.48**	2.30**

Statistical significance *$P < 0.05$, **$P < 0.001$.

Carpathian group for the *Rag-1* locus and *cyt b*, but only when performed with Fu and Li's statistic. McDonald and Kreitman's test only rejected the hypothesis of neutrality for *cyt b* (Table S6, Supporting information).

Gene flow between the Carpathian and Balkan B. variegata

All analysed data sets and independent runs gave similar results indicating that MCMC converged to stationary distribution and that sampling procedure had no effect on parameter estimates. The estimated marginal posterior distribution curves and maximum-likelihood estimates, together with the lower and upper bounds of the estimated 95% highest posterior density (HPD) intervals of all model parameters, are given in Figs 6 and 7 and Table 6. The estimates of θ for two *Bombina* groups amounted to 3.47 (2.09–5.57) and 7.63 (5.00–11.41) for C and B group, respectively, indicating that the population size of the Balkan group was two times larger than that of the Carpathian group. The migration rate from B to C group equalled 0.148 (0.025–0.442) and 0.106 (0.000–0.340) in the opposite direction. The HPD interval of migration rate from the C group includes zero, implying no gene flow from this direction. Indeed, the likelihood ratio test did not reject the hypothesis of zero migration from C to B group, but confirmed significant gene flow into the Carpathians. Other parameter estimates had large confidence intervals precluding the reliable estimation of divergence time and ancestral population size.

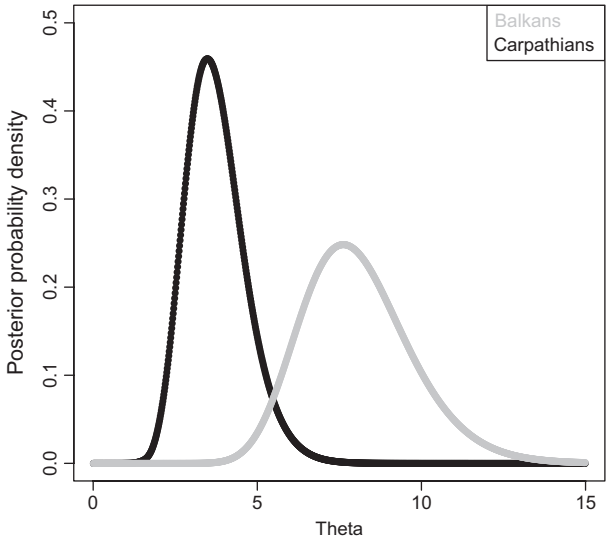

Fig. 6 Posterior probability distribution of θ values for Carpathian and Balkan populations of *Bombina variegata* estimated by using *cyt b*, *Ncx-1* and *Rag-1* sequences.

Table 5 Neutrality tests calculated for only synonymous and only replacement mutations data sets and significance values from heterogeneity test carried out on the three mtDNA clades: *Bombina bombina* (Bb), and *Bombina variegata* Carpathian (C) and Balkano-Western (BW) for three DNA markers

	cyt b			*Ncx-1*			*Rag-1*		
	n	Tajima	Fu & Li	*n*	Tajima	Fu & Li	*n*	Tajima	Fu & Li
Bb									
Synonymous	62	−1.77*	−4.89**	2	1.31	0.7	2	−0.28	0.73
Replacement	25	−2.12**	−6.38**	0			1	−0.21	0.52
P-value		0.301	0.095					0.495	0.689
C									
Synonymous	52	−1.46	−1.71	8	0.8	0.65	11	1.31	−1.10
Replacement	22	−2.29*	−6.50*	0			3	−1.64	−3.28*
P-value		0.112	0.001					0.002	0.03
BW									
Synonymous	103	0.4	−0.36	13	1.28	0.52	17	0.17	0.22
Replacement	28	−1.45	−2.33*	1	−0.6	0.55	5	0.60	0.02
P-value		0.004	0.036		0.066	0.572		0.689	0.498

Statistical significance *$P < 0.05$, **$P < 0.01$.

Table 6 Maximum-likelihood estimates (MLE), lower (HPD95Lo) and higher (HPD95Hi) bound of the estimated 95% highest posterior density interval for population sizes and migration rates calculated for Carpathian (C) and Balkan (B) populations of *Bombina variegata*

	θ_C	θ_B	$m_{B>C}$	$m_{C>B}$
MLE	3.473	7.628	0.1485	0.1065
HPD95Lo	2.092	5.003	0.0255	0
HPD95Hi	5.572	11.41	0.4425	0.3405

θ_C, Theta for Carpathian population; θ_B, Theta for Balkan population; $m_{B>C}$, migration rate from Balkan to Carpathian population; $m_{C>B}$, migration rate from Carpathian to Balkan population.

Coalescent simulations

The present mutation-scaled effective population size of northern and southern *B. bombina* populations was estimated from the $\theta(\pi)$ value, which equalled 2.08 (northern) and 2.25 (southern) for mtDNA and 0.38 (northern) and 0.69 (southern) for *Ncx-1*. F_{ST} between the observed northern and southern populations was 0.67 for mtDNA and 0.45 for *Ncx-1*. For all parameter sets, the F_{ST} distributions were not distinguishable between the competing hypotheses. Thus, depending on the parameter values, both models had to be either rejected or accepted. Results of several simulations are presented in Table S7 (Supporting information). In general, the change in the ancestral population size and mutation rate had a negligible effect on the F_{ST} values, whereas the strongest influence on genetic diversity was generated by a strong and sufficiently long bottleneck. Therefore, our data do not allow for distinction between the one or two refugia hypotheses for *B. bombina*.

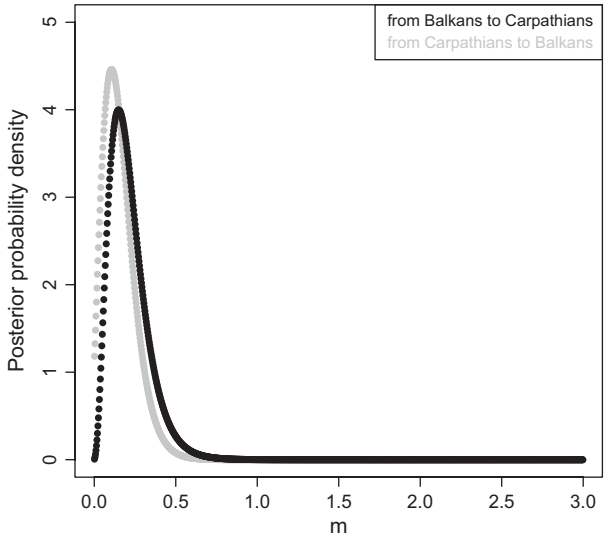

Fig. 7 Posterior probability distribution of migration rates between Carpathian and Balkan populations of *Bombina variegata* estimated by using *cyt b*, *Ncx-1* and *Rag-1* sequences.

Discussion

This study provided a comprehensive reconstruction of the phylogeographic patterns of the European *Bombina* species, by using substantially extended mtDNA sampling and incorporating sequences of two nuclear genes. First, no evidence for gene flow was detected between *B. bombina* and *B. variegata* outside the narrow hybrid zones. Second, new data have provided unequivocal support for the hypothesis that the Carpathians and lowlands northwest of the Black Sea constituted important refugial areas for *Bombina* toads during glacial maxima. We also identified additional refugia located in the southern Apennines and the Balkans, typical refugial areas postulated for multiple species (Schmitt 2007; Varga 2010). In contrast, no evidence for refugia located in other east European plains has been found. The distribution of mtDNA and *Ncx-1* variation in *B. bombina* in the Black Sea region suggests the presence of two local refugia there; however, coalescent simulations did not allow us to distinguish between the one and two refugia hypotheses. Strong genetic drift apparently accompanied postglacial expansions, as evidenced by reduced genetic diversity in the areas of putative expansion of both species. Although the general pattern shows the presence of two almost nonoverlapping *B. variegata* populations, one situated in the Carpathians and another situated in the Balkans, we presumably found postglacial gene flow into the Carpathians, as estimated by IM.

Genetic diversity in glacial refugia and postglacial colonization

A very dense sampling and incorporation of nuclear markers enabled us to test hypothesis concerning location of glacial refugia postulated by Hofman *et al.* (2007), by examining patterns of genetic diversity throughout the entire distribution ranges of *B. bombina* and *B. variegata*. With particularly extended sampling in the putative glacial refugia, we were able to capture the actual features of the distribution and structuring of genetic variation in refugial areas. In spite of phylogeographic incongruence between the analysed markers, all proved useful in detecting areas of increased genetic diversity.

The Carpathian region has been regarded as an extra-Mediterranean ice-age refugium for 'continental species' according to the classification presented by Schmitt

(2007). This region was suggested as a glacial refugium for several species (Provan & Bennett 2008), including *B. variegata*, although previous bio- and phylogeographic studies (Arntzen 1978, Szymura 1993; Hofman *et al.* 2007) consider the Balkan Peninsula an essential Pleistocene refugium for this species. The highest nucleotide diversity we found in the southeastern Carpathians in both *cyt b* and *Rag-1*, as well as allozymes (Szymura 1998), indeed supports the importance of the southeastern Carpathians as a source area for subsequent colonization. Nucleotide diversity of *cyt b* in the Balkans is similar to that in the Carpathians. Nuclear genes displayed exceptionally high diversity in the Balkan toads. In *B. v. pachypus*, generally, nucleotide diversity was very low, suggesting that these toads have expanded from a small population. Variation in nuclear sequences was limited to the southern part of the Apennines, consistent with the location of a separate refugium there, a view also supported by allozyme data (Szymura 1998; Canestrelli *et al.* 2006).

The centre of *B. bombina* genetic diversity is located in the lowlands adjacent to the Black Sea, a refugial area for Mediterranean fauna (Schmitt 2007). Although it was not possible to determine whether one or two refugia were present there, the western and northwestern shores of the Black Sea are very likely the only areas where this species survived glaciations. Several studies support the existence of amphibian refugia in the Ponto-Caspian basin (Tarkhnishvili *et al.* 2000; Veith *et al.* 2003; Babik *et al.* 2005; Gvoždík *et al.* 2010), but they were all confined southwards to the Anatolia or the Caucasus. One exception appears to be the spadefoot toad, whose eastern lineage could have originated east of the Dnieper River delta (Crottini *et al.* 2007). Terrestrial ectothermic species distributed farther to the east such as the moor frog (Babik *et al.* 2004) or adder (Ursenbacher *et al.* 2006) had their refugia either near the Carpathians or in southern Russia. Therefore, western and northwestern Black Sea lowstand shores, together with paleodeltas, represent newly recognized northern glacial refugia for semi-aquatic animals.

There are two common consequences of expansion from a single refugium: (i) the lack of genetic structure in the expansion area, as a result of the relatively recent common origin of populations, and (ii) a the relative excess of rare nucleotide variants that confirms demographic expansion.

Patterns of both weak genetic structure and relative excess of rare variants were observed in all but one (southern *B. bombina*) putative expansion area. There was a gradient of decreasing genetic diversity along each putative migration route, and populations distant from glacial refugia were characterized by low F_{ST} values across large areas. A significant excess of rare polymorphisms was detected in most of the examined regions except for the southern (B3-1) lineage of *B. bombina*, where no clear pattern of expansion along the Danube could be observed. In *B. variegata*, high differentiation among the populations in the Balkans and Carpathians suggests the presence of 'refugia within refugia' (Gómez & Lunt 2006). The Balkan region was characterized by strong differentiation among localities, and we distinguished several groups with SAMOVA. One such differentiation centre, not detected in previous studies, was a small range in southern Greece where distinct *cyt b* and *Rag-1* haplotypes were revealed. A similar pattern applied to the southern Carpathians, although high within-population variation in some localities may also be attributed to a mixing of the two distinct clades, Balkano-Western and Carpathian.

Genetic variation in B. variegata *and testing gene flow between the Carpathian and Balkan populations*

The phylogeographic relationships of mitochondrial and nuclear markers in *B. variegata* conflict in some respects. The population size of nuclear genes is larger than mtDNA, making genetic drift a weaker force in shaping genetic structure; hence, polyphyletic relationships in nuclear markers are not rare. Sharing similar haplotypes between different populations may be attributed to ancestral polymorphisms, which failed to sort out and have persisted together with less-related alleles (Maddison 1997; Degnan & Rosenberg 2009). A similar picture can also result from recent migration processes between previously isolated populations (Pinho & Hey 2010).

The clearest manifestation of this discordance is the discrepancy between mitochondrial and nuclear markers in populations of *B. variegata* in Western Europe. mtDNA haplotypes fall into the BW3-2 lineage present in the western Balkans, whereas nuclear alleles are shared with the populations from the Carpathians. Such distribution of nuclear diversity is consistent with the pattern revealed by allozymes (Szymura 1993, 1998). Hofman *et al.* (2007) explained the discrepancy between the mitochondrial and nuclear genomes as introgression of mtDNA from the Balkan toads into populations carrying a nuclear genome of predominantly Carpathian origin. This introgression may have occurred during or after the LGM.

Bombina variegata populations from the Balkans and Carpathians carry highly distinct mtDNA. Nevertheless, some populations in the area of the Iron Gate, which separates the Carpathians from the Balkan Mountains, harbour haplotypes from both mtDNA groups. We tested whether the observed distribution of haplotypes is a result of uni- or bidirectional gene flow between

the Balkan and Carpathian *B. variegata* population groups. IM analysis confirmed gene flow into the Carpathians; however, we did not find evidence for gene flow in the opposite direction. These results suggest a continuation of the range expansion of the two populations after LGM and corroborate the hypothesis of separate refugia in the Carpathians and Balkans. Although the IM model was designed to recover parapatric divergence with constant gene flow, a secondary contact after allopatric divergence may also generate nonzero migration estimates (Becquet & Przeworski 2009). This is probably the case in our study, because the shared haplotypes only occurred in a narrow zone centred on the Danube where the two populations overlapped.

We were unable to estimate the time of divergence, yet previous calculations based on mtDNA suggest a pre-Pleistocene split of the Balkan and Carpathian populations of *B. variegata* (Hofman *et al.* 2007). Given such an old time of divergence, as well as their current distribution and genetic distinctness, the populations appear to have largely independent histories with limited gene flow. More detailed information about the divergence process between *Bombina* species and within the major phylogeographic groups within the species may be obtained by examining a larger set of nuclear sequence markers. Here, an application of a number of relatively slow-evolving exons, with shared polymorphisms predating the split of the major lineages, could be particularly informative in this respect.

Conclusions

We identified three major glacial refugial areas for *B. variegata* distributed in the southern Carpathians, Balkan Peninsula and southern Apennines, whereas a single refugial area for *B. bombina* was located in the lowlands west and northwest of the Black Sea. From here, *B. bombina* colonized large expanses of the Central and Eastern European lowlands. If, as appears to be the case with two *Bombina* species, a vast part of Europe has been recolonized postglacially from either the Carpathians or the west and northwest Black Sea coast, then even ectothermic terrestrial animals were able to survive the glacial maxima in the northern glacial refugia. The patterns of genetic diversity and population structure show the loss of variation along the recolonization migration routes from glacial refugia towards the edge of the distribution area of the species and indicate that postglacial expansion was accompanied by strong genetic drift. The multilocus analysis of historical gene flow between the Carpathian and Balkan *B. variegata* revealed unidirectional gene flow from the Balkans northwards into the southern Carpathians.

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A.F. is a PhD student with J.M.S studying divergence of populations and species on genomic level. K.N. uses molecular tools to study the effects of demographic events, gene flow and natural selection on differentiation of populations and closely related species. S.H. is an Associate Researcher in J.M.S. laboratory focusing on sequence variation and phylogeography. S.N.L.'s research concerns mainly speciation of amphibians of Eastern Europe. W.B. is interested in historical inference from molecular markers and in understanding adaptation on the molecular level. M.S. is a PhD student at Jagiellonian University developing and applying bioinformatics tools for next generations sequencing projects. G.G. is interested in evolution and ecology of amphibians. L.C., a postdoctoral fellow at Liběchov, studies speciation, hybridization and origin of asexuality in vertebrates. D.C. is a Professor at the University Ovidius Constanta interested in the life history, evolution and conservation of European amphibians. T.V. and G.D. are a Research Associate and Principal Scientist at Institute for Biological Research, Belgrade; they are interested in evolution, phylogeny, ecology, morphology and conservation of amphibians. J.M.S. is a Professor at Jagiellonian University; his fields are hybrid zones, hybridization and speciation in amphibians.

Data accessibility

DNA Sequences: GenBank accessions: JF898320–JF898491, sampling locations, individual haplotypes and genotypes: Table S1, Data S1 (Supporting information).

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 (a–c) Sampling sites of *B. bombina* and *B. variegata* (a) *cyt b*, (b) *Ncx-1*, (c) *Rag-1*.

Fig. S2 Partitioning of sampling sites into populations with SAMOVA within the main clades of *B. bombina* and *B. variegata*.

Table S1 Sample localities used in the study are keyed to Fig. S1a–c by site number.

Table S2 Summary characteristics of the three analyzed loci.

Table S3 (a) F_{ST} values between seven *B. bombina* population groups (Bb) for the *cyt b* gene, (b) F_{ST} values between five Carpathian population groups of *B. variegata* (C) for the *cyt b* gene, (c) F_{ST} values between *B. variegata* population groups in Western Europe (W), Balkan Peninsula (B), and Apennine *B. v. pachypus* (A) for the *cyt b* gene.

Table S4 AMOVA—percentage of variation for the *Ncx-1* and *Rag-1* genes in three mtDNA clades, *B. bombina* (Bb), and Carpathian (C) and Balkano-Western (BW) *B.variegata*, estimated among groups, among populations within groups, and within populations.

Table S5 (a) F_{ST} values between six *B. bombina* population groups (Bb) for the *Ncx-1* gene. (b) F_{ST} values between four Carpathian population groups of *B. variegata* (C) for the *Ncx-1* and *Rag-1* genes. (c) F_{ST} values between *B. variegata* population groups in Western Europe (W), Balkan Peninsula (B), and Apennine *B. v. pachypus* (A) for the *Ncx-1* and *Rag-1* genes.

Table S6 McDonald and Kreitman's test—number of synonymous (S) and nonsynonymous (NS) substitutions of fixed and polymorphic mutations in *cyt b*, *Ncx-1*, and *Rag-1*, compared among three mtDNA clades: *B. bombina* (Bb), and Carpathian (C) and Balkano-Western (BW) *B. variegata*.

Table S7 Example simulation results for different values of ancestral population (N_A), mutation rate (u), and extension of bottleneck (N_B).

Data S1 Sampling sites of *Bombina* species.

Data S2 Captions of all supporting informations used.

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