

# Phylogeography of the fire-bellied toads *Bombina*: independent Pleistocene histories inferred from mitochondrial genomes

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## Abstract

The fire-bellied toads *Bombina bombina* and *Bombina variegata*, interbreed in a long, narrow zone maintained by a balance between selection and dispersal. Hybridization takes place between local, genetically differentiated groups. To quantify divergence between these groups and reconstruct their history and demography, we analysed nucleotide variation at the mitochondrial cytochrome *b* gene (1096 bp) in 364 individuals from 156 sites representing the entire range of both species. Three distinct clades with high sequence divergence ( $K2P = 8\text{--}11\%$ ) were distinguished. One clade grouped *B. bombina* haplotypes; the two other clades grouped *B. variegata* haplotypes. One *B. variegata* clade included only Carpathian individuals; the other represented *B. variegata* from the southwestern parts of its distribution: Southern and Western Europe (Balkano–Western lineage), Apennines, and the Rhodope Mountains. Differentiation between the Carpathian and Balkano–Western lineages,  $K2P \sim 8\%$ , approached interspecific divergence. Deep divergence among European *Bombina* lineages suggests their preglacial origin, and implies long and largely independent evolutionary histories of the species. Multiple glacial refugia were identified in the lowlands adjoining the Black Sea, in the Carpathians, in the Balkans, and in the Apennines. The results of the nested clade and demographic analyses suggest drastic reductions of population sizes during the last glacial period, and significant demographic growth related to postglacial colonization. Inferred history, supported by fossil evidence, demonstrates that *Bombina* ranges underwent repeated contractions and expansions. Geographical concordance between morphology, allozymes, and mtDNA shows that previous episodes of interspecific hybridization have left no detectable mtDNA introgression. Either the admixed populations went extinct, or selection against hybrids hindered mtDNA gene flow in ancient hybrid zones.

**Keywords:** *Bombina*, hybrid zones, mtDNA, phylogeography, Pleistocene refugia

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## Introduction

Multiple cycles of range contraction and expansion linked to climatic oscillations were a notable feature of the Pleistocene history of temperate and boreal species (Avice 2000; Hewitt 2004). During short interglacials, the

expanding ranges of populations previously isolated in separate refugia met and genetic exchange was possible. With the onset of the following glacial cycle, such secondary contacts disappeared at higher latitudes through extinction of the populations involved. In the milder south, contacts could have persisted or at least the populations involved in them could have existed in close proximity, meeting occasionally in response to minor climatic and environmental oscillations (Hewitt 1999, 2004).

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Analyses of contemporary transitions, or hybrid zones, between genetically differentiated population and species provide insight into processes that take place upon contacts. For any pair of taxa, genetic and environmental factors interact in a complex way, creating spatial heterogeneity of the hybrid zones at various locations (Szymura 1993; Saetre *et al.* 1999; Vines *et al.* 2003; Božíková *et al.* 2005; Hoskin *et al.* 2005). This in turn reflects the relative importance of endogenous vs. exogenous selection against hybrids along the zone (MacCallum *et al.* 1998; Vines *et al.* 2003; Yanchukov *et al.* 2006), and geographical scale (Arntzen 1996). For species that had multiple Pleistocene refugia, genetic differences between populations acquired in those refugia may be an important factor contributing to this heterogeneity. Accordingly, detailed phylogeographical information concerning the geographical distribution of genealogical lineages, the amount of genetic variation present, and the extent of divergence in various parts of the range, is indispensable for unravelling the evolutionary history of hybridizing forms and the origin of their hybrid zones.

Although clearly distinguished by morphology, anatomy, ecology, and behaviour, the fire-bellied toads *Bombina bombina* and *Bombina variegata* hybridize in a long but narrow zone wherever their parapatric ranges meet at the transition between lowlands and mountains in central Europe (Szymura 1993; Fig. 1). The zone appears to be maintained by a balance between selection and dispersal (Szymura & Barton 1986, 1991). Introgression is limited by endogenous selection resulting from negative epistatic interactions between divergent genomes (Szymura & Barton 1991; Kruuk *et al.* 1999) and exogenous, habitat-dependent selection against hybrids (Nürnberg *et al.* 1995; MacCallum *et al.* 1998). Moreover, analyses of widely separated transects (in Poland, Ukraine, Romania, Slovakia, Hungary, Austria, and Croatia) indicate that the genetic structures of the hybrid zone in its various geographical segments differ substantially (Szymura & Barton 1986, 1991; Gollmann *et al.* 1988; Szymura 1993; Gollmann 1996; MacCallum *et al.* 1998; Vines *et al.* 2003; Yanchukov *et al.* 2006). Allozyme (Szymura 1993, 1998) and limited mitochondrial DNA (mtDNA) variation data (Szymura *et al.* 2000; Spolsky *et al.* 2006; Vörös *et al.* 2006) showed that local contacts are established between a more genetically uniform lowland *B. bombina* and genetically differentiated groups of the higher altitude *B. variegata*.

The European fire-bellied toad species have broad geographical distributions with mutually exclusive ranges, which mirror complex geographical features of Central Europe (Fig. 1). In *B. bombina*, two closely related groups were delineated by allozyme variants: a northern one, inhabiting lowlands north of the Carpathian Mountains, and a southern one, distributed along the Danubian plains. These two groups probably intergrade east of the Carpathians. In *B. variegata*, four units were distinguished by allozyme studies, three of which correspond to recognized

subspecies: *Bombina variegata pachypus* from the Apennines in Italy (often regarded as a separate species; Lanza & Vanni 1991; Canestrelli *et al.* 2006); *Bombina variegata scabra* from the Balkan Peninsula; and the nominal form, *Bombina variegata variegata*, which was further divided into the Carpathian and western groups (Szymura 1993, 1998).

Digestion of *Bombina* mtDNA molecules with restriction endonucleases confirmed the distinctness of the *B. bombina* lineage. It also revealed a surprisingly large divergence within *B. v. variegata*, between the toads from the Carpathians and the rest of the range. The western haplotypes are highly similar to the haplotypes present in the Balkan *B. v. scabra*, suggesting that mtDNA transfer has occurred between these two *B. variegata* groups (Szymura *et al.* 2000; Spolsky *et al.* 2006). This apparent transfer contrasts with limited mtDNA introgression demonstrated in several transects across *Bombina* hybrid zones. Negative cytonuclear interactions and/or low female dispersal could account for this outcome (Yanchukov *et al.* 2006; Hofman & Szymura 2007). A range-wide mtDNA study in the European fire-bellied toads is obviously needed to obtain a broader perspective.

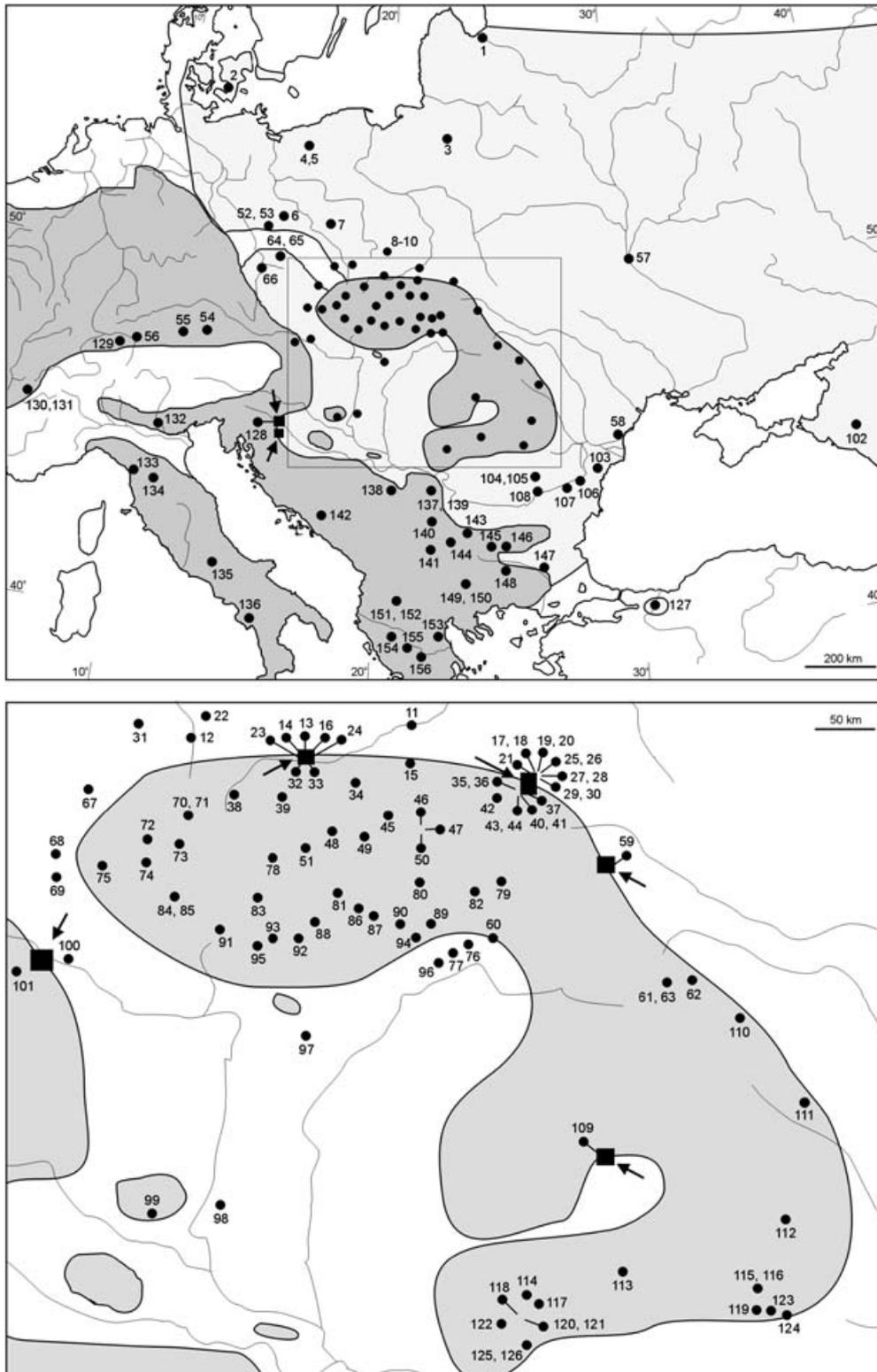
We aimed specifically to: (i) quantify the extent of nucleotide variation, identify maternal lineages present in each species, and assess their phylogenetic relationships; (ii) delineate geographical ranges of the mitochondrial lineages and pinpoint regions of contact between them; (iii) identify glacial refugia and reconstruct the routes of postglacial recolonization of Europe; (iv) assess the long-term effect of interspecific hybridization on mtDNA lineage specificity and examine allozyme–mtDNA discordance in some parts of the range of *B. variegata*; (v) estimate past population size changes in population groups as defined by major mtDNA lineages.

## Materials and methods

### Sampling, DNA extraction and sequencing

We analysed a total of 364 individuals of *Bombina bombina* and *Bombina variegata* from 156 localities representing the entire range of each species except for the easternmost range of *B. bombina* (Appendix S1, Supplementary material; Fig. 1). As outgroups, we used East Asian congeners: *Bombina orientalis*, *Bombina maxima* and *Bombina microdeladigitora*.

Total DNA was extracted from tissue biopsies, mostly toe-tips preserved in 96% ethanol, using the phenol-chloroform method. Mitochondrial cytochrome *b* (*cyt b*) sequences were obtained by amplifying and then sequencing a 1200-bp fragment with the primer pair L16245 and H17444 (Hofman & Szymura 2007) based on a cloned *B. variegata* mtDNA sequence (GenBank Accession no. AY971143). Laboratory procedures were detailed by Hofman & Szymura (2007). For all individuals, a sequence of 1096 nucleotides (beginning at codon 16) was obtained.



**Fig. 1** Location of *Bombina* sampling sites. Light and dark grey areas indicate *Bombina bombina* and *Bombina variegata* ranges, respectively. Seven intensively studied transects are marked by squares and arrows. Higher resolution of the central insert is shown in a separate map. Site numbers refer to Appendix S1.

### Phylogenetic analyses

Sequences were visually inspected and edited using BIOEDIT 7. Using MODELTEST 3.5 (Posada & Crandall 1998), we determined that the Tamura–Nei model of nucleotide substitution with rate heterogeneity (TrN +  $\Gamma$ ) was most appropriate, with the following parameter settings: nucleotide frequencies 0.266 (A), 0.310 (C), 0.137 (G), 0.286 (T); gamma shape parameter  $\alpha = 0.24$ . A maximum-likelihood (ML) tree was constructed with PHYML using Guindon & Gascuel's (2003) method. The robustness of the topology was tested with 1000 bootstrap replicates. A Bayesian tree was also constructed, using MRBAYES 3.1 (Ronquist & Huelsenbeck 2003). The likelihood settings corresponded to the general time-reversible model of sequence evolution with rate heterogeneity (GTR +  $\Gamma$ ), which is the closest approximation of the TrN +  $\Gamma$  model available in MRBAYES; priors were set to default values. Four Metropolis-coupled Monte Carlo Markov chains (three of them 'heated', temperature = 0.20) were run for  $2 \times 10^6$  generations and sampled every 1000 generations. The first half of the 'forest' was discarded as burn-in, resulting in  $10^3$  sampled trees. This analysis was repeated five times to evaluate the congruence of the likelihood values. Log-likelihood values were plotted against generation time to ensure that no trees were retained prior to the run reaching stationarity. All analyses started with trees that were randomly generated. In order to calculate the *a posteriori* probability of each bipartition, the majority-rule consensus tree was constructed over 5000 trees, representing all five runs.

For the major clades, we computed nucleotide diversities ( $\pi$ ) and net sequence divergences ( $D_a$ ) using Kimura 2-parameter distances (*K2P*) with MEGA2 2.1 (Kumar *et al.* 2001). Standard errors were obtained through 1000 bootstrap replicates. The TrN model of sequence evolution was used for the construction of the phylogenetic trees but the *K2P* model for presenting divergence estimates, because this simple correction for multiple hits has lower variance than more complex measures and is useful for comparative purposes as many studies report this measure. Also, molecular-clock calibrations used in our study (see Discussion) are based on this measure. Thus, we combine the more sophisticated model of sequence evolution chosen through the formal procedure for the phylogenetic reconstruction, with a simpler distance measure for showing sequence divergence between groups.

### Neutrality tests, nested clade analysis and demographic analyses

The conformation of our data set to neutral expectations was tested using two approaches. We applied McDonald & Kreitman's (1991) test (MK) to compare the ratio of fixed differences to polymorphisms between phylogenetic groups

for synonymous and nonsynonymous substitutions. To separate effects of demographic changes and selection on the mutation frequency spectrum, we used a modification (Hahn *et al.* 2002) of Tajima's (1989) and Fu & Li's (1993) tests (*H* test), which compares the values of the respective selection statistics for synonymous and nonsynonymous substitutions. This heterogeneity test was performed using software available from [www.bio.indiana.edu/%7Ehahnlab/Software.html](http://www.bio.indiana.edu/%7Ehahnlab/Software.html).

We combined three complementary methods to gain insight into historical demographic processes, such as population size and range changes, as well as into contemporary patterns of female-mediated gene flow. Nested clade analysis (NCA; Templeton 1998, 2004) was used to infer the patterns of dispersal and gene flow that have led to the current distribution of hierarchically nested haplotype clades within major mtDNA lineages. Coalescent-based analysis of demographic growth (Kuhner *et al.* 1998) and mismatch analysis (Schneider & Excoffier 1999) were used to estimate basic demographic parameters:  $\theta$ , the product of the female effective population size and the mutation rate;  $g$ , the exponential growth parameter; and  $\tau$ , time since the expansion.

Because the genetic distances among *B. bombina*, *B. variegata* from Carpathians, and *B. variegata* from the Balkans and Western Europe (Balkano–Western lineage) greatly exceeded the 95% parsimony limit (14 mutational steps), NCAs were performed separately for each group. Italian (Apennine) and South-East Bulgarian (Rhodopean) haplotypes were excluded from all demographic analyses because of their small sample sizes. Statistical parsimony (SP) cladograms were constructed using tcs 1.18 (Clement *et al.* 2000).

For resolving cladogram ambiguities we applied three criteria derived from coalescent theory (Pfenninger & Posada 2002). The frequency, topological, and geographical criteria state that in case of ambiguities, haplotypes are more likely to be connected to haplotypes that (i) have a higher frequency, (ii) are interior in cladograms rather than exterior, and (iii) come from the same population or region. The last criterion fits well with the limited dispersal of *Bombina* documented in Bulgaria (Beshkov & Jameson 1980), Poland (Szymura & Barton 1986), and Austria (Gollmann & Gollmann 2002). The nesting procedure was performed following the standard rules (Crandall 1996; Templeton 1998). NCA was performed using GEODIS 2.4 (Posada *et al.* 2000). Two statistics were estimated: the clade distance ( $D_c$ ), which measures the geographical spread of a clade, and nested clade distance ( $D_n$ ), which measures how a clade is geographically distributed relative to other clades in the same higher-order category. The null hypothesis of no geographical association of clades was tested by comparing  $D_c$  and  $D_n$  values with those obtained after 10 000 random permutations of clades against sampling

**Table 1** Haplotype diversity ( $h$ ) and net sequence divergence  $D_a$  between groups based on Kimura 2-parameter distance (below diagonal) and standard errors of the estimates (1000 bootstrap replicates, above diagonal). Nucleotide diversities ( $\pi$ ) within groups on the diagonal, with standard errors (1000 bootstrap replicates) in parentheses. All values are expressed as percentages

	$n$	$h$	$Bb$	$Bv$ Carp	$Bv$ BW	$Bv$ Italy	$Bv$ Rhod
$Bb$	111	0.84 (0.01)	0.39 (0.10)	0.86	1.09	1.09	1.01
$Bv$ Carp	170	0.78 (0.01)	8.98	0.30 (0.08)	0.93	1.03	0.92
$Bv$ BW	71	0.84 (0.01)	11.39	7.87	0.38 (0.11)	0.61	0.48
$Bv$ Italy	7	0.86 (0.06)	11.31	9.28	4.56	0.33 (0.10)	0.67
$Bv$ Rhod	5	0.80 (0.10)	10.33	7.89	2.45	4.97	0.09 (0.06)

$n$ , number of sequences;  $Bb$ , *B. bombina*;  $Bv$  Carp, *B. variegata* Carpathian group;  $Bv$  BW, *B. variegata* Balkano–Western group;  $Bv$  Italy, *B. v. pachypus* Italy;  $Bv$  Rhod, *B. v. scabra* Rhodopean group.

locations. Biological inferences for clades with significant geographical association were drawn from the patterns of significant distance measures using the most recent version of the NCA inference key ([http://inbio.byu.edu/faculty/kac/crandall\\_lab/computer.html](http://inbio.byu.edu/faculty/kac/crandall_lab/computer.html); Templeton 2004).

The demographic histories of the three major mtDNA clades defined by NCA nesting pattern were assessed with two other approaches. ML-based estimators of theta ( $\theta_{ML}$ ,  $\theta = 2N_f\mu$  for mitochondrial genes, where  $N_f$  is the female effective population size and  $\mu$  is the mutation rate) and exponential population growth parameters ( $g$ ) were computed jointly with LAMARC 2.02 (Kuhner *et al.* 2005). This coalescent-based method takes into account genealogical relationships among haplotypes (Kuhner *et al.* 1998). The estimates of  $\theta$  and  $g$  were obtained by Markov chain Monte Carlo searches through the genealogy space. The final estimates were based on a run of 10 short chains sampling 500 trees each and 2 long chains sampling 10 000 trees each; the sampling interval was set to 20. Approximate confidence intervals (CIs) for these parameters were obtained using the percentile method. Because these CIs tend to be too narrow (Kuhner *et al.* 2005), we used 99% instead of 95% CIs for statistical inferences.

The second approach was a mismatch distribution analysis (Schneider & Excoffier 1999), performed with ARLEQUIN 3.1 (Excoffier *et al.* 2005). Mismatch analysis performs well in cases of population subdivision and when the demographic history of the populations is more complex than assumed by simple expansion models. In any real data set, both of these factors are likely to occur. Goodness-of-fit to the sudden expansion (SudEx) and the spatial expansion (SpatEx) (Excoffier 2004) models was tested using a parametric bootstrap approach (10 000 replicates). Here, the reported values of  $\theta_0$  and  $\theta_1$  are estimators of theta before and after expansion according to the SudEx model,  $\theta_{spatial}$  is theta before expansion from the SpatEx model and  $\tau_{sudden}$  and  $\tau_{spatial}$  are the times since expansion estimated from the SudEx and SpatEx models, measured in mutational time units. Their approximate confidence intervals were obtained through parametric bootstrapping (10 000 replicates).

## Results

A total of 104 different haplotypes were identified among 364 ingroup sequences (GenBank Accession nos. AY971143, DQ146949, EF212448–EF212810). No indels or stop codons were present. Two hundred and forty-three nucleotide positions (22.2%) were variable, of which 206 (18.8%) were parsimony informative. There were 225 positions with two variant nucleotides and 18 positions with three variants. Forty-seven variable sites (19.3%) were in the first, 12 (4.9%) in the second, and 184 (75.7%) in the third codon position. Forty-three of 366 amino acid sites (11.8%) were variable.

All three outgroup sequences represented unique haplotypes (GenBank Accession nos. EF212811–EF212813). Including outgroup taxa, 363 nucleotide positions (33.1%) were variable, and 272 (24.8%) were parsimony informative; 58 amino acid sites (16.0%) were variable.

Our analysis distinguished five haplogroups: *Bombina bombina* (B), Carpathian *Bombina variegata* (C), Balkano–Western *B. variegata* (BW), Rhodopean *B. variegata* (R) and Italian *B. variegata* (I). Haplotype diversities within and pairwise  $K2P$  sequence divergences between these groups are given in Table 1. Each of the first three groups was subdivided, as defined by higher-level clades in the nested cladograms, into subgroups: B3-1 and B3-2; C4-1 and C4-2; and BW3-1 and BW3-2 (Figs 2 and 3).

### Phylogenetic analysis and sequence divergence among major groups

Both the maximum-likelihood and Bayesian trees supported the same phylogenetic grouping of haplotypes (Fig. 2). The sister status of *B. bombina* and *B. variegata* was supported with 99% bootstrap (BS) and 100% posterior probability (PP). *Bombina bombina* (B) received 100% BS and PP support. In *B. variegata* (86% BS, 100% PP) two main clades are evident; one (99% BS and 100% PP) groups a number of closely related haplotypes and includes individuals from the Carpathian part of *B. variegata*'s range (C), the other (100% BS and PP) is itself divided into three well supported

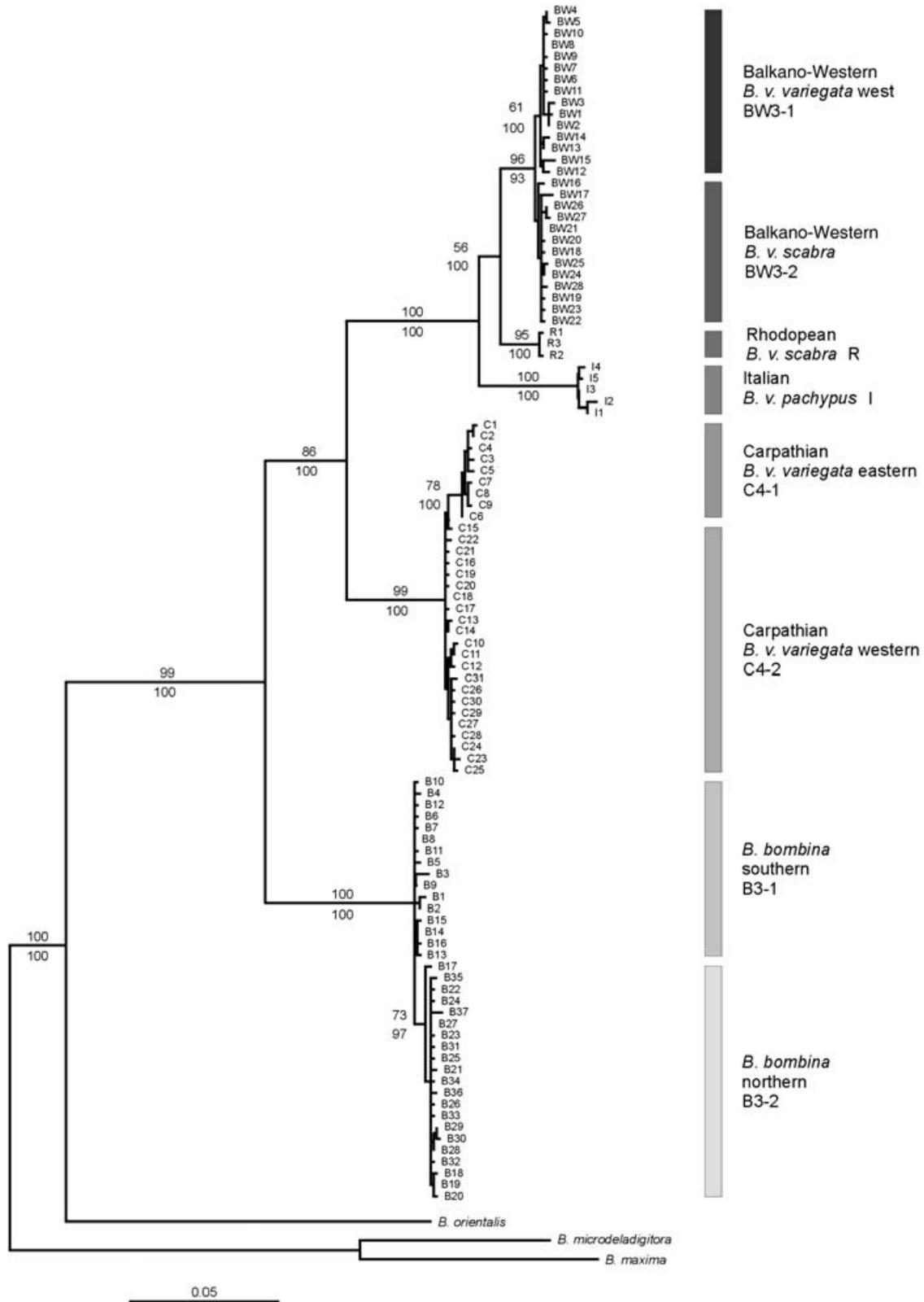
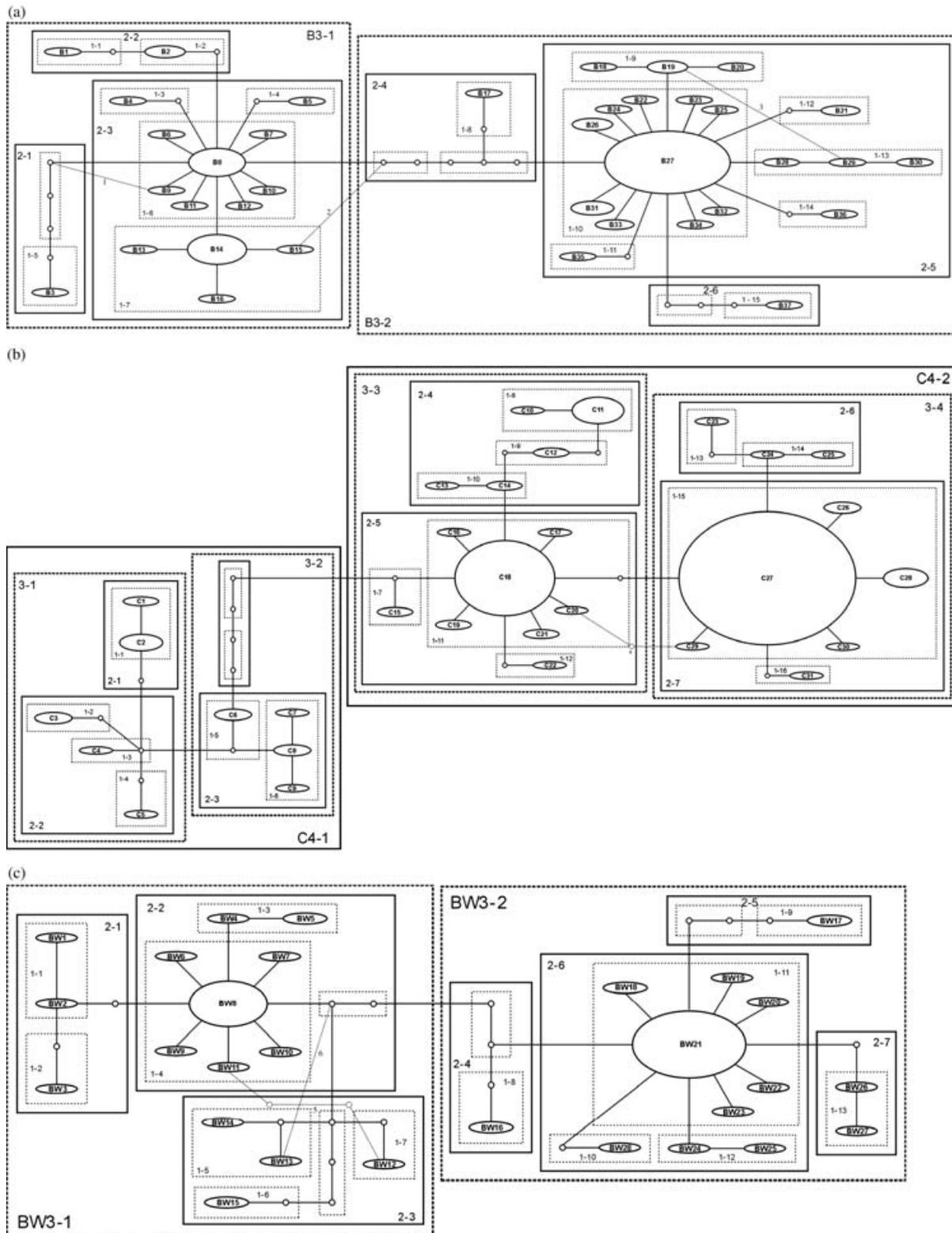
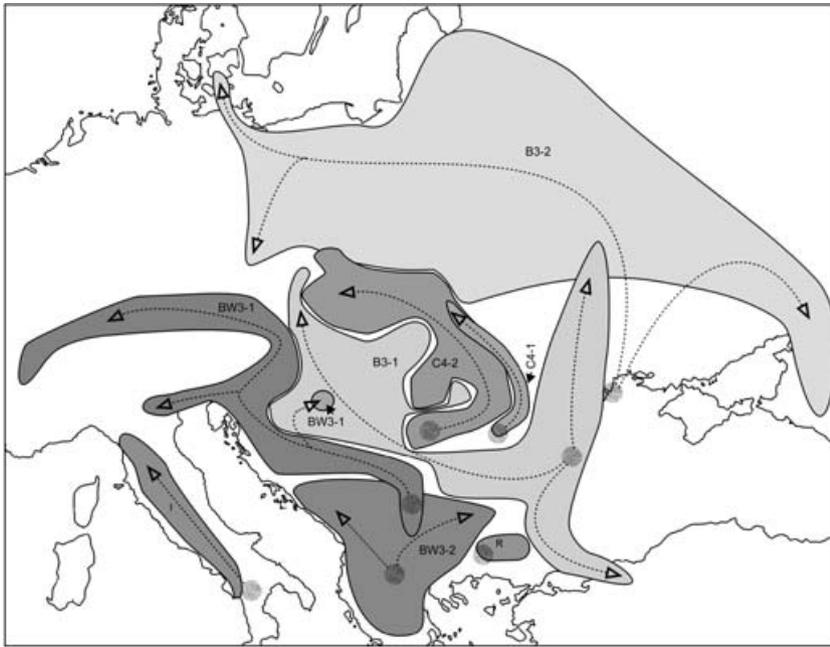


Fig. 2 Phylogenetic relationships among *Bombina* haplotypes. The topology and branch lengths from the maximum-likelihood analysis are shown. Numbers above branches indicate percent bootstrap values for ML, numbers below branches indicate posterior probabilities from the Bayesian analysis.



**Fig. 3** Nested clade designs for *cyt b* haplotypes for *Bombina* haplogroups. Each solid line in the network represents a single mutational change. A haplotype, marked by a number (see Appendix S1), is represented by an oval with the surface area proportional to the number of individuals bearing this particular haplotype. Circles are missing haplotypes. Broken lines with numbers 1 through 6 linking some haplotypes indicate resolved cladogram ambiguities. (a) *Bombina bombina*. (b) the Carpathian group of *Bombina variegata*. (c) the Balkano-Western group of *B. variegata*.



**Fig. 4** Geographical overlay of the nested clades for *Bombina bombina* (three-step level, B3-1 and B3-2), Carpathian group of *Bombina variegata* (four-step level, C4-1 and C4-2), and Balkano–Western group of *B. variegata* (three-step level, BW3-1 and BW3-2), along with distribution of the Italian (I) and Rhodopean (R) *B. variegata* groups. Grey circles indicate probable location of refugia for each *Bombina* group, and dashed arrows mark inferred routes of postglacial colonization.

branches: Italian *B. variegata* (I), toads from the Rhodope range in Bulgaria (R), and the major branch comprising *B. variegata* from the Balkans and Western Europe (BW).

#### Geographical distribution of the mtDNA clades

The mtDNA phylogeographical pattern is best shown by the distribution of higher-level nested clades (Figs 3 and 4).

The two three-step clades of *B. bombina*, B3-1 and B3-2, corresponded to the southern and northern lineages, which are separated by the Carpathians and the intervening range of *B. variegata* (Figs 3a and 4). The southern lineage was found along the Danube lowlands from the Black Sea up to Moravia. The northern clade occupies the vast European plains north and east of the Carpathians. This lineage also penetrated the western Bohemian Plateau along the Elbe River from the north. The two *B. bombina* clades apparently overlap in the lowlands east of the Carpathians, a region not sampled by us, because a single southern group haplotype, B11, was observed in Kiev (Site 57, Appendix S1), among the northern group haplotypes (Figs 1 and 4).

The Carpathian *B. variegata* haplotypes group into two four-step clades, the eastern C4-1 and western C4-2 (Fig. 3b): the first is limited to the easternmost part of the Carpathian arch, whereas the second occupies nearly all of the Carpathians but the Carpathian bend (Fig. 4).

On a larger geographical scale, the Carpathian *B. variegata* are surrounded by *B. bombina*. The contact of the two species results in a narrow hybrid zone. A number of samples came from transects (Fig. 1), of which three cross the

northern foothills of the Carpathians (Kraków transect: 7 sites,  $N = 15$  individuals; Przemyśl transect: 20 sites,  $N = 45$ ; and Stryi transect: 14 sites,  $N = 19$ ) and one their southern slopes in Romania ( $N = 6$ ). There was close correspondence between the *bombina* and *variegata* haplotypes found in the transects and the clades present in nearest proximity to the hybrid zone. Sites where haplotypes of both species co-occurred were limited to a few central positions within the transects (cf. Yanchukov *et al.* 2006; Hofman & Szymura 2007).

In the Balkano–Western group, two three-step clades were distinguished (Fig. 3c). Clade BW3-2 (the Balkan haplogroup), distributed throughout most of the Balkan Peninsula, co-occurs with clade BW3-1 (the western haplogroup) in the north-central Balkans, which is spread over an extensive area towards the west including the forelands of the Alps, as well as an isolated enclave in the Mecsek Mountains (Site 99).

The Rhodopean group (R) is limited to the Rhodope Mountains in South-East Bulgaria, whereas the Italian group (I) is limited to the Apennine Mountains. The latter group forms an allopatric clade separated by the Po River Valley from the distinct Balkano–Western clade of *B. variegata* that is present on the southern slopes of the Alps in northern Italy.

In sum, five main mtDNA haplotype groups revealed in European *Bombina* have patchy, parapatric distributions (Fig. 4). The only exceptions were samples from hybrid populations in the contact zone, and a sample from Toszek (southern Poland, Site 22) where a single *variegata* haplotype was discovered among *B. bombina*.

**Table 2** Interpretation of the results of the nested clade analysis using the inference key of Templeton (2004). Clades with a significant association are shown

Clades nested with	Permutational $\chi^2$ statistic	<i>P</i>	Chain of inference	Inference
<i>bombina</i>				
1-7	36.88	0.036	19-20-NO	IGS
1-10	411.23	0.000	2-3-5-6- too few clades	RE or RGF
2-5	240.05	0.035	2-3-5-6- too few clades	RE or RGF
3-1	76.00	0.001	2-3-5-6- too few clades	RE or RGF
Total cladogram	107.71	0.000	2-11-17-4-NO	RGFwIbD
Carpathian <i>variegata</i>				
2-4	27.39	0.018	2-3-4-NO	RGFwIbD
3-1	13.00	0.029	19-20-2-3-4-NO	RGFwIbD
3-3	46.06	0.027	2-3-5-6- too few clades	RE or RGF
4-2	110.91	0.000	2-3-4-NO	RGFwIbD
Total cladogram	148.01	0.000	2-11-17-4-NO	RGFwIbD
Balkano-Western <i>variegata</i>				
2-2	21.00	0.013	19-NO	AF
3-1	57.19	0.000	2-3-5-6-too few clades	RE or RGF
3-2	114.00	0.000	2-3-4-NO	RGFwIbD
Total cladogram	66.32	0.000	2-11-17-4-NO	RGFwIbD

AF, allopatric fragmentation; IGS, inadequate geographical sampling; RE, range expansion; RGF, restricted gene flow or dispersal; RGFwIbD, restricted gene flow with isolation by distance.

### Testing for selection, patterns of gene flow and historical demography

The MK test rejected neutrality in all three lineages (B and C:  $P = 0.0033$ , Fisher's exact test; B and BW:  $P = 0.014$ ; BW and C:  $P = 0.0034$ ); in all tests there was an excess of nonsynonymous polymorphisms suggesting weak purifying selection. The *H* test with Tajima's statistic was significant only in lineage BW ( $P = 0.0339$ ), whereas with Fu and Li's statistic it was significant only in lineage C ( $P = 0.0062$ ).

In the nested clade analysis, statistically significant geographical associations were detected within each of the three major lineages: B, C and BW (Table 2, Fig. 4). For each of these, restricted gene flow with isolation by distance was inferred for the total cladogram. The limited number of clades prevented us from distinguishing between range expansion and restricted gene flow in the southern *B. bombina* clade, B3-1, and in the northwestern haplotype groups (B2-5, B1-10) dominating in the northern *B. bombina* branch. In Carpathian *B. variegata*, restricted gene flow with isolation by distance was inferred for all but one clade, C3-3; its haplotype distribution pattern could be explained either by range expansion or restricted gene flow. In the Balkano-Western lineage, the Balkan clade (BW3-2) showed restricted gene flow with isolation by distance; the western clade (BW3-1) was subject either to range expansion or restricted gene flow. Clade BW2-2

underwent allopatric fragmentation restricting clade BW1-3 to an enclave in the Mecsek Mountains.

Confidence intervals for theta from the LAMARC and mismatch analyses overlapped for all clades; in most cases, however, average estimates differed substantially (Table 3). Highest thetas in LAMARC analysis were generally inferred for those clades for which the highest growth parameter values were estimated. For all but one clade significant demographic growth was inferred, the exception being the East Carpathian C4-1, for which the *g* value was not significantly different from zero. This was the only clade for which both the SudEx and SpatEx models were rejected in the mismatch analysis, so estimation of demographic parameters by this method was unwarranted.

## Discussion

### Phylogenetic relationships among major lineages

Phylogenetic analyses yielded a clear and well-supported pattern of relationships among the three major mtDNA lineages: *Bombina bombina*, Carpathian *Bombina variegata*, and *B. variegata* from the rest of its range in southwestern Europe (Fig. 2). Whereas *B. bombina* and the Carpathian *B. variegata* show only shallow internal differentiation, the remaining *B. variegata* haplotypes are grouped into three well-defined lineages: from the Apennine Mountains of Italy, from the Rhodope Mountains of Bulgaria, and from

**Table 3** The results of demographic analyses in major population groups as revealed by NCA higher-level nesting pattern (Fig. 3).  $n$ , number of sequences;  $\theta_{ML}$ , the coalescent based estimator of theta;  $g$ , the exponential growth rate; CI, confidence interval;  $P_{\text{sudden}}$ ,  $P_{\text{spatial}}$  probabilities, based on 10 000 bootstrap replicates, that the mismatch distribution conforms to the models of, respectively, sudden (SudEx) and spatial (SpatEx) expansion;  $N$ , mean number of pairwise differences;  $\tau_{\text{sudden}}$ ,  $\tau_{\text{spatial}}$ , time since expansion inferred for the SudEx and SpatEx models ( $\tau = \mu t$ , where  $\mu$  is per generation mutation rate and  $t$  is the number of generations);  $\theta_0$  and  $\theta_1$  are theta values before and after expansion, respectively;  $\theta_{\text{spatial}}$  is theta value from the SpatEx. All theta values are given as percentages per site

Group	$n$	$\theta_{ML}$ (99% CI)	$g$ (99% CI)	$P_{\text{sudden}}$	$N$ (95% CI)	$\tau_{\text{sudden}}$ (95% CI)	$\theta_0$ (95% CI)	$\theta_1$ (95% CI)	$P_{\text{spatial}}$	$\tau_{\text{spatial}}$ (95% CI)	$\theta_{\text{spatial}}$ (95% CI)
B3-1	39	51.56 (4.36–960.84)	8462.4 (4605.7–15496.8)	0.56	1.883 (1.174–2.305)	1.714 (0.511–2.325)	0.00 (0.00–0.12)	1.10 (0.86–648.58)	0.591	1.325 (0.653–2.844)	0.04 (0.00–0.13)
B3-2	72	9.74 (2.91–43.26)	6486.9 (3800.6–10427.4)	0.86	1.194 (0.270–2.257)	1.368 (0.125–3.275)	0.00 (0.00–0.07)	0.29 (0.04–452.87)	0.817	0.491 (0.307–3.574)	0.06 (0.00–0.14)
BW3-1	34	1.90 (0.61–9.10)	1471.1 (106.5–4430.8)	0.92	2.280 (0.515–5.093)	5.824 (1.705–10.078)	0.00 (0.00–0.37)	0.24 (0.02–11.48)	0.822	2.410 (0.259–14.170)	0.21 (0.00–0.34)
BW3-2	38	3.03 (0.85–38.18)	3810.6 (1598.9–9360.7)	0.87	1.548 (0.211–4.619)	0.000 (0.000–6.203)	0.16 (0.00–0.039)	0.21 (0.06–350.68)	0.885	1.728 (0.166–7.247)	0.11 (0.00–0.21)
C4-1	21	0.59 (0.16–3.39)	726.1 (–1116.5–3959.5)	0.0005					0.021		
C4-2	148	1.53 (0.64–2.12)	3128.9 (1158.2–5850.3)	0.18	1.774 (0.678–3.698)	2.820 (0.689–6.250)	0.00 (0.00–0.10)	0.35 (0.05–347.13)	0.567	2.464 (0.366–4.802)	0.00 (0.00–0.15)

Western Europe and the Balkans. Nesting of the Italian *Bombina* within the *B. variegata* lineages strongly suggests that *Bombina pachypus* is not, as argued by Lanza & Vanni (1991) and Canestrelli *et al.* (2006), a distinct species.

Currently there is no reliable calibration of a molecular clock for *Bombina cyt b*. Calibrations for the closest relatives (García-París & Jokusch 1999) give pairwise *K2P* divergence rates of 1.4–1.6%/million years (*Alytes*) or 1.3–1.5%/million years (*Discoglossus*). If these rates hold for *Bombina*, then sequence divergence values indicate an Upper Miocene or Lower Pliocene split between *B. bombina* and *B. variegata*, as suggested by earlier immunological and allozyme data (Szymura 1993). Palaeontological evidence does not contradict this dating: fossils identified as *Bombina* in Europe extend well into the Miocene (Sanchiz 1998). More recent remains, present in Central Europe during the interglacials, are difficult to assign to either species because skeletons of the two species are poorly differentiated. These fossils, although they cannot be used to calibrate a molecular clock, point to repeated interglacial colonization of the deglaciated areas (Szymura 1993).

A consequence of using divergence rates that suggest an Upper Miocene or Lower Pliocene divergence between the European *Bombina* species is that the divergence between the Carpathian lineage and all other *B. variegata* mtDNA lineages is only slightly younger and definitely pre-Pleistocene (*K2P* divergence: 7.9–9.3%; Table 1). High mtDNA divergences for *cox1* and *nad4* among *Bombina* populations from the Carpathian Basin (Vörös *et al.* 2006), corresponding to our clades B3-1, C4-1 and BW3-1, are consistent with our estimates. The pre-Pleistocene origin of both European *Bombina* species as well as of the two major *B. variegata* lineages is hardly surprising given comparative data from other organisms. Although the role of Pleistocene glaciations in speciation is controversial and the evidence contradictory (Avice *et al.* 1998; Johnson & Cicero 2004; Zink *et al.* 2004), the pre-Pleistocene origin for the three major European *Bombina* lineages suggests that Pleistocene climatic fluctuations at most remodelled the distributions of these pre-existing lineages, as postulated for other amphibians (e.g. Nielson *et al.* 2001; Babik *et al.* 2005). This remodelling may have promoted geographical isolation and induced differentiation within different isolates. In *B. variegata*, such differentiation is apparent at the external phenotypic level (Gollmann & Gollmann 2002; Vukov *et al.* 2006) and seems to correlate with major geographical features of Central Europe.

#### Phylogeographical pattern and glacial refugia

A major aspect of range remodelling for European *Bombina* during Pleistocene climatic fluctuations was restriction of various clades to refugia during the glacial period, followed by postglacial expansion. Our extensive geographical

mtDNA sampling combined with available allozyme data, pointing to higher variation in the south (Szymura 1993, 1998), restricted the possible refugial areas for each major clade and suggested routes for postglacial recolonization. The lowland species, *B. bombina*, exhibits only shallow mtDNA *cyt b* differentiation in agreement with differentiation at allozyme loci. Two three-step clades (B3-1, B3-2) have largely nonoverlapping distributions, suggesting an origin during the last glacial maximum (LGM) in separate refugia in the lowlands near the Black Sea, or possibly in the now inundated shelf in the northwestern part of the Black Sea (Ryan *et al.* 2003; Popescu *et al.* 2004). When this shelf was exposed, the Black Sea was a freshwater lake. High haplotype diversity and variable allozymes point to the southern Romanian lowlands as a refugium for clade B3-1, whence one branch would have expanded westwards up along the Danube through the Iron Gate and into the Hungarian Plains, while the other branch expanded to the northeast.

Populations representing clade B3-2, now inhabiting most of the species' range, may have originated in a refugium at the northern shores of the Black Sea, where at least pockets of forest vegetation were present during the LGM (Tarasov *et al.* 2000). Clade B3-2 penetrated into the western part of the Bohemian Plateau along the Elbe River, a possibility mentioned by Arntzen (1978).

Mitochondrial DNA differentiation in *B. variegata* is more complex. A major dichotomy exists between populations in the Carpathian Mountains and the rest of the range (Fig. 2). The present mtDNA perspective, supported by allozyme data, provides strong evidence that *B. variegata* survived the LGM in the Carpathian Mountains. Earlier interpretations failed to identify a Carpathian refugium or placed the refugium for Carpathian *B. variegata* in the Balkans (i.e. Arntzen 1978; Szymura 1993).

Within the Carpathians, the distribution of mtDNA clades and allozyme variants suggest two separate refugia. Clade C4-1 probably had its refugium in the southeast bend of the Carpathians as evidenced by the presence of multiple nested clades up to the 3-step level in this region. Only a subset of these clades is found farther to the north (haplotype diversity,  $h$ , is 0.88 in the postulated refugium vs. 0.43 in the rest of the range). Allozyme data provide additional support, because only in this part of the Carpathians are *Ldh-1* and *Mdh-1* polymorphic (Szymura 1998). Clade C4-2 has a broader distribution, encompassing nearly the entire mountain range except for its southeastern corner. The likely refugium for clade C4-2 was in the southern Carpathians, where the haplotype diversity is highest ( $h = 0.76$  vs. 0.63 in the remaining part of the clade's range).

Recent work has documented the importance of Central Europe as a refugial area for numerous taxa. Evidence comes from palaeoclimatology (Sümegei & Krolopp 2002), palynology (Willis & van Andel 2004), plant and animal fossils (Willis *et al.* 2000; Stewart & Lister 2001; Willis & van

Andel 2004) as well as from phylogeographical analyses of both plant and animal species (Wallis & Arntzen 1989; Taberlet & Bouvet 1994; Babik *et al.* 2004, 2005). Climatic conditions in the eastern Alps, the Carpathian Basin, and in the southern Carpathians permitted the survival of temperate organisms, including ectothermic animals, well to the north of the 'canonical' refugial regions in the southern European peninsulas (Tzedakis *et al.* 2002; Huntley *et al.* 2003). Among amphibians, a Carpathian refugium has been postulated for an endemic newt (*Triturus montandoni*, Babik *et al.* 2005), and in this study for the yellow-bellied toad, *B. variegata*. Despite discordance between allozymes and mtDNA in the Balkano-Western group (see below), the distribution of mtDNA and allozyme variation makes the identification of plausible refugia for this group and routes of its postglacial colonization possible. For the Western group (BW3-1, corresponding to *B. v. variegata*), the highest haplotype diversity is found in the upland areas of eastern Serbia ( $h = 0.89$  vs. 0.60 in the rest of the clade's range), so we postulate a refugium for BW3-1 in this region, from which both northeastern and northwestern expansion could have occurred. NCA inference for clade BW3-1 agrees with this scenario.

A wide clinal transition in allozyme allele frequencies and high heterozygosity in the central Balkans suggest postglacial contact between the more southern *Bombina variegata scabra* (BW3-2) and the western *B. v. variegata* (Szymura *et al.* 2000; Vukov *et al.* 2006). Within clade BW3-2, which includes most *B. v. scabra* populations, a more complex pattern occurs. One broadly distributed 2-step clade (BW2-6) overlaps several other 2-step clades in its distribution. This suggests that in addition to a recent expansion, multiple populations survived in the southern Balkans at least during the LGM. The complex geography of the Balkans would facilitate long-term isolation of such populations, as exemplified by the Rhodope *B. variegata* group. Polymorphic allozymes in the southern Balkans support the location of a refugium in this region (Szymura 1998).

The Italian or Apennine *Bombina variegata pachypus* has been apparently effectively isolated from the rest of the *B. variegata* range even longer ( $K2P = 4.56\%$  to the Balkano-Western group), and survived glacial periods within the Apennine Peninsula, most likely in the southernmost part as suggested by allozyme and mtDNA polymorphisms (Szymura 1993, 1998; Canestrelli *et al.* 2006).

#### *Discordance between mtDNA, allozymes and morphology*

On the largest geographical scale, there is a high concordance between phenotypic traits, allozymes, and mtDNA haplotypes: *B. bombina* and *B. variegata* can be distinguished by any of an impressive list of diagnostic traits characterizing the two species (Szymura 1993).

Although absence of premating isolation between the species would plausibly facilitate genetic exchange along their convoluted contact zones, concordance of morphology, allozymes, and mtDNA across the species implies that, over a long evolutionary timescale, either introgression has not occurred or that its effects have been eliminated by extinction of admixed populations. Evidence from modern hybrid zones suggests that selection has prevented introgression. Only in populations in the centre of a narrow hybrid zone separating the two species are *bombina* and *variegata* mtDNA haplotypes found together. In fact, the width of mtDNA clines does not exceed that of the phenotypic or allozyme clines (Yanchukov *et al.* 2006) and may even be narrower (Hofman & Szymura 2007). This pattern is consistent with negative epistatic interactions between divergent genomes of the species, sharpened by environment-dependent selection, preventing introgression. Further tests of possible introgression of specific genomic segments between these species could be obtained by testing the isolation-with-migration model using multiple nuclear sequence markers (e.g. Machado *et al.* 2002; Hey & Nielsen 2004).

Although interpreted by the authors as repeated mtDNA introgression, lineage independence applies also to the Carpathian Basin (Vörös *et al.* 2006). In areas of overlap, or hybrid zones, a wide range of nuclear recombinants are generated, so occasionally 'pure' morphotypes may carry mtDNA haplotypes of the other species (Hofman & Szymura 2007).

While concordance of morphology, allozymes, and mtDNA holds across the species and most geographical groups within *B. variegata*, there is an important discordance between mtDNA haplotypes of the western European toads and their allozymes and morphology. Morphological and allozyme data link them to the Carpathian group, but *B. v. variegata* around the Southern Morava River valley in eastern Serbia and westward possess haplotypes related to the Balkan lineage. A plausible explanation involves the transfer of Balkan mtDNA into western European toads, which, considering the more recent divergence of these lineages, may be unaffected by the negative effects of the breakdown of epistatic interactions between mtDNA and nuclear genomes in hybrids. Free recombination of mtDNA from its nuclear background facilitates mtDNA introgression (Barton 1986) and many examples of this phenomenon have been described (e.g. Bachtrog *et al.* 2006). An alternative explanation involving transfer of nuclear genes alone from the Carpathian *B. variegata* into *B. v. scabra* populations seems less likely.

Transfer of the Balkan mtDNA onto the nuclear background shared by Carpathian and western European *B. variegata* implies repeated backcrossing. Initial matings had to involve females of *B. v. scabra* and males of western European *B. v. variegata*, and then mating of the female F<sub>1</sub> progeny to the Carpathian/western type. The contact

between the two groups took place over a broad area, and in a geographically complex region of the eastern Serbian Mountains, creating multiple opportunities for repeated intergroup matings. The contact occurred in highly dynamic postglacial landscapes, characterized by local extinctions and colonizations with leptokurtic dispersal paralleling an oscillating climate. Under such conditions, local genetic patches are easily achieved (Ibrahim *et al.* 1996). The transfer must have preceded expansion of this group to the west.

At present, the Balkan *B. v. scabra* and the northern *B. v. variegata* occupy dissimilar habitats in xeric and mesic climates, respectively (Vukov *et al.* 2006). *Bombina v. scabra* inhabits mostly small, slowly flowing shallow streams, often deeply cut into the underlying rock, whereas *B. v. variegata* shows preferences for temporary rain puddles and small ponds found at lower mountain elevations, making it a better colonizer of newly formed transient habitats.

### Demographic history

Tests of selective neutrality were not conclusive. Whereas the MK test was significant in all three lineages, the *H* test was largely insignificant. If anything, these tests suggest weak purifying selection as often inferred for mtDNA (Gerber *et al.* 2001; Elson *et al.* 2004). Such selection is likely to affect the estimates of demographic parameters, but is not likely to significantly bias qualitative results of NCA and demographic analyses. This form of selection is consistent with the absence of introgression of mtDNAs between the two *Bombina* species, despite repeated episodes of hybridization in interglacials. Sequence data from other parts of the genome would be important for detecting selective sweeps (Gerber *et al.* 2001). However, selective sweeps are unlikely in our case, because the same pattern of shallow divergence within major clades with multiple closely related haplotypes was revealed repeatedly in all major mtDNA lineages.

The most conspicuous feature of the demographic history, as revealed by mtDNA variation patterns, was substantial population expansion in all but one main clade. As may be inferred from the mode of mismatch distribution centred around two pairwise differences (Table 3, Fig. 5), the expansion was apparently recent. We attribute this to the postglacial colonization of vast areas previously inhospitable for *Bombina*. Thus, the demographic expansion *sensu stricto* was accompanied by substantial geographical expansion but the effects of these two processes may be difficult to separate.

Unfortunately, the pattern of nucleotide variation within major clades precludes drawing any conclusions with regard to the demographic history predating the LGM. Any prior information was apparently erased by the bottlenecks during the last glacial period as evidenced by the shallow mtDNA genealogies.

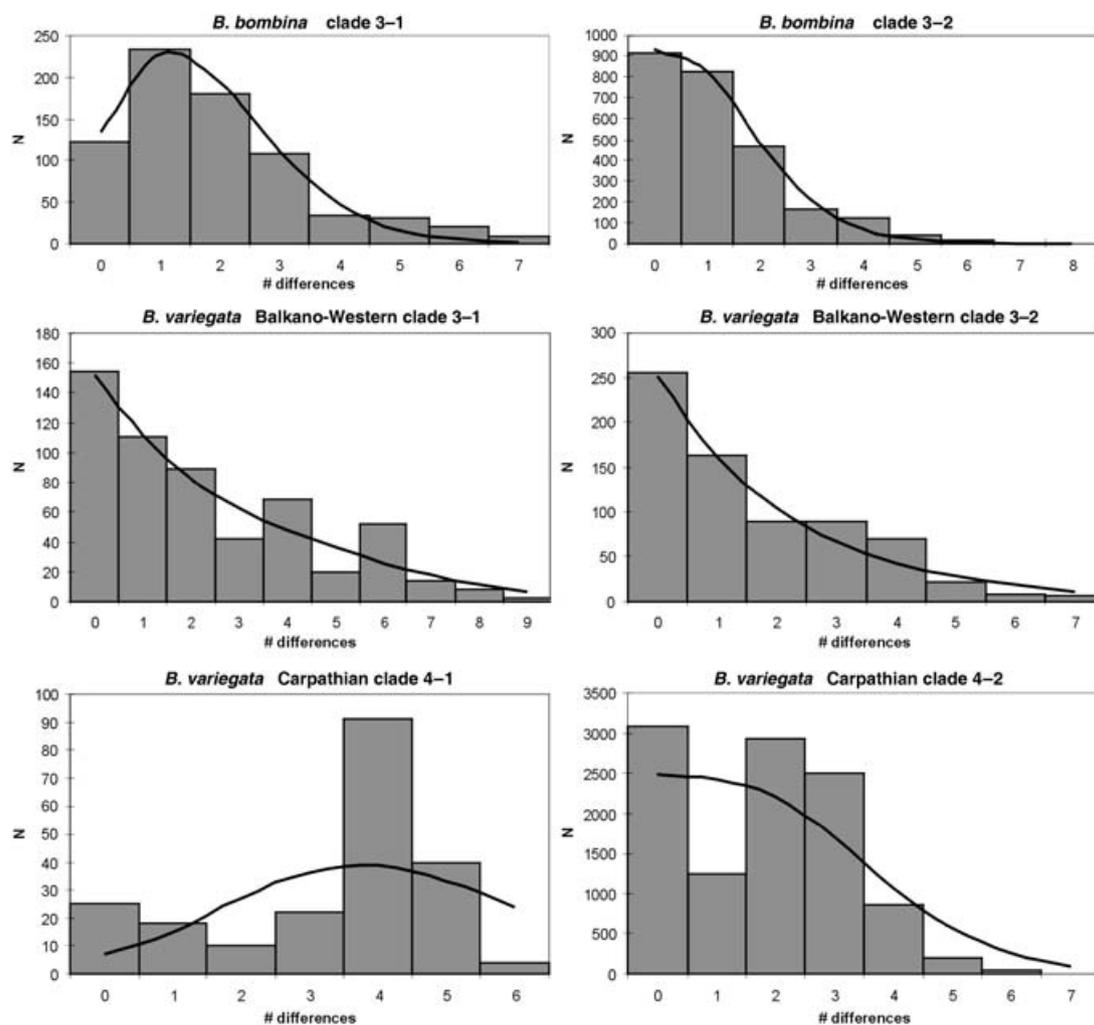


Fig. 5 Mismatch distributions classes in the higher level nested clade analyses with at least 20 sequences. Black curves show shapes of theoretical distributions according to the sudden expansion model.

## Conclusions

Our study demonstrated deep divergence of the *cyt b* mtDNA within *Bombina*, suggesting an ancient, pre-Pleistocene split of the species, soon followed by a division of *Bombina variegata* into two groups, now inhabiting the Carpathians, and the Balkans and Western Europe, respectively. The Pleistocene glacial refugia of the European *Bombina* were located both in the classical refugial areas of Italy and the Balkans as well as more to the north in the Carpathians and adjoining lowlands. Population growth and expansion after the LGM resulted in a geographical mosaic of parapatrically distributed mtDNA clades. Possible repeated episodes of hybridization in the previous interglacials have not resulted in mtDNA introgression between the species. The complex past history reconstructed in the present phylogeographical study and supported by allozyme variation patterns and the fossil record differs from earlier ideas founded on biogeography (Mertens 1928; Arntzen 1978) or allozymes

alone (Szymura 1993). Our results provide answers and new insight into specific questions raised by long-term research on hybrid zone dynamics, species identity, and history. It complements and expands on the pattern of 'northern uniformity and southern richness' (Hewitt 2004). This broad geographical survey provides a basic framework against which the complexities of *Bombina* evolutionary history can be organized.

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### Supplementary material

The following supplementary material is available for this article:

**Appendix S1** Sample localities used in the study Keyed to Fig. 1, their geographical coordinates, sample sizes (*n*), taxa present, and haplotypes recorded at each site, as named in Fig. 2 and Fig. 3.

This material is available as part of the online article from:

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