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Mitochondrial DNA variation in the hybridizing fire-bellied toads, *Bombina bombina* and *B. variegata*

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Abstract

Using five restriction enzymes, geographical variation of mitochondrial DNA (mtDNA) in *Bombina bombina* and *B. variegata* was studied in samples from 20 locations. Each restriction enzyme produced a species-specific fragment pattern. *B. bombina* haplotypes A and B were closely related to each other. In contrast, haplotypes A and B of *B. variegata* formed two distinct lineages. A very distinctive haplotype (C) was found in the Carpathian Mountains, whereas two other haplotypes, D and E (differing by a single *Ava*I site), were present in western Europe and the Balkans, respectively. Populations polymorphic for haplotypes D and E occurred in the central Balkans where the haplotypes could replace each other clinally. mtDNA sequence divergence between *B. bombina* and *B. variegata* was estimated as 6.0–8.1% and 4.7–5.2% between type C and types D/E of *B. variegata*. The latter divergence is contrary to allozyme and morphological data that place the western and Carpathian *B. v. variegata* together (Nei's *D* = 0.07) and separate them from the Balkan subspecies *B. v. scabra* (Nei's *D* = 0.18). Broad interspecific correlation among morphology, allozymes and mtDNA types in European fire-bellied toads argues that, despite continuous hybridization (interrupted perhaps during Pleistocene glacial maxima), little or no mtDNA introgression between the species has occurred outside the narrow hybrid zones that separate these parapatric species.

Keywords: Anura, *Bombina*, clinal variation, hybrid zone, mtDNA

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Introduction

The European fire-bellied toads, *Bombina bombina* and *B. variegata*, have a parapatric distribution that is related to their ecological requirements and postglacial dispersal from southern refuges (Arntzen 1978; Szymura 1993). *B. bombina* occupies lowlands of central and eastern Europe, whereas *B. variegata* is found at higher elevations in western Europe, in the Apennine, Balkan and Carpathian Mountains as well as in isolated ridges on the Hungarian Plain (Fig. 1). Although the species are well differentiated in morphology, anatomy, behaviour and life-history traits, as well as at the molecular level, they hybridize wherever they meet along a zone of contact several thousand km long (Szymura 1993). The zone lies at the border between lowland and upland terrain. In the Danube Basin it stretches from Bohemia to the Black Sea. It also surrounds *B. variegata* enclaves in the Hungarian Plains and encircles the Carpathian Mts. The species are in close allopatry in Germany, but indications of past hybridization have been found (J. M. Szymura & S. Möller, unpublished). The narrow transition from one species to the other, which involves parallel change in many characteristics, suggests a barrier to genetic exchange between the species (Szymura & Barton 1986, 1991). The zone in southern Poland, at the north edge of the Carpathian Mts, has been regarded as a classic example of a clinal hybrid zone (Futuyma 1998). South of the Carpathian Mts, however, there is considerable variation in the structure of the hybrid zone (Gollmann 1984, 1987; Gollmann & Szymura 1986; Gollmann *et al*. 1988; Szymura 1993). In regions that are environmentally complex, mosaic zones rather than smooth clinal zones may be the norm, because the species show active habitat preferences (Bugter *et al*. 1997; MacCallum *et al*. 1998). This has an important consequence for the dynamics

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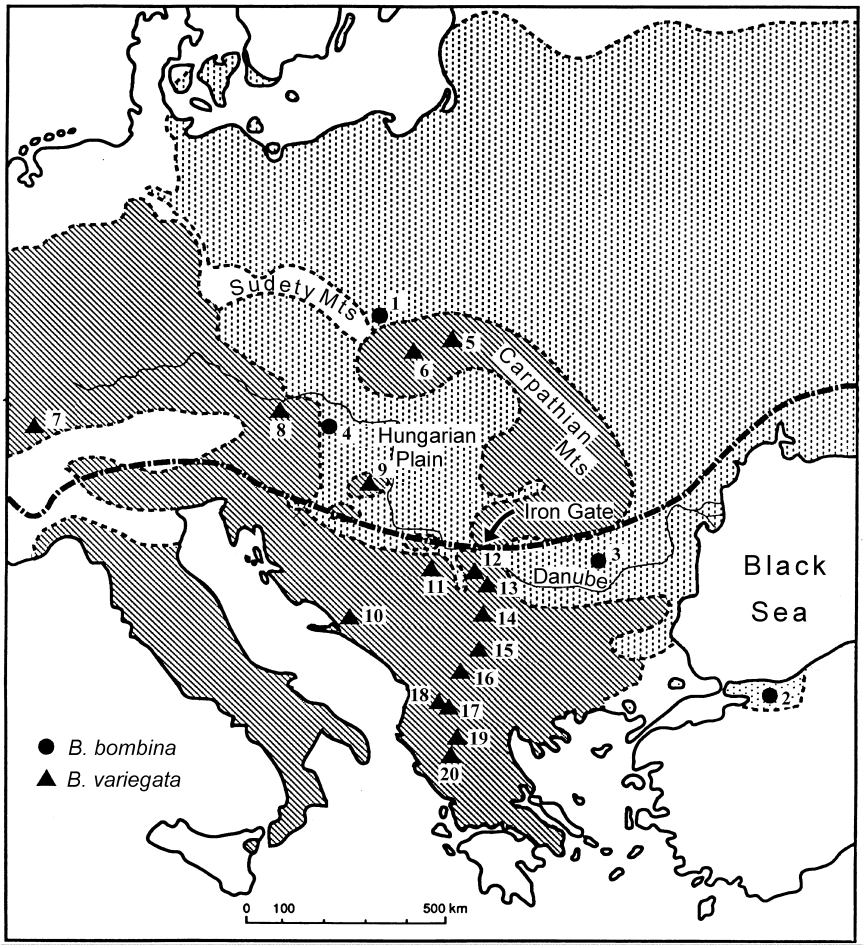

Fig. 1 Distribution of *Bombina bombina* and *B. variegata* in Europe and location of samples. The thick broken line indicates the southern margin of permafrost during the last glacial maximum.

of hybrid zones, which may widen in belts of complex environments (Arntzen & Wallis 1991; Arntzen 1996), increasing overlap between the species and thus creating greater opportunity for gene exchange between them.

Electrophoretic study delimits two groups of *B. bombina* (Szymura 1988). A 'southern' group occupies the Danube basin south of the Sudety and Carpathian Mts, while a 'northern' group consists of populations located north and east of them. The two groups meet in intermediate populations along the lower Danube. Disjunct populations of *B. bombina* occur in Greece and Turkey; the former are isolated from the latter by a sea strait. The electrophoretic studies revealed greater subdivision in *B. variegata*. A Carpathian group inhabits the Carpathian Mts. A western group is spread over a large area from France through Germany, Alpine countries and eastwards to the northern Balkans where it meets the rather similar Carpathian group across the Danube River. These two *B. variegata* groups comprise *B. v. variegata*. The more distinctive Balkan group (*B. v. scabra*) occupies the southern region of the Balkan peninsula. It intergrades with the nominal subspecies in the central Balkans. The Italian group (*B. v. pachypus*) is restricted to the Apennine Mts of Italy. It is isolated from the western group of *B. variegata* by the Po River Valley. This considerable intraspecific differentiation has been interpreted as resulting from periodic contractions of *Bombina* ranges during Pleistocene glacial periods followed by expansions during interglacial periods (Szymura 1988, 1993).

Because the species are not reproductively isolated and their divergence is placed in the Pliocene (Szymura 1983), they probably have exchanged genes in the past. The fossil record from warm interglacials shows that their ranges were subject to periodic contractions and expansions during Pleistocene climatic perturbations. Moreover, the present distribution of the species on the Hungarian Plain argues for a large geographical shift in one hybrid zone during early postglacial expansion of *B. bombina*. This species, which entered the plain from the southeast through the narrow Iron Gate along the Danube River, restricted *B. variegata* to enclaves on the isolated mountain tops scattered in the plain (Arntzen 1978; Szymura 1988).

Electrophoretic studies that revealed such extensive intraspecific variation in allele frequencies also showed that the interspecific differences between the species are

consistently maintained across hybrid zones despite a long history of hybridization. The fire-bellied toads thus offer a system for assessing the influence of long-term hybridization on species identity (Szymura 1996). Because mitochondrial DNA (mtDNA) is a powerful tool for studying evolutionary history across hybrid zones (Avise 1993), we investigated mtDNA variation in *Bombina*, both to assess the amount of variation within and between species and to compare patterns of mtDNA variation to patterns observed with allozymes.

Materials and methods

Fire-bellied toads (*Bombina bombina*, *B. v. variegata*, *B. v. scabra*) were collected at the following localities, which are represented in the map (Fig. 1) by number.

B. bombina. Poland: 1. Żory (four individuals); Turkey: 2. Adapazari (four); Romania: 3. Bucharest (one); Austria: 4. Marchegg (five).

B. variegata. Poland: 5. Stróże (three), 6. Stawek Zawadowski, Gorce Mts (five); Switzerland: 7. Aadorf near Zürich (eight); Austria: 8. Breitenfurt (four); Hungary: 9. Budafa near Pécs (eight); Bosnia and Hercegovina: 10. Mostar (three); Yugoslavia: 11. Rudnik (four), 12. Leskovo (eight), 13. Brestovacka Banja (seven), 14. Niška Banja (six), 15. Vranska Banja (five); Macedonia: 16. Matka near Skopje (one), 17. Peštani, Galičica Mts (one), 18. Ohrid (one); Greece: 19. Metsovon (two), 20. Melissopetra (three).

Each animal was anaesthetized using MS 222 (ethyl-m-aminobenzoate). The procedure used for isolating purified mtDNA has been described previously (Szymura *et al*. 1985). Briefly, a tissue homogenate was centrifuged through a sucrose-step gradient in which the mitochondrial fraction collected at the 0.9/1.6 M interface. A crude mtDNA preparation was obtained by sodium dodecyl sulphate (SDS) lysis of the mitochondrial fraction followed by potassium acetate precipitation of the lysate. The clear supernatant was either frozen at this point or further purified. Further purification involved standard organic extraction with phenol followed by extraction with chloroform. The sample was then digested with RNAse followed by proteinase K. This solution was again extracted: once with phenol, once with 1 : 1 phenol–chloroform, then twice with chloroform. DNA was ethanol precipitated and the purified mtDNA pellet dissolved in 50–100 μL of TE/10 buffer (1 mM Tris, 0.1 mM EDTA, pH 8.0). Five-microlitre aliquots were run on a mini gel alongside 0.1 and 0.5 μg of λ phage DNA to estimate the amount and quality of mtDNA present. Our procedure yielded 3–5 μg of mtDNA per animal. *Bombina* are rather small (large adults are 4–5 cm snout-to-vent), but even 2-cm animals yielded sufficient mtDNA for analysis.

Digestion of mtDNA was carried out under conditions recommended by the supplier. Each of the 83 mtDNA samples was digested using five restriction enzymes that recognize specific hexanucleotide sequences: *Ava*I (CPyCGPuG), *Bcl*I (TGATCA), *Hin*dIII (AAGCTT), *Pst*I (CTGCAG) and *Pvu*II (CAGCTG). Details of ^{32}P labelling and gel electrophoresis were as described by Brown (1980) with later modifications (Wright *et al*. 1983). Digestion profiles were revealed by autoradiography of gels dried onto Whatman 3 MM paper. Fragment sizes were determined by comparisons with molecular size markers provided by *Hin*dIII digests of λ DNA, *Hin*cII digests of ϕX174 DNA or *Alu*I digests of pBR322.

Using the fragment patterns generated by restriction enzymes, the proportion of shared fragments, *F*, was calculated for each pair of mtDNA haplotypes as $F = 2N_{xy}/(N_x + N_y)$, where N_x and N_y are the numbers of fragments in genotypes *X* and *Y*, and N_{xy} is the number of fragments shared. Fragments were considered homologous or shared if they migrated the same distance in side-by-side comparisons. Fragments that in most digests were routinely fuzzy, indicating heteroplasmy (marked with an asterisk in Table 1), were also considered homologous in certain clearcut cases, despite differences in migration. Homology in uncertain cases was confirmed by mapping sites. Values of *F* were used to estimate nucleotide sequence divergence (p) of mtDNA types by the method of Nei & Li (1979) using Upholt's (1977) formula.

A phenogram was constructed from p using FITCH in Felsenstein's (1989) PHYLIP package, and rooted at the midpoint of the longest path.

Results

Variation of Bombina *mtDNA*

Fragment sizes produced by cleaving mtDNA of *Bombina* with each of the five restriction enzymes are shown in Table 1. Each enzyme cuts mtDNA into one to 10 fragments that range in size from ≈ 120 to 19 000 bp. By summing fragments produced by *Ava*I, *Bcl*I or *Hin*dIII, we estimated that mtDNA of different haplotypes differed in size by ≈ 500–1000 bp. In digests with most of the enzymes, one of the fragments appeared either fuzzy or separated into numerous faint bands (marked with an asterisk in Table 1). Fuzziness is a result of size heteroplasmy caused by a variable number of short tandem repeats within the D-loop (J. M. Szymura, unpublished). The 'average' length of such a heteroplasmic fragment varied among individuals of a particular haplotype, as well as among haplotypes.

Based on combined fragment patterns, we distinguished seven separate mtDNA haplotypes (Table 2) among the 83 individuals; these fell into three major groups that corresponded well with the geographical origin of the samples (*B. bombina* and two groups of *B. variegata*). Each of the restriction enzymes distinguished mtDNA of the three

Table 1 Restriction fragment patterns observed with five endonucleases in *Bombina bombina* and *B. variegata*

*Ava*I						
Pattern:	1	2	3	4		
	6000	6000	3300	3600		
	3300	2600	2700	2500*		
	2700	2100	2400*	2400		
	2300*	2000	2350	1800		
	2100	2000*	2300	1700		
	1800	1800	2200	1500		
	800	1200	1800	1400		
	120	800	1300	1250		
		120	800	1100		
				800		
*Bcl*I						
Pattern:	1′	1	2	3	4	4′
	6100	4400	5700	5600*	5100*	8900*
	3800	3800	4500*	4400	4400	4400
	2200*	2200*	3800	3800	3800	3400
	1600*	1700	1800	1800	3400	1300
	1300	1600*	1700	1650	1300	
	1250	1300	1000	1300		
	1200	1250	600			
	1100	1200				
		1100				
*Hin*dIII						
Pattern:	1	2	3	4		
	6000*	4800*	6200*	5900*		
	3600	3600	3600	3600		
	2600	3200	2600	2900		
	2400*	2400*	1900	2600		
	2300	2300	1200	1200		
	1100	1100	1000	900		
	650	950	900	580		
	580	650	580	300		
			300			
*Pst*I						
Pattern:	1	2	3			
	11 000	9200	8100			
	3700	4100	5800			
	2900	3700	2000			
	1300	2200	1300			
			800			
*Pvu*II						
Pattern:	1	2	3			
	19 000	6800	9600			
		4700	4800			
		3500	3700			
		3200				

*, variable-size fragments.
′, minor *Bcl*I variants.

major groups. This is consistent with results of a previous study (Szymura *et al*. 1985) showing considerable divergence between mtDNA of the two species in Poland.

Within *B. bombina*, three patterns of mtDNA were disclosed by restriction with *Hin*dIII and *Bcl*I. Type A was found in Poland and Turkey (Fig. 1, localities 1, 2), type B was found in Austria (locality 4) and type Z in Romania (locality 3). These three mtDNA types differed from each other by only one or two restriction sites. Type Z was intermediate in the sense that it had a *Bcl*I restriction pattern of type B, but a *Hin*dIII restriction pattern of type A. Another mtDNA variant was found in a hybrid zone between *B. bombina* and *B. variegata* in southern Poland. This mtDNA differed from type A only in having an 8900-bp *Bcl*I fragment rather than the 5100- and 3800-bp fragments found in the standard A type (Szymura *et al*. 1985).

In contrast, mtDNA types within *B. variegata* fell into two very different groups (Table 2). One group, type C, characterized *B. variegata* from the western Carpathians in Poland (Fig. 1, localities 5, 6). The other group contained two mtDNA types (D, E) that were distinguished by a single *Ava*I site. Type D was found in western Europe (localities 7, 8), whereas type E was found in the southern Balkans (localities 15–20). Geographically intermediate populations (localities 10–14) contained both of these mtDNA types. A minor *Bcl*I variant (type D′), with a 6100-bp fragment instead of the 4400- and 1700-bp fragments typical of type D mtDNA, was found in an individual of *B. variegata* from Budafa (locality 9). The other seven individuals in this sample had type D mtDNA.

Divergence among mtDNAs of Bombina

The five restriction enzymes employed in this study yielded a total of 66 distinct mtDNA fragments. All mtDNAs examined shared three of these: the 1800- and 800-bp *Ava*I fragments and the 3600-bp *Hin*dIII fragment (Table 1). These shared fragments indicated both that 9.5% of the fragments were conserved and that all 83 mtDNA samples in both species were ultimately derived from a single female ancestor.

Among pairs of mtDNA haplotypes, the proportion of shared fragments (Table 3) varied greatly, ranging from a high of 95.2% (between type D and type E *B. variegata* mtDNA) to a low of 25.8% (between type A of *B. bombina* and type C of *B. variegata*). This corresponded to sequence divergences of 0.3–8.1%, an ≈ 30-fold range (Table 3).

There was a surprisingly large divergence within mtDNA of *B. variegata*: the Carpathian type C differed from the western and Balkan types D and E by 4.7–5.2% of the nucleotides, only slightly less than the divergence between mtDNA of *B. bombina* and any *B. variegata* mtDNA (6.0–8.1% nucleotide difference). A phenogram rooted at the midpoint of the longest path based on divergences (Fig. 2) clustered the two *B. variegata* haplotypes, but showed a large, and presumably ancient, divergence within *B. variegata*; in contrast, divergences within *B. bombina* were small and presumably recent.

Table 2 Distribution of mitochondrial DNA (mtDNA) restriction patterns and haplotypes in samples of *Bombina bombina* and *B. variegata*

Locality	*N*	*Ava*I	*Bcl*I	*Hin*dIII	*Pst*I	*Pvu*II	Haplotype
B. bombina							
1. Żory	4	4	4	4	3	3	A
2. Adapazari	4	4	4	4	3	3	A
3. Bucharest	1	4	3	4	3	3	Z
4. Marchegg	5	4	3	3	3	3	B
B. variegata							
5. Stróże	3	3	2	2	2	2	C
6. St.Zawadowski	5	3	2	2	2	2	C
7. Aadorf/Zürich	8	1	1	1	1	1	D
8. Breitenfurt	4	1	1	1	1	1	D
9. Budafa/Pecs	8	1	1,1′	1	1	1	7:D, 1:D′
10. Mostar	3	1,2	1	1	1	1	1:D, 2:E
11. Rudnik	4	1,2	1	1	1	1	2:D, 2:E
12. Leskovo	8	1,2	1	1	1	1	5:D, 3:E
13. Brestovacka Banja	7	1,2	1	1	1	1	6:D, 1:E
14. Niška Banja	6	1,2	1	1	1	1	2:D, 4:E
15. Vranska Banja	5	2	1	1	1	1	E
16. Matka/Skopje	1	2	1	1	1	1	E
17. Peštani	1	2	1	1	1	1	E
18. Ohrid	1	2	1	1	1	1	E
19. Metsovon	2	2	1	1	1	1	E
20. Melissopetra	3	2	1	1	1	1	E

N, no. of individuals.
Primed symbols indicate minor variants.

Table 3 No. of fragments shared/no. of fragments compared, coefficient of similarity (*F*) (above the diagonal) and per cent sequence divergence (p) and its SD (in parenthesis) among mitochondrial DNA (mtDNA) haplotypes of *Bombina bombina* and *B. variegata* (below the diagonal)

		B. bombina			*B. variegata*		
		A	Z	B	C	D	E
B. bombina	A	—	28/61	28/62	8/62	9/60	11/61
			0.918	0.903	0.258	0.300	0.361
	Z	0.5	—	29/63	9/62	9/61	11/62
		(0.5)		0.921	0.286	0.295	0.355
	B	0.6	0.5	—	9/64	9/62	11/63
		(0.5)	(0.5)		0.281	0.290	0.349
B. variegata	C	8.1	7.5	7.6	—	14/62	13/63
		(1.5)	(1.5)	(1.5)		0.452	0.413
	D	7.2	7.3	7.4	4.7	—	29/61
		(1.5)	(1.5)	(1.5)	(1.2)		0.952
	E	6.0	6.1	6.2	5.2	0.3	—
		(1.4)	(1.4)	(1.4)	(1.3)	(0.4)	

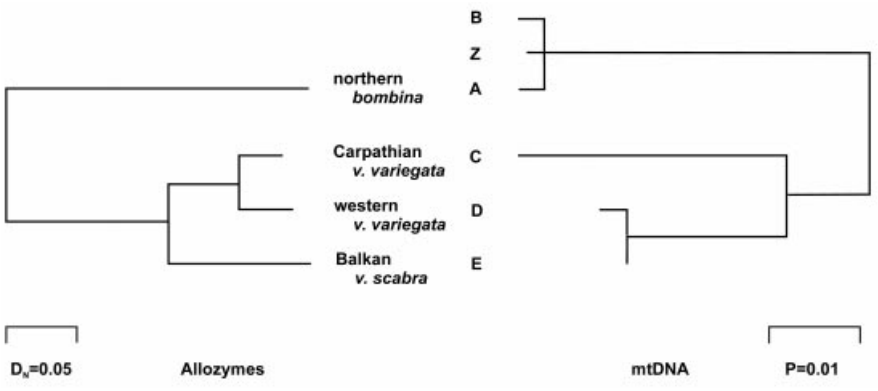

Fig. 2 Maximum likelihood trees obtained using FITCH in Felsenstein's PHYLIP 3.57 for mitochondrial DNA (mtDNA) and allozymes in *Bombina*. Phenograms are based on sequence similarity of mtDNA haplotypes (p) and Nei's *D* calculated from 29 loci studied in *B. bombina* and representative populations of *B. variegata* from the Carpathian, western and Balkan groups. The trees were rooted at the midpoint of the longest path.

A cline in mtDNA types in B. variegata

Samples of *B. variegata* from western Europe (localities 7–9) contained only type D mtDNA, whereas those from the southern Balkans (localities 15–20), three of which consisted of single individuals, contained only type E mtDNA. Samples from the northern Balkans (localities 10–14) contained both. These two mtDNA types, which differed by a single *Ava*I site ($P = 0.3\%$), may replace each other along a cline from Greece towards western Europe and also towards the Carpathian Mts. Such a cline would parallel clines observed at three allozyme loci (cf. Szymura 1988; Fig. 6).

Discussion

Our samples of *Bombina bombina* from Poland and Austria represent electrophoretically distinguishable groups of this species (Szymura 1988). The mtDNAs of the two groups differ at 0.6% of their nucleotides (Table 3). The sample from Turkey (Adapazari, locality 2), although geographically distant, is electrophoretically more similar to Polish than to Austrian *B. bombina* (Szymura 1988). This striking relationship is also supported by the present mtDNA analysis, which revealed no differences at 30 restriction sites between samples from Turkey and Poland (Table 2).

One of the two distinct haplotype groups in *B. variegata* is known only from the Carpathians. Few Carpathian samples were available so it is not known how widespread type C is. All Polish *B. variegata*, however, have this or a very similar mtDNA. Including samples reported in this paper, 101 mtDNA preparations from Polish *B. variegata* have been classified on the basis of *Hin*dIII and *Bcl*I digestion profiles; all are type C (Szymura *et al*. 1985).

The other distinct haplotype group in *B. variegata* is found in western Europe (haplotype D) and the southern Balkans (haplotype E). Populations polymorphic for mtDNA types D and E inhabit northern regions of the Balkans. The transition between these mtDNA types is paralleled by electrophoretic differences that separate *B. v. scabra* from *B. v. variegata*. Clines detected at three enzyme loci in the northern Balkans have been interpreted as resulting from a Pleistocene separation of *B. variegata* into northern and southern groups. Narrow transition zones that broadly overlap the clinal variation found in *B. variegata* have also been described for other species in the Balkans: *Natrix natrix natrix* (grass snake; Thorpe 1984), *Triturus vulgaris* (smooth newt; Kalezić 1984) and *Mus musculus/domesticus* (house mouse; Vanlerberghe *et al*. 1986). Coincident transition in these taxa suggests that both isolation and renewed, or secondary, contact had a similar basis in all of them (cf. Remington 1968; Taberlet *et al*. 1998).

In *B. variegata*, electrophoretic subgroups correspond to subspecific categories. The Balkan, western and Carpathian groups also have characteristic mtDNA haplotypes, but the relationships among these groups inferred from mtDNA and electrophoretic comparisons disagree (Fig. 2). A study of 29 allozyme loci showed that the Carpathian and western samples (*B. v. variegata*) are close to each other (Nei's $D = 0.07$) and approximately equidistant from the Balkan group, *B. v. scabra* (Nei's $D = 0.18–0.19$; Szymura 1988). In contrast, mtDNA places the western and Balkan groups together ($P = 0.3\%$), whereas the Carpathian *B. variegata* form a separate lineage that differs from the western and Balkan groups at 4.7–5.2% of its mtDNA nucleotides.

The discordant genealogy revealed among these groups of *B. variegata* at the nuclear and cytoplasmic level may have several causes: (i) the dissimilar mode of inheritance of mtDNA and nuclear alleles and the different rules governing the dynamics of these genetic elements in populations; (ii) lineage sorting; (iii) the past geological history of *B. variegata*; and (iv) the small number of endonucleases employed. Relating this mitochondrial/nuclear discrepancy to the past geological history of *B. variegata* is difficult, but the large divergence between the western and Carpathian groups (types D and C), almost as great as that between *B. bombina* and *B. variegata*, suggests a long independence of these two mitochondrial lineages. Similar intraspecific divergences in mtDNA have been observed within other European animal groups: *Crocidura suaveolens* ($P = 6.4\%$), *Arvicola* spp. (7.6%; Taberlet *et al*. 1998), *Erinaceus europaeus* and *E. concolor* (5.1% and 6.0%, respectively; Santucci *et al*. 1998), as well as within the *Triturus cristatus* superspecies (range 3.9–7.1%; Wallis & Arntzen 1989), indicating that many lineages within other species may also stem from the Pliocene (Taberlet *et al*. 1998).

Clear geographical trends in heterozygosity within *Bombina* were discovered in a study of allozyme variation (Szymura 1988, 1993). Northern populations of both species of *Bombina* are less variable than are southern populations, probably reflecting loss of alleles as the two species expanded following the last glaciation. At the same time, the two subspecific groups of *B. variegata* came into contact in the central Balkans. The time of contact between the subspecies can be inferred using cline theory. Assuming that the contact is neutral, as is highly probable between subspecific groups, the width of a cline (w) should be proportional to time (T) and gene flow (l) related to dispersal of animals: $w = (1.68\sqrt{T})/l$ (Endler 1977). The width of the cline in mtDNA and three allozyme loci, measured along a single transect as the distance between a frequency change from 0.2 to 0.8, is at most 125 km. The parameter l can be approximated by σ, the dispersal distance between parents and offspring. For the hybrid zone in Poland, σ is estimated to be 0.99 km gen-1/2 (Szymura & Barton 1991). Thus $T = 5648$ generations. Because generation time in *Bombina*

is ≈ 3 years, the absolute age of the contact is ≈ 17 000 years. This time estimate is consistent with the contact starting during a warm period following the last glacial maximum.

Palaeontological, electrophoretic and immunological evidence suggests that *B. bombina* and *B. variegata* diverged 2.5–6.8 million years ago (Ma) (Szymura 1983). Taking an average divergence between mtDNAs of *B. bombina* and *Bombina variegata* as 7.0%, it appears that mtDNA in *Bombina* evolved at a rate of 1–2.8% per million years (Myr), a rate comparable to that of mammalian mtDNA (Wilson *et al*. 1985). In a separate study using 17 endonucleases, mtDNA of *B. bombina* and *B. variegata* in Poland shared 21% of 124 fragments, which corresponds to a sequence divergence of 9.4 ± 1% (Szymura *et al*. 1985). This is higher than the 7.0% reported in the present work, and gives divergence rate estimates of 1.4–3.8% per Ma.

It has been suggested that mtDNA can cross species boundaries more easily than can nuclear genes both because it is not directly linked to genes that are involved in reproductive isolation and because its cytoplasmic location allows free recombination from its nuclear background (Barton & Jones 1983; Takahata & Slatkin 1984). In addition, mtDNA may have different selective values related to nuclear background (MacRae & Anderson 1988; Ballard & Kreitman 1995; Kilpatrick & Rand 1995). Given the asymmetry in initial matings between *B. bombina* and *B. variegata* (nearly all such matings are between female *B. bombina* and male *B. variegata*; Michałowski 1966) and higher fecundity of *B. bombina* females (Rafińska 1991), we would expect interspecies transfer of mtDNA from *B. bombina* into *B. variegata*. Although several range contractions and expansions apparently took place in the history of European *Bombina* (Szymura 1983, 1993), and hybridization seems to occur regularly when the two species are in contact, there is no indication of mtDNA leakage between *B. bombina* and *B. variegata* (cf. Szymura *et al*. 1985). If introgression took place soon after deglaciation, introgressed variants could spread as populations grew and spread. Because *Bombina* moved into areas virtually empty of competitors as the ice sheets receded, the spread may have been extremely rapid, comparable perhaps to the spread of *Bufo marinus* in Australia (Easteal 1981). Our mtDNA data were, however, unambiguous: we found no mtDNA leakage in either direction between *B. bombina* and *B. variegata*.

Of special interest in this respect was a population of *B. variegata* from Budafa (locality 9) in the Mecsek Mountains of Hungary. This isolated population, restricted to a small area, is surrounded by *B. bombina*, which inhabits the lowlands around the mountains (Fig. 1). All samples of *B. variegata* from this region had low frequencies of *B. bombina* electrophoretic alleles, indicating that the Budafa samples are located within a hybrid zone but on the *B. variegata* side of it (Szymura 1988). Nevertheless, all eight individuals from Budafa had type D mtDNA seen only in *B. variegata* (Table 2), suggesting insufficient backcrossing or movement of hybrid females carrying *Bombina* mtDNA to *B. variegata* to be detectable in our sample.

Given repeated opportunities for hybridization followed by introgression, it is striking that such effects have not been seen. It is possible that some shared allozymes (such as CK or IDH-1) reflect introgression of alleles between the species, although quite possibly they are convergent or inherited from a common ancestor.

The reasons for introgression of mtDNA through a hybrid zone in some areas (Ferris *et al*. 1983) but not in others (Sage *et al*. 1986; Vanlerberghe *et al*. 1986) are not clear, although both genetic background and a unique sequence of historic events may be involved. Even the dynamics of a hybrid zone may limit genetic exchange across the zone. Introgression may be considerably impeded by selection acting in two ways that are not mutually exclusive: either against unfit hybrids or favouring alternate genotypes in different environments (Szymura & Barton 1991). Indeed, both types of selection have been demonstrated in a *Bombina* hybrid zone (MacCallum *et al*. 1995; Kruuk *et al*. 1999). Moreover, because selection is spread over the whole genome, linkage disequilibria between loci will be maintained even when the zone moves in space tracking local environment. Initial associations between nuclear loci and mtDNA type can be further strengthened by mate choice or habitat preferences in areas where hybrid zones with a more patchy distribution have formed (Bugter *et al*. 1997; MacCallum *et al*. 1998).

Range expansions of European *Bombina* apparently took place after each Pleistocene glacial episode (Szymura 1983, 1993), and hybridization seems to occur regularly when the two species are in contact. During the glacial maxima, *B. bombina* and *B. variegata* were probably restricted to allopatric refuges located south of the permafrost area (cf. Figure 1), *B. bombina* perhaps along the northwest coast of the Black Sea, which was then a freshwater lake, *B. variegata* to one or two refugia in the western and southern Balkans. At such times, the hybrid zones between the two species would probably have disappeared. If this process was repeated after each interglacial period, the chance that an introgressed mtDNA or a nuclear allele would become established is low. In this sense, incoming glaciers acted as 'cold purgatory' and may have removed evidence of previous introgression at both nuclear and mtDNA levels.

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