Hybridization between the fire-bellied toads *Bombina bombina* and *Bombina variegata* in the karst regions of Slovakia and Hungary: morphological and allozyme evidence

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**Key words:** Hybrid zone; allozymes; *Bombina bombina*; *Bombina variegata*; amphibia.

**Abstract**

Geographic variability and genetic interactions in the contact zone between the fire-bellied toads, *Bombina bombina* and *B. variegata*, were studied using analysis of morphological and genetic variation in sixteen samples from the Slovak Karst and Aggtelek Karst regions. Genotype frequencies at four marker loci (*Ldh-I, Mdh-I, Adh, Hem*) demonstrate the existence of a hybrid zone with highly variable population structures. While some samples appear to represent panmictic hybrid populations, other samples are very heterogeneous. Pure individuals of both species occurred together with hybrids at one site. Habitat segregation among genetically differentiated demes probably causes this heterogeneity of population structures. Increased frequencies of the allele *Ldh-I*M, which is present in low proportion in *B. bombina* south of the contact zone, were found in some hybrid populations.

**Introduction**

The analysis of natural hybridization is one of the central and most active fields in the study of speciation. Conjectured dynamics of hybrid zones are of great impor-

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tance in several pertinent hypotheses, including the classical assumption of reinforcement of premating reproductive isolation ("adaptive speciation theory" sensu Moore and Buchanan, 1985) as well as the theories of parapatric and stasipatric speciation (Endler, 1977; White, 1978). Two decades ago it was a widely accepted opinion, termed the "ephemeral-zone hypothesis" by Moore (1977), that zonal hybridization was a short-lived phenomenon which would eventually lead either to fusion of the hybridizing taxa or to perfection of isolating mechanisms (see Wilson, 1963; Remington, 1968). However, more recent studies have produced ample evidence indicating that many hybrid zones are apparently stable and of ancient origin (see Moore, 1977; Barton and Hewitt, 1985). Explanations for this stability have been sought along three main lines of argumentation:

a) Hybrids are less fit than both parental forms; hybrid zones persist owing to a balance between dispersal and selection against hybrids. Hybrid zones maintained by this mechanism have been termed "tension zones" (Key, 1981; Barton and Hewitt, 1985).

b) Hybrids have greater fitness than either parental form in a restricted ecotonal region.

c) Different alleles are selected for in different environments on both sides of a hybrid zone.

Moore (1977) and Moore and Buchanan (1985) contrasted the first two models as (a) "dynamic-equilibrium hypothesis" and (b) "bounded hybrid superiority hypothesis", emphasizing the differences in selective regimes (endogenous vs. exogenous) thought to be crucial in determining the fitness of the hybrids.

The hybrid zone between the European fire-bellied toads, Bombina bombina and B. variegata, has already been studied by electrophoretic analysis of allozyme markers in some parts of Central Europe (Szymura, 1976a, b; Gollmann, 1984, 1986, in press; Szymura and Barton, 1986). Characteristic features of this hybrid zone are its great length and a considerable variation in population structures among different geographic regions. The position of the zone is clearly related to environmental factors and distinct adaptations of both species: B. variegata inhabits hilly and mountainous areas and usually spawns in small, intermittent pools, while B. bombina is a lowland species breeding in larger, more permanent bodies of water.

In an investigation on distribution and hybridization of both species in Slovakia, utilizing only morphological methods, Lác (1961) reported a peculiar situation from the Slovak Karst area: The lowland form B. bombina lived on the plateau of Slíka (Sličská planina) at higher altitudes than B. variegata, which was found in the neighbouring valley of the river Slaná. Lác (1961) suggested that the warmer climate of the plateau, in connection with recent human activities, would create favourable conditions for B. bombina.

This paper presents a new investigation of the fire-bellied toads from the Slovak Karst area and adjacent parts of Hungary (Aggtelek Karst), studying both morphological and allozymic variation. A preliminary compilation of a part of these data has already been published (Gollmann, 1986). Here we give full details of the results, compare them with the findings of Lác (1961), and discuss their implications for some general questions in the study of hybrid zones.
Material and Methods

A total of 176 specimens (32♂, 85♀, 59 juv.) was collected in August 1983 (Slovakia) and July 1984 (Hungary). Sample sites are indicated in the map (Fig. 1) and characterized in the following list. For each location name (usually that of a nearby village), approximate altitude, sample size and a brief description of the habitat are given.
A. Krásnohorská (300 m), n = 15, wheel rut puddles in Krásnohorská-Dlhá Lúka, immediately north of the bridge over river Čremošná.

B. Jovice (280 m), n = 15, wheel rut puddles on a country road and a moderately vegetated ditch near Jovice, in agricultural land south of river Čremošná.

C. Gombasek (260 m), n = 16, a pool above the curbed spring near Gombasek.

D. Bohuňovo (210 m), n = 7, a stream about 1 km north of Bohuňovo, in the section between its passage under the road from Plešivec to Bohuňovo and where it joins the river Slaná.

E. Silická Jablonica (300 m), n = 10, shallow pools near Brezinovský brook north of Silická Jablonica.

F. Silica I (580 m), n = 15, a few richly vegetated pools in a ditch about 600 m east of the lake Jaštěrčie jazero. One specimen collected in a shallow puddle very close to Jaštěrčie jazero and another one, caught in a puddle on the road about 200 m east of Jaštěrčie jazero, are also included in this sample.

G. Silica II (500 m), n = 7, wheel rut puddles in a meadow beside the road, about 1500 m east of locality J.

H. Silica III (490 m), n = 11, a drainage ditch about 1 km southeast of Silica.

I. Silica IV (500 m), n = 8, the bank of a large pond about 500 m southeast of Silica.

J. Silická Brezová (500 m), n = 15, hoofprint puddles at a spring in a dolina, about 1 km west of Silická Brezová.

K. Silická Vrbka (500 m), n = 8, small puddles with hoof prints, near the stream Ménes-patak.

L. Ménes-patak (340 m), n = 10, shallow pools with hoof prints, near the stream Ménes-patak.

M. Dlhá Ves (340 m), n = 7, two drainage ditches in an agricultural cooperative in Dlhá Ves.

N. Vörös-tő (320 m), n = 13, a large pond, vegetated with reeds and pond-weed.

P. Imola (260 m), n = 8, hoofprint puddles at a draw-well at Avas-oldal west of Imola.

Q. Szőlősardó (250 m), n = 11, a well southeast of Szőlősardó.

R. Rudabánya (300 m), n = 10, a large pond north of Rudabánya, with reeds and pond-weeds growing close to the bank.

Bombina tadpoles (including metamorphosing specimens) were observed at locations B, F, G and M, juveniles were present at sites A, B, D, G, J and K.

Colour pattern and skin structure characters were recorded using the classification tables of Lác (1961) and Gollmann (1984). As both species exhibit considerable overlap in morphometric relations, and because heterogeneity in the composition of sex and size classes among the samples and small sample sizes would introduce additional bias, no morphometric data will be given in this paper.

Electrophoretic investigations followed standard procedures (see Gollmann, 1984). Variation of the following proteins was studied: lactate dehydrogenase (LDH, EC 1.1.1.27), malate dehydrogenase (MDH, EC 1.1.1.37), creatine kinase (CK, EC 2.7.3.2), adenylate kinase (ADK, EC 2.7.4.3), phosphoglucomutase (PGM, EC 2.7.5.1), glucosephosphate isomerase (GPI, EC 5.3.1.9) and hemoglobin (HEM).
Results

Results obtained by both morphological classification tables were in good agreement. The heterogeneity of several samples precludes meaningful comparison of average values of populations, as presented in earlier papers (Gollmann, 1984, in press). To allow direct comparison of morphological and electrophoretic data, individual values at a morphological character index (MCI), which is based on the four characters that discriminate best between the two species (for details see Gollmann, in press), are indicated in the genetic hybrid index diagram (see below, Fig. 2). Detailed data on individual colour pattern variation are available on request from the first author.

![Fig. 2. Distribution of individuals over the hybrid index (HI) in all samples. Values at the morphological character index (MCI) are shown by shading of the rectangles representing single individuals: black = bombina-like (0–2), hatched = intermediate (3–5), white = variegata-like (6–8).](image)

Electrophoretic phenotypes found in this investigation were the same as described in previous studies (Szymura, 1976a; Gollmann, 1984, in press). The nomenclature of loci and alleles follows these papers. Ldh-2 and Pgm were monomorphic for the same alleles in all samples, at Mdh-2 a few individuals showed variant patterns, the genetic basis of which could not be unambiguously resolved. Allele frequencies at six polymorphic loci and average heterozygosity at these loci, determined by actual counts, are given in Table 1. To examine the statistical significance of increased frequencies of the rare allele Ldh-1H in some hybrid populations (see also Fig. 4 and discussion) these data were checked against approximations, which were estimated using the assumptions of a normal distribution of alleles and a linear decrease of both...
Table 1. Allele frequencies at six polymorphic loci and average heterozygosity (H) in 16 *Bombus* samples (n. s. = not sampled).

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"bombina"-alleles \((Ldh-1^M, Ldh-1^s)\) with increasing shares of the "variegata"-allele \(Ldh-1^F\) across the hybrid zone. Frequencies of \(Ldh-1^M\) were found to be significantly higher than the approximated values in samples H \((\alpha < 1\%)\) and J \((\alpha < 3\%)\).

To describe differentiation within and among localities a hybrid index (HI) was calculated based on the genotypes at four marker loci (see Gollmann, in press). The presence of each "variegata-allele" \((Ldh-1^F, Mdh-1^F, Adk^F, Hem^F)\) counts as one point on this index, which thus ranges from zero (pure \(B. bombina\)) to eight (pure \(B. variegata\)). Histograms of HI scores at single locations are shown in Fig. 2. Note that for samples A and B, where no hemoglobin data are available, HI ranges only from zero to six. Three individuals (two from sample I and one from sample E) are not included in Fig. 2, as data on them are incomplete. Six specimens (two from sample H and one from samples B, J, K and L) were heterozygous at all marker loci, thus representing potential \(F_1\) hybrids. However, their individual morphologies as well as the absence of pure parental forms at most of these locations indicate that they are more likely recombinants than \(F_1\) hybrids.

**Discussion**

The structure of the hybrid zone

Láč (1961) reported the occurrence of \(B. bombina\), showing only minor traces of introgression, at the exceptionally high altitude of about 600 m on the plateau of Silica (Silická planina). In the narrow valley of the river Slaná, northwest of this plateau, he found \(B. variegata\) near the villages of Gombasek and Vidová, while \(B. bombina\), bearing signs of hybridization, lived in this valley only south of Plešivec (see Fig. 1).

The new samples collected in the valleys of Slaná (C, Gombasek) and its tributary Čremošná (A, Krásnohoršká; B, Jovice) consist mainly of \(B. variegata\), exhibiting very low levels of introgression of "bombina-alleles". However, in samples B (Jovice) and C (Gombasek) a few \(bombina\)-like and intermediate hybrids are also present (see Fig. 2). Sample E (Silická Jablonica), likewise collected at the foot of the steep slope bordering the plateau, but in the Turná drainage system, represents a population of \(B. variegata\), too. In the area south of Plešivec, where Láč (1961) found a \(bombina\)-like population, the habitat has been largely destroyed by construction of a road and installation of a refuse pit. Sample D (Bohuňovo), collected south of this place, is composed of \(variegata\)-like hybrids.

Sample site F (Silica I) is closest to the location given for the \(B. bombina\) population on Silická planina in the map of Láč (1961, p. 20). \(Variegata\)-like hybrids prevail in this sample, but pure \(B. variegata\) individuals and intermediate hybrids are also present. Sample J (Silica IV) is even more heterogeneous, containing pure specimens of both species as well as intermediate hybrids. On the other hand, genotype frequencies and poor correlation of HI and MCI values in samples H (Silica III) and K (Silická Brezová) suggest that these samples represent panmictic hybrid populations. Samples G (Silica II) and L (Ménes-völgy) contain various hybrid genotypes with a slight preponderance of "\(variegata\)-alleles", while in the southern parts of the
Sloako-Hungarian Karst region *bombina*-like populations were found, which showed decreasing frequencies of introgressed “*variegata*-alleles” towards the south (samples M, N, P, Q and R).

These data require cautious interpretation, as sampling was carried out in late summer after the main breeding period. Seasonal migrations of *Bombina* are known (see Madej, 1973; Beshkov and Jameson, 1980; Kapfberger, 1984), although not well understood. Meteorological conditions causing filling or desiccation of ponds and puddles certainly affect these migrations strongly. The summer of 1983 was exceptionally dry. So, it is possible that many toads were not collected at their breeding sites; rather, individuals spawning at different places may have been concentrated in a few remaining pools.

![Map of Slovak Karst region](image)

**Fig. 3.** Average frequencies of “*bombina*-alleles” (black sectors) at four (for samples A and B three) marker loci.

Nevertheless, some conclusions on the structure of the contact zone in the Slovak Karst/Aggtelek Karst area can be drawn. Populations of almost pure *B. variegata* and almost pure *B. bombina* are separated by a hybrid zone of about 20 km width (see Fig. 3), which is roughly the same as found in other parts of Central Europe (see Karaman, 1922; Szymura, 1976b; Gollmann, 1984). But whereas in most other
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regions zones of allopatric hybridization (sensu Woodruff, 1973) exist, showing transition of gene frequencies through sequences of panmictic hybrid populations in “stepped” clines (Gollmann, 1984; Szymura and Barton, 1986), the genetic structure of the hybrid zone in the Slovako-Hungarian Karst area is more complicated. Pure individuals of both species are present in the centre of this zone, although extensive hybridization occurs. At some sites hybrid populations may have reached genetic equilibrium. Altitude is a poor predictor of gene frequencies in this area.

To explain the heterogeneity of population structures in this hybrid zone is a matter of speculation. Habitat qualities are undoubtedly of great importance in determining position and character of the contact zones in *Bombina*. The patchy environmental structure of the karst region creates possibilities for habitat segregation among genetically differentiated demes. Thus, choice of different breeding places may account for the sympatric coexistence of both species in the presence of a majority of hybrids.

The data given by Lác (1961) are too sparse to allow any valid inference on changes in the hybrid zone in the course of the last 25 years. However, a retreat of *B. bombina*, as was observed in other parts of Slovakia (Gollmann, 1986), seems to have taken place in this region, too.

A gloss on the “rare allele phenomenon”

Increased frequencies of otherwise rare alleles and the occurrence of unique alleles in hybrid populations have been noted in many investigations of hybrid zones (see Case and Williams, 1984; Murphy et al., 1984; Barton and Hewitt, 1985; Kat,

![Fig. 4. Frequencies of $Ldh-1$ alleles in 19 Bombina populations (Closed circles: data from this study; open circles: hybrid populations from the Labeorec valley in eastern Slovakia, see Gollmann, 1986) given as attitudes of the triangle. The line close to the basis indicates the expected mean frequencies of allele $M$ in hybrid populations assuming neutral diffusion.](image-url)
1986). Hunt and Selander (1973) suggested that selective values of alleles may be altered in the new genetic environment created by introgression. Other possible explanations for increased frequencies of minor alleles in hybrid populations include higher mutation rates in hybrids (Woodruff and Thompson, 1980), intracistronic recombination (Golding and Strobeck, 1983) and genetic drift (Szumura and Święs, 1979). The small sample sizes of the present study are not well suited for detecting rare alleles or accurately determining their frequencies. However, the variation in frequencies of \( \text{Ldh-I}^M \) allows some inferences to be drawn on this topic. This allozyme obviously did not arise by mutation or intragenic recombination in the hybrid zone, as it is present in \( B. \) bombina outside this zone, usually in frequencies of about 10% in northern Hungary (Szumura, in prep.; Gollmann, in press; locations N, P, Q, and R of this study). It is striking that the highest frequencies of \( \text{Ldh-I}^M \) are found in hybrid populations showing an intermediate genetic constitution (Table 1, Fig. 4). Sampling error and genetic drift may contribute to this pattern of variation, yet it seems to fit the assertion of modified selective regimes in hybrid populations better than a purely neutralistic interpretation of the “rare allele phenomenon”.

**Dynamics of hybridization in Bombina**

The narrow hybrid zones in \( B. \) bombina appear to be stable, even if their position may have been subjected to large changes during postglacial range expansion of both species (Arntzen, 1978) and to smaller shifts in historical time. So far, no indications of either incipient fusion of the parental gene pools or of character displacement have been found in any area of contact between \( B. \) bombina and \( B. \) variegata.

The many obviously adaptive differences between the two species (see Szumura and Barton, 1986, p. 1142) suggest that differential selection of alleles on both sides of the contact zone is a significant component in the dynamics of hybridization. Given the existence of intermediate habitats, this dissimilarity would also provide a basis for regarding a hybrid superiority model. However, increased embryonic mortality and a higher incidence of morphological aberrations in the centre of the hybrid zone near Cracow lead Szumura and Barton (1986) to the conclusion that selection against hybrids was the primary force in maintaining this hybrid zone. Considering the pronounced local differentiation among hybrid populations in Lower Austria, Gollmann (1984) assumed that gene flow between these populations was unimportant; like populations H (Silica III) and K (Silická Brezová) from Silická planina they possibly represent stabilized hybrid swarms, which have regained genetic balance in a process resembling the “founder-flush” model of Carson (1975; Carson and Templeton, 1984). Circumstantial evidence from other vertebrate species shows that re-establishment of genomic coadaptation may indeed proceed rapidly in hybrid populations (see Graham and Felley, 1985). Unfortunately, sufficient genetical and ecological data on \( B. \) bombina, which could allow a profound synthetic approach to integrate these partly conflicting findings and ideas, are still missing.

At best, a comparative assessment of geographic variation may provide some clues to the underlying mechanisms maintaining these hybrid zones. Modifications
of evolutionary interactions along contact zones have been detected in a number of taxa (see Harrison and Arnold, 1982; Littlejohn and Watson, 1985; Howard, 1986). Various factors have been discussed in attempts to account for this variability: duration of the contact, genetic variation in the hybridizing taxa, and variation in ecological conditions. In Bombina, neither the time which has elapsed since the first contact nor genetic variation in either species can explain the pattern of variation among different parts of the hybrid zone; thus, population structures seem to be determined by local environmental conditions and by casual events during colonization of particular habitats (Gollmann, 1984; Gollmann and Szymura, 1986; Szymura in prep.).

The scope of the present study is restricted by small sample sizes and the limited number of electrophoretic markers and morphological characters that have been analysed. Nevertheless, it may hold as a general conclusion that even more elaborate morphological and genetic investigations may help in sharpening questions on the dynamics and evolutionary significance of hybrid zones, rather than answering them. To achieve the goal of understanding these hybrid zones, detailed field studies of behavioural ecology and population dynamics will be necessary.

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