

Exploration of a hybrid zone between two toad species in Central Europe's Carpathian region with a new molecular marker

Zoltán GÁL^{1a*}, Tibor KOVÁCS^{2a}, János UJSZEGI³, Brandon P. ANTHONY⁴,
Balázs VÁGI⁵ and Orsolya I. HOFFMANN¹

1. Hungarian University of Agriculture and Life Sciences, Gödöllő, Pest County, Hungary.

2. Hungarian Biodiversity Research Society, Budapest, Hungary.

3. Lendület Evolutionary Ecology Research Group, Plant Protection Institute, Centre for Agricultural Research, Budapest, Hungary.

4. Department of Environmental Sciences and Policy, Central European University, Vienna, Austria.

5. University of Debrecen, Department of Evolutionary Zoology and Human Biology, Debrecen, Hajdú-Bihar County, Hungary.

a. These authors contributed equally to this work.

* Corresponding author, Z. Gál, E-mail: zoltan.gal89@gmail.com

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Abstract. Analyzing hybrid zones allows insight into interactions between previously isolated lineages. The distributions of fire-bellied toads (*Bombina orientalis*) and yellow-bellied toads (*B. orientalis*) meet in the Carpathian Basin. We aimed to explore the genetic composition of a transient zone between the two sister species along a transect in the Carpathian foothill region. We collected 230 specimens from 21 locations and sampled them using buccal swabs. We used mitochondrial markers and designed a novel test based on the restriction of the nuclear *Ncx-1* gene to distinguish between the two species and determine hybrid individuals. According to our results, the complete Börzsöny Hills and many locations in Krupinská Planina were colonized by *B. orientalis*, including typical *B. orientalis* habitats at higher elevations. However, in Krupinská Planina, the most remote sites still harbor *B. orientalis* populations, often mixed with hybrid individuals. This pattern may indicate the northward and altitudinal range expansion of *B. orientalis*. Our results warrant enhanced attention to hybrid zones, where introgression and other changes in the populations' genetic composition may reflect recent rapid environmental alterations.

Keywords: *Bombina orientalis*, *Bombina orientalis*, contact zone, distribution mapping, introgression.

Introduction

Hybridization between closely related species often occurs when previously isolated sister lineages come into secondary contact (Barton & Hewitt 1985, Harrison 1993). Analyzing hybrid zones of genetically and ecologically differentiated but not reproductively isolated taxa allows insight into admixture processes determining the fate of the closely related lineages after the re-encounter (Arntzen et al. 2020).

In Europe, several hybridization cases are considered a consequence of warming periods after glacial ages, when previously allopatric populations of sister lineages became parapatric due to area expansions (Hewitt 2000). The European fire-bellied and yellow-bellied toads (*Bombina orientalis* and *B. orientalis*; Bombinatoridae) form a narrow hybrid zone extending far across several regions within Central Europe (Szymura & Barton 1986, Gollmann et al. 1988, Vörös et al. 2006, Szymura 2009). The two species have otherwise parapatric distribution: *B. orientalis* occupies the lowlands of Europe from Germany to Russia, while *B. orientalis* occurs mainly in the highlands of the Balkan Peninsula, Central and Western Europe, and is absent east of the Carpathian Ridges (Arnold 1995).

Due to transitional morphological characteristics expressed by hybrid individuals, reliable identification of the sister species and their hybrids is only possible with the application of molecular methods, including protein electrophoresis (Gollmann 1987, Gollmann et al. 1988, Szymura 1983, 2009) and DNA-based techniques such as mitochondrial sequencing (Hofman et al. 2007, Vörös et al. 2006), single copy nuclear gene sequencing (Fijarczyk et al. 2011), and microsatellite analysis (Nürnberg et al. 2005, Vines et al. 2003). Employing molecular methods also enabled tracing the post-glacial area expansions of the two species.

Based on mtDNA analyses, the range expansion of *B. orientalis* initiated from one refugium near the Black Sea coast. In contrast, the recent distribution of the more diverse haplotypes of *B. orientalis* (Pröhl et al. 2021) reflects post-glacial radiation from Balkanian, Mediterranean, and Carpathian refugia. The larger genetic variation of the latter species is not surprising, as it is a mountain-dweller (Cogălniceanu et al. 2013, Szabolcs et al. 2017) with more isolated populations while *B. orientalis* is restricted mainly to plains and hilly regions (Cogălniceanu et al. 2013) with enhanced opportunities for gene flow.

In Central Europe, the complex topography of the Carpathians and its surrounding basin created a complicated and diverse contact zone between the two species. The inner part of the Carpathian Basin is predominantly a lowland, which is populated mainly by connected populations of *B. orientalis* (100-300 m and rarely above, up to 400-500 m) (MacCallum et al. 1998, Indykiewicz 2011). In contrast, *B. orientalis* occurs in the surrounding mountain range (up to 2000 m and rarely below 300) and forms isolated populations in inner mountainous enclaves. The two species come into contact along the foothill regions, creating an extended contact zone in the Carpathian Basin, resulting in hybrid individuals in many populations (Gollmann 1987, Vörös et al. 2006).

The Carpathian contact zone of the *Bombina* species is only partially explored. Gollmann et al. (1988) studied a shorter section of the ridges on the border of northern Hungary and southern Slovakia in the Aggtelek Karst region and revealed a hybrid zone between the two species with highly variable population structures. Sas et al. (2005) reported a hybrid population around Oradea, Romania. Evidence of hybridizing populations from other sections of the Carpathians was also shown (Vines et al. 2003, Yanchukov et

al. 2006, Hofman et al. 2007).

We aimed to explore a possible transient zone between *B. bombina* and *B. variegata* and assess the distribution of the two species and their hybrids from the inner part of the Carpathian Basin to the southern edge of the Carpathian Ridges along an approximately 50 km long north-south transect (Figure 1). We also aimed to test a novel molecular marker based on the nuclear *Ncx-1* gene to distinguish between the two species and determine hybrid individuals. We hypothesized that the distribution of the species would follow an altitudinal separation according to species preferences, and hybrid individuals would appear only at transient sites between lowland and mountainous habitats.

Material and Methods

Study area

Börzsöny Hills are located in northern Hungary, separated from Krupinská Planina in Slovakia by the valley of the Ipoly River, which also demarcates the border between the two countries (Figure 1). The highest elevation in Börzsöny is 938 m a.s.l. Due to its rugged terrain, most valleys are strongly shaded with cold microclimates. Krupinská Planina is a sibling range of Börzsöny in Slovakia consisting of identical rock material, a Miocene andesite. Its highest point is only 535 m a.s.l., but its terrain is heavily rugged. Published herpetofaunistic data from Börzsöny is outdated (Szabó 1960). At the same time, plenty of data were collected in an online database (<https://herppterkep.mme.hu>), but no systematic assessment was made for *Bombina* species prior to this study. Herpetofauna data of Krupinská Planina is also lacking, as the only record available is a summary of a 2-year (2008–2009) data collection. Both *Bombina* species had been observed in the survey, but the author did not analyze their distributions (Urban 2009).

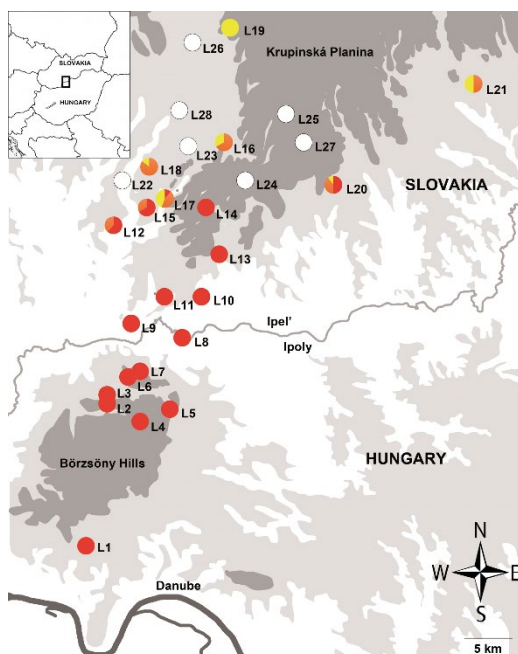


Figure 1. Map of the sampling area. Circles indicate the locations (L1-21). White, light grey and dark grey areas represent the relief < 200 m, between 200-400 m and > 400 m, respectively. Pie chart of the locations represents the distribution of the species; red is *B. bombina*, orange is hybrid, and yellow is *B. variegata*. Empty circles represent sites where no *Bombina* were observed. For location names, see Table 1; for altitudes, see Supplementary Table S1.

Sample collection

We searched for *Bombina* habitats based on literature sources and an online database (herppterkep.mme.hu). Potential habitats were marked on satellite maps (Google Earth). We visited 21 sites (Supplementary Table S1) and sampled 230 toads between 2014 and 2018. Individuals were caught by hand, and DNA samples were collected using a non-invasive buccal swabbing. Each individual was placed into a transparent plastic box and fixed by gently pressing a plastic sponge against their back. The ventral pattern of each toad was photographed for further analyses (not presented here, Supplementary Fig. S1), and they were then released at the capture site.

Amplification and analysis of the nuclear *Ncx-1* gene

The collected swabs were stored in 70% ethanol until the DNA extraction by the phenol-chloroform method (Sambrook & Russell 2001). The following primers were used to amplify an 846 base pair (bp) long region of the *Ncx-1* gene: *NcxF* (5'-TCATCCGCTCCTGAAATTC-3') and *NcxR* (5'-CACAGTCCCACAGTTTTCCA-3') (Fijarczyk et al. 2011). All PCR reactions were implemented with MyTaq Ready Mix (Bioline) according to the manufacturer's instructions. The reactions contained 80 ng DNA and 8 μ M of each primer. The PCR conditions were the following: an initial denaturation step at 95 °C for 5 min, 30 cycles with denaturation at 95 °C for 20 sec, an annealing temperature of 62 °C for 20 sec, and elongation at 72 °C for 20 sec, and a final elongation at 72 °C for 2 min. The 846 bp long PCR product was analyzed in 1% agarose gel.

To find restriction fragment length polymorphism (RFLP) loci in the *Ncx-1* gene, haplotypes were downloaded from the National Centre for Biotechnology Information (NCBI) and aligned to a consensus sequence both in *B. bombina* and *B. variegata* with ClustalW2 (EMBL-EBI). Three were obtained from *B. bombina*, and 16 haplotypes originated from *B. variegata* according to Fijarczyk et al. (2011). The GenBank accession numbers can be found in Supplementary Table S2. The created *B. bombina* and *B. variegata* *Ncx-1* consensus sequences were compared pairwise with MultAlign (Corpet 1988). With NEBCutter (Vincze et al. 2003) and dCAPS Finder (Neff et al. 2002), four HpyCH4V restriction enzyme sites (TG[^]CA) were detected in *B. bombina*, with one out of four missing from the *B. variegata* sequences (Supplementary Fig. S2). The amplified PCR fragments were digested with the HpyCH4V enzyme (New England Biolab) at 37 °C for 3 hours. Ten μ l of each digestion reaction were loaded into 2.5 % TAE agarose gel, and GeneRuler™ 1 kb Plus DNA Ladder (Fermentas) was used as a molecular weight marker. The digestion results in a 694 bp long fragment in the case of *B. variegata*. *B. bombina* samples cleave to 369 bp and 325 bp long fragments. The samples of hybrid individuals carry each of the 694 bp, 369 bp, and 325 bp long fragments. The remaining 152 bp split into 96 bp, 32 bp, and 24 bp long fragments in both genotypes (Figure 2).

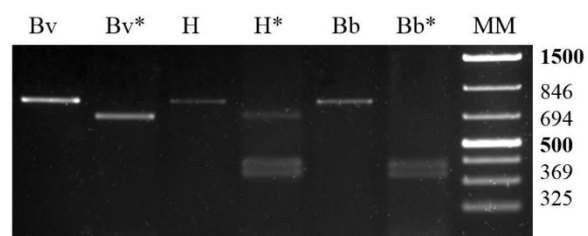


Figure 2. Electrophoretic differentiation of the digested PCR fragments specific to the *Ncx-1* gene. The amplified PCR product from the *Ncx-1* gene before enzymatic digestion: *B. variegata* (Bv; identifier of the individual: 101), *B. bombina* (Bb; identifier of the individual: 189) and hybrid (H (Bv \times Bb); identifier of the individual: 169) Fragments resulted by the digestion with HpyCH4V restriction enzyme: Bv* (101), H* (Bv \times Bb) (169), Bb* (189). MM - molecular weight marker.

Sequencing

Thirty-seven of the PCR amplified *Ncx-1* gene fragments were sequenced by Sanger sequencing (Eurofins Genomics, Ebersberg, Germany) to verify the digesting method. The resulting chromatograms were analyzed with Chromas 2.6.5 software (Technelyium).

Amplification of the mitochondrial fragments

Six samples were processed at each location where only one species occurred according to the nuclear marker. From the other locations, all samples were analyzed with the following primers designed to the mitochondrial sequence of *Bombina* species. The primers were based on the total *B. bombina* and *B. variegata* mitochondrial genome at NCBI (EU115993.1, NC_009258.1). Two mitochondrial primer pairs were used to distinguish the two species: RadF (5'-CAGCTAGTATCAACCCACCAGAT -3') and RadR (5'-TTGATCTGTGTCTGGGTACGCTCTG -3') primers are specific for *B. bombina*, TynF (5'-CAATAAAATTCAACCGCCAACAAT -3') and TynR (5'-AAGTTGATCTGTGTCTGGGTAIGTCTA -3') primers are specific for *B. variegata* mitochondria (Pabijan et al. 2008). The PCR reactions were completed with Mytaq Red Mix (Bioline) according to the manufacturer's instructions. The PCR cycles were the following in the case of the Rad primer pair: an initial denaturation step at 95 °C for 5 min, 35 cycles with denaturation at 95 °C for 20 sec, an annealing temperature of 63 °C for 20 sec, and elongation at 72 °C for 20 sec and a final elongation at 72 °C for 2 min. The same PCR protocol was used with the Tyn primer pair, except that the annealing temperature was 60 °C. The resulting DNA fragments were analyzed at 2.5 % agarose gel containing ethidium-bromide with GeneRuler™ 1 kb Plus DNA Ladder (Fermentas). The RadF-R primers amplify a 173 bp long fragment; the amplicon with the TynF-R primers is 196 bp long (Figure 3).

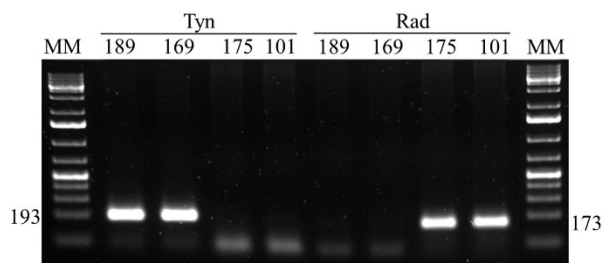


Figure 3. The amplification of mitochondrial regions with Tyn and Rad primer pairs specific for *B. bombina* (Bb) and *B. variegata* (Bv), respectively. According to the nuclear *Ncx-1* marker, the individual 189 carries Bb genotype, while the individual 101 carries Bv genotype. Individuals 169 and 175 are hybrids. MM - molecular weight marker.

Ethics approval

We confirm the collection of samples was conducted in accordance with all applicable laws, guidelines, and regulations. The permits were issued by the Közép-Duna-völgyi Környezetvédelmi és Természetvédelmi Felügyelőség, No: KTF11887-3/2014 (Hungary) and Odbor Státnej Správy Ochrany Prírody, No: 4175/2016-2.3 (Slovakia).

Results

Identifying a new nuclear marker in the *Ncx-1* gene

Altogether, we analyzed 230 samples from 21 sampling sites in Hungary and Slovakia (Table 1). We could successfully amplify an 846 bp long fragment of the *Ncx-1* gene in all 230

cases. The designed restriction enzyme digestion with HpyCH4V could distinguish between the two species and the hybrid individuals. We sequenced 13 *B. variegata*, 9 *B. bombina*, and all the 26 hybrid individuals to validate our digestion results. The sequencing confirmed the result of the *Ncx-1* enzymatic digestion in all cases, as the heterozygous locus was specific to the individuals who were considered hybrids based on the digestion fragment composition (Supplementary Fig. S2).

Analyzing of mtDNA

We analyzed the mitochondrial DNA of 119 samples from 14 locations (including all 7 locations with hybrids and half of the locations where only *B. bombina* was found) with the two primer pairs that can distinguish between the *B. bombina* and *B. variegata* mitochondria. The samples showed that the mitochondrial markers agreed with the *Ncx-1* gene results at all 7 tested locations where we found only *B. bombina* individuals (Table 1). Among the 7 sites with hybrids, at one location, the mitochondrial markers revealed *B. bombina* genotype; at five locations, the results showed *B. variegata* mitochondria. There was one location in the hybrid zone - Plášťovce (L12) - where we detected both *B. bombina* and *B. variegata* mitochondrion (Supplementary Table S3). Table 2 summarizes the analyses based on the two mitochondrial markers in 22 hybrid individuals (in 4 individuals, we failed to amplify mtDNA; see Supplementary Table S4 for more details).

Distribution of the two species in the study area

Based on our species identification method, *Bombina bombina* occurred at 17 locations (including all locations in Börzsöny Hills), while *B. variegata* occurred at six locations. In 13 locations, all individuals proved to have *B. bombina* genotype. At one location (Čekovce), we found only the *B. variegata* genotype. We identified 7 locations where hybrid individuals (to the *Ncx-1* nuclear marker) occurred, covering an approximately ten kilometers wide zone. In total, we caught 26 hybrid individuals identified with the *Ncx-1* nuclear gene marker at seven locations: Plášťovce, Brezovo, Čabrad, Litava, Medovarce, Pribelce, Chrťany. At Litava and Pribelce we found *B. bombina*, *B. variegata* and hybrid toads.

The examined toad populations in the Börzsöny Hills and the Ipoly Valley consist of *B. bombina* genotype specimens, and both species were found in the Krupinská Planina area. *B. variegata* genotype appears exclusively in the latter region's coolest, remote highland areas, while *B. bombina* occupied the edges of the plateau at even higher elevations. On average, *B. variegata* genotype individuals occupied higher elevations than *B. bombina* genotypes, but the two reached the same maximum altitude. Hybrids occurred at an intermediate altitude between the two genotypes' median elevations (Figure 4). However, there was no significant difference between the altitudinal distributions of the homozygous individuals and the hybrids (one-way ANOVA, for all locations: $F_{2,27} = 0.473$; $P = 0.63$; only for locations in Krupinská Planina, excluding pure *B. bombina* sites in Börzsöny and Ipoly Valley: $F_{2,18} = 1.064$; $P = 0.37$). In the northern half of the Krupinská Planina, we investigated 7 more locations (all artificial ponds), where we did not find any *Bombina* specimens (Figure 1).

Table 1. Species identification results according to nuclear marker Ncx-1 at each location. Bb: *Bombina bombina*, Bv: *Bombina variegata*, H: hybrid individual.

Location number	Location name	Altitude (m)	Number of samples	Nuclear marker		
				Bb	Bv	H
L1	Kapitány-rét	259	29	29	-	-
L2	Kemence 1	329	1	1	-	-
L3	Kemence 2	232	2	2	-	-
L4	Királykút	409	11	11	-	-
L5	Pénzásás	419	15	15	-	-
L6	Hármashatár	347	5	5	-	-
L7	Hont	200	10	10	-	-
L8	Drégelypalánk	127	10	10	-	-
L9	Tešmák	124	15	15	-	-
L10	Sečianky	163	5	5	-	-
L11	Berínček	184	6	6	-	-
L12	Plášťovce	150	19	14	-	5
L13	Vinica	203	20	20	-	-
L14	Hrušov	405	15	15	-	-
L15	Brezovo	261	3	2	-	1
L16	Čabrad	252	9	-	6	3
L17	Litava	208	34	6	14	14
L18	Medovarce	360	7	-	6	1
L19	Čekovce	420	2	-	2	-
L20	Príbelce	267	10	5	4	1
L21	Chrťany	231	2	-	1	1
Sum			230	171	33	26

Table 2. Combined mitochondrial and nuclear genotypes of individuals from locations where hybrids occurred.

Nuclear marker	Mitochondrial marker	Sample size	Location ID
Bb	Bb	14	L12, L15, L17
Bb	Bv	7	L17, L20
Bv	Bv	27	L16, L17, L18, L20, L21
H	Bb	3	L12, L15
H	Bv	19	L12, L16, L17, L18, L20

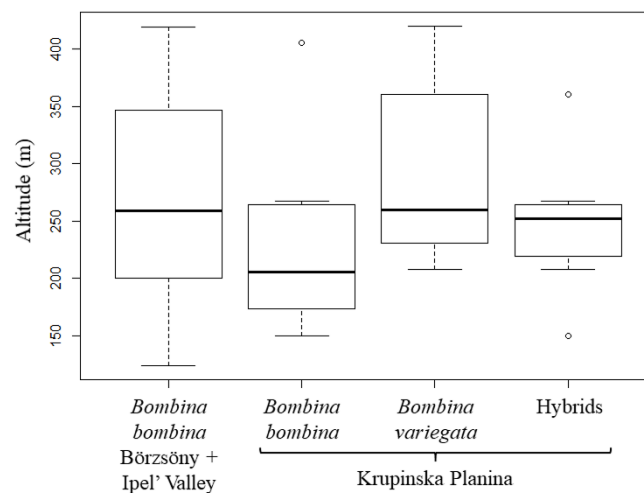


Figure 4. The altitudinal distribution of the two species and their hybrids in the two areas. The horizontal line is the median; whiskers represent range, boxes represent interquartiles, and the circle indicates an outlier (deviating from the boundary of the interquartile range (IQR) by more than $1.5 \times \text{IQR}$).

Discussion

In this study, we used a genetic test based on the restricted digestion of a nuclear gene, which was never applied to identify *B. bombina* and *B. variegata* and their hybrids. We successfully identified the two homozygous and heterozygous genotypes from a transient area of the distribution of the two species. Then, we compared the nuclear genotypes of the sampled individuals with their mitochondrial markers and found various genomic combinations in the actual hybrid zone. Our molecular analyses showed that, in contrast to Börzsöny Hills, which harbors only *B. bombina* genotype populations, Krupinská Planina is inhabited by both species, which produce hybrids in sympatry.

Since we did not detect introgression outside of a 10-12 kilometers wide range between the populations of the sister species, we assume this hybrid zone to be relatively narrow, similarly to other *Bombina* hybrid zones reported previously (Yanchukov et al. 2006, Fijarczyk et al. 2011, Dufresnes et al. 2020). Furthermore, the distribution patterns we explored are characteristically different on the two sides of the Ipoly Valley, and we could not confirm the supposed altitudinal divergence in neither area. While in Börzsöny Hills, we found only *B. bombina* populations even at the highest locations and in the smallest wheel track water bodies, in Krupinská Planina, the distribution of the two species and hybrids show a geographic and altitudinal overlap. While there was no significant difference between the altitudes occupied by each species, in general, *B. bombina* occupies the lowest and more southern locations, while *B. variegata* occurs at the most northern location and highest altitude. Intermediate locations (both in altitudinal and geographic terms) are inhabited by hybrid populations, where *B. variegata* appear to be the more abundant parent species.

In the Krupinská Planina, the greatest frequency of hybrid individuals has *B. variegata* mitochondrial haplotype. This pattern can be explained by the greater abundance of *B. variegata* in these habitats and sampling bias due to low sample sizes in certain locations. As hybrids occurred mostly alongside *B. variegata* individuals, it seems likely that *B. bombina* genes introgressed into these populations via occasional dispersal of *B. bombina* individuals, similar to the findings by Dufresnes et al. (2020), indicating that the invasion of *B. bombina* alleles into the range of *B. variegata* is more frequent than gene flow in the opposite direction. In *B. variegata*, there is a limited dispersion of individuals among wetlands, and the species shows site fidelity (Beshkov 1980). According to Beshkov (1980), *B. variegata* shows sex-specific bias in dispersal (with larger distances covered by the males), while Hartel (2008) did not detect bias. Much less is known about dispersal in *B. bombina* as this species tends to inhabit large, continuous wetlands, where dispersal patterns are more difficult to determine. However, the fact that most hybrids have *B. variegata* maternal origins implies that hybridization arose from matings between *B. variegata* females and *B. bombina* males. This is consistent with the pattern found in other anuran species, where males disperse further than females (Wang et al. 2020) and that indiscriminate, coercive males readily mate with heterospecific females under both experimental and natural

conditions (Hettyey et al. 2014, Vági & Hettyey 2016).

It is interesting whether Börzsöny Hills harbored *B. variegata* populations in the past, which were replaced by its congener. Alternatively, as we did not detect hybridization introgression and have no historical data about the occurrence of *B. variegata* from Börzsöny Hills, we can only speculate if the species never reached this area. Several factors may delimit species dispersal even into favorable habitats. In addition to the 21 sampling sites, we visited 7 ponds in the northernmost portion of our study site, where we found no *Bombina* individuals, despite the habitats appearing suitable for at least one of the species. The absence of either species in the artificial ponds may be a result of (i) barriers to dispersal within the landscape matrix, including unsuitable microclimates and habitat for facilitating movement (Cayuela et al. 2020), (ii) the relatively short time since these ponds have been established coupled with relatively short dispersal distances of *Bombina* species (Barandun & Reyer 1998), and/or (iii) the fact that these permanent ponds were artificial, deeper and moderate in size which are less likely to be utilized by *B. variegata* (Hartel & von Wehrden 2013). Similar obstacles could have impeded the colonization of Börzsöny Hills by *B. variegata* in historic times.

The unexpected occurrence of *B. bombina* populations at *variegata*-like habitats in the Börzsöny Hills is similar to the findings by Gollmann (1987) in Mátra Hills. It suggests that *B. bombina* may have a broader ecological niche and can colonize more isolated habitats with higher altitudes in the absence of interspecific competition than expected based on its typical range, as may have happened in other regions in the Carpathian Basin (Arntzen 1978, Gollmann 1987, Vörös et al. 2006).

Climate change may have also contributed to altering the species' distribution ranges (McCarty 2001, Lindner et al. 2010, Walther 2010, Bellard et al. 2012). Although many studies revealed a rapid and sometimes irreversible decline in amphibian populations due to global warming (Carey & Alexander 2003, Lips et al. 2008, Blaustein et al. 2010), some species will respond by dispersing and expanding their ranges (Araújo et al. 2006). Of the two *Bombina* species, *B. variegata* seems to be more susceptible to these changes, as it reproduces predominantly in temporal wetlands (Barandun & Reyer 1998), and desiccation of its breeding habitats is the main regulator of its metapopulation dynamics (Hartel et al. 2007). The lack of connectivity between its habitats can cause severe inbreeding depression in its populations (Pröhl et al. 2021), which may cause higher susceptibility to diseases (Oswald et al. 2020). In contrast, *B. bombina* can find refuge in more permanent, larger wetlands during droughts. Possible area expansion of *B. bombina* has been recently documented or suggested at the northern edge of its range (Pupina & Pupins 2013; Dolgener et al. 2014). The higher genetic diversity of *B. bombina* populations than its sister species (Dolgener et al. 2014, Pröhl et al. 2021) reflects more favorable prospects for this lowland-dwelling species. In line with these findings, area expansion of *B. bombina* is more likely in the Carpathian Basin due to the climate shifting in a hotter and drier direction (Bartholy & Pongrácz 2007, Bartholy et al. 2009, Pongrácz et al. 2011).

Both *Bombina* species are considered reliable indicators of habitat quality and are listed under the European Union's

Council Directive 92/43/EEC on the conservation of natural habitats and wild fauna and flora (<https://eur-lex.europa.eu>). Being species of community interest, their population sizes should be regularly monitored. Our research highlights that special attention should be paid to hybrid zones, where population composition changes should be investigated in more detail, as they may reflect the effects of recent rapid environmental alterations. As morphological identification of individuals could be problematic due to the large overlap between phenotypes, emerging genetic methods provide a useful tool to track the hybrid population's genetic composition.

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+ Supplementary material (available online)

Supplementary table S1

Supplementary table S2

Supplementary table S3

Supplementary table S4

Supplementary figure S1

Supplementary figure S2