

## Revisiting a speciation classic: Comparative analyses support sharp but leaky transitions between *Bombina* toads

Christophe Dufresnes<sup>1\*</sup>, Tomasz Suchan<sup>2</sup>, Nazar A. Smirnov<sup>3</sup>, Mathieu Denoël<sup>4</sup>, Juriy M. Rosanov<sup>5</sup>, Spartak N. Litvinchuk<sup>5,6</sup>

<sup>1</sup> *LASER, College of the Biology and the Environment, Nanjing Forestry University, Nanjing, People's Republic of China*

<sup>2</sup> *W. Szafer Institute of Botany, Polish Academy of Sciences, Kraków, Poland*

<sup>3</sup> *Department of Nature, Chernivtsi Regional Museum, Chernivtsi, Ukraine*

<sup>4</sup> *Laboratory of Ecology and Conservation of Amphibians (LECA), Freshwater and Oceanic science Unit of reSearch (FOCUS), University of Liège (ULiège), Liège, Belgium*

<sup>5</sup> *Institute of Cytology, Russian Academy of Sciences, St. Petersburg, Russia*

<sup>6</sup> *Department of Biology, Dagestan State University, Makhachkala, Russia*

\* *corresponding author*

### Abstract

**Aim** – The amount of gene flow between parapatric species can be greatly variable depending on how species boundaries are maintained in respect to numerous genetic and ecological factors that affect the strength of reproductive isolation. We quantified this variability to understand its effect on the genetic integrity of a well-studied pair of hybridizing amphibians.

**Location** – Central and Eastern Europe

**Taxa** – The fire- and yellow-bellied toads *Bombina bombina* and *B. variegata*.

**Methods** – We first complemented the mitochondrial phylogeography of European *Bombina* by barcoding additional populations and built MaxEnt species distribution models to identify the routes of post-glacial colonization that led to hybrid zone formation. Second, we targeted the areas of parapatry in Poland and Ukraine to assess nuclear admixture by population genetics (PCA, Bayesian clustering) of allozyme and genomic markers (RAD-seq). Third, we harvested the rich *Bombina* literature to thoroughly compare the extent of geographic introgression across eleven transitions altogether, using cline analyses when possible.

**Results** – We found sharp (2-11km wide) but leaky transitions (geographically extensive traces of introgression) in all parapatric areas, either measured from a few allozymes or thousands of species-diagnostic SNPs, and irrespective of the intraspecific lineages involved or the relative ages since first contact.

**Main conclusion** – *Bombina* species are permeable to gene flow despite selection against hybrids that retards but cannot ultimately prevent putatively neutral introgression into the parental gene pools. Although local hybrid swarms face genetic assimilation, the species' integrities remain intact due to their large geographic ranges and ecological differentiation that enforces tight boundaries regardless of the biogeographic attributes of the contacts. Opposing the great variability of introgression patterns found between cryptic species, these results support that gene flow should not be viewed as a force of despeciation when reproductive isolation involves multiple intrinsic and extrinsic barriers.

**Key words:** Cline Analyses, Hybrid Zone, Hybridization, Introgression, RAD sequencing

## Introduction

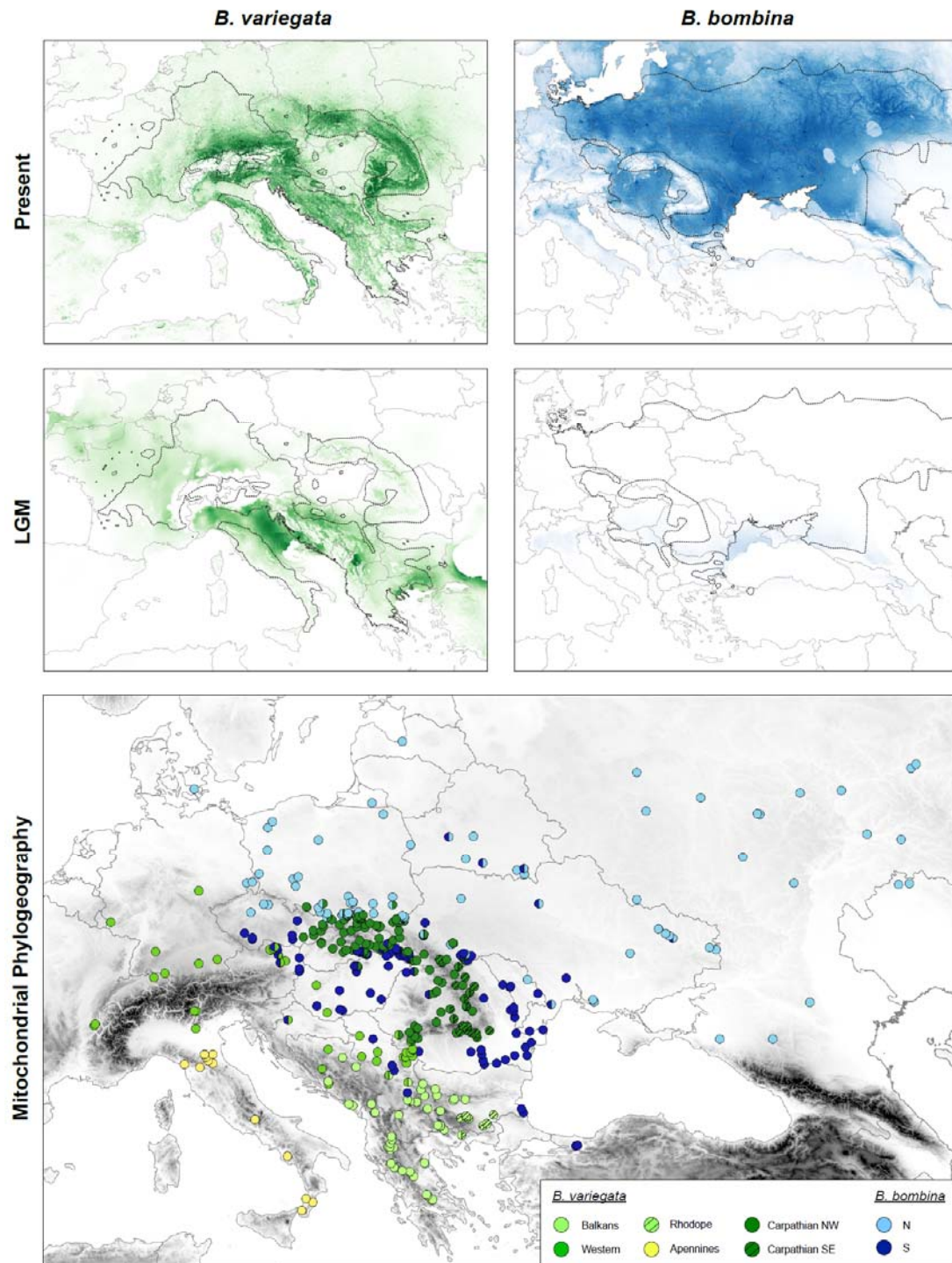
Hybrid zones offer natural laboratories where to gauge the progress and mechanisms of reproductive isolation between incipient species (Hewitt, 1988; Barton & Hewitt, 1989). As such, hybrid zone analyses have become useful models for speciation (Harrison, 1993), phylogeographic (Avice, 2000) and systematic studies (Dufresnes, Pribille, et al., 2020). However, hybrid zones can expand, collapse, or become isolated from parental ranges, resulting in wide admixture or narrow tension zones for the same species pair (Dufresnes, Berroneau, Dubey, Litvinchuk, & Perrin, 2020). For instance, hybridization events during post-glacial range expansions have left traces of introgression over extensive geographic areas (Wielstra & Arntzen, 2012; Wielstra, Burke, Butlin, Avci, et al., 2017). Hybrid zones often move (Buggs, 2007; Wielstra, 2019), as the parental species shifted distributions since the initial contact, and foreign alleles may persist hundreds of kilometers away from the current transitions (Wielstra, Burke, Butlin, & Arntzen, 2017; Dufresnes, Litvinchuk, et al., 2020). At the regional level, geographic or ecological changes altering population connectivity, such as large river displacements or human-induced habitat disturbances, can further contribute to dynamically promote or disrupt species boundaries over short timescales (e.g. Hasselman et al., 2014; Burbán et al., 2020). Finally, admixture patterns at secondary contacts also depend on intrinsic factors: reproductive isolation is sometimes variable, as the different intraspecific lineages of hybridizing species may not have the same level of compatibility (Barnard-Kubow & Galloway, 2017).

Given the complex evolutionary processes of hybrid zone formation (e.g. Arntzen, de Vries, Canestrelli, & Martínez-Solano, 2017), the limited sets of nuclear loci traditionally used in phylogeography and population genetics may not accurately capture their geographic and genomic pictures of introgression. For instance, this low resolution hampers the reliability to detect admixed individuals beyond the first generations of backcrossing (Boecklen & Howard, 1997), especially if selection against hybrids is strong and only small portions of the genome pass reproductive barriers. Moreover, because the loci cross-amplifying between diverged taxa often retain ancestral polymorphism, shared alleles might be misinterpreted as traces of introgression, hence blurring true species boundaries. We recently highlighted this matter when re-investigating European amphibian hybrid zones with hundreds of SNPs obtained from RAD-sequencing data, which yielded drastically different results compared to previous surveys based on conventional markers (e.g. Dufresnes, Nieceza, et al., 2020; Dufresnes, Litvinchuk, et al. 2020). Therefore, targeting replicate transects with multilocus datasets that represent the entire genome appears necessary to fully grasp the outcome of secondary contacts between hybridizing species (Harrison & Larson, 2016).

In the light of these issues, we revisit a pioneer model of speciation, the fire-bellied toads *Bombina orientalis* and *B. variegata*, which form hybrid zones running hundreds of kilometers along mountain-lowland transitions in Central and Eastern Europe. Since the first genetic evidence of

hybridization (Szymura, 1976a, 1976b), this system inspired many theoretical advances on hybrid zone research, including cline theory (Barton & Hewitt, 1985; Szymura & Barton, 1986; Barton & Gale, 1993). Following original analyses of a transect near Kraków in southern Poland (Szymura & Barton, 1986), *B. bombina* / *variegata* contacts were extensively characterized elsewhere in Poland (Szymura & Barton, 1991), Romania (Vines et al., 2003), Ukraine (Yanchukov et al., 2006), Austria (Gollmann, 1984), Croatia (Nürnberger, Barton, MacCallum, Gilchrist, & Appleby, 1995), Slovakia and Hungary (Gollmann, 1987; Gollmann, Roth, & Hödl, 1988) (Fig. 1). The two species diverged since the Miocene (>6My of divergence; Pabijan et al., 2013) and their transitions are mediated by intrinsic genetic incompatibilities (Szymura & Barton, 1986) that translate into hybrid dysfunctions (Kruuk, Gilchrist, & Barton, 1999), together with other factors such as mating calls (Sanderson, Szymura, & Barton, 1992), morphological and developmental traits (Nürnberger et al., 1995), and habitat preferences (MacCallum, Nürnberger, Barton, & Szymura, 1998) associated with different ecological pressures (e.g. tadpole predation, Kruuk & Gilchrist, 1997). The remarkable maintenance of these adaptations in the face of gene flow has made fire-bellied toads a rare model at the interplay between allo-parapatric and ecological speciation.

In spite of their significance in the field, the surveyed *Bombina* hybrid zones were only analyzed with limited sets of markers, notably allozymes (Table 1). The parapatric ranges investigated all consist of narrow transitions located at ecotones that connect the respective habitats of the species (Szymura & Barton, 1986; Yanchukov et al., 2006), such as lowland vegetated ponds for *B. bombina*, and mountain meadows and forests with temporary puddles for *B. variegata* (e.g. MacCallum et al., 1998; Hartel & von Wehrden, 2013). Genetic analyses concur that *Bombina* hybrid zones are maintained by a stable balance between dispersal and selection against hybrids, which should protect the parental genomes from introgression. Yet, remarkable differences in the patterns of admixture were reported, including swarms of backcrossed individuals (Gollmann, 1987; Vines et al., 2003), smooth (Nürnberger et al., 1995; Gollmann et al., 1988) or stepped clinal transitions (Szymura & Barton, 1991; Yanchukov et al., 2006), where foreign alleles often reach tens of kilometers outside the contact. However, coalescent analyses of transcriptomic data from pure populations flanking the Kraków transect revealed negligible contemporary gene flow from the nearby hybrid zone, although the species continuously exchanged genes since their initial split (Nürnberger, Lohse, Fijarczyk, Szymura, & Blaxter, 2016). These puzzling observations, together with the fact that previous inferences rely on a few loci, call for a reassessment of *Bombina* hybrid zones using genome-wide data. Moreover, given the intraspecific diversification of both species during the Quaternary (Hofman et al., 2007; Fijarczyk et al., 2011) and the complex mitochondrial distributions in Central Europe (e.g. Vörös, Alcobendas, Martínez-Solano, & García-París, 2006), this system provides opportunities to quantify the variability of admixture depending on the biogeographic attributes of secondary contacts.



**Fig. 1: Species distribution modelling and mitochondrial phylogeography of *Bombina* in Europe.** Top panels project species occurrence probabilities (as shades of green and blue colors) under current and last glacial maximum (LGM) conditions. The bottom panel maps the *cytochrome-b* lineages identified by Fijarczyk et al. (2011), complemented by our new data. While the *cyt-b* structure informs on Quaternary roots of post-glacial colonization, shallow lineages were not recovered from mitogenome analyses (Fig. 2).

**Table 1: A summary of the 11 *Bombina* contact zones analyzed with nuclear markers.** Transitions are either mosaic, hybrid swarms or clinal, in which case we performed cline analyses on the genome average and report the selected cline model and the cline width (km). The biogeographic attributes of each contact are given as follows: distance (in km) from the putative Black Sea refugium and the intraspecific lineage of *B. variegata* contacted (C or BW for Carpathian or Balkano-Western, respectively). Geographic locations are displayed in Fig. 2.

Code	Area	Year	Diagnostic nuclear loci	Type/Cline	Width	Biogeography	Display	Reference
A	Kraków	1974-1981	5–6 allozymes	2-tails	6.7 (5.9-7.7)	1000 - C	Fig. 5	Szymura & Barton, 1986
B	SE-Austria	1979-1982	6 allozymes	mosaic?	-	1400 - BW	File S6	Gollmann, 1984
C	Karst	1983-1984	6 allozymes	1-tail ( <i>B. variegata</i> )	7.1 (3.6-14.9)	1200 - C	Fig. 5	Gollmann, et al., 1988
D	Mátra	1984	4 allozymes	hybrid swarm	-	1100 - C	File S7	Gollmann, 1987
E	Przemysł	1985-1986	5–6 allozymes	1-tail ( <i>B. variegata</i> )	8.5 (6.9-10.4)	800 - C	Fig. 5	Szymura et al., 1991
F	Peščenica	1991-1992	4 allozymes	1-tail ( <i>B. variegata</i> )	5.1 (2.0-9.1)	1200 - BW	Fig. 5	Nürnberger et al., 1995
G	Transcarpathians	1993-2002	6 allozymes	2-tails	11.4 (8.7-16.9)	1200 - C	Figs. 4, 5	this study
H	Apahida	2000-2001	4 $\mu$ sats	mosaic	-	1100 - C	-	Vines et al., 2003
I	Stryi	2000-2003	1-4 allozymes/ $\mu$ sats	2-tails	2.1 (1.3-3.3)	700 - C	Fig. 5	Yanchukov et al., 2006
J	Chernivtsi	2008-2017	653 SNPs	mosaic	-	500 - C	Fig. 3	this study
K	Bochnia	2017	1,883 SNPs	no tail	6.2 (0.0-12.5)	1000 - C	Figs. 3, 5	this study

In this study, we build upon forty years of genetic work to develop a comparative framework on *Bombina* hybrid zones. We first traced the glacial and post-glacial ranges of *B. bombina* and *B. variegata* using species distribution models to document how and when hybrid zones were putatively formed, in respect to the mitochondrial phylogeography of species. Second, we assess patterns of introgression across three different contact zones from the Carpathian ranges, in southern Poland and southwestern Ukraine (Cisrpathian and Transcarpathian). Genotyping thousands of RAD-seq loci, we expect narrow transitions between pure populations if reproductive isolation is strong enough to prevent introgression into the opposite gene pools. Hence, combining data for eleven transitions altogether, our aim was to assess the variability of admixture among the ecologically-diverged *B. bombina* and *B. variegata*, and to test whether it was affected by their biogeographic history.

## Methods

### *Phylogeography and glacial refugia*

To get insights into the origin of *Bombina* hybrid zones in time and space, we mapped the main mitochondrial clades reported by Fijarczyk et al. (2011) for the gene *cytochrome-b* (*cyt-b*). We complemented their extensive dataset by our own *cyt-b* barcoding for 41 new localities covering three parapatric areas (primarily surveyed with nuclear markers, see below) and additional locations from central and eastern Europe (Table S1). Samples consisted of tissues taken from live adults (non-invasive buccal swabs preserved at -20°C or toe clips preserved in 96% ethanol) or specimens of the ZISP (Zoological Institute of Russian Academy of Sciences, St. Petersburg) herpetological collection (muscle pieces preserved in 70% ethanol). DNA was extracted with the Biosprint Robotic workstation (Qiagen). A ~500bp *cyt-b* fragment was then amplified with newly designed primers (Bomb-F1: 5'-GGATCYTTTATTGACCTCCCC-3'; Bomb-R2: 5'-TTGATCCTGTTTCGTGAAG-3'). PCR were carried out in 25µL reactions, including 7.5µL of Qiagen MPMM (a mix containing hot start polymerase, buffer and dNTPs), and 1µL of each primer (10mM), and were run as follow: 95°C for 15'; 35 cycles of 94°C for 30", 53°C for 45", 72°C for 1'; 72°C for 10'. Amplicons were sanger-sequenced in a single direction (Bomb-F1) and sequences were manually aligned in Seaview (Gouy, Guindon, & Gascuel, 2010). The final dataset included eight *cyt-b* clades (six for *B. variegata* and two for *B. bombina*) across 429 localities, four of which correspond to robust monophyletic clades in phylogenies built from full mitogenomes (Pabijan et al., 2013).

To trace the location of glacial refugia, we also reconstructed ecological niche models for each species and predicted their ranges under current and LGM conditions. Models were built with MaxEnt 3.4.1 (Phillips, Anderson, & Schapire, 2006), with the full procedures detailed in Appendix II. Briefly, for each species, we filtered occurrence records (1461 *B. bombina* and 508 *B. variegata*; available in Geofile S1) and environmental variables (23 in *B. bombina* and 25 in *B. variegata*) to be retained in the final models, which parameters were calibrated with the R package *kuenm* (Cobos, Peterson, Barve, & Osorio-Olvera, 2019). We then projected the niches obtained according to two LGM atmospheric circulation models: the Community Climate System Model (CCSM; <http://www2.cesm.ucar.edu/>) and the Model for Interdisciplinary Research on Climate (MIROC; Watanabe et al., 2011).

### *Genomic survey of the Polish and Ciscarpathian contact zones*

A total of 85 and 80 adult *Bombina* samples collected from parapatric ranges in southern Poland (14 localities, near Bochnia, Lesser Poland) and southwestern Ukraine (10 localities, near Chernivtsi, Ciscarpathians), respectively, were analyzed by RAD-seq (Table S1). Two genomic libraries (one per contact zone) were prepared following the double digest RAD (ddRAD) protocol detailed in Brelsford

et al. (2016). Each was sequenced on two lanes of Illumina Hi-Seq 2500 (single read 125). Raw sequences were demultiplexed and processed with stacks version 1.48 (Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013), using default  $-m -n$ , and  $-M$  parameters. We then called polymorphic tags sequenced in all individuals of all populations of the Ukrainian contact ( $-p 10 -r 1$ ), which yielded 4,294 SNPs from 2,993 tags (100% of matrix completeness). In Poland, we retained those sequenced in at least 75% of individuals of each population ( $-p 14 -r 0.75$ ), which yielded 6,010 SNPs from 3,263 tags (96.3% of matrix completeness).

To evaluate the genetic structure among the initial datasets, we computed pairwise  $F_{st}$  distances (*hierfstat*, Goudet, 2005) and performed Principal Component Analyses (PCA) on individual allele frequencies (*adegenet*, Jombart, 2008). In both study areas, most of the genetic variance was explained by the differentiation between the two species, which corresponded to the first component (PC1) of the analyses (see Results). In the absence of reference samples from allopatric ranges in the RADseq libraries, we maximized the species diagnosticity of our dataset by selecting loci which contributed most to build these components, i.e. with PC1 scores above 90% (attribute  $co > 0.9$ ). We further retained a single SNP per tag to avoid physically linked loci. The final datasets included 653 SNPs (Ukraine) and 1,883 SNPs (Poland). From these, we estimated the individual ancestry to *B. bombina* and *B. variegata* using the Bayesian clustering algorithm of STRUCTURE (Pritchard, Stephens, & Donnelly, 2000), focusing on analyses with  $K = 2$  with the admixture model, uncorrelated allele frequency and no prior assignment of individuals. Ten replicates were run, each of 100,000 iterations after 10,000 of burnin, and combined with CLUMPP (Jakobsson & Rosenberg, 2007).

#### *Genetic analyses of the Transcarpathian parapatric ranges*

From 1993 to 2002, 491 *Bombina* individuals were collected from 42 Transcarpathian localities (southwestern Ukraine), anaesthetized (immersion 1% MS222), and sampled for blood and muscles (stored at ZISP, details in Table S1). The genome of *B. bombina* is about 7% larger than *B. variegata* and can be reliably diagnosed as a barcoding tool (Borkin, Litvinchuk, Rosanov, Džukić, & Kalezić, 2005; Litvinchuk, Rosanov, Borkin, & Skorinov, 2008). To this end, the genome size (nuclear DNA content) was measured for all but one individual ( $n = 490$ ) using DNA flow cytometry following the procedures in Borkin, Litvinchuk, Rosanov, & Milto (2001).

In addition, 392 individuals from 33 populations were genotyped for six allozymes with species-diagnostic polymorphism: Ldh-B (lactate dehydrogenase), Mdh-2 (malate), Est-1, Est-2, EstD-1, EstD-3 (esterase). Pieces of femur muscle (stored at  $-80^{\circ}\text{C}$ ) were used as tissue samples and vertical polyacrylamide (6–8%) gel electrophoresis was performed using Tris-boric-EDTA pH 8.3 (for Ldh-B, Est-1, EstD-1, and EstD-3) and Tris-citric pH 8.0 (Mdh-2 and Est-2) buffers. The proteins were visualized by standard techniques (Shaw & Prasad, 1970).

## *Review of Bombina contact zones and cline analyses*

To visualize and compare patterns of admixture with previously studied *Bombina* contact zones, we extracted the average frequency of *B. variegata* alleles genotyped along the Kraków and Przemyśl transects in Poland (Table 1 in Szymura & Barton, 1991), the Pešćenica transect in Croatia (Table 1 in Nürnberger et al., 1995), the Stryi transect in Ukraine (Yanchukov et al., 2006, data kindly provided by A. Yanchukov), as well as from northeastern Austria (Table 2 in Gollmann, 1984), the Mátra Mountains in Hungary (Table 1 in Gollmann, 1987) and the Karst region in Hungary/Slovakia (Table 1 from Gollmann et al., 1988). For the mosaic hybrid zone of Apahida in Romania, we refer to the original study by Vines et al. (2003).

We fitted sigmoid clines to the average nuclear ancestry of populations sampled along geographic transects, which was possible for seven hybrid zones: Kraków, Przemyśl, Stryi, Pešćenica, Karst, Transcarpathian and Bochnia. For the first three, we followed the transect directions and distances given in the original studies. For Pešćenica, we set up a transect running northeast-southwest along localities 1053, 1039, 1040, 2143, 1104, 1110, 2011, 1113, 2146, 2147, 1099, 2163, 2200, 2165 in Nürnberger et al. (1995), which goes from pure *B. bombina* in the Sava valley to pure *B. variegata* in the Pešćenica hills, but limits the influence of *B. bombina* contacting from the southeastern side. For the Karst area, Gollmann (1987) sampled the hybrid zone along a northeast-southwest direction (localities A–R). Like in Stryi (Yanchukov et al., 2006), the Transcarpathian hybrid zone extends along an ecotone at the mountain foothills and our sampling was two-dimensional (see Results). We thus followed a similar approach by delineating the predicted center of the transitions along the foothills and calculating the distances from this line in Google Earth (<https://earth.google.com>). For the Bochnia contact zone, we selected localities 1–7 and 12–14, which broadly follow a linear south-north transect. For the remaining four contact zones, it was not adequate to fit clines as the sampling did not target specific transects (Chernivtsi, NE-Austria) or the transition had no clinal direction (Apahida, Mátra).

Clines were estimated using the maximum-likelihood algorithm of *hzar* (Derryberry, Derryberry, Maley, & Brumfield, 2014). The MCMC chains were run 100,000 iterations, after 10,000 of burnin. The direction from *B. bombina* to *B. variegata* was chosen in all seven transects for comparison. In each, we tested cline models involving only two parameters (the center  $c$  and a width  $w$ ) and up to eight parameters, including the frequencies in edge populations ( $P_{min}$  and  $P_{max}$ ), and the position and steepness of the exponential tails on either side of the cline. A total of 12 models were tested per transect, and we reported those with the lowest AIC criterion.



## Results

### *Glacial refugia of Bombina*

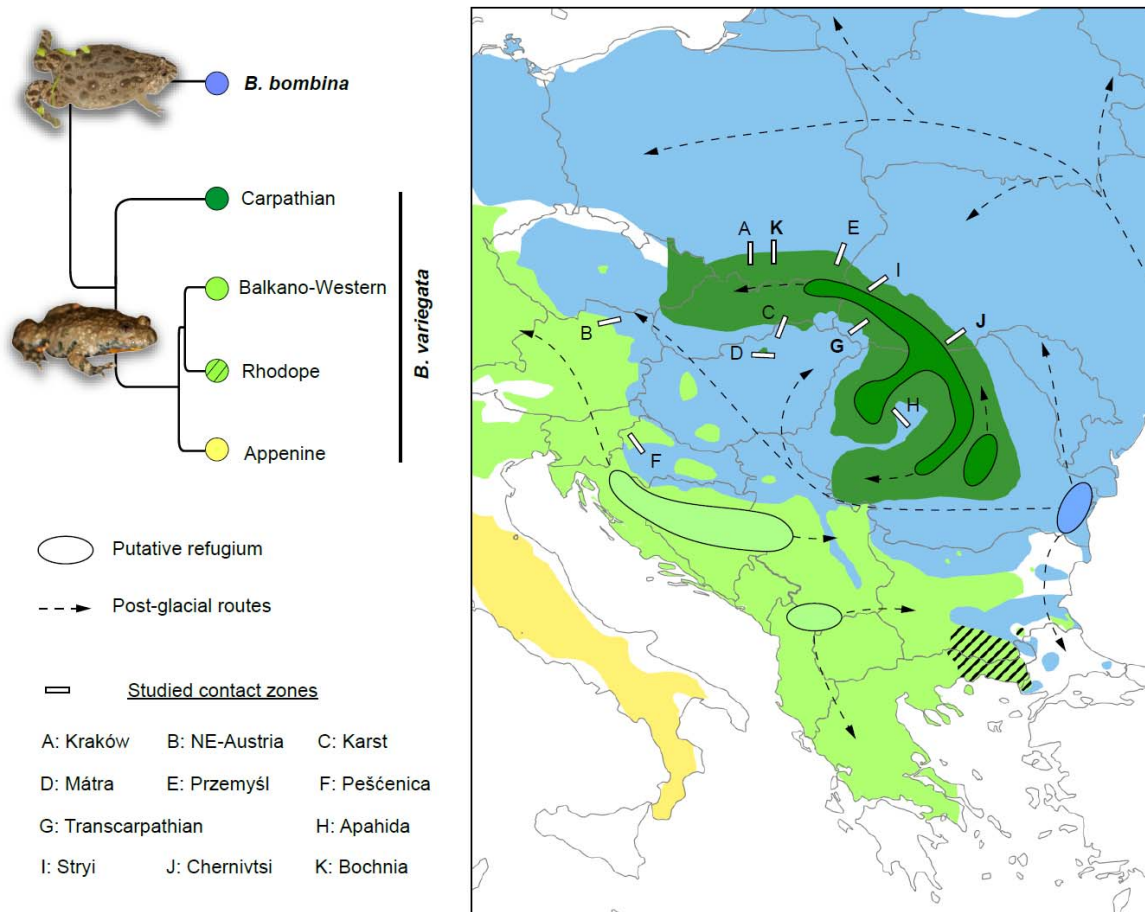
The distribution models under current climatic conditions properly depicted the ranges presently inhabited by each species (Fig. 1). Variable contribution and performance metrics are available in Table S2. Precipitation during the driest month was the most important variable for *B. bombina* (51%). For *B. variegata*, the most important variables were the coverage by broadleaf forests (12%), and precipitation in the warmer quarter (10%).

The projected distributions under the MIROC and CCSM models were broadly similar, so we report only the latter (Fig. 1). Most of Europe was unsuitable for *B. bombina* during the LGM. The only areas with non-zero probabilities of occurrence border the Black Sea, i.e. where the two shallow mitochondrial lineages identified in that species putatively originate from (Fijarczyk et al., 2011). LGM conditions were more suitable for *B. variegata*, and some refugia can be attributed to distinct lineages (Fig. 1): northeastern Greece and southern Bulgaria (Rhodope lineage), southwestern Serbia and northeastern Albania (Balkan lineage), and along the Dinaric Mountains (Western lineage). The Apennine lineage seems to have survived in northern Italy, notably the Po plain, which formerly extended into the present Adriatic Sea and provided mild LGM conditions. Finally, no clear refugia stand out for the Carpathian clade of *B. variegata*, which was potentially isolated in mountain patches.

As summarized in Fig. 2, all *Bombina* contact zones in Central Europe presumably established when *B. bombina* arrived from its distant Black Sea refugia, i.e. after the end of the last glaciation. Their respective age should thus roughly correlate with their distance from the Black Sea. In addition, we can distinguish the hybrid zones involving the diverged Balkano-Western *versus* Carpathian clades of *B. variegata*.

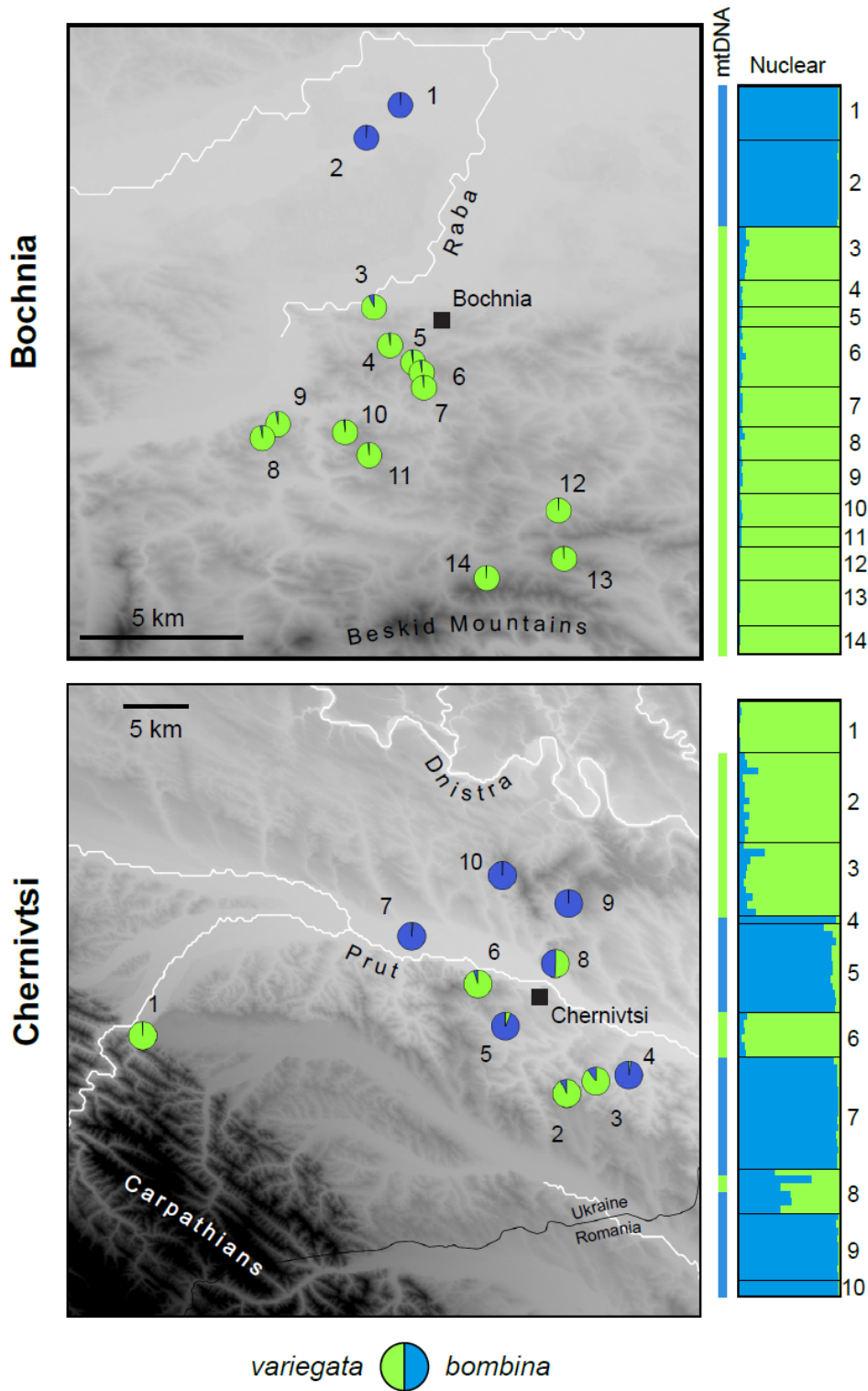
### *Bochnia and Chernivtsi contact zones*

The RAD-seq data accurately dissected the patterns of admixture in the transitions identified in the surroundings of Bochnia and Chernivtsi (Fig. 3). For Bochnia (6,010 SNPs), interspecies  $F_{st}$  ranged 0.72–0.82 (Fig. S2), and species differentiation accounted for 58.4% of the variance by the first axis of the PCA (Fig. S3), from which we selected 1,883 species-diagnostic SNPs. The STRUCTURE analysis confirmed the diagnosticity: with  $K = 2$ , the allele frequency divergence  $D$  between the two groups was 0.98. Our analyses uncovered the nuclear and mitochondrial transition between loc. 2 (*B. bombina*) and loc. 3 (*B. variegata*). Populations on the *B. variegata* side (loc. 3–11) featured 2–7% of *B. bombina* ancestry (Fig. 3), which reflects introgression rather than a lack of resolution: the 90% confidence intervals (CI) of individual estimates never reached 0% of *B. bombina* ancestry in these populations.



**Fig 2: Mitochondrial phylogeny and dynamics of Central European ranges of *Bombina* during the Late-Quaternary.** The tree shows the relationships among lineages recovered by the full mitogenome analyses by Pabijan et al. (2013). Shallow *cytochrome-b* lineages that were not monophyletic in the mitogenomes are not detailed. The map displays the approximate location of glacial refugia and routes of post-glacial colonization according to the LGM predictions and the distribution of the mitochondrial diversity and structure (Fig. 1). The locations of studied contact zones are indicated. Photo credit: CD.

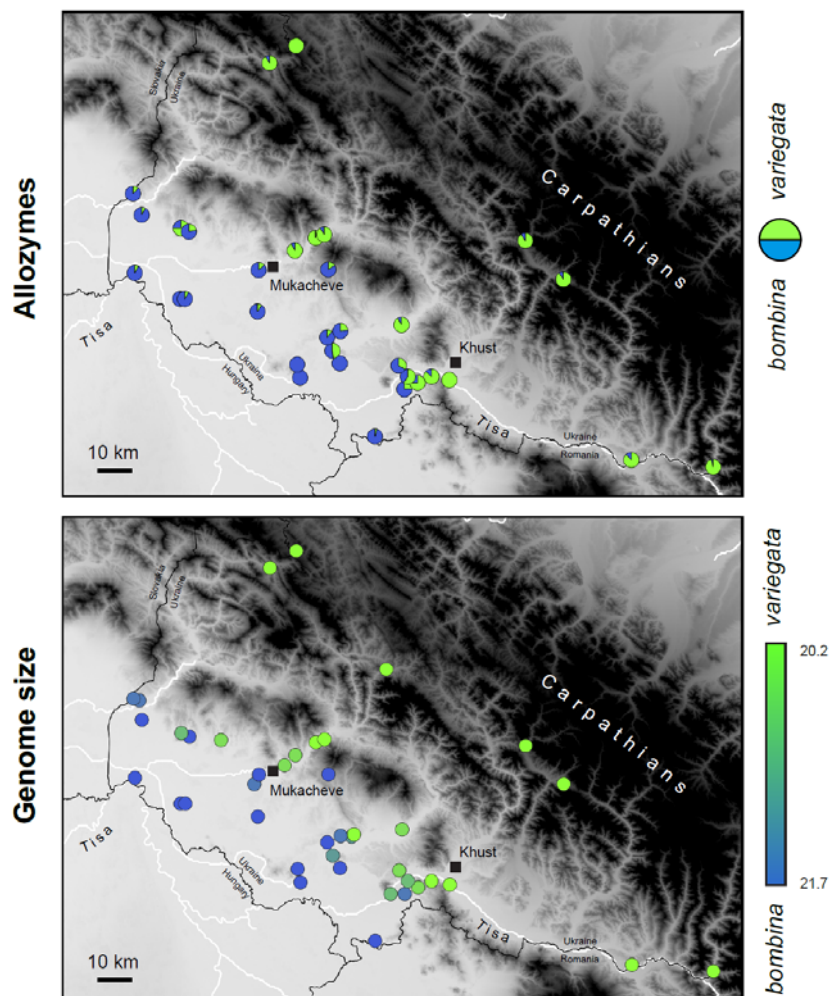
For Chernivtsi, (4,294 SNPs), interspecies  $F_{st}$  ranged 0.41–0.71 (Fig. S4), and species differentiation accounted for 27.2% of the variance by the first axis of the PCA (Fig. S5). From this, we selected 653 SNPs that were highly species-diagnostic in subsequent STRUCTURE analyses ( $D = 0.99$ ). In this area, we identified a mosaic of *B. bombina* and *B. variegata* populations (Fig. 3), most of which featured >1% of admixture (loc. 2–8), again with high confidence (90% CI > 0% of foreign ancestry). In one population (loc. 8), both mtDNA were found and all individuals received ancestries around 50%, consistent with recent hybridization events. All other populations fixed the mtDNA of the species they were predominantly assigned to with the RAD-seq data.



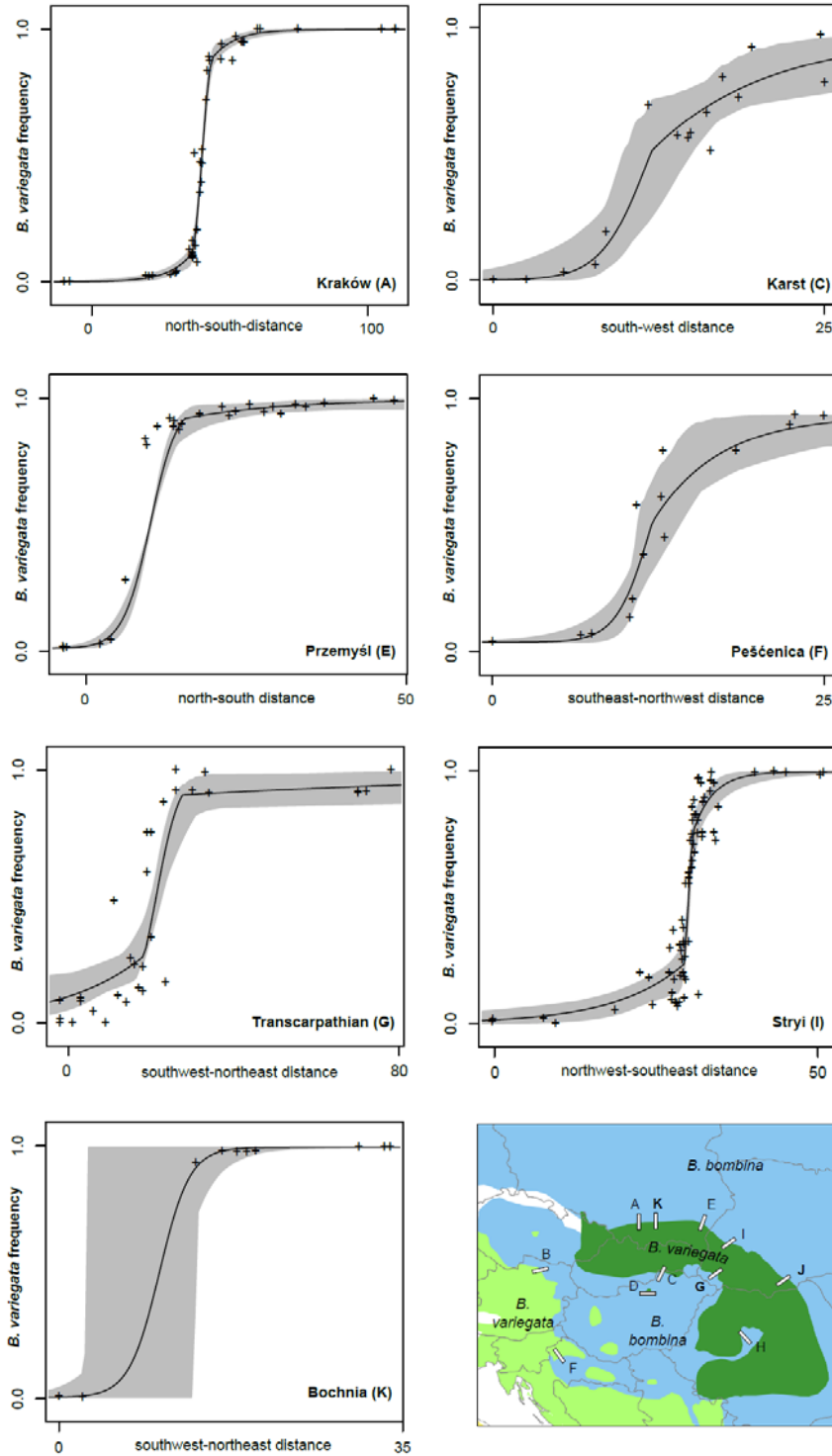
**Fig. 3: Population genomic analyses of the Bochnia and Chernivtsi *B. bombina*/*variegata* contact zones.** The map shows the average population ancestry obtained with 1,883 and 653 species-diagnostic SNPs obtained from RAD-seq for Bochnia and Chernivtsi, respectively. The barplots show mitochondrial identity and nuclear ancestry estimated by STRUCTURE for each individual.

### *Transcarpathian contact zone*

Our data (six allozymes and genome size) accurately mapped the distribution of *B. bombina* and *B. variegata* in the Ukrainian Transcarpathians (Fig. 4). The two datasets were largely congruent: genome size correlates with the average frequency of allozyme alleles (Fig. S6) and admixed populations show the highest variation in genome size (Fig. S7). Several sharp transitions were located at the Carpathian foothills, i.e. localized hybrid populations flanked by populations predominantly assigned to one of the species, with a few alleles of the other. In fact, only one *B. variegata* and two *B. bombina* populations lacked foreign allozyme alleles over this whole area (Fig. 4).



**Fig. 4: Analysis of the *B. bombina* / *variegata* hybrid zone from the Ukrainian Transcarpathian.** The top map shows allele proportions for each population, based on six species-diagnostic loci. The bottom map displays the average genome size (pg) of the individuals for each population, which is diagnostic (Fig. S6).



**Fig. 5: Cline analyses on the nuclear *B. variegata* ancestry across seven *Bombina* contact zones sampled along transects.** The best-fitting models were reported according to the lowest AIC (Table 1). Crosses show the empirical data, and the shaded areas show the 95% confidence intervals of clines.

### *Comparisons of Bombina hybrid zones*

The seven clinal hybrid zones analyzed with *hzar* were all narrow (Fig. 5, Table 1). Our analyses for Bochnia and Transcarpathia yielded cline widths of 6.2 and 11.4km, respectively, which broadly correspond to the ranges of values obtained in previously studied areas (2.1–8.5km). The best cline models featured introgression tails in all but one case, indicative of allele diffusion within the ranges of the other species. Interestingly, the introgression was biased on the *B. variegata* side in three cases (Table 1). The only two-parameter cline was Bochnia, which must be taken with caution given our scattered sampling near the center of the transect (Fig. 3). Although not quantified by clines, the remaining transitions shared similar geographic patterns of admixture, i.e. localized hybrid populations but introgression reaching distant parental populations: Chernivtsi (Fig. 3), NE-Austria (Fig. S8), the Mátra Mountains (Fig. S9) and Apahida (Vines et al. 2003).

There was no apparent association between the biogeographic attributes of the hybrid zones and their steepness. Cline width and the distance from the putative Black Sea refugial area of *B. bombina* (as a proxy for the age of the hybrid zone) were not correlated (linear regression,  $P = 0.26$ , adjusted  $R^2 = 0.09$ ,  $df = 5$ ). Likewise, the outcome of secondary contacts (similar across the ranges) appears irrespective of the intraspecific lineages involved (Table 1).

### **Discussion**

Admixture estimations based on hundreds of species-diagnostic SNPs confirmed sharp but leaky transitions between *B. bombina* and *B. variegata*. On the one hand, genome average clines were narrow, indicative of strong reproductive isolation. On the other hand, we evidenced weak allele diffusion into parental populations. These results are in full congruence with the classic studies based on a few markers (Table 1, Fig. 5) where narrow clines translated into high selection estimates (Szymura & Barton, 1986, 1991; Yanchukov et al., 2006), even though foreign variants reached some of the most distant populations analyzed, e.g. ~40km south of the hybrid zone center in Przemyśl (Szymura & Barton, 1991), ~60km uphill in the Transcarpathian mountains (Fig. 3). In his pioneer work, G. Gollmann already noted the rarity of truly pure populations at parapatric ranges from Austria, Hungary and Slovakia (Gollman 1984; Gollmann et al., 1988).

Across densely sampled transects, such pattern is typically depicted by introgression tails flanking the sharp clines, which were favored for most datasets (Table 1). However, tails were only explicative on the *B. variegata* side in half of the cases, suggesting that at the local scale, *B. bombina* alleles may invade more easily the range of *B. variegata* than vice-versa. Such apparent asymmetry could be due to habitat connectivity, likely higher in the forest (where *B. variegata* can be found continuously), compared to the human-impacted lowlands (where *B. bombina* is more localized). This

issue is well-illustrated in southern Poland, where the plains bordering the Carpathian hills have suffered strong landscape alteration over the past decades, even leading to the disappearance of the original Kraków sites (M. Pabijan and J. Szymura pers. com.). Alternatively, the asymmetry could have selective causes. Reciprocal crossing experiments did not reveal a directional bias in the intrinsic fitness of hybrid clutches (Kruuk et al., 1999), but the tadpole of *B. variegata* was shown to be more sensitive to the predators abundantly found in the semi-permanent ponds used by *B. bombina* (Kruuk & Gilchrist, 1997).

While the steep transitions confirm that the two species are isolated, selection against hybridization remains insufficient to fully permeabilize their genomes. Even if linkage disequilibrium imposes genome-wide selection on neutral loci, the generated barrier to gene flow delays but cannot ultimately prevent their diffusion into the opposite gene pool (Yanchukov et al., 2006). Weak but geographically extensive introgression is characteristic of sharp tension zones (e.g. Phillips, Baird, & Moritz, 2004; Macholán et al., 2007; Baldassarre, White, Karubian, & Webster, 2014). In this respect, it is somewhat surprising that the transcriptome-based coalescent analyses of Nürnberger et al. (2016) did not reveal traces of contemporary admixture in their specimens of *B. bombina* and *B. variegata*, which were sampled only 60km from the Kraków transect. As argued by these authors, gene flow might be too low for the spread of neutral (or even slightly adaptive) alleles beyond the outskirts of this hybrid zone, especially given the recent establishment of the contacts (see below). Habitat destruction near Kraków could have also completely interrupted interspecies migration, hence precipitating the full backcrossing of the edge populations. Alternatively, it is also possible that neutral variants did diffuse outside the parapatric ranges, but were not linked to coding genomic regions, and hence could not be detected by transcriptomic analyses.

Were species boundaries between *B. bombina* and *B. variegata* affected by their biogeographic history? Variation in the extent of introgression between replicate transects is often interpreted as differences in the strength of reproductive isolation, in respect to genetic and ecological factors, or a combination of both (Harrison & Larson, 2016). Based on observations between cryptic tree frog taxa, Dufresnes, Berroneau, et al. (2020) proposed that hybrid zones from glacial refugia could be narrower than those from post-glacial or introduced ranges, as they presumably had more time to reach a selection-dispersal equilibrium and promote pre-mating barriers via reinforcement. Genetic incompatibilities emerging independently within single species can also differentially affect the outcome of hybridization (Barnard-Kubow & Galloway, 2017). Here however, the transitions appear irrespective of the relative age of contacts and the intraspecific (mitochondrial) lineages involved (Table 1). Species boundaries in *Bombina* remained remarkably well-defined, perhaps as their ecological differentiation limits spatial overlap at range margins, and hence hybridization, compared to cryptic species. Moreover, their strong evolutionary divergence – the two species split during the Late-Miocene (Pabijan et al., 2013) – is probably enough to trigger genetic incompatibilities that drastically constrain

introgressive hybridization (e.g. Dufresnes et al., 2019; Dufresnes, Pribille, et al. 2020). Note that all our contacts were established at roughly the same period (Holocene), which can also account for the low variability in the extent of introgression. In parallel, it remains unclear whether the deep lineages of *B. variegata* in Central Europe truly represent evolutionary divergences (Pabijan et al., 2013) – that could potentially bear distinct incompatibilities – or just ghost mitochondrial clades (Dufresnes, Nicieza, et al. 2020). Anyhow, the remarkable replicability of the geographic transitions between *B. bombina* and *B. variegata* confirms their reliability for hybrid zone analyses (Szymura & Barton, 1986), which will be worth re-examining in a comparative context to characterize the genomic architecture of introgression underlying reproductive isolation.

Rather than unique biogeographic attributes, the seemingly leakier genetic barriers in areas where *B. bombina* and *B. variegata* seem to admix “freely” probably stem from local landscape characteristics. In Apahida, the mosaic of forest puddles and open swamps connects both species in every direction, hence promoting swarms of hybrids of various levels of backcrossing (Vines et al., 2003). In the Mátra Mountains, *B. variegata* is separated from the main Carpathian ranges, which explains its progressive assimilation by *B. bombina* (Gollmann, 1987), a process that probably occurs in other isolated pockets (e.g. the Bakony Mountains, Vörös et al., 2006). Local populations may thus lose their genetic integrity by episodic bursts of introgression, even if selection against hybrids is strong. At the species’ level, however, the extensive ranges should buffer the slow diffusion of neutral variants, especially if introgression occurs in post-glacial populations and never reaches the refugial areas – where populations persist in the long-term. Hence, while gene flow is often viewed as a force reversing speciation (Seehausen, Takimoto, Roy, & Jokela, 2008), this is not necessarily the case when reproductive isolation involves strong and multiple intrinsic and extrinsic barriers, regardless of the numerous opportunities to hybridize offered by the dynamic biogeography of species.

### *Conclusions*

Despite multiple opportunities to hybridize along thousands of kilometers across heterogeneous environments, the fire-bellied toads *B. bombina* and *B. variegata* always form steep transitions of a few kilometers, with only weak allele diffusion outside the hybrid zones. This suggests that strong reproductive isolation is maintained regardless of the biogeographic dynamics of the contacts, potentially due to the ecological differentiation between the two species. Therefore, introgressive hybridization should not necessarily be viewed as antagonist to speciation.



**Acknowledgments** – We thank I. Danilov, I. Skilsky and K. Kolenda for help with the fieldwork, M. Khalturin for help with the allozyme analysis, L. Borkin and N. Ananjeva for providing access to the ZISP herpetological collections, H. Belefonte for accompanying us in the field, as well as M. Pabijan and J. Szymura for discussions. Fieldwork was conducted under permit no.DPZ-WG.6401.02.2.2017.dł issued by the General Directorate of Environmental Protection in Poland. We are grateful to N. Perrin for support via a grant from the Swiss National Science Foundation (SNSF, 31003A\_166323). CD was funded by a SNSF fellowship (P2LAP3\_171818). TS was supported by the statutory funds of W. Szafer Institute of Botany, Polish Academy of Sciences. SNL and JMR were supported by a grant from the Russian Foundation for Basic Research (RFBR.20-04-00918). MD is a Research Director at Fonds de la Recherche Scientifique – FNRS (Belgium).

**Data availability statement** – The mitochondrial and nuclear barcoding information is provided in Table S1. The RAD nuclear sequences (individual raw sequence reads) are archived in the NCBI SRA under bioproject PRJNA542138.

## ORCID

Christophe Dufresnes <https://orcid.org/0000-0002-8497-8908>

Tomasz Suchan <https://orcid.org/0000-0002-0811-8754>

Nazar A. Smirnov <https://orcid.org/0000-0001-9731-6404>

Mathieu Denoël <https://orcid.org/0000-0002-3586-8323>

Spartak N. Litvinchuk <https://orcid.org/0000-0001-7447-6691>

## References

- Arntzen, J. W., de Vries, W., Canestrelli, D., & Martínez-Solano, I. (2017). Hybrid zone formation and contrasting outcomes of secondary contact over transects in common toads. *Molecular Ecology*, 26, 5663–5675. doi: 10.1111/mec.14273
- Avise, J. (2000). *Phylogeography: The history and formation of species*. Cambridge, MA: Harvard University Press.
- Baldassarre, D. T., White, T. A., Karubian, J., Webster, M. S. (2014). Genomic and morphological analysis of a semipermeable avian hybrid zone suggests asymmetrical introgression of a sexual signal. *Evolution*, 68, 2644–2657. doi: 10.1111/evo.12457
- Barnard-Kubow, K., & Galloway, L. F. (2017) Variation in reproductive isolation across a species range. *Ecology & Evolution*, 7, 9347–9357. doi: 10.1002/ece3.3400
- Barton, N. H., & Hewitt, G. M. (1985). Analysis of hybrid zones. *Annual Review of Ecology and Systematics*, 16, 113–148. doi: 10.1146/annurev.es.16.110185.000553

- Barton, N. H., & Hewitt, G. M. (1989). Adaptation, speciation and hybrid zones. *Nature*, 341, 497–503. doi: 10.1038/341497a0
- Barton, N., & Gale, K. S. (1993). Genetic analysis of hybrid zones. In R. Harrison (Ed.), *Hybrid zones and the evolutionary process* (pp. 13–45). New York: Oxford University Press
- Boecklen, W. J., & Howard, D. J. (1997). Genetic analyses of hybrid zones: numbers of markers and power of resolution. *Ecology*, 78, 2611–2616. doi: 10.1890/0012-9658(1997)078[2611:GAOHZN]2.0.CO;2
- Borkin, L. J., Litvinchuk, S. N., Rosanov, J. M., & Milto, K. D. (2001). Cryptic speciation in *Pelobates fuscus* (Anura, Pelobatidae): evidence from DNA flow cytometry. *Amphibia-Reptilia*, 22, 387–396. doi: 10.1163/15685380152770354
- Borkin, L. J., Litvinchuk, S. N., Rosanov, J. M., Džukić, G., & Kalezić, M. L. (2005). Genome size variation in the Balkan anurans. In: Ananjeva, N., Tsinenko, O. (eds.), *Herpetologia Petropolitana*. St. Petersburg: Societas Europaea Herpetologica. 16–19.
- Brelsford, A., Dufresnes, C., & Perrin, N. (2016). High-density sex-specific linkage maps of a European tree frog (*Hyla arborea*) identify the sex chromosome without information on offspring sex. *Heredity*, 116, 177–181. doi: 10.1038/hdy.2015.83
- Buggs, R. J. A. 2007. Empirical study of hybrid zone movement. *Heredity*, 99, 301–312. doi: 10.1038/sj.hdy.6800997
- Burban, C., Rocha, S., Leblois, R., Rossi, J.-P., Sauné, L., Branco, M., & Kerdelhué, C. (2020). From sympatry to parapatry: a rapid change in the spatial context of incipient allochronic speciation. *Evolutionary Ecology*, 34, 101–121. doi: 10.1007/s10682-019-10021-4
- Catchen, J., Hohenlohe, P., Bassham, S., Amores, A., & Cresko, W. (2013). Stacks: An analysis tool set for population genomics. *Molecular Ecology*, 22, 3124–3140. doi: 10.1111/mec.12354
- Cobos, M. E., Peterson, A. T., Barve, N., Osorio-Olvera, L. (2019). kuenm: an R package for detailed development of ecological niche models using Maxent. *PeerJ* 7, e6281. doi: 10.7717/peerj.6281
- Derryberry, E. P., Derryberry, G. E., Maley, J. M., & Brumfield, R. T. (2014). HZAR: hybrid zone analysis using an R software package. *Molecular Ecology Resources*, 14, 652–663. doi: 10.1111/1755-0998.12209
- Dufresnes, C., Strachinis, I., Suriadna, N., Mykytynets, G., Cogălniceanu, D., Székely, P., Vukov, T., Arntzen, J.W., Wielstra, B., Lymberakis, P., Geffen, E., Gafny, S., Kumlutaş, Y., Ilgaz, Ç., Candan, K., Mizsei, E., Szabolcs, M., Kolenda, K., Smirnov, N., Géniez P., Lukanov, S., Crochet, P.-A., Dubey, S., Perrin, N., Litvinchuk, S.N., Denoël, M. (2019). Phylogeography of a cryptic speciation continuum in Eurasian spadefoot toads (*Pelobates*). *Molecular Ecology*, 28, 3257–3270. doi: 10.1111/mec.15133
- Dufresnes, C., Pribille, M., Alard, B., Gonçalves H., Amat, F., Crochet, P.-A., Dubey, S., Perrin, N., Fumagalli, L., Vences, M., & Martínez-Solano, I. (2020a). Integrating hybrid zone analyses in species delimitation: lessons from two anuran radiations of the Western Mediterranean. *Heredity*, 124, 423–438. doi: 10.1038/s41437-020-0294-z
- Dufresnes, C., Nicieza, A. G., Litvinchuk, S. N., Rodrigues, N., Jeffries, D. L., Vences, M., Perrin, N., & Martínez-Solano, I. (2020b). Are glacial refugia hotspots of speciation and cytonuclear discordances? Answers from the genomic phylogeography of Spanish common frogs. *Molecular Ecology*, 29, 986–1000. doi: 10.1111/mec.15368
- Dufresnes, C., Berroneau, M., Dubey, S., Litvinchuk, S.N., & Perrin, N. (2020c). The effect of phylogeographic history on species boundaries: a comparative framework in *Hyla* tree frogs. *Scientific Reports*, 10, 5502. doi: 10.1038/s41598-020-62382-4
- Dufresnes, C., Litvinchuk, S. N., Rozenblut-Kościsty, B., Rodrigues, N., Perrin, N., Crochet P.-A., & Jeffries, D.L. (2020d). Hybridization and introgression between toads with different sex chromosome systems. *Evolution Letters* In press. doi: 10.1002/evl3.191

- Fijarczyk, A., Nadachowska, K., Hofman, S., Litvinchuk, S. N., Babik, W., Stuglik, M., Gollmann, G., Choleva, L., Cogălniceanu, D., Vukov, T., Džukić, G., Szymura, J. M. (2011). Nuclear and mitochondrial phylogeography of the European fire-bellied toads *Bombina bombina* and *Bombina variegata* supports their independent histories. *Molecular Ecology*, 20, 3381–3398. doi: 10.1111/j.1365-294X.2011.05175
- Gollmann, G. (1984). Allozymic and morphological variation in the hybrid zone between *Bombina bombina* and *Bombina variegata* (Anura, Discoglossidae) in northeastern Austria. *Journal of Zoological Systematics and Evolutionary Research*, 22, 51–64. doi: 10.1111/j.1439-0469.1984.tb00562.x
- Gollmann, G. (1987). *Bombina bombina* and *Bombina variegata* in the Mátra mountains (Hungary): New data on distribution and hybridization (Amphibia, Anura, Discoglossidae). *Amphibia-Reptilia*, 8, 213–224.
- Gollmann, G., Roth, P., & Hödl, W. (1988). Hybridization between the fire-bellied toads *Bombina bombina* and *Bombina variegata* in the karst regions of Slovakia and Hungary: morphological and allozyme evidence. *Journal of Evolutionary Biology*, 1, 3–14. doi: 10.1046/j.1420-9101.1988.1010003.x
- Gouy, M., Guindon, S., & Gascuel, O. (2010). SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Molecular Biology & Evolution*, 27, 221–224. doi: 10.1093/molbev/msp259
- Harrison, R. G. (1993). *Hybrid Zones and the Evolutionary Process*. Oxford University Press: Oxford.
- Harrison, R. G., & Larson, E. L. (2016). Heterogeneous genome divergence, differential introgression, and the origin and structure of hybrid zones. *Molecular Ecology*, 25, 2454–2466. doi: 10.1111/mec.13582
- Hartel, T., & von Wehrden, H. (2013). Farmed areas predict the distribution of amphibian ponds in a traditional rural landscape. *PLoS ONE*, 8, e63649. doi: 10.1371/journal.pone.0063649
- Hasselman, D. J., Argo, E. E., McBride, M. C., Bentzen, P., Schultz, T. F., Perez-Umphrey, A. A., & Palkovacs, E. P. (2014). Human disturbance causes the formation of a hybrid swarm between two naturally sympatric fish species. *Molecular Ecology*, 23, 1137–1152. doi: 10.1111/mec.12674
- Hewitt, G. M. (1988). Hybrid zones – natural laboratories for evolutionary studies. *Trends in Ecology & Evolution*, 3, 158–167. doi: 10.1016/0169-5347(88)90033-X
- Hofman, S., Spolsky, C., Uzzell, T., Cogălniceanu, D., Babik, W., & Szymura, J. M. (2007). Phylogeography of the fire-bellied toads *Bombina*: independent Pleistocene histories inferred from mitochondrial genomes. *Molecular Ecology*, 16, 2301–2316. doi: 10.1111/j.1365-294X.2007.03309.x
- Jakobsson, M. & Rosenberg, N. A. (2007). CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics*, 23, 1801–1806. doi: 10.1093/bioinformatics/btm233
- Jombart, T. (2008). adegenet: A R package for the multivariate analysis of genetic markers. *Bioinformatics*, 24, 1403–1405. doi: 10.1093/bioinformatics/btn129
- Kruuk, L. E. B., & Gilchrist, J. (1997). Mechanisms maintaining species differentiation: predator-mediated selection in a *Bombina* hybrid zone. *Proceedings of the Royal Society B: Biological Sciences*, 264, 105–110. doi: 10.1098/rspb.1997.0016
- Kruuk, L. E. B., Gilchrist, J. S., & Barton, N. H. (1999). Hybrid dysfunction in fire-bellied toads (*Bombina*). *Evolution*, 53, 1611–1616. doi: 10.1111/j.1558-5646.1999.tb05425.x
- Litvinchuk, S. N., Rosanov, J. M., Borkin, L. J., & Skorinov, D. V. (2008). Molecular, biochemical and cytogenetic aspects of microevolution in anurans of Russia and adjacent countries. In: Ananjeva N. B. (ed.), *The Problems of Herpetology*, St. Petersburg: 235-245.

- MacCallum, C. J., Nürnbergger, B., Barton, N. H., & Szymura, J. M. (1998). Habitat preference in the *Bombina* hybrid zone in Croatia. *Evolution*, 52, 227–239. doi: 10.1111/j.1558-5646.1998.tb05156.x
- Macholán, M., Munclinger, P., Sugerková, M., Dufková, P., Bímová, B., Božíková, E., Zima, J., & Piálek, J. (2007). Genetic analyses of autosomal and X-linked markers across a mouse hybrid zone. *Evolution*, 61, 746–771. doi: 10.1111/j.1558-5646.2007.00065.x
- Nürnbergger, B., Barton, N., MacCallum, C., Gilchrist, J., Appleby, M. (1995). Natural selection on quantitative traits in the *Bombina* hybrid zone. *Evolution*, 49, 1224–1238. doi: 10.1111/j.1558-5646.1995.tb04449.x
- Nürnbergger, B., Lohse, K., Fijarczyk, A., Szymura, J. M., Blaxter, M. L. (2016). Para-allopatry in hybridizing fire-bellied toads (*Bombina bombina* and *B. variegata*): Inference from transcriptome-wide coalescence analyses. *Evolution*, 70, 8003–8018. doi: 10.1111/evo.12978
- Pabijan, M., Wandycz, A., Hofman, S., Węcek, K., Piwczyński, M., & Szymura, J. M. (2013). Complete mitochondrial genomes resolve phylogenetic relationships within *Bombina* (Anura: Bombinatoridae). *Molecular Phylogenetics & Evolution*, 69, 63–74. doi: 10.1016/j.ympev.2013.05.007
- Phillips, B. L., Baird, S. J. E., Moritz, C. (2004). When vicars meet: a narrow contact zone between morphologically cryptic phylogeographic lineages of the rainforest skink, *Carlia rubrigularis*. *Evolution*, 58, 1536–1548. doi: 10.1111/j.0014-3820.2004.tb01734.x
- Phillips, S. J., Anderson, R. P., Schapire, R. E. (2006). Maximum entropy modeling of species geographic distributions. *Ecological Modelling*, 190, 231–259. doi: 10.1016/j.ecolmodel.2005.03.026
- Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, 155, 945–959
- Sanderson, N., Szymura, J. M., & Barton, N. H. (1992). Variation in mating call across the hybrid zone between the fire-bellied toads *Bombina bombina* and *B. variegata*. *Evolution*, 46, 595–607. doi: 10.1111/j.1558-5646.1992.tb02068.x
- Seehausen, O., Takimoto, G., Roy, D., Jokela, J. (2008). Speciation reversal and biodiversity dynamics with hybridization in changing environments. *Molecular Ecology*, 17, 30–44. doi: 10.1111/j.1365-294X.2007.03529.x
- Shaw, C. R., & Prasad, R. (1970). Starch gel electrophoresis of enzyme - A compilation of recipes. *Biochemical Genetics*, 4, 297–320. doi: 10.1007/BF00485780
- Szymura, J. M. (1976a). Hybridization between discoglossid toads *Bombina bombina* and *Bombina variegata* in southern Poland as revealed by the electrophoretic technique. *Zeitschrift für zoologische Systematik und Evolutionsforschung*, 14, 227–236
- Szymura, J. M. (1976b). New data on the hybrid zone between *Bombina bombina* and *Bombina variegata* (Anura Discoglossidae). *Bulletin de l'Académie Polonaise des Sciences*, 11, 355-363.
- Szymura, J. M., & Barton, N. H. (1986). Genetic analyses of a hybrid zone between the fire-bellied toads, *Bombina bombina* and *B. variegata*, near Cracow in southern Poland. *Evolution*, 40, 1141–1159. doi: 10.1111/j.1558-5646.1986.tb05740.x
- Szymura, J. M., & Barton, N. H. (1991). The genetic structure of the hybrid zone between the fire-bellied toads *Bombina bombina* and *B. variegata*: comparisons between transects and between loci. *Evolution*, 45, 237–261. doi: 10.1111/j.1558-5646.1991.tb04400.x
- Vines, T. H., Köhler, S. C., Thiel, M., Ghira, I., Sands, T. R., MacCallum, C. J., Barton, N. H., & Nürnbergger, B. (2003). The maintenance of reproductive isolation in a mosaic hybrid zone between the fire-bellied toads *Bombina bombina* and *B. variegata*. *Evolution*, 57, 1876–1888. doi: 10.1111/j.0014-3820.2003.tb00595.x

- Vörös, J., Alcobendas, M., Martínez-Solano, I., & García-París, M. (2006). Evolution of *Bombina bombina* and *Bombina variegata* (Anura: Discoglossidae) in the Carpathian Basin: A history of repeated mt-DNA introgression across species. *Molecular Phylogenetics & Evolution*, 38, 705–718. doi: 10.1016/j.ympev.2005.08.010
- Watanabe, S., Hajima, T., Sudo, K., Nagashima, T., Takemura, H., Okajima, H., Nozawa, T., Kawase, H., Abe, M., Yokohata, T., Ise, T., Sato, H., Kato, E., Takata, K., Emori, S., Kawamiya, M., 2011. MIROC-ESM 2010: Model description and basic results of CMIP5-20c3 m experiments. *Geoscientific Model Development*, 4, 845–872. doi: 10.5194/gmd-4-845-2011
- Wielstra, B. (2019). Historical hybrid zone movement: More pervasive than appreciated. *Journal of Biogeography*, 46, 1300–1305. doi: 10.1111/jbi.13600
- Wielstra, B., & Arntzen, J. W. (2012). Postglacial species displacement in *Triturus* newts deduced from asymmetrically introgressed mitochondrial DNA and ecological niche models. *BMC Evolutionary Biology*, 12, 161. doi: 10.1186/1471-2148-12-161
- Wielstra, B., Burke, T., Butlin, R. K., & Arntzen, J. W. (2017). A signature of dynamic biogeography: enclaves indicate past species replacement. *Proceedings of the Royal Society B: Biological Sciences*, 284, 20172014. doi: 10.1098/rspb.2017.2014
- Wielstra, B., Burke, T., Butlin, R. K., Avci, A., Üzümlü, N., Bozkurt, E., Olgun, K., & Arntzen, J. W. (2017). A genomic footprint of hybrid zone movement in crested newts. *Evolution Letters*, 1, 93–101. doi: 10.1002/evl3.9
- Yanchukov, A., Hofman, S., Szymura, J. M., Mezhzherin, S. V., Morozov-Leonov, S. Y., Barton, N. H., & Nürnberger, B. (2006). Hybridization of *Bombina bombina* and *B. variegata* (Anura, Discoglossidae) at a sharp ecotone in western Ukraine: comparisons across transects and over time. *Evolution*, 60, 583–600. doi: 10.1111/j.0014-3820.2006.tb01139.x

## Biosketch

The authors share broad interests in ecology, phylogeography, evolution and systematics. CD and SNL are evolutionary biologists specialists of the Palearctic herpetofauna, which they use as models to study biogeographic and speciation processes. TS is a population geneticist and phylogeographer who develops genomic methods (including on historical DNA) to measure biodiversity in a vast array of plants and animals. JMR, NAS, and MD are conservation biologists and ecologists whose research focuses on amphibian diversity and distributions at multiple scales. Authors contributed to this work as follows.

Author contributions: CD and SNL designed the study; CD, TS, NAS, MD and SNL participated in the fieldwork; CD, JMR and SNL performed molecular labwork and analyses; CD drafted the manuscript, which was critically improved by all coauthors.

## Supporting Information

**Appendix I:** *Bombina* localities analyzed in this study (contains Table S1)

**Appendix II:** Extended methods and results for the species distribution modelling analyses (contains Table S2 and Fig. S1; Geofile S1 is provided as an external file).

**Appendix III:** Additional genetic analyses of the hybrid zones (contains Figs. S2-S9)