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Genetic data to describe the hybrid zone between *Bufo bufo* (Linnaeus, 1758) and *Bufo verrucosissimus* (Pallas, 1814) in northeastern Türkiye

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Abstract. Hybrid zones are geographic areas where individuals from distinct taxa meet, mate, and hybridize. These zones may have complex histories, but many of them originated relatively recently, during climatic oscillations in the Quaternary, following range shifts of formerly isolated, well-differentiated lineages. The *Bufo bufo* species group comprises four species distributed over the Western Palearctic. Whereas the contact zone between *Bufo bufo* and *B. spinosus* in western Europe has been well characterized, little is known about other species contacts. Here we focused on the contact between *B. verrucosissimus* and *B. bufo* in northeastern Türkiye, using mtDNA and microsatellite markers to describe genetic structure and patterns of admixture in the hybrid zone based on Bayesian clustering and cline analyses. Both species meet in a narrow contact zone at Rize province, with restricted introgression suggesting barriers to hybridization consistent with species status. Spatial population genetic analyses of microsatellite data pinpoint a possible enclave population of *B. bufo* at the Borçka district in Artvin province, isolated within the *B. verrucosissimus* range. The centers of the microsatellite and mtDNA-based clines are slightly displaced, with *B. verrucosissimus* mtDNA introgressing about 33 km W of the nuclear contact. Hybrid zone dynamics seem to be associated with range shifts mediated by Pleistocene glacial cycles and/or sex-biased dispersal.

Keywords: Anatolia, Caucasia, cline analysis, hybridization, introgression, microsatellites, mtDNA.

Introduction

Hybrid zones are the geographic areas where distinct species set contact, mate, and hybridize (Barton and Hewitt, 1985; Barton and Gale, 1993). These zones are considered natural laboratories of speciation (Hewitt, 1988; Harrison, 1990). They allow inferring the strength of evolutionary forces favouring or hindering species divergence, for instance by quantifying gene flow and introgression in the gene pools of interacting taxa (Harrison and Larson, 2016; Gompert, Mandeville and Buerkle, 2017). Hybrid zones generally tend to be stable in space and time due to an equilibrium between ongoing dispersal of parental individuals into the contact zone and selection against hybrids. These zones are called tension zones and are characterized by a low frequency of hybrids and a narrow spatial dimension, associated with the presence of reproductive barriers to hybridization (Kawakami and Butlin, 2012;

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Abbott et al., 2013). Alternatively, hybrid zones can also shift their spatial location through time, a phenomenon that may be more frequent than currently acknowledged (Wielstra, 2019).

Amphibians are suitable models for the study of the relative roles of intrinsic and extrinsic factors in shaping patterns of hybridization along the speciation process because of some of their biological characteristics, including low dispersal capacity and general lack of prezygotic isolating mechanisms in closely related species (Littlejohn and Watson, 1985; Malone and Fontenot, 2008). They also show strong dependence on environmental variables like temperature and humidity which, in interaction with landscape features, constrain their potential distribution ranges (Whitton et al., 2012). For example, the succession of glacial and interglacial periods in the Pleistocene caused extensive range shifts in many taxa across Europe, originating areas of secondary contact and tension zones both in anurans (Dufresnes et al., 2019; Dufresnes and Martínez-Solano, 2020; Dufresnes et al., 2020a, 2020b) and salamanders (Mikulíček et al., 2012; Wielstra et al., 2017a, 2017b; Ficetola et al., 2019).

The *Bufo bufo* species group comprises four distinct species distributed throughout the Western Palearctic realm. *Bufo bufo* has the broadest distribution, with up to seven mtDNA lineages across its European range (Arntzen et al., 2017). Molecular studies have characterized the hybrid zone between *Bufo spinosus* and three different lineages of *B. bufo* along ca. 900 km in NW to SE France (Arntzen et al., 2016; Trujillo et al., 2017; Arntzen et al., 2018; Arntzen, 2019; van Riemsdijk et al., 2019, 2023; Arntzen, Canestrelli and Martínez-Solano, 2020; Arntzen et al., 2020). However, other contact zones have received less attention, especially at the eastern range of the species group, where *B. bufo* meets its sister species *Bufo verrucosissimus*. Since *B. bufo* and *B. verrucosissimus* are more closely related than *B. bufo* and *B. spinosus*, the potential eastern hybrid zone might behave differently from the well-studied western *Bufo* hybrid zone.

Initial surveys based on a limited set of samples suggested hybridization between *B. bufo* and *B. verrucosissimus*, although the actual extent of introgression differed among studies, raising disagreement about their taxonomic status (Garcia-Porta et al., 2012; Recuero et al., 2012; Arntzen et al., 2013). Özdemir et al. (2020) revised the taxonomy of these species with a comprehensive molecular and morphological study including samples from all across Türkiye. Accordingly, they delimited the distribution of *B. verrucosissimus* in two allopatric nuclei: one in Artvin province in the north and another one in the Mediterranean region in the south. They also delimited the northeastern distribution of *B. bufo* to Rize, but a geographic gap remained between Artvin and Rize provinces, where they may meet and hybridize with *B. verrucosissimus* (fig. 1). Finally, Dursun, Özdemir and Gül (2023) used mtDNA sequences to analyse the distribution of the two species in northeastern Türkiye. They extended the geographic range of *B. verrucosissimus* up to the Pazar district of Rize and revealed mitochondrial contact with *B. bufo* in that area. However, to date no study has analysed patterns of variation in nuclear markers to characterize fine-scale patterns of hybridization in this contact zone in a cline analysis framework, which is relevant to assess their taxonomic status (Mayr, 1969; Dufresnes et al., 2021b).

In this study, we present a comprehensive molecular analysis of the hybrid zone between *B. bufo* and *B. verrucosissimus* in northeastern Türkiye, where their distribution ranges meet. We describe the genetic structure and the degree of admixture in the hybrid zone using microsatellite markers and compare observed patterns with published mtDNA data under a geographic cline framework.

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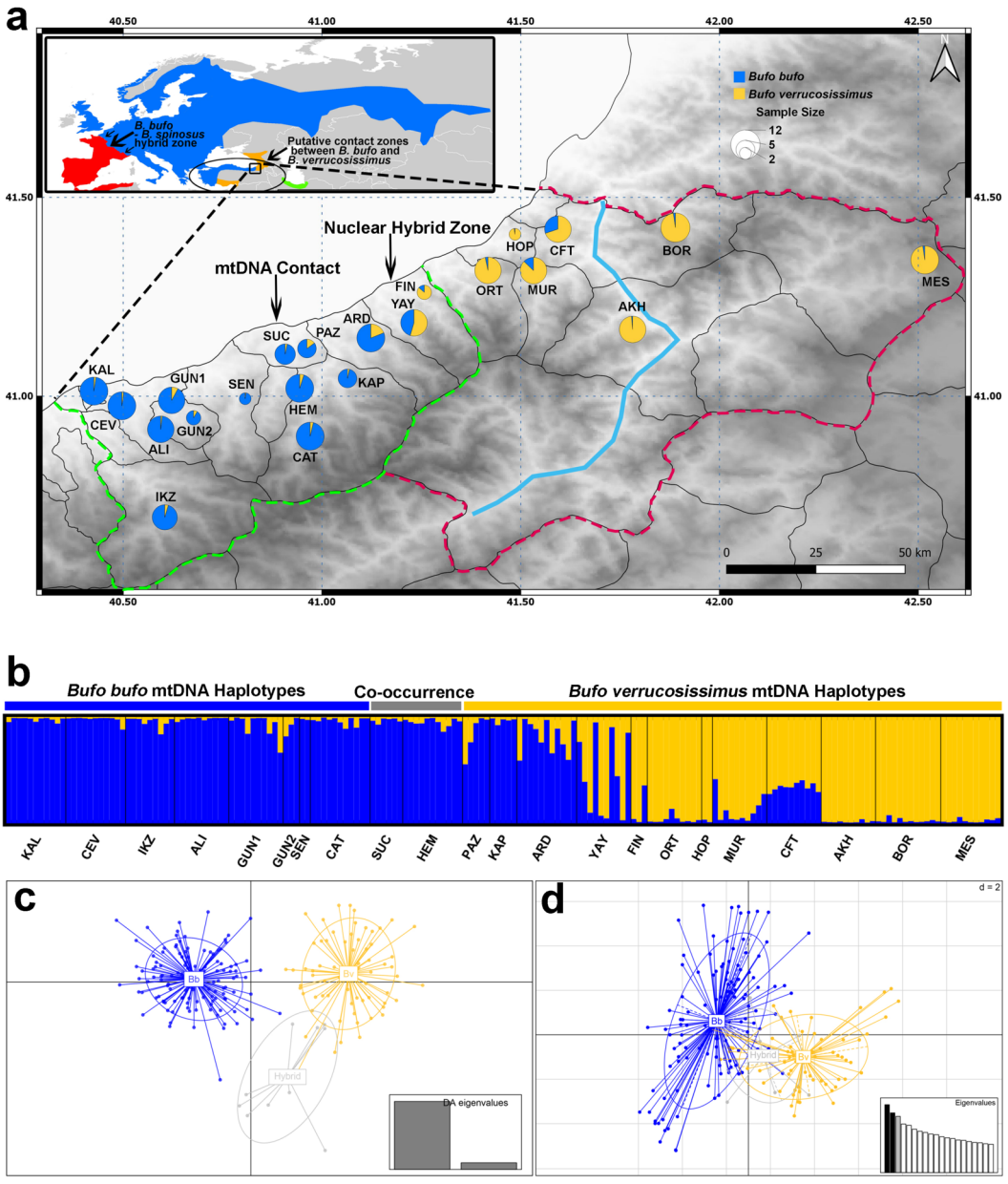


Figure 1. Admixture proportions of each population as illustrated by pie charts on the map of the study area (a) according to STRUCTURE results (b), with information on mtDNA haplotypes observed in each population displayed over the STRUCTURE barplot. The inset map, modified from Özdemir et al. (2020), represents the geographic range of the *Bufo bufo* species group, with arrows pinpointing the western hybrid zone between *Bufo spinosus* (Iberian Common Toad, in red) and *Bufo bufo* (Common Toad, in blue) and potential contact areas between *Bufo bufo* and *Bufo verrucosissimus* (Caucasian Toad, in yellow) in Türkiye. The scatterplots of PCA (c) and DAPC (d) represent three groups (Bb: *Bufo bufo*; Bv: *Bufo verrucosissimus*; Hybrid: YAY population). Green dashed lines: Rize borders; Ruby red dashed lines: Artvin borders; Cyan line: Çoruh River along Artvin.

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Table 1. Geographic coordinates of sampling sites, population codes and the number of genotyped samples. The distance to the western end of the linearized sampling transect (see Methods) is also shown for each population.

Location	Code	Distance (km)	Latitude	Longitude	Sample size
Rize, İyidere, Kalecik Village	KAL	0	40.977265	40.391197	11
Rize, Kalkandere, Cevizlik Village	CEV	6	40.940342	40.461885	11
Rize, İkizdere, Ballıköy Village Road	IKZ	9	40.662801	40.573095	9
Rize, Karasu Village, Alişor District	ALI	15	40.882399	40.561415	10
Rize, Güneysu, Ballıdere Village	GUN1	21	40.955632	40.597200	10
Rize, Güneysu, Başköy Village	GUN2	26	40.926408	40.659281	3
Rize, Çayeli, Kaptanpaşa Village Road	SEN	42	40.977914	40.792446	10
Rize, Çamlıhemşin, Çat Village	CAT	53	40.863810	40.935220	11
Rize, Pazar, Suçatı (Başköy)	SUC	55	41.079027	40.888264	6
Rize, Hemşin, Hemşin Waterfall	HEM	56	41.045670	40.908900	11
Rize, Pazar, Akbucak Village	PAZ	61	41.095719	40.938826	10
Rize, Çamlıhemşin, Kaplıca Village	KAP	69	41.020292	41.040828	10
Rize, Ardeşen, Yukarıdurak Village	ARD	77	41.110750	41.087906	11
Rize, Fındıklı, Yaylacılar Village	YAY	90	41.183889	41.198333	10
Rize, Fındıklı, Beydere Village	FIN	96	41.242491	41.239790	3
Artvin, Arhavi, Ortacalar District	ORT	113	41.282146	41.384842	10
Artvin, Hopa, Yoldere Village	HOP	125	41.391390	41.471040	2
Artvin, Murgul, Başköy Village	MUR	125	41.281769	41.499082	10
Artvin, Borçka, Çifteköprü Village	CFT	134	41.385898	41.560632	10
Artvin, Kafkasör, Hatila Road	AKH	146	41.133934	41.748394	10
Artvin, Borçka, Lake Karagöl	BOR	165	41.387000	41.853000	12
Artvin, Şavşat, Lake Karagöl	MES	228	41.307000	42.481771	11

Materials and methods

Sampling

Fieldwork was carried out in 22 sampling sites along the border of Artvin and Rize provinces, where the distribution ranges of both species come together (fig. 1). A total of 201 tissue samples was obtained from toeclips of adult individuals, roadkill carcasses, and tail tips of tadpoles. Before taking tissue samples, live individuals were anesthetized with 250 mg/l MS222 and, after recovery, they were released at the place of capture. Tissues were stored in 96% ethanol prior to DNA extraction. Detailed information on sampling sites is presented in table 1.

Molecular data

Total genomic DNA was extracted from tissue samples using the NucleoSpin Tissue Kit (Macherey-Nagel) following the manufacturer's instructions. A total of twenty nuclear microsatellite loci, including markers characterized for *B. bufo* and *B. spinosus* (Brede et al., 2001; Trujillo et al., 2017) and some unpublished primers were initially screened, from which we finally retained fourteen polymorphic loci for further analyses (table 2), based on cross-amplification success in both target species *B. bufo* and *B. verrucosissimus*. For the design of multiplex PCRs, selected loci were grouped in three different reactions using Multiplex Manager v1.0 (Holleley and Geerts, 2009). Forward primers were labelled with fluorescent dyes 6-FAM, VIC, NED, and PET. Multiplex PCR reactions were run using Type-it Microsatellite PCR (Qiagen) kits in a final volume of 15 μl including 7.5 μl of Master Mix, 1.2 μl of Primer Mix, 5.3 μl of RNase-free H_2O, and 1 μl of template DNA.

Thermocycling conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 30 denaturation cycles for 30 s at 95°C, annealing for 90 s at 60°C, and extension for 30 s at 72°C, with a final extension phase of 30 min at 60°C for each multiplex reaction. The amplification of PCR products was verified by electrophoresis in 2% agarose gels. Negative controls were included in all PCRs to test for possible contaminations. The amplicons were commercially genotyped by SECUGEN (Madrid, Spain) in an ABI PRISM 3730 sequencer with the GeneScan 500 LIZ size standard (Applied Biosystems). Allele scoring of each individual, based on the size of the amplified fragments, was assigned using Genemapper v4.0 (Applied Biosystems).

Molecular analyses

The possible presence of null alleles, scoring errors, and large-allelic dropout was explored using Micro-Checker v.2.2.3 (Van Oosterhout et al., 2004). Significant departures from Hardy-Weinberg proportions (HWP) and linkage disequilibrium (LD) in the dataset were tested with package *genepop* (Rousset, 2008), setting a dememorization number of 10 000 with 1000 batches and 10 000 iterations per batch. The sequential Bonferroni correction was applied to correct the significance of p-values in multiple tests (Rice, 1989). The packages *diveRsity* (Keenan et al., 2013), *hierfstat* (Goudet, 2005), and *poppr* (Kamvar, Tabima and Grünwald, 2014) were used to calculate genetic diversity indices (observed and expected heterozygosity, allelic richness, number of private alleles and F_{IS}), and estimates of

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Table 2. Descriptive table of the 14 microsatellite loci used in this study, including: repeat motifs, primer sequences, fluorescent dye, multiplex group, allelic size range (in base pairs), number of alleles (NA) per locus, observed (H_O) and expected (H_E) heterozygosity in the dataset.

Locus	Repeat motif	Forward and reverse primers (5′-3′)	Dye	Multiplex group	Allelic range	NA	H_O	H_E	Reference
Bbufμ47	CA	GGATCAAGCCCTCAGACAACTC CACAGCAGCAGAAATTTTGACCAG	6-FAM	M1	130-148	12	0.72	0.80	Brede et al. (2001)
Bspi3.23	AAT	ATGCCCTGGGTACATATCATAGG TGTACAGCGCTATGGAATGAATG	VIC	M1	143-152	5	0.54	0.58	This study
Bspi4.14	GATA	ATGAGTCTGCTAGGAATTGTCTC CTGTAGCAATCATCTTCTCCTGC	VIC	M1	324-492	40	0.95	0.95	Trujillo et al. (2017)
Bbufμ13	CA	AGCCTACATTGCTCAATCCGATAC GTGGCGTGGAGGGTCATAAAATC	NED	M1	146-156	5	0.21	0.20	Brede et al. (2001)
Bspi4.28	CTAT	GGCATGGGTGAATAAAGAAGTCC AAACTTAGCTCACCTGGTCAGC	PET	M1	179-223	17	0.83	0.90	Trujillo et al. (2017)
Bbufμ49	GT	GATCTGGGCAGTGTTGGATTG ATTCCGTCTGCTAAATGTCTCTTG	6-FAM	M2	208-230	12	0.73	0.85	Brede et al. (2001)
Bspi4.29	TAGA	CCCTTTCTATGTCACCTCTGTAC ACAGTGCCATATCTTCAGTGTTG	VIC	M2	192-216	7	0.74	0.80	Trujillo et al. (2017)
Bspi3.02	GGT	GGATTAGGGCATAGACAACTGAC CCGTCACAGAGAAATCAAAGGG	NED	M2	288-297	5	0.28	0.33	Trujillo et al. (2017)
Bspi4.27	GATA	GAGACACGTAATCCAGACTTTCC TTAGGACTTGTGTGACATCTGAG	PET	M2	220-276	20	0.84	0.91	Trujillo et al. (2017)
Bspi4.05	AGAT	TGATCTCAGCAGGTCCTTTAATG CAAAGTACCTCTGCAGCTAATGG	PET	M2	382-402	8	0.48	0.73	This study
Bspi3.11	CTT	TTTCCTGCCTTCTTGTAACGTTG CTCACTGTCAGCAATGTATGACC	6-FAM	M3	258-261	2	0.18	0.26	Trujillo et al. (2017)
Bspi4.24	GATA	AAATTTGGGAGCAATCCTGTAGG GTCACAACTGTCCTGTTACCTTG	VIC	M3	234-270	10	0.74	0.84	Trujillo et al. (2017)
Bspi4.07	AGAT	ACCCACCTCACGTTAATTATTGC ATCTTCAATTCCATAGCTGCACC	NED	M3	347-419	29	0.90	0.93	Trujillo et al. (2017)
Bbufμ11	CA	GTCACATGGATAATAAATGAGACC TCTAATATTGATGACCAGACAACC	PET	M3	99-129	16	0.78	0.84	Brede et al. (2001)

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pairwise population differentiation F_{ST}, Hedrick's G_{ST} and Jost's D (Gerlach et al., 2010; Edelaar, Burraco and Gomez-Mestre, 2011).

Multivariate discriminant analysis of principal components (DAPC) was conducted with package *adegenet* (Jombart, 2008) to summarize genetic differences between populations. Sampling localities were set as pre-defined groups, and the number of retained principal components was determined by optimizing the α-score to avoid over-fitting.

Because overrepresentation of siblings within population samples can introduce biases in model-based genetic structure analyses (Sánchez-Montes et al., 2017), we used the full likelihood model implemented in COLONY ver. 2.0.6.4 (Jones and Wang, 2010) to identify related individuals in each sampling population. For these analyses, we used all the genotyped samples of the corresponding population as the "offspring" sample, without specifying any candidate parent, and set prior genotype error rates to 0.05 for each marker. To reduce the potential excess of siblings in each locality, all but two individuals from each family group identified were removed from the dataset for downstream analyses.

The Bayesian clustering algorithm implemented in STRUCTURE v2.3 (Pritchard, Stephens and Donnelly, 2000) was applied to assign individuals to clusters according to their membership coefficients (Q-scores) and identify admixed individuals. Analyses were run under the admixture model with correlated allele frequencies and with no prior information on locality of origin. Chains were run with 500 000 burn-in cycles followed by 500 000 MCMC steps. Ten independent replicates were performed for each K value ranging from 1 to 10. The optimal K value was estimated by calculating ΔK (Evanno, Regnaut and Goudet, 2005) as implemented in STRUCTURE HARVESTER (Earl and von Holdt, 2012). The results were combined and visualized using Clumpak server (Kopelman et al., 2015). A threshold of $0.2 < Q < 0.8$ was used to classify admixed individuals as hybrids (Endler, 1977; Arntzen et al., 2020). We also took higher K values into consideration to explain the underlying genetic substructure in each species based on geographic separation.

To interpolate genetic structure and reveal putative hybrid zone geography, the Bayesian model-based clustering algorithm implemented in *tess3r* package was applied (Caye et al., 2016). Ancestry proportions were mapped using the output Q-matrix from STRUCTURE analyses.

To characterize the spatial characteristics of the hybrid zone, geographic cline analyses were conducted with package *hzar* (Derryberry et al., 2014), using STRUCTURE Q-scores for $K = 2$ and mtDNA frequencies as reported in Dursun, Özdemir and Gül (2023), corresponding to all localities in this study. Because sampling areas did not follow a straight transect, geographic coordinates were linearized along a west-east axis using package *geosphere* (Hijmans, 2021) based on Euclidean distances. Fifteen model variants were tested for each marker modifying the scripts provided by van Riemsdijk et al. (2019) with relevant parameters. Besides, Pmin and Pmax values were determined for each model. The convergence of parameter scores was visually assessed in trace plots. Selection of the best cline model was based on corrected AIC (AICc) scores.

Results

Analysis with Micro-Checker did not show evidence of systematic anomalies in scored genotypes across populations for any marker (just identified potential null alleles in locus *Bspi3.23* in population PAZ, *Bspi4.5* in population YAY, and *Bbufμ11* in population MES), so all 14 loci were retained for downstream analyses. Apart from the biallelic locus *Bspi3.11*, the rest of markers of the dataset showed high polymorphism, with the number of alleles per locus ranging from 5 (*Bspi3.23*, *Bbufμ13*, and *Bspi3.02*) to 40 (*Bspi4.14*). The mean number of alleles per locus was 13.4. The rate of missing data across the dataset was 0.92%. We found no significant departure from Hardy-Weinberg proportions or linkage disequilibrium in any population or locus or combinations of loci. The basic genetic diversity indices for each population are summarized in supplementary material S1.

Pairwise genetic distances among populations were largely concordant across the three indices used (F_{ST}, G_{ST}, and D, supplementary tables S3-S4). Regarding pairwise F_{ST} distances, values ranged from 0 (SUC-GUN2) to 0.383 (FIN-CFT). Most of the genetic differentiation estimates were significantly >0. Global F_{ST} values for the *B. bufo* and the *B. verrucosissimus* clusters (based on STRUCTURE results, see below) were 0.119 and 0.151, respectively, reflecting stronger genetic structure among *B. verrucosissimus* populations.

DAPC analysis produced 40 PCs explaining 80.30% of total genetic variance. This analysis assigned 81.15% of individuals to groups consistent with their actual sampling populations, and 96.51% of individuals were assigned to the same genetic clusters as in the STRUCTURE analyses (supplementary figs S1-S6).

Among the 201 genotyped individuals, 3 pairs of full siblings were identified in populations SEN, KAP, and PAZ, which were maintained in the dataset. All individuals in the SEN sample were identified as full siblings.

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and thus only two randomly selected individuals were retained in this population for downstream analyses. In PAZ and KAP, five individuals were discarded per population for the same reason. Therefore, the pruned dataset for downstream model-based genetic structure analyses included the remaining 183 individuals.

The optimal number of clusters in STRUCTURE analyses was identified as $K = 2$ according to the ΔK method ($\mathrm{LnP}(K) = -8935.07$; $\Delta K = 106.80$, see supplementary tables S5-S6). STRUCTURE results at $K = 2$ showed a clear separation of two genetic clusters corresponding to *B. bufo* and *B. verrucosissimus*, with population YAY showing evidence of admixture (fig. 1). From west to east, populations KAL to ARD were assigned to pure *B. bufo*, and populations FIN to MES were classified as pure *B. verrucosissimus*. Population CFT, well within the pure *B. verrucosissimus* range, showed high levels of admixture under $K = 2$. Analyses at increasing K values (5 to 10) showed evidence of genetic substructure within both species, with some differentiated populations at increasing K values, including KAL, IKZ, GUN1+GUN2, HEM, KAP and ARD in *B. bufo*, and FIN+ORT+HOP+MUR, CFT, AKH and BOR+MES in *B. verrucosissimus* (supplementary fig. S7).

Spatial population analysis in TESS under $K = 2$ showed the same major genetic division, with the cluster representing *B. bufo* dominating in the western end of our transect, and the second cluster (*B. verrucosissimus*) occupying the eastern side of the study area. The population YAY showed an intermediate location within the transition between the two major genetic groups (fig. 2). Based on the genetic interpolation map, the admixed population CFT is represented as a *B. bufo* isolate within the *B. verrucosissimus* range (fig. 2).

Given the peculiar genetic ancestry of CFT, we performed cline analyses both including and excluding this population. The best-fit cline models and cline parameters for each marker dataset are presented in table 3. Based on STRUCTURE Q-scores, the *B. bufo-B. verrucosissimus* contact zone is centered near population YAY (c = 89.4 km, 95%CI: 82.3-96.7 km), with a width (w) of 17.43 km (95%CI: 6-63.8 km) (fig. 3). Results were similar when population CFT was excluded (c = 88.9 km, 95%CI: 81.4-97.5 km; w = 38.6 km, 95%CI: 17.1-64.9 km).

The mtDNA cline showed a westward position with respect to the center inferred for the microsatellite cline (fig. 3). The optimal model yielded a sharp, narrow contact zone with c = 56.2 km (95% CI: 54.5-59.7 km) and w = 3.8 km (95% CI: 1-16.8 km). The best model located the center of the hybrid zone between populations SUC and PAZ in the Pazar district of Rize. Excluding CFT produced very similar results (c = 56.2 km, 95% CI: 54.5-59.7 km; w = 3.7 km, 95% CI: 1-16.8 km; table 3).

Discussion

This study is the first detailed characterization of the hybrid zone between *B. bufo* and *B. verrucosissimus* in northeastern Türkiye. Our comprehensive geographic sampling and the analysis of highly polymorphic microsatellite data in a cline analysis framework provide new insights about the structure of the hybrid zone, with implications for the taxonomy of the group.

Genetic analyses of microsatellite genotype data identified two well-differentiated gene pools corresponding to *B. bufo* and *B. verrucosissimus*, which replace one another over the study area along a west-east axis, with one documented geographically intermediate admixed population (YAY). Most populations in our transect were robustly identified as pure populations of either species: pure *B. bufo* ($Q > 0.8$) west of the admixed YAY population, and pure *B. verrucosissimus* ($Q < 0.2$) on the eastern area, except for the CFT population. According to these results, the geographic distribution of *B. verrucosissimus* in the area centers around the Çoruh River (populations MES, AKH, and BOR, fig. 1 and supplementary fig. S7). This biogeographic

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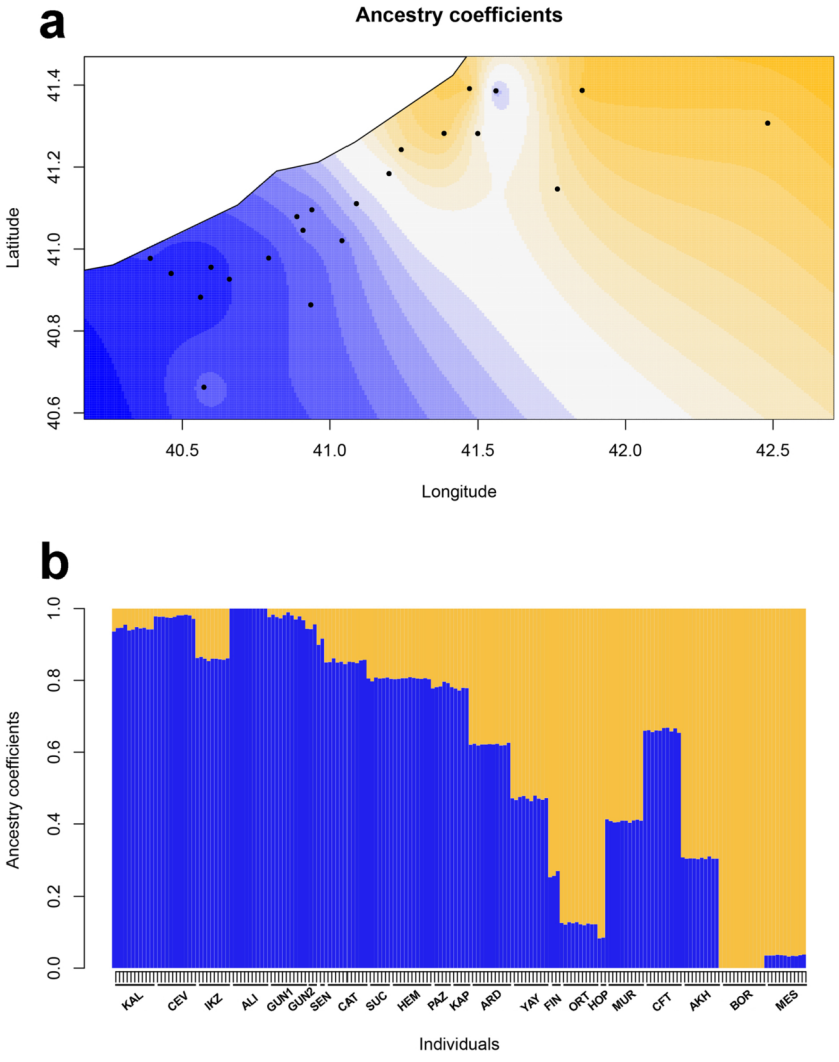


Figure 2. Interpolation map (a) and individual assignment barplot (b) constructed under $K = 2$ in Structure corresponding to the genetic pools of *Bufo bufo* (Common Toad) and *Bufo verrucosissimus* (Caucasian Toad). Different shadings show probabilities of belonging to the respective species (blue for *Bufo bufo* and yellow for *Bufo verrucosissimus*). Dark shades represent pure populations of each species whereas lighter shades indicate higher admixture. The white range indicates maximum admixture in the center of the hybrid zone.

Table 3. Maximum likelihood estimates of cline centre (Km from the western end of the linearized transect) and width for microsatellites (based on Structure Q-scores) and mtDNA, including/excluding population CFT. Mean and 95% confidence intervals (in brackets) are presented. The optimum models were selected based on AICc scores. Pmin = Minimum estimated frequency; Pmax = Maximum estimated frequency.

Marker	Model	Centre (km)	Width (km)	AICc	Pmin	Pmax
Structure	typM	89.40 (82.26-96.73)	17.43 (6.02-63.79)	17.09	0	1
Structure (CFT excluded)	fixN	88.86 (81.43-97.49)	38.62 (17.08-64.85)	9.63	0.01	0.98
mtDNA	typN	56.22 (54.50-59.70)	3.81 (1.01-16.78)	4.80	0	1
mtDNA(CFT excluded)	typN	56.20 (54.51-59.72)	3.74 (0.98-16.79)	4.81	0	1

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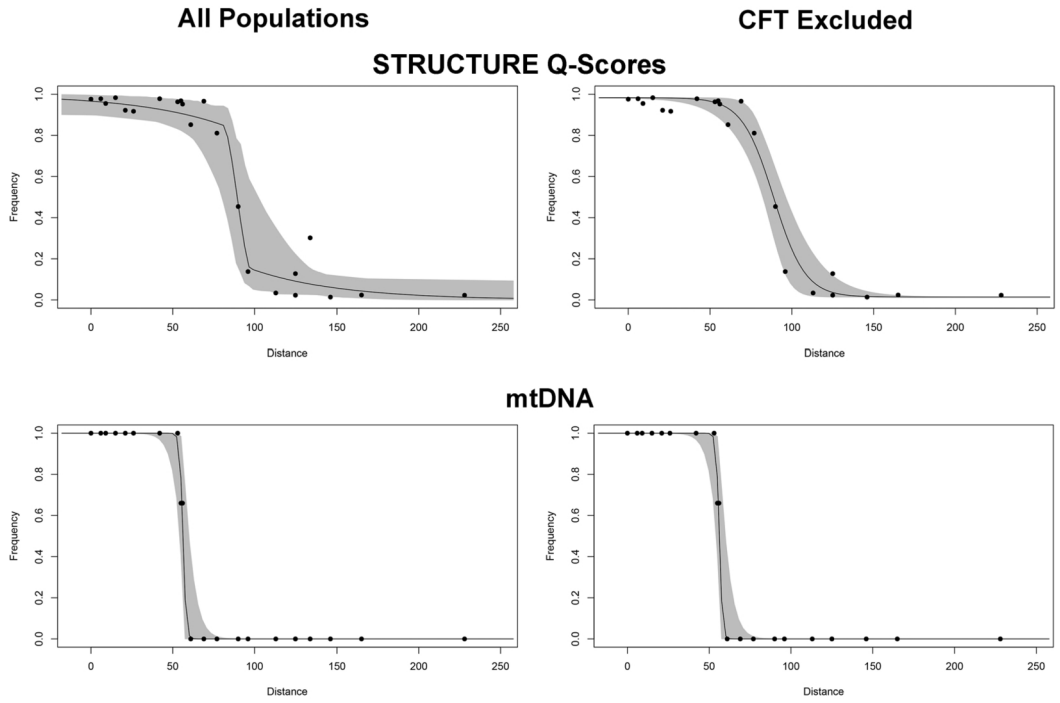


Figure 3. Cline analysis of nuclear (based on STRUCTURE Q-scores) and mitochondrial data across the *Bufo bufo*/*Bufo verrucosissimus* (Common Toad/ Caucasian Toad) hybrid zone along a west-east transect. Each point represents one population and grey shades represent 95% confidence intervals of fitted clines.

pattern has parallel examples in other taxonomic groups, including *Phonochorion* crickets (Sağlam, Küçükyıldırım and Çağlar, 2014) and *Darevskia* lizards (Kurnaz et al., 2019).

Cline analyses consistently recovered very narrow and steep clines describing the genetic transition from *B. bufo* to *B. verrucosissimus*, with a center in the Fındıklı district (YAY population). This bimodal hybrid zone (Jiggins and Mallet, 2000) shows tension zone features suggesting reproductive isolation after secondary contact (Smith et al., 2013; Dufresnes et al., 2014; Maier et al., 2019). Steep clines across narrow hybrid zones like those inferred in this study generally indicate strong selection against hybrids and reduced hybrid fitness due to post-zygotic isolation, which is consistent with previous reports on artificial interspecific crosses. Pisanets (2001, 2002) obtained fertile F_1 hybrids of *B. bufo* and *B. verrucosissimus* in a mating laboratory experiment, but reported reduced F_2 hybrid viability, suggesting post-zygotic mechanisms may contribute to reproductive isolation.

Among western Palearctic anurans, species that diverged <3 Mya tend to admix and hybridize extensively under natural conditions (Colliard, 2010; Dufresnes et al., 2015, 2017, 2021b). Previous studies have estimated that *B. bufo* and *B. verrucosisimus* diverged around 2.75 Mya, in the Pleistocene (Garcia-Porta et al., 2012; Recuero et al., 2012; Dursun, Özdemir and Gül, 2023), yet admixture is quite limited in this case. The climatic oscillations during the Quaternary strongly affected species distributions, triggering the formation of secondary contact zones (Hewitt, 1999, 2011). In the northern hemisphere, major glacial refugia were located in the Iberian Peninsula, the Balkans, Italy, Anatolia, and Caucasia (Taberlet et al., 1998; Stewart et al., 2010; Sánchez-Montes et al., 2019). During warmer periods with suitable habitat conditions, post-glacial

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range expansion of refugial populations led to the establishment of secondary contacts among well-differentiated lineages, resulting in hybridization and introgression events (Stöck et al., 2012; Litvinchuk et al., 2013; D'Aoust-Messier and Lesbarrères, 2015; Benkovský et al., 2021).

The southern shore of the Black Sea region is a suture zone, where closely related species meet and hybridize following post-glacial range expansion (Bilgin, 2011), including other amphibians, like banded newts in genus *Ommatotriton* (van Riemsdijk et al., 2022; Kalaentzis et al., 2023). Additionally, the studied region is a central unit of the eastern Pontides (Yilmaz et al., 2000; Rangin et al., 2002). This region, including Artvin and Rize provinces, is also a crossing point between populations from Anatolian and Caucasian refugia. The area is characterized by a complex topography, with high mountains and major rivers likely promoting population divergence, as exemplified by studies in multiple taxa, including *Lissotriton* newts (Wielstra, Bozkurt and Olgun, 2015), *Vipera* snakes (Mebert et al., 2015; Gül, 2020), and *Darevskia* lizards (Arribas et al., 2022), as well as our target species.

A remarkable feature of the *B. bufo-B. verrucosissimus* hybrid zone in NE Türkiye is that the nuclear and mitochondrial clines are nearly coincident, with centers displaced approximately 33 km in the Fındıklı and Pazar districts of Rize province. Populations in this area are characterized by mito-nuclear discordances, with ARD, PAZ, and KAP being assigned to *B. bufo* based on microsatellites but bearing mitochondrial haplotypes of *B. verrucosissimus* (Dursun, Özdemir and Gül, 2023). Non-coincident clines in mtDNA vs nuDNA have generally been explained by differential introgression, for instance in scenarios where the demographic expansion of one species leads to range overlap and admixture in the advancing front, or when sex-biased dispersal promotes faster diffusion of the mitochondrial genome (Currat et al., 2008; Wielstra and Arntzen, 2012). Dursun, Özdemir and Gül (2023) hypothesized that *B. verrucosissimus* expanded its range along north-eastern Türkiye following the Last Glacial Maximum (20 Kya) and that *B. bufo* expanded the range approximately in the same period and in the same region. Besides, Garcia-Porta et al. (2012) noted that a narrow forest band might have provided suitable refugial habitats in the same geographic area 18 Kya ago. Thus, non-coincident clines can be the result of ongoing range expansion and/or sex-biased dispersal of *B. bufo* over the *B. verrucosissimus* range. Asymmetric mtDNA introgression is common in hybrid zones (Toews and Brelsford, 2012), often resulting in narrower mtDNA clines compared to the nuDNA (Qi et al., 2014; Johnson et al., 2015). In this respect, patterns of cyto-nuclear discordance in the north-eastern hybrid zone between *B. bufo* and *B. verrucosissimus* are similar to those observed in secondary contact zones of other amphibians (Wielstra et al., 2017a, 2017b; Dufresnes et al., 2020c; Sequeira et al., 2020).

The results of spatial genetic analyses showed a gradual transition in the gene pools from *B. bufo* to *B. verrucosissimus*, with the center of the hybrid zone located near population YAY. However, the CFT population, geographically well within the *B. verrucosissimus* range, is admixed, with predominating *B. bufo* ancestry (Q-score: 66%). Dursun, Özdemir and Gül (2023) found a mitochondrial haplotype of *B. verrucosissimus* in this population, raising questions about its genetic ancestry. Two possible scenarios are to be considered. First, the geographic distribution of *B. bufo* and *B. verrucosissimus* in northeastern Türkiye may represent a mosaic hybrid zone. These zones occur when hybridizing taxa are associated with patchily distributed habitats and show non-clinal transitions associated to environmental factors, as exemplified by *Bombina* toads (Arntzen, 1996; Vines et al., 2003; Dufresnes

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et al., 2021a) and *Triturus* newts (Arntzen and Wallis, 1991; Arntzen, 1996, 2023; Babik, Szymura and Rafiński, 2003; Arntzen, 2018) in Europe.

In the second scenario, the CFT population could represent an enclave, a genetically isolated population of a species surrounded by populations of a closely related species. Enclave populations can originate due to demographic expansion of an invading species over the range of an invaded species, or they can represent relict populations representing a broader distribution of the replaced species in the past (Arntzen, 1978; Themudo and Arntzen, 2007; Vörös et al., 2016; Wielstra et al., 2017b; Arntzen, 2019; Wielstra, 2019; Arntzen et al., 2021; López-Delgado, van Riemsdijk and Arntzen, 2021; Sequeira et al., 2022). Considering the observed clinal transitions in nuclear and mtDNA and the fact that CFT is located well outside the regular dispersal distance of *Bufo* toads with respect to the centre of the hybrid zone, the unusually deep genetic footprint of the CFT population may pinpoint an eroded enclave, although the presence of pure *B. bufo* populations further north, in unsampled areas in Georgia, cannot be ruled out.

The presence of a *B. bufo* enclave within the range of *B. verrucosissimus* (an "island of introgression" sensu Macholán et al., 2011) could result from westward hybrid zone movement, with *B. verrucosissimus* outcompeting *B. bufo* in the study region. mtDNA data supports a westward demographic expansion of *B. verrucosissimus* in northern Türkiye in the past 20 Kya, while *B. bufo* populations in the area remained more stable, but also increasing in the past 50 Kya (Dursun, Özdemir and Gül, 2023). The asymmetric introgression of *verrucosissimus* mtDNA towards the west, ahead of the nuclear contact zone, could be explained by range expansions followed by hybridization. While neutral processes can cause such asymmetric patterns, the role of selection cannot be dismissed (Wielstra and Arntzen, 2014).

In summary, our study supports recognition of *B. bufo* and *B. verrucosissimus* as distinct species based on narrow, nearly coincident mitochondrial and microsatellite clines suggestive of barriers to hybridization. Future studies using high-coverage genomic data may clarify fine-scale temporal and spatial dynamics of the hybrid zone, including the role of landscape and environmental features in patterns of admixture and in generating cyto-nuclear discordances. These studies should also extend to the contact zone in southwestern Türkiye, which provides an exciting opportunity to analyse an independent replicate of the evolutionary processes at play.

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