BRILL
Amphibia-Reptilia (2026) DOI:10.1163/15685381-bja10249
brill.com/amre

The position, shape and dynamics of the hybrid zone between the Danube and Italian crested newt based on genome-wide data, with a cautionary tale on the discovery of a ‘distinct’ mtDNA lineage

Arilah J. van Eden[1,2], Anagnostis Theodoropoulos[1,2], Jan W. Arntzen[1,2], Janis Czurda[3], Róbert Dankovics[4], Krisztián Harmos[5], Daria Kranželić[6], Zdeněk Mačát[7], Peter Mikulíček[8], Antonín Reiter[9], Bruno Schmidt[6,10], David Stanković[11], Mojca Vek[11,12], Judit Vörös[13], Ben Wielstra[1,2,*]

1 - Institute of Biology Leiden, Leiden University, P.O. Box 9505, 2300 RA Leiden, The Netherlands
2 - Naturalis Biodiversity Center, P.O. Box 9517, 2300 RA Leiden, The Netherlands
3 - Institute of Vertebrate Biology of the Czech Academy of Sciences, Brno, Czech Republic
4 - Savaria Museum, Kisfaludi Sándor u. 9., 9700 Szombathely, Hungary
5 - Bükk National Park Directorate, Sánc u. 6., 3304 Eger, Hungary
6 - Association Hyla, Lipovac I no. 7, 10 000 Zagreb, Croatia
7 - Podyjí National Park Administration, Na Vyhlídce 1581/5, 669 02 Znojmo, Czech Republic
8 - Department of Zoology, Faculty of Natural Sciences, Comenius University in Bratislava, Ilkovicova 6, 842 15 Bratislava, Slovakia
9 - South Moravian Museum in Znojmo, Přemyslovců 129/8, 669 02 Znojmo, Czech Republic
10 - Public Institution for the management of protected areas and other protected parts of nature at the Zagreb County level “Zeleni prsten”, Zagreb, Croatia
11 - National Institute of Biology, Večna pot 121, 1000 Ljubljana, Slovenia
12 - Jožef Stefan International Postgraduate School, Jamova cesta 39, 1000 Ljubljana, Slovenia
13 - HUN-REN Balaton Limnological Research Institute, Klebersberg Kuno u. 3., 8237 Tihany, Hungary
*Corresponding author; e-mail: b.m.wielstra@biology.leidenuniv.nl
ORCID iDs: van Eden: 0009-0000-5278-0887; Theodoropoulos: 0009-0003-0272-6358; Arntzen: 0000-0003-3229-5993; Czurda: 0009-0000-9151-3482; Kranželić: 0000-0001-6291-0593; Mačát: 0000-0002-5697-159X; Mikulíček: 0000-0002-4927-493X; Schmidt: 0000-0003-3544-4739; Stanković: 0000-0002-3312-382X; Vek: 0009-0007-6977-4943; Vörös: 0000-0001-9707-1443; Wielstra: 0000-0002-7112-5965

Received 11 September 2025; final revision received 29 December 2025; accepted 1 January 2026;

Associate Editor: Iñigo Martínez-Solano

Abstract. When species that are reproductively incompletely isolated meet in parapatry and reproduce, a hybrid zone is established. The strength of reproductive isolation between these species and the dynamics of their ranges can be inferred from the extent and direction of gene flow across the hybrid zone. We determine the position, shape and dynamics of the relatively poorly studied hybrid zone between the Danube (*Triturus dobrogicus*) and Italian (*T. carnifex*) crested newts, using genome-wide nuclear DNA data (24 species-diagnostic SNPs) and an mtDNA marker (ND4). Overall, the hybrid zone is narrow, signifying strong selection against hybrids. In the north of the hybrid zone, asymmetric introgression of *T. dobrogicus* alleles into *T. carnifex* indicates postglacial expansion by *T. carnifex*. We also report a highly distinct haplotype lineage that presumably reflects ‘cryptic pseudogenes’; while the relevant sequences do not bear the hallmarks of a pseudogene, their apparent distinctiveness is not supported by an independent mtDNA marker (ND2).

Keywords: geographical cline analysis, KASP genotyping, mitochondrial DNA, nuclear DNA, pseudogene, *Triturus*.

Published with license by Koninklijke Brill BV | DOI: 10.1163/15685381-bja10249
| ISSN: 0173-5373 (print) 1568-5381 (online)

Introduction

Hybrid zones are regions where distinct species meet, mate and produce genetically admixed (i.e. hybrid) offspring (Hewitt, 1988; Wielstra, 2021). The climate fluctuations during the Pleistocene Ice Age helped shape today's hybrid zones (Hewitt, 2011). Species whose ranges were contracted to refugia during cold glacial spells could expand their ranges during warm interglacial spells, facilitating secondary contact and hybrid zone formation. Most hybrid zones observed today formed during the Holocene.

Increased genetic divergence between species is typically manifested as reduced fitness of their hybrid offspring (Wu and Ting, 2004; Stankowski and Ravinet, 2021). The shape and permeability of hybrid zones reflect the strength of reproductive barriers between species (Barton and Hewitt, 1985, 1989). In a so-called tension zone, the hybrid zone is maintained by a balance between selection against hybrids and the influx of pure parental species (Hewitt, 1988; Wielstra, 2021). The narrower the width of the hybrid zone in relation to the dispersal distance of the species involved, the stronger the reproductive isolation (Barton and Gale, 1993).

While a hybrid zone may remain stable at the position it originally formed upon secondary contact (Barton, 2001), if one of the species involved would be able to outcompete the other, this would lead to hybrid zone movement (Wielstra, 2019). When the fitness of hybrid offspring is non-zero, alleles of one parental species may introgress into the other, while the two preserve their overall genetic integrity (Mallet, 2005). Potential movement of the hybrid zone can be inferred from introgression patterns: a hybrid zone that shifts its position is predicted to leave a trail of introgressed selectively neutral markers behind (Buggs, 2007; Wielstra, 2019).

The genus *Triturus*, distributed in Europe and western Asia, encompasses approx. 10 marbled and crested newt species that hybridise where their ranges meet (Arntzen et al., 2014; Wielstra et al., 2014). Most *Triturus* hybrid zones have been studied in detail and differ in the extent of reproductive isolation and spatial dynamics (e.g., Arntzen and Wallis, 1991; Arntzen et al., 2009, 2014, 2021a,b; Espregueira Themudo et al., 2012; Wielstra et al., 2017a,b). North of the Balkan Peninsula, *Triturus* hybrid zones are relatively understudied.

The Danube crested newt (*T. dobrogicus*) and the Italian crested newt (*T. carnifex*) meet each other around the eastern foothills of the Alps (fig. 1; Wielstra et al., 2014). Hybridisation between the two species has been reported in the Czech Republic, Austria, Croatia and Hungary and is expected to also occur in Slovenia (Vörös and Major, 2007; Mikulíček et al., 2012; Stanković and Delić, 2012; Gubányi et al., 2010; Arntzen et al., 2014; Vörös et al., 2016; Mačát et al., 2019; Wielstra et al., 2021). However, due to the scattered nature of the sampling in previous studies, the position and shape of the hybrid zone along its entire >400 km length remains unclear. In this study, we combine mtDNA barcoding and KASP genotyping of 24 species-diagnostic nuclear DNA SNPs (single nuclear polymorphisms) from across the genome to delineate the hybrid zone, determine its width and characterise its dynamics.

Materials and methods

Sampling and DNA extraction

We included 383 *Triturus* individuals from 172 localities, with a maximum of three individuals per locality (Fig. 1; sample details in supplementary table S1). We obtained 41 DNA samples (with already established mtDNA haplotype) from previous studies (Wielstra et al., 2013, 2019, 2021); the remainder was newly collected. DNA was extracted using the Wizard® Genomic DNA purification kit (Promega). In the Czech Republic the *T. cristatus* range approaches the northern side of the hybrid zone between *T. carnifex* and *T. dobrogicus*. To reduce the chance of genetic admixture with that third species, we only included samples that do not show genetic admixture with *T. cristatus* based on a panel of microsatellites (Mikulíček et al., 2012; Mikulíček, unpublished data).

MtDNA barcoding

We amplified a 658 bp fragment of the ND4 (NADH dehydrogenase subunit 4) mtDNA gene using the primers KARF4 and KARR1 (Wielstra et al., 2013). PCRs were performed in 12 μl reactions. These contained 0.06 μl of

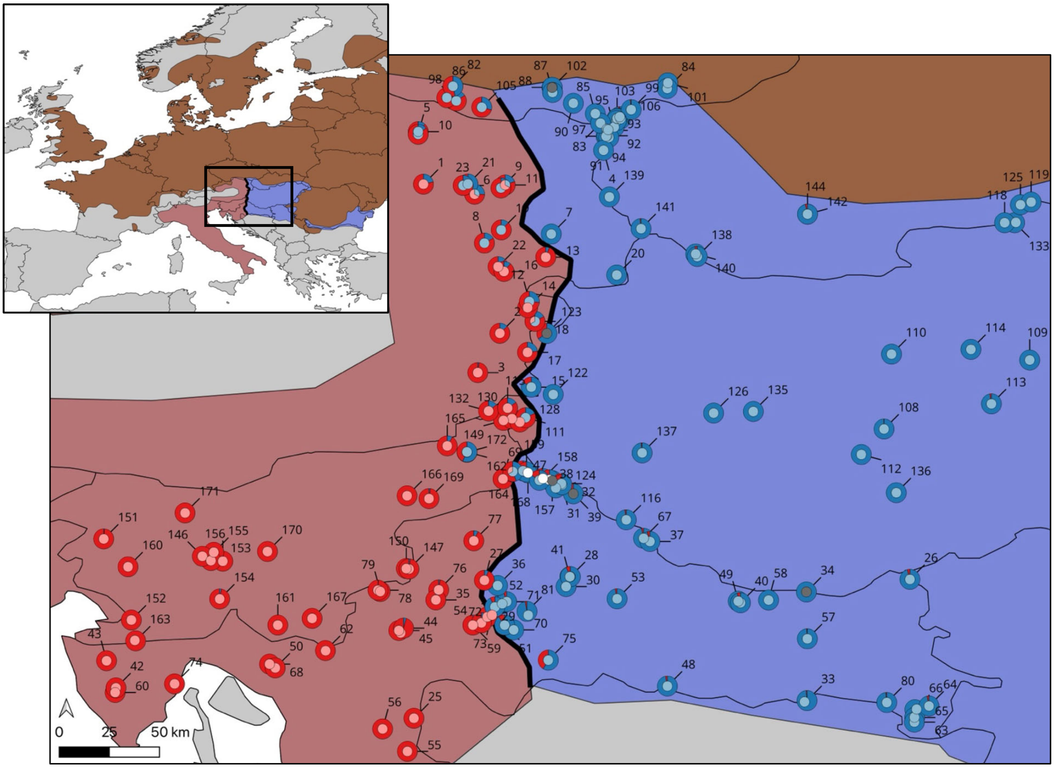


Figure 1. Distribution map and genetic composition of localities for the Italian (*Triturus carnifex*) and Danube (*T. dobrogicus*) crested newt. Background colours indicate a rough outline of the geographic ranges of different *Triturus* species (*T. carnifex* in red, *T. dobrogicus* in blue and *T. cristatus* in brown). The large (outer) pie charts represent the proportion of *T. carnifex* (red) and *T. dobrogicus* (blue) nuclear DNA alleles based on 24 nuclear DNA SNP markers (details in table S1 in the Supplementary material). The small (inner) pie charts represent the proportion of *T. carnifex* (red) and *T. dobrogicus* (blue) mtDNA based on the ND4 gene; grey reflects failed mtDNA sequencing. We also identified two highly distinct haplotypes (white) that appear to reflect 'cryptic pseudogenes' (see Discussion). The solid black line represents the inferred centre of the hybrid zone, where genetic admixture is interpolated to be 0.5.

each primer (0.05 μM end concentration), 6 μl Qiagen multiplex PCR master mix, 4.88 μl purified water and 1 μl of DNA extract. PCR conditions were: a hot start for 15 minutes at 95°C, followed by 35 cycles of denaturation for 30 seconds at 95°C, annealing for 1 minute at 55°C and extension for 1 minute at 72°C, and extension at 72°C for ten minutes. Sanger sequencing was outsourced to Macrogen (Amsterdam, The Netherlands). The sequences were edited and trimmed in Geneious Prime 2025.0.2 (https://www.geneious.com).

The sequences were aligned against a database of *Triturus* ND4 haplotypes (Wielstra et al., 2013). To determine the corresponding haplotypes (if any) of the newly sequenced individuals in our database, we used the 'DNA to haplotype collapser and converter' function in FaBox (Villesen, 2007). To determine to which *Triturus* species any new haplotypes belonged, we conducted maximum likelihood phylogenetic inference using the IQ-TREE webserver (Trifinopoulos et al., 2016). We used *Calotriton asper* as an outgroup and also included haplotypes representing two marbled and five additional crested newt species (taken from Wielstra et al., 2010). We used ModelFinder (Kalyaanamoorthy et al., 2017) to determine the most appropriate partitioning scheme and model of sequence evolution (TPM2+F+G4 for codon 1, HKY+F+I for codon 2 and TN+F+G4 for codon 3). To determine branch support we used 1,000 ultrafast bootstrap replicates (Hoang et al., 2017). Individuals that carried mtDNA of *T. cristatus*, which both focal species meet at the northern end of their ranges (Fig. 1), were removed from downstream analyses.

We identified two highly distinct ND4 haplotypes in several individuals (see Results), which we confirmed by resequencing at a different sequencing company, BaseClear (Leiden, The Netherlands). Therefore, we also amplified a 1,035 bp fragment containing the entire ND2 (NADH dehydrogenase subunit 2) mtDNA gene, using the primers L3780 and H5018 (Babik et al., 2005). We conducted phylogenetic inference for ND2 as above (with selected models being K3Pu+F+G4 for codon 1, TN+F+I for codon 2 and TN+F+G4 for codon 3). Note that haplotypes for *Calotriton asper* and the two marbled newts are two triplets longer and we removed these 6 bp from our analysis.

SNP identification from target capture data

For SNP marker design we followed the procedure of Meilink et al. (2025). First, to obtain genome-wide data, we followed the target capture protocol NewtCap (de Visser et al., 2025a). We included 11 *T. carnifex* and 11 *T. dobrogicus* individuals, covering the entire distribution range (sample details in table S1 in the Supplementary material). Data for nine of these 22 individuals were available from other studies (Wielstra et al., 2019; de Visser et al., 2025b) and the remainder was newly generated (sample details in table S2 in the Supplementary material). In brief, libraries were constructed using the NEB Next® Ultra™ II FS DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA). Target capture was then performed with a custom probe set that targets 7139 exons based on *Triturus* transcriptome data (MyBaits v4.0 kit, Arbor Biosciences Ref. No. 170210–32; Wielstra et al., 2019). Sequencing was conducted by BaseClear (Leiden, The Netherlands) on the NovaSeq 6000 platform (Illumina, San Diego, CA, USA) using 150 bp paired-end sequencing. We retrieved a mean of 5 533 554 read pairs per sample (range 2 710 747–9 933 423 million).

Upstream bioinformatics closely followed the NewtCap pipeline (de Visser et al., 2025a) and cleaned reads were mapped to a reference set of 7139 *T. dobrogicus* sequences. We filtered raw VCF files to include only bi-allelic single nucleotide polymorphisms (SNPs) with a mapping quality greater than 10 using VCFtools version 0.1.16 (Danecek et al., 2011). Next, the *-consensus* function of BCFtools version 1.18 (Danecek et al., 2021) was used to generate sequences in FASTA format for each sample. For each of the two species, we imported and aligned the available sequences and built consensus sequences for each marker. Sites that showed intraspecific variation were denoted with the appropriate IUPAC code. Samples had a mean of 97.68% coverage (min = 93.86%, max = 98.53%), with a mean depth of 85.1× (min = 23.05×, max = 191.55×). We then aligned the consensus sequences for *T. dobrogicus* and *T. carnifex* to identify presumed species-specific SNPs in Geneious Prime v2025.0.3 (https://www.geneious.com) after confirming the diagnosticity of these SNPs in the raw reads in IGV version 2.12.3 (Robinson et al., 2011).

The position on the genome of 4226 exons is known, based on a *Triturus* linkage map (France et al., 2025). We designed primers to target SNPs for 24 of these markers: two on each of the 12 chromosomes, one near the ‘beginning’ (at approx. 25% of the total map length of the chromosome) and one near the ‘end’ (at approx. 75%). Primers for the 24 SNP markers (details in table S3 in the Supplementary material) were designed in Kraken 23.11.7 (LGC Biosearch Technologies).

KASP genotyping

SNP genotyping was conducted using KASP (Kompetitive Allele-Specific PCR), which involves fluorescence-based genotyping (Semagn et al., 2014). For each marker for each individual, the SNP variant present is determined in uniplex assays. These assays include a common reverse primer and two allele-specific forward primers that have a final base complementary to one of the two potential SNP variants. The allele-specific primers also possess unique tail sequences. Two distinctly fluorescently labelled sequences present in the KASP master mix are complementary to each tail sequence. While originally quenched, these labels are activated when incorporated in subsequent PCR cycles, with further cycling causing signal intensity to increase. Fluorescent signals are measured using a PHERAstar plate reader. Depending on which signal is emitted, it can be determined which SNP variant is present (both in the case of a heterozygote). Genotypes were automatically called using Kraken 23.11.7 (LGC Biosearch Technologies), followed by visual confirmation and manual correction when necessary (see fig. A1 in the Appendix for an example of plotted output). Individuals with over 50% missing data were removed from downstream analyses.

Hybrid index and geographical cline analysis

The hybrid index was calculated as the proportion of *T. dobrogicus* alleles, averaged per locality. We fitted one-dimensional geographical clines by transforming the two-dimensional hybrid zone setting into a one-dimensional transect as follows. Hybrid index values were interpolated using the Akima package v.0.6-3.4 (Akima et al., 2025) in R v2024.12.1 (R Core Team, 2025). The 0.5 contour of the hybrid index was used to define the theoretical centre of the hybrid zone. Next, minimal distances from each locality to the centre of the hybrid zone were calculated with the R package geosphere v1.5-20 (https://cran.r-project.org/web/packages/geosphere/index.html), assigning negative values to *T. carnifex* and positive values to *T. dobrogicus*. Geographical clines were then fitted for 24 nuclear SNP markers, mtDNA, and the hybrid index using the R package HZAR (Derryberry et al., 2014). Allele frequencies were calculated as the proportion of *T. dobrogicus* alleles per individual and averaged per locality. For each marker, five cline models of different complexities were tested, namely no tails (typN), left tail only (typL), right tail only (typR), mirrored tails (typM), and independent tails on both sides (typB), with frequencies fixed at the ends of the cline to 0 for pure *T. carnifex* and 1 for pure *T. dobrogicus*. All models estimated the cline centre and the cline width, while those with tails additionally estimated the tail slope (τ) and the distance from the cline centre to the tail (δ). Model convergence was assessed using trace plots and model selection was based on the lowest Akaike Information Criterion (AICc). Because geographical cline models draw information from adjacent populations, the effect of small per-site sample sizes on overall cline estimation is limited.

Effective selection

Effective selection was estimated following Barton and Gale (1993) using the R script provided by van Riemsdijk et al. (2019), by comparing observed cline widths with those expected under neutrality, using lifetime dispersal distances inferred from admixture linkage disequilibrium based on the hybrid index. Linkage disequilibrium was estimated from the variance in the hybrid index and allele frequencies across markers (equation 2b in Barton and Gale, 1993). Lifetime dispersal distance per generation (σ) was estimated, while accounting for both pre-metamorphic (n_{pre}) and post-metamorphic (n_{post}) individuals in the total sample set (n), as $\sigma = \frac{npre}{n} * \sqrt{rD\omega_0^2} + \frac{npost}{n} * \sqrt{rD\omega_0^2/(1+r)}$. The recombination rate, r, was estimated as 0.431 following equation 6 in Macholán et al. (2007), considering that *Triturus* has 12 chromosomes, using 27.5 chiasmata based on the average observed in *T. carnifex* (Wallace et al., 1997), and setting r_0 to 0.001, D was the estimated linkage disequilibrium at the cline centre, and ω_0 the observed cline width from the geographical cline analysis. Expected cline width under neutrality was calculated using a generation time of 3.5 years and a time since secondary contact of 12,000 years, following Wielstra et al. (2017b). Effective selection was determined as $s* = (2\sigma/\omega_0)^2$ (Barton and Gale, 1993). Scripts are available online at https://zenodo.org/records/17094645.

Results

MtDNA barcoding

We obtained ND4 mtDNA sequences for 358 out of 383 individuals (summarized in table S1 in the Supplementary material). Six of these possessed *T. cristatus* mtDNA (two different haplotypes). Across the remaining 352 individuals we found 30 *T. dobrogicus* haplotypes, of which 15 were newly identified, and 27 *T. carnifex* haplotypes, of which 22 were newly identified. GenBank Accession numbers for haplotypes are in table S4 in the Supplementary material.

Cytonuclear discordance was most pronounced in the northern part of the hybrid zone (fig. 1). We found mtDNA introgression of *T. dobrogicus* into *T. carnifex* (as identified from nuclear DNA data, see below) for five haplotypes and the reverse for two haplotypes (fig. 2). In the same region (locality 32 in fig. 1), we also found a relatively distinct basal haplotype for *T. dobrogicus* (Tdob45, see fig. 2).

We identified two highly distinct *Triturus* haplotypes (T-?-01 and T-?-02, see fig. 2) present in three *T. dobrogicus* localities close to the hybrid zone (localities 148, 158 and 168 in fig. 1). We confirmed that these sequences translated properly, without stop codons, and did not deviate in GC content. However, based on the mtDNA marker ND2, these same individuals do not stand out and simply cluster with *T. dobrogicus* (see the phylogeny in fig. A2 in the Appendix).

KASP genotyping and hybrid index

After excluding six individuals that carried *T. cristatus* mtDNA and another nine individuals with over 50% missing data for KASP genotyping, we obtained data for 94% of the 24 × 374 = 8976 potential nuclear SNP genotype calls (genotyping data provided in table S5 in the Supplementary material). The theoretical centre of the hybrid zone based on the 0.5 contour of the hybrid index runs roughly south to north, straight through Croatia, easternmost Slovenia and Austria, up to the southernmost part of the Czech Republic (fig. 1). Overall, genetic admixture is geographically restricted to close to the 0.5 hybrid index contour, but it is more extensive in the northern section of the hybrid zone, on the western side, in north-eastern Austria (fig. 1).

Geographical cline analysis

The geographical cline based on the hybrid index cline has a mean width of 3.08 km (fig. 3; details on selected models in table S6 in the Supplementary material). The average of the mean widths for the 24 nuclear DNA SNP markers is 9.6 km and for mtDNA the mean width is 28.8 km. The confidence intervals for the position of the centre overlaps for the mtDNA marker and all but three nuclear SNP markers (SNP3, SNP9 and SNP24; fig. 3; table S6 in the Supplementary material) with those for the hybrid index cline. Of the displaced nuclear SNP marker clines, two are shifted towards *T. carnifex* and one towards *T. dobrogicus*. Inspection of the tail slopes shows that 15 out of 24 nuclear DNA marker clines, as well as the mtDNA and hybrid index clines, exhibit shallower slopes for the left compared to the right tail, whereas two nuclear DNA marker clines show the opposite pattern (fig. 3).

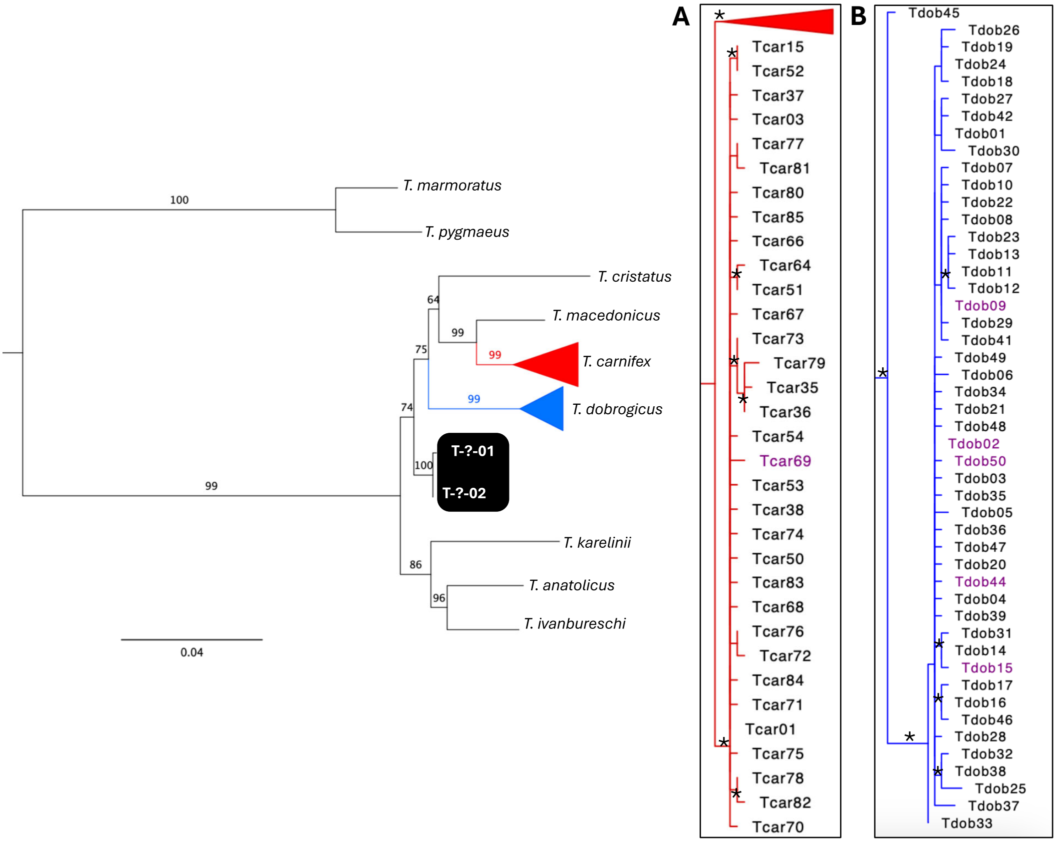


Figure 2. Maximum likelihood phylogeny for the Italian (*Triturus carnifex*) and the Danube (*T. dobrogicus*) crested newt based on the mtDNA gene ND4. The *Calotriton asper* outgroup is not shown. The inset shows a simplified phylogeny including all crested newt species. Note the highly distinct haplotypes (T-?-01 and T-?-02) found in *T. dobrogicus*. The red and blue triangles represent the collapsed clade of all *T. carnifex* haplotypes (shown in detail in panel A) and *T. dobrogicus* haplotypes (shown in detail in panel B). For *T. carnifex* (A) only the distinct Balkan mtDNA clade relevant to the present study is shown in full, other clades are collapsed (red triangle). Haplotypes highlighted in purple are found introgressed in the opposite species. In A and B ultrafast bootstrap support values $\geqslant 95$ are denoted with an asterisk (*).

Effective selection

The average effective selection against hybrids ($s*$) is estimated at 0.018. The lifetime dispersal (σ) is estimated at 0.209 km per generation. The cline width under neutrality (ω_n) is estimated at 30.72 km.

Discussion

Delineation of a narrow hybrid zone

We delineate the hybrid zone between *T. dobrogicus* and *T. carnifex* along its entire length, based on genome-wide nuclear DNA data and a mitochondrial DNA marker. The hybrid zone as defined by the 0.5 contour of the hybrid index runs from south to north, from central Croatia through eastern Slovenia and Austria, up to the south of the Czech Republic (fig. 1). The position of the hybrid zone roughly corresponds to the western boundary of the Pannonian Basin, in line with the species having distinct ecological preferences (Wielstra et al., 2012), with the Danube crested newt being a lowland specialist (Arntzen et al., 1997; Vörös et al., 2016).

The geographical cline analysis reveals clines that are generally concordant, sharing a steep, narrow transition from one species to the other.

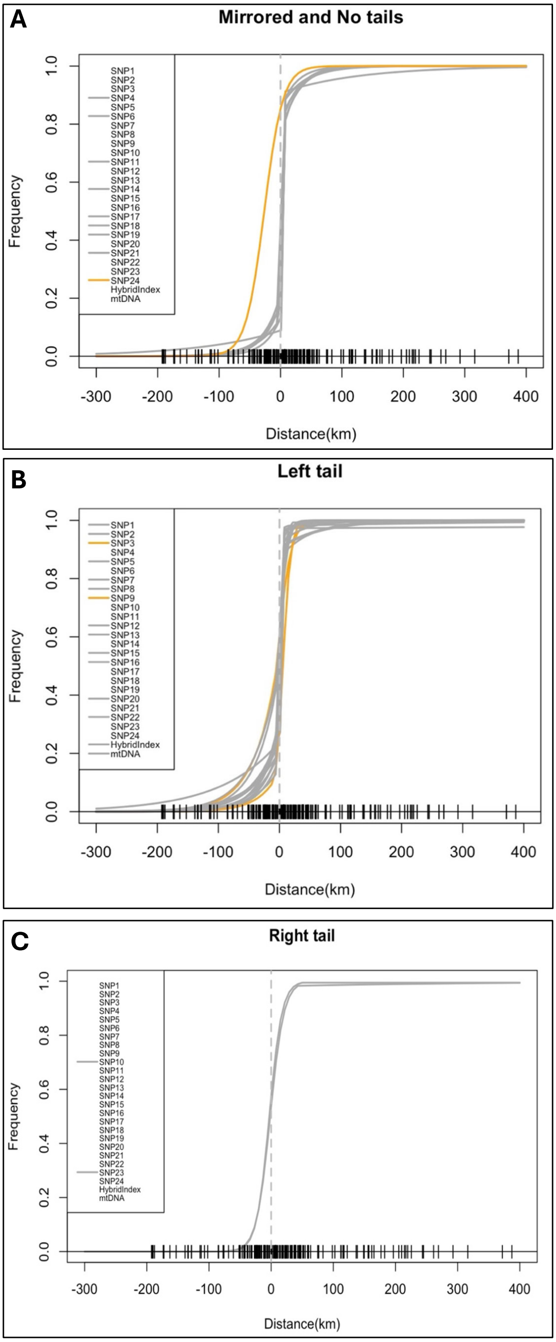


Figure 3. Geographical clines for mtDNA, 24 nuclear DNA SNP markers and the hybrid index for the hybrid zone between the Italian (*Triturus carnifex*) and Danube (*T. dobrogicus*) crested newt. The x-axis shows the distance in km with the black tick marks indicating the position of sampled localities along the transect. The y-axis shows the allele frequency/proportion from 0 (pure *T. carnifex*) to 1 (pure *T. dobrogicus*). The dotted line indicates the inferred centre of the hybrid zone at a distance of 0 km. Panel (A) shows nine nuclear DNA SNP markers for which no separate left and right tails were fitted, panel (B) 13 nuclear DNA SNP markers, as well as the mtDNA and hybrid index cline, that show a shallower slope of the left tail compared to the right tail, and panel (C) two nuclear DNA SNP markers that show a shallower slope of the right tail compared to the left tail (although visually this is hard to discern). Three nuclear DNA SNP markers in orange have a centre for which the confidence interval does not overlap with that of the hybrid index.

This sharp geographical turnover is in line with a tension zone, maintained by a balance between dispersal and selection against hybrids (Barton and Hewitt, 1985; Barton and Gale, 1993). The steepness of the clines observed suggests strong reproductive barriers and limited gene flow between the two species, which is reflected by the estimate of a relatively strong effective selection against hybrids of $s* = 0.018$. Compared to other amphibian systems, our findings fall between those showing weaker effective selection, such as the toad species *Bufo bufo* and *B. spinosus* (as low as 0.0022 in the northern transect sampled, but a comparable 0.020 in the southern transect; van Riemsdijk et al., 2023) and the two subspecies comprising the golden-striped salamander *Chioglossa lusitanica* (0.0063; Sequeira et al., 2022), which exhibit wider and often displaced clines, and those under stronger selection, such as *Ommatotriton* newts (0.14; Kalaentzis et al., 2023) and fire-bellied toads *Bombina* (0.17; Szymura and Barton, 1986), where clines are coincident and steep. Our estimate is similar to that reported in another *Triturus* hybrid zone involving *T. anatolicus* and *T. ivanbureschi* (0.011; Wielstra et al., 2017b).

Hybrid zone dynamics

In the south of the hybrid zone genetic admixture is confined to close to the 0.5 hybrid index contour, in line with a locally stable hybrid zone. Yet, we notice that in the north of the hybrid zone genetic admixture is geographically more extensive and runs westwards from the 0.5 hybrid index contour (Fig. 1). Here, individuals regularly, and in some localities exclusively, carry *T. dobrogicus* mtDNA. However, from the nuclear DNA perspective these individuals are predominantly *T. carnifex*, albeit genetically admixed with *T. dobrogicus*. This mtDNA introgression from *T. dobrogicus* into *T. carnifex* is in line with previous studies (Mikulíček et al., 2012; Mačát et al., 2019; Wielstra et al., 2021).

Overall, the geographical clines are coincident, mostly exhibiting overlapping cline centres, and there is no obvious bias in the few markers for which the clines are displaced (fig. 3). However, we do observe an asymmetry in the slopes of the tails of the geographical clines: the majority of nuclear DNA SNP markers, the mtDNA marker and the hybrid index (which is based on all nuclear DNA SNP markers together) exhibit shallower left tail slopes. This pattern reflects that there is more introgression to the west of the hybrid zone.

The asymmetric introgression observed would be expected under a scenario of local hybrid zone movement, with *T. carnifex* displacing *T. dobrogicus* at the northern side of the hybrid zone (Currat et al., 2008; Petit and Excoffier, 2009; Wielstra, 2019). Alternatively, an expanding population of *T. carnifex*, already genetically admixed with *T. dobrogicus*, could have transported *T. dobrogicus* alleles here via ‘allele surfing’ (Klopfstein et al., 2006; Currat et al., 2008), without species displacement. We cannot distinguish between these two different scenarios and they are not mutually exclusive. Species distribution modelling suggests neither species was present in the relevant region at the Last Glacial Maximum (Wielstra et al., 2013, 2021), so it depends on the extent to which *T. dobrogicus* had already colonized the area postglacially, as *T. carnifex* expanded its range northwards around the Alps. Future work could explore if *T. dobrogicus* alleles have moved even further west, into an area that is clearly outside of the ecological amplitude of *T. dobrogicus* (i.e. into foothills and mountains, beyond the lowland to which *T. dobrogicus* is confined).

MtDNA structure and a ‘cryptic pseudogene’

The geographical genetic structure of mtDNA, based on the ND4 gene, in both *Triturus* species reveals a complex evolutionary history, close to the present-day hybrid zone. While *T. dobrogicus* mtDNA has been studied intensively before (Vörös et al., 2016; Wielstra et al., 2016), the distinct Balkan mtDNA clade of *T. carnifex* has been relatively understudied (Canestrelli et al., 2012; Wielstra et al., 2021). Therefore, the discovery of many new *T. carnifex* mtDNA haplotypes is not surprising. However, we also uncovered several new *T. dobrogicus* mtDNA haplotypes. The rich genetic diversity observed suggests that both species had a glacial refugium in the neighbourhood of the hybrid zone (see also Vörös et al., 2016). This fits with the inferred long-term hybrid zone stability in the area (see above).

Furthermore, two highly distinct haplotypes (T-?-01 and T-?-02) stand out that are found in *T. dobrogicus* (localities 148, 158 and 168 in fig. 1). These cluster with the crested newt species *T. carnifex*, *T. cristatus*, *T. dobrogicus* and *T. macedonicus*, but bootstrap support is not particularly high (fig. 2). At first glance this fits the scenario of a 'ghost lineage', with these haplotypes reflecting mtDNA captured from a distinct, now extinct, *Triturus* lineage. The haplotypes do not bear the hallmarks of a pseudogene: there are no stop codons or frameshift mutations (and only 3–4 non-synonymous substitutions) and their GC content does not deviate from that of other haplotypes. However, from the perspective of an independent mtDNA marker, ND2, the affected individuals do not appear genetically distinct at all (fig. A2 in the Appendix). Therefore, we consider them 'cryptic pseudogenes', i.e. pseudogenes that are not readily distinguishable from functional mitochondrial DNA sequences.

Hybrid zone analysis with KASP genotyping

Our study illustrates that KASP genotyping provides a cost-effective and scalable way to target a limited set of species-diagnostic bi-allelic SNPs, and rapidly screen a large number of individuals, across many sites around a hybrid zone between two species. Evidently, this provides sufficient genetic resolution to unveil general aspects of the position, shape and dynamics of the *T. carnifex*–*T. dobrogicus* hybrid zone. However, if one is interested in more intricate patterns that require orders of magnitude more markers, such as subtle patterns of introgression, then other techniques become more appropriate. For *Triturus* we would recommend the target capture approach NewtCap, with which approx. 7000 genes can be sequenced (de Visser et al., 2025a). An admittedly rough estimate is that costs per individual and the time spent in the laboratory and for analysis would increase tenfold compared to those required for the small panel of markers targeted in the present study.

Another consideration is that KASP genotyping is not geared towards studying admixture between more than two species. *Triturus cristatus* meets both *T. carnifex* and *T. dobrogicus* in hybrid zones (Mikulíček et al., 2012; Arntzen et al., 2014; Wielstra et al., 2014; Mačát et al., 2019). To minimize the influence of genetic admixture with *T. cristatus*, we sampled away from the hybrid zones with that species and excluded samples that carried *T. cristatus* mtDNA. Additionally, the samples from the Czech Republic, taken from the area where the *T. cristatus* range approaches the northern side of the hybrid zone between *T. carnifex* and *T. dobrogicus*, do not show genetic admixture with *T. cristatus* based on microsatellites (Mikulíček et al., 2012; Mikulíček, unpublished data). Therefore, the influence of admixture with *T. cristatus* in our dataset, if any, should be minimal. While separate SNP panels, one for each possible pairwise comparison, could be designed to study the three-way *Triturus* hybrid zone, this would defeat the purpose of using KASP genotyping, which is supposed to be efficient and cost-effective. Rather, for a future study aiming to unravel the triad hybridisation in *Triturus*, we again recommend NewtCap (de Visser et al., 2025a).

Supplementary materials. Data is available on https://doi.org/10.1163/15685381-bja10249 under Supplementary Materials.

Acknowledgements. Arilah J. van Eden and Anagnostis Theodoropoulos contributed equally to this work. G. Babocsay, D. Cogalniceanu, M. García-París, B. Halpern, D.

Jandzik, I. Kiss, J. B. Kiss, K. Koller Šarić, T. Koren, T. Kovács, D. Kujundžić, B. Lauš, L. Libusová, Đ. Majetić, L. Mikuskovics, J. Nédli, L. Petrokov, V. Salehi-Vogler, L. Somai, G. Szövényi, Zs. Török and J. Vers. helped during fieldwork. Fam. Esterhazy, Ettmar, Hauerhof, Hudak, Kühberger and Lhotka allowed us to sample on their properties. Permits for sampling were provided by the provincial governments of Lower Austria (Nr. RU5-BE-1847/001-2022) and Burgenland (Nr. A4/NR.AB-10117-8-2022) for Austria; the Ministry of Environmental Protection and Green Transition (UP/I-352- 04/22-08/75, and UP/I-352-04/24-08/87, URBROJ 517-10-1-1-22-3 and 571-10-1-2-24-2) for Croatia; the Nature Conservation Agency of the Czech Republic (7166/SOPK/19; 05153/SOPK/20; 05656/SOPK/22) and the Regional Authority of the South Moravian Region (JMK076894/22/OŽP) for the Czech Republic; the Ministry of Environment and Water (now Ministry of Agriculture) (KJHF-848/3/2005) for Hungary; the Ministry of Environment of the Slovak Republic (519/2022 6.3) for Slovakia; and the Slovenian Environmental Agency (35606-84/2022-2550-10) for Slovenia. L. Jeřábková coordinated financial and permit support from the Nature Conservation Agency of the Czech Republic. D. Stankovic and M. Vek were supported by the Slovenian Research and Innovation Agency (P1-0255).

References

Akima, H., Gebhardt, A., Petzoldt, T., Maechler, M. (2025): Akima: interpolation of irregularly spaced data. R package version 0.6-3.4. R Foundation for Statistical Computing, Vienna.

Arntzen, J.W., Wallis, G.P. (1991): Restricted gene flow in a moving hybrid zone of the newts *Triturus cristatus* and *T. marmoratus* in western France. Evolution **45**: 805-826.

Arntzen, J.W., Bugter, R.J.F., Cogalniceanu, D., Wallis, G.P. (1997): The distribution and conservation status of the Danube crested newt, *Triturus dobrogicus*. Amphibia-Reptilia **18**: 133-142.

Arntzen, J.W., Jehle, R., Bardakci, F., Burke, T., Wallis, G.P. (2009): Asymmetric viability of reciprocal-cross hybrids between crested and marbled newts (*Triturus cristatus* and *T. marmoratus*). Evolution **63**: 1191-1202.

Arntzen, J.W., Wielstra, B., Wallis, G.P. (2014): The modality of nine *Triturus* newt hybrid zones, assessed with nuclear, mitochondrial and morphological data. Biol. J. Linn. Soc. **113**: 604-622.

Arntzen, J.W., Jehle, R., Wielstra, B. (2021a): Genetic and morphological data demonstrate hybridization and backcrossing in a pair of salamanders at the far end of the speciation continuum. Evol. Appl. **14**: 2784-2793.

Arntzen, J.W., López-Delgado, J., van Riemsdijk, I., Wielstra, B. (2021b): A genomic footprint of a moving hybrid zone in marbled newts. J. Zool. Syst. Evol. Res. **59**: 459-465.

Babik, W., Branicki, W., Crnobrnja-Isailovic, J., Cogalniceanu, D., Sas, I., Olgun, K., Poyarkov, N.A., Garcia-Paris, M., Arntzen, J.W. (2005): Phylogeography of two European newt species – Discordance between mtDNA and morphology. Mol. Ecol. **14**: 2475-2491.

Barton, N.H., Hewitt, G.M. (1985): Analysis of hybrid zones. Annu. Rev. Ecol. Syst. **16**: 113-148.

Barton, N.H., Hewitt, G.M. (1989): Adaptation, speciation and hybrid zones. Nature **341**: 497-503.

Barton, N.H., Gale, K.S. (1993): Genetic analysis of hybrid zones. In: Hybrid zones and the evolutionary process, p. 13-45. Harrison, R.G., Ed., Oxford University Press, New York.

Barton, N.H. (2001): The role of hybridization in evolution. Mol. Ecol. **10**: 551-568.

Buggs, R.J.A. (2007): Empirical study of hybrid zone movement. Heredity **99**: 301-312.

Canestrelli, D., Salvi, D., Maura, M., Bologna, M.A., Nascetti, G. (2012): One species, three Pleistocene evolutionary histories: phylogeography of the Italian crested newt, *Triturus carnifex*. PLoS ONE **7**: e41754.

Currat, M., Ruedi, M., Petit, R.J., Excoffier, L. (2008): The hidden side of invasions: massive introgression by local genes. Evolution **62**: 1908-1920.

Danecek, P., Auton, A., Abecasis, G., Albers, C.A., Banks, E., DePristo, M.A., Handsaker, R.E., Lunter, G., Marth, G.T., Sherry, S.T., McVean, G., Durbin, R., Genomes Project Analysis, G. (2011): The variant call format and VCFtools. Bioinformatics **27**: 2156-2158.

Danecek, P., Bonfield, J.K., Liddle, J., Marshall, J., Ohan, V., Pollard, M.O., Whitwham, A., Keane, T., McCarthy, S.A., Davies, R.M., Li, H. (2021): Twelve years of SAMtools and BCFtools. GigaScience **10**: giab008.

de Visser, M.C., France, J., McCartney-Melstad, E., Bucciarelli, G.M., Theodoropoulos, A., Shaffer, H.B., Wielstra, B. (2025a): NewtCap: An efficient target capture approach to boost genomic studies in Salamandridae (true salamanders and newts). Ecol. Evol. **15**: e71835.

de Visser, M.C., France, J., Paulouskaya, O., Brown, T., Fahrbach, M., van der Ploeg, C., Wielstra, B. (2025b): Conserved gene content and unique phylogenetic history characterize the 'bloopergene' underlying *Triturus*' balanced lethal system bioRxiv: 2024.10.25.620277.

Derryberry, E.P., Derryberry, G.E., Maley, J.M., Brumfield, R.T. (2014): HZAR: hybrid zone analysis using an R software package. Mol. Ecol. Resour. **14**: 652-663.

Espregueira Themudo, G., Nieman, A., Arntzen, J.W. (2012): Is dispersal guided by the environment? A comparison of interspecific gene flow estimates among differentiated regions of a newt hybrid zone. Mol. Ecol. **21**: 5324-5335.

France, J., de Visser, M., Arntzen, J.W., Babik, W., Cvijanović, M., Ivanović, A., Smith, J., Vučić, T., Wielstra, B. (2025): The balanced lethal system in *Triturus* newts originated in an instantaneous speciation event. bioRxiv: 2024.10.29.620207.

Gubányi, A., Vörös, J., Kiss, I., Dankovics, R., Babocsay, G., Kovács, T., Molnár, P., Somlai, T. (2010): Contribution to knowledge of the distribution of Italian Crested Newt (*Triturus carnifex*), Danube Crested

New (*T. dobrogicus*) and European Fire-bellied Toad (*Bombina bombina*) in Hungary. Állattani Közlemények **95**: 253-279.

Hewitt, G.M. (1988): Hybrid zones – natural laboratories for evolutionary studies. Trends Ecol. Evol. **3**: 158-167.

Hewitt, G.M. (2011): Quaternary phylogeography: the roots of hybrid zones. Genetica **139**: 617-638.

Hoang, D.T., Chernomor, O., von Haeseler, A., Minh, B.Q., Vinh, L.S. (2017): UFBoot2: Improving the Ultrafast Bootstrap Approximation. Mol. Biol. Evol. **35**: 518-522.

Kalaentzis, K., Arntzen, J.W., Avcı, A., van den Berg, V., Beukema, W., France, J., Olgun, K., van Riemsdijk, I., Üzüm, N., de Visser, M.C., Wielstra, B. (2023): Hybrid zone analysis confirms cryptic species of banded newt and does not support competitive displacement since secondary contact. Ecol. Evol. **13**: e10442.

Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K.F., von Haeseler, A., Jermiin, L.S. (2017): ModelFinder: fast model selection for accurate phylogenetic estimates. Nature Methods **14**: 587-589.

Klopfstein, S., Currat, M., Excoffier, L. (2006): The fate of mutations surfing on the wave of a range expansion. Mol. Biol. Evol. **23**: 482-490.

Mačát, Z., Rulík, M., Jablonski, D., Reiter, A., Jeřábková, L., Rada, S., Mikulíček, P. (2019): Species-specific habitat preferences do not shape the structure of a crested newt hybrid zone (*Triturus cristatus* x *T. carnifex*). Ecol. Evol. **9**: 12446-12458.

Macholán, M., Munclinger, P., Šugerková, M., Dufková, P., Bímová, B., Božíková, E., Zima, J., Piálek, J., Nürnberger, B. (2007): Genetic analysis of autosomal and X-linked markers across a mouse hybrid zone. Evolution **61**: 746-771.

Mallet, J. (2005): Hybridization as an invasion of the genome. Trends Ecol. Evol. **20**: 229-237.

Meilink, W.R.M., Cvijanović, M., de Visser, M.C., France, J., Ivanović, A., Theodoropoulos, A., Vučić, T., Wielstra, B. (2025): Exposing selection and genetic linkage in the evolutionary enigmatic balanced lethal system in *Triturus* newts. Ecol. Evol. **15**: e71591.

Mikulíček, P., Horák, A., Zavadil, V., Kautman, J., Piálek, J. (2012): Hybridization between three crested newt species (*Triturus cristatus* superspecies) in the Czech Republic and Slovakia: Comparison of nuclear markers and mitochondrial DNA. Folia Zool. **61**: 202-218.

Petit, R.J., Excoffier, L. (2009): Gene flow and species delimitation. Trends Ecol. Evol. **24**: 386-393.

R Core Team (2025): R: A language and environment for statistical computing. Vienna, Austria.

Robinson, J.T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E.S., Getz, G., Mesirov, J.P. (2011): Integrative genomics viewer. Nature Biotechnol. **29**: 24-26.

Semagn, K., Babu, R., Hearne, S., Olsen, M. (2014): Single nucleotide polymorphism genotyping using Kompetitive Allele Specific PCR (KASP): overview of the technology and its application in crop improvement. Mol. Breed. **33**: 1-14.

Sequeira, F., Arntzen, J.W., van Gulik, D., Hajema, S., Diaz, R.L., Wagt, M., van Riemsdijk, I. (2022): Genetic traces of hybrid zone movement across a fragmented habitat. J. Evol. Biol. **35**: 400-412.

Stanković, D., Delić, T. (2012): Morphological evidence for the presence of the Danube Crested Newt, *Triturus dobrogicus* (Kiritzescu, 1903), in Slovenia. Natura Sloveniae **14**: 23-29.

Stankowski, S., Ravinet, M. (2021): Defining the speciation continuum. Evolution **75**: 1256-1273.

Szymura, J.M., Barton, N.H. (1986): Genetic analysis of a hybrid zone between the fire-bellied toads, *Bombina bombina* and *B. variegata*, Near Cracow in Southern Poland. Evolution **40**: 1141-1159.

Trifinopoulos, J., Nguyen, L.-T., von Haeseler, A., Minh, B.Q. (2016): W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. Nucleic Acids Res. **44**: W232-W235.

van Riemsdijk, I., Butlin, R.K., Wielstra, B., Arntzen, J.W. (2019): Testing an hypothesis of hybrid zone movement for toads in France. Mol. Ecol. **28**: 1070-1083.

van Riemsdijk, I., Arntzen, J.W., Bucciarelli, G.M., McCartney-Melstad, E., Rafajlović, M., Scott, P.A., Toffelmier, E., Shaffer, H.B., Wielstra, B. (2023): Two transects reveal remarkable variation in gene flow on opposite ends of a European toad hybrid zone. Heredity **131**: 15-24.

Villesen, P. (2007): FaBox: an online toolbox for fasta sequences. Mol. Ecol. Notes **7**: 965-968.

Vörös, J., Major, Á. (2007): Phylogeograhy and species composition of the two *Bombina* species and the *Triturus cristatus* species complex in the Carpathian Basin. In: The origin of the fauna of the Carpathian Basin, pp. 269-282. Hungarian Natural History Museum. Forró, L., Ed., Budapest.

Vörös, J., Mikulíček, P., Major, Á., Recuero, E., Arntzen, J.W. (2016): Phylogeographic analysis reveals northerly refugia for the riverine amphibian *Triturus dobrogicus* (Caudata: Salamandridae). Biol. J. Linn. Soc. Lond. **119**: 974-991.

Wallace, H., Wallace, B., Badawy, G. (1997): Lampbrush chromosomes and chiasmata of sex-reversed crested newts. Chromosoma **106**: 526-533.

Wielstra, B., Espregueira Themudo, G., Güclü, Ö., Olgun, K., Poyarkov, N.A., Arntzen, J.W. (2010): Cryptic crested newt diversity at the Eurasian transition: the mitochondrial DNA phylogeography of Near Eastern *Triturus* newts. Mol. Phylogenet. Evol. **56**: 888-896.

Wielstra, B., Beukema, W., Arntzen, J.W., Skidmore, A.K., Toxopeus, A.G., Raes, N. (2012): Corresponding mitochondrial DNA and niche divergence for crested newt candidate species. PLoS ONE **7**: e46671.

Wielstra, B., Crnobrnja-Isailović, J., Litvinchuk, S.N., Reijnen, B.T., Skidmore, A.K., Sotiropoulis, K., Toxopeus, A.G., Tzankov, N., Vukov, T., Arntzen, J.W. (2013): Tracing glacial refugia of *Triturus* newts based on mitochondrial DNA phylogeography and species distribution modeling. Front. Zool. **10**: 13.

Wielstra, B., Sillero, N., Vörös, J., Arntzen, J.W. (2014): The distribution of the crested and marbled newt species (Amphibia: Salamandridae: *Triturus*) – an addition to the New Atlas of Amphibians and Reptiles of Europe. Amphibia-Reptilia **35**: 376-381.

Wielstra, B., Vörös, J., Arntzen, J.W. (2016): Is the Danube crested newt *Triturus dobrogicus* polytypic? A review and new nuclear DNA data. Amphibia-Reptilia **37**: 167-177.

Wielstra, B., Burke, T., Butlin, R.K., Arntzen, J.W. (2017a): A signature of dynamic biogeography: enclaves indicate past species replacement. Proc. Roy. Soc. Lond. B: Biol. Sci. **284**: 20172014.

Wielstra, B., Burke, T., Butlin, R.K., Avcı, A., Üzüm, N., Bozkurt, E., Olgun, K., Arntzen, J.W. (2017b): A genomic footprint of hybrid zone movement in crested newts. Evol. Lett. **1**: 93-101.

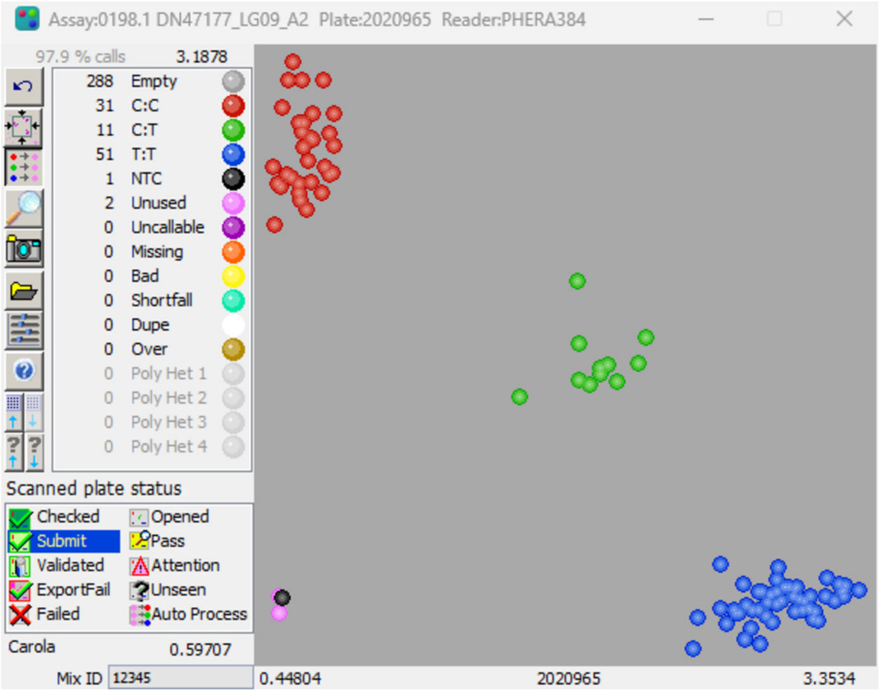
Wielstra, B. (2019): Historical hybrid zone movement: more pervasive than appreciated. J. Biogeogr. **46**: 1300-1305.

Wielstra, B., McCartney-Melstad, E., Arntzen, J.W., Butlin, R.K., Shaffer, H.B. (2019): Phylogenomics of the adaptive radiation of *Triturus* newts supports gradual ecological niche expansion towards an incrementally aquatic lifestyle. Mol. Phylogenet. Evol. **133**: 120-127.

Wielstra, B. (2021): Hybrid zones. Curr. Biol. **31**: R108-R109.

Wielstra, B., Salvi, D., Canestrelli, D. (2021): Genetic divergence across glacial refugia despite interglacial gene flow in a crested newt. Evol. Biol. **48**: 17-26.

Wu, C.-I., Ting, C.-T. (2004): Genes and speciation. Nature Rev. Genet. **5**: 114-122.



Assay:0198.1 DN47177_LG09_A2 Plate:2020965 Reader:PHERA384

97.9 % calls 3.1878

288	Empty	●
31	C:C	●
11	C:T	●
51	T:T	●
1	NTC	●
2	Unused	●
0	Uncallable	●
0	Missing	●
0	Bad	●
0	Shortfall	●
0	Dupe	○
0	Over	●
0	Poly Het 1	●
0	Poly Het 2	●
0	Poly Het 3	●
0	Poly Het 4	●

Carola 0.59707

Mix ID 12345

Figure A1. Example of plotted KASP (Kompetitive Allele-Specific PCR) output. If an individual is a homozygote (i.e. only has only one or the other allele) for a particular SNP, it shows up as either ‘red’ or ‘blue’ because only one signal is emitted. If an individual is a heterozygote (i.e. has both alleles) for a particular SNP, it shows up as ‘green’ because both signals are emitted.

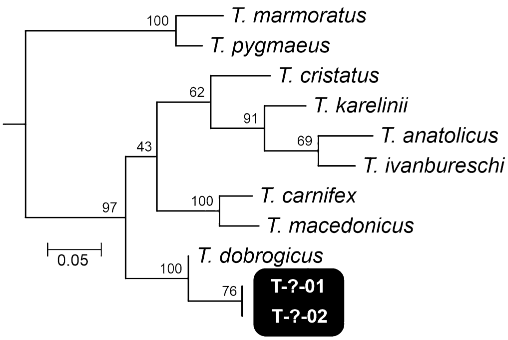


Figure A2. Maximum likelihood phylogeny for *Triturus* newts based on the mtDNA gene ND2; the *Calotriton asper* outgroup is not shown. This shows the phylogenetic position of a highly distinct 'ghost lineage', found introgressed into *T. dobrogicus*.