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Phylogeographic analysis reveals northerly refugia for the riverine amphibian *Triturus dobrogicus* (Caudata: Salamandridae)

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We investigated the recent evolutionary history of the Danube crested newt, *Triturus dobrogicus* through reconstructions of: (1) the number and position of refugia at the last glacial maximum, (2) the role of major central European rivers in pattern of post-glacial dispersal, and (3) the present-day distribution pattern. We analysed sequences of mitochondrial DNA (ND2, 1065 bp) and six microsatellite loci in 363 *T. dobrogicus* individuals from 58 populations covering the range of the species. Our analyses suggested that *T. dobrogicus* survived the last glacial maximum in two separate refugia positioned in northwestern Pannonia and in Southern Pannonia from where its range expanded along the Danube and Tisza Rivers. Our findings also confirmed that rivers played an important role in shaping the evolutionary history of amphibian species in Central Europe. We compared the *T. dobrogicus* range with another lowland amphibian, the fire-bellied toad *Bombina bombina*, using species distribution modelling. In line with these models, the isolated mountains inside Pannonia are occupied not by *B. bombina*, but by its mountain-dwelling sister-species *B. variegata*. However, in contrast to the model, crested newts (including *T. dobrogicus*) are absent from these mountains. We attribute this biogeographical discrepancy to the positioning of the species' refugia at the last glacial maximum. © 2016 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **119**, 974–991.

KEYWORDS: Danube River – microsatellites – mitochondrial DNA – newt – riverine barrier – riverine dispersal – Sava River.

INTRODUCTION

Rivers can play an active role in the evolutionary history of species by influencing population dynamics in different ways, depending on the lifestyle of the organism involved (Robinson, Tockner & Ward, 2002; Lyttle & Poff, 2004). Specifically, rivers can act as barriers to dispersal for terrestrial species, and as dispersal corridors for riverine species, promoting or counteracting geographical isolation and diversification (Eriksson *et al.*, 2004; Brandley *et al.*, 2010; Vernesi *et al.*, 2015). For amphibians, rivers can be both barriers and dispersal routes. The barrier effect may be particularly pronounced for pond-dwelling species (Martínez-Solano *et al.*, 2004), and for species that typically inhabit small streams (García-París & Jockusch, 1999; Alexandrino, Arntzen &

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Ferrand, 2002). Evolutionary scenarios in which river drainages support dispersal of amphibian species are less frequently documented but some examples are known for semi- or fully aquatic riverine salamanders (Carstens *et al.*, 2005; Jones *et al.*, 2006).

In Europe, the Balkan Peninsula and the Black Sea coast are thought to have been southern refugia for a wide variety of animal and plant species during the Pleistocene glaciations (Hewitt, 1999). From these refugia the species could disperse northwards e.g. following the Danube Basin, eventually reaching their present-day positions (Varga, 2010). The Danube River has been an important factor in the biogeographic history of Europe affecting the distribution patterns and evolutionary histories of many taxa ever since its formation at 5 Mya (Englbrecht *et al.*, 2000; Nagy *et al.*, 2010). Genetic data, however, mostly reflect Holocene events, because the Pleistocene glaciations caused southwards range shifts that were followed by northerly recolonizations, thereby erasing the older phylogeographic information (Hewitt, 1999). For example the European pond turtle (*Emys orbicularis*) colonized parts of Northern and Central Europe via the Danube River from a refugium in the Balkan Peninsula (Fritz *et al.*, 2005). Part of the western lineage of the spadefoot toad (*Pelobates fuscus*) expanded from a glacial refugium located in the Lower Danube system, colonizing the Upper-Danube plain while utilizing the river as a riparian corridor (Crottini *et al.*, 2007). In the northern Pannonian Basin the Danube separates two divergent lineages of *Bombina variegata* (Vörös *et al.*, 2006), while in the Southern Carpathians a *Triturus cristatus* mtDNA clade is restricted to the south of the Danube suggesting long-term isolation by the river (Wielstra, Babik & Arntzen, 2015).

To further investigate the importance of rivers in shaping the evolutionary history of European amphibians we here focus on the Danube crested newt, *Triturus dobrogicus* (Kiritzescu, 1903). *Triturus dobrogicus* inhabits vegetated and unspoiled stagnant waters, ponds, lakes, floodplains and marshes, and may also be present in rivers and their associated oxbows (Arntzen *et al.*, 1997) and is the most aquatic and most riverine member of its genus (Arntzen, 2003). *Triturus dobrogicus* has a range restricted to the floodplains of the Danube, Tisza, Drava and Sava rivers on the Pannonian and Dobrogean plains, from the Vienna basin to the Black Sea. The distribution is narrowed or interrupted at the Iron Gate where the Danube runs through the southern Carpathians. It also has an isolated population in the Dnepr Delta at the northern coast of the Black Sea (see insert on Fig. 1) (Arntzen *et al.*, 1997; Litvinchuk, 2005), and at Lake Durankulak in Bulgaria, where the existence of a past population is supported by a few museum specimens with unknown collection date (Ghergehel & Iftime, 2009).

The evolutionary history of *T. dobrogicus* has previously been discussed in several contexts. Arntzen, Themudo & Wielstra (2007) identified the vicariant events that differentiated *T. dobrogicus* from other crested newt species. Vörös & Arntzen (2010) used 40 enzyme loci to study the genetic differentiation of *T. dobrogicus* populations along the Sava, Tisza and Danube rivers. They hypothesized that southern Pannonia was a refugium for the *T. dobrogicus* during the last glacial maximum. Wielstra *et al.* (2013a) applied mtDNA phylogeography and species distribution modelling to reconstruct the position of glacial refugia and post-glacial dispersal of the species, but failed to reconstruct suitable conditions during the last glacial maximum for *T. dobrogicus*.

We here use mitochondrial and nuclear microsatellite genetic markers, plus species distribution models to set a scenario for the recent historical biogeography of a riverine species, *T. dobrogicus*. In particular we aim to:

1. Reconstruct the position of one or more refugia at the last glacial maximum (LGM). We specifically tested for the presence of refugia (1) previously hypothesized in southern Pannonia along the Sava River (Vörös & Arntzen (2010), (2) at the north- and northwestern shores of the Black Sea region, which long have been recognized as glacial refugia for a variety of plant and animal species (Hewitt, 1999) and (3) the Middle- and Upper-Danube section in the Pannonian plains, where several northerly refugia have recently been identified (Schmitt & Varga, 2012).
2. Understand the patterns of dispersal from the LGM refugia to form the present-day range. We hypothesized that the *T. dobrogicus* used the major central European rivers and associated lowlands as dispersal corridors.
3. Further deepen the understanding of recent evolutionary history of *T. dobrogicus* by comparing our results with published phylogeographic data for the fire-bellied toad, *Bombina bombina*, a species with similar ecological characteristics and distribution in Central Europe and the northern Balkans. While both *T. dobrogicus* and *B. bombina* occupy the lowlands associated to the Danube River and its tributaries, their parapatric congeners (*T. carnifex*, *T. cristatus*, *T. ivanbureschi* and *T. macedonicus* and *B. variegata*) live in the more mountainous areas. We compare present-day distribution of both species with current potential distribution models and seek to link these with the observed patterns of genetic

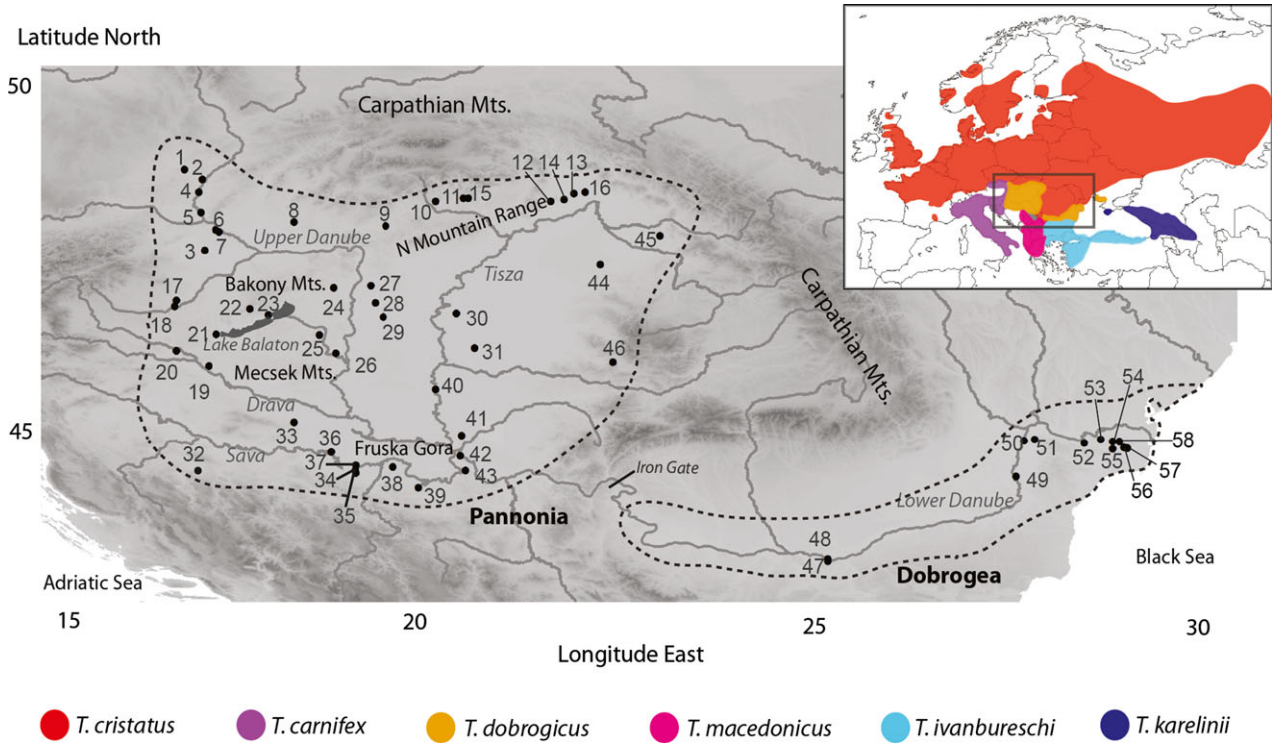


Figure 1. Distribution of the Danube crested newt, *Triturus dobrogicus*, over Central Europe, shown by dotted line (schematic). Note that the species' range is composed of a western, Pannonian part and an eastern, Lower Danube Plain part that are connected by the Danube River through the southern Carpathian Mountains at the Iron Gate. The isolated population at the Dnepr Delta is not shown. Numbered dots represent sampled populations in line with Table 1. The insert shows distribution of the five congeneric *Triturus* species, following Wielstra *et al.* (2013b).

variation and with the phylogeographic interpretations that are either our own (*T. dobrogicus*, present paper) or published [*B. bombina* (Fijarczyk *et al.*, 2011; Pabijan *et al.*, 2013)].

MATERIAL AND METHODS

Sampling, mtDNA sequencing and genotyping of microsatellites

We collected tissue samples from 363 individuals in 58 populations covering the documented range of *T. dobrogicus* (Fig. 1 and Table 1). Total genomic DNA was extracted from ethanol preserved and frozen tissues using Qiagen DNeasy Blood and Tissue kit. We amplified a 1065 base pair (bp) stretch of which 43 bp correspond to the tRNA-Met and the remainder to the NADH dehydrogenase subunit 2 gene (ND2), using the primer pair L3780/H5018 (Babik *et al.*, 2005). PCR reactions were performed as per se methods described by Babik *et al.* (2005). The amplified fragments were sequenced on an Applied Biosystem/Hitachi 3130 Genetic Analyzer. Consensus sequences were compiled using BioEdit version 7.0.9.0 (Hall, 1999) and aligned manually. Sequence data for the outgroup taxa (*T. pygmaeus*, *T. marmoratus*, *T. karelinii*, *T. macedonicus*, *T. carnifex*, *T. cristatus*) were obtained from GenBank (accession numbers HQ697280, GQ258948, GU982516, GU982461, GU982459, GQ258949, respectively).

We analyzed six microsatellite loci that were originally developed for *T. cristatus* (Krupa *et al.*, 2002). PCR reactions were performed following Vörös & Major (2007). Fragment length analysis was run on an Applied Biosystem/Hitachi 3130 Genetic Analyzer under the FragmentAnalysis 36_POP7 protocol. Data collection and scoring were performed with the Software GeneMapper v4.0 (Applied Biosystems).

Analysis of mitochondrial DNA

Estimates of mean nucleotide and haplotype diversities within *T. dobrogicus*, within the two main lineages found and within the four main river sections (Upper–Middle Danube section, Tisza, Sava and Lower Danube) were calculated with DNASP 4.0 (Rozas and Rozas, 1999).

Table 1. Population numbers, locality names, countries, geographic coordinates, sample sizes, haplotype numbers and GenBank accession numbers for mitochondrial DNA along with sample sizes for microsatellite analyses

Population	Locality	Country	Longitude east	Latitude north	MtDNA sample size	mtDNA haplotypes	Genbank accession codes	Nr. individuals msats
1	Nové Mlýny	Czech Republic	16.73	48.85	1	H2	JN831518	–
2	Lanžhot	Czech Republic	16.97	48.72	3	H2, H7	JN831511–JN831513	5
3	Tadten	Austria	17.00	47.77	2	H6, H2	JN831543–JN831544	5
4	Drösing	Austria	16.92	48.55	3	H2, H7	JN831547–JN831549	6
5	Devínske Jazero	Slovakia	16.95	48.28	4	H2, H7	JN831490–JN831493	21
6	Bratislava-Rusovce	Slovakia	17.15	48.05	2	H2, H8	JN831525–JN831526	23
7	Bratislava-Čunovo	Slovakia	17.20	48.02	2	H2, H14	JN831467–JN831468	–
8	Torozlín	Slovakia	18.20	48.15	1	H2	JN831536	–
9	Malý Kiarov	Slovakia	19.43	48.10	3	H2, H21	JN831533–JN831553	–
10	Veľký Blh	Slovakia	20.10	48.43	1	H24	JN831537	–
11	Domica	Slovakia	20.47	48.47	1	H2	JN831492	–
12	Svätá Mária	Slovakia	21.82	48.45	3	H28	JN831527–JN831529	–
13	Beša	Slovakia	21.95	48.53	1	H29	JN831466	–
14	Veškovce	Slovakia	22.10	48.55	2	H28	JN831538–JN831539	–
15	Aggtelek, Vörös-tó	Hungary	20.54	48.47	3	H23, H20	JN831462–JN831463	–
16	Sátoraljaújhely	Hungary	21.64	48.43	3	H28	JN831540–JN831542	–
17	Egyházasrádóc	Hungary	16.62	47.10	2	H13	JN831486, JN831524	–
18	Körmend	Hungary	16.60	47.02	1	H27	JN831550	–
19	Dráva, Lankóci-erdő	Hungary	17.06	46.23	3	H35, H2	JN831494–JN831496	8
20	Letenye, Murcsák, Mura	Hungary	16.62	46.43	3	H34, H33, H35	JN831514–JN831516	8
21	Kis-Balaton	Hungary	17.16	46.65	3	H35, H2	JN831500–JN831502	10
22	Öcs, Nagy-tó	Hungary	17.61	46.90	2	H12, H3	JN831519, JN83152	4
23	Tihany, Külső-tó	Hungary	17.86	46.91	2	H2	JN831509, JN831510	6
24	Baracska	Hungary	18.73	47.27	1	H2	JN831465	–
25	Tolna, Kapszeg-tó	Hungary	18.76	46.40	3	H9, H2, H17	JN831530–JN831532	8
26	Matild-tó, Dunaföldvár	Hungary	18.54	46.64	1	H2	JN831517	–
27	Felső-Babád	Hungary	19.23	47.30	3	H2	JN831521–JN831523	9

Table 1. *Continued*

Population	Locality	Country	Longitude east	Latitude north	MtDNA sample size	mtDNA haplotypes	Genbank accession codes	Nr. individuals msats
28	Kunpeszér	Hungary	19.29	47.07	4	H4, H2	JN831503–JN831506	17
29	Fülöpháza	Hungary	19.40	46.88	3	H2	JN831497–JN831499	18
30	Öcsöd	Hungary	20.38	46.93	1	H25	JN831546	6
31	Kardoskút, Fehér-tó	Hungary	20.62	46.47	2	H27	JN831507–JN831508	14
32	Dugo Selo	Croatia	16.25	45.82	1	H18	JN831580	–
33	Podgorač	Croatia	18.20	45.48	2	H23, H35	JN831560, JN831563	–
34	Županja	Croatia	18.70	45.08	3	H35	JN831565–JN831567	5
35	Jamena	Serbia	19.03	44.90	4	H18, H19	JN831574, JN831576–JN831578	6
36	Vršani	Bosnia-Hercegovina	19.02	44.83	3	H28, H35	JN831545, JN831561–JN831562	–
37	Donja Ĉadjavica	Bosnia-Hercegovina	19.03	44.80	5	H35	JN831570–JN831573, JN831575	–
38	Glušci	Serbia	19.53	44.88	3	H36, H35	JN831555–JN831557	6
39	Debrc	Serbia	19.87	44.60	2	H35	JN831558–JN831559	–
40	Senta	Serbia	20.10	45.92	3	H27, H22, H23	JN831551–JN831552, JN831568	7
41	Ečka	Serbia	20.45	45.30	3	H23, H3, H27	JN831457–JN831459	6
42	Opovo	Serbia	20.43	45.03	2	H1, H26	JN831453, JN831454	6
43	Beograd	Serbia	20.50	44.83	3	H5, H2	JN831453–JN831455	4
44	Pişcolt	Romania	22.30	47.58	–	–	–	–
45	Băbeşti	Romania	23.10	47.97	2	H27	JN831584–JN831585	
46	Virfuri	Romania	22.47	46.28	1	H2	JN831564	–
47	Zimnicea	Romania	25.35	43.65	2	H27	JN831456–JN831561	2
48	Svistov	Romania	25.35	43.62	2	H27, H1	JN831460–JN831469	2
49	Giurgheni	Romania	27.87	44.75	4	H2, H1, H31	JN831586–JN831589	–
50	Island S of Braila	Romania	27.98	45.23	1	H23	JN831559	10
51	Măcin - Braila	Romania	28.12	45.25	8	H23, H2, H27, H30	JN831590–JN831597	–
52	Danube Delta, Şontea	Romania	29.01	45.25	3	H31, H22, H27	JN831470–JN831472	12

Table 1. *Continued*

Population	Locality	Country	Longitude east	Latitude north	MtDNA sample size	mtDNA haplotypes	Genbank accession codes	Nr. individuals msats
53	Danube Delta, Mila23	Romania	28.79	45.20	2	H30, H31	JN831473–JN831474	–
54	Danube Delta, Razboinita	Romania	29.17	45.22	3	H11, H31	JN831475–JN831477	19
55	Danube Delta, Mahmudia	Romania	29.17	45.12	3	H35, H15, H18	JN831478–JN831480	9
56	Danube Delta, Caraorman1	Romania	29.37	45.13	2	H10, H11	JN831481–JN831482	18
57	Danube Delta, Caraorman2	Romania	29.32	45.14	3	H37, H16, H23	JN831483–JN831485	18
58	Danube Delta, Olguţa	Romania	29.26	45.22	3	H11, H38	JN831487–JN831489	15

We used PartitionFinder v1.1.1 (Lanfear *et al.*, 2012) to determine the optimal partitioning strategy and models of nucleotide substitution for the mtDNA sequences, which resulted in three partitions: the tRNA-Met fragment plus second codon positions (HKY + I), first codon positions (HKY + G) and third codon positions (TrN + G). A gene tree was built from mtDNA haplotypes with BEAST v1.8.1 (Drummond *et al.*, 2012), under the uncorrelated lognormal relaxed clock model and a Birth and Death tree prior, with 200 million generations, sampling every 20 000 generations and discarding the initial 25% of trees as burn-in. The analyses were time-calibrated with reference to the fossil record, setting a lognormal prior at the root of the tree, considering 24 Myr as the minimum age of the most recent common ancestor of *Triturus* (Arntzen *et al.*, 2015), and by setting a prior distribution with a lognormal distribution and a standard deviation of 1 Myr (Wiens, Sparreboom & Arntzen, 2011). Parameter estimates were inspected to check for convergence and adequate Effective Sample Sizes (ESSs, larger than 200) in Tracer v1.6 (Rambaut *et al.*, 2014). All parameters in the analysis reached stable results and ESSs values larger than 200.

Secondly, a haplotype network was built using the median-joining algorithm implemented in the software PopArt (French *et al.*, 2014). We arranged the haplotypes in the network using the information gained by the individual-based, spatially explicit model of BAPS 6.0 (Corander, Siren & Arjas, 2008; Cheng *et al.*, 2013) in five independent runs with K varying from 1 to 57 (the number of populations studied).

To learn about the recent demographic history of *T. dobrogicus* lineages, mismatch distribution and raggedness index (r) (Harpending, 1994) were calculated under a model of sudden expansion with 1000 bootstrap replicates using Arlequin 3.5 (Excoffier & Lischer, 2010). This analysis compares the frequency distribution of pairwise differences between haplotypes in a population with that expected by simulated models fitted to the data. The distribution is unimodal when a lineage has undergone recent population expansion and multimodal in case of constant population size or geographical subdivision during its evolution (Marjoram & Donnelly, 1994). Additionally Fu's F_s test (Fu, 1997) and Tajima's D statistic (Tajima, 1989) were used to test for the possibility of recent population size changes. Significant negative values of these tests can indicate signature of population expansion. Both tests were performed with Arlequin 3.5 (Excoffier & Lischer, 2010) using 10 000 simulations.

Analysis of microsatellites

Because initial results indicated the potential for interspecific hybridization affecting the results with nuclear markers, populations situated along the northern and north-eastern fringe of the *T. dobrogicus* range were omitted and analyses of

microsatellites were run with 31 populations (Table 1). To calculate the total number of alleles, number of private alleles (per population), inbreeding coefficient (F_{IS}) and expected (H_E) and observed (H_O) heterozygosity, we used the software GenAlEx 6 (Peakall & Smouse, 2006) and included only the 28 populations with at least five individuals. Allelic richness (AR), which enables a direct comparison of populations with unequal sample sizes, was calculated using FStat 2.9.3 (Goudet, 2001). Null alleles and scoring errors were estimated with Micro-Checker 2.2.3 (Van Oosterhout *et al.*, 2004) in 15 populations with $N > 12$. The 95% confidence intervals with Bonferroni correction were used to calculate the expected frequency of homozygotes. Deviations from the Hardy–Weinberg equilibrium for each population and locus and across all loci per population were tested by the score test (*U*-test for heterozygote deficiency) using the software GenePop 3.3 (Raymond & Rousset, 1995). The same software was used to estimate linkage disequilibria (LD) over all samples. LD within populations was not calculated because of small sample sizes. Population differentiation was measured using F_{ST} statistics (Weir & Cockerham, 1984), corrected for the presence of null alleles with the program FreeNA (Chapuis & Estoup, 2007). The 95% confidence intervals for the global (over all loci and populations) and interpopulation F_{ST} values were estimated using 10 000 replicates.

The level of genetic structure of the *T. dobrogicus* populations was estimated using a Bayesian approach implemented in the program Structure 2.3.3 (Pritchard, Stephens & Donnelly, 2000). Posterior probabilities for *K* were estimated using the *ad hoc* statistic ΔK (Evanno, Regnaut & Goudet, 2005), assuming uniform prior values and *K* ranging from 1 to 32. Admixture and uncorrelated allele models were applied. We chose these models because we assumed that there was gene flow among *T. dobrogicus* populations, and we expected allele frequencies to vary among populations across the species range. The analyses were based on five runs of 10^6 iterations, following a burn-in of 10^5 iterations.

As the presence of null alleles – through the excess of perceived homozygotes – can lead to deviations from Hardy–Weinberg expectations (Pemberton *et al.*, 1995), in addition we used the software Geneland (Guillot, Mortier & Estoup, 2005), which offers the option to account for the presence of null alleles, to infer spatial genetic structure of our populations. Ten independent runs of 10^5 iterations (thinning every 100 simulations) were conducted and the first 10% discarded as burn-in. The spatially explicit model was run assuming uncorrelated allele frequencies.

We applied approximate bayesian computation (ABC) using microsatellite data from the 11 selected populations (populations 5, 6, 21, 28, 29, 31, 52, 54, 56, 57, 60) with sample sizes of ten or more to estimate the relative likelihood of possible scenarios for the *T. dobrogicus* population histories. We used software diyABC v2.0.4 (Cornuet *et al.*, 2008), which compares observed values with simulated datasets in reference tables associated with predetermined scenarios. We grouped populations according to Geneland results plus the geographic isolation of Pannonia and Dobrogea (i.e. two genetic groups, one of them further divided in two geographic groups, for a total of three groups). We first generated six different scenarios considering alternative split options between the three *a priori* defined population groups (Fig. 2): (1) single refugium; (2) two refugia, one for each geographic group (Pannonia and Dobrogea); (3) two refugia, one for each genetic group (western and eastern) as inferred by Geneland; (4) three refugia for the genetic plus geographic groups; (5) up to three refugia, one for each genetic group (western and eastern), plus one more recent split event between both geographic areas (Pannonia and Dobrogea) within the eastern genetic group, keeping southern Pannonia as ancestral; and (6) same as the previous scenario with the Danube Delta as the ancestral population (Fig. 2). As suggested in the diyABC software manual we performed some preliminary runs in order to adjust some of the

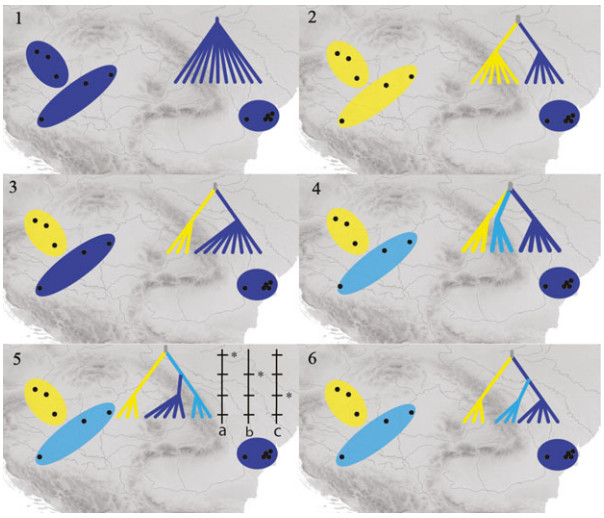


Figure 2. Spatio-temporal representation of six scenarios for *T. dobrogicus* refugia. Note the additional temporal detail of split events in scenarios 5. Scenario numbers are shown in the top left corner of each of the panels. Scenarios 5b and 5c are equal to scenario 5a, but with a more compressed temporal scale. Time events marked with * correspond to the Last Glacial Maximum. Background map corresponds to map in Figure 1.

prior values and then generated a 6×10^6 simulated data set. The scenario with the highest likelihood (scenario 5) was selected to further test the temporal framework of the different split events. To do this we constrained coalescence time to not exceed the start of the last glacial maximum, for three different scenarios: (5a) the split between both genetic groups occurred no later than the start of the last glacial maximum; (5b) the split between geographic groups within the eastern genetic group occurred no later than the start of the last glacial maximum; (5c) the split within each of the three defined groups occurred no later than the start of the last glacial maximum (Fig. 2). We assessed the fit of the model to the data using principal components analysis (PCA) and compared the relative likelihoods of the scenarios by the logistic regression approach, with a 5% subset of the closest simulated data.

Present-day distribution modelling

The ranges of *T. dobrogicus* and *B. bombina* in Central Europe and the northern Balkans are bounded by mountains that are occupied by closely related congeners (*T. carnifex*, *T. cristatus*, *T. ivanbureschi* and *T. macedonicus* for the newt, and *Bombina variegata* for the toad). In both systems the ranges are essentially parapatric (Pabijan *et al.*, 2013; Arntzen, Wielstra & Wallis, 2014), and we modelled the species distributions by contrasting congeners with presence-only data as the dependent variable, and altitude and relief as explanatory variables. We refrained from reconstructing the distribution models of the past, because we have no data to support the past parapatry of species ranges.

For the area of interest, 2000 locality data points were available for *Triturus* and 4342 for *Bombina* (Sillero *et al.*, 2014; Wielstra *et al.*, 2014). Records duplicated in 30 arc-second grid cells were removed. We retained 1463 records for *Triturus* of which 470 (32.1%) referred to *T. dobrogicus*, and we retained 3109 records for *Bombina* of which 1513 (48.7%) referred to *B. bombina*. In a logistic regression analysis a weight factor was then applied such that the number of records for both groups were equal, without affecting total sample size. Altitude data were obtained from the WorldClim version 1.4 database at 30 arc-seconds (*c.* 1 km) resolution (Hijmans *et al.*, 2005). We calculated the parameter relief from altitude through a set of filter operations in ILWIS 3.8 (ILWIS, 2008). Both variables were standardized to a mean of zero and standard deviation of unity, to facilitate the interpretation of the results. The accuracy of the species distribution models was assessed with the Area Under the Curve statistic (AUC) (Guisan & Zimmermann, 2000).

RESULTS

Mitochondrial genetic markers

The ND2 mtDNA fragment contained 44 nucleotide positions that were variable, of which 20 were parsimony informative. We observed 38 different haplotypes of which 13 were shared across populations and 25 were found only once. Haplotypes potentially representing other crested newt species were not found. Ten haplotypes were exclusive to Dobrogean populations, 21 haplotypes were exclusive to Pannonian populations, and seven were found in both areas. The most common haplotype (H2) was found in 35 individuals and has a wide distribution (insert to Fig. 3 and Table 1). Overall haplotype diversity (H_d) was 0.896 ± 0.016 (SD) and overall nucleotide diversity (π) was 0.003 ± 0.000 (SD). Haplotype and nucleotide diversity was the highest within the Lower Danube ($H_d = 0.927 \pm 0.020$, $\pi = 0.003 \pm 0.002$), slightly lower along the Sava River ($H_d = 0.709 \pm 0.090$, $\pi = 0.002 \pm 0.001$) and lowest for the Tisza River ($H_d = 0.831 \pm 0.044$, $\pi = 0.001 \pm 0.001$) and Upper–Middle Danube ($H_d = 0.647 \pm 0.075$, $\pi = 0.001 \pm 0.000$). When analysing the two mtDNA lineages, populations associated with the Tisza and Sava River had higher haplotype and nucleotide diversity ($H_d = 0.875 \pm 0.029$, $\pi = 0.003 \pm 0.001$) than populations associated to the Pannonian Danube section ($H_d = 0.770 \pm 0.044$, $\pi = 0.002 \pm 0.001$).

The phylogenetic reconstruction yielded no convincing evidence for geographically bounded monophyletic units within *T. dobrogicus*, other than for *T. dobrogicus* as a whole (Supporting Information, Fig. S1). Age estimates of the most recent ancestor for the extant *T. dobrogicus* mtDNA lineages dates to the Late Pleistocene, with a mean estimated value of 0.99 Ma (highest posterior density distribution, 95% confidence interval 1.44–0.59).

BAPS identified two haplotype groups, one containing populations from the northern Pannonian Danube section (H1–H17), and another including populations from Sava and Tisza regions (H18–H38). The haplotype network constructed with PopArt was more structured in the Sava-Drava, Tisza and Lower Danube region than in the northern Pannonian Danube section (Fig. 3).

The mismatch distributions for both lineages are presented in Figure 3. The model for population expansion was not rejected in any of the lineages (northern Pannonian Danube section, SSD = 0.30, Raggedness index = 0.43; Sava and Tisza regions, SSD = 0.47, Raggedness index = 0.78). Both Tajima's D statistic and Fu's F_s resulted in negative values, and according to Fu's F_s statistic there were significant departures from equilibrium in both lineages (northern Pannonian Danube section, $F = -6.970$,

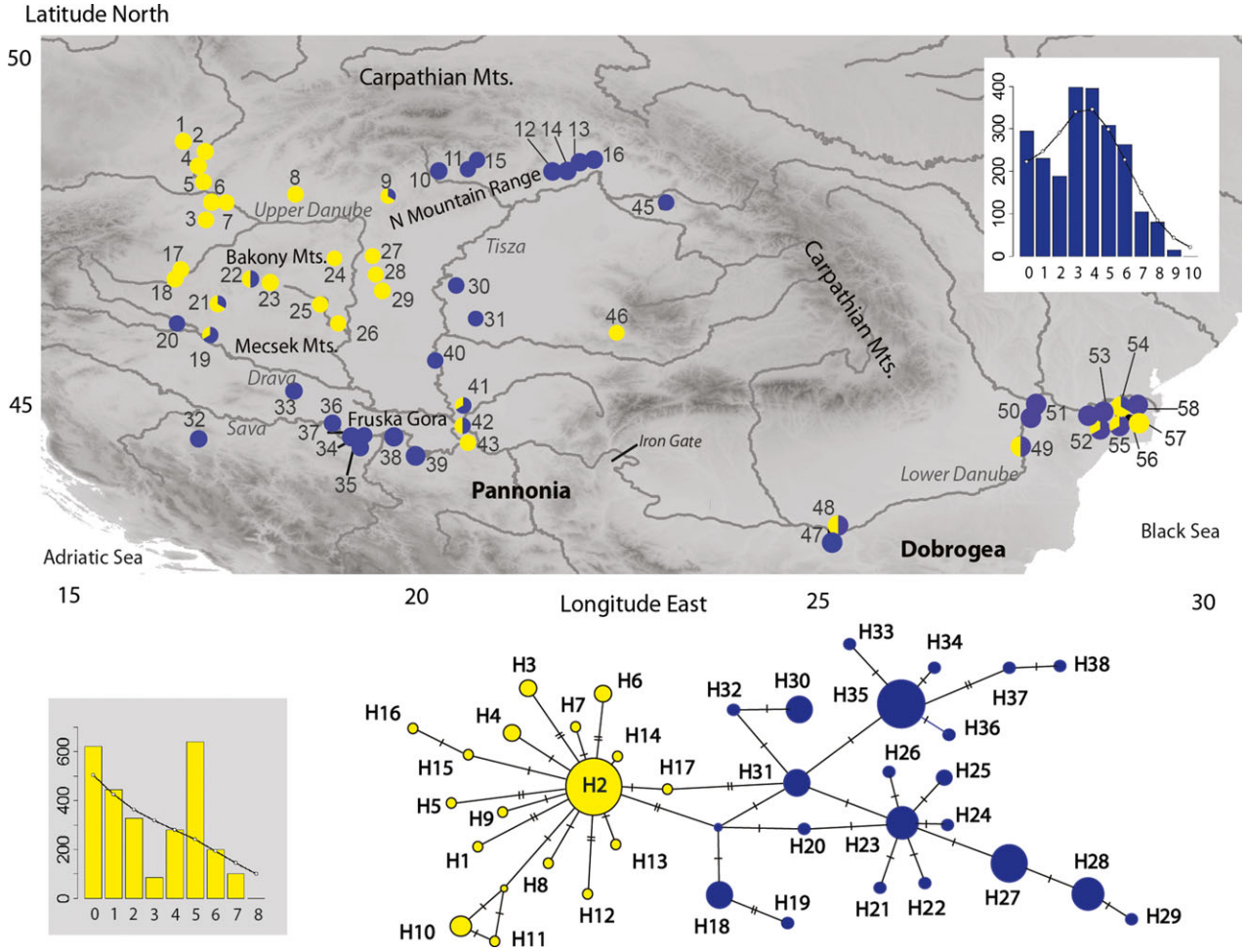


Figure 3. Median-joining haplotype network of 142 mitochondrial DNA sequences. The size of the circles represents haplotype frequency. Each line segment represents a single nucleotide substitution. Yellow circles represent haplotype group 1 (numbers H1–17) and a blue circles show haplotype group 2 (numbers H18–38). Histograms show mismatch distribution for the two lineages. Bars indicate the observed values and black lines show the expected distribution under the sudden expansion model. For sampling details see Table 1.

$P = 0.014$; Sava and Tisza regions, $F = -8.504$, $P = 0.002$) ($P < 0.001$).

Microsatellite markers

The six microsatellite loci employed were all highly polymorphic. The total number of alleles was 274, of which 55 were private. The average number of alleles per locus and population was 9.5 (range = 2.8–16.8). Mean allelic richness per population (based on the minimum sample size of one individual) was 1.85 (range = 1.56–1.94). The observed heterozygosity was slightly lower (average $H_O = 0.67$, range = 0.41–0.85) than the expected heterozygosity ($H_E = 0.80$, range = 0.50–0.89) (Table 2). Pairwise LD values estimated between the six loci were not significant and indicated that the studied microsatellite loci recombine freely (Supporting Information, Table S1). Significant deviations from the Hardy–Weinberg equilibrium were found in 16 out of 28 analyzed populations in locus *Tcri13* (two populations), *Tcri27* (five populations), *Tcri35* (nine populations), *Tcri36* (two populations) and *Tcri46* (12 populations). Inbreeding coefficient (F_{IS}) ranged from −0.2 to 0.3 (Table 2). Negative F_{IS} values were found only in one population (population 50). Null alleles were inferred in five loci (all but *Tri13*) and in all ten analyzed populations. The highest frequency of null alleles was found in the locus *Tcri46* (in ten populations), followed by loci *Tcri35* (six populations), *Tcri27* (five populations), *Tcri36* (one populations) and *Tcri29* (one population). Global F_{ST} with the 95% confidence intervals using

Table 2. Average number of alleles (*NA*), allelic richness (*AR*, calculated based on the minimum sample size of one individual), observed (H_O) and expected (H_E) heterozygosity and inbreeding coefficient (F_{IS}) for 28 *T. dobrogicus* populations

Population	*NA*	*AR*	H_O	H_E	F_{IS}
2	2.8	1.6	0.4	0.5	0.2
3	4.0	1.7	0.6	0.6	0.0
4	5.0	1.8	0.7	0.7	0.1
5	10.2	1.8	0.5	0.8	0.3
6	9.0	1.8	0.5	0.8	0.3
19	8.2	1.9	0.7	0.8	0.2
20	6.8	1.8	0.6	0.7	0.2
21	8.5	1.9	0.6	0.8	0.3
23	6.0	1.8	0.7	0.8	0.1
25	9.2	1.9	0.8	0.8	0.1
27	9.7	1.9	0.7	0.8	0.2
28	15.0	1.9	0.7	0.9	0.2
29	13.7	1.9	0.7	0.9	0.2
30	7.8	1.9	0.8	0.8	0.0
31	11.0	1.9	0.7	0.8	0.2
35	7.3	1.9	0.7	0.8	0.1
38	6.3	1.8	0.5	0.7	N.A.
40	7.8	1.9	0.6	0.8	0.2
41	8.7	1.9	0.7	0.8	0.1
42	8.7	1.9	0.8	0.9	0.1
47	6.8	1.9	0.7	0.8	0.1
50	5.7	1.9	0.9	0.8	-0.2
52	14.0	1.9	0.8	0.9	0.1
54	16.8	1.9	0.7	0.9	0.2
55	10.0	1.9	0.7	0.8	0.1
56	16.5	1.9	0.7	0.9	0.1
57	16.8	1.9	0.7	0.9	0.2
58	14.7	1.9	0.7	0.9	0.1
Average	9.5	1.9	0.7	0.8	0.1

N.A., not analyzed.

the ENA correction revealed the value 0.058 (0.031–0.096), showing low population differentiation. Inter-population F_{ST} values ranged from 0 to 0.289 (populations 2 and 35) (results not shown).

The Structure analysis yielded $K = 2$ as the optimal number of groups. Individuals of populations 2–6 in northwest Pannonia grouped together, while all other populations were more or less strongly admixed (Fig. 4). [Correction added on 5th August, 2016 after first online publication: In the preceding sentence, the Pannonia's region was wrongly identified as 'northeast' and has been corrected in this version]. In accordance with Structure results, the number of clusters simulated from posterior distribution by Geneland displayed a clear mode at $K = 2$ [Ln P(D) = −10 730], clustering populations from northwestern Pannonia (populations 2–6, populations 19–23) and a second group encompassing the remainder of populations (Fig. 5).

When comparing scenarios including different split histories with diyABC we found highest probability (0.5–0.9) for scenario 5 (Fig. 2). Probability for other scenarios were the followings: s1 = 0, s2 = 0, s3 = 0, s4 = 0–0.1, s6 = 0.1–0.3. When constraining coalescent times on scenario 5, the highest probability (pp = 1.0) is found for scenario 5a (Fig. 2) and alternative scenarios considering older times had negligible probabilities.

Species distribution modelling

Species distribution models for the *Triturus* and *Bombina* species groups are described by the following logistic equations, in which P is the probability that a particular spatial unit would be occupied by *T. dobrogicus* (P_T) or by *B. bombina* (P_B):

$$P_T = (1/(1 + \exp(4.284 * \text{Altitude} + 1.625 * \text{Relief} + 2.602))) \text{ and}$$

$$P_B = (1/(1 + \exp(4.900 * \text{Altitude} - 0.119 * \text{Relief} + 1.459))).$$

For a spatial representation of these models (see Fig. 6). AUC values were 0.906 ($P < 0.0001$) for the *Triturus* model and 0.937 ($P < 0.0001$) for the *Bombina* model. Ignoring the parameter relief, the models correspond to a lowland/upland transition point at 156 m above sea level (a.s.l.) in *Triturus* and at 269 m a.s.l. in *Bombina*. As suggested by this difference, the distribution of *B. bombina* in Central Europe and the northern Balkans is wider than that of *T. dobrogicus*. Both models indicate the presence of suitable habitat for congeneric species inside Pannonia, at mountain ranges such as the Bakony Mountains, the Northern Mountain Range, Mecsek Mountains and Fruška Gora. The actual distribution data indicate that these mountains are occupied by *B. variegata*, and are unoccupied by (non-*dobrogicus*) crested newts (Trócsányi, Schäffer & Korsós, 2007; Gubányi *et al.*, 2010; Vukov *et al.*, 2013).

DISCUSSION

A comprehensive investigation with molecular data enabled us to test hypothesized locations of glacial refugia of *T. dobrogicus*. Our findings indicate that the phylogeographic pattern observed is more complex than previously recognized. With our mitochondrial DNA data two haplogroups are identified, one in the Pannonian Danube region and one along the Tisza and Sava Rivers, while the Lower Danube

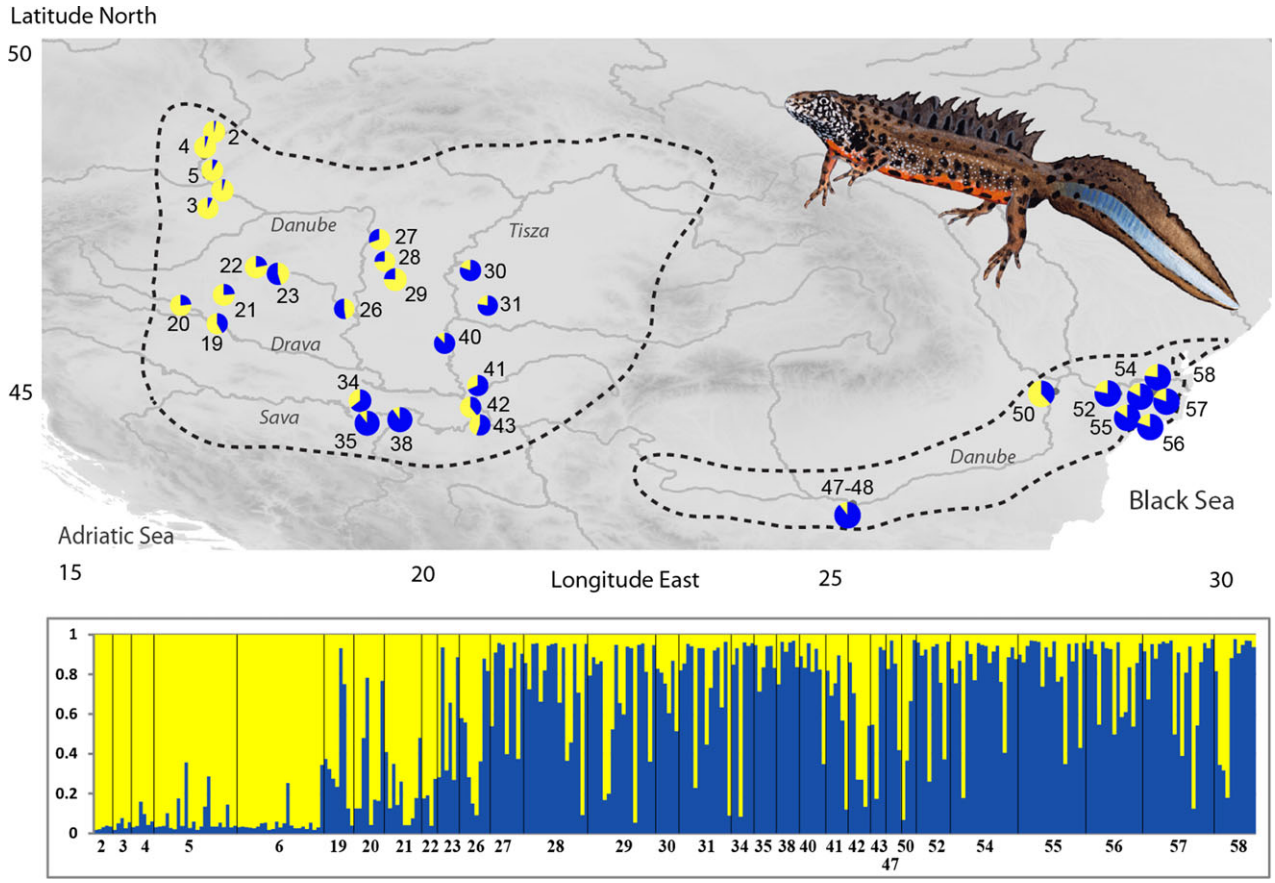


Figure 4. Population structure of 313 *T. dobrogicus* individuals from 32 populations, based on the Bayesian clustering of six microsatellites analyzed with Structure. Bar plots and pie charts represent the assignment probability at the level of the individual and the population, respectively. For sampling details see Table 1. Newt drawing courtesy of Márton Zsoldos.

seems to be admixed. The highest haplotype diversity was found in the Danube Delta and along the Sava River (around and after the confluence of Danube-Sava-Tisza). Increased genetic variation downstream of the confluence of headwaters in dendritic systems have been reported earlier (Selkoe, Scribner & Galindo, 2015) and, moreover, a slightly asymmetric (passive) migration induced by elevation of the landscape, can have a marked influence on the genetic structure of riverine organisms (Morrissey & de Kerckhove, 2009).

Although we did not analyze samples from the two isolated *T. dobrogicus* populations known from the Black Sea coast north and south of the main Danube Delta range of the species, considering the genetic homogeneity all along the Middle and Lower Danube sections and because of their proximity to the sampled locations we presume them to be genetically submerged into the Delta population. This theory is supported by another study which based on large scale nuclear DNA data has shown that the Dnepr Delta population is not separated genetically from the Lower Danube (Wielstra, Vörös & Arntzen, 2016).

The combined molecular genetic data suggest a genetic split between populations in northwestern Pannonia and the remainder of range. The diyABC analyses indicates an additional split between southern Pannonia and Danube Delta at a younger age (Fig. 2, scenario 5a).

The molecular genetic data also suggest that *T. dobrogicus* survived the LGM in two separate refugia, positioned in northwestern Pannonia and in Southern Pannonia from where the range pockets expanded along the Danube and Tisza Rivers. Demographic expansion is confirmed by the mismatch distribution analysis and by Fu's F_s and Tajima's D tests for the two mtDNA lineages (Upper-Middle Danube and Sava-Tisza River). The highly significant Fu's F_s value suggests that the population group presently distributed along the Sava–Tisza–Lower Danube regions might have experienced a rapid growth from a small ancestral population.

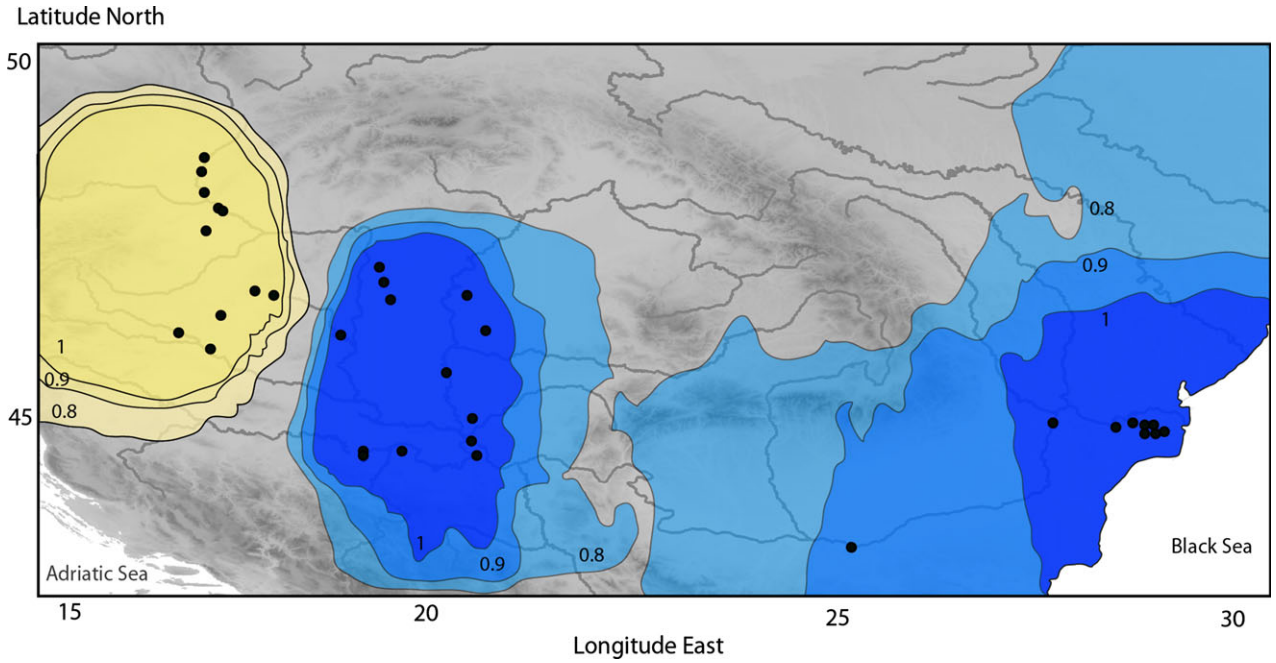


Figure 5. Maps of Geneland individual assignments to clusters for $K = 2$. The two plots represent the assignment to each cluster: Northwest Pannonia (yellow) and South Pannonia and Dobrogea (blue) cluster. Black dots represent localities analysed in this study. The greatest probability of inclusion are indicated in dark blue and yellow and the level curves illustrate the spatial changes in assignment values. (Original figures obtained with Geneland are attached as Supporting Information, Fig. S2.)

The Balkan Peninsula and the Black Sea coast have long been recognized as refugia for a wide variety of animal and plant species during the Würm glaciation. From these refugia the northern regions of Europe and western Asia were recolonized following the retreat of the ice sheets (Taberlet *et al.*, 1998; Hewitt, 1999). For riverine and lowland species the Danube River and the Danube Basin are obvious routes along which northward dispersal into Central Europe probably took place (Varga, 1995). No potential refugium areas could be resolved by modelling the range of *T. dobrogicus* species at LGM conditions (Wielstra *et al.*, 2013a: fig. 7.). However in addition to long-recognized biogeographical arguments, recent genetic data suggest the presence of northerly refugia for some species, especially in the Carpathian Mountains and within Pannonia (Babik *et al.*, 2005; Varga, 2010; Schmitt & Varga, 2012; Ruiz-González *et al.*, 2013). This appears to apply to *T. dobrogicus* also. Being a highly aquatic organism, its persistence in seemingly inhospitable regions extending over glacial maxima may have been attributed to a more stable aquatic microhabitat (Seifertová *et al.*, 2012; Pârvulescu *et al.*, 2013).

However, it seems that persistence of *T. dobrogicus* in separate refugia and rapid population expansions did not affect nuclear genetic diversity of newts since the microsatellite loci yielded a large number of alleles, a high level of polymorphism, and a high overall genetic variability across the range of *T. dobrogicus*. A high level of nuclear genetic diversity was also found with allozymes (Vörös & Arntzen, 2010), and for seven microsatellite loci in the northern part of the Pannonian Basin (Mikulícek, Crnobrnja-Isailovic & Piálek, 2007). In contrast, Wallis & Arntzen (1989) observed low diversity of mitochondrial DNA across the range of *T. dobrogicus*, explaining the inferred high dispersal by the regular flooding conditions of lowland rivers.

What caused population groups of *T. dobrogicus* to become genetically differentiated over Northwest Pannonia? As pointed out by Gábris & Mari (2007), some 140 000 ybp the direction of flow of rivers in this area changed from north to south, which separated the North and Middle Danubian regions. With the formation of Lake Balaton at the end of the Pleistocene the rivers turned northwards again to form 'intra-valley drainage divides' to the west and the south of the lake (Sümegi, Gulyás & Jakab, 2008). This scenario seems to hold for the stream-dwelling gudgeons (*Gobio* spp.) (Takács *et al.*, 2014) and may apply to *T. dobrogicus* also.

The significant deficit of heterozygotes and positive F_{IS} values in some populations and loci may have been caused by the presence of null alleles. While

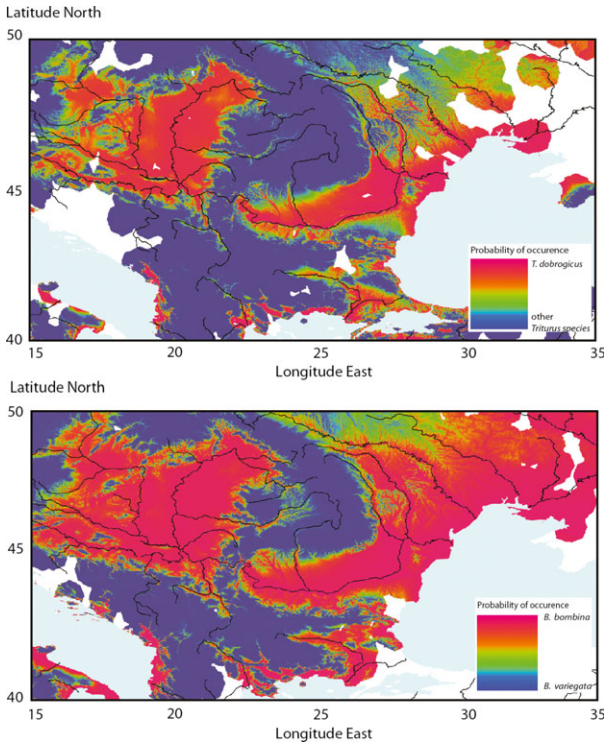


Figure 6. Maps showing the species distribution modelling for (top panel) *T. dobrogicus* and (bottom panel) *B. bombina*. Red areas show the target species distributions, blue areas represent the distribution of congeneric parapatric species (A) *T. carnifex*, *T. cristatus*, *T. ivanbureschi* and *T. macedonicus* and (B) *B. variegata*.

the presence of null alleles will affect population genetic analyses that rely on Hardy–Weinberg expectations, they have only a moderate effect on model-based clustering and Bayesian assignment methods such as Structure analyses (Carlsson, 2008).

Species distributions and comparison with *Bombina*

Modelling the present-day distribution of *T. dobrogicus* is based upon the essentially parapatric relationship of this species with each of four neighbouring congeners (Arntzen *et al.*, 2014: fig. 1). Indeed, the modelled outer range border of *T. dobrogicus* with its congeners is sharp and without large areas of sympatry (Fig. 6A). The parallel distribution pattern of the newts and two *Bombina* species is striking (Fig. 6B), with the fire-bellied toad *B. bombina* in the lowlands (along with *T. dobrogicus*) and the yellow-bellied toad *B. variegata* in the more mountainous areas (along with the newts *T. carnifex*, *T. cristatus*, *T. ivanbureschi* and *T. macedonicus*).

An important difference between the *Bombina* and *Triturus* distribution models and their actual distributions is the presence of *Bombina variegata* in the isolated mountain ranges of the Pannonia, such as the Bakony Mountains, the Northern Mountain Range, Mecsek Mountains and Fruška Gora; whereas – although predicted to occur there by the distribution models – non-*dobrogicus* crested newts are not known from these areas. This is surprising as the modelled range of *T. dobrogicus* is smaller than that of *B. bombina* (Fig. 6A, B), with predicted enclaves larger for mountain *Triturus* than for mountain *Bombina*. We suggest that the reason for this biogeographical discrepancy lies in the different positions of refugia at the LGM, and the subsequent species dispersal histories.

As one of us pointed out (Arntzen, 1978), the presence of the Pannonian enclaves of *B. variegata* cannot be explained by recent *B. variegata* dispersal, as the species would have had to cross terrain that is occupied by *B. bombina*, and, as suggested by field observations, these are ecologically incompatible species with mutually exclusive, parapatric ranges. Moreover, *B. bombina* and *B. variegata* form a hybrid zone of considerable length across Central Europe, inside as well as outside Pannonia. Detailed genetic studies have shown that this hybrid zone is at its centre composed of a variety of hybrid classes with few individuals of either parental species (Szymura & Barton, 1986, 1991; Harrison, 1993; MacCallum *et al.*, 1998; Kruuk, Gilchrist & Barton, 1999; Vines *et al.*, 2003). Extensive interspecific hybridization makes it unlikely that *B. variegata* would have reached the Pannonian isolated mountains genetically uncompromised, yet no *B. bombina*-specific alleles are found in the enclaves (Gollmann, 1987; Gollmann, Roth & Hödl, 1988).

A more probable scenario is the relatively recent arrival of *B. bombina* in Pannonia that displaced *B. variegata* from the low lying areas via competition and hybridization (Arntzen, 1978; Vörös *et al.*, 2006). While the concept of moving hybrid zones has been supported by theoretical and empirical data (Barton & Hewitt, 1985; Buggs, 2007), the presence of enclaves as crucial support for moving hybrid zones, is limited [e.g. *Limnodynastes* frogs in Australia (Littlejohn & Roberts, 1975) and *Triturus* newts in France (Arntzen & Wallis, 1991), Portugal (Themudo & Arntzen, 2007) and in the Balkans (Wielstra & Arntzen, 2012)]. Following the moving hybrid zone and enclave formation argument we suggest that *T. dobrogicus*, with a refugium at the Sava region in southern Pannonia, spread over the lowland portions of the entire lowland prior to the post-glacial arrival of any of its congeners, so that these could not reach the isolated mountains of Pannonia. Conversely, in *Bombina* the LGM refugium of the lowland species (*B. bombina*) was at a considerable distance in the

Black Sea coastal area (*c.* 600 km) (Fijarczyk *et al.*, 2011). Pannonia was reached by *B. variegata* first, which colonized the area including the mountains, and was replaced by *B. bombina* in the surrounding lowlands.

CONCLUSIONS

Using mitochondrial and nuclear markers and applying distribution modelling we were able to reconstruct the recent evolutionary history of the riverine newt species *Triturus dobrogicus*. We identified two main Pleistocene refugia: one in the northwestern and another one in the southwestern regions of Pannonia along the Danube and Sava rivers. The position of refugia could explain the current distributions of the lowland species but shed light also on the dispersion history of now parapatric species present in the neighboring mountains. The phylogeographical analyses suggested that newts could use the central European rivers and their tributaries as dispersal corridors to form their present-day distribution pattern, highlighting the potential role of rivers as important geographical elements shaping evolutionary history of amphibians.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Bayesian Maximum Clade Credibility phylogram for *T. dobrogicus* mtDNA haplotypes. The blue bars at the nodes refer to the 95% HPD range for estimated divergence times. The bottom scale represents time in millions of years (Myr).

Figure S2. Original figures obtained by Geneland showing assignments to clusters for K = 2. Black dots represent localities analysed in this study.
Table S1. Pairwise LD values estimated between the six loci.

SHARED DATA

Data available from the Dryad Digital Repository: doi:10.5061/dryad.n6g99 (Judit Vörös *et al.*, 2016)