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# Is the Danube crested newt Triturus dobrogicus polytypic? A review and new nuclear DNA data

Short title: **Is the Danube crested newt Triturus dobrogicus polytypic?**

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**Abstract**

The Danube crested newt Triturus dobrogicus has been proposed to comprise two subspecies: T. d. dobrogicus and T. d. macrosoma. Uncertainty exists in the literature over their distribution and diagnosability. We conduct a multilocus phylogeographical survey and review published data to determine whether a two taxon treatment is warranted. Newly produced and published nuclear DNA data suggest intraspecific variation in the Pannonian Plain part of the range, but with extensive genetic admixture, whereas mitochondrial DNA data shows a lack of geographical structuring in T. dobrogicus altogether. None of the studied morphological characters suggest the presence of two geographical groups in T. dobrogicus unequivocally. Although Danube Delta newts do have relatively short bodies compared to the remainder of the range (the Pannonian and Lower Danube Plains and the Dnepr Delta), we argue that this finding can be explained by phenotypic plasticity – particularly in light of the incongruent evolutionary scenario suggested by genetic data. We conclude that the total body of evidence does not support the two subspecies hypothesis and recommend that T. dobrogicus is treated as a monotypic species.

**Key words:** Ion Torrent, Next-generation sequencing, Subspecies, Taxonomy, Triturus cristatus superspecies, Triturus dobrogicus macrosoma

## Introduction

The taxonomy of crested newts (Triturus cristatus superspecies) has been regularly updated. Currently six species are recognized, with a seventh awaiting formal description (Wielstra et al., 2013b). All crested newt species are considered monotypic, except for T. dobrogicus (Kiritzescu, 1903), for which Litvinchuk and Borkin (2000) recognized two subspecies. They reinstated T. d. macrosoma (Boulenger, 1908) to reflect a perceived geographical differentiation within T. dobrogicus.

Litvinchuk and Borkin (2000) analyzed a suite of body measurements with principal component and discriminant analysis. In the formal description they mentioned two indices as most informative in separating the two subspecies, namely the 'Wolterstorff-index' (forelimb length divided by interlimb distance) and Ltc/L (head width divided by body length), that are both on average higher in the nominotypical subspecies. The authors noted that the two subspecies showed differences in the number of pre-sacral rib-bearing vertebrae (NRBV; with T. d. dobrogicus characterized by an NRBV count of 16, and T. d. macrosoma by an NRBV count of 17). Litvinchuk and Borkin (2000) also noted differences in coloration of the belly, which tends towards red in T. d. dobrogicus and orange or yellow in T. d. macrosoma and noted that the nominotypical subspecies has a more polished skin, more obvious costal grooves on the sides of the body and smaller and sparser rounded spots on the belly. Larvae of T. d. dobrogicus were said to be usually darker than those of T. d. macrosoma. Based on a crossing experiment the authors suggested a reduced fitness for offspring from crosses between representatives of the two subspecies.

According to Litvinchuk and Borkin (2000), the nominotypical subspecies is distributed in the Danube Delta "along the lower Danube, probably, eastward to Reni [Romania]" and ssp. T. d. macrosoma in the Pannonian and Lower Danube Plains (Fig. 1A). Other authors have suggested that the Iron Gate (the gorge where the Danube river makes its way through the Southern Carpathians) separates the ranges of the two subspecies (Raffaëlli, 2007; Thiesmeier et al., 2009). An allopatric crested newt population in the Dnepr Delta (Fig. 1A) was attributed to T. dobrogicus by Litvinchuk (2005), but not at the level of the subspecies.

The study by Litvinchuk and Borkin (2000) did not include samples from across the entire Danube crested newt range. Notably, material from the Lower Danube Plain and the Dnepr Delta (the latter not known to harbor T. dobrogicus at the time) was not included. This hampers determining the distribution of the two T. dobrogicus taxa. Furthermore, subsequent studies have casted doubt on the diagnosability of the two subspecies based on either genetic or morphological data (e.g. Vörös and Arntzen, 2010; Naumov and Biserkov, 2013) and the taxonomy of Litvinchuk and Borkin (2000) has not universally been adopted (e.g. Sparreboom, 2014).

In an attempt to shed light on the intraspecific taxonomy of T. dobrogicus we conduct a phylogeographic survey using an Ion Torrent next-generation sequencing protocol that provides large scale nuclear DNA data for crested newts (Wielstra et al., 2014a). We interpret the new results in the context of a literature review on intraspecific genetic and morphological variation in T. dobrogicus and use the total body of evidence to decide whether to support or contradict the two subspecies hypothesis. This raises the question on how to delineate a subspecies in the first place. For background on the difficulties of defining subspecies and applying the subspecies rank in taxonomy we refer to Mayr (1969). We here take a pragmatic approach. To accept the two subspecies hypothesis we would require to find geographically consistent intraspecific variation that cannot be explained by environmental plasticity.

## Material and methods

### Sampling and laboratory methods

Our sampling covered the entire range of T. dobrogicus (Fig. 1A; Table 1). We included 46 populations and 121 individuals (1-3 individuals with on average 2.6 individuals per population). Twenty-nine populations with 83 individuals were from the Pannonian Plain, 12 populations with 27 individuals were from the Lower Danube Plain, four populations with ten individuals were from the Danube Delta and one population with one individual was from the Dnepr Delta. We obtained data for 52 nuclear DNA markers following the protocol of Wielstra et al. (2014a). In brief, we amplified markers of c. 140 bp in length (excluding primers, see Table S1), positioned in 3' untranslated regions, in five multiplex PCRs. We pooled the multiplexes for each individual and ligated unique tags to be able to recognize the product belonging to each individual. We sequenced the amplicons on

the Ion Torrent next-generation sequencing platform and processed the output with a bioinformatics pipeline that filters out poor quality reads, identifies alleles and converts data to a format directly usable for population genetic analysis. Sequence data for a mtDNA marker (ND4, 658 bp) were taken from, or newly produced following the protocol in Wielstra et al. (2013a). For six individuals we did not manage to obtain mtDNA sequence data.

Nuclear DNA: testing for interspecific gene flow

Because crested newt species hybridize at their contact zones (Arntzen et al., 2014) we aimed to exclude potentially confounding effects of interspecific gene flow. Therefore we included data from Wielstra et al. (2014a) for the four species with which T. dobrogicus is in spatial contact, namely T. carnifex in the west, T. cristatus in the north, T. ivanbureschi in the southeast and T. macedonicus southwest. We analyzed the data with BAPS 6 (Corander et al., 2008; Cheng et al., 2013) and, considering that five crested species were involved in the comparison, enforced the number of distinct gene pools to five (i.e. k = 5). This should highlight any T. dobrogicus individuals showing introgression from other Triturus species. We conducted ten replicate runs and tested for admixture between gene pools. Newts ascribed to T. dobrogicus with a probability less than unity were excluded from further analyses.

Nuclear DNA: testing for intraspecific genetic structuring

We used FSTAT (Goudet, 1995) to determine gene diversity and the number of alleles per marker and the percentage of missing data. We conducted a spatially explicit Bayesian clustering analysis with BAPS 6 and TESS 2.3.1 (Chen et al., 2007; Durand et al., 2009). We determined the optimal number of gene pools k over a range of 1-44 (the upper limit is defined by the number of populations included), using ten replicates per k value. BAPS determines the optimum k value internally. In TESS the optimum k value was defined as the one where the average deviance information criterion value reached a plateau (as advocated in the TESS manual). In BAPS we incorporated the geographical origin of individuals (‘spatial clustering’) and tested for admixture between gene pools. In TESS we modelled admixture using the conditional autoregressive model, with 20,000 sweeps of which 5,000 were discarded as burn-in. The spatial interaction parameter was kept at the default with the option to update this parameter activated. The estimated admixture proportions for independent runs were averaged using CLUMPP 1.1.2 (Jakobsson and Rosenberg, 2007). All output was visualized with DISTRUCT (Rosenberg, 2004).

Nuclear DNA: population differentiation in T. dobrogicus

We constructed a population tree with the program POPTREE2 (Takezaki et al., 2010). To determine the position of the root we added T. cristatus as outgroup. We used the Neighbour Joining method based on uncorrected $F_{ST}$ distance and the robustness of interpopulational relationships was tested with 1000 bootstrap replicates. Eight markers had to be excluded in this exercise because data were missing for one or more populations.

Mitochondrial DNA analyses

Mitochondrial DNA haplotypes were identified by comparison against the mtDNA haplotype database presented in Wielstra et al. (2013a). To determine to which Triturus species newly identified haplotypes belonged we constructed a Neighbour-Joining tree with 1000 bootstrap replicates in MEGA6 (Tamura et al., 2013). A Median Joining network (Bandelt et al., 1999) was created using Network 4.6.11 (www.fluxus-engineering.com).

Data accessibility

Sampling details are presented in Table S2 and GenBank Accession numbers for mtDNA are in Table S3. Raw Ion Torrent reads in FASTQ format; scripts associated with the bioinformatics pipeline; BWA alignments in SAM format; raw SNP reports in VCF and BCF format; the filtered SNP report used to construct consensus sequences; an overview of the number of reads in total and per marker and/or individual; nuclear DNA data in genotypic format; nuclear DNA sequence alignments; and input and output files for Network, FSTAT, BAPS, TESS and POPTREE; are available via Dryad Digital Repository entry doi:10.5061/dryad.b9765.

## Results

Results of the analysis testing for interspecific gene flow of nuclear DNA are summarized in Table 1 with details in Table S2. Five crested newt populations have T. dobrogicus in syntopy with another Triturus species (T. carnifex once, T. macedonicus once and T. cristatus three times) and seven populations have T. dobrogicus individuals showing genetic admixture with other Triturus species. Altogether we excluded 18 individuals from ten populations in further analyses, of which two populations in their entirety (Table S2).

144 The markers in the dataset used for the intraspecific analyses on average had 6.0 ± 3.1
145 (standard deviation) alleles and an average gene diversity of 0.35 (details in Table S1). The results for
146 the analyses testing for intraspecific genetic clustering show the same overall pattern, with minor
147 differences in details (Fig. 2, Table S2). BAPS yields k = 3 as the most probable number of gene pools
148 present in T. dobrogicus. Although TESS also identifies k = 3 as the most likely solution, support for
149 allocation of individuals to the third group is negligible. Hence we also provide results for TESS
150 under the next lowest meaningful k value, i.e. k = 2 (Fig. 2; Table S2). The third TESS group under k
151 =3 is then subsumed in the first TESS group under k = 2 whereas the distinction between a first and a
152 second group is similar under both k values (details in Table S2). From here on we only consider
153 TESS results under k = 2.
154 Two of the three BAPS groups roughly correspond to one of the two groups in TESS. The
155 overall pattern is one group occupying the southwest of the Pannonian Plain range (populations 12-21
156 and partially populations 7 and 10 in Fig. 1A) and another group occupying the remainder of the
157 range (including the Pannonian and Lower Danube Plains and the Danube and Dnepr Deltas). The
158 results differ in the amount of admixture inferred between these two groups (Fig. 2; details in Table
159 S2). TESS generally suggests a more pronounced admixture between these groups than does BAPS.
160 Conversely, BAPS suggests admixture in one individual from the Danube Delta (from population 42),
161 which was not found by TESS.
162 The population tree based on nuclear DNA genetic distances constructed with POPTREE
163 suggests that the four range sections, Pannonian and Lower Danube Plains and Danube and Dnepr
164 Deltas, do not constitute reciprocally monophyletic groups (Fig. 1B). The populations comprising the
165 southwest Pannonian Plain group identified by BAPS and TESS (populations 12-21 in Fig. 1A)
166 cluster together, but bootstrap support for monophyly of this assemblage is low (Fig. 1B).
167 We observed one instance of a pure T. dobrogicus individual based on nuclear DNA
168 possessing mtDNA originating from another species, namely T. ivanbureschi (Table S2). The
169 haplotype network for the T. dobrogicus mtDNA indicates a lack of geographical structuring (Fig. 3)
170 as e.g. illustrated by the central haplotype Tdob02, which is found all across the range of T.
171 dobrogicus, in the Pannonian and Lower Danube Plains and in the Danube and Dnepr Deltas.

## Discussion

### Interpretation of published and newly produced genetic data

Mitochondrial DNA shows a shallow genetic divergence across the T. dobrogicus range, with identical haplotypes present in the Pannonian, Lower Danube and Dnepr range sections (Wallis and Arntzen, 1989; Wielstra et al., 2013a; this study). Allozyme data suggest a higher level of intraspecific genetic variation than mtDNA, but agree on a lack of geographical structure between the Pannonian and Lower Danube Plain populations (Vörös and Arntzen, 2010). Rather, populations from the southwest of the Pannonian Plain are relatively distinct from those in the remainder of the range. No material from the Danube Delta is included in Vörös and Arntzen (2010). However, the allozyme data presented in Litvinchuk et al. (1994) show a Nei's genetic distance of zero between a Danube Delta and Transcarpathian (northeast Pannonian Plain) population sample (Litvinchuk and Borkin, 2000; Arntzen, 2003; Naumov and Biserkov, 2013).

The newly produced nuclear DNA data improve upon previous studies in the number of markers analyzed and the geographical coverage of population samples. Although the results of the cluster-based analysis of the new data are in line with the presence of two genetic clusters within T. dobrogicus, the spatial arrangement differs distinctly from the one proposed by Litvinchuk and Borkin (2000). One group is distributed in the southwest of the Pannonian Plain range (mainly populations 12-21 in Fig. 1A) and another group occupies the remainder of the range, i.e. the rest of the Pannonian Plain, the Lower Danube Plain and the Danube and Dnepr Delta. These findings are similar to those based on allozyme data (Vörös and Arntzen, 2010). The new nuclear DNA data agree with all previous genetic studies that genetic admixture between intraspecific genetic groups in T. dobrogicus is extensive. Furthermore, we do not find significant bootstrap support for either group identified in the population tree based on the new data.

Crucially, our genetic results do not support long term limitations to gene flow between the Danube Delta and the remainder of the range (including the currently allopatric Dnepr Delta). This finding is not in line with the crossing experiment of Litvinchuk and Borkin (2000), which suggests

relatively low survival resulting from the cross of T. d. dobrogicus and T. d. macrosoma (it should be note that half of the offspring in the genus Triturus dies during embryonic development due to the peculiar 'chromosome 1 syndrome'; Macgregor and Horner, 1980; Ridley, 2004). However, it should be noted that sample sizes in this experiment are extremely low, with n = 2 for T. d. dobrogicus x T. d. dobrogicus, n = 2 for T. d. dobrogicus x T. d. macrosoma and n = 0 for T. d. macrosoma x T. d. macrosoma. The replicates for the T. d. dobrogicus x T. d. macrosoma differ widely in embryo and larval survival (1.9% versus 13.3% and 100% versus 51.1%). Embryo survival for T. d. dobrogicus x T. d. macrosoma is unrealistically low, even lower than for a cross between two distinct Triturus species (T. carnifex x T. karelinii; see Table 4 in Litvinchuk and Borkin, 2000). Because, in contrast to the two T. dobrogicus subspecies, the different Triturus species are characterized by distinct average genome sizes (Litvinchuk et al., 1999), genetic incompatibilities would a priori be expected to play a larger role in interspecific crosses. Considering the low sample size in the crossing experiment, alternative explanations for low survival (e.g. disease outbreak, a problem with temperature control, etc.) cannot be safely excluded. We consider the results of the crossing experiment unconvincing and put our trust in the molecular genetic data.

Interpretation of published morphological data

Litvinchuk and Borkin (2000) mention in their formal diagnosis of the two subspecies in T. dobrogicus that two indices, the ‘Wolterstorff-index’ (forelimb length divided by interlimb distance) and Ltc/L (head width divided by body length), are both on average higher in the nominotypical subspecies (the former for males only). These findings indicate that newts from the Danube Delta possess relatively short bodies. Naumov and Biserkov (2013) conducted a morphological survey for T. dobrogicus, adding material from the Lower Danube Plain that was not represented by Litvinchuk and Borkin (2000) and concluded that the new material is closer to T. d. macrosoma from the Pannonian Plain than it is to T. d. dobrogicus from the Danube Delta.

We summarized data for the Wolterstorff and Ltc/L indices for individual newts from Litvinchuk and Borkin (2009) and Naumov and Biserkov (2013) and Wolterstorff-index data only from Arntzen and Wallis (1999). We excluded populations located near the contact zones with other

Triturus species to minimize confounding effects of interspecific gene flow (see Table S4 for the total dataset). Data for males and females are plotted in Fig. 4 for the Pannonian Plain, the Lower Danube Plain, the Danube Delta and the Dnepr Delta separately. Student's t-tests confirm that for the Wolterstorff-index males ($t=5.93$, $P<0.01$) but not females ($t=1.24$, $P>0.05$) and for the Ltc/L both males ($t=4.80$, $P<0.001$) and females ($t=4.624$, $P<0.001$) from the Danube Delta on average show higher values – and therewith shorter bodies – compared to those from the Pannonian plus Lower Danube Plains.

One of us (JWA, pers. obs.) has noted a marked phenotypic plasticity in body shape in T. dobrogicus larvae, with particular stout bodies in water bodies with fish predators present, versus a more regular, elongated form typical for the genus as a whole. We suggest a similar phenotypic plasticity may apply to adults as well. This would explain why the genetic data suggest an incongruent evolutionary scenario, with the deepest intraspecific divergence found within the Pannonian Plain. In this light we doubt the taxonomical relevance of the observed body shape differentiation in T. dobrogicus.

The use of indices hampers interpretation of the diagnosticity of individual characters. Arntzen and Wallis (1994) find the number of rib-bearing vertebrae (NRBV) to better discriminate the different Triturus species. Litvinchuk and Borkin (2000) suggest that NRBV differs between T. d. dobrogicus and T. d. macrosoma on average, with T. d. dobrogicus more inclined to show an NRBV count of 16 and T. d. macrosoma an NRBV count of 17. However, their sample sizes are small, overlap in NRBV count is rampant (Table 3 in Litvinchuk and Borkin, 2000) and a G-test for independence does not suggest a significant difference between the two subspecies ($G=0.50$, $P>0.05$). A more comprehensive sampling shows that in the Pannonian Plain an NRBV count of 16 or 17 occurs at the same frequency (Arntzen et al., 2015). The limited sampling in the Lower Danube Plain shows two individuals with an NRBV count of 16 and two with 17.

Although NRBV is known to show intraspecific plasticity (Slijepčević et al., 2015), Litvinchuk and Borkin (2000) hypothesize that the frequency of an NRBV count of 16 in their material of T. d. macrosoma could be inflated due to genetic admixture with T. cristatus (and implicitly that this is not the case for T. d. dobrogicus). This hypothesis has some merit because

Arntzen et al. (2014) show that T. dobrogicus individuals from the contact zone with T. cristatus more often show an NRBV count of 16 and those away from contact zones an NRBV count of 17. However, we argue that without additional evidence that different mechanisms underlie an NRBV count of 16 in T. d. macrosoma and T. d. dobrogicus, we should stick to the simplest explanation of the data. We conclude that the currently available data do not support a frequency differentiation in NRBV count across the T. dobrogicus range.

Litvinchuk and Borkin (2000) consider the coloration of the belly, almost red in T. d. dobrogicus and orange or yellow in T. d. macrosoma, to distinguish the two subspecies (although raw data is not provided and it is not stated how color was quantified). Considering that the 'redness' of the belly in amphibians is influenced by diet (Matsui et al., 2003) and UVB exposure (Michaels et al., 2015), we question its applicability in crested newt taxonomy.

Although not mentioned in the taxonomic account as distinguishing the two subspecies, Litvinchuk and Borkin (2000) mention additional perceived differences in the main text. The frequency with which black spots merge on the belly and form a dorso-ventral black stripe is suggested to be lower in the Danube Delta than in the Pannonian Plain. As both the size and density of belly spots increase with age (Lantz, 1953; Arntzen and Teunis, 1993) we advise against using these characters in crested newt taxonomy. The obviousness of costal grooves seems to us to depend in the first place on the feeding condition of animals and we doubt its relevance for taxonomy. Without rigorous quantification it is impossible to interpret the relevance of differences in how polished the skin is. Triturus dobrogicus larvae stand out from the larvae of other Triturus species by a deep black coloration at large (> 50 mm) size (Arntzen, 2003), but we cannot confirm a geographical pattern within T. dobrogicus. The pigmentation of Triturus larva is most likely subject to phenotypic plasticity (Cvijanović et al., 2015) and we do not consider darkness of larvae taxonomically informative.

Taxonomy of T. dobrogicus

Based on the accumulated data from multiple molecular marker systems and morphological characters we can address the question whether the data so far support the two subspecies treatment of T.

dobrogicus. All molecular genetic data disagree with the treatment by Litvinchuk and Borkin (2000) of a distinct group inhabiting the Danube Delta. Furthermore, the molecular genetic data show that the Iron Gate, which has sometimes been interpreted as separating the ranges of two T. dobrogicus subspecies (Raffaëlli, 2007; Thiesmeier et al., 2009), does not pose a barrier to gene flow between the Pannonian and Lower Danube range sections (for further discussion see Arntzen et al., 1997; Gherghel and Papeş, 2015). Rather, for nuclear DNA the bulk of intraspecific genetic variability is to be found within the Pannonian range section of T. dobrogicus, with one distinct genetic cluster inhabiting the southwest of the Pannonian Plain and the other inhabiting the remainder of the range, including the Danube Delta. However, genetic admixture between these two genetic groups is rampant. Furthermore, we contest that the morphological data point towards a distinct taxon inhabiting the Danube Delta. We believe that other potential explanations for intraspecific variation within T. dobrogicus – related to age, environment and interspecific gene flow – have not been sufficiently excluded and doubt that some characters express geographical differentiation at all. Hence we conclude that there is insufficient support for the two subspecies treatment proposed by Litvinchuk and Borkin (2000) and recommend that T. dobrogicus is treated as monotypic.

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411
412 **Tables**

413

414 **Table 1.** Sampled Triturus dobrogicus populations. Population numbers correspond to Fig. 1 and

415 more details can be found in Table S2.

416

**Figure legends**

**Fig. 1.** Sampling scheme and population tree for T. dobrogicus. (a) The distribution is outlined with a black line (based on Wielstra et al., 2014b). Populations are from four range sections: the Pannonian (yellow in the online version of this figure) and Lower Danube (green online) Plains and the Danube (blue online) and Dnepr (red online) Deltas. The Pannonian and Lower Danube Plain are separated by the Iron Gate and the Lower Danube Plain and the Danube Delta are bounded by populations 41 and 45 following Litvinchuk and Borkin (2000). Grey background shading reflects elevation above 150 meters above sea level. The Danube River is shown as a dark line (blue in the online version) running through the center of the range. Population numbers correspond to Table 1 and Table S2. (b) Genetic differentiation of populations based on nuclear DNA. In the online version populations are color coded according to the region of origin. Only bootstrap support values over 50 are shown. Populations with a thick line in (a) and in boldface in (b) are consistently identified as predominantly belonging to a relatively distinct genetic cluster in the intraspecific spatial Bayesian clustering analyses (see Fig. 2).

**Fig. 2.** Spatial Bayesian clustering results for Triturus dobrogicus individuals according to the programs BAPS and TESS. Numbers at the top are population numbers and in the online version colors reflect regions.

**Fig. 3.** Haplotype network for Triturus dobrogicus mtDNA. Only individuals identified as pure T. dobrogicus are included and one mtDNA haplotype introgressed from another crested newt species is excluded. Pies (or pie slices) are color coded according to the region of origin of haplotypes: the Pannonian (white in print and yellow in the online version) and Lower Danube (light grey in print, green online) Plains and the Danube (dark grey in print, blue online) and Dnepr (black in print, red online) Deltas. Numbers refer to haplotype code (with the suffix “Tdob” not shown) and correspond to Table S3.

**Fig. 4.** Body shape measurements for Triturus dobrogicus. Shown from left to right are the average, range and standard deviation for (a) the Wolterstorff-index (forelimb length divided by interlimb distance) and (b) Ltc/L (head width divided by body length) for females and males from the Pannonian (yellow in the online version) and Lower Danube (green online) Plains and the Danube (blue online) and Dnepr (red online) Deltas. Note that for females there are no data from the Dnepr Delta. Sample sizes are noted in italics below box plots and raw data are in Table S4. The thick grey lines show the optimal cut-off value as determined with weighted logistic regression, for the Wolterstorff-index – males (1/(1+exp(0.161*WI-8.375))), for Ltc/L – females (1/(1+exp(0.811*HI-10.475))) and Ltc/L – males (1/(1+exp(0.915*HI-12.669))). For Wolterstorff-index – females no significant model was found.

**Online appendices**

**Table S1.** Marker details for intraspecific analysis.

**Table S2.** Sampling details for Triturus dobrogicus.

**Table S3.** GenBank Accession numbers for mtDNA.

**Table S4.** Morphological data for Triturus dobrogicus.