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# Where to draw the line? A nuclear genetic perspective on proposed range boundaries of the crested newts *Triturus karelinii* and *T. arntzeni*

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**Abstract.** The group of crested newts distributed from the southern Balkans to the southern shore of the Caspian Sea, *Triturus karelinii* sensu lato, comprises two species, *T. karelinii* in the east and *T. arntzeni* in the west. Three hypotheses have been forwarded defining the range of *T. arntzeni*, namely from northern Serbia eastwards i) in to Thrace, ii) up to the Aegean-Black Sea waterway including the Bosphorus, or iii) into western Anatolia. We study 130 newts from 22 populations across this area with a panel of 40 enzyme nuclear genes. A combined analysis with the computer programs Structure and NewHybrids reveals the existence of two groups with admixture at two localities. The ‘western group’ comprises all European populations and a population at the southern shore of the Sea of Marmara in Asiatic Turkey, whereas the ‘eastern group’ is found at the Sakarya river valley in northern Anatolia, Asiatic Turkey. The admixed populations are also located in northern Anatolia. An analysis with the computer program BAPS resolves six genetic clusters, of which three represent the ‘western group’ and the other three coincide with the ‘eastern group’ and the two admixed populations. These analyses indicate that the species transition from *T. arntzeni* to *T. karelinii* is not in Thrace but in northern Anatolia. The presence of ‘western’ *T. arntzeni* to the east of ‘eastern’ *T. karelinii* indicates that the species’ contact zone has a convoluted shape. Moreover, the spatial distribution of diagnostic allozymes only roughly coincides with that of two deeply divergent mitochondrial DNA haplotypes. A more detailed survey on the crested newt distribution in Anatolia is required to elucidate the picture further.

**Keywords:** allozymes, Anatolia, Bosphorus, contact zone, hybridization, mitochondrial DNA, Thrace, *Triturus cristatus* superspecies.

## Introduction

The crested newt *Triturus cristatus* superspecies has a fuzzy taxonomic history. The number of recognized taxa increased from two in Laurenti’s days, to four early in the 20<sup>th</sup> century and six at the present day. A breakthrough came with the study by Wolterstorff (1923), who demonstrated that four taxa could be distinguished based on relative appendage size (the ‘Wolterstorff-Index’, describing body shape as ‘forelimb length divided by interlimb distance’) and that such a classification was in line with coloration characteristics. Moreover, his classification gave rise to a largely geographically coherent distribution pattern, with one taxon

in the northern Palearctic, one in the Pannonian and Dobrogean plains, one in the Apennine Peninsula and northern Balkans and one in the southern Balkans eastwards to include Crimea, the Caucasus and the southern shore of the Caspian Sea. Bucci-Innocenti et al. (1983), summarizing the mounting evidence from previous work, proposed that the four forms warrant recognition as distinct species: *T. cristatus* (Laurenti, 1768), *T. dobrogicus* (Kiritzescu, 1903), *T. carnifex* (Laurenti, 1768) and *T. karelinii* (Strauch, 1870). Since these four taxa meet up in the Balkan Peninsula, this is where research into crested newts subsequently had a focal point and here the species ranges are well documented (Arntzen, 2003).

For crested newts, overall body shape appears to reflect ecology, with a gradient running from the more terrestrial species with a short and sturdy body and a low vertebrae count as in *T. karelinii* to the more aquatic species with a long and slender body and a high vertebrae

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count as in *T. dobrogicus*. Arntzen and Wallis (1999) found the number of rib-bearing presacral vertebrae (NRBV) to represent a taxonomically discriminating character, superior to the 'Wolterstorff-Index' (with which NRBV is negatively correlated). The modal NRBV count increases from 13 in *T. karelinii*, via 14 in *T. carnifex* and 15 in *T. cristatus*, to 16 or 17 in *T. dobrogicus*. The geographical distribution of morphotypes largely corresponds to that of mitochondrial DNA phylogroups (Wallis and Arntzen, 1989; Arntzen and Wallis, 1999). Although deep genetic divergence has been revealed within *T. carnifex* and within *T. karelinii* (Wallis and Arntzen, 1989), this is not accompanied by a differentiation in NRBV (Arntzen and Wallis, 1999). Considering the genetic distinction and qualitative morphological differentiation, the 'Balkan form' of *T. carnifex*, was first reinstated as a subspecies and subsequently elevated to species level (Arntzen and Wallis, 1999; Arntzen et al., 2007): *Triturus macedonicus* (Karaman, 1922).

Litvinchuk et al. (1999) described a western subspecies of *T. karelinii* based on differences in genome size, protein variation and morphological characteristics. Espregueira Themudo et al. (2009), employing mitochondrial and nuclear DNA sequence data, found this taxon to be as distinct as the other crested newt taxa and gave it species status: *Triturus arntzeni* Litvinchuk, Borkin, Džukić and Kalezić, 1999 (in Litvinchuk et al., 1999). However, it is as yet unclear if, where and how the ranges of the constituent species meet. The type localities of *T. karelinii* and *T. arntzeni* are from the far ends of the *T. karelinii* sensu lato range; from Iran and Serbia, separated by approximately 2700 kilometer as the crow flies. Attempts to narrow down on the position of the species border have yielded conflicting interpretations (fig. 1). Firstly, Wallis and Arntzen (1989) found that two types of mitochondrial DNA occur in syntopy at the Sakarya river valley in the west of Asiatic Turkey. Secondly, according to Litvinchuk et al. (1999), *T. karelinii* reaches into

European Turkey, as *T. arntzeni* specific alleles are absent in the Thracian population studied (see also Sotiropoulis, 2004). Thirdly, a recent handbook suggests that the marine corridor connecting the Aegean and Black Sea (composed of the Dardanelles, Sea of Marmara and Bosphorus) separates the species ranges (Raffaëlli, 2007). In other words, the boundary between *T. arntzeni* and *T. karelinii* has been proposed to be situated in Anatolia, Asia, in Thrace, Europe, and at the boundary between the two continents, following the Aegean-Black Sea waterway. We here i) explore the spatial genetic structuring over the western part of the *T. karelinii* sensu lato range by employing a panel of allozyme loci and ii) compare the results with morphological and genetic data already available.

## Material and methods

### *Sampling strategy and laboratory methods*

We studied 130 crested newts from 22 populations in south-eastern Europe and northwestern Turkey, spanning the area of interest (fig. 1 and table 1). Genetic profiles were established for 40 nuclear gene loci by protein electrophoresis, following published protocols (Arntzen, 2001). Data on 23 individuals in three populations were taken from Arntzen et al. (2007). Conditions of Hardy-Weinberg equilibrium and linkage disequilibrium were analyzed with GenePop v. 4 (Rousset, 2008), without Bonferroni correction due to mostly small sample sizes. In view of the evidence for introgressive hybridization at or near the various crested newt contact zones, we analyzed published data for the three *Triturus* species with which *T. karelinii* sensu lato is in spatial contact, namely *T. cristatus* ( $N = 153$ ), *T. dobrogicus* ( $N = 152$ ) and *T. macedonicus* ( $N = 94$ ) (Arntzen, 2001; Vörös and Arntzen, 2010).

### *The number of distinct gene pools and introgressive hybridization*

Analyses on introgressive hybridization were made for pairwise interspecific combinations, using the computer program NewHybrids 1.1b3 (Anderson and Thompson, 2002). NewHybrids infers for every individual the Bayesian posterior probability with which it belongs to a purebred (either of the species under consideration) or a hybrid class (F1, F2 or a backcross with one of the two parental species). Short initial runs, conducted to ensure convergence on the same result, were followed by one long run of 100 000 iterations after stabilization of likelihood values. Structure 2.2 (Pritchard et al., 2000) was used to determine the number of distinct ancestral gene pools ( $K$ ) in *T. karelinii* sensu



**Figure 1.** Upper figure – The approximate range of *T. karelinii* sensu lato with the type localities of *T. karelinii* ('southern shore of the Caspian Sea in Iran', solid triangle) and *T. arntzeni* (Vrtovač, Serbia, open triangle) and three *a priori* hypotheses on the location of the of *T. karelinii*-*T. arntzeni* species boundary: A – Thrace (cf. Litvinchuk et al., 1999), B – the Europe/Asia continental divide (cf. Raffaelli, 2007), and C – Sakarya river (cf. Wallis and Arntzen, 1989). Lower figure – Populations studied, with those identified as 'western' in white, as 'eastern' in black, and as 'intermediates' in black and white.

lato, over a  $1 < K < 22$  range. We used the admixture model, which recognizes that individuals may have a mixed ancestry, in combination with the correlated allele frequency model. We ran five independent simulations for each  $K$  value (100 000 MCMC replications, after 50 000 initial burn-in iterations). The  $K$  value which showed the highest increase in likelihood compared to its precursor was considered optimal (as suggested by Evanno et al., 2005). This analysis was complemented with a NewHybrids run with the procedure as described above. The criteria used to classify individual newts as belonging to a particular species were  $P > 0.8$  in Structure and  $P > 0.9$  in NewHybrids. Individuals were considered 'intermediate' when they had a Structure score of  $0.2 \leq P \leq 0.8$  and a pooled hybrid and backcross hybrid score in NewHybrids of  $P \geq 0.1$ .

The data for *T. karelinii* sensu lato were also analyzed with the computer program BAPS v. 3.2 (Corander et al., 2005), which under default settings includes (amongst others)  $K$  as a parameter to be estimated. We used a two-step procedure, with first the clustering of individuals and second the determination of the level of admixture.

## Results

We observed 100 alleles over 40 protein loci. The number ranges from one allele in ten monomorphic loci to four alleles in five loci (*Gpi*, *Lap*, *Nadhdh-2*, *6-Pgd* and *Xdh*) and five alleles in four loci (*Est-1*, *Est-2*, *Pep-1* and *Trf*). For the distribution of allele frequencies see Appendix. One individual from population 1 (#654), from the border region with *T. dobrogicus* and *T. cristatus* (Wallis and Arntzen, 1989; Arntzen, 2003), showed signs of genetic admixture with the latter species at the level of  $P = 0.19$  in NewHybrids. It was excluded from further analysis.

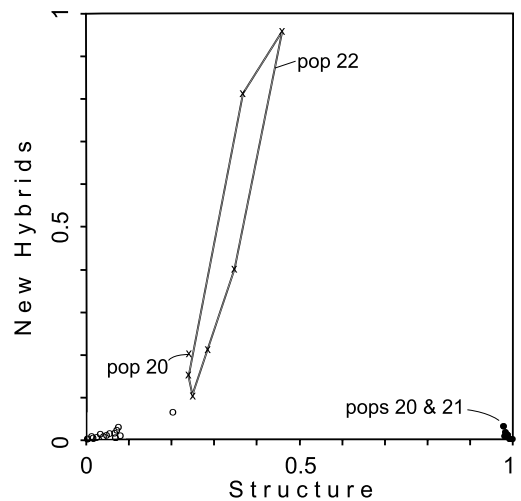
No population was found to be out of Hardy-Weinberg equilibrium across loci ( $P > 0.05$ ).

**Table 1.** *Triturus karelinii* sensu lato populations studied, with coordinates and sample size. ZMA-Herp. is the number under which vouchers have been stored at the Herpetological collection of the Zoological Museum Amsterdam.

Number	Population		Coordinates		Sample size	ZMA Herp.	
	Name	Country	Latitude	Longitude		Jar	Individual
1	Trešnja	Serbia	44°36'N	20°35'E	10	9164	321-323, 649-651, 654-657
2	Djurinci	Serbia	44°30'N	20°38'E	2	9099	931-932
3	Arandjelovac	Serbia	44°19'N	20°35'E	9	9086	390-395, 763-765
4	Grivac	Serbia	43°58'N	20°40'E	9	9115	817-824, 893
5	Guberevac	Serbia	43°49'N	20°46'E	6	9116	320, 617, 631-632, 648, 779
6	Vitanovac	Serbia	43°43'N	20°48'E	5	9169	868-871, 934
7	Gornja Sabanta	Serbia	43°54'N	21°00'E	1	9113	935
8	Resavica	Serbia	44°03'N	21°36'E	3	9150	825-826, 882
9	Sisevac	Serbia	43°56'N	21°37'E	4	9157	827-828, 874-875
10	Bigla	Macedonia	41°56'N	22°40'E	5	9092	794-796, 892, 895
11	Mitrašinci	Macedonia	41°45'N	22°46'E	12	9140	797-807, 894
12	Bansko	Macedonia	41°23'N	22°46'E	1	9088	867
13	Kentriko	Greece	41°10'N	22°54'E	13	9127	808-814, 885-890
14	Dafnohori	Greece	40°57'N	22°48'E	3	9096	815-816, 891
15	Sevlievo	Bulgaria	43°28'N	25°13'E	2	9154	460-461
16	Levski	Bulgaria	43°25'N	25°08'E	9	9134	442-445, 769-773
17	Karlovo	Bulgaria	42°38'N	24°49'E	4	9125	377-379, 719
18	Rakovski	Bulgaria	42°16'N	24°58'E	4	9149	515, 766-768
19	Karacabey	Turkey	40°15'N	28°18'E	3	9121	483-485
20	Yakacık	Turkey	40°55'N	29°12'E	9	9118	953-961
21	Adapazari	Turkey	40°45'N	30°23'E	6	9082	402-404, 774-776
22	Bartin-Çaycuma	Turkey	41°33'N	32°13'E	10	7564	116-125

At individual loci no significant excess of heterozygotes was found while a significant heterozygote deficit ( $P < 0.05$ ) was found at two loci (*Mdh-1*, *Nadhdh-2*) in population Yakacık and at one locus in populations Bartın-Çaycuma (*Pep-1*), Guberevac (*Nadhdh-2*), Kentriko (*G-6-pd*) and Mitrasinci (*Np*). Significant linkage disequilibrium ( $P < 0.05$ ) was found for six locus pairwise comparisons in population Levski, four times in population Yakacık and another five times across the other populations.

In the Structure analysis, the model assuming two ancestral gene pools in *T. karelinii* sensu lato was preferred. These two groups we call 'eastern' and 'western' in line with their approximate geographical position (fig. 1). Seven individuals constitute a third category of 'intermediates' as shown by Structure and NewHybrids (fig. 2). Populations falling in more than one category are Yakacık (population 20, with one intermediate and eight eastern type) and Bartın-Çaycuma (population 22, with six intermediates and four western type). When ignoring

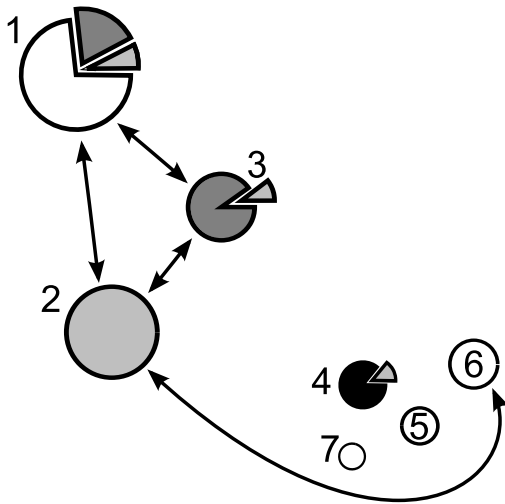


**Figure 2.** Scatterplot for 129 *Triturus karelinii* sensu lato crested newts in 22 populations from southeastern Europe and adjacent Asia. The horizontal axis gives the probability of belonging to the western group *T. arntzeni* (open symbols) versus the eastern group *T. karelinii* (solid symbols), as established with the software Structure. The vertical axis gives the probability of hybrid origin, as determined with the software NewHybrids. Intermediate individuals are marked with a cross. Pop(s) is population(s).

the intermediate individuals, the populations 20 and 21 from Adapazari and Yakacık classify as eastern and the other 20 populations classify as western.

With BAPS six genetic clusters are resolved, showing a strong spatial structure; individuals from cluster 1-6 are found in spatial groups with the same number. However, the correspondence is not complete for spatial groups 1, 3 and 4, which at moderate or low frequency carry individuals from clusters 2 and 3 (fig. 3). An aberrant result is found for population Karabey, Asia: all three investigated individuals are placed in cluster 1, which is otherwise restricted to the European part of the *T. karelinii* sensu lato range.

The mean expected heterozygosity (weighted for sample size,  $H_e$ ) is markedly lower in the western populations ( $H_e = 0.074$ , range 0.038-0.118) than in the eastern populations ( $H_e = 0.139$ , range 0.122-0.161; for values per population see Appendix). Genetic distance between



**Figure 3.** Six genetic clusters in *T. karelinii* sensu lato representing the best partition among individual newts as found with the software BAPS, with their distribution over seven spatial groups (schematic). Individuals in clusters 1-4 are represented by white, light grey, dark grey and black shadings, respectively and clusters 5 and 6 by their numbers. Note that individuals of spatial group 7 (Karabacey) also belong to cluster 1. Sample size is reflected by the size of the circles. Admixture proportions with values in excess of 2% are shown by arrows.

the two groups are  $D_{\text{Nei-unbiased}} = 0.078$  and  $D_{\text{Rogers-modified}} = 0.262$ . Discriminating allelic profiles for the eastern and western groups at the loci *Ldh-2* and *Xdh* show single locus genetic distances of 3.26 and 3.01 ( $D_{\text{Nei-unbiased}}$ ) and 0.963 and 0.764 ( $D_{\text{Rogers-modified}}$ ). The highest genetic differentiation is found between the population from Adapazari and all others at  $D_{\text{Nei-unbiased}} = 0.138$  and  $D_{\text{Rogers-modified}} = 0.353$ , followed by Yakacık against the remaining populations at  $D_{\text{Nei-unbiased}} = 0.095$  and  $D_{\text{Rogers-modified}} = 0.298$ . The remaining populations are grouped together with genetic distance values of up to  $D_{\text{Nei-unbiased}} = 0.067$  and  $D_{\text{Rogers-modified}} = 0.261$ .

## Discussion

We employed a large panel of nuclear genetic markers to explore if, where and how the contact zone of *T. arntzeni* and *T. karelinii* is located over the western part of the *T. karelinii* sensu lato range. We demonstrate the presence of a western and an eastern group that we interpret as corresponding to *T. arntzeni* and *T. karelinii*. At  $D_{\text{Nei}} = 0.08$  the level of genetic differentiation is modest relative to the species pairs *T. carnifex*-*T. macedonicus* and *T. marmoratus*-*T. pygmaeus* ( $D_{\text{Nei}} = 0.19$  for both; Arntzen et al., 2007). Our observations do not support the positioning of the *T. arntzeni* and *T. karelinii* species boundary in Thrace. Instead, the species border appears to be located in western Anatolia. We first evaluate the contribution of published data sets to elucidate the biogeographical pattern.

### Evaluation of published and new data

**Morphology** – The modal count of the number of rib-bearing vertebrae (NRBV) is a powerful tool to discriminate between *Triturus* species, but does not help to distinguish between *T. karelinii* and *T. arntzeni* (cf. Arntzen, 2003). Also the multivariate statistical analysis on a suite of morphological characters evaluated

by Litvinchuk et al. (1999) did not yield a clear species discrimination nor did it reveal diagnostic character states for *T. arntzeni*-*T. karelinii*.

**Genome size** – The potential of genome size as a source of taxonomic information has been assessed by Kron et al. (2007) and discussed for amphibians in particular by Green and Sessions (2007). A difference in genome size between *T. arntzeni* and *T. karelinii*, with non-overlapping confidence intervals, was observed by Litvinchuk et al. (1999). Interestingly, an extensive overlap in genome size was observed among *T. arntzeni* from Serbia and its neighbor *T. macedonicus* from Montenegro (see populations 14 and 13 of Litvinchuk et al., 1999). Unfortunately, ‘population 14’ represents seven different localities over an area covering ca. 2.5 degrees longitude and 2.5 degrees latitude, precluding a more accurate assessment of the likelihood of signal distortion due to e.g. interspecific introgressive hybridization. Given the sampling regime of *T. arntzeni* populations close to the range of *T. macedonicus* and the widespread occurrence of mitochondrial DNA haplotypes typical for *T. arntzeni* in *T. macedonicus* (Arntzen and Wallis, 1999), this possibility cannot be excluded.

**Mitochondrial and nuclear DNA loci** – Two distinct mitochondrial DNA clades were found syntopic near Adapazari at the Sakarya River valley in northwestern Anatolia (Wallis and Arntzen, 1989). Further eastwards at Bartın-Çaycuma and in the Crimea, the Caucasus and Iran ‘eastern’ mitochondrial DNA was found, whereas populations from Yakacık and across the Balkans had ‘western’ mitochondrial DNA (Wallis and Arntzen, 1989; Arntzen et al., 2007; Espregueira Themudo et al., 2009). Variation at five nuclear DNA markers (Espregueira Themudo et al., 2009) also supports a ‘western’ (Balkan) versus ‘eastern’ (the Caucasus and Iran) pattern, but the restricted sampling (not including Turkey) precludes a detailed comparison. The mitochondrial DNA molecular clock for *Triturus* (based on a  $d_{K80} \approx 0.06$ ) suggests

speciation to have occurred at 6.1 Ma (Arntzen et al., 2007).

**Enzyme loci** – Protein loci that were identified as diagnostic for *T. arntzeni* versus *T. karelinii* are *GP-4* and *Pgd* by Litvinchuk et al. (1999) and *Ldh-2* and *Xdh* in the present study. It is important to note that alleles at *GP-4* and *Pgd*, considered typical for *T. arntzeni*, were observed fixed in Serbia/Macedonia, whereas the alternate alleles, considered typical for *T. karelinii*, were found from Thrace eastwards. However, our results for *6-Pgd* (= *Pgd*) do not coincide with the above, with the two most common alleles (*6-Pgd*<sup>bc</sup>) found in several European and Anatolian populations. Of the two loci that we identified as diagnostic (*Ldh-2* and *Xdh*), the bi-allelic locus *Ldh-2* is common to both studies, with largely similar results, such that the *T. karelinii* specific allele is fixed in Anatolia, Georgia and Krasnodar (Litvinchuk et al., 1999) and in Adapazari (present study) whereas *T. arntzeni* populations has the alternate allele at low (0.21, range 0.18-0.50; Litvinchuk et al., 1999) or high mean frequency (0.96, range 0.75-1.00; present study). Based on the allozyme molecular clock for *Triturus*, a genetic distance of  $D_{Nei} = 0.08$  equates to speciation at 2.8 Ma (Arntzen et al., 2007).

### *Biogeographical pattern*

The new allozyme data provide convincing spatial interpretations when analyzed with modern analytical approaches. BAPS recognizes six non-hierarchical groups with admixture between them more prominent in the west than in the east. This is in line with the level of genetic variability, which is moderate ( $H_e = 0.07$ ) and high ( $H_e = 0.14$ ), respectively. The ‘eastern’ and ‘western’ groups resolved by Structure include the signature of admixture at two populations located at either side (east and west) of the Sakarya river valley. The distribution pattern of *T. arntzeni*-*T. karelinii* is more complex than the separation over a longitudinal axis suggested by mitochondrial DNA data. In the absence of independent criteria for species recog-

dition (e.g., from morphology) an interpretation remains ambiguous. However, the phenomenon that a species contains mitochondrial DNA typical of a related one has commonly been observed across the genus *Triturus*. The area of discrepancy can be narrow or wide and the pattern can be symmetric or asymmetric. Examples include *T. cristatus*-*T. dobrogicus* in which the zone is narrow and symmetric and *T. arntzeni*-*T. macedonicus* in which the zone is wide and asymmetric (Wallis and Arntzen, 1989; Arntzen and Wallis, 1999).

The mitochondrial DNA data indicate syn-  
 tophy of *T. arntzeni* and *T. karelinii* at the Sakarya River valley. This valley was part of the waterway through the İzmit Gulf and Lake Sapanca, which connected the Marmara and Black Seas during the Pleistocene from 2.59 Ma-11.7 Ka (Elmas, 2003). A scenario of allopatric speciation at 2.59 Ma is in line with the protein molecular clock estimate of 2.8 Ma. Secondary spatial contact between *T. arntzeni* and *T. karelinii* originated at 11.7 Ka and was followed by range expansions, with *T. karelinii* moving to the northwest at the expense of *T. arntzeni*. Dispersal into Europe was prevented by the Bosphorus which formed as an alternative to the İzmit Gulf-Lake Sapanca-Sakarya Valley waterway (Elmas, 2003; Kerey et al., 2004). At the same time *T. arntzeni* expanded its range at the expense of *T. karelinii* eastwards upto Bartın-Çaycuma (or further into the area for which we have no data). This tentative explanation for the *T. arntzeni* and *T. karelinii* contact zone classifies as 'wide' and 'symmetrical'. An intriguing aspect is the marked discrepancy in the level of genetic differentiation at mitochondrial and allozyme loci. Since the mutual distribution of *T. arntzeni*-*T. karelinii* has turned out to be much more complex than previously thought, but remains as yet unclear, we propose to survey Anatolia – and indeed the entire range of *Triturus karelinii* sensu lato – in more detail, for example with the help of diagnostic allozyme and mitochondrial genetic markers in eggs and embryos, which are more readily collected than larvae,

juveniles or adults. This work could be paralleled by a search for diagnostic features in the species morphology, e.g. in the cranial osteology of adults.

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## Appendix. (Continued).

Locus	Allele	Sabanta																					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
<i>Gpi</i>	c	0.00	0.25	0.00	0.11	0.08	0.20	0.00	0.33	0.13	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.50	0.06	0.00	0.05
	e	1.00	0.75	1.00	0.89	0.92	0.80	1.00	0.67	0.88	1.00	1.00	1.00	1.00	0.83	1.00	1.00	0.25	1.00	0.50	0.94	0.67	0.90
	f	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.75	0.00	0.00	0.00	0.00	0.00
	g	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.05
<i>G-6-pd</i>	a	0.00	0.00	0.00	0.11	0.17	0.10	0.00	0.17	0.25	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.17	0.17	0.00	0.00
	b	1.00	1.00	1.00	0.89	0.83	0.90	1.00	0.83	0.75	1.00	1.00	1.00	0.92	1.00	1.00	1.00	1.00	0.83	0.83	1.00	1.00	
<i>Icd-2</i>	a	1.00	1.00	0.94	1.00	1.00	1.00	1.00	1.00	1.00	0.90	0.88	1.00	0.96	0.67	0.75	1.00	0.75	0.88	1.00	0.89	1.00	1.00
	b	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.13	0.00	0.04	0.33	0.25	0.00	0.25	0.13	0.00	0.11	0.00	0.00
<i>Ldh-1</i>	a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	c	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.83	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
<i>Ldh-2</i>	b	0.94	1.00	0.94	1.00	0.75	0.90	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.94	1.00	1.00	1.00	0.11	0.00	0.85
	d	0.06	0.00	0.06	0.00	0.25	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.89	1.00	0.15
<i>Lap</i>	a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	b	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.88	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.94	0.92	1.00
	c	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.00
	d	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00
<i>Mdh-1</i>	b	0.94	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.50	0.89	1.00	0.75	1.00	0.56	1.00	0.75
	c	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.25	0.00	0.39	0.00	0.00
	d	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.06	0.00	0.25
	a	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.67	0.50	1.00	1.00	0.50	0.92	1.00	0.25	0.61	0.25	0.25	0.67	0.83	0.67
<i>Mdh-2</i>	b	0.06	0.00	0.06	0.06	0.00	0.10	0.00	0.00	0.33	0.50	0.00	0.00	0.50	0.00	0.75	0.39	0.75	0.75	0.33	0.17	0.33	0.00
	c	0.94	1.00	0.94	0.94	1.00	0.90	1.00	1.00	0.67	0.50	1.00	1.00	0.50	0.92	1.00	0.25	0.61	0.25	0.67	0.83	0.67	1.00
	a	0.00	0.00	0.00	0.00	0.00	0.30	0.00	0.50	0.17	0.13	0.20	0.00	0.00	0.04	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	d	0.78	1.00	1.00	0.72	0.92	0.60	0.00	0.00	0.83	0.88	0.80	1.00	1.00	0.96	0.83	1.00	1.00	1.00	1.00	1.00	1.00	0.00
<i>Me</i>	b	0.06	0.00	0.00	0.28	0.00	0.30	0.00	0.50	0.17	0.13	0.20	0.00	0.00	0.04	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	c	0.17	0.00	0.00	0.00	0.08	0.10	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	a	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	d	0.78	1.00	1.00	0.72	0.92	0.60	0.00	0.00	0.83	0.88	0.80	1.00	1.00	0.96	0.83	1.00	1.00	1.00	1.00	1.00	1.00	0.00

Locality: Trešnja Džurinci Arandjelovac Grivac Guberevac Vitanovac Gorinja Resavica Sisevac Bigla Mitrašinci Banske Kentriko Dafnohori Sevljevo Levski Karlovo Rakovski Karacabey Yakacak Adapazari Bartin-Çayuma

## Appendix (Continued).

Locus	Locality: Trešnja Djurmci-Arandjelovac Grivac Guberevac Vitanovac Gornja Resavica Sisevac Bigla Mitrašinci BANSKO Kentriko Dafnohori Sevljevo Levski Karlovo Rakovski Kanacabey Yakaack Adapazari Bartin-Çayuma																							
	Population: Allele	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
<i>Mpi</i>	b	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.13	0.00	0.00	0.50	0.04	0.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	c	1.00	1.00	0.89	1.00	1.00	1.00	1.00	1.00	0.88	1.00	1.00	0.50	0.96	0.67	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.61	0.00	0.00
	b	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.28	0.50	0.45
<i>Nadhdh-2</i>	c	0.11	1.00	1.00	0.67	0.67	0.10	1.00	0.00	0.38	0.50	0.33	0.00	0.27	0.50	1.00	1.00	1.00	0.88	1.00	1.00	0.11	0.50	0.55
	d	0.83	0.00	0.00	0.33	0.33	0.90	0.00	1.00	0.63	0.50	0.67	1.00	0.73	0.50	0.00	0.00	0.00	0.13	0.00	0.00	0.00	0.00	0.00
	a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00
	b	0.00	0.00	0.17	0.00	0.00	0.40	0.00	0.00	0.25	0.10	0.08	0.00	0.15	0.33	0.00	0.00	0.06	0.00	0.50	0.00	0.22	0.58	0.05
<i>Np</i>	c	1.00	1.00	0.83	1.00	1.00	0.60	1.00	1.00	0.75	0.90	0.92	1.00	0.85	0.67	1.00	0.89	1.00	0.50	1.00	0.78	0.42	0.95	
	a	0.00	0.25	0.06	0.00	0.08	0.00	0.00	0.00	0.13	0.10	0.00	0.00	0.12	0.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	b	1.00	0.75	0.94	1.00	0.92	1.00	1.00	1.00	0.88	0.90	1.00	1.00	0.89	0.67	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
<i>Pg-1</i>	a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00
	b	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.94	1.00	1.00	1.00	1.00	1.00	1.00
<i>Pg-2</i>	a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	b	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.94	1.00	1.00	1.00	1.00	1.00	1.00
<i>Pg-3</i>	a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.00	0.00	0.00	0.00	0.25	0.00
	b	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.78	1.00	1.00	1.00	1.00	0.75	1.00
<i>Pep-1</i>	b	0.00	0.00	0.50	0.00	0.17	0.00	0.50	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.50	0.83	0.75	0.75	0.00	0.06	0.58	0.05	0.00
	c	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	d	0.83	1.00	0.50	0.89	0.58	0.80	0.50	1.00	0.88	1.00	1.00	1.00	1.00	0.83	0.50	0.00	0.25	0.25	0.00	0.33	0.42	0.60	0.60
	e	0.00	0.00	0.00	0.00	0.11	0.00	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.61	0.00	0.35	0.00
	g	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>Pep-2</i>	a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	
	b	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.67	1.00	0.85
	c	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.00	0.00	0.15

**Appendix.** (Continued).

Locality: Trešnja Džurinci Aranđjelovac Grivac Guberevac Vitanovac Gornja Resavica Sisevac Bigla Mitrašinci Banskó Kentriko Dafnóhori Sevljevo Levski Karlovo Rakovski Karacabey Yakacak Adapazari Bartin-Çaycuma		21	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
Population:																						
Allele																						
Subanta																						
<i>6-Pgd</i>	a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.50	0.12	0.17	0.00	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.00
	b	0.78	1.00	1.00	0.90	1.00	1.00	1.00	1.00	0.96	0.50	0.89	0.83	0.75	1.00	0.88	0.88	1.00	1.00	0.88	1.00	0.58
	c	0.22	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.13	0.00	0.00	0.00	0.00	0.45
	f	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>Sdh</i>	a	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.29	0.00	0.12	0.00	0.00	0.00	0.13	0.00	0.00	0.00	0.13	0.00	0.00
	b	1.00	1.00	0.89	1.00	1.00	1.00	1.00	0.88	0.67	1.00	0.81	0.83	1.00	1.00	0.88	0.88	1.00	1.00	0.88	1.00	0.85
	c	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.08	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10
<i>Sod-1</i>	a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	b	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.83	1.00	1.00	0.96	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
<i>Trf</i>	a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	b	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	c	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.85	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	d	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	e	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>Xdh</i>	a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	b	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.19	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25
	c	0.78	1.00	0.78	1.00	0.80	0.75	1.00	1.00	0.83	1.00	0.77	1.00	1.00	1.00	0.78	0.88	1.00	0.50	0.00	0.00	0.00
	d	0.22	0.00	0.22	0.00	0.20	0.25	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.22	0.13	0.00	0.50	0.00	0.00	0.00
H <sub>e</sub>		0.076	0.038	0.054	0.056	0.102	0.093	0.050	0.090	0.112	0.058	0.055	0.100	0.055	0.118	0.071	0.082	0.087	0.052	0.122	0.161	0.071
SE		0.023	0.021	0.018	0.021	0.027	0.027	0.035	0.030	0.028	0.022	0.021	0.048	0.017	0.032	0.030	0.027	0.026	0.025	0.031	0.037	0.027

Monomorphic loci *Acpb-2*, *Ada*, *Ata-2*,  $\alpha$ -*Gly1*, *Icd-1*, *Nadhdh-1*, *Pg-4*, *Pgm-1*, *Pgm-2* and *Sod-2* not shown.