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Is mitochondrial DNA divergence of Near Eastern crested newts (*Triturus karelinii* group) reflected by differentiation of skull shape?

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ABSTRACT

The Eurasian *Triturus karelinii* group of crested newts comprises three distinct, geographically coherent mitochondrial DNA lineages, designated as the eastern, central and western lineage. These three lineages are genetically as diverged as other, morphologically well-differentiated crested newt species. However, on the ground of restricted morphological studies the three lineages have been considered morphologically uniform. We analyze skull shape in the *T. karelinii* group using geometric morphometric techniques and interpret the results in a phylogenetic context. We found a high divergence between populations and variable patterns of sexual dimorphism within mitochondrial DNA lineages, significant divergence in skull shape including significant divergence in allometry of the ventral skull side in females, and lack of concordance between the pattern of morphological and genetic variation within lineages and between lineages. The observed pattern indicates that ecologically mediated divergences could play an important role in the evolution of skull shape. Reconstruction of the evolutionary trajectory of the *T. karelinii* group indicates that the eastern lineage largely retains the ancestral skull shape and that the central and western lineages possess a derived skull shape. Skull shape does not clearly support the presence of three discrete geographical groups as suggested by the mitochondrial DNA data, but the amount of shape changes between *T. karelinii* lineages is similar to that between *T. karelinii* lineages and the outgroup species, *T. macedonicus*.

1. Introduction

Crested newts (*Triturus cristatus* superspecies) are a well-supported monophyletic group of closely related species for which the phylogeny has recently been resolved (Wielstra and Arntzen, 2011). Crested newts provide an example of a rapid adaptive radiation (Espregueira Themudo et al., 2009; Wielstra and Arntzen, 2011; Ivanović et al., 2012a). The crested newts species differ in the slenderness of their bodies, a feature correlated to discrete differences in the number of rib bearing pre-sacral vertebrae. The correlation between body built and species-specific duration of the annual aquatic period indicates that this morphological differentiation was accompanied by ecological diversification (Arntzen, 2003; Ivanović et al., 2012a). Furthermore, the differentiation in the number of rib bearing pre-sacral vertebrae reflects the crested newt phylogeny (Wielstra and Arntzen, 2011).

The basal split in the crested newts separates the *T. karelinii* group, distributed in the Balkans, western and northern Turkey, the Caucasus and the Crimea, from the other four crested newt species, distributed across the remainder of Europe. Mitochondrial DNA sequences unveiled the presence of three distinct lineages within the *T. karelinii* group which, in line with their distribution, are referred to as the eastern, central and western lineage (Fig. 1; Wielstra et al., 2010; Wielstra and Arntzen, 2011). These three lineages are genetically as diverged as other crested newt species. However, this deep genetic divergence does not coincide with differences in the number of rib bearing pre-sacral vertebrae (the typical count is 13 for the *T. karelinii* group, 14 for *T. carnifex–T. macedonicus*, 15 for *T. cristatus* and 16 or 17 for *T. dobrogicus*), or any other morphological characteristics that have been studied up to now (Arntzen, 2003; Litvinchuk and Borkin, 2009). This could indicate that either the *T. karelinii* group shows a remarkably deep intraspecific mitochondrial DNA variation or, at the other side of

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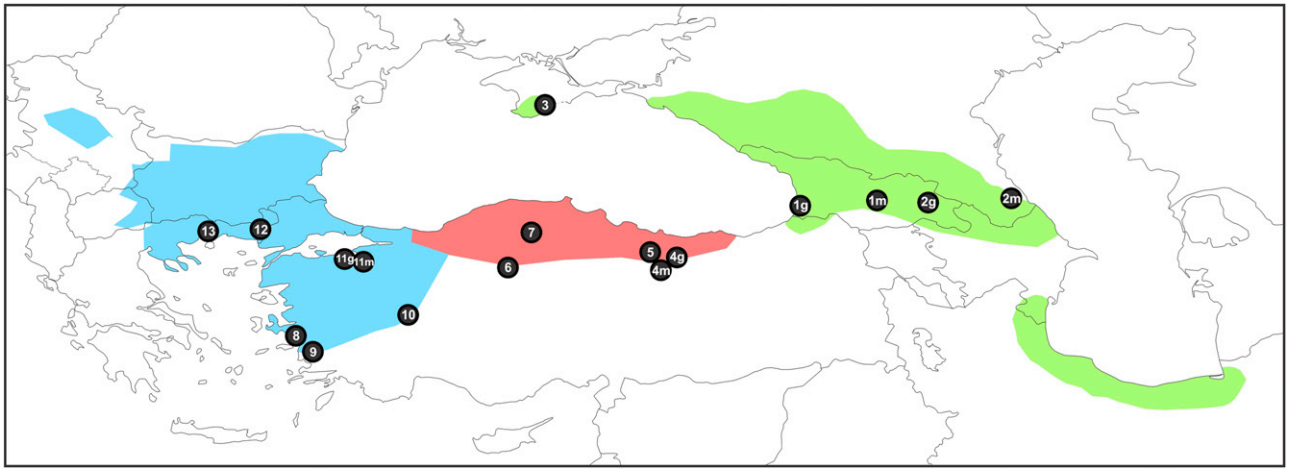

Fig. 1. Geographic position of 13 populations of *Triturus karelinii* group and their affiliation to the eastern (green), central (red) and western (blue) mitochondrial DNA lineage (see Table 1 for details). The distribution covers the Balkans, western and northern Turkey, the Caucasus and the Crimea and the southern Caspian Sea shore. If study populations for genetic and morphological data were not the same this is marked (g – genetic; m – morphology). Note that populations 3g and 3m are geographically too close to show separately. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the coin, that the three mitochondrial DNA lineages reflect morphologically cryptic species.

The vertebrate skull is a developmentally and functionally complex morphological structure that plays a crucial role in the perception of the environment (sight, hearing, olfaction), protection of the brain and food acquisition and processing. This suggests that morphological variation of the skull has adaptive significance. Therefore, the vertebrate skull has been used as a model system in evolutionary studies, including some that deal with phylogenetic signal in skull shape (e.g. in mammals Marroig and Cheverud, 2001; Nicola et al., 2003; Monteiro and dos Reis, 2005; Goswami, 2006; Cardini and Elton, 2008a,b). Recent work on several newt genera (*Triturus, Ichthyosaura*, *Lissotriton*; Ivanović et al., 2008, 2009, 2012b) indicates that skull shape retains phylogenetic signal. As the marked genetic differentiation within the *T. karelinii* group is not paralleled by any documented eco-morphological changes, a study on skull shape is a promising avenue for research.

Geometric morphometrics involves the comparative statistical analysis of shape variables (e.g. Klingenberg, 2010). The method allows: (i) to reconstruct a group's consensus shape, (ii) to visualize the change from one shape to another and interpret the change anatomically, and (iii) to infer the shape of the most recent common ancestor of that group. As the phylogeny is the basic reference in evolutionary events, we use the geometric morphometric approach for the *T. karelinii* group in a phylogenetic context, to (i) explore the presence of as yet unrecognized morphological divergence among the three mitochondrial DNA lineages, to (ii) test for phylogenetic signal in skull shape, and (iii) to visualize the evolution of skull shape.

2. Methods

2.1. Sampling

We analyzed 13 newt samples, covering the mitochondrial phylogeographic diversity of the *T. karelinii* group (Table 1 and Fig. 1). We aimed to obtain mitochondrial DNA sequences from the very populations that were analyzed for morphology and where we did not accomplish this we used geographically proximate ones. As outgroup we employed *T. macedonicus* (morphology and mitochondrial DNA) and *T. marmoratus* and *Calotriton asper* (mitochondrial DNA only).

2.2. Genetic divergence and phylogeny

To obtain a measure of genetic divergence among populations, we gathered part (658 bp) of the NADH dehydrogenase subunit 4 gene (ND4) for 50 *T. karelinii* group individuals. Part of the sequences used ($n = 41$) were taken from Wielstra et al. (2010) and Wielstra and Arntzen (submitted for publication) while the remainder ($n = 9$) was newly produced following their protocol. In three of the populations, more than one haplotype was found and we only included the most abundant one (the excluded haplotypes were highly similar, data not shown).

In order to test for phylogenetic signal in skull shape, we constructed a phylogeny based on full mitogenomic sequences for three mitochondrial DNA lineages comprising the *T. karelinii* group and the three outgroup species (GenBank accession numbers HQ697275–HQ697279 and EU880307; data from Wielstra and Arntzen, 2011; Zhang et al., 2008). The most appropriate model of sequence evolution (GTR + G + I) was determined with MrModeltest 2.2 (Nylander, 2004), based on the Akaike Information Criterion. A phylogeny was constructed under Bayesian inference with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) via the CIPRES Science Gateway (http://www.phylo.org/sub_sections/portal). Two runs of fifty million generation were conducted with a heating parameter of 0.02 and a sample frequency of 0.001. Tracer 1.5 (Rambaut and Drummond, 2007) was used to check for stabilization of overall likelihood within and convergence between runs. The first quarter of sampled trees was discarded as burn-in and the inference was drawn from the remaining 'forest'.

2.3. Morphology

Adult newts were cleared with trypsin and KOH, stained with Alizarin Red S for bone depositions (Dingerkus and Uhler, 1977) and stored in glycerin. The number of vertebrae was counted. Populations from all three lineages have the same median number ($n = 13$) of rib bearing vertebrae.

Images of the ventral and dorsal skull side were obtained with a Sony DSCF828 digital camera with the palate and skull roof positioned parallel to the photographic plane. A set of 47 landmarks (three median landmarks and 44 pairs on the left and right sides; Fig. 2) was digitized on the ventral and dorsal side of each skull using TpsDig software (Rohlf, 2005), all by the same person (AI). We analyzed 232 individuals with a complete landmark set for

Table 1
Overview of *Triturus* salamanders used in the present study, with locality, sample sizes, haplotypes recoded and GenBank accession numbers. No.F, number females; No.M, number of males. Latitude (Lat) is given in degrees north; longitude (Long) is given in degrees east.

Population, locality, country		Lat	Long	No.F	No.M	Sequencing	Haplotype	GenBank accession number
Triturus karelinii – eastern lineage								
1	Kobuleti, Georgia	41.822	41.814			5	TkarA09	GU982399
	Tbilisi vicinity, Georgia	Not recorded		4	2			
2	Telavi, Georgia	41.903	45.475			3	TkarA09	GU982399
	Ersi Lake, Dagestan, Russia	41.983	47.983	9	9			
3	Alushta, Krym, Ukraine	44.682	34.384			5	TkarA16	GU982406
	Kutuzovskoe Lake, Krym, Ukraine	44.750	34.350	6	10			
Triturus karelinii – central lineage								
4	Şebinkarahisar, Turkey	40.286	38.126			4	TkarB02	GU982409
	Şerefiye, Turkey	40.116	37.750	10	10			
5	Reşadiye, Turkey	40.450	37.483	10	10	3	TkarB04	GU982411
6	Kalecik, Turkey	40.083	33.350	10	5	5	TkarB11	GU982418
7	Tosya, Turkey	41.017	34.033	9	9	4	TkarB10	GU982417
Triturus karelinii – western lineage								
8	Klaros, Turkey	38.000	27.183	10	10	2	TkarC12	GU982435
9	Mersinbeleni, Turkey	37.650	27.683	8	9	4	TkarC12	GU982435
10	Büyük Kalecik, Afyon, Turkey	38.687	30.455	3	8	5	TkarC18	GU982441
11	Yenişehir, Turkey	40.268	28.605			1	TkarC05	GU982428
	Uludağ, Bursa, Turkey	40.116	29.033	9	10			
12	Dadia, Greece	41.120	26.147	5	6	5	TkarC54	JQ240230
13	Saint Kosmas, Greece	41.084	24.669	6	4	4	TkarC19	GU982442
Triturus macedonicus								
Og1	Bjeloši, Montenegro	42.374	18.907			2	Tmac11	JQ240248
	Rid, Montenegro	42.366	18.950	12	8			
Og2	Elafotopos, Greece	39.900	20.669	9	6	3	Tmac02	GU982388

the dorsal side; data for the ventral side was missing in two of the specimens. The specimens used in this study were part of two herpetological collections (Institute of Biological Research "Siniša Stanković" Belgrade, Serbia and Adnan Menderes University, Aydin, Turkey). For the purpose of this study, the specimens were cleared and stained; the skeletons were stored in glycerol and deposited in the herpetological collection of the Institute of Biological Research "Siniša Stanković" (IBISS) (the specimens voucher numbers are given in Appendix A).

2.4. Statistics

We applied a Generalized Procrustes Analysis (GPA; Rohlf and Slice, 1990; Bookstein, 1996; Dryden and Mardia, 1998), to obtain a matrix of shape coordinates (also known as Procrustes coordinates) from which differences due to position, scale and orientation had been removed. General size was computed as the centroid size (CS), which reflects the amount of dispersion around the centroid of the landmark configuration. To explore the variation in skull shape, we used the averages of original and mirrored configuration of each specimen, which constitute the symmetric component of shape variation (Klingenberg et al., 2002). The shape variables (symmetric component of shape variation) were obtained using MorphoJ software (Klingenberg, 2011).

2.4.1. Sexual dimorphism

To explore the pattern of sexual dimorphism in skull size, the analysis of variance (ANOVA) with sex and population as factors, including their interaction were performed within each mitochondrial DNA lineage separately; the pattern sexual dimorphism in skull shape within lineages was explored through multivariate analysis of variance (MANOVA) with sex and population as factors including their interaction. Analyses were conducted in SAS (SAS Institute Inc., Cary, NC, version 9.1.3) using PROC GLM procedure.

2.4.2. Divergence in skull size and shape

The divergence in skull size was analyzed through analysis of variance (ANOVA) with lineage as a fixed effect and population nested within the lineage as a random effect. The divergence in skull shape among lineages was analyzed through multivariate analysis of variance (MANOVA), with lineage and population (nested within lineage) as factors using PROC GLM procedure in SAS (SAS Institute Inc., Cary, NC, version 9.1.3). Matrices of morphological differences between populations (Procrustes distances) were calculated. To estimate the degree of correspondence between morphometric and genetic divergence within lineages, we used matrix correlations (Monteiro and dos Reis, 2005; Ivanović et al., 2009; Cardini and Elton, 2008b). Matrices of morphological differences between populations (Procrustes distances) were compared with matrices of genetic distances (p-distance) based on ND4 haplotypes (calculated in MEGA 5.05; Tamura et al., 2011). A Mantel test with 1000 random permutations (PopTools, version 2.7), was used for testing the significance of the observed matrix correlation. The null hypothesis states that the correlation between these two matrices is no larger than expected by chance.

2.4.3. Allometry

Allometry, the dependence of shape on size, tends to be a dominant factor of variation across taxa (e.g. Emerson and Bramble, 1993; Marroig, 2007; Cardini and Elton, 2008a; Kulemeyer et al., 2009; Ljubisavljević et al., 2010). We analyzed the size related shape changes by regressing shape variables on size variables (lnCS) within each population and sex separately. Due to a relatively small sample size per population and sex, there was no statistically significant regression slope after Bonferroni correction

Fig. 2. Configuration of 47 two-dimensional landmarks identified for the ventral and dorsal side of *Triturus karelinii* skulls: 1, 2, tip of occipital condyle; 3, 4, cranial base and posterior pterygoideum; 5, 6, anterior tip of pterygoideum; 7, 8, most lateral point of quadratum; 9, 10, vomeral teeth – posterior; 11, 12, vomeral teeth – anterior; 13, 14, most anterior point of vomer; 15,16, tip of maxilla; 17, 18, most posterior point of premaxilla; 19, 20, anterior end of suture between premaxilla and maxilla; 21, 22, most lateral points of vomer that form the posterior margin of choana; 23, tip of the snout. 24, 25, most posterior point of occipital; 26, 27, suture between squamosal and occipital; 28, 29, tip of squamosal; 30, 31, tip of the occipital; 32, 33, suture between parietal and squamosal; 34, 35, suture between parietal and frontal; 36, 37, suture between prefrontal and frontal; 38, 39, distal point of suture between prefrontal and maxilla; 40, 41, suture between prefrontal and maxilla; 42, 43, maxilla at nasal opening; 44, 45, premaxilla at nasal opening; 46, suture between frontals and 47, suture between frontal and parietal.

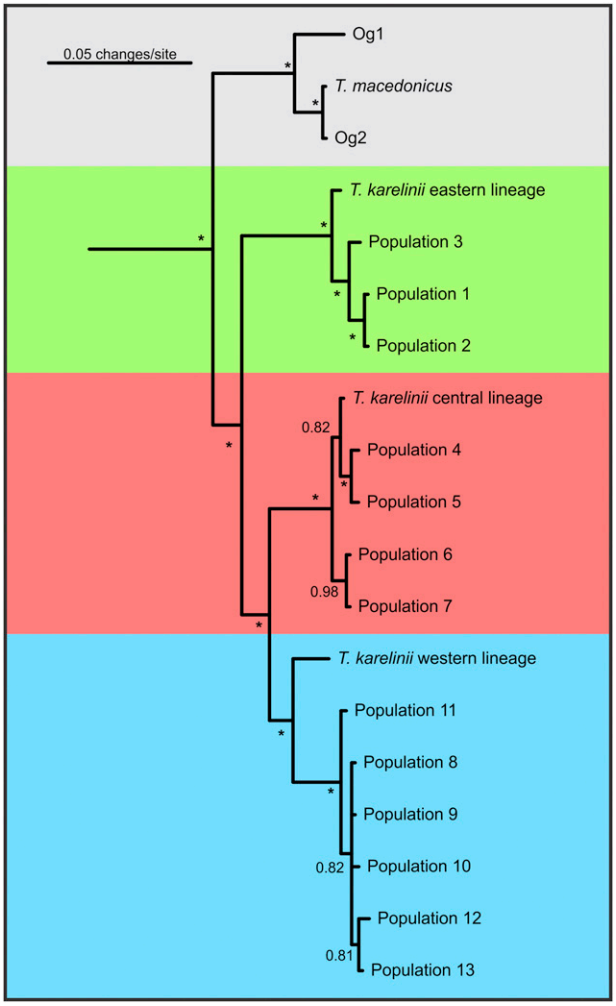

Fig. 3. A phylogeny showing the full mitochondrial DNA backbone (used for the analyses for genetic signal), together with the ND4 haplotypes representing the 13 *Triturus karelinii* and two *T. macedonicus* populations. The three *T. karelinii* lineages are shown with a different background color, which corresponds to Fig. 1. The outgroup species *Calotriton asper* and *T. marmoratus* used for rooting the phylogeny are not shown. See Table 1 for sampling details. Node support is indicated as Bayesian posterior probability, with those nodes with a support of 1.0 denoted with an asterisk. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(results not shown). We pooled populations within lineages and calculated lineage-specific allometric slopes. The divergence between slopes was explored through MANCOVA with DNA lineage as factor, lnCS as a covariate and lineage × lnCS interaction, using PROC GLM procedure in SAS (SAS Institute Inc., Cary, NC, version 9.1.3). The statistically significant lineage × lnCS interaction indicates that lineage-specific allometric slopes significantly diverge. The divergence between lineages specific allometric slopes was further explored by comparing the angles between vectors of allometric coeffcients using MorphoJ software (Klingenberg, 2011). The statistical significance of divergence was tested against the null hypothesis that the vectors have random directions in shape tangent space.

2.4.4. Phylogenetic signal

The null hypothesis of a complete absence of phylogenetic signal in skull shape was tested by randomly permuting the landmark configurations of skull shape over the terminal units of the phylogenetic tree (10,000 iterations) using MorphoJ software (Klingenberg, 2011), in which the test statistic is the total amount of squared change summed over all branches of the tree. Rejection of the null hypothesis indicates that there is phylogenetic signal in the analyzed dataset.

The character states for the internal nodes were estimated from the average values for terminal units under the criterion of squared-change parsimony (Maddison, 1991; McArdle and Rodrigo, 1994;

Table 2
Analysis of variance on ventral skull size (top panel) and dorsal skull size (lower panel) in female and male *Triturus karelinii*, with percentage of variation explained. df, degrees of freedom; MS, mean squares; *F*, *F*-test statistic; *P*, statistical significance.

Source of variation	Females					Males				
	df	MS	*F*	$P^{\#}$	Variance explained	df	MS	*F*	$P^{\#}$	Variance explained
Ventral side					52.0%					52.8%
Lineage	2	0.00880	2.56	0.0829	2.9%	2	0.01356	4.74	0.0113	4.5%
Populations within lineage	10	0.02999	8.73	**<0.0001**	49.3%	10	0.02293	8.01	**<0.0001**	48.4%
Dorsal side					52.3%					41.8%
Lineage	2	0.01261	3.94	0.0232	5.4%	2	0.01590	5.54	**0.0055**	5.4%
Populations within lineage	10	0.02691	8.40	**<0.0001**	45.4%	6	0.01366	4.76	**<0.0001**	32.9%

$^{\#}$ Significances with Holm-Bonferroni correction (Holm, 1979) at a tablewide critical level of alpha = 0.05 are shown in boldface.

Table 3
Multivariate analysis of variance for ventral skull shape (top panel) and dorsal skull shape (lower panel) in females and males of *Triturus karelinii*, with Wilks' lambda and generalized η^2 as a measure of the strength of association between dependent and independent variables. df_1, model degrees of freedom; df_2, error degrees of freedom; *F*, *F*-test statistics; *P*, statistical significance.

Source of variation	Females						Males					
	Wilks' lambda	df_1	df_2	*F*	$P^{\#}$	η^2	Wilks' lambda	df_1	df_2	*F*	$P^{\#}$	η^2
Ventral side												
Lineage	0.01731012	42	130	20.43	**<0.0001**	0.98269	0.06211424	42	128	9.18	**<0.0001**	0.937886
Populations within lineage	0.00008056	210	612.55	5.33	**<0.0001**	0.99992	0.00004467	210	603.48	5.80	**<0.0001**	0.999955
Dorsal side												
Lineage	0.03573897	44	122	11.89	**<0.0001**	0.964261	0.06949216	44	126	8.00	**<0.0001**	0.930508
Populations within lineage	0.00015407	220	581.26	4.26	**<0.0001**	0.999846	0.00014141	220	600	4.46	**<0.0001**	0.999859

$^{\#}$ Significances with Holm-Bonferroni correction at a tablewide critical level of alpha = 0.05 are shown in boldface.

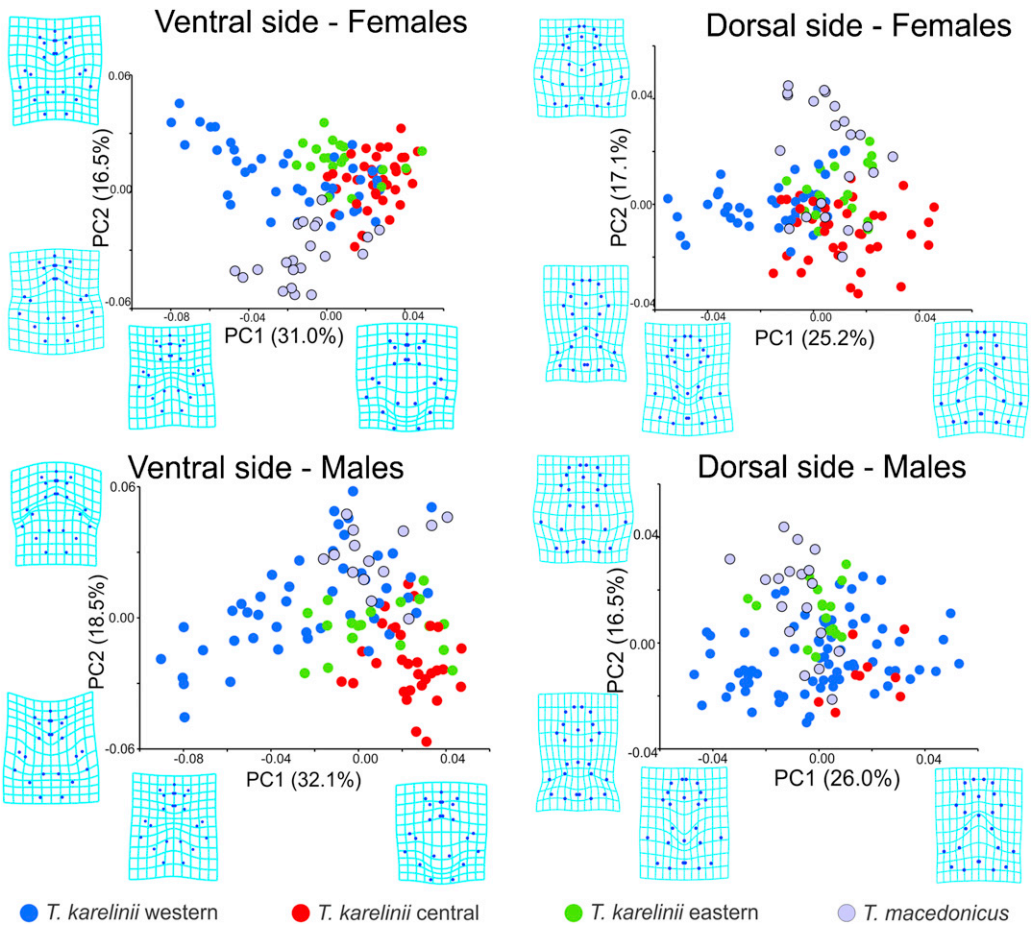

Fig. 4. Ordination of individuals belonging to the *Triturus karelinii* lineages (eastern – green; central – red; and western – blue) and *T. macedonicus* (gray) over the first (PC1) and second (PC2) principle component axes obtained from the PCA of skull shape variables, for females (top diagrams) and males (bottom diagrams). Ventral skull shape morphospace is shown on the left and dorsal morphospace on the right. The thin-plate spline deformation grids illustrate the skull shape changes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Rohlf, 2001, 2002; Klingenberg and Gidaszewski, 2010), weighted by molecular change on the respective branches of the tree.

3. Results

3.1. Sexual dimorphism

A phylogeny showing the distinction among the three lineages is shown in Fig. 3. We explored the pattern of sexual dimorphism in skull size and skull shape within each lineage separately (Tables S1 and S2). The statistically significant sexual dimorphism in skull size and shape was recorded in two lineages (central and western lineage), while no significant sexual dimorphism in skull size and shape within eastern lineage was recorded. Due to a statistically significant but inconsistent pattern of sexual dimorphism within and between analyzed lineages, males and females were treated separately in all further analyses.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcz.2012.08.005.

3.2. Divergence in skull size and shape

The variation in skull size within lineages (among populations) is much more pronounced than variation between lineages. The among lineage variation in skull size is statistically significant only for the dorsal side in males while among population variation is significant for both sexes (Table 2 and supplementary information in Tables S3 and S4).

The three *T. karelinii* lineages differ significantly in skull shape with significant divergences among populations within lineages ($P < 0.0001$ in all tests, Table 3). Pairwise comparisons of skull shape at the population level indicate that statistically significant differences between populations exist within all three lineages, and that divergences in skull shape are more pronounced between males from different populations than between females (supplementary information in Table S5). We found no statistically significant concordance between skull shape distances and genetic distances within lineages (Mantel test, $P > 0.05$ for dorsal and ventral skull side of both sexes).

Table 4
Procrustes distances between groups, *in casu* the eastern, central and western lineages of *Triturus karelinii* and the outgroup *T. macedonicus*. Values for females below the diagonal and values for males to the right of the diagonal. All distances are statistically significant ($P < 0.001$) after Bonferroni correction.

	T. karelinii			*T. macedonicus*
	Eastern	Central	Western	
Ventral side				
T. karelinii				
Eastern lineage		0.0377	0.0397	0.0572
Central lineage	0.0383		0.0553	0.0600
Western lineage	0.0368	0.0520		0.0512
T. macedonicus	0.0572	0.0578	0.0483	
Dorsal side				
T. karelinii				
Eastern lineage		0.0277	0.0307	0.0267
Central lineage	0.0277		0.0318	0.0340
Western lineage	0.0307	0.0318		0.0325
T. macedonicus	0.0267	0.0340	0.0343	

The differences in shape between the lineages are highly statistically significant (Table 4). The shape dissimilarity of the central and western lineage compared with the eastern lineage is about equally marked as that of *T. macedonicus* with any of the *T. karelinii* lineages (Table 4). Bivariate scatterplots indicate that the central and western lineages are differentiated along the first PCA axis, with the eastern lineage taking an intermediate position (Fig. 4). On the second axis, *T. macedonicus* differentiates from the *T. karelinii* group. Shape changes shown in thin-plate spline deformation grids indicate that the central lineage has a shorter skull base (described by landmarks 1–8 and 24–33) and an elongated palatal and rostral skull portion (described by landmarks 11–23 and 34–47) compared to the western lineage. The western lineage has the shortest skull base, and shorter rows of vomeral teeth (described by landmarks

Table 5
The effects of mitochondrial DNA lineage, size and their interaction for ventral skull shape (top panel) and dorsal skull shape (lower panel) in females and males of *Triturus karelinii*, tested by a multivariate analysis of covariance (MANCOVA). df_1, model degrees of freedom; df_2, error degrees of freedom; *F*, *F*-test statistics; *P*, statistical significance.

Source of variation	Females					Males				
	Wilks' lambda	df_1	df_2	*F*	$P^{\#}$	Wilks' lambda	df_1	df_2	*F*	$P^{\#}$
Ventral side										
Lineage	0.29547055	42	144	2.88	**<0.0001**	0.48394366	42	142	1.48	0.0475
Size (ln CS)	0.35489663	21	72	6.23	**<0.0001**	0.46306805	21	71	3.92	**<0.0001**
Lineage × size (lnCS)	0.30162257	42	144	2.81	**<0.0001**	0.49298384	42	142	1.43	0.0620
Dorsal side										
Lineage	0.34641619	44	136	2.16	**0.0004**	0.35580681	44	140	2.15	**0.0004**
Size (ln CS)	0.34673315	22	68	5.82	**<0.0001**	0.36462389	22	70	5.54	**<0.0001**
Lineage × size (ln CS)	0.35234249	44	136	2.12	**0.0006**	0.35857938	44	140	2.13	**0.0005**

[#] Significances with Holm–Bonferroni correction at a tablewide critical level of alpha = 0.05 are shown in boldface.

Table 6
The significance of allometry and percentage of shape changes explained by size in each mitochondrial DNA lineage. *N*, number of specimens; *P*, statistical significance against the null hypothesis of independence of size and shape.

Lineage	Females			Males		
	N	$P^{\#}$	% explained	*N*	$P^{\#}$	% explained
Ventral side						
Eastern	19	**<0.0001**	25.88	19	0.0328	14.11
Central	38	**0.0061**	7.70	32	**0.0006**	10.57
Western	32	**0.0006**	10.57	46	**0.0050**	8.79
Dorsal side						
Eastern	18	**0.0009**	17.48	18	**0.0011**	14.69
Central	37	**0.0011**	9.53	32	0.7611	2.03
Western	40	**<0.0001**	12.73	47	**0.0014**	7.15

[#] Significances with Holm–Bonferroni correction at a tablewide critical level of alpha = 0.05 are shown in boldface.

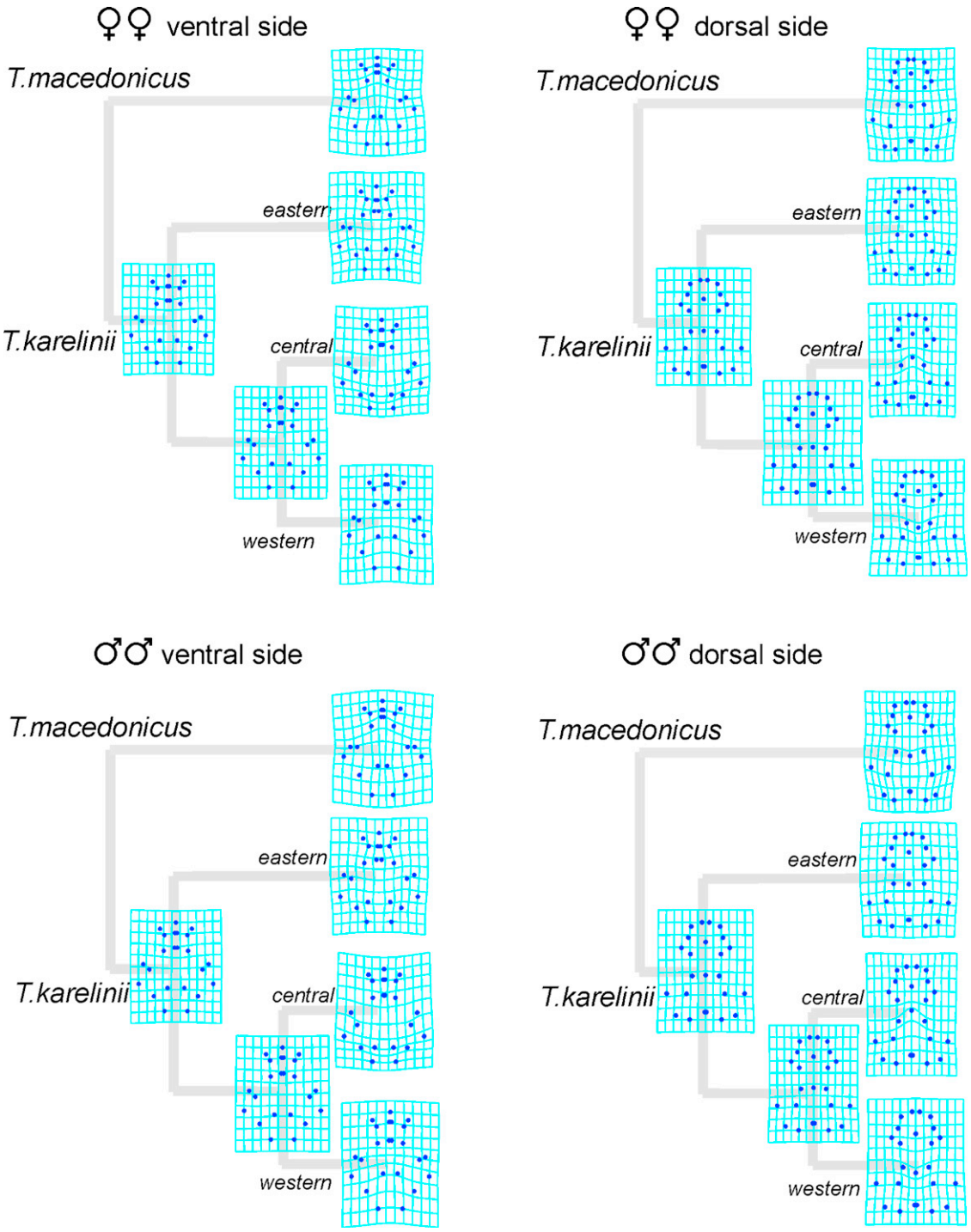

Fig. 5. The skull shape changes mapped onto the phylogeny of the *Triturus karelinii* group. Top diagrams – females; bottom diagrams – males. The thin-plate spline deformation grids are exaggerated by a factor 3.

9–12) comparing to central and eastern lineage. The central lineage stands apart from both other lineages by the longest rows of vomeral teeth (described by landmarks 9–12).

3.3. Allometry

The MANCOVA (Table 5) revealed a significant lineage × size interaction in all comparisons except for the ventral skull shape changes in males. The multivariate regression of shape on log-transformed CS within each lineage separately revealed that shape changes were significantly correlated with changes in size (Table 6). The allometry is statistically significant and explains a relatively high percentage of shape changes within each lineage (from 7 to 25%). Exceptions are ventral skull side of males (eastern lineage), and dorsal skull side of males (central lineage) – in these two cases no statistically significant relationship between size and shape was found (Table 6).

We performed pairwise comparisons and calculated angles between statistically significant allometric vectors. This analysis revealed that lineage-specific allometric vectors of ventral skull side in females significantly diverge: the allometric vector of the western lineage significantly diverges from the allometric vector

of the eastern lineage (87.63°) and from the allometric vector of the central lineage (72.84°).

3.4. Phylogenetic signal in skull shape

The permutation tests for phylogenetic signal in skull size and skull shape with the *T. karelinii* lineages as terminal units, weighted by molecular change on the respective branches of the tree, yielded no statistically significant results ($P > 0.05$ in all cases). We mapped skull shape on the tree. (Because lineages do not differ in skull size, the size was not mapped on the mitochondrial DNA phylogenetic tree.) The difference in skull shape among the three *T. karelinii* lineages is reflected by differentiation in the dorsal shape of the skull roof, in particular in the relative size of frontal bones, prefrontal bones and the occipital region (Fig. 5). The central lineage is characterized by shorter frontal bones (described by landmarks 34–37, 46, 47), elongated parietal bones and squamosums positioned closer to the mid-sagittal plane (described by landmarks 24–35) compared to the western lineage, which has larger and laterally moved squamosal bones.

4. Discussion

Our results show that the pattern of variation in skull size and shape in the *T. karelinii* group is very complex and it is characterized by: (i) a high divergence between populations and variable patterns of sexual dimorphism within mitochondrial DNA lineages, (ii) significant divergence in skull shape but not in skull size between mitochondrial DNA lineages, including significant divergence in allometry of the ventral skull side in females, and (iii) a lack of concordance between the pattern of morphological and genetic variation within lineages and the absence of phylogenetic signal in skull size and skull shape.

The observed pattern of high morphological variation between populations along with variable patterns of sexual dimorphism is not surprising. In newts, a marked sexual dimorphism in overall body size (Arntzen, 2000; Ivanović et al., 2008) and in skull shape and size (Ivanović and Kalezić, 2012) exists. Previous studies of skull size and shape variation in crested newts (Ivanović et al., 2009; Ivanović and Kalezić, 2012) show that high variation in size and shape exists within and between crested newt species. In taxa with indeterminate growth, body size is largely dependent on individual age, environmental conditions and habitat resources (Arntzen, 2000; Gotthard, 2001; Cogălniceanu and Miaud, 2003). Therefore, high variation in size between populations within *T. karelinii* lineages is to be expected. Also, newts have a low dispersal ability and they are tied to their aquatic habitat (e.g. Sinsch, 1990). The combination of low mobility and philopatric behavior of newts (e.g. Kupfer and Kneitz, 2000; Maletzky et al., 2010; see also Arntzen, 2003, and references therein), would promote interpopulation differentiation at a small spatial scale. On the other hand, phenotypic differentiation could reflect phenotypic plasticity (West-Eberhard, 1989; Adams and Rohlf, 2000; Muschick et al., 2011). In general, the observed pattern of divergence in skull size and shape among populations could reflect a response to spatial variation in resources and/or community structure (see Butler, 2007). Numerous environmental factors such as habitat size and population density and food availability, predation and competition by other species can affect skull morphology (Adams and Rohlf, 2000; Muschick et al., 2011). Clearly, more detailed studies, especially those addressing functional morphology of the feeding apparatus, are needed to explore potential influences of adaptation and phenotypic plasticity on skull shape.

The *T. karelinii* mitochondrial DNA lineages differ in skull shape and in allometric relations of the ventral skull, i.e. the observed divergences in ventral skull shape between lineages are not the result of shape changes along a common allometric slope. This finding indicates divergence in the covariation pattern of structures which are more directly related to foraging. Unfortunately, the small sample size per population and per sex prevents us to analyze population-specific allometric slopes. Therefore, we cannot exclude the possibility that allometric slopes could diverge between populations as well.

The pattern of skull shape divergence among *T. karelinii* mitochondrial DNA lineages is not concordant with the phylogeny. In terms of morphometrics, phylogenetic signal exists if closely related taxa occupy a similar portion of morphometric space, whereas more distantly related taxa are further removed in morphometric space (Klingenberg and Gidaszewski, 2010). The phenetic similarity of the mitochondrial DNA lineages could have been inherited from a common ancestral lineage. However, factors besides phylogenetic dependence could reflect the geographical proximity of lineages resulting from a shared history, such as drift, selection and gene flow between populations (Stone et al., 2011). Again, the absence of concordance between genetic and morphological variation among lineages point to ecological specialization.

The visualization of skull shape at internal nodes of the phylogeny provides insight into the evolution of this complex morphological structure in the *T. karelinii* group. According to the reconstructed shape changes, the eastern lineage largely retains the ancestral shape. The divergence in skull shape in the central and western lineage is related to changes in the relative size of the palatal and rostral region relative to skull base. These changes show opposite pattern in the central and western lineages, with the central lineage showing shorter rostral region and elongated skull base, and the western lineage showing elongated rostral region and shorter skull base. Further studies of the pattern of shape changes on a broader range of crested newt taxa, using an increased sample density while paying close attention to the phylogenetic context, can be used to test whether observed evolutionary changes in the *T. karelinii* group is associated with lineages-specific changes, such as founder events or the adaptation to new habitats. Studies of ontogenetic shape changes could point to the underlying mechanisms and processes that lead to the divergence between populations and between lineages, and indicate whether the same processes and mechanisms that produce intraspecific morphological variation also produce morphological variation among crested newts species.

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Appendix A.

A.1. Specimen data – Institute for Biological Research, "Siniša Stanković" (IBISS) herpetological collection. Number of specimens are given in parentheses

Triturus karelinii – eastern lineage: Tbilisi vicinity, Georgia (6) 22242–22247; Ersi Lake, Dagestan (18) 22802, 22804–22821; Lake Kutuzovskoe, Krym, Ukrain (16) 22822–22837. *Triturus karelinii* – central lineage: Şerefiye, Turkey (20) 22439–22458;

Reşadiye, Turkey (20) 22500–22519; Turkey, Kalecik (15) 22479–22493; Tosya, Turkey (18) 22828–22855. *Triturus karelinii* – western lineage: Klaros, Turkey (20) 22510–22539; Mersinbeleni, Turkey (17) 22459–22476; Büyük Kalecik, Afyon, Turkey (11) 22876–22887; Uludağ, Bursa, Turkey (19) 22856–22874; Dadia, Greece (11) DA1–DA11; Saint Kosmas, Greece (10) SK1–SK10. *Triturus macedonicus*: Rid, Montenegro (20) C30_1M/C30_4M/C30_5M/C30_6M/C30_8M-C3015M/C30_2F/C30_4F–C30_7F/C30_10F–C30_18F; Elafotopos, Greece (15) ZG1–ZG15.

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