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A phylogenetic view on skull size and shape variation in the smooth newt (*Lissotriton vulgaris*, Caudata, Salamandridae)

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Abstract

In this study, we explore skull size and shape variation in the smooth newt, a taxon with substantial morphological differentiation and complex phylogeographic relations. By projecting phylogenies into the morphospace of the skull shape, we explore the variation in and differentiation of this complex morphological structure within a phylogenetic framework. For these analyses, we used a dataset that covers the most southern part of the species' distribution range, including all conventionally recognized subspecies. The study revealed different patterns of divergence in skull shape between sexes, which is paralleled by intraspecific differentiation. The divergence in dorsal skull shape is concordant with the phylogenetic divergence, as the most diverged clades of the smooth newt (*Lissotriton vulgaris kosswigi* and *Lissotriton vulgaris lantzi*) exhibit a skull shape that significantly diverges from the smooth newt's mean shape configuration. The results of this study also indicate that ventral skull portion, which is more directly related to feeding and foraging, shows higher variation between populations than dorsal skull portion, which appears to be less variable and phylogenetically informative.

Key words: Geometric morphometrics – morphospace – phylogenetic signal – tailed amphibians

Introduction

Analyses grounded within a phylogenetic context extend our knowledge of evolutionary changes, as a phylogenetic framework is essential in assessing evolutionary trends as well as to ascertain whether observed similarities result from common descent or were acquired independently. Testing for phylogenetic signal, defined as the degree to which phenotypic similarities are congruent with phylogeny, can help to discern between these two possibilities (e.g. Blomberg et al. 2003; Cardini and Elton 2008).

Regarding the object of our investigations, we focus on the smooth newts (*Lissotriton vulgaris*). These small-bodied newts belong to the group of Modern Eurasian newt (sensu Steinfartz et al. 2007). As other tailed amphibians, these newts exhibit a marked homing behaviour and a relatively small dispersal range and they are particularly deme-structured organisms. Such characteristics indicate an island model of speciation (Wright 1931; Maruyama 1970). The smooth newt demonstrates substantial genetic differences not only among different subspecies (Nadachowska and Babik 2009), but also among local populations as well (Kalezić and Tucić 1984). It is very likely that the observed pattern of phenotypic variation might be driven mostly by environmental factors, sexual selection or drift.

The monophyletic group of the smooth newt (*Lissotriton vulgaris*, Linnaeus, 1758) displays the broadest geographic range among all Modern Eurasian newts, encompassing both formerly glaciated and unglaciated areas of north-west, central and eastern Europe, including southern Scandinavia, western Asia and Asia Minor (Kuzmin and Zuiderwijk 1997). According to recent reviews based on external morphological traits (Raxworthy 1990; Schmidtler and Franzen 2004), seven subspecies are considered to be valid (see Fig. 1): *Lissotriton vulgaris ampelensis* (Fuhn, 1951); *Lissotriton vulgaris graecus* (Wolterstorff, 1905); *Lissotriton vulgaris kosswigi* (Freytag, 1955); *Lissotriton vulgaris lantzi* (Wolterstorff, 1914); *Lissotriton vulgaris meridionalis* (Boulenger, 1882); *Lissotriton vulgaris schmidtlerorum* (Raxworthy, 1988); and *Lissotriton vulgaris vulgaris*, (Linnaeus, 1758). The nominotypical subspecies occupies most parts of the species range while most of the smooth newt evolutionary diversification occurred within the Apennine and Balkan peninsulas, Turkey and Caucasus region where subspecies are confined to much smaller ranges. Contrary to the other Modern Eurasian newt species, subspecific differentiation of the smooth newt is based almost exclusively on male epigamic integumental traits (Raxworthy 1990; Griffiths 1996). Smooth newts display some rather complex phylogeographic relations. The most recent mtDNA phylogenetic analysis (Babik et al. 2005) revealed 12 major clades. Their age was estimated at *c.* 4.5–1.0 million years. Most of the older clades were found in the southern part of the range, and also in central Europe, mainly in Romania. Some significant discrepancies between morphological data versus allozymic and molecular data (Kalezić 1984; Babik et al. 2005, respectively) have been also reported.

The skull represents a developmentally and functionally complex morphological structure and as such it is of special interest in phylogenetic studies (e.g. Marroig and Cheverud 2001; Nicola et al. 2003; Monteiro and Dos Reis 2005; Cardini and Elton 2008). Numerous biological functions of the skull (e.g. food acquisition and processing, sight, hearing, olfaction

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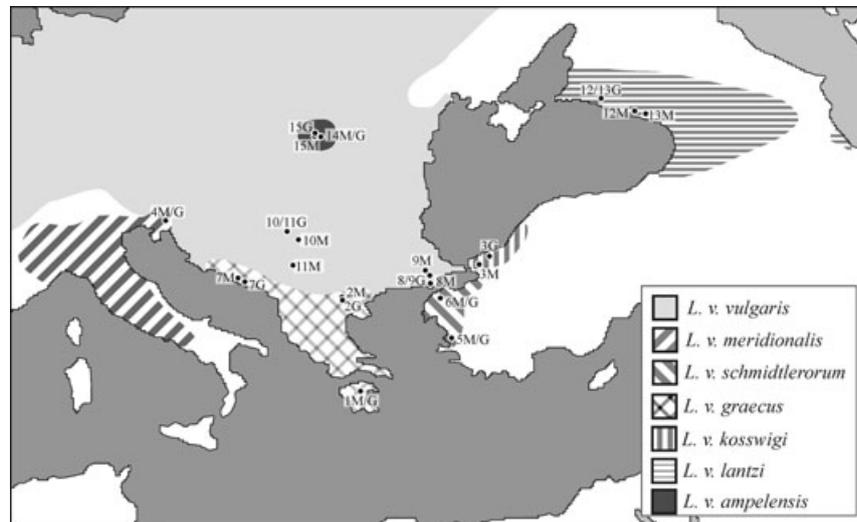
Fig. 1. Geographic positions of the 15 analysed populations and their affiliations to the conventionally recognized subspecies (Raxworthy 1990; Schmidtler and Franzen 2004). Locality designations are provided in Table 1. M, locality for morphological data; G, locality for genetic data

and protection of the brain) indicate that the morphological variation in skulls has often adaptive significance. Different patterns of skull shape variation within a phylogenetic context have been previously found in the crested newts (Ivanović et al. 2008) and the alpine newts (Ivanović et al. 2009). In this paper, we explored skull size and shape variation in the smooth newts (*L. vulgaris*) within a phylogenetic framework. For this purpose, we used a dataset covering the southern part of species' distribution range including all conventionally recognized subspecies. We projected smooth newt's phylogenies onto the morphospace of skull size and shape in order to (1) explore for shape differences; and (2) test the significance of the phylogenetic signal in cranial morphology.

Materials and Methods

Sample collection

For the purpose of this study, we were able to provide and to analyse population samples of adult individuals that originated from the very same or nearby populations that had been used for previous phylogenetic analysis (Babik et al. 2005). A total of 328 individuals with a complete landmark set for the dorsal skull view and 330 individuals with a complete landmark set for the ventral skull view were included in the analysis (Fig. 1; Table 1). The specimens used in this study were part of two herpetological collections (Institute of Biological Research "Siniša Stanković" Belgrade 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" Belgrade. All individuals used for skeletal preparations were adults, with well-developed secondary sexual characteristics and gonads.

Skeleton preparation

Due to their small body size and fragile skeletons, the specimens were first cleared with trypsin and KOH, stained with Alizarin Red S for bone depositions (Dingerkus and Uhler 1977) and stored in glycerine. Images of the ventral and dorsal skull, with the palate and skull roof positioned parallel to the photographic plane, were obtained with a Nikon SMZ1000 stereoscopic loupe with mounted digital camera (2592 × 1944 pixels resolution). The skulls were positioned in the centre of the optical field to reduce and equalize distortion. The configuration of 13 two-dimensional landmarks on the dorsal and 13

landmarks on the ventral skull side, which have been previously used to describe skull shape in newts (Ivanović et al. 2007, 2008, 2009), was digitized by the same person (A. I.) using TPSDIG software (Rohlf 2005) on the right side of each specimen (Fig. 2).

Phylogenetic analysis

To produce a molecular phylogeny with exact branch lengths of all the morphologically analysed populations (Fig. 1, Table 1), we used published sequences of the respective or geographically proximate populations from two mitochondrial DNA fragments: a 1016-bp fragment comprising 951 bp of the ND2 gene and almost the entire tRNA-Trp gene (further referred to as 'ND2' after Babik et al. (2005)), and an approximately 835-bp fragment covering 683 bp of the ND4 gene, the whole tRNA-His and tRNA-Ser genes and 15 bp from the 5′ end of the tRNA-Leu gene (further referred to as 'ND4' after Babik et al. 2005). For two of the examined populations, only 'ND4' was available (Nestani: JF681230, and Strumičko polje: JF681231; K. Sotiropoulos and K. Eleftherakos unpublished data). Since these two populations bear ND4 sequences of the previously recognized smooth newt clades C and K, respectively (Babik et al. 2005), we used clade-specific ND2 sequences to complement the available ND4 sequences (for Nestani, haplotype C1 ND2: AY951493; Babik et al. 2005; for Strumičko polje, haplotype K1 ND2: AY951343; Babik et al. 2005). We used *Lissotriton helveticus* as the outgroup (ND2: AY951503; ND4: AY951648, Babik et al. 2005).

The alignment of the concatenated ND2 and ND4 sequences was performed with Clustal X (Thompson et al. 1997) and corrected by eye. Phylogenetic inference was analysed using the maximum-likelihood (ML) method (Felsenstein 1981). Nucleotides were used as discrete, unordered characters. To examine whether the sequences from the two gene fragments should be combined in a single analysis, a partition homogeneity test was run in PAUP (v.4.0b10, Swofford 2002), and significance was estimated by 10 000 repartitions. This test, which was described as the incongruence length difference test by Farris et al. (1995), indicated no conflicting phylogenetic signals between the datasets (p = 0.99), and given that the mtDNA genes are linked, datasets from both gene fragments were analysed together. The best-fit model of DNA substitution and the parameter estimates employed for tree construction were chosen under the Akaike Information Criterion (AIC; Akaike 1974) in Modeltest (v.3.7, Posada and Crandall 1998). The general time reversible (GTR) model with rate heterogeneity and a zero proportion of invariable sites had the highest likelihood score (−ln L = 5631.2720) and showed a significantly better fit than the other models (model parameters: GTR + G, G-shape parameter with four discrete rate categories = 0.176; base frequencies A = 0.3293, C = 0.2504, G = 0.1297, T = 0.2905). A ML tree with

Table 1. Samples for skeleton analyses and available genetic data

Locality morphology (M)		Coordinates	♀♀	♂♂	Locality genetics (G)		Coordinate	Haplotype	Clade	Subspecies	GenBank accession number (ND2/ND4)
1M	Nestani	37°37'N 22°28'E	12	9	1G	Nestani	—	'Nestani'	C	L. v. graecus	AY951493/JF681230
2M	Strumičko polje	41°28'N 22°43'E	13	7	2G	Dojran	41°13'N 22°42'E	'Dojran'	K	L. v. graecus	AY951343/JF681231
3M	Gebze Kocaeli	40°47'N 29°25'E	10	10	3G	Adapazari	40°47'N 30°24'E	B1[a]	B	L. v. kosswigi	AY951342/AY951510
4M	Podstrmec	45°48'N 14°34'E	14	13	4G	Podstrmec	—	L4[a]	L	L. v. meridionalis	AY951462/AY951614
5M	Efes Izmir	37°55'N 27°20'E	15	15	5G	Efes Izmir	—	E4[a]	E	L. v. schmitlerorum	AY951400/AY951531
6M	Lapseki	40°21'N 26°41'E	13	14	6G	Lapseki	—	E12[a]	E	L. v. schmitlerorum	AY951436/AY951591
7M	Domanovići	43°08'N 26°41'E	11	12	7G	Domanovići	—	D1[a]	D	L. v. graecus	AY951501/AY951646
8M	Gelibolu	40°24'N 26°40'E	15	14	8G	Bolayir	40°31'N 26°46'E	E3[a]	E	L. v. vulgaris	AY951363/AY951534
9M	Keşan Edirne	40°51'N 26°37'E	10	10	9G	Bolayir	40°31'N 26°46'E	E9[a]	E	L. v. vulgaris	AY951362/AY951533
10M	Mutalj	44°07'N 20°28'E	15	15	10G	Jamena	44°52'N 19°04'E	L17[a]	L	L. v. vulgaris	AY951418/AY951640
11M	Tutin	42°59'N 20°19'E	11	12	11G	Jamena	44°52'N 19°04'E	L43[a]	L	L. v. vulgaris	AY951408/AY951573
12M	Krasnaya Polyana	43°41'N 40°12'E	15	15	12G	Kaluzhskaya	44°46'N 38°58'E	A6[a]	A	L. v. lantzi	AY951425/AY951584
13M	Malaya Ritsa	43°28'N 40°30'E	15	10	13G	Kaluzhskaya	44°46'N 38°58'E	A7[a]	A	L. v. lantzi	AY951426/AY951584
14M	Zlatna	46°07'N 23°13'E	–	3	14G	Zlatna	—	J1[a]	J	L. v. ampelensis	AY951500/AY951645
15M	Gârda de Sus	46°27'N 22°49'E	6	2	15G	Briheni	46°30'N 22°24'E	F3[a]	F	L. v. ampelensis	AY951367/AY951537

The localities are numbered: M, morphology; G, genetic data.
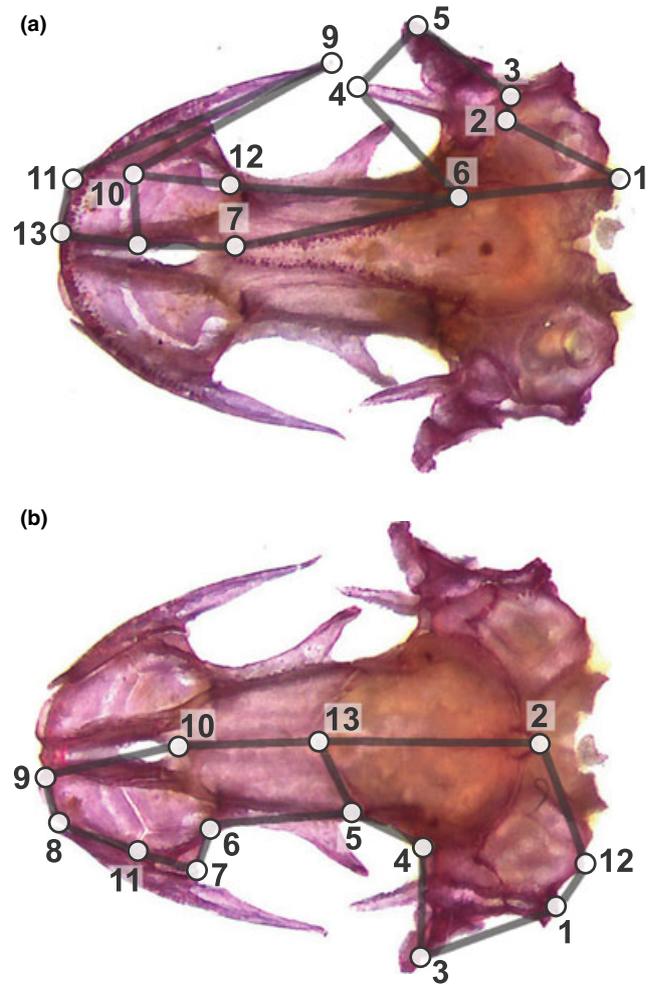[a]From Babik et al. (2005).

Fig. 2. The configuration of 13 two-dimensional landmarks that were identified for the ventral (a) and dorsal (b) skull view. (a) Ventral view: 1, tip of occipital condyle; 2, cranial base and posterior pterygoideum; 3, posterior pterygoideum; 4, anterior tip of pterygoideum; 5, most lateral point of quadratum; 6, vomeral teeth – posterior; 7, vomeral teeth – anterior; 8, most anterior point of vomer; 9, tip of maxilla; 10, most posterior point of premaxilla; 11, anterior end of suture between premaxilla and maxilla; 12, most lateral points of vomer that form the posterior margin of choana; 13, tip of the snout. (b) Dorsal view: 1, suture between squamosum and occipital; 2, tip of the occipital; 3, tip of squamosum; 4, suture between parietal and squamosum; 5, suture between parietal and frontal; 6, suture between prefrontal and frontal; 7, distal point of suture between prefrontal and maxilla; 8, maxilla at nasal opening; 9, premaxilla at nasal opening; 10, suture between frontals; 11, suture between prefrontal and maxilla; 12, most posterior point of occipital; 13, suture between frontal and parietal

Modeltest-derived parameters was constructed with the PHYML v.3.0 program using the method of Guindon and Gascuel (2003). We tested the robustness of the topology with 1000 bootstrap replicates. The same procedure was followed to construct a second phylogenetic tree without the population from Zlatna, to map skull size and shape of females. Again GTR + G had the highest likelihood score (−ln L = 5467.1802) and showed a significantly better fit than the other less complicated models (model parameters: GTR + G, G-shape parameter with four discrete rate categories = 0.179; base frequencies A = 0.3291, C = 0.2498, G = 0.1299, T = 0.2912).

Morphometric data analyses

To analyse variation in skull size and shape of ventral and dorsal part of the newt skull described by landmark configuration, we applied

standard geometric morphometric approaches. A generalized procrustes analysis (GPA) was applied (Rohlf and Slice 1990; Bookstein 1996; Dryden and Mardia 1998) to obtain a matrix of shape coordinates from which differences because of position, scale and orientation had been removed.

Skull size was computed as centroid size (CS), which is a measure of the amount of dispersion of landmarks around the centroid of the landmark configuration. To explore variation in CS, a geometric morphometric measure of size was calculated for each specimen. An ANOVA on CS with population, sex and their interaction as factors was performed. A significant interaction indicates differences in the magnitude or direction of sexual dimorphism in skull size across populations.

Skull shape variables were obtained by thin-plate spline analysis (Bookstein 1989). These shape variables were used to explore the variation in skull shape (ventral and dorsal skull shape). The variations in shape were analysed by MANOVA with population, sex and their interaction as factors. To investigate and to visualize variation in cranial shape, a PCA on populations' mean shapes was performed.

We applied a new procedure for testing the existence of a phylogenetic signal in the morphometric data, which is based on mapping geometric morphometric data onto a known phylogeny and extends a test described in Klingenberg and Gidaszewski (2010). The generalized method of least squares (Martins and Hansen 1997; Rohlf 2001) was used to find values for the internal nodes of the phylogeny from the shape averages of the terminal taxa (Maddison 1991; McArdle and Rodrigo 1994; Rohlf 2001, 2002). The phylogenetic signal in the shape data was tested following the procedure described by Klingenberg and Gidaszewski (2010). This test uses a permutation approach to simulate the null hypothesis of the complete absence of phylogenetic structure by randomly reassigning the phenotypic data to the terminal nodes of the phylogeny. The visualization of skull shape changes in the evolutionary morphospace, along with testing and quantification of phylogenetic signals in skull shape, was performed in the MORPHOJ software (Klingenberg 2011). All standard statistical procedures were performed using SAS statistical package (SAS statistical package vers. 9.1.3; SAS Institute 2009).

Results

Phylogenetic analysis

Within the 1851 sites examined, there were 180 variable ND2 sites, 92 of which were parsimony-informative (278 and 114, respectively, when the outgroup was included in the analysis), and 110 variable ND4 sites, 67 of which were parsimony-informative (209 and 81, respectively, including outgroup). Maximum-likelihood analysis of the complete population set under the GTR + G model resulted in a topology with ln L = −5633.3852 (G-shape parameter with four discrete rate categories = 0.176; nucleotide frequencies: A = 0.3314, C = 0.2464, G = 0.1299, T = 0.2922). The second analysis of the reduced population set under the GTR + G model resulted in a topology with ln L = −5467.2649 (G-shape parameter with four discrete rate categories = 0.179; nucleotide frequencies: A = 0.3318, C = 0.2464, G = 0.1296, T = 0.2923).

The obtained phylogenetic relations and tree topologies are concordant with the original tree of Babik et al. (2005). All the examined populations, each represented by a single haplotype (Table 1), represent all the major Balkan clades of the smooth newt as described in Babik et al. (2005): populations Krasnaya Polyana and Malaya Ritsa (ssp. *lantzi*) represent clade A, Gebze Kocaeli (ssp. *kosswigi*) clade B, Nestani (ssp. *graecus*) clade C, Domanovići (ssp. *graecus*) clade D, Efes Izmir, Lapseki, Gelibolu and Keşan Edirne (ssp. *schmitlerorum* and ssp. *vulgaris*) represent clade E, Gârda de Sus and Zlatna (ssp. *ampelensis*) represent clade F and J, respectively, Strumičko
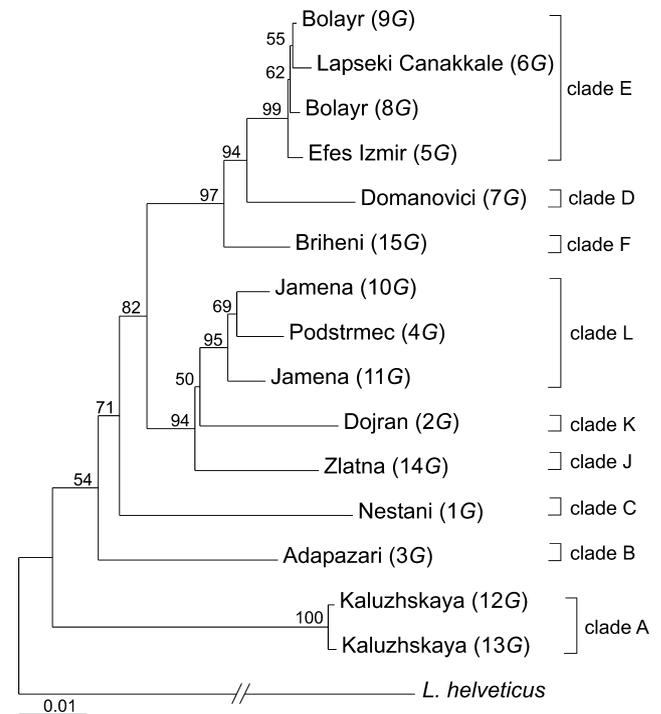

Fig. 3. Maximum-likelihood tree for the 15 smooth newt populations based on the combined ND2/ND4 mtDNA fragments. In cases where sequences from the morphologically analysed populations were lacking, we used geographically proximate ones (for population correspondence see Table 1). Bootstrap values (%) are shown on nodes

polje (ssp. *graecus*) clade K, while the remaining populations Podstrmec (ssp. *meridionalis*), Mutalj and Tutin (ssp. *vulgaris*) represent clade L (Table 1, Fig. 3).

Variation in skull size and shape

The two-way ANOVA on skull size revealed statistically significant variation among populations and a statistically significant interaction between population and sex (Table 2). However, statistically significant sexual dimorphism in skull size was found for dorsal skull only. To analyse variation in skull shape, a factorial MANOVA analysis was performed with population, sex and population × sex interaction as factors. A significant variation in both ventral and dorsal skull shape between populations and between sexes was found (Table 3). The population × sex interaction was also found to be significant, which indicated that the pattern of sexual dimorphism, for both dorsal and ventral skull shape, differed between populations (Table 3). Pairwise comparison between female and male mean shape configurations (after 10 000 permutations) found that females and males significantly differ in ventral skull shape. After correction for multiple testing (Bonferroni correction), the females and males significantly differ in four of the 14 populations (Efes, Lepseki, Podstrmec, Strumičko Polje), and in dorsal skull shape in tree out of the 14 populations (Efes, Lepseki, Strumičko Polje). Our results clearly indicate that population, sex and their interaction affect skull shape (Table 3). Therefore, all further analyses were performed on females and males separately.

Pairwise comparisons in dorsal skull size and shape (Table S1) revealed that populations Krasnaya Polyana (ssp.

Table 2. Results from the two-way ANOVA on skull size (centroid size) based on the landmark configurations of the ventral and dorsal skull view

Source of variation	df, error df	MS	F	p
Ventral skull				
Population	14 301	17.19	52.88	**< 0.0001**
Sex	1301	1.16	3.58	0.0595
Population × sex	13 301	1.11	3.43	**< 0.0001**
Dorsal skull				
Population	14 299	16.08	51.84	**< 0.0001**
Sex	1299	2.29	7.41	**< 0.01**
Population × sex	13 299	1.07	3.46	**< 0.0001**

Table 3. Multivariate Analysis of Variance for ventral and dorsal skull shape variables

Source of variation	Wilks' Lambda	F	df1	df2	p
Ventral skull					
Population	0.0025	5.93	364	3459.6	< 0.0001
Sex	0.5972	7.16	26	276.0	< 0.0001
Population × sex	0.2169	1.35	338	3260.6	< 0.0001
Dorsal skull					
Population	0.0049	5.56	336	3393.8	< 0.0001
Sex	0.6986	4.96	24	276.0	< 0.0001
Population × sex	0.2655	1.26	312	3205.4	< 0.01

Table 4. Permutation test against the null hypothesis of no phylogenetic signal. Tree lengths computed with weighted squared-change parsimony, and p values for the permutation test of phylogenetic signal

	Skull size		Skull shape	
	Ventral skull	Dorsal skull	Ventral skull	Dorsal skull
Females	0.1037	0.1063	0.0095	0.0059
p	0.2451	0.2150	0.4506	**0.0243**
Males	0.1030	0.1116	0.0095	0.0060
p	0.0762	0.0950	**0.0295**	**0.0282**

lantzi; clade A), Podstrmec (spp. *meridionalis*; clade L) and Domanovići (spp. *graecus*; clade D) diverge in both, skull size and skull shape from all other populations. Populations Gelibolu (spp. *schmitlerorum*) and Lapseki (ssp. *vulgaris*), both from clade E, also show significant divergence in skull shape from each other and from the remaining populations. For ventral skull size and shape (Table S2), populations Efes Izmir, Gebze Kocaeli, Nestani and Podstrmec found to be the most divergent ones based on ventral skull shape.

To explore the existence of any phylogenetic signal in smooth newt skull shape, we applied the evolutionary principal component analysis (Klingenberg 2010). The permutation test against the null hypothesis of no phylogenetic signal revealed no phylogenetic structure in skull size, and the existence of phylogenetic signal in skull shape, although marginally significant (Table 4). Since the phylogenetic signal was not significant for skull size, we further explored the patterns of shape variation in relation to phylogeny only.

To illustrate the patterns of shape variation in relation to phylogeny (Fig. 3), we superimposed the molecular phylogeny in the morphospace defined by the first two principal components of the ventral and dorsal skull shape. The first two axes described 62% of the total variation in ventral skull shape for females and 51% for males (Fig. 4).

The first principal axis summarized shape changes mostly related to change in the relative palate size and skull base. In both females and males, specimens from clade A, Malaya Ritsa and Krasnaya Polyana populations (spp. *lantzi*), clade F and J, Gârda de Sus and Zlatna populations (spp. *ampelensis*), and specimens from clade L, Podstrmec population (assigned to spp. *meridionalis*), display elongated vomers, laterally shifted palatines and shorter skull base compared to clade C – Nestani and K – Strumičko polje populations (spp. *graecus*), and clade E – Efes Izmir population (spp. *schmitlerorum*), which display short vomers and elongated skull base. The second axis summarized skull shape changes that were largely different between females and males. In females, these changes were mostly related to a narrowing of the snout, change in size of maxilla bones as well as change in the relative size of vomers and the position of choana. Individuals from Domanovići (clade D), Krasnaya Polyana (clade A) and Lapseki (clade E) display smaller praemaxillae, shorter maxillae and a wider skull comparing to individuals from Efes, Tutin, Gelibolu that cluster in clades E and L (spp. *vulgaris*), which exhibit narrower and elongated skulls, with wider snout, and elongated maxillae and vomers.

Individuals from Malaya Ritsa (clade A – spp. *lantzi*) and Nestani (clade C – spp. *graecus*) were clearly discriminated in the morphospace defined by the first two axes that describe variation in male skull shape. The specimens from Malaya Ritsa population displayed the shortest skull base. Males from Nestani (clade C) were characterized by the most elongated skull base and shortest vomers comparing to all other smooth newt populations, while males from Podstrmec population (clade L – spp. *meridionalis*) had the most elongated maxillary bones comparing to the remaining populations.

The first two axes summarized 54% of the total variation in dorsal skull shape for females and 52% for males (Fig. 5). In the phylo-morphospace defined by the first two PC axes, all specimens with a *vulgaris/schmidtlerorum* haplotype (clades E and L) were grouped together. These specimens exhibit a relatively narrower skull at jaw articulation point, narrower prefrontal bones and a posteriorly displaced lateral suture between frontal and parietal bones comparing to specimens from Gebze Kocaeli (clade B – spp. *kosswigi*). The specimens of clade A populations, Malaya Ritsa and Krasnaya Polyana (spp. *lantzi*), clade L (Podstrmec) and populations from Romania (clade J – Zlatna and clade F – Gârda de Sus),separate from the remaining populations mostly along the second axis. The specimens from these populations display elongated prefrontal and longer frontal bones compared to the other smooth newt populations.

Discussion

Skull size and shape variation in smooth newts

Our data suggest that clades A and B (ssp. *lantzi*, and ssp. *kosswigi*) largely diverge in skull shape. These two clades also diverge from clades E and L (ssp. *vulgaris* and ssp. *schmidtlerorum*). The populations of ssp. *vulgaris* and ssp. *schmidtlerorum* mostly cluster together. There may also be some parallel trends between main smooth newt phylogenetic lineages (Fig. 5). Population Domanovići (clade D), previously described as a subspecies (*Lissotriton vulgaris graecus* forma *tomasinii* Wolterstorff 1908), and clade L population Podstrmec (ssp. *meridionalis*) show parallel evolution in skull shape
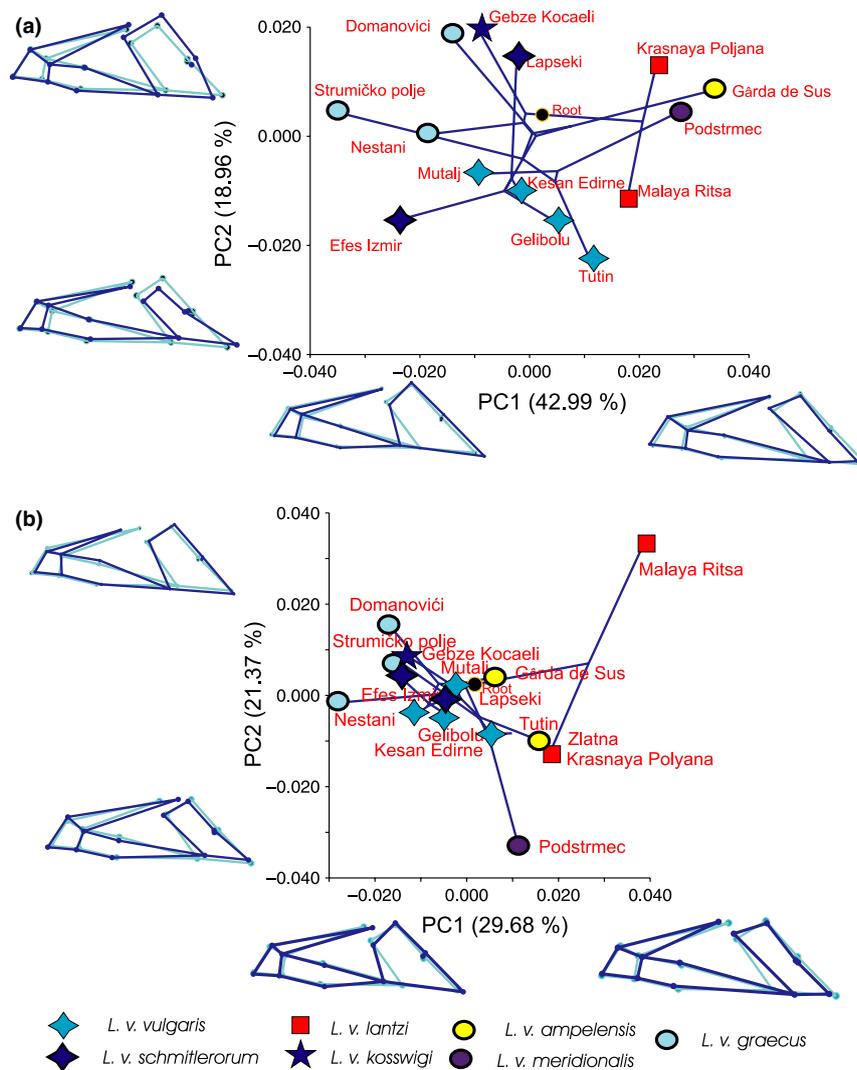
Fig. 4. Phylogenetic PCA with wireframe diagrams for the extremes of each axis. (a) Smooth newt females. (b) Smooth newt males

with the clade B Gebze Kocaeli population (ssp. *kosswigi*). These specimens display wider skulls at the otic region and larger prefrontal bones comparing to other smooth newt populations. Such parallelisms of variation may be due to the selection and adaptation, or a shared constraint such as allometry (see Klingenberg 2010). Our analyses reveal that most of the variation in smooth newt's skull shape, as described by landmark configuration of the ventral skull, is related to the variation in the relative size of maxilla and vomers, as well as to the changes in the relative size of the skull base. The variation in dorsal skull shape is related to the change in shape of prefrontal bones, and the changes in the skull width at the jaw articulation point.

The taxonomic implications of our results are apparently straightforward. Our results strengthen the validity of the taxonomic position of ssp. *lantzi* and ssp. *kosswigi*, which have been separated from the main stock of the smooth newt well before Pleistocene (Nadachowska and Babik 2009). Later, during Pleistocene climate shifts, *graecus*, *meridionalis* and *ampelensis* diverged from the nominotypical subspecies. Regarding skull shape, *schmidtlerorum* and *vulgaris* group together supporting the view of Olgun et al. (1999) that these clades should not be recognized as separate subspecies.

The presence of a phylogenetic signal in smooth newt skull shape variation is interesting for several reasons. First, this group is characterized by very complex phylogenetic relationships, which are not concordant with differentiation in external morphology (Babik et al. 2005). Second, recent studies dealing with the reconstruction of the temporal distribution of past gene flow have shown that, despite the deep pre-Pleistocene divergence, asymmetric introgression from *L. v. vulgaris* to *L. v. kosswigi* has occurred (Nadachowska and Babik 2009). Transition zones characterized by gradients of allozyme allele frequencies have been described between *L. v. vulgaris* and *L. v. meridionalis* (Kalezić 1984), and between *L. v. vulgaris* and *L. v. ampelensis* (Rafiński et al. 2001). Moreover, this is the first indication of morphological differentiation among subspecies, which was based, until now, only on secondary sexual characters and thus limited mostly to males.

Phylogenetic signal in skull size and shape

As it now stands, skull size seems phylogenetically uninformative in the Modern Eurasian newts (Ivanović et al. 2008, 2009; this study) and can be excluded of being a potential factor in the evolutionary shaping of the skull (for the issue of
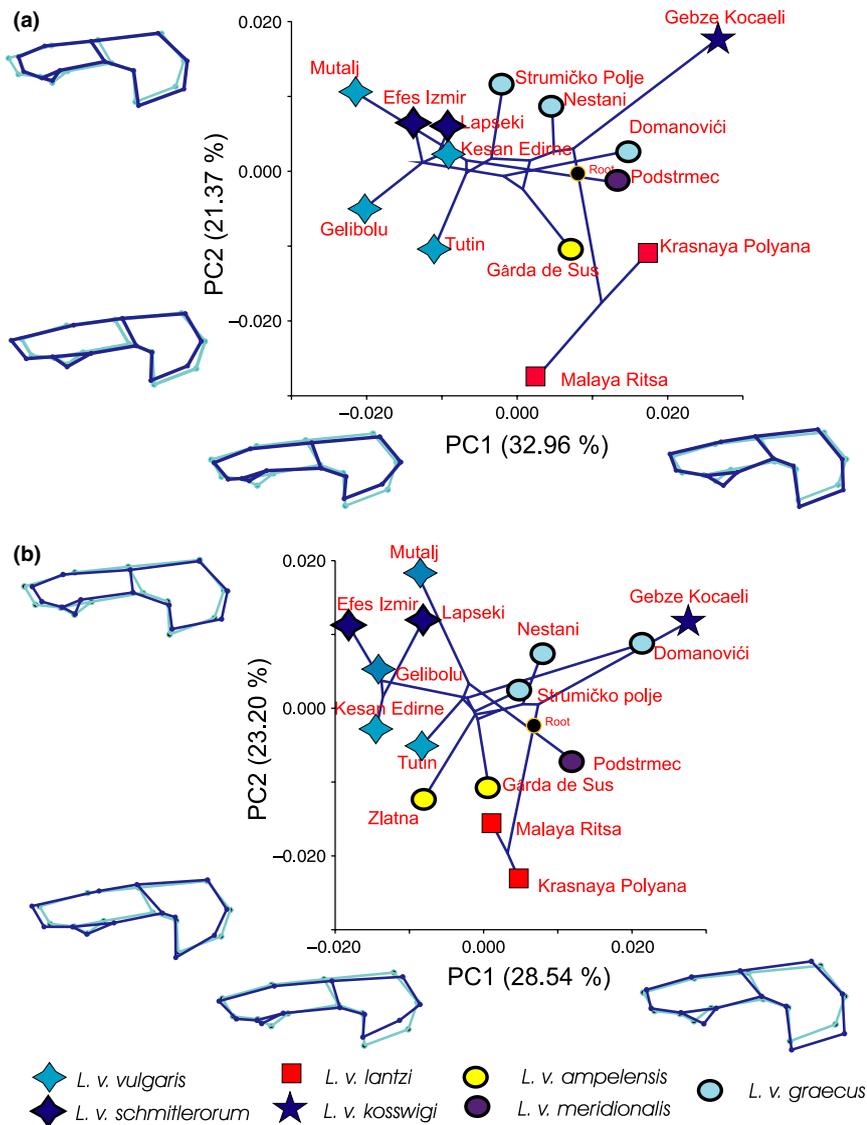
Fig. 5. Phylogenetic PCA with wireframe diagrams for the extremes of each axis. (a) Smooth newt females. (b) Smooth newt males

the relation between size and phylogenetic signal see Marroig et al. 2004; Cardini et al. 2005). The skull shape appears to be a phylogenetically informative trait in *Triturus* (Ivanović et al. 2008), *Ichthyosaura* (Ivanović et al. 2008) and *Lissotriton* (this study). Generally, Modern Eurasian newts, including the smooth newt, are characterized by strongly deme-structured population structure. Previous analyses on variation in skull shape in crested and alpine newts (Ivanović et al. 2008, 2009; respectively) and the results of this study showed high level of variation in skull shape between populations. Such pattern of variation between populations indicates ecological specialization. Ecological specialization and other evolutionary forces, such as drift, may trigger an uncoupling of molecular and phenotypic evolution, and the departure of phenotypic from phylogenetic patterns (Renaud et al. 2007). However, the phylogenetic signal in skull shape appears to be present in three studied genera of Modern Eurasian newts. The explanations for the phylogenetic signal in complex morphological entities, such as the vertebrate skull, were searched for in the interplay of development and numerous epigenetic factors affecting its integration level (e.g. Monteiro and Dos Reis 2005; Cardini and Elton 2008). By exploring the structure and patterns of morphological integration in newt skull, it is

possible to identify the trait associations and mechanisms (e.g. developmental and functional) that underlie and produce morphological integration, providing more insight in the pattern of morphological and ecological differentiation within and between Modern Eurasian newts.

Acknowledgements

Zusammenfassung

Eine phylogenetische Betrachtung von Schädelgröße und –form beim Molch Lissotriton vulgaris (Caudata, Salamandridae).

In dieser Studie untersuchen wir die verschiedenen Schädelgrößen und –formen von *Lissotriton vulgaris*, ein Taxon mit erheblicher morphologischer Differenzierung und komplexen phylogenetischen Beziehungen. Wir transformierten die Stammesgeschichte in die Schädelform und waren so in der Lage, Variation und Differenzierung

dieser komplexen morphologischen Struktur innerhalb eines phylogenetischen Rahmens zu erkunden. Für diese Analysen benutzten wir eine Datenbank, die den südlichsten Teil der Arten und deren Verbreitungsgebiet einschließlich aller bekannten Unterarten abdeckte. Die Studie zeigt, dass es unterschiedliche Muster in der Schädelform bei den Geschlechtern gibt, die durch intraspezifische Differenzierung gleichgerichtet verlaufen. Die Unterschiede im dorsalen Schädel stimmen mit phylogenetischen Unterschieden überein, da die unterschiedlichen Claden der Molche (*L. v. kosswigi* und *L. v. lantzi*) eine Schädelform aufweisen, die signifikant von den Durchschnitsswerten der Molche abweicht. Die Ergebnisse zeigen auch, dass der ventrale Schädel mit seiner größeren Beziehung zur Nahrungsaufnahme und Futtersuche eine höhere Variation zwischen den Populationen zeigt als der dorsale Schädel.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. The divergences in dorsal skull size and dorsal skull shape between populations.

Table S2. The divergences in ventral skull size and ventral skull shape between populations.

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