Morphological variability without geographical structuring in the fire salamander (Salamandra salamandra, Salamandridae) from the Central Balkans

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Abstract. In order to establish patterns of phenotypic variability, 15 quantitative and 3 qualitative body traits of 74 populations grouped in 14 geographic areas from the Central part of the Balkan Peninsula were examined. Although results of our multivariate analysis indicate existence of significant differences among populations belonging to different geographical groups, clear patterns of variation have not been found. Reason for the absence of geographical structuring of morphological variation in the fire salamander could be due to palaeogeographic situation of the Balkan Peninsula during Pleistocene.

Key words: morphometry, color pattern, Pleistocene, glaciations.

Introduction

The fire salamander, Salamandra salamandra (Linnaeus 1758), inhabits southern and central parts of Europe, from the Iberian Peninsula to the Black Sea, and from Italy to northern Germany. Fire salamander populations are absent in Great Britain, Ireland and Scandinavia (Griffiths 1996, Veith 1997). In the central Balkans, the fire salamander could be found in the highland area, up to 1700 m asl (Džukić 1993, Kalezić et al. 2000).

It is widely accepted that the fire salamander is a polytypic species (Griffiths 1996, Veith 1997, Steinfartz et al. 2000, García-París et al. 2003, Thiesmeier & Grossenbacher 2004, Martínez-Solano et al. 2005, Köhler & Steinfartz 2006). Intraspecific differentiation is most pronounced in the Iberian Peninsula where ten subspecies were recognized (Steinfartz et al. 2000, García-París et al. 2003, Köhler & Steinfartz 2006), of which nine are endemic. The central parts of Europe are inhabited by S. s. terrestris populations, the east part of Europe by the nominotypic S. s. salamandra, while S. s. gigliolii occurs in Italy (Steinfartz et al. 2000). The Balkan Peninsula is inhabited by three subspecies (S. s. salamandra, S. s. beschkovi and S. s. werneri, Džukić & Kalezić 2004). S. s. beschkovi and S. s. werneri are the endemic taxa of the southwestern part of Bulgaria and Peloponnesse, respectively, while S. s. salamandra occurs in the rest of the Balkans, extending in the east to the western parts of Ukraine (Pisane 2005). Establishing taxonomic validity and evolutionary distinctiveness of salamander subspecies is still a task in progress.

The three southern Mediterranean peninsulas (the Iberian, Apennines and the Balkans) are recognized as important glacial refugial regions and biodiversity hotspots (e.g. Hewitt 2000). However, in contrast to the other two regions, the Balkans remain understudied regarding amphibian diversity (Džukić & Kalezić 2004). For example, concerning the fire salamander, a lot of attention has been given to the Iberian Peninsula, where initially a number of subspecies was recognized on the basis of their morphological distinction, and afterwards were intensively genetically studied (Alcobendas et al. 1996, García-París et al. 2003, Martínez-Solano et al. 2005, Steinfartz et al. 2000). Up to now, some of the Balkan populations of the fire salamander have been included in genetic studies (see Joger & Steinfartz 1995, Steinfartz et al. 2000), while morphological characteristics were analyzed in populations from Bulgaria (Beškov & Cončev 1963) and Greece (Grillitsch & Grillitsch 1991, Veith 1994). To the best of our knowledge, comparative morphological analyses of populations from the Central Balkans (Serbia and Montenegro) have not been carried out.

When other parts of the distribution area of the fire salamander were taken into consideration, it is notable that most of the studies were devoted to revealing morphological differences between subspecies and had taxonomical connotation (e.g. Bosch & López-Bues 1994, Montori & Franceschi 1995, Degani 1986, Baran & Öz 1994).

Considering the small amount of data from
the Central Balkans and lack of studies that evaluate morphological variability within subspecies level, our main goal was to characterize the pattern of morphological variation and to determine the degree of differentiation between the populations of the fire salamander in the central part of the Balkan Peninsula.

Materials and methods

Population samples

We examined the specimens of the fire salamander deposited in the herpetological collections of the University of Pristina, temporarily seated in Kosovska Mitrovica, Faculty of Science and Mathematics, Biology Department and Institute for Biological Research, University of Belgrade. The specimens were previously collected from 74 localities (Fig. 1, Appendix) in different times and therefore, no animals were sacrificed for this study.

Figure 1. Location of the fire salamander population samples and population’s grouping.

The samples were pooled into 14 groups (see Fig. 1) according to their geographic position in order to obtain adequate sample size for statistical analyses. Geographic groups generally correspond to regional geographic division and to phyto- and zoogeographic characterization of the former Yugoslavia (Hadži 1931, Šegota 1967, Stesanović 1992). The locality data, i.e. the group affiliation, the name, coordinates, and the numbers of females and males collected are listed in the Appendix. In total, 415 sexually mature and 42 subadult ethanol-preserved specimens were analyzed.

Multivariate morphometrics

The analyses included only sexually mature individuals. Since many morphometric (quantitative) traits of the fire salamanders demonstrate significant sexual dimorphism (Kalezić et al. 2000), analyses were performed separately for females and males. The variability of 15 morphometric traits was studied: Svl – snout-vent length, Tl – tail length, Lc – head length, Ltc – head width, Ac – head height, D – interlimb distance, Lpa – forelimb length, Lpp – hindlimb length, Dn – distance between exterior nostrils, Do – eye diameter, Spp – minimal distance between orbits, Lgr – parotid gland length, A – forefoot length (measured from the base of foot to the end of 3rd toe), P – hind foot length (measured from the base of foot to the end of 4th toe), Lm – jaw length (measured from the snout to the corner of the mouth). To avoid observer bias, the 1st author took all measurements with a digital caliper to a precision of 0.01 mm.

The MANOVA and ANOVA tests with sex and groups as sources of variability were used to test for differences between the mean values of the morphometric traits of population samples.

To reduce the impact of overall size, the Mosimann’s approach of obtaining the shape variables were used (Darroch & Mosimann 1985). The CDA on Mosimann shape variables was applied to maximize differences between predefined groups. Standardized coefficients of the canonical variables were examined in order to obtain an estimate of their respective contributions to the general morphometric variability and separation of the groups of the fire salamander.

In order to test the degree of correspondence between morphometric and geographic distance, matrix correlation test (Mantel 1967) was used. Significant correlation between matrices suggests a non-negligible association between them. Mahalanobis distances between localities were calculated, and the obtained morphometric matrix was compared to straight line distances in kilometers between localities. The statistical significance for the matrix correlations was obtained by a permutation test (10,000 replications) using popTools, 2.5.5 version (Hood 2000).

Qualitative traits

Three qualitative traits with several states were examined for males and females, separately: I – pattern of dorsal blotches, a. diffuse b. medio-dorsally fused c. symmetrical; II – ventral side color, a. black b. with yellow dots; III – position of ventral blotches, a. only on throat b. large number on throat, abdomen and tail c. small number on throat, abdomen and tail.

Preliminary analysis revealed no significant age related variations in the frequencies of qualitative traits in the representative sample of the Group 1 (I: G = 2.70; II: G = 0.67; III: G = 5.39; P > 0.05, for all comparisons). Therefore, qualitative data from adult and subadult specimens of the same group were pooled for further analyses.

Correspondence analysis was performed to examine differences in qualitative traits between predefined groups of the fire salamander populations.

The analyses were carried out using the computer package Statistica® (STATISTICA for Windows. StatSoft, Inc., Tulsa, OK), considering p < 0.05 as the level for significance.
Results

Significant variations between the sexes (MANOVA, Wilks’ lambda = 0.48, df1 = 15, df2 = 377, \( p < 0.0001 \)) and groups (MANOVA, Wilks’ lambda = 0.24, df1 = 195, df2 = 3754, \( p < 0.0001 \)) were observed.

In the CDA, three significant functions in males and five in females were obtained. The last one explained 14.2% and 6.4% of the variance for males and females respectively. If we take into account the first two canonical axes, 57.0% of the total discrimination in males and 53.2% in females were obtained (Table 1). Ordination of groups of populations along the first two canonical axes showed that there was no distinctive geographical grouping of populations for both sexes (Fig. 2).

The values of the standardized coefficients for males indicated that the greatest contributions to discrimination on the 1st canonical axis were made by differences in head width (Ltc), eye diameter (Do), head height (Ac), hind foot length (P) and snout-vent length (Svl) (Table 1). Groups of males were distributed along the first axis from those with individuals characterized by small Ltc, Svl and large Do, Ac, P (e.g. East Serbia and Vlasina - Kukavica) to the populations with individuals with large Ltc, Svl and small Do, Ac, P (e.g. group Vršački breg). The greatest contribution to discrimination on the 1st canonical axes for females was made by differences in snout-vent length (Svl), head height (Ac), head width (Ltc) and fore foot length (A) (Table 1). Groups of females are distributed along the first axis from those with individuals characterized by large Svl, Ltc and small Ac, A (e.g. East Serbia and Vlasina - Kukavica). Discrimination on the second canonical axis were made by differences in forelimb length (Lpa) and hindlimb length (Lpp) for males and eye diameter (Do) and interlimb distance (D) for females (Table 1). Groups were distributed along the second axis from those with individuals characterized by large Lpa and small Lpp (males), small Do and large D (females) (e.g. Vršački breg, Despotovac – Plažane, Vlasina – Kukavica in males, Vršački breg, Western Serbia, Eastern Serbia for females), to the groups with individuals with small Lpa and large Lpp (males) and large Do and small D (females) (e.g. Jastrebač Group for males, Vlasina – Kukavica for females).

<table>
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<th>Table 1. Standardized canonical discriminant function coefficients (CD1, CD2) for morphometric traits.</th>
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Morphometric distances and geographic distances showed no significant correlation for both sexes (Mantel test: males: \( r = -0.01, p = 0.49 \); females: \( r = 0.10, p = 0.15 \)) which indicate absence of geographical structuring of morphometric traits.

If we take into account the first two correspondence axes, 81.4% of the total correspondence in males and 76.0% in females were obtained. Ordination of groups of populations along the first two correspondence axes showed that there was no distinctive geographical grouping of populations for both sexes (Fig. 3). Absence of specimens in male group 13 (central Serbia) with medio-dorsally fused dorsal blotches (Ib), absence of specimens with black ventral side (IIa) and the presence of ventral blotches only on throat (IIIa) separated this group from others. Discrimination of the male group 3 (East Serbia) in the upper right quadrant is caused by the predominance of specimens with ventral blotches only on throat (IIa) (Fig. 3). Female group 11 (south-western Serbia) was separated from others because of prevailing number of specimens with medio-dorsally fused dorsal blotches (Ib) (Fig. 3).

Discussion

Morphometric and qualitative traits

Results of our multivariate analysis of morphological variation of the fire salamander from the
Morphological variability in *Salamandra salamandra* Central Balkans indicated differences between populations belonging to different geographical groups. Although tests showed that morphometric differences between the groups are statistically significant, a clear pattern of variation could not be found, for both males and females. In other words, population grouping is not based on geographic distance, so we had cases where geographically very distinct groups showed substantial morphological similarity. For instance, males and females from population groups one (Vršački breg) and five (Jastrebac group) were delimited from other geographic groups based on snout-vent length, head length and head width. However, males from group five showed substantial similarity with males from southwestern and western Serbia (groups eleven and twelve) regarding small length of fore and hind limbs. Larger males with long body, wide and deep head, and long fore and hind limbs were found in the eastern part of Serbia (groups two and three), but also in the south of Serbia (groups four and seven). Largest females came from populations from eastern and southern parts of Serbia but also in populations from the western part (group 12). Similar absence of clear geographical pattern in morphometric variation was found for several Iberian populations of the fire salamander (Montori & Francesch 1995).

Similarly to morphometric characters, analysis of qualitative traits confirmed the absence of a clear pattern of group variation in both sexes. Geographical differentiation and perhaps local adaptations could be expected for species with limited ability to move long distances and due to their close ties to a particular habitat, as with most tailed amphibian species (Smith & Green 2005). However, low migration ability was ruled out for the fire salamander populations (Steinfartz et al. 2000), which can explain lack of morphological
differentiation in the Central Balkans.

Although we could not expect that morphological characters and mtDNA genes evolve in tandem, genetic studies could give us a glimpse about possible morphological structuring (see Ivanović & Kalezić 2009). Previous knowledge about the low genetic variability in the fire salamander populations from Europe (Steinfartz et al. 2000) and the palaeogeographic situation of the Balkan Peninsula gave us a possibility to presuppose reasons for absence of morphological geographic patterning of the fire salamanders from the Central Balkans.

Palaeogeography
The reason for the absence of geographical structuring of morphological variation in the fire salamander could be palaeogeographic situation of the Balkan Peninsula during Pleistocene. In that period (400,000 years ago) a large population of the fire salamander that inhabited a large part of Europe differentiated into the current populations of Central and Eastern Europe, as well as the populations from the Balkans (Steinfartz et al. 2000).

The Pleistocene climate was marked by repeated glacial cycles, with the fluctuation of ice cover in a north-south direction. Even during the coldest period when 2/3 of Europe was covered by ice, the Balkan Peninsula was without the ice coverage. However, the Balkans mountain peaks were covered by ice and snow from the top down to 700-800 asl. Parts without the ice coverage were the river valleys and basins (Rabrenović et al. 2003). Although the large parts of the Central Balkans were ice-free, they were under the strong influence of ice sheets, which has been reflected by the position of vegetation zones, from tundra to the north to the forests to the south. The largest area of the Central Balkans was covered by different types of tundra: moss, meadow, shrub, and

Figure 3. Ordination of group centroids along the first and second correspondence axis for males and females, respectively. For abbreviations for the groups and qualitative traits’ states, see Fig. 1 and Material and methods section, respectively.
forest tundra. The forest tundra occupied river valleys, and other spaces were without forest. Mild slopes and low hills were covered by grass. Forests could be found in the south of Macedonia, and near the Aegean Sea they became deciduous (Lazarević 1994).

Climatic condition and vegetation during the glacial periods did not give possibility of survival of populations of the fire salamander in the broad area of the Central Balkans. There were no small isolated forest areas that could be their shelter. That was a reason of the retreat of the fire salamander population to the southern refugium in front of the coming cold from the north. Although the Pontic region was given as a most probable refugium (Eiselt 1958, Nicklas et al. 1994), we suggest the southern part of the Balkans near the Aegean Sea as the more probable refugium. This presupposition is supported by the fact that this area was covered by the mixed deciduous forests during the Würm glaciation (Lazarević 1994), which is natural habitat for the fire salamander population. Therefore, migrations of the fire salamanders and other animals toward the south and afterward recolonization during the interglacial periods, was aggravated by the natural Dinaric-Carpethian barrier with west-east extension. Therefore, migrations were taken along the valleys of the Great Morava River and the Vardar River (Džukić & Kalezić 2004, Forsten & Dimitrijević 2004). Fossil deposits of the fire salamander found in the valley of the Raška River from the upper Pleistocene, also act in favor of this migration route (Paunović & Dimitrijević 1990). During the recolonization, the Balkan Peninsula has been populated first, and then Eastern and Central Europe. This scenario has been repeated several times during the Pleistocene. The absence of natural barrier between the Balkan Peninsula and middle Europe, in contrast to the Iberian and Apennine Peninsulas, enabled intensive migration in both directions during the Pleistocene.

Because of that, nowadays populations of the fire salamander from the central Balkans should be considered in broader context, as a part of a large European population which inhabited a large area of Europe 400,000 years ago, and which is ancestral to the Balkans’ populations.

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Appendix

Group 1 (Vršački breg): Vršački breg (45°-08' N, 21°-25' E, 28
adult + 8 subadult males, 6 adult + 3 subadult females), Vršački
(45°-06' N, 21°-18' E, 0 + 0, 3 + 0).
Group 2 (Drenovacka-Plažana): Drenovacka (44°-05' N, 21°-26'
E, 5 + 0, 13 + 0), Plažana (44°-08' N, 21°-24' E, 7 + 0, 7 + 0).
Group 3 (Eastern Serbia): Miroče (44°-38' N, 22°-19' E, 3 + 0, 8 +
0, Gorjana Reka Reka (43°-46' N, 22°-12' E, 4 + 2, 3 + 0), Jakovac
(43°-39' N, 22°-18' E, 0 + 0, 1 + 0), Niklovac (43°-25' N, 21°-51' E,
0 + 0, 1 + 0).
Group 4 (Vlasina-Kukavica): Vlasina (44°-50' N, 22°-16' E, 4 + 0,
1 + 0, 2 + 0, Prokuplje (44°-12' N, 22°-00' E, 1 + 0, 3 + 0), Bara
(43°-52' N, 22°-01' E, 5 + 0, 0 + 0).
Group 5 (Jastrebac Group): Volek (43°-23' N, 21°-27' E, 8 + 7 + 12 +
5), Pješkovo (43°-10' N, 21°-17' E, 1 + 0, 0 + 0), Prokuplje
(43°-14' N, 21°-35' E, 1 + 0, 1 + 0), Bresnik (43°-09' N,
21°-25' E, 1 + 0, 0 + 0, Tovrljane (43°-07' N, 21°-25' E, 1 + 0, 0 + 0).
Group 6 (Western Morava valley): Treštenik (43°-37' N, 21°-00'
E, 1 + 0, 1 + 0), Bela Voda (43°-37' N, 21°-11' E, 2 + 0, 5 + 0),
Ugljarevo (43°-40' N, 20°-34' E, 0 + 0, 5 + 0), Vitancovac (43°-43'
N, 20°-47' E, 1 + 0, 0 + 0), Baranovac (43°-43' N, 20°-43' E, 0 +
0, 2 + 0), Goč (43°-34' N, 20°-50' E, 0 + 0, 2 + 0).
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