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Genetic structure of the fire salamander *Salamandra salamandra* in the Polish Sudetes

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Abstract. We analysed genetic variation within and differentiation between nineteen populations of the fire salamander *Salamandra salamandra* inhabiting the north-eastern margin of the species range in the Sudetes Mountains (south-western Poland). The results were compared with those obtained recently for the Polish part of the Carpathians. Variation of 10 nuclear microsatellite loci was analysed in 744 individuals to estimate genetic structure, gene flow, isolation and to test for a geographic gradient of genetic variation. Mitochondrial DNA control region (D-loop) of 252 specimens from all localities was used to identify the origin of populations currently inhabiting its north-eastern range. We found little genetic differentiation among populations in the Sudetes indicating substantial recent or ongoing gene flow. The exceptions were one isolated peripheral population located outside the continuous distribution range which displayed extremely reduced genetic variation probably due to a combination of long term isolation and low population size, and one population located at the eastern margin of the Polish Sudetes. Populations inhabiting the Sudetes and the Carpathians formed two separate clusters based on microsatellite loci. In accordance with available phylogeographic information, single mitochondrial haplotype (type IIb) fixed in all populations indicates their origin from a single refugium and may suggest colonization from the Balkan Peninsula. The analysis of geographic gradient in variation showed its decline in the westerly direction suggesting colonization of Poland from the east, however, alternative scenarios of postglacial colonization could not be rejected with the available data.

Keywords: genetic variation, isolation, microsatellites, mitochondrial DNA, peripheral populations, *Salamandra salamandra*, species range.

Introduction

Land use change e.g. habitat conversion, isolation or loss, is one of the main causes of global amphibian decline (Collins and Storfer, 2003). Such processes lead to increase of unstable and unfavourable environmental conditions and often result in lower population density and decrease in effective population size. As a consequence, populations experience stronger drift rapidly producing genetic differentiation and are vulnerable to extinction (Lesica and Allendorf, 1995; Frankham, 1996; Andersen, Fog and Damgaard, 2004). However, diverse environmental conditions may also promote adaptation to specific local conditions providing a potential source of adaptative variation (García-Ramos and Kirkpatrick, 1997) and therefore play an important role in the maintenance of biological diversity (Mayr, 1970; Channell and Lomolino, 2000). But not only adverse environmental factors, but also the population history, e.g. postglacial dispersal plays a role in forming the population structure. Direction of colonization may be reflected in a gradient of decreasing variation caused by repeated bottlenecks during colonization (Hewitt, 1996; Ibrahim, Nichols and Hewitt, 1996; Schmitt, 2007). Such genetic patterns have been observed in animal species (e.g. Johnson, 1988), including European amphibians e.g. *Rana latastei* (Garner, Pearman and Angelone, 2004), *Epidalea calamita* (Beebee and Rowe, 1999; Oromi et al., 2012), *Triturus cristatus* (Wielstra, Babik and Arntzen,

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2015) or *Lissotriton montandoni* (Zieliński et al., 2014).

Terrestrial salamanders have generally been considered philopatric, not capable of long-range dispersal (Sinsch, 1990; Beebee, 2005; Smith and Green, 2005; Zakrzewski, 2007). However, recent studies support the notion that the potential to migrate has been underestimated in some species (Bar-David et al., 2007; Schmidt, Schaub and Steinfartz, 2007; Schulte, Küsters and Steinfartz, 2007). Nonetheless, salamanders show a tendency for higher genetic differentiation than other taxa at comparable geographic scale (Highton, 1999; Spear et al., 2005; Blank et al., 2013).

The Polish part of the fire salamander *Salamandra s. salamandra* (Linnaeus, 1758) range represents the north-eastern limit of the species distribution in Europe and encompasses the Sudetes and the Carpathian Mountains (Zakrzewski, 2007; Kuzmin, 2009). Increasing habitat fragmentation in the region of Lower Silesia (south-western Poland), caused mainly by expansion of agriculture and road network, may constitute strong anthropogenic barriers to dispersal (Ogrodowczyk et al., 2010). On the other hand, salamanders have also been found in unsuitable habitats, such as fields, meadows or even gardens, so it is likely that they are able to migrate throughout such habitat, and thereby maintain gene flow between populations.

Colonization of Europe by *S. salamandra* after the last glaciation had been reconstructed by Veith (1992), Joger and Steinfartz (1994), Veith et al. (1996), Steinfartz et al. (2000) and Weitere et al. (2004). These studies revealed that *S. salamandra* colonized northern Europe from both the Iberian and the Balkan Peninsulas, which is reflected in the geographic distribution of two major mtDNA haplotype groups (I and II). The presence of haplotype IIb was revealed in populations inhabiting Polish part of the Carpathians, suggesting its colonization from the Balkans (Najbar et al., 2015).

Here we used nuclear microsatellite markers to study genetic variation and structure of *S. salamandra* at the northern margin of the species range in the Polish Sudetes. We predicted that populations which experienced less favourable environmental conditions, recent habitat fragmentation or isolation will exhibit significantly lower genetic variation than populations from the continuous part of the range. The Sudetes dataset was compared with already published results for the Carpathians (Najbar et al., 2015). Mitochondrial DNA control region (D-loop) was sequenced to identify the origin of *S. salamandra* inhabiting the Sudetes. Microsatellite variation was also used to assess the direction of colonization of Poland.

Material and methods

Sampling

744 samples were collected in spring and summer 2008 from 18 sampling sites (fig. 1, table 1) and in 2014 from one site (population 19) in the Polish part of the Sudetes and their Piedmont (Lower Silesia), constituting the northern margin of the species range (fig. 1). Additionally, 356 samples collected in 2014 from 10 sampling sites in the Carpathians (previously published in Najbar et al., 2015) were included for mountain ranges comparison. In total, 1100 individuals were analysed.

Small fragments of caudal folds were cut off from larvae and the second phalanx of the right hind limb was amputated from the metamorphosed individuals using microsurgical scissors. 4% aqueous potassium permanganate solution was used to disinfect the injured part. Immediately after sampling, salamanders were released to their habitat. Tissues were preserved in 70% ethanol.

Laboratory methods

Total DNA was isolated according to the standard proteinase K-phenol-chloroform method (Steinfartz, Veith and Tautz, 2000). Agarose gel electrophoresis was used to check for the presence and quality of DNA stained with ethidium bromide (EtBr). Extracted DNA was stored in Tris-EDTA buffer.

We analysed 11 microsatellite loci, one consisting of dinucleotide (*SalE2*) and 10 of tetranucleotide repeats (*Sal3*, *Sal23*, *Sal29*, *SalE5*, *SalE6*, *SalE7*, *SalE8*, *SalE11*, *SalE12*, *SalE14*). Ten forward and one reverse primers were labelled with FAM, HEX and NED fluorescent dyes (Steinfartz, Kuesters and Tautz, 2004) (see online supplementary table S1). Three multiplexes were used for the multilocus amplification (supplementary table S1). Polymerase Chain Reaction followed the standard protocol (Steinfartz, Kuesters and Tautz, 2004) in the volume of 10 μl consisting of Multiplex Master Mix including HotStarTaq DNA Polymerase (Qiagen), Q Solution (Qiagen), 0.2 μM of primers,

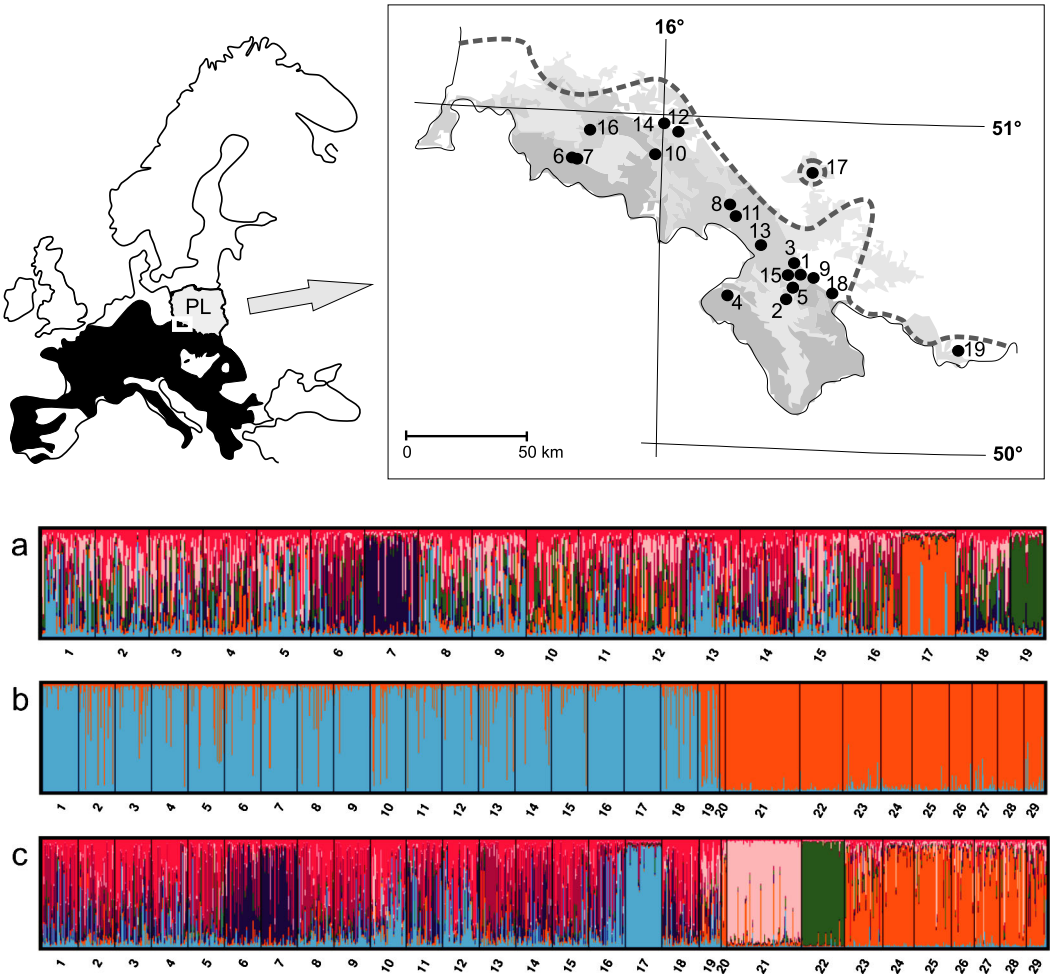

Figure 1. Top: *Salamandra salamandra* distribution in Europe (left) according to IUCN (Kuzmin et al., 2009) and sampling sites located in the Sudetes (right). Under: the genetic structure in the Sudetes based on STRUCTURE results (1-19) for: a) K = 7, and the Sudetes with the Carpathians (20-29) for b) K = 2, and c) K = 7. This figure is published in colour in the online version.

RNase Free-Water and salamander DNA. The reaction conditions were as follows: 95°C for 15 min, followed by 40 cycles of: 95°C (30 s), 54°C for MP1, 59°C for MP2 and 60°C for MP3 (1 min) and 72°C (1 min), and the final extension at 60°C (30 min). PCR products were checked by gel electrophoresis, then diluted with water and mixed with the size standard GeneScanTM-500 ROX (Thermo Fisher). Genotyping was performed on an ABI 3100-Avant genetic analyser (Applied Biosystems).

Two primers L-Pro-ML (5′-GGCACCCAAGGCCAAAATTCT-3′) and H-12S1-ML (5′-CAAGGCCAGGACCAAACCTTTA-3′) were used to amplify and sequence 758 bp of mitochondrial DNA control region (D-loop) (Steinfartz, Veith and Tautz, 2000). PCR reactions was performed in the total volume of 20 μl under the standard conditions (according to Kocher et al., 1989) with TopTaq polymerase (Qiagen). Products were checked on agarose gel (stained with EtBr, then visualised under UV light) and purified with ExoSAP-IT®. Symmetric cycle sequencing with Ready Reaction kit (Amersham, Freiburg, Germany) was performed on an ABI 3100-Avant genetic analyser (Applied Biosystems).

Microsatellite loci analysis

Microsatellite loci were genotyped using GeneMapper 4.0. Genetic data were checked for misprint, scoring errors and presence of null alleles using MICRO-CHECKER V2.2.3 (van Oosterhout et al., 2004). Deviations from Hardy-Weinberg equilibrium were assessed in GENEPOP 4.4.3 (Rousset, 2008); the Markov chain method (5000 iterations) was used for estimation of P-values. The Bonferroni procedure (Rice, 1989) was applied to control type I error while using multiple tests. Allele frequencies were adjusted to accommodate the presence of null alleles in FreeNA (Chapuis

Table 1. Sampling sites of *Salamandra salamandra* in the Sudetes, sample size (N_T: total, N_A: number of adults, N_L: number of larvae) and genetic variation parameters: mean expected heterozygosity (H_E), allelic richness (R_S) and inbreeding coefficient (F_{IS}). Isolated populations are shown in bold.

Population number	GPS coordinates	N			H_E	R_S	F_{IS}
		N_T	N_A	N_L			
1	50°30′11.50″N 16°44′11.51″E	40	40	0	0.58	5.04	0.073
2	50°27′41.95″N 16°40′48.96″E	40	40	0	0.59	5.36	0.037
3	50°31′53.38″N 16°42′33.49″E	40	40	0	0.61	5.78	0.035
4	50°26′49.25″N 16°19′20.00″E	40	40	0	0.58	5.07	0.084
5	50°28′57.44″N 16°42′47.14″E	40	40	0	0.61	5.53	0.085
6	50°43′04.72″N 16°19′30.55″E	40	40	0	0.61	4.65	0.050
7	50°51′32.78″N 15°33′40.26″E	40	40	0	0.53	3.94	0.089
8	50°51′36.61″N 15°32′53.62″E	40	28	12	0.63	5.17	0.074
9	50°30′03.30″N 16°45′49.47″E	40	40	0	0.55	4.91	0.045
10	50°53′23.38″N 15°56′35.23″E	39	30	9	0.54	4.38	−0.092
11	50°43′53.55″N 16°18′56.08″E	40	2	38	0.61	5.25	0.082
12	50°59′05.80″N 16°02′39.83″E	40	10	30	0.57	4.70	0.031
13	50°37′31.16″N 16°27′43.61″E	40	40	0	0.59	4.56	0.085
14	51°00′24.37″N 15°59′24.40″E	40	40	0	0.56	4.45	0.117
15	50°30′29.97″N 16°43′25.97″E	40	40	0	0.58	5.51	0.056
16	50°58′04.68″N 15°38′16.44″E	40	4	36	0.57	4.60	0.225
17	50°52′25.14″N 16°40′47.39″E	40	38	2	0.39	2.82	0.105
18	50°26′11.57″N 16°51′47.25″E	41	41	0	0.62	5.10	0.065
19	50°16′22.07″N 17°25′08.01″E	24	24	0	0.58	4.60	0.029

and Estoup, 2007) based on the Expectation-Maximization (EM) algorithm (Dempster, Laird and Rubin, 1977).

Number of alleles, allelic richness (R_S), inbreeding coefficient (F_{IS}), and number of private alleles (PA) per locus and population were estimated in FSTAT 2.9.3.2 (Goudet, 2002). Allelic richness in the Sudetes populations was estimated using the minimum number of 24 individuals representing population 19. However, in comparison of the two mountain ranges, we used the minimum sample size of 6 individuals to maximize the number the Carpathian populations included in the analysis. ARLEQUIN 3.5 (Excoffier and Lischer, 2010) was used to calculate observed heterozygosity (H_O), expected heterozygosity (H_E) and pairwise F_{ST} between populations. Nonmetric Multidimensional Scaling (NMDS) in STATISTICA 12 (Statsoft, USA) was used to visualize the F_{ST} matrix for a) the Sudetes, b) the Sudetes and the Carpathians.

Isolation-by-distance (IBD) analysis was assessed by Mantel test in ARLEQUIN 3.5 with 10 000 permutations (Slatkin, 1993); results were visualized in Microsoft Office Excel 2007. Recent effective population size reduction was tested in BOTTLENECK 1.2.02 (Cornuet and Luikart, 1997). We performed the sign and Wilcoxon tests for the stepwise mutation model (S.M.M), infinite alleles model (I.A.M) and two-phased model (T.P.M) of microsatellite mutation with 90% proportion of I.A.M (according to Cristescu et al., 2009).

Locus-by-locus molecular variance analysis (AMOVA) in ARLEQUIN 3.5 was used to partition the total variance in allele frequency data into the among groups, among populations within groups and within populations components. We examined 4 groupings of populations: a) western (populations 6, 7, 10, 12, 14, 16) and eastern (populations 1, 2, 3, 4, 5, 8, 9, 11, 13, 15, 18) Sudetes with populations 17 and 19 representing separate groups, b) western and eastern Sudetes excluding populations 17 and 19, c) the Sudetes and the Carpathians, and d) the Sudetes an the Carpathians excluding differentiated populations 17, 19, 21 and 22.

We also conducted an individual-based Bayesian clustering in STRUCTURE 2.3.4 (Pritchard et al., 2000) for two data sets: 1) only the Sudetes populations and 2) the Sudetes and the Carpathians. We used models of genetic admixture and correlated allele frequencies. Burnin of 2×10^5 steps and post-burnin analysis of 2×10^6 Markov chain Monte Carlo simulations were performed for 10 independent iterations for K 1 to 18. Estimation of K was conducted in on-line software: CLUMPAK (Kopelman et al., 2015) and STRUCTURE HARVESTER (Earl and von Holdt, 2012; http://taylor0.biology.ucla.edu/structureHarvester/) based on L(K) and ΔK (Evanno et al., 2005). STRUCTURE output was visualised in CLUMPAK.

To compare genetic variation between the Sudetes and the Carpathians and to test for the presence of geographic gradients of variation we used general linear models in R (R Core Team, 2013). We performed two separate analyses with expected heterozygosity (H_E) and allelic richness (R_S) as the dependent variable. The position of populations along the NW-SE axis (see online supplementary fig. S1) was a continuous explanatory variable and the mountain range was a fixed categorical factor.

Mitochondrial DNA analysis

Mitochondrial DNA control region was sequenced in 252 individuals (14 individuals from each sampling site) from

the Sudetes. Sequences were analysed in Sequence Navigator software (Perkin Elmer), then checked by eye and aligned in SEQSCAPE v2.5 (Applied Biosystems, 2004). MtDNA fragments were compared with haplotypes of several *S. salamandra* subspecies (described by Steinfartz, Veith and Tautz, 2000) and haplotype obtained in our previous study (Najbar et al., 2015) using ClustalW multiple alignment (Thompson, Higgins and Gibson, 1994) in BioEdit (Hall, 1999). Sequences were deposited in GenBank: KY055013 (the Polish Sudetes), KY055014 (the Polish Carpathians), KT335882-KT335887 and KT335891-KT335928 (analysed in Steinfartz, Veith and Tautz, 2000).

Results

Variation of microsatellite loci was examined in 744 individuals from 19 localities in the Sudetes (table 1). The Sudetes and the Carpathians were compared using data from a total of 29 populations (supplementary fig. S1; the Carpathian populations described in detail by Najbar et al., 2015).

Genetic variation in the Sudetes populations

Locus *SalE2* was excluded from the dataset due to the problem with preferential amplification of one allele; all genetic analyses were thus conducted for 10 loci. All microsatellite loci exhibited polymorphism in all populations except *Sal3* which was monomorphic in population 17 and 19. The number of alleles per locus ranged from 3 (*SalE5*) to 36 (*SalE12*); the total number of alleles across all loci was 101. Significant deviations from the Hardy-Weinberg equilibrium (after Bonferroni correction) were observed in 21 of 187 tests, only at 2 loci (*Sal3*, *SalE12*) suggesting the presence of null alleles. For these loci allele frequencies were adjusted to accommodate the presence of null alleles in FreeNA. However, the correction did not significantly change the results so they are presented here without the ENA correction. In addition to loci *Sal3* and *SalE12*, MICRO-CHECKER indicated the presence of null alleles also in *SalE11* (population 11), *Sal29* (population 13), *SalE14* and *Sal23* (both in population 16).

Genetic variation within populations was measured as the mean expected heterozygosity (H_E) and mean allelic richness (R_S). The expected heterozygosity ranged from 0.39 (population 17) to 0.63 (population 8) (table 1) with mean $H_E = 0.57 \pm$ (SD) 0.05. The allelic richness ranged from 2.82 (population 17) to 5.78 (population 3) (table 1) with mean $R_S = 4.81 \pm$ (SD) 0.7. With respect to both measures, isolated population 17 was a clear outlier showing much lower variation. Overall 20 private alleles were found, they were most numerous in population 2 (4 private alleles). BOTTLENECK suggested significant excess of heterozygosity only in population 18 ($P = 0.001$, Wilcoxon test for I.A.M and T.P.M).

Population structure

The pairwise F_{ST} values between the populations in the continuous part of the distribution ranged from very low 0.003 (1-3, 1-15, 3-15) to moderate 0.121 (7-10). The differentiation between the isolated population 17 and the remaining populations was notably higher, with F_{ST} between 0.118 and 0.245 (table 2). Higher F_{ST} vales were also observed in comparisons involving population 19 (table 2), with the highest $F_{ST} = 0.269$ between population 17 and 19. Overall 166 out of 171 pairwise F_{ST}s were significant at the 0.05 level. There was a significant positive correlation between F_{ST} and geographic distance between all populations (Mantel test: $r = 0.383$, $P = 0.012$); when population 17 was excluded, the correlation became stronger ($r = 0.577$, $P < 0.001$) (fig. 2).

Analysis of molecular variance (AMOVA) was performed for two groupings of the Sudetes populations (supplementary table S2). Variation at all hierarchical levels was highly significant, however the between group level explained only ca. 3.90% or even 1.30% (with populations 17 and 19 excluded) of variation, while variation among populations within groups explained ca. 4% of variation. Overwhelming majority of microsatellite variation was found within populations (supplementary table S2).

Inspection of the ln probability of data (supplementary fig. S2a) for various values of K

Table 2. Population pairwise F_{ST}; significant values are shown in bold.

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
2	**0.021**																	
3	0.003	0.005																
4	**0.027**	**0.029**	**0.013**															
5	**0.023**	**0.034**	**0.018**	**0.050**														
6	**0.038**	**0.034**	**0.023**	**0.030**	**0.071**													
7	**0.079**	**0.056**	**0.042**	**0.060**	**0.096**	**0.043**												
8	**0.010**	**0.018**	0.004	**0.028**	**0.025**	**0.044**	**0.075**											
9	**0.010**	**0.026**	**0.017**	**0.034**	**0.026**	**0.064**	**0.096**	**0.022**										
10	**0.066**	**0.060**	**0.042**	**0.059**	**0.091**	**0.064**	**0.121**	**0.041**	**0.074**									
11	**0.023**	**0.021**	**0.011**	**0.027**	**0.036**	**0.026**	**0.068**	**0.026**	**0.037**	**0.057**								
12	**0.052**	**0.046**	**0.036**	**0.054**	**0.091**	**0.051**	**0.106**	**0.044**	**0.061**	**0.040**	**0.035**							
13	**0.046**	**0.029**	**0.032**	**0.067**	**0.048**	**0.053**	**0.101**	**0.040**	**0.056**	**0.082**	**0.023**	**0.054**						
14	**0.049**	**0.047**	**0.031**	**0.022**	**0.081**	**0.027**	**0.073**	**0.051**	**0.067**	**0.070**	**0.030**	**0.053**	**0.066**					
15	0.003	**0.018**	0.003	**0.032**	**0.023**	**0.046**	**0.076**	**0.014**	**0.009**	**0.073**	**0.026**	**0.052**	**0.038**	**0.054**				
16	**0.040**	**0.049**	**0.034**	**0.055**	**0.080**	**0.044**	**0.105**	**0.047**	**0.050**	**0.063**	**0.042**	**0.024**	**0.064**	**0.060**	**0.049**			
17	**0.154**	**0.170**	**0.135**	**0.153**	**0.166**	**0.181**	**0.245**	**0.140**	**0.137**	**0.143**	**0.143**	**0.118**	**0.156**	**0.185**	**0.149**	**0.130**		
18	**0.043**	**0.019**	**0.019**	**0.030**	**0.052**	**0.036**	**0.050**	**0.028**	**0.051**	**0.068**	**0.041**	**0.064**	**0.052**	**0.047**	**0.040**	**0.063**	**0.198**	
19	**0.119**	**0.100**	**0.932**	**0.105**	**0.121**	**0.117**	**0.160**	**0.089**	**0.120**	**0.122**	**0.121**	**0.129**	**0.148**	**0.136**	**0.122**	**0.107**	**0.269**	**0.243**

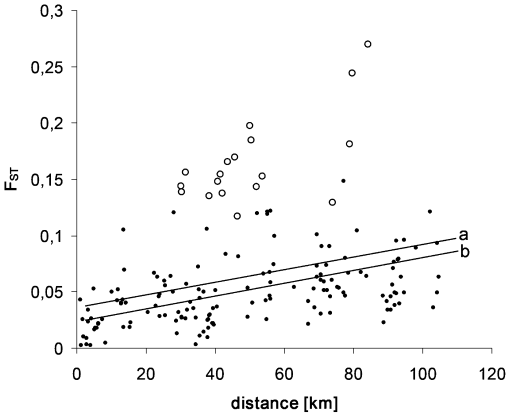

Figure 2. Correlation of the geographic (km) and genetic distance (F_{ST}) for: a) all the studied Sudetes populations (correlation coefficient = 0.383, $P = 0.012$) and b) the Sudetes populations excluding isolated population 17 (correlation coefficient = 0.577, $P < 0.001$). White points represent correlations with population 17.

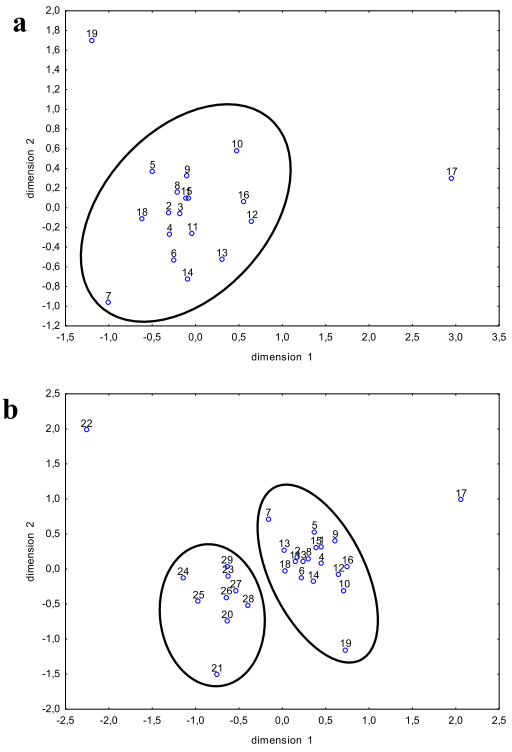

Figure 3. Nonmetric Two-dimensional Scaling of F_{ST} matrix of the fire salamander *Salamandra salamandra* populations from a) the Sudetes (1-19; stress = 0.13) and b) the Sudetes and the Carpathians (20-29; stress = 0.12). Numbers 17 and 22 represent distinct populations influenced by genetic drift. This figure is published in colour in the online version.

suggest the existence of 7 genetic group within the Sudetes (fig. 1a). The Δ(K) method (supplementary fig. S2a) supported K = 7, which distinguished population 17 and 19 as a separate clusters and showed some signs of differentiation of population 7 as well (fig. 1a). However the general lack of concordance between the STRUCTURE clusters and localities together with results of AMOVA indicate that genetic structuring of *S. salamandra* within the continuous part of the Sudetes range is very subtle.

Two-dimensional scaling of the pairwise F_{ST} matrix did not show clear substructuring within the Sudetes, with the exception of the highly differentiated populations 17 and 19 and slightly differentiated population 7 (fig. 3a).

Genetic variation and differentiation among the Sudetes and the Carpathians

The AMOVA estimated that more than 6% of the overall variation was distributed among the mountain ranges, while over 7% was distributed among populations within ranges (supplementary table S2). Differentiation between the Sudetes and the Carpathians was confirmed in the two-dimensional scaling of the pairwise F_{ST} matrix (fig. 3b). Four populations (17, 19, 21 and 22) were outliers, two of them (17 and 22) showing extreme differentiation of allele frequencies. The STRUCTURE analysis supported 2 (ΔK) and 7 (L(K)) genetic clusters (fig. 1b-c, supplementary fig. S2b). For K = 2, the clusters corresponded to the Sudetes and the Carpathians with the easternmost population in the Sudetes (19) admixed (fig. 1b). Assuming K = 7, the three populations (17, 21, and 22) were assigned to separate clusters (fig. 1c).

Comparison of variation between the two mountain ranges and tests of the geographic gradient in variation were performed both including all populations (full dataset) and excluding the two populations showing extremely

reduced variation (17 and 22, no_outliers dataset). No difference between the two ranges were observed for either measure of variation in the full dataset (H_E: $F_{1,27} = 0.52$, $P = 0.48$; R_S: $F_{1,27} = 1.38$, $P = 0.25$), while in the no_outliers dataset variation in the Carpathians was higher (H_E: $F_{1,25} = 3.67$, $P = 0.067$; R_S: $F_{1,25} = 6.81$, $P = 0.015$). A closer look into the model including the mountain range and the position of populations along the NW-SE axis (supplementary fig. S1) indicates that these differences between the Sudetes and the Carpathians are due to the geographic gradient of genetic variation. For the full dataset neither interaction nor any of the main factors were significant. However for the no_outliers dataset, while interaction was not significant for either H_E or R_S, both measures of variation increased towards SE (H_E: $F_{1,24} = 4.47$, $P = 0.045$; R_S: $F_{1,24} = 8.87$, $P = 0.007$), and the effect of the mountain range was not significant.

Mitochondrial DNA analysis

The analysis of mitochondrial DNA control region showed complete lack of variation. Only haplotype IIb was found in the Sudetes, similarly as in the Carpathians (see Najbar et al., 2015).

Discussion

Microsatellite loci variation in the Sudetes

Variation of microsatellite loci estimated by H_E and R_S was relatively similar among the Sudetes populations (see table 1) with exception of population 17 which showed markedly lower variation (table 1). Similar results were obtained for *Ichthyosaura alpestris* from this locality (Pabijan and Babik, 2006). Population 17 inhabits extremely peripheral Ślęża Massif and is separated from the main Sudetes range (approximately 30 km) by extensive agricultural areas. The lowest microsatellite variation, lack of private alleles and high divergence were probably caused by strong genetic drift due to long-term isolation (table 1). In addition, slightly reduced variation (table 1) and elevated values of F_{ST} (table 2) were also observed in population 7 (fig. 1a, table 1). In this case, the results may suggest the start of differentiation processes caused by progressive habitat fragmentation, isolation and reduction of population size.

We expected to find a bottleneck signature in isolated populations but it was detected only in large and stable population 18. Molecular markers and methods for such analysis may be unreliable in some cases (compare e.g. Andersen, Fog and Damgaard, 2004; Burns, Eldridge and Houlden, 2004), but this result may also reflect the situation from the 1970s when, due to water contamination by arsenic (Marszałek and Wąsik, 2000), not contaminated streams – a major aquatic habitat for larvae deposition in Poland (Zakrzewski, 2007) – were used as a source of drinking water which led to their desiccation in the area where population 18 is located.

Population structure in the Sudetes

Despite limitation of the habitat to the mountainous areas and its progressive fragmentation in recent years (Ogrodowczyk et al., 2010 and personal observation), populations of *S. salamandra* in the Sudetes still show genetic homogeneity (fig. 1a, fig. 3a, supplementary tables S2 and S3) and demonstrate no significant historical barriers to gene flow. Similar conclusions refer to the Carpathian populations of this species (Najbar et al., 2015), but also Polish populations of *I. alpestris* (Pabijan and Babik, 2006) and *Lissotriton montandoni* (Zieliński et al., 2014). Stable genetic variation in small or fragmented populations may be the result of large effective population size (Beebee, 2005; Ficetola et al., 2010). Álvarez et al. (2015) studied small and isolated population of *S. salamandra* trapped in the city of Oviedo (Spain). Using *capture-mark-recapture* method, the authors obtained high ratio of N_e/N, which suggests that e.g. multiple

paternity, which has been demonstrated for *S. salamandra* (Steinfartz et al., 2006; Caspers et al., 2014) may contribute to the maintenance of variation and to some extent prevent the inbreeding depression in small populations.

Indeed, the time of isolation resulting from habitat fragmentation may still be too short to detect any genetic differentiation among the Sudetes populations. Steinfartz, Weitere and Tautz (2007) did not find any evidence for genetic differentiation of *S. salamandra* in a population isolated for 40 years, whereas genetic differences were visible in other population isolated for at least 150 years. On the other hand, Lesbarrères et al. (2006) studied the effects of 20 years highway barrier on French populations of *Rana dalmatina* and argued for the effect of recent isolation on the genetic differentiation as F_{ST} varied from 0.022 (non-fragmented populations) to 0.238 (fragmented populations).

In contrast to the homogeneous group of populations (fig. 1a), genetic consequences of geographic isolation and adverse habitat conditions were noticed in the extremely peripheral population 17 which showed the lowest H_E and R_S and was identified as divergent by STRUCTURE (fig. 1a) and NMDS (fig. 3a). In addition, Bayesian analysis also identified population 7 as slightly differentiated, but greater admixture was observed in this population than in population 17 (fig. 1a).

Genetic differences, structure and gradient of microsatellite variation among the Sudetes and the Carpathians

Analysis of genetic structure among the Sudetes and the Carpathians (supplementary fig. S1) led to the conclusion that these main Polish mountain ranges represent two different genetic groups (see fig. 1b). Three populations (17, 21 and 22) were pointed as outliers (fig. 1c, fig. 3b) and revealed reduced genetic variation (supplementary table S3). Moreover, population 19 showed some evidence of the admixture between mountain ranges. The signal of admixture revealed by STRUCTURE (see fig. 1b) may indicate more recent ancestry between this population and the Carpathians or actual recent admixture. Currently, the Moravian Gate separating the two ranges most likely constitutes a natural barrier to gene flow and in our opinion, recent migration between the Sudetes and the Carpathians is unlikely. Populations of the Alpine newt also showed structuring between the Sudetes and the Carpathians (Pabijan and Babik, 2006).

Assuming the Balkan origin of *S. salamandra*, we hypothesized that after the last glacial maximum the species could migrate to Eastern Europe from the North-Western Carpathians, then colonizing Poland in the easterly direction from the centre of its range – also suggested for *I. alpestris* (Pabijan and Babik, 2006). Therefore we expected a gradient of microsatellite variation declining to the east. However, we found slightly but significantly higher variation in the Carpathians suggesting colonization in the opposite direction. Thereby, it might be hypothesized that the Sudetes have been colonized from the Carpathians. Admixture in the Opawskie Mountains (population 19) obtained in STRUCTURE for $K = 2$ (fig. 1b) may define the contact zone of these two genetic groups. Understanding of *S. salamandra* phylogeography in the Balkans and in the Carpathians is still insufficient and scenarios such as colonization through the Moravian Gate or the existence of glacial refugia in the Carpathians should be tested.

In conclusion, we found little differentiation within *S. salamandra populations* in the Polish part of the Sudetes indicating recent or ongoing gene flow, situation similar to that reported earlier for the Polish Carpathians. The exceptions were populations located outside the continuous range or inhabiting isolated sites that revealed lower genetic variation caused by strong genetic drift. To estimate genetic condition of marginal populations and to determine effective conservation plan for the species, further analysis of genetic variation between marginal and central

populations in its entire European range are required.

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