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Genetic structure and differentiation of the fire salamander *Salamandra salamandra* at the northern margin of its range in the Carpathians

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Abstract. Amphibian populations occurring at the margin of the species range exhibit lower genetic variation due to strong genetic drift and long-term isolation. Limited mobility and site fidelity together with habitat changes may accelerate genetic processes leading to local extinction. Here, we analyze genetic variation of the fire salamander subspecies *Salamandra s. salamandra* inhabiting the Outer Carpathian region in Poland, at the northern border of its distribution. Nuclear DNA polymorphism based on 10 microsatellite loci of 380 individuals sampled in 11 populations were analysed to measure gene flow between subpopulations and possible long-term isolation. Mitochondrial DNA control region analysis among 17 individuals representing 13 localities was used to detect the origin of populations which colonized Northern Europe after the last glaciation. Overall, pairwise F_{ST}'s and AMOVA test of 'among group' variation showed little differences in the allele frequencies and relatively high local gene flow. However, Bayesian clustering results revealed subtle structuring between eastern and western part of the studied region. Two extreme marginal populations from the Carpathian Piedmont revealed reduced genetic variation which may be attributed to strong influence of genetic drift. Only one mitochondrial DNA haplotype (type IIb) was found in all individuals and suggest that after the Last Glacial Maximum *Salamandra salamandra* migrated to the North-Western Europe from the single glacial refugium placed in the Balkan Peninsula.

Keywords: gene flow, isolation, microsatellites, mtDNA, peripheral populations, population structure, *Salamandra salamandra*.

Introduction

Genetic structure of population is shaped by both historical and contemporary processes (Hewitt, 1999; Dyer and Nason, 2004; Schmitt, 2007; Excoffier, Foll and Petit, 2009). Gene flow is a major process opposing the effect of genetic drift and is essential to maintain high genetic variation and adaptive potential of populations (García-Ramos and Kirkpatrick, 1997; Frankham, 2005). 'Central-marginal' theory posits that ecological and genetic processes in populations inhabiting fragmented habitats or located at the margin of species range usually resemble those observed in small and isolated populations (Hampe and Petit, 2005; Eckert, Samis and Lougheed, 2008; Sexton et al., 2009). The reduction of the effective population size and lower density compared to the populations in the center of the range lead to the loss of variation, and in extreme cases to inbreeding depression (Beebee, 2005). Such genetic patterns were documented in several European amphibian species, i.e. *Hyla arborea* in Denmark (Andersen, Fog and Damgaard, 2004), *Bufo calamita* in Britain (Rowe and Beebee, 2003) or *Triturus* (*Ichthyosaura*) *alpestris* (Pabijan, Babik and Rafiński, 2005; Pabijan and Babik, 2006) in Poland. Thus, previous studies of amphibians indicated that isolated populations are susceptible to the loss of genetic variation and adaptive potential, which may lead to their extinction (Beebee, 2005; Frankham, 2010). Eastern part of Europe still requires detailed genetic studies to identify both genetically diverse and genetically depauperate, and thus possibly endangered, amphibian populations (Hartel et al., 2010).

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Genetic structuring of populations is often a reflection of the historical processes, related for example to the rapid colonization of new areas (Schmitt, 2007). The major expectation of the species expansion is the gradual decline of variation in populations towards the margin of the range (Ibrahim et al., 1995; Schmitt, 2007; Excoffier, Foll and Petit, 2009; Allendorf, Luikart and Aitken, 2013). Gradient in the diversity of microsatellite loci, as a result of postglacial colonization, had been confirmed for instance in the Italian agile frog *Rana latastei* (Garner et al., 2004) or in European populations of the natterjack toad *Bufo calamita* (Beebee and Rowe, 1999).

The fire salamander *Salamandra salamandra* (Linnaeus, 1758) is a polytypic species comprising multiple evolutionary lineages classified into 13 subspecies widely distributed in Western, Central and Northern Europe (Thiesmeier, 2004; Kuzmin et al., 2009). Populations inhabiting Poland represent the nominative subspecies *S. s. salamandra* (Thiesmeier, 2004; Zakrzewski, 2007). Although not described as typically mountainous species (Thiesmeier, 2004), its occurrence in Poland is restricted to uplands and mountainous areas in the Sudetes and Carpathian Mountains, which represent the northeastern border of the species geographical range (Najbar, 1995; Głowaciński and Rafiński, 2003; Zakrzewski, 2007). Because of its inability of long-range migrations, although usually shorter than approximately 1300 meters (see Bar-David et al., 2007; Schmidt, Schaub and Steinfartz, 2007; Schulte, Küsters and Steinfartz, 2007), relatively low fertility and breeding site fidelity, the fire salamander may be sensitive to adverse environmental conditions (Zakrzewski, 2007; Ogrodowczyk et al., 2010; Ogrodowczyk, 2011). The longevity of up to 20 years (Zakrzewski, 2007) and secretive lifestyle prevent rapid detection of negative changes in the environment leading to reduction of the effective population size (Ogrodowczyk et al., 2010; Ogrodowczyk, 2011). Increasing habitat fragmentation, also observed in the marginal Carpathian populations of *S. salamandra* (Najbar A., Najbar B., personal observations), may lead to partial or complete isolation of local populations, reduction of genetic variation caused by genetic drift or bottleneck effect, and even to local extinctions (Slatkin, 1987; Manel et al., 2003). Use of genetic methods based on polymorphism of neutral molecular markers is primarily needed to determine the current genetic variability and historical gene flow level, as well as to identify populations affected by adverse genetic phenomena (Jehle and Arntzen, 2002; Arif et al., 2011; Blank et al., 2012). In Poland, genetic variation and structuring of the fire salamander has recently been studied in the Sudetes Mountains. Generally, microsatellite DNA analysis revealed lack of significant differentiation between subpopulations with the evident isolation of a single marginal population (Ogrodowczyk, 2011).

During the Last Glacial Maximum, the northern Carpathian area was uninhabitable for most amphibians (but see Zieliński et al., 2014) and thus, colonization by *S. salamandra* must have occurred after the last glaciation (Steinfartz, Veith and Tautz, 2000; Weitere et al., 2004; Pabijan, Babik and Rafiński, 2005). Phylogeographic analysis of mitochondrial DNA showed that European populations of the fire salamander constitute a homogeneous clade C (Steinfartz, Veith and Tautz, 2000), represented by three types of haplotype I (I a-c, found in the Iberian Peninsula) and two variants of haplotype II (II a-b, representing the fire salamanders from the Balkans) (Weitere et al., 2004). According to Weitere et al. (2004), *S. salamandra* colonized Europe from both the Iberian and Balkan refugia and its movement may be related to the Holocene expansion of deciduous trees about 6000 to 8000 years ago, especially of the European beech *Fagus sylvatica* (Tinner and Lotter, 2001), typical fire salamander habitat. Ogrodowczyk (2011) identified haplotype IIb in all Polish populations from the Sudetes and concluded that *S. salamandra* from this region belongs to the eastern evolutionary lineage.

In the present study we used microsatellite markers to assess genetic variation and differentiation among populations of the fire salamander from the Polish part of the Carpathian Mountains. We hypothesize that populations inhabiting the species' range may exhibit reduced genetic variation and interrupted gene flow as a consequence of habitat fragmentation and isolation. We also used mitochondrial DNA (mtDNA) control region to investigate the origin of *S. salamandra* in that region of Poland. We hypothesize that after the last glaciation, the species colonized North-Eastern Europe from the Balkan Peninsula and therefore we expect a fixed haplotype IIb.

Material and methods

Study sites and sampling

Samples were collected between July and October 2014. The study area is located in Southern Poland and represents the Polish part of the external Carpathian range (the Outer Carpathians). In total, 382 individuals from 13 locations were sampled along the west-east axis: the Carpathian Piedmont, the Beskids and the Bieszczady Mountains (fig. 1). We examined microsatellite variation in 380 individuals from 11 sampling sites (1-11, see table 1); 17 individuals from 13 sampling sites (1-13, see table 1) were randomly selected for mitochondrial DNA study. Population number 11, belonging to the Eastern Sudetes, was also included in the analysis (table 1). Genetic material was obtained from larvae and adults. In larvae, 5 mm fragment of caudal fold was cut off using microsurgical scissors (sterilized between uses). Three buccal swabs were collected from each adult. To minimize possibility of larvae collection which could have been siblings, individuals were collected on the long

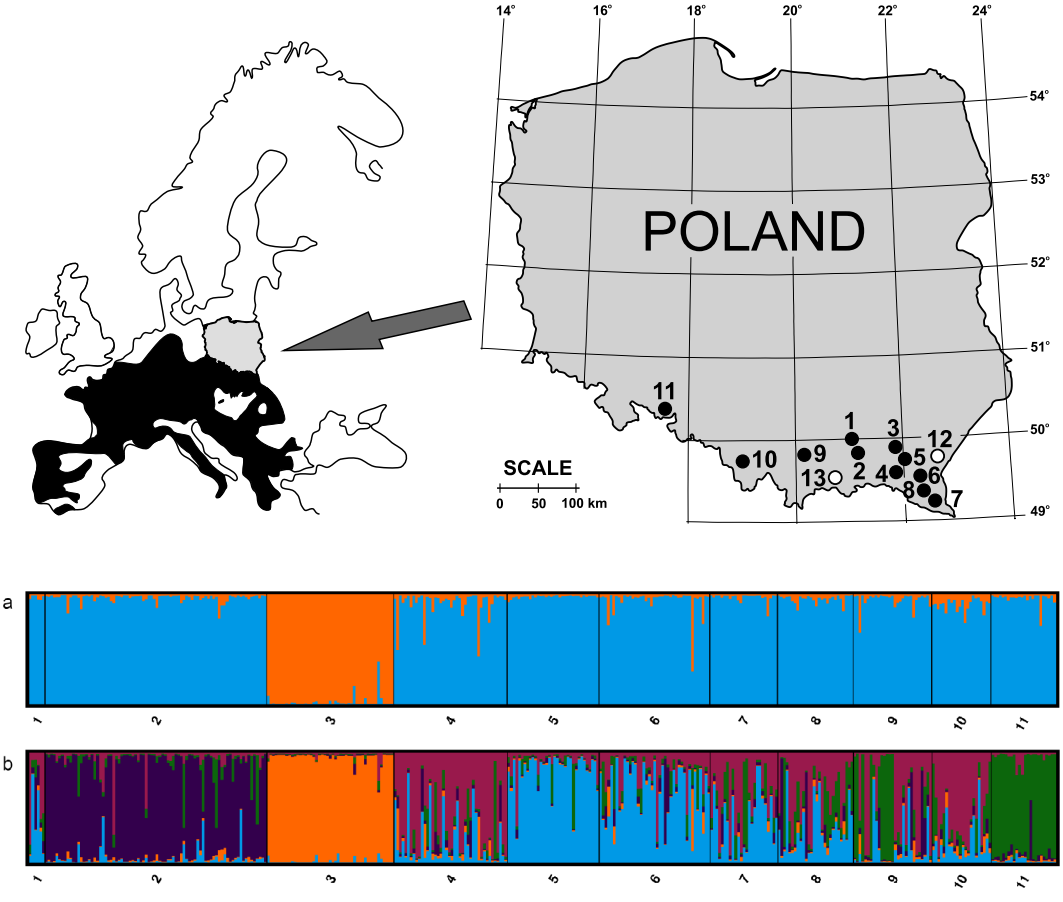

Figure 1. Top: Distribution of *Salamandra salamandra* according to the International Union for Conservation of Nature (left) and sampling localities in Poland (right). Black points (1-11) represent populations taken for microsatellite study; white points (12-13) represent additional localities included for mitochondrial DNA analysis. Population numbers refer to table 1. Bottom: Genetic structuring for (a) K = 2 and (b) K = 5. This figure is published in colour in the online version.

Table 1. Sampling locations of *Salamandra salamandra* in the Carpathian Mountains, Poland. Sampling site 11 represents the Easternmost Sudetes. Locations 12 and 13 were additionally taken for the mitochondrial analysis. N: number of individuals taken for microsatellite analysis, R_S: allelic richness; H_E: mean expected heterozygosity (for 10 loci), PA: number of private alleles.

Collection site number	Name of locality	GPS position	N	R_S	H_E	PA
1	the Rożnów Piedmont	49°54′58.2″N; 020°55′20.4″E	6	4.40	0.64	5
2	the Ciężkowice Piedmont	49°49′15.4″N; 021°21′06.5″E	82	3.29	0.52	1
3	the Strzyżów Piedmont	49°54′35.3″N; 021°50′07.8″E	47	2.39	0.41	0
4	the Lower Beskids	49°30′41.0″N; 021°41′08.1″E	42	3.85	0.62	6
5	the Lower Beskids	49°45′10.1″N; 021°47′58.7″E	34	3.68	0.56	3
6	the Bieszczady Mountains	49°41′29.4″N; 022°26′44.7″E	41	3.65	0.60	3
7	the Bieszczady Mountains	49°14′40.5″N; 022°34′14.3″E	25	4.09	0.67	3
8	the Bieszczady Mountains	49°16′35.1″N; 022°29′15.8″E	28	3.88	0.64	3
9	the Island Beskids	49°41′33.7″N; 020°18′14.4″E	29	3.84	0.64	4
10	Bielsko-Biała City	49°48′37.9″N; 019°05′21.5″E	22	3.61	0.61	0
11	the Opawskie Mountains	50°16′22.7″N; 017°25′08.1″E	24	3.45	0.58	1
12	the Przemyśl Piedmont	49°42′16.8″N; 022°31′53.1″E	1			
13	the Rożnów Piedmont, the Sącz Beskids	49°29′57.7″N; 020°40′33.3″E	1			

stretch of the streams, in various water pools. Animals were released immediately after sampling. Tissue samples and swabs were preserved in 96% ethanol and stored at 4°C.

Molecular markers

We used polymorphism of 10 nuclear microsatellite loci consisting of tetranucleotide repeats: *Sal3*, *Sal23*, *Sal29*, *SalE5*, *SalE6*, *SalE7*, *SalE8*, *SalE11*, *SalE12*, *SalE14* (Steinfartz, Küsters and Tautz, 2004) to investigate genetic variation, structure within populations, and to test the hypothesis that peripheral populations may reveal loss of genetic variation or long-term isolation. 758-bp fragment of mitochondrial DNA control region (*D-loop*) was sequenced to determine the affiliation of the Polish fire salamanders comparing to the European evolutionary lineages described by Steinfartz, Veith and Tautz (2000).

Laboratory methods

Total genomic DNA was extracted using Gene MATRIX Tissue DNA Purification Kit (EURx) according to the manufacturer's protocol. The presence of DNA and its quality were checked by agarose gel electrophoresis; ethidium bromide-stained DNA was visualized under UV light.

10 microsatellite loci were amplified using Polymerase Chain Reaction (PCR) method. To visualize products during capillary electrophoresis, forward primers were labelled with 4 fluorescent dyes: PET (*SalE12*, *SalE14*), VIC (*SalE5*, *SalE7*, *Sal23*), FAM (*Sal3*, *SalE6*, *SalE8*, *SalE11*) and NED (*Sal29*). Multilocus amplification was performed in 3 multiplexes. The first two (MP1, MP2) contained 4 loci each (*Sal3*, *SalE7*, *Sal23*, *Sal29* and *SalE8*, *SalE5*, *SalE12*, *SalE6*) and the third (MP3) was composed of 2 loci (*SalE14*, *SalE11*). PCR was performed in the total volume of 10 μl following the standard protocol used by Ogrodowczyk (2011). The reaction mixture contained Q Solution (Qiagen), 0.2 μM of the forward and reverse primers, dH_2O, PCR Multiplex Master mix (Qiagen) and salamander DNA. PCR reactions were performed as follows: 1 cycle of 15 min at 95°C, 40 cycles of 95°C for 30 s, primer annealing temperature (54°C for MP1, 59°C for MP2 and 60°C for MP3) for 1 min, 72°C for 1 min and final extension at 60°C for 30 min. The products were checked by agarose gel electrophoresis stained with GelRed and visualized under UV light. PCR product was diluted 1:10, then mixed with highly deionized formamide and GeneScan™-500LIZ® size standard (Thermo Fisher Sci.). Genotyping was performed on an 3130*xl* Genetic Analyzer (Applied Biosystems).

Mitochondrial DNA control region was sequenced in 17 individuals from 13 populations (from 11 localities used for microsatellite analysis and 2 additional localities as shown in the fig. 1 and table 1). Amplification and sequencing were carried out using the primers L-Pro-ML 5′-GGCACCCAAGGCCAAAATTCT-3′ and H-12S1-ML 5′-CAAGGCCAGGACCAAACCTTTA-3′ (Steinfartz, Veith and Tautz, 2000). PCR was performed in a total volume of 15.8 μl containing 3.2 μM of primers, Taq polymerase (Qiagen), 10 mM dNTPs, isolated DNA, 25 mM $MgCl_2$, 10× $(NH_4)_2SO_4$ and dH_2O. PCR reaction scheme was as follows: 1 cycle of 3 min at 94°C, 35 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 80 s, and final extension step at 72°C for 3 min. The presence of PCR products was checked by agarose gel electrophoresis. The products were purified using enzymes Endonuclease I (Exo I) and Thermosensitive Alkaline Phosphatase (FastAP) (Thermo Fisher Sci.). Both strands were sequenced with BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Sci.). Sequences were checked and analysed using CHROMAS LITE software version 2.1.1 (Technelysium Pty Ltd, http://technelysium.com.au/). Alignment and comparison of haplotypes were performed using BIOEDIT software version 7.2.5 (Hall, 1999). Haplotype classification of post-Pleistocene colonization lineage followed Weitere et al. (2004).

Microsatellite data analysis

MICRO-CHECKER 2.2.3. (Van Oosterhout et al., 2004) was used to detect genotyping errors, null alleles, large allele dropout and stuttering. We also checked for the presence of null alleles for each population and locus in FreeNA software package (Chapuis and Estoup, 2007) using EM algorithm (Dempster, Laird and Rubin, 1977). GENEPOP 4.3 software (Rousset, 2008, 2014) was used to test for deviations from Hardy-Weinberg equilibrium at each locus within populations. Estimation of exact *P*-values was performed by the Markov Chain method. To detect deficit of heterozygotes, inbreeding coefficient F_{IS} within all loci and subpopulations was calculated in GENEPOP 4.3; the significance of F_{IS} values was tested using 10 000 iterations. To control the type I error level of multiple tests, the Bonferroni correction was applied (Rice, 1989). Observed (H_O) and expected (H_E) heterozygosity were calculated for each subpopulation and regions in ARLEQUIN 3.5 (Excoffier and Lischer, 2010). Allelic richness (R_S) per locus and sample was calculated using FSTAT 2.9.3.2 (Goudet, 2011); in calculations of allelic richness we used the sample size of 6 individuals corresponding to the sample size from the population 1 (table 1). In comparison to initial analysis including 22 individuals, smaller number of individuals proved to be sufficient for correct indication of differences between populations, therefore we did not exclude population 1. F_{ST} values between pairs of localities were calculated in ARLEQUIN and their significance was tested using 10 000 permutations. An analysis of molecular variance (AMOVA) implemented in ARLEQIUN 3.5 was used to partition overall genetic variation into the among group, among population within groups, and within population levels; we used *locus-by-locus* AMOVA test and the variance significance level was estimated by 10 000 permutations.

The Bayesian clustering method implemented in STRUCTURE 2.3.4 (Pritchard, Stephens and Donelly, 2000; Porras-Hurtado et al., 2013) was used to determine the most likely number of genetically differentiated groups in the study area. We used correlated allele frequency and implemented admixture models. Analyses were performed for K values ranging from 1 to 12, with 10 independent runs for each K value. Burnin of 100 000 steps and post-burnin analysis of 100 000 steps were used. In order to select the most probable number of existing genetic groups (clusters), L(K) and ΔK (Evanno, Regnaut and Goudet, 2005) were calculated in software available on-line: STRUCTURE HARVESTER (Earl and von Holdt, 2012) and CLUMPAK (Kopelman et al., 2015; http://clumpak.tau.ac.il/).

Isolation by distance (IBD) (Slatkin, 1993) was assessed by Mantel test (10 000 permutations) implemented in ARLEQIUN 3.5 and visualised in Microsoft Office Excel 2007. Distances between sites were measured using GOOGLE EARTH software (Google Inc.). To define similar groups of sampled subpopulations, Nonmetric Multidimensional Scaling (NMDS) of the F_{ST} matrix for 2 dimensions was performed in STATISTICA 12 (StatSoft, USA). BOTTLENECK 1.2.02 software (Cornuet and Luikart, 1997) was used to detect recent effective population size reduction based on the distribution of allele frequencies. The Wilcoxon test was carried out (1000 iterations) for the two-phased model (T.P.M), recommended for microsatellite data (Di Rienzo et al., 1994), which allows multiple-step mutations and includes stepwise mutation model (S.M.M) and the infinite allele model (I.A.M). Test was performed with 70% proportion of S.M.M (Piry, Luikart and Cornuet, 1999). We also checked for bottleneck effect using only S.M.M model.

Mitochondrial DNA analysis

MtDNA control region sequences from the clade C (Steinfartz, Veith and Tautz, 2000) were aligned in Clustal W (Thompson, Higgins and Gibson, 1994) implemented in MEGA6 (Tamura et al., 2013). A sequence of *S. s. bernardezi*, classified into clade B (Steinfartz, Veith and Tautz, 2000), was used as an out-group. The total of 730 *D-loop* positions were used for phylogeny reconstruction with the Tamura 3-parameter model of nucleotide substitution and the Neighbor-Joining method in MEGA6. Robustness of the relationships among haplotypes was tested with 1000 bootstrap replicates.

Results

Microsatellite variation within populations

All loci were polymorphic at the level of the entire Carpathian dataset. The population from the Sudetes (11) was monomorphic in locus *Sal3*. The number of alleles ranged from 5 (*SalE5*) to 29 (*SalE12*) and overall 109 alleles were observed. In most cases genotype frequencies followed closely the Hardy-Weinberg expectations; 19 of 109 tests were significant at the nominal 0.05 level, but only one test remained significant after the Bonferroni correction (*Sal11* in population 6). MICRO-CHECKER analyses indicated that null alleles might be present at 4 loci but only in some populations: *Sal3* (1, 2, 8, 9), *Sal29* (1, 3), *SalE14* (4), *SalE11* (6). High frequency of null alleles was estimated in FreeNa only for locus *Sal3* in population 1 (0.333; supplementary table S1). Therefore, it appears that null alleles are not a major issue in our dataset and thereby results of the analyses are presented without correction for null alleles. Genetic variation, measured both as allelic richness (R_S, mean 3.7 $\pm$ (SD) 0.5) and as expected heterozygosity (H_E, mean 0.58 $\pm$ 0.07) was similar across populations. The two

outliers with respect to both measures of variation were population 2 ($R_S = 3.3$, $H_E = 0.52$), and particularly population 3 ($R_S = 2.4$, $H_E = 0.41$), both showing reduced microsatellite variation (table 1). The highest number of private alleles (6) was observed in population 4. No private alleles were found in populations 3 and 10 (table 1).

Population structure

Pairwise F_{ST} values between the Carpathian populations varied from 0.005 to 0.274 and all but two (7-8 and 1-9) were significant (table 2). The highest F_{ST} were noticed in comparisons involving populations 3 and 2. Including population 11 from the Sudetes, all pairwise F_{ST} ranged from 0.099 (9) to 0.323 (3) (table 2). The hierarchical analyses of genetic variation (AMOVA) for two groupings of populations indicated that most variation resides within populations; higher level grouping, although significant, explained only a minor fraction of variation (table 3). When population 3 was considered as a separate group, the differences between groups increased from 1.82% to 7.63% (table 3).

The STRUCTURE analysis supported the presence of 2 or 5 genetic clusters. The values of ln probability of data given K ($\ln \Pr(D|K)$) suggested the occurrence of 5 genetic groups; the ΔK method indicated the presence of 2 clusters. Because both these results may reflect underlying biological reality, we interpreted both clustering variants. For both 2 and 5 clusters, analysis clearly distinguished population 3 from the remaining populations (fig. 1a, b). Assuming 5 genetic groups, STRUCTURE distinguished additionally populations 2 and 11 as distinct clusters and suggested the existence of 2 genetic clusters corresponding to the Eastern and Western Carpathians (fig. 1b). However, admixture

Table 2. Population pairwise F_{ST} (below diagonal); values in bold were significant ($P < 0.05$). Above diagonal values present the geographic distance [km] between populations.

	1	2	3	4	5	6	7	8	9	10	11
1	–	32	63	67	65	113	139	135	51	135	255
2	**0.034**	–	36	37	34	182	108	104	74	165	286
3	**0.273**	**0.274**	–	41	19	65	92	89	109	198	318
4	**0.041**	**0.111**	**0.178**	–	23	49	72	69	98	192	318
5	**0.087**	**0.148**	**0.268**	**0.060**	–	51	78	75	106	198	321
6	**0.055**	**0.123**	**0.257**	**0.057**	**0.054**	–	27	24	146	240	367
7	**0.032**	**0.097**	**0.247**	**0.027**	**0.052**	**0.028**	–	4	168	262	390
8	**0.041**	**0.110**	**0.216**	**0.019**	**0.046**	**0.017**	0.005	–	165	286	387
9	0.036	**0.115**	**0.229**	**0.041**	**0.094**	**0.065**	**0.032**	**0.030**	–	94	220
10	**0.081**	**0.140**	**0.198**	**0.022**	**0.068**	**0.060**	**0.032**	**0.026**	**0.042**	–	128
11	**0.160**	**0.164**	**0.323**	**0.156**	**0.180**	**0.173**	**0.135**	**0.130**	**0.099**	**0.141**	–

Table 3. AMOVA results for two variants of populations grouped into Eastern (populations 5, 6, 7 and 8) and Western (populations 1, 2, 4, 9 and 10) districts of the Carpathian Mountains: (A) excluding population 3; (B) with population 3 treated as a separate group.

Source of variation	d.f.	Percentage of variation
(A) Excluding population 3		
among groups	1	1.82
among populations within groups	7	6.58
within populations	609	91.60
(B) With population 3 treated as a separate group		
among groups	2	7.63
among populations within groups	7	6.49
within populations	702	85.88

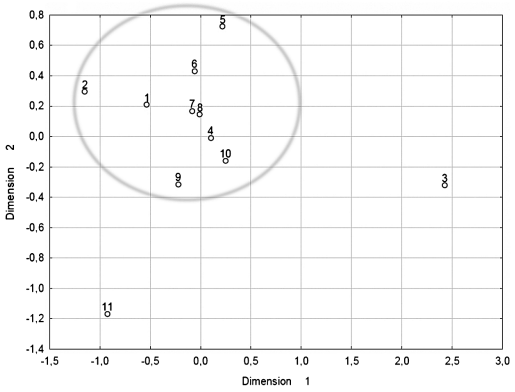

Figure 2. Nonmetric two-dimensional scaling of the F_{ST} matrix for all populations (stress value = 0.0416).

of these two clusters was detected in most populations. Populations 3 and 11 were also clearly separated in the NMDS analysis (fig. 2).

The Mantel tests showed positive, but statistically not significant, correlation between genetic and geographical distances for all populations. For the Carpathian mountain range the correlation was negative and not significant (supplementary fig. S1). The analysis in BOTTLENECK 1.2.02 did not provide evidence for recently bottlenecked populations.

Mitochondrial DNA analysis

The analysis of mitochondrial control region showed that all sequenced mtDNA fragments represented the same haplotype IIb (fig. 3).

Discussion

Microsatellite variation

Nuclear microsatellites used for the study varied in the number of alleles from 1 (*Sal3*, population 11) to 16 (*SalE12*, population 7) and suggest differences in mutation rate among loci, which is probably influenced by the tandem repeat structure, which affects the rate of polymerase slippage, or their position in the genome (Li et al., 2002). The number of alleles identified among loci corresponded to the variation level reported originally for populations from Germany (Steinfartz, Küsters and Tautz, 2004). The highest level of polymorphism was found in the locus *SalE12* (29 alleles). Locus *SalE5* was the least variable with the total of 5 alleles. The largest number of private alleles had been found in populations 6, 5 and 4, demonstrating their higher variability in contrast to populations 3 and 10 where no private alleles were observed.

While most of the Carpathian populations exhibit relatively high and similar level of genetic variation, two populations (3 and 2) inhabiting the extreme margin of the species range had considerably reduced variation which we attribute to the strong influence of the genetic drift. All analyses clearly identified the population 3 as the least variable and most differentiated from the remaining populations. The population, which inhabits the area of about 3 hectares, was discovered in 2008 and represents the extreme peripheral area of the taxon's range (Nowak, 2010). STRUCTURE analysis (fig. 1a) and NMDS plot of pairwise F_{ST} (fig. 2) identified this population as significantly distinct from the others. This divergence is most likely not a result of long-term isolation, but appears rather the consequence of low effective population size and recent habitat fragmentation. Under such conditions, drift would cause both substantial reduction of variation and considerable divergence of allele frequencies setting the population apart from the others in analyses based on allele frequencies. The lack of private alleles in this population supports this scenario.

Population 2 was also found as genetically differentiated. Variation in this population is reduced (see table 1), but admixed individuals were identified in the STRUCTURE analysis (fig. 1b). We suspect that this population may have become isolated more than 50 years ago due to extension of farmlands. According to Steinfartz, Weitere and Tautz (2007), no differences were found in population of *Salamandra salamandra* (Germany) isolated approximately 40 years ago, but more than 150-year-old isolation caused significant differences in genetic variation. However, the rate of differentiation

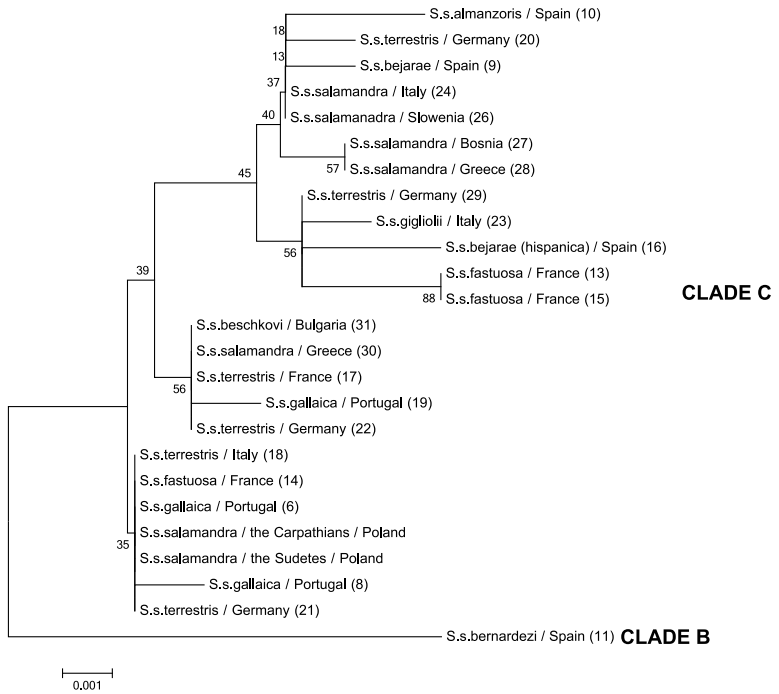

Figure 3. The neighbour joining tree of mitochondrial DNA haplotypes of the fire salamander *Salamandra salamandra* subspecies belonging to clade C, described and numbered as previously published in Steinfartz, Veith and Tautz (2000), including the Sudetes (described by Ogrodowczyk, 2011) and populations of Polish part of the Carpathians. Numbers at nodes represent the bootstrap values.

will primarily depend on the effective population size (Beebee, 2005). Patterns of genetic impoverishment or isolation in marginal populations of urodeles had been found in *Mesotriton alpestris* in Greece (Sotiropoulos et al., 2008) or *Salamandra infraimmaculata* in Northern Israel (Blank et al., 2012).

Population structure and gene flow

Under the influence of unfavourable environmental conditions, populations especially occurring at the periphery of the species range may experience multiple disruptions to gene pool exchange, and therefore are expected to show significantly lower genetic variation caused by genetic drift, inbreeding or bottleneck effect (Slatkin, 1987; Manel et al., 2003). Generally, analysis of the Carpathian populations of *S. salamandra* led to the main conclusion that most populations show little genetic differentiation and no evidence of strong barriers to gene flow (see fig. 1a). The Alpine newt *Ichthyosaura alpestris* (Pabijan and Babik, 2006), the Carpathian newt *Lissotriton montandoni* (Zieliński et al., 2014) and most of the Sudetes populations of the fire salamander *S. salamandra* (Ogrodowczyk, 2011) show the same pattern. The genetic effects of factors such as fragmentation or geographical isolation often occurring at the margin of the species range and leading to population differentiation can be observed in population 3 and 2. Similarly, Ogrodowczyk (2011) detected one peripheral and isolated population in the Sudetes, inhabiting the Ślęża Massif, which showed reduced variation consistent with long-term isolation and small effective population size.

In our study, Mantel test did not confirm genetic isolation by distance between the Carpathian populations; low F_{ST} and 'among group' molecular variance analyses implied a substantial gene flow among studied locations.

The NMDS assigned most of the studied populations to a single cluster (fig. 2). STRUCTURE results for K = 5 showed subtle structure in the eastern and western part of the Carpathians (fig. 1b) with many populations showing admixture of two or more clusters.

Statistical analysis also provided some evidence of differentiation between populations inhabiting the Sudetes and Carpathians (see population 11 separated from the others in the fig. 1b or fig. 2). Such significant differences between regions were also confirmed in populations of the Alpine newt from the same area (Pabijan and Babik, 2006). However, in this study we considered only one population from the Sudetes and further studies of both mountain ranges are required.

The STRUCTURE analysis revealed that genotypes of several specimens from population 9 appeared to be similar to those observed in population 11 (see fig. 1b). Therefore, we followed clustering for K = 8, suggested as most probable in Prob(K) chart generated in CLUMPAK, which showed genotype differentiation. The explanation for the contentious proportion of genotypes observed in population 9 may be the origin of individuals belonging to 2 different families.

Post-glacial colonization of the Carpathians

To investigate the origin of the fire salamanders which colonized the Carpathian Mountains after the last glaciation, we sequenced the mitochondrial control region from various localities (table 1). All sampled individuals possessed haplotype IIb, also fixed in the populations from the Sudetes (see Ogrodowczyk, 2011) (fig. 3). Mitochondrial DNA homogeneity supported our hypothesis that colonization proceeded from the Balkan refugium.

Following the linear model of animal migration, higher genetic variation is expected in population occurring closer to the original founder population, while the effect of bottlenecks will be more visible along the colonization routes (Schmitt, 2007). Bayesian clustering for K = 5 suggested possible differentiation between the Western and Eastern Carpathians (fig. 1b). However, patterns of the post-glacial colonization require further analysis including remaining parts of the Carpathians and the Sudetes. Assuming the significantly higher variation in the Sudetes (and the Carpathians representing the subset of the Sudetes alleles), we would presume that *S. salamandra* migrated in the west-east direction. Following the post-glacial colonization of numerous European herpetofauna, such as the European pond turtle *Emys orbicularis* (Sommer et al., 2009; Prusak et al., 2013), we also propose the hypothesis of the expansion route from the Western Balkans through the Middle Europe (present-day Austria, the Czech Republic and through the Moravian Gate), with the north-west orientation, and then split into two colonizing directions: western of the Sudetes and eastern of the Carpathians.

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