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Population genetics of fire salamanders in a pre-Alpine urbanized area (Salzburg, Austria)

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Abstract. Habitat alteration has been identified as one of the major causes of amphibian decline. In this study, the genetic structure of seven fire salamander subpopulations in the urbanized area of Salzburg (Austria) was investigated based on seven polymorphic microsatellite loci. We combined Bayesian clustering approaches (STRUCTURE, TESS) with the traditional F-statistics to evaluate the effect of potential barriers on gene flow. Both clustering approaches suggested that all sampled individuals belong to a single genetic pool (K = 1). While no clear-cut sign of genetic differentiation could be detected, pairwise F_{ST}-values suggest that the city of Salzburg potentially has an effect, but the effect of the highway leading to it remains hypothetical. This study corroborates that habitat alteration effects might take several generations before leading to isolated genetic pools, particularly in long-lived species. Such delayed effects have to be taken into account for population genetic analyses, in particular when it comes to conservation management and planning.

Key words. Bayesian clustering, *Salamandra salamandra*, habitat fragmentation, pairwise F_{ST}, microsatellite.

Introduction

Habitat fragmentation, mostly resulting from processes of urban and agricultural development, has been named as one of the major reasons for the current loss in biodiversity (Fahrig 2003). Affected species experience habitat loss and a decrease in population size together with a reduction of habitat connectivity, leading to restricted gene flow between populations. This results in lower genetic diversity, increased inbreeding and even local extinctions in extreme cases (e.g., Cushman 2006, Delaney et al. 2010). Habitat alteration might consequently have a strong negative influence on the long-term persistence of species. Identifying the effects of habitat fragmentation at the population-level for amphibians, which are considered as one of the most threatened vertebrate classes on earth (Beebee & Griffiths 2005), is of particular interest for the development of conservation plans (e.g., design of corridors to mitigate negative impacts of urbanization and transportation).

Several studies have shown that constantly increasing urbanization is a strong limiting factor for the gene flow between populations and leads to genetic differentiation between (sub-)populations (e.g., Dubey et al. 2009, Delaney et al. 2010, Emaresi et al. 2011). However, the extent of genetic changes varies and depends on various factors like the vagility of the species, population parameters, time elapsed, type of barrier (partial, complete), and the quality of the remaining habitats (e.g., Landguth et al. 2010). In addition, the ability to detect linear barriers to gene flow also depends on the analytical techniques employed (Balkenhol & Waits 2009, Safner et al. 2011, Blair et al. 2012).

This study focuses on the potential effect of the progressing urbanization on the population structure of fire salamanders in the greater Salzburg area. The fire salamander, *Salamandra salamandra* (Linnaeus, 1758), has a complex life cycle, undergoing aquatic larval and terrestrial adult stages. It is a common species in most parts of its distribution range, but listed as Near Threatened on the Red List of Austria (Gollmann 2007). Fire salamanders show strong site fidelity and have small home ranges, nonetheless recent mark-recapture studies have suggested that fire salamanders are much more motile than previously assumed (Schmidt et al. 2007, Schulte et al. 2007). Consequently, the effect of habitat fragmentation on this species is difficult to foresee. In a preliminary attempt to evaluate this effect, a limited sampling strategy was applied, focusing on the urbanized region of Salzburg, where anthropogenic barriers (urbanized area, highway, diked river) were expected to affect the genetic structure of fire salamanders.

Table 1. Sampling location, number of sampled individuals (N_S; A – adults; L = larvae), locality coordinates (based on WGS84 geographic coordinate system), number of alleles (N_A), allelic richness (Ar; based on a minimum sample size of 5), within population gene diversity (H_S) and observed heterozygosity (H_O; derived from H_S and F_{IS} values as $H_O = H_S - (F_{IS} \times H_S)$), F_{IS} values (none was significant) and average pairwise F_{ST} values. Overall F_{ST} and F_{IS} following Weir & Cockerham (1984) were 0.039 (*) and 0.031 (NS).

Location (code)	N_S (A/L)	Coordinates	N_A	Ar	H_S	H_O	F_{IS}	F_{ST}
Aigen (Aig)	7 (7/0)	47.786067 13.093283	25	3.123	0.534	0.462	0.134	0.033
Gersberg (Ger)	8 (8/0)	47.805167 13.088883	27	3.287	0.554	0.594	-0.073	0.024
Heuberg (Heu)	6 (6/0)	47.826600 13.128667	21	2.920	0.532	0.486	0.087	0.030
Kapuzinerberg (Kap)	9 (9/0)	47.801350 13.050783	21	2.755	0.565	0.528	0.066	0.063
Maria Plain (Mpl)	10 (1/9)	47.839617 13.046117	28	3.471	0.632	0.672	-0.063	0.060
St. Wolfgang (Stw)	15 (0/15)	47.742300 13.458150	33	3.382	0.573	0.596	-0.04	0.031
Untersberg (Unt)	15 (0/15)	47.722417 13.036200	30	3.081	0.543	0.464	0.145	0.020

Besides traditional F-statistics (Wright 1943, 1950), which are commonly used to evaluate genetic differentiation within and among a priori defined subpopulations, two different Bayesian clustering approaches (spatial and non-spatial) were used to identify the genetic structure of fire salamanders (*Salamandra salamandra salamandra*, Linnaeus, 1758) in the urbanized region of Salzburg.

Material and methods

Study area

The study area (476 km²) is located in two provinces of northwestern Austria (Salzburg, Upper Austria). Six sampling sites (Table 1) were chosen to cover the whole Salzburg Basin, dominated by the river Salzach and bordered by two mountain ranges: Untersberg in the west and Osterhorngruppe in the east. This region shows typical habitat characteristics suitable for fire salamanders; deciduous or mixed forest with several little brooks or streams that are used for depositing larvae (on the Kapuzinerberg only ponds are found). A seventh sampling site (Table 1) was located in St. Wolfgang (StW, Upper Austria), which lies around 30 km east of Salzburg. Regarding potential barriers to gene flow, a six-lane highway built in the 1970s separates Untersberg (Unt) and Maria Plain (MPl) from the others (Fig. 1). Kapuzinerberg (Kap) and Maria Plain (MPl) lie in a densely urbanized area. Finally, the Salzach River known to act as a barrier for crested newts (Maletzky et al. 2009), separates Untersberg in the west from all other sites.

Sampling

From March to September 2011, a total of 70 individuals were sampled, including 30 adults in close proximity (less than 100 m) of streams used as breeding sites and 40 larvae in small streams or ponds. To avoid any possible spread of chytridiomycosis, individuals were handled according to the instructions given in Schmidt et al. (2009). For adults, DNA samples were taken with sterile swabs (buccal or cloacal/skin swabbing: Model 501C201, Copan, Italy) or by collecting a small piece of tissue from road-killed individuals. Larvae were collected in different sections of a stream to minimize the risk of sampling siblings and ventral and dorsal skin swabs (Model 516CS01, Copan, Italy) were taken. All samples were stored in 1.5-ml Eppendorf tubes and frozen at -20°C until DNA extraction. At each sampling site, 6 to 15 samples were collected (Table 1).

Genotyping

DNA extractions were performed using the DNEasy Blood & Tissue Kit (QIAGEN) with an adapted protocol following Emaresi et al. (2011). Samples were genotyped using nine microsatellite markers (SALE2, SALE5, SALE6, SALE7, SALE8, SALE11, SALE12, SAL3, SAL29 developed by Steinfartz et al. (2004); SALE14 & SAL23 could not be efficiently optimised). Loci SALE5 & SAL29 had to be removed from the final dataset due to peak readability problems and low (< 50%) amplification success, respectively. A Polymerase Chain Reaction was performed in a total reaction volume of 20 µl with a final concentration of 1x Qiagen PCR buffer, 0.5 µM for each primer, 0.2 mM dNTP's, 0.5 U (1 U for SALE2/SALE7/SAL3) QIAGEN Taq Polymerase, 4 µl extracted DNA (11.8–266 ng/µl DNA) and 1.5 mM $MgCl_2$ (2 mM for SALE2). Forward primers were labelled with fluorochromes: HEX, FAM or ATTO 550. The following thermal profiles were used: initial denaturation for five minutes at 95°C, followed by 40 cycles (45 cycles for SALE2/SALE11, 35 cycles for SALE8/SALE12) of 30 sec of denaturation at 94°C, 1 min of annealing at 53°C (59°C for SALE6, 58°C for SALE8/SALE12 and 50°C for SALE11), elongation at 72°C for 1 min, and final elongation at 72°C for 10 min. Genotyping runs were performed on a MegaBACE™ 750 (Amersham Biosciences) and allele sizes were scored using MegaBACE™ Fragment Profiler Version 1.2.

Statistical analyses

The following summary statistics were computed with FSTAT 2.9.3.2 (updated from Goudet 1995) for each pop-

ulation for the seven microsatellite loci (Table 1) and for each locus independently (Table 2): number of alleles (N_A), allelic richness (Ar), within population gene diversity (H_S), F_{IS} per population, as well as overall F_{IS} and F_{ST} (following Weir & Cockerham 1984). Pairwise F_{ST}-values for all populations were estimated, and p-values were obtained after 21,000 permutations. Tests for pairwise linkage disequilibrium (21,000 permutations) and departure from the Hardy-Weinberg equilibrium (10,000 randomisations) between all loci were carried out using FSTAT. Micro-checker 2.2.3 (van Oosterhout et al. 2004) was used to detect the presence of null alleles for each marker based on 3,000 randomisations and using the Bonferroni correction. The software COLONY 2.0.1.4 (Jones & Wang 2010) was used to detect full and half siblings.

The correlation between genetic distance and geographical distance between populations was tested with a Mantel test (Mantel 1967) to check for isolation by distance. Pairwise $F_{ST}/(1-F_{ST})$ (Rousset 1997) values were calculated with SPAGeDi 1.3 (Hardy & Vekemans 2002) and regressed against the natural logarithm of the Euclidian geographical distances (km) among each pair of sampling sites based on 10,000 permutations.

Two different Bayesian clustering programs were used to investigate the genetic structure. STRUCTURE 2.2.3 (Pritchard et al. 2000, Falush et al. 2003) uses a Markov Chain Monte Carlo (MCMC) simulation based on allele frequencies to detect K, a true number of clusters that are in both Hardy-Weinberg (HWE) and linkage equilibria. The admixture model was run with correlated allele frequency, using no a priori information about sampling sites (using no admixture or uncorrelated allele frequencies did not change the results, data not shown). Ten runs were generated, each with 100,000 MCMC iterations (10,000 burn-in) from K = 1 to K = 7. To determine the most likely K-value in our dataset, the logarithmic probability of the data (Ln P(D)) was computed for each K. TESS 2.3.1 (Chen et al. 2007) is a spatial clustering method that estimates population structure using multilocus genotypic data together with geographical coordinates (individual spatial coordi-

Table 2. Number of alleles (N_A), allelic richness (Ar; based on a min. sampling size of five individuals), observed heterozygosity (H_O) and intrapopulation gene diversity (H_S) according to Nei (1987), F_{ST} and F_{IS} following Weir & Cockerham (1984), for each locus. Levels of significance (*P < 0.05, **P < 0.01, and ***P < 0.001) of F_{ST} (based on 10,000 randomisations of genotypes among samples) and F_{IS} (based on 10,000 randomisations of alleles within samples) are indicated.

Locus	N_A	Ar	H_O	H_S	F_{ST}	F_{IS}
SALE12	9	4.521	0.837	0.737	0.044***	-0.102
SALE7	7	2.574	0.359	0.335	0.050**	-0.087
SALE8	6	3.864	0.661	0.697	0.046*	0.052
SALE2	9	3.690	0.513	0.579	-0.027	0.111
SAL3	11	3.734	0.584	0.627	0.112***	0.052
SALE11	3	2.472	0.449	0.433	0.021	-0.045
SALE6	4	2.452	0.399	0.525	0.006	0.209*
Overall	49		0.543	0.562		

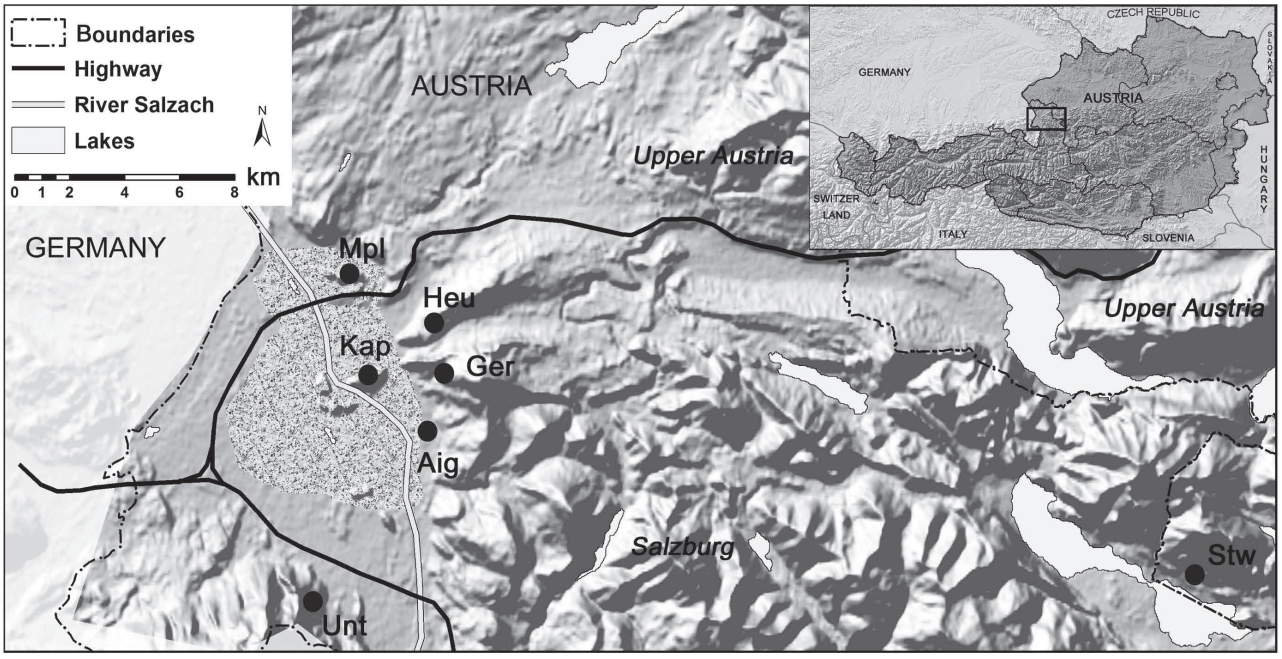

Figure 1. Locations of the seven sampling sites (abbreviations as in Table 1) within Salzburg and Upper Austria and potential barriers to gene flow (the densely urbanized area in dark grey, shaded area). The inset gives a general overview of the study area.

Table 3. Table showing pairwise F_{ST}-values in the upper right corner (levels of significance are indicated: * P < 0.05, ** P < 0.01, and *** P < 0.001) and pairwise geographical distances in km in the lower left corner

	Aig	Ger	Heu	Kap	Mpl	StW	Unt
Aig		0.0303	0.0601	0.0072	0.0623*	0.0368	0.0026
Ger	2.15		-0.0149	0.0592	0.0794*	-0.0028	-0.0067
Heu	5.23	3.81		0.1009*	0.0283	-0.0005	0.0088
Kap	3.61	2.89	6.47		0.0779*	0.0959***	0.0394
Mpl	6.92	4.99	6.35	4.27		0.0453	0.068**
StW	27.78	28.54	26.40	31.23	32.71		0.0087
Unt	8.27	10.01	13.50	8.84	13.05	31.72	

nates were derived from the locations coordinates using the "Generate Spatial Coordinates" option in TESS). The "admixture model" was chosen to determine the number of clusters. Ten runs for each number of K_{max} from 2 to 7 were carried out with a burn-in of 20,000 sweeps and a total of 100,000 sweeps. For each run, the Deviance Information Criterion (DIC: Spiegelhalter et al. 2002) was computed to infer the number of clusters. As the DIC value did not reach a clear plateau, we computed the mean assignment value of each sampling site to a given cluster (by averaging individuals' assignment values, over the 10 replicated runs for each K = 2 to K = 7) to determine the most likely K.

Finally, as sample sizes were unbalanced (Table 1) and the number of used microsatellites limited, the statistical power of our setting for revealing population differentiation, quantified as F_{ST}, and the α error probability (type I; probability to detect genetic differentiation while there is none), were evaluated using the simulation-based software POWSIM version 4.1 (Ryman & Palm 2006). The results indicated that our setting was powerful enough to detect levels of true genetic differentiation of 0.02 and higher (probability 85–90 % for 0.02 and of 99–100 % for F_{ST}-values above 0.032, with the Fisher method providing slightly lower values than the chi-square approach). The α error probability was lower than 0.05 with the chi-square approach (only slightly above using the Fisher method: 0.06), suggesting that there was a low risk for false significance. In respect to the power of clustering analyses, a simulated dataset of 10 codominant loci of a population structured into five subpopulations showed that STRUCTURE can correctly identify the true number of subpopulations at low levels of population differentiation (F_{ST} = 0.03) (Latch et al. 2006). We consequently think that our study design is powerful enough to detect any deviation from genetic homogeneity.

Results

Pairwise geographical Euclidian distances between the different sites ranged from 2.15 to 32.71 km (Table 3). A total of 70 individuals from seven different sampling sites were genotyped at seven microsatellite loci (genotyping success: 97%). The number of alleles per sampling site ranged from 21 to 33 with a total of 49 alleles across the seven sites. Allelic richness (based on a minimum sample size of 5) ranged between 2.755 and 3.471 per site. Expected (H_S) and observed (H_O) heterozygosities per population ranged from 0.532 to 0.632 and 0.462 to 0.672, respectively. No significant inbreeding coefficient (F_{IS}) values were found at sampling site level (Table 1). The information concerning each locus is provided in Table 2. No evidence for significant linkage disequilibrium was found between any pair of loci. Null alleles were detected for SALE6, SAL3, and SALE2, causing a significant departure from the HW equilibrium only for SALE6 (F_{IS} = 0.209; P = 0.0378). All loci were kept for further analysis nevertheless, as a simulation analysis of the effect of null alleles on estimates of population differentiation by Chapuis & Estoup (2007) found that F_{ST} would not be biased because of null alleles when lacking population substructure. This is indeed the case here, as is indicated by a significant low overall F_{ST}-value of 0.039. COLONY did not find any evidence of full siblings.

Average pairwise F_{ST}-values between sampling sites were low and ranged from 0.020 to 0.063 (Table 1). The correlation between geographical (ln (km)) and genetic distance ($F_{ST}/(1 - F_{ST})$) was not significant (P = 0.87, r_2 = 0.014), suggesting no isolation by distance. Both Bayesian clustering methods identified K = 1 as the true number of clusters for our dataset. With STRUCTURE, the highest log-likelihood probability was obtained for K = 1 (Ln P(D) = -1146.98). For TESS, all sampling sites were assigned to a single cluster with average assignment values greater than 0.93 for all K_{max}-values tested.

Discussion

Contrary to our expectations of observing an effect of anthropogenic barriers on the spatial genetic structure of fire salamanders, our study found no clear overall genetic differentiation, and Bayesian clustering methods suggested that all individuals belonged to one genetic pool. The absence of a clear genetic differentiation might suggest that either (i) high gene flow between sampling locations is still maintained despite geographical habitat fragmentation,

or (ii) potentially large population sizes avoid or delay the effect of genetic drift (i.e., time lag between the establishment of a barrier to gene flow and its genetic signature; Landguth et al. 2010).

Regarding the first hypothesis, recent studies have shown that dispersal is not as limited as was commonly perceived for amphibians (Smith & Green 2005). In particular, salamander species might migrate over longer distances than previously presumed (e.g., fire salamanders: Schmidt et al. 2007, Schulte et al. 2007; Alpine salamanders: Helfer et al. 2012).

Concerning the second hypothesis, fire salamander densities can reach up to 400 individuals/hectare (Steinfartz et al. 2007) and assuming that the fire salamanders in our study area had high population densities before habitat alteration occurred, population size together with the longevity of the species (up to 20 years; Feldmann 1987) could indeed have delayed the effect and signature of genetic drift (Anderson et al. 2010). Thus, the impact of real barriers to gene flow might not be detectable yet, because fragmentation happened too recently.

While no clear sign of genetic differentiation could be detected by the clustering approaches, the effect of habitat alteration should nonetheless be evaluated with care and not underestimated. Urbanization, including the increase in human density and infrastructure, significantly changes the ecological conditions of amphibian habitats by either changing habitat availability (fragmentation, isolation or loss) or habitat quality (water quality, disease, vegetation) (Hamer & McDonnell 2008). A negative correlation between urbanization and amphibian richness and abundance has been observed in anurans (e.g., Knutson et al. 1999). Moreover, several studies based on F_{ST}-values produced evidence of negative effects of landscape change (roads, urbanization) on the spatial genetics of amphibians (e.g., in *Rana arvalis*: Arens et al. 2007, *Hyla arborea*: Dubey et al. 2009, *Ichtyosaura alpestris*: Emaresi et al. 2011). In this study, F_{ST}-statistics appear to be more effective than Bayesian clustering approaches in detecting genetic differentiation. Kapuzinerberg and Maria Plain, two forest islands located within the densely populated Salzburg area, with the latter being also isolated by the highway, have several significant and high pairwise F_{ST}-values (Table 3; see Table 1 for averaged values).

The absence of isolation-by-distance suggests that at our spatial scale drift effects are more influential than gene flow (Hutchison & Templeton 1999), as is suspected for Kapuzinerberg and Heuberg according to their low allelic richness (Table 1), or that other factors (like anthropogenic barriers) than geographic distance might affect the genetic structure.

For Kapuzinerberg, the significant F_{ST} together with the low allelic richness suggests that this subpopulation might have experienced a population decline that has accelerated a local genetic drift. For Maria Plain, the underlying process remains unclear, as this subpopulation exhibits the highest allelic richness with an observed heterozygosity higher than the overall population (H_O = 0.672, overall H_O = 0.543). Maybe here, the genetic differentiation stems mainly from a reduction in gene flow due to the highway. The potential effect of the highway as a barrier to gene flow remains hypothetical though, as no significant genetic differentiation could be observed for the Untersberg population, which is also geographically isolated from the other sampling sites by the highway and the Salzach River. A microsatellite study of *Salamandra salamandra* in the Kottenforst near Bonn, Germany, found that a highway built 40 years ago had no impact on the genetic structure (Steinfartz et al. 2007), while empirical evidence of negative effects of roads on the genetic diversity and differentiation of amphibians has been shown by several other studies (e.g., Holderegger & di Giulio 2010).

The lack of evidence of an effect of the Salzach is surprising, as Maletzky et al. (2009) found that the Salzach acted as a migration barrier for crested newts, and other rivers have been reported to affect other amphibians' genetic structures (e.g., Angelone & Holderegger 2009). This could be explained by a passive gene flow caused by larval drift, as fire salamanders in the study area deposit their larvae mainly in tributary streams of the Salzach, which regularly experience strong currents and high water levels after heavy rains. Larval drift rates of up to 41% were reported by Thiesmeier & Schuhmacher (1990).

According to our study, an effect of habitat alteration on the genetic structure of *S. salamandra* cannot be excluded, but it is not strong enough to lead to clear independent genetic populations. To better understand the underlying causes and mechanisms leading to the observed genetic structure, further studies should be performed to estimate population densities and directly test for the effect of landscape features on genetic structure using landscape genetics approaches. These should be based on a more robust dataset, increasing the sample size and making use of more, already developed, microsatellite loci (Hendrix et al. 2010) to test our preliminary study. More generally, theoretical studies based on simulation analysis in landscape genetics will help to better evaluate the potential effect of habitat alteration on the genetic structure of species with different life history traits, such as dispersal ability or longevity (Anderson et al. 2010, Landguth et al. 2010, Blair et al. 2012).

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