Short Notes

Genetic differentiation in two European tree frog (*Hyla arborea*) metapopulations in contrasted landscapes of western Switzerland

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Abstract. The survival of threatened species as the European tree frog (*Hyla arborea*) is strongly dependent on the genetic variability within populations, as well as gene flow between them. In Switzerland, only two sectors in its western part still harbour metapopulations. The first is characterised by a very heterogeneous and urbanized landscape, while the second is characterised by a uninterrupted array of suitable habitats. In this study, six microsatellite loci were used to establish levels of genetic differentiation among the populations from the two different locations. The results show that the metapopulations have: (i) weak levels of genetic differentiation (F_{ST} within metapopulation ≈ 0.04), (ii) no difference in levels of genetic structuring between them, (iii) significant ($p = 0.019$) differences in terms of genetic diversity (*Hs*) and observed heterozygozity (*Ho*), the metapopulation located in a disturbed landscape showing lower values. Our results suggest that even if the dispersal of *H. arborea* among contiguous ponds seems to be efficient in areas of heterogeneous landscape, a loss of genetic diversity can occur.

Keywords: amphibians, conservation, fragmentation, *Hylidae*, microsatellite markers.

As a result of growing urbanization and subsequent habitat degradation and fragmentation, numerous animal species currently face extinction or have declined drastically during the last century. These disturbances commonly result in population size reduction and decrease the possibility of migration and subsequent gene flow between populations (e.g. Frankham et al., 2002; Cushman, 2006). The genetic consequences of population fragmentation are complex and critically depend upon levels of gene flow between fragments. When it is restricted, population fragmentation is expected to reduce within-population genetic polymorphism and increase genetic differentiation among populations, typically leading to a loss of genetic diversity within fragments (e.g. Hitchings and Beebee, 1997). Consequently, understanding the effects of population fragmentation is crucial in conservation biology, since extinction risks are more elevated in fragmented populations with low levels of genetic variability (Frankham et al., 2002; Frankham, 2005).

The studied species, The European tree frog (*Hyla arborea*), is a pond-breeding species which possess a distribution area extending from Portugal to southern Sweden, and from the Balkans to west Asia (Gasc et al., 1997). As in several other amphibian species (Wake, 1991), a strong decline across its entire distribution range has been recorded during the last decades (Gasc et al., 1997). In Switzerland, once widespread across this country, the species has been reduced to less than one dozen sec-

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tors, which undergo local extinctions (Grossenbacher, 1988, 1994). This severe decline is thought to be mainly due to anthropogenic activities. Studies carried out on the European tree frog in Sweden (Carlson and Edenhamn, 2000), as well as in Switzerland (Pellet et al., 2006), highlighted regular extinction and recolonisation events characteristic of a metapopulation dynamics (Hanski and Gilpin, 1997). Extensive theory has been developed to model genetic processes within metapopulation structures. Due to frequent extinctions and bottlenecks during recolonisations, metapopulations are likely to suffer more rapidly from inbreeding and fitness reduction than single large populations with the same total size (Gilpin, 1991; Hanski and Gilpin, 1997).

In this study we sampled the two remnant metapopulations of the western portion of Switzerland (Dubey et al., 2006): (i) the first lies on the northern shore of the lake of Geneva (LG metapopulation, fig. 1), a region characterised by a very heterogeneous and mixed agricultural and urbanized landscape, and present a low number of occupied ponds (less than 25; Pellet et al., 2002); (ii) the second is located on the southern bank of the lake of Neuchâtel (LN metapopulation), which on the opposite is an uninterrupted landscape of suitable habitats, including marshes and wet meadows, as well

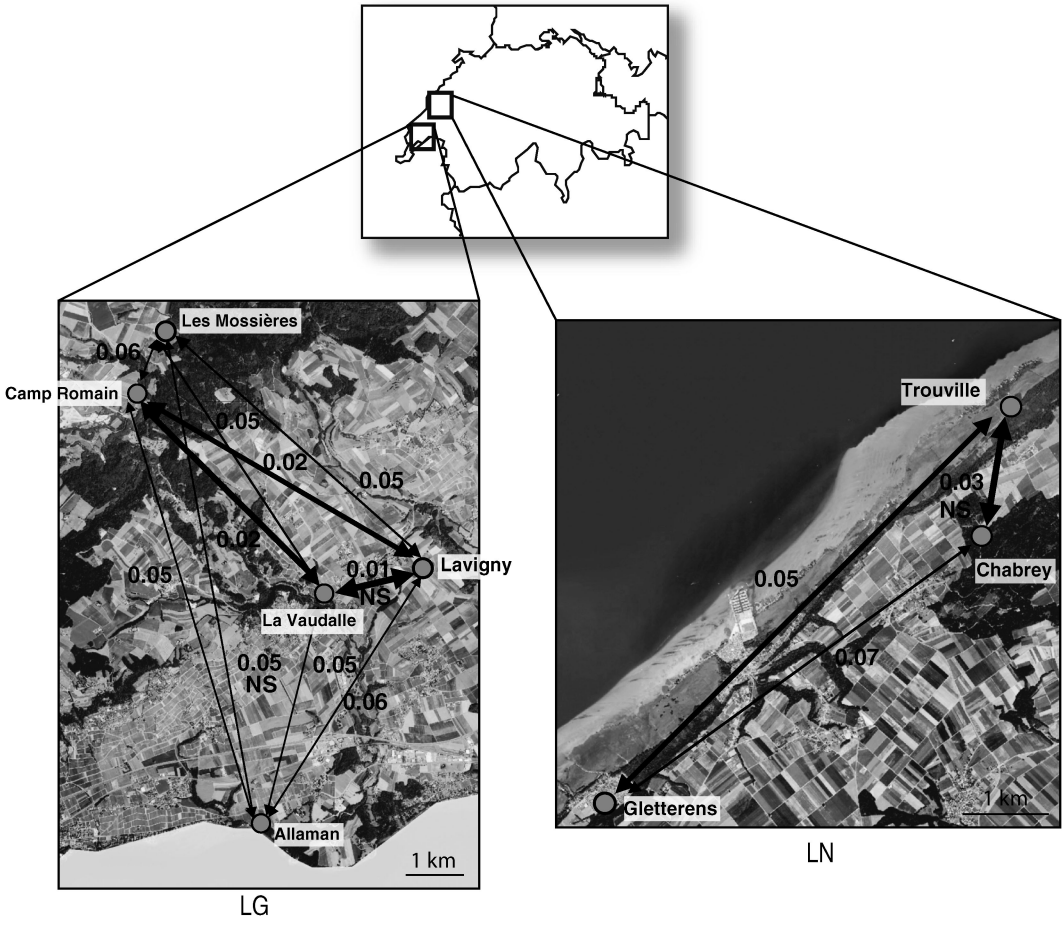

Figure 1. Localisation of demes within the northern shore of the lake of Geneva (LG) and the southern bank of the lake of Neuchâtel (LN), with arrows corresponding to pairwise Fst values between demes within metapopulations (all values significant at 0.05 level are indicated; the thickness of arrows indicates the level of gene flow).

as woodland bordering the lake in a continuous way. In the year 2000, 40 calling ponds were detected, totalling several thousand frogs (Pellet and Neet, 2001). This area is considered as one of the largest metapopulation in Switzerland (Grossenbacher, 1988).

Information concerning the extent of population fragmentation is critical to determine whether a species requires proactive management plans to reduce extinction risks associated with genetic stochasticity. Hence, to estimate the effect of habitat fragmentation on the genetic structure and variability of tree frog populations, we investigated the two isolated metapopulations in western Switzerland with six microsatellite loci. We hypothesized that in metapopulations embedded in continuous habitats genetic structure would be lower, while genetic variability and gene flow higher compared to metapopulations characterized by lower densities and where heterogeneous habitat may act as a barrier to gene flow.

During spring 2002 and 2003, a total of 235 samples of *Hyla arborea* (tadpoles and eggs) were collected in 8 ponds in the two studied areas (fig. 1). Five ponds were sampled within the LG metapopulation (Allaman, Camp Romain, Lavigny, Mossières, Vaudalle) and three ponds in the LN metapopulation (Chabrey, Gletterens, Trouville). When eggs were collected, only one egg per clutch was used for genetic analyses.

DNA extraction from tissues and egg samples was carried out using the QIAamp DNA Mini Kit (QIAGEN), or with a CTAB protocol (Milligan, 1992). Six microsatellite loci isolated and characterized for *Hyla arborea* (Wha1-9, Wha1-20, Wha1-25, Wha1-103, Wha1-104, Wha1-140; Arens et al., 2000) were amplified and scored (see Arens et al. (2000) for the specific PCR profiles). Amplified products were genotyped with an ABI PRISM 377 DNA Sequencer using genescan analysis 2.1 software (Applied Biosystems).

Gene diversities comprising observed (*Ho*), expected (*Hs*) within-deme (the populations will be treated as demes in the different sections of the manuscript) and expected overall heterozygosities (*Ht*) were estimated following (Nei and Chesser, 1983). Genotypic disequilibrium between loci in each sample and deviations from Hardy-Weinberg equilibrium (HWE) within samples were tested based on 2400 permutations and 10 000 randomizations, respectively. Wright's fixation indices for within-deme deviation from random mating (F_{IS}), as well as pairwise deme differentiation (F_{ST}), were estimated following Weir and Cockerham (1984). Deviation from random mating within demes (F_{IS}) per locus and sample were computed with a bootstrap procedure (2000 randomizations). Statistical support for pairwise deme differentiation was obtained through exact G-tests on allelic frequencies as described by Goudet et al. (1996) with 2000 randomizations. All summary statistics and tests mentioned above have been computed using FSTAT Version 2.9.3.2 (Goudet, 1995). Permutation tests were carried out in order to detect significant differences in allelic richness, expected (*Hs*) and observed (*Ho*) heterozygosities and F_{ST} indices among the two studied metapopulations.

Genetic isolation by distance at the metapopulational level and overall was tested by using a partial Mantel test (Mantel, 1967); p-value were given after 10 000 randomizations.

A Bayesian model-based clustering method (Pritchard et al., 2000) for inferring population structure and assigning individuals to populations was used as implemented in structure version 2.1 (Falush et al., 2003). Based on allele frequencies, individuals are assigned, through the use of a Markov chain Monte Carlo (MCMC) simulation, a membership coefficient for each of K populations. We performed 10 runs of $6 \cdot 10^5$ iterations (the first 10^5 considered as burn-in) for K = 1 to K = 10 (i) including all the populations and (ii) within the two metapopulations. The number of populations best fitting our data set was defined as described in Evanno et al. (2005). The latter statistics compares the rate of change in the log probability of data between successive K and the corresponding variance of log probabilities.

We used the software migrate 2.0.6 (Beerli and Felsenstein, 2001; Beerli, 2004) to estimate the scaled migration rate (M) between demes within metapopulations. This software is based on a coalescence model with mutation and migration, and estimates a measure of effective population size, θ, defined as $4Ne\mu$, where μ denotes mutation rate, and migration M, defined as m/μ, where m denotes migration rate. We assumed a stepwise mutation model and based estimates on 15 short [10^4 Markov Chain Monte Carlo (MCMC) steps] and five long (10^5 MCMC steps) chains. To ensure convergence, we used the 'adaptive heating' option with one 'cold' and three 'hot' chains.

Tests for HWE indicated that all loci tested were at HWE and in genotypic equilibrium. For the six microsatellite loci, the number of alleles per locus ranged from 7 to 17 (average = 9.83), with a total of 59 alleles across 6 loci. The allelic richness within deme ranges from 4.41 to 5.29, with an overall mean of 6.25 (table 1). Expected heterozygosities per locus within demes (*Hs*) ranged from 0.16 to 0.90, with an average of 0.58, whereas expected overall heterozygosity (*Ht*) averaged 0.62 (range per locus: 0.35-0.87). Observed heterozygosity (*Ho*) values varied from 0.44 to 0.68, with an average of 0.51 (see table 1). There was a significant deviation from random mating in the analyzed demes (overall $F_{IS} = 0.12$, $p > 0.001$;

LN $F_{IS} = 0.06$, LG $F_{IS} = 0.16$), suggesting the occurence of a within-sample substructure. The genetic differentiation between demes (pairwise F_{ST}) within each metapopulation was low, ranging from 0.01 to 0.07, all values being significant, except for the pairs of demes: Mossière-Allaman, Vaudalle-Lavigny, and Trouville-Chabrey ($p > 0.05$). Comparable overall F_{ST} values were found in the LN and LG metapopulations (respectively 0.046, 95% CI: 0.017-0.05 and 0.039, 95% CI: 0.025-0.079; $p = 0.86$; see fig. 1). The pairwise F_{ST} values among demes from separated metapopulations varies from 0.086 to 0.142 (mean = 0.11), for geographical distances ranging from 59.2 to 69.7 km.

Table 1. Genetic diversities for eight *Hyla arborea* demes in two distinct metapopulations based on 6 microsatellite loci. N = maximum number of singers in the population in 2002 (Pellet et al., 2002); n = sample size; *Ho* = observed heterozygosity; *Hs* = expected heterozygosity; A_R = allelic richness.

Site	N	n	*Ho*	*Hs*	A_R
1. Allaman	20	20	0.45	0.54	4.62
2. Camp romain	27	24	0.45	0.59	5.30
3. Lavigny	149	24	0.44	0.49	5.12
4. Mossières	25	12	0.47	0.51	5.22
5. Vaudalle	11	20	0.44	0.55	5.24
Mean LG (1 to 5)	232	100	0.45	0.54	5.10
6. Chabrey	26	21	0.58	0.66	5.29
7. Gletterens	11	19	0.63	0.60	4.41
8. Trouville	99	22	0.68	0.63	4.96
Mean LN (6 to 8)	136	62	0.60	0.64	4.89
Total/Total Mean (1 to 8)	368	162	0.51	0.58	6.25

No isolation by distance within the LG metapopulation was detected ($p = 0.40$, $r^2 = 0.09$), indicating the absence of a pattern of genetic isolation with geographical distance (not tested on the three populations of LN). Significant differences between metapopulations were observed for *Ho* (LN $Ho = 0.60$, LG $Ho = 0.45$, $p = 0.019$) and *Hs* (LN $Hs = 0.64$, LG $Hs = 0.54$, $p = 0.019$), whereas allelic richness (A_R), F_{ST} and F_{IS} were not significantly different (respectively $p = 0.43$, $p = 0.86$ and $p = 0.11$; see table 1).

The analyses performed with structure, revealed that the number of populations best fitting our data set is K = 2. No substructure was revealed within the metapopulations.

Concerning the pairs of unidirectional migration rates (M) estimated between demes within metapopulations, 11 of the 13 in total were asymmetric (i.e. where 95% CI did not overlap; table 2). In addition, the analysis clearly showed that recent migrations occurred between demes within both metapopulations.

Table 2. Gene flow between populations within demes ($M = m/\mu$, with 95% confidence interval).

Polulation (i)	Allaman $\rightarrow i$	C. romain $\rightarrow i$	Lavigny $\rightarrow i$	Mossières $\rightarrow i$	Vaudalle $\rightarrow i$	Chabrey $\rightarrow i$	Gletterens $\rightarrow i$	Trouville $\rightarrow i$
1. Allaman	–	7.49 6.42-8.67	2.47 2.09-2.90	0.86 0.63-1.12	0.74 0.58-0.91			
2. Camp romain	2.55 2.07-3.11	–	0.87 0.65-1.13	1.26 0.99-1.58	0.42 0.38-0.56			
3. Lavigny	3.58 3.00-4.24	1.80 1.30-2.41	–	2.29 1.91-2.71	1.04 0.86-1.25			
4. Mossières	1.77 1.37-2.23	1.30 0.88-1.82	1.24 0.97-1.55	–	1.21 1.01-1.44			
5. Vaudalle	4.40 3.75-5.12	2.97 2.32-3.73	2.12 1.76-2.52	1.41 1.12-1.75	–			
6. Chabrey						–	4.26 3.62-4.98	2.89 2.50-3.33
7. Gletterens						2.25 1.91-2.65	–	0.49 0.34-0.67
8. Trouville						1.39 1.14-1.68	3.67 3.08-4.34	–

The main result stemming from this study is the absence of substantial differences in terms of genetic differentiation between the two metapopulations, despite an important contrast in habitat fragmentation and demes densities, as shown by (i) the low global structure observed within metapopulations (mean $F_{ST} \approx 0.04$), with pair-wise F_{ST} ranging from 0.01 to 0.07; (ii) the absence of substructure within metapopulations, as revealed by clustering analyses and (iii) the unidirectional indices of migration (M) observed between demes within metapopulations, with some very high values observed (M max. for LN: 4.26 and for LG: 7.49). Therefore, our data suggest a high overall rate of dispersal within both metapopulations. Though, the pairs of unidirectional indices of migration (M) were mostly asymmetric within both metapopulations (11 of 13 pairs), with values varying from 0.42 to 7.49, revealing that the demes contributed differently to the low structure observed between them. Therefore, the genetic diversity of demes could be reduced by the effect of asymmetric gene flow.

In contrast with the low structure observed within both areas, the analyses revealed significant ($p = 0.019$) lower gene diversity (Hs) and observed heterozygosity (Ho), within LG metapopulation, as well as a higher, but not significantly different, substructure (F_{IS}).

Consequently, the results pointed out that the landscape of LG is less suitable for the European tree frog than that of LN and as a result, dispersal is less effective to maintain gene flow among local demes, and as consequence genetic diversity within demes. This pattern is illustrated within LG by a strong variability of pairwise F_{ST} values between spatially closer demes distant of 1.5 and 1.8 km, with F_{ST} varying from 0.01 to 0.06, respectively. Thus, it confirmed that other independent factors than distance alone must be taken into account in the dispersion of the tree frog, such as roads, urban areas, as well as natural obstacles and historical factors (Pellet et al., 2004).

Overall and despite significant differences observed in term of genetic diversity between our two metapopulations, these values are comparable to genetic variation at microsatellite loci found in other amphibian species, e.g. from Arens et al. (2007) on *Rana arvalis*, or from Scribner et al. (2001) on *Bufo bufo*. In addition, our estimates of genetic variability *Hs*, varying from 0.49 to 0.66, are slightly in contrast with the data from Andersen et al. (2004; 0.35-0.53) and Arens et al. (2006; 0.39-0.59) on *H. arborea* metapopulations in Denmark and the Netherlands, respectively, or e.g. from Hitchings and Beebee (1997) on *Rana temporaria* and Newman and Squire (2001) on *Rana sylvatica*, where heterozygosity levels are lower.

Mostly based on the same microsatellite markers, genetic differentiation is in opposition substantially higher within the Danish and Dutch metapopulations (Andersen et al., 2004, Arens et al., 2006), characterized by F_{ST} values ranging from 0.03 to 0.33 (overall $F_{ST} = 0.22$) and from 0 to 0.35 (overall = 0.19), respectively. Several mutually non-exclusive hypotheses could explain the substantial difference in genetic differentiation between our results and the two available studies on *H. arborea*: (i) in contrast with the area studied by Andersen et al. (2004) and Arens et al. (2006), some natural structuring elements such as riparian ecosystem exist in the LG metapopulation landscape, and such complementary terrestrial habitats could act as potential dispersal corridors for *H. arborea*; (ii) geographical scale is smaller in our study (groups of ponds in Andersen et al. (2004) are for instance separated by several km); (iii) the Danish and Dutch studied areas being situated in the northern margins of the distribution of the species, phenomena as local drift and local extinction could be more significant, leading to an increase of genetic structure; (iv) when sampling tadpoles, potential sampling of siblings (individuals from the same clutches) may upwardly bias F_{ST} estimates. Although the occurrence of closely related tadpoles could not be excluded, our sam-

pling was performed in order to minimize their presence. On the contrary, the Danish sampling possibly included related individuals, as four different tadpoles per clutch were analyzed (no information was given concerning the Dutch sampling strategy).

Overall, our results suggest that even if the dispersal of *H. arborea* among contiguous ponds is high in areas of heterogeneous landscape, as shown in LG, a loss of genetic diversity can occur. Although no signs of inbreeding depression have been highlighted within the fragmented LG metapopulation of *H. arborea*, the small size of the demes, coupled with a lower genetic diversity compared to LN metapopulation, might increase the risk of demographic and genetic stochasticity. Thus it is likely that the size and the level of connectivity among demes is a crucial factor for the survival of the metapopulations of *Hyla arborea*. Consequently, in order to protect the European tree frog populations in an efficient way, priority should be given to conserving areas of suitable habitat to promote population connectivity and maintain genetic diversity and evolutionary potential. In particular, conservation management actions should be focused on: (i) increasing the metapopulation sizes and the number of demes; (ii) encourage the connectivity within the metapopulations. In this context, the relatively high migration potential of the European tree frog should permit a rapid natural colonisation of newly created ponds. Although the two metapopulations have been separated for only 20 years, they are genetically well differentiated (mean F_{ST} between the two metapopulations 0.11). Consequently, environment improvements between them might be undertaken in order to reconnect each other and to recreate gene flow between both regions. Since the European tree frog is a mobile species, the connection between the two metapopulations could probably be realised with the creation of only a limited group of ponds with favourable surrounding habitats.

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