

# The effects of 20 years of highway presence on the genetic structure of *Rana dalmatina* populations<sup>1</sup>

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**Abstract:** Habitat destruction and fragmentation caused by highways can negatively affect animal populations, but a better understanding of the effects of highways on population genetic structure is still needed to improve conservation plans in urbanized landscapes. We investigated the degree of genetic variability and differentiation within and among seven *Rana dalmatina* populations located far from highly trafficked roads (non-fragmented populations) and four populations sampled on both sides of a major highway (fragmented populations). The degree of population subdivision was significantly higher among fragmented ( $F_{ST} = 0.238$ ) as compared to non-fragmented populations ( $F_{ST} = 0.022$ ). Furthermore, in the four fragmented populations, significantly lower allelic richness as compared to non-fragmented populations was observed. Together with potential high levels of road mortality leading to smaller population size, these results suggest that separation by highways not only has reduced the genetic diversity and polymorphism in local populations over two decades, but also has resulted in a higher degree of population differentiation, most likely due to genetic drift.

**Keywords:** agile frog, allelic richness, fragmentation, genetic differentiation, highway, *Rana dalmatina*.

**Résumé :** La destruction des habitats et la fragmentation causées par les autoroutes peuvent s'avérer néfastes pour les populations animale et une meilleure compréhension des effets des autoroutes sur la génétique des populations est nécessaire pour améliorer les plans de conservation dans les paysages urbains. Nous avons examiné le degré de variabilité génétique et de différenciation génétique pour sept populations de *Rana dalmatina* éloignées de toute route ayant un fort trafic (populations non fragmentées) et quatre populations situées à proximité d'une autoroute (populations fragmentées). Le degré de divergence des populations est significativement plus grand entre les populations fragmentées ( $F_{ST} = 0.238$ ) qu'entre celles non fragmentées ( $F_{ST} = 0.022$ ). Par ailleurs, au sein des quatre populations fragmentées, on observe une richesse allélique significativement inférieure à celle des populations non fragmentées. En combinaison avec le haut taux de mortalité routière réduisant probablement la taille des populations, ces résultats suggèrent qu'en une vingtaine d'années, la séparation due à l'autoroute a non seulement réduit la diversité génétique et le polymorphisme des populations locales mais a également conduit à un plus grand degré de différenciation génétique vraisemblablement dû à la dérive génétique.

**Mots-clés :** grenouille agile, richesse allélique, fragmentation, différenciation génétique, autoroute, *Rana dalmatina*.

**Nomenclature:** Arnold & Owenden, 2002.

## Introduction

Understanding the effects of habitat fragmentation on genetic variation and population structure is one of the key challenges and aims of conservation genetics (Frankham, Ballou & Briscoe, 2002). Dispersal barriers caused by habitat fragmentation may result in loss of genetic variation and an increased degree of population differentiation due to genetic drift (Fahrig & Merriam, 1985; Hedrick, 2000). Habitat fragmentation will also reduce the re-introduction of lost alleles back into the gene pool via immigration and hence inhibit a genetic rescue effect (Slatkin, 1987). In fact, loss and reduction in habitat size as well as interruption of gene flow among populations are now recognized as some of

the major threats to animal populations (Primack, 1993; Vos & Chardon, 1998). Furthermore, with regard to their migration rate and distances, populations are sensitive to the fragmentation of their habitat, due to the reduction in gene flow and consequent increased divergence. Thus, habitat fragmentation may lead to population differentiation if critical habitats are destroyed or if remaining patches of suitable habitat become too isolated to allow recolonization by dispersing individuals (Semlitsch & Bodie, 1998). In such circumstances, remaining populations may suffer from reduction in size, which increases the risk of inbreeding (Saccheri *et al.*, 1998; Madsen *et al.*, 1999) and accelerates loss of genetic variability through random genetic drift by altering the effective metapopulation structure (Fahrig & Merriam, 1985; Hanski & Gilpin, 1991).

Traffic may be destructive to populations in two ways, directly in the sense of actually killing individuals and

<sup>1</sup>Rec. 2006-01-23; acc. 2006-06-12.

Associate Editor: Patrick Gregory.

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indirectly by causing habitat fragmentation (Mader, 1984; Lodé, 2000). Worries about habitat fragmentation caused by transportation infrastructure have gained importance during the last couple of decades, resulting in some special policies for protecting areas (Dufek & Adamec, 1999). For many species, roads are barriers to dispersal owing to physical obstacles such as fences, or the deterrence by the unknown artificial habitat (Mader, 1984). On many highways, compensatory measures have been recommended to reduce the impact of habitat loss and the partitioning of populations (Lesbarrères & Lodé, 2000). While the impact of roads on the movement of individuals or on genetic diversity has been assessed in several studies (Baur & Baur, 1989; Fahrig *et al.*, 1995; Vos & Chardon, 1998; Haxton, 2000; Vallan, 2000; Lesbarrères, Lodé & Merilä, 2004), the reduction in gene flow among populations resulting from such damage still needs to be investigated to improve conservation plans in urbanized landscapes (Hitchings & Beebee, 1997; Keller & Largiadèr, 2003).

Roads result in habitat destruction (Blaustein & Kiesecker, 2002) and pollution (Hecnar & M'Closkey, 1996), and their impact on population substructure is one of the factors responsible for the global amphibian decline (Alford & Richards, 1999; Hels & Buchwald, 2001; Kiesecker, Blaustein & Belden, 2001; IUCN, Conservation International and NatureServe, 2004). Regular and significant road mortality and consequent interruption of gene flow greatly affect species that undergo terrestrial seasonal migrations. For instance, most amphibian species are expected to be affected by road presence as they have an annual cycle that requires migration between habitats with different ecological properties (Wilbur, 1980). Therefore, habitat fragmentation may increase population substructure and consequently the extinction risk of amphibian populations (Sjögren, 1991a).

The main aim of this study was to investigate the genetic consequences of habitat fragmentation induced by a large highway on an amphibian, the agile frog *Rana dalmatina*. In particular, we investigated whether highway-induced habitat fragmentation had resulted in an increased genetic differentiation and potential reduction in migrants among populations living in fragmented landscape as compared to populations living in a more continuous habitat.

## Methods

In order to assess the genetic consequences of road presence, we sampled 11 ponds where the agile frog, *R. dalmatina*, was breeding, in environments with different degrees of road proximity in western France (Figure 1). The populations were divided into two groups. First, along a transect covering 45 km, seven populations (1–7) were investigated in ponds far ( $\geq 550$  m, Mean = 2173 m, SD = 1092) from any physical barriers, such as high-traffic roads, that could act as barriers to gene flow. These ponds were situated in meadows between Angers and Cholet, western France (Figure 1). All roads around the ponds were of low traffic density ( $\leq$  two lanes,  $< 500$  vehicles per hour, Association des Sociétés Françaises d'Autoroutes et d'ouvrages à péage, 2001). These populations were con-

sidered non-fragmented. They were separated by an average distance of 17 km (SD = 11). The second group, comprising four additional populations (A–D), were located close ( $\leq 180$  m, Mean = 94 m, SD = 62) to the A11 highway (four lanes  $> 2000$  vehicles per hour and with scarce protection fences and no specific tunnel underneath the road, Association des Sociétés Françaises d'Autoroutes et d'ouvrages à péage, 2001) between Angers and Nantes, western France (Figure 1). These populations were considered fragmented. They were separated by an average distance of 32 km (SD = 16). Five additional ponds investigated along a 60-km transect of the highway were inspected but did not support breeding *R. dalmatina* populations.

In the studied area, the agile frog is usually the first breeding anuran at the end of the winter (Arnold & Owenden, 2002). The males arrive first and space out in breeding aggregations by defending territories, each male occupying a calling site of about 1–2 m<sup>2</sup> in which females lay their eggs (Geisselmann, Flindt & Hemmer, 1971; Baumgartner *et al.*, 1996). Females mature at two to three years old and lay only one clutch during the breeding season (Strömberg, 1995). As one of the potential factors influencing the presence and size of amphibian populations is their habitat, we compared pond characteristics for all ponds. Using recent studies on habitat features and amphibian diversity (Lesbarrères & Lodé, 2002; Ficetola & De Bernardi, 2004), we restricted our analyses to the variables known to influence our study species. Proportion of vegetation cover was estimated by eye to describe pond and shoreline cover. Values for this variable range from 0 (no vegetation cover and diversity) to 1 (full vegetation cover and high diversity, with more than three physical types represented). We measured sun exposure in February and May during sunny days (between 20 to 24 d for each pond). Sun exposure was estimated as the proportion of the pond lighted between 1100 and 1300 (UT). We used the proportion of sun exposure averaged across all days sampled to get a mean sun exposure over the breeding season. Finally, surface area and maximum depth of each pond were estimated using a

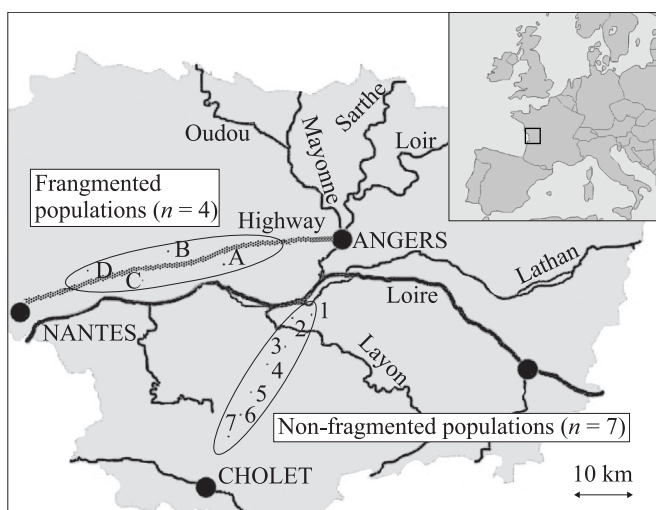


FIGURE 1. A map showing the location of “fragmented” and “non-fragmented” populations of *Rana dalmatina* included in this study.

decimeter ( $\pm 0.1$  m). Fish presence has been shown to be an important factor influencing amphibian diversity, but we did not include it in our analyses as we did not record any fish in any of the ponds during the study period.

Population size was estimated by counting clutches (equivalent to female population size) in each pond and multiplying by three as we assumed a 2:1 sex ratio (Lodé *et al.*, 2004). In May 1999, approximately 50 random eggs were sampled from every clutch in each pond. Each egg mass from each clutch was reared separately in 48-L aquaria under constant environmental conditions ( $T_{\text{water}} = 19^\circ\text{C}$ ) until the eggs hatched. The water in each aquarium was changed every third day, and the tadpoles were fed *ad libitum* with lettuce. After emergence from eggs (Gosner stage 25; Gosner, 1960), 30 randomly chosen larvae from each population were preserved at  $-23^\circ\text{C}$  until analyzed with horizontal starch-gel electrophoresis. Samples were homogenized in equal volumes of distilled water and centrifuged at 12,000 G for 5 min at  $4^\circ\text{C}$ . Electrophoresis was performed in 12% continuous Tris citrate starch gels, for 3–5 h at 300 V and  $4^\circ\text{C}$ . Slices were stained to reveal specific enzymes using standard procedures (Hillis & Moritz, 1990), and nine enzymes encoded by 12 polymorphic loci were successfully resolved (AK, EC 2.7.4.3; CK-1 and CK-2, EC 2.7.3.2; 6-PGDH, EC 1.1.1.44; GPI, EC 5.3.1.9;  $\alpha$ GDH, EC 1.1.1.8; LDH-1 and LDH-2, EC 1.1.1.27; MDH-1 and MDH-2, EC 1.1.1.37; MPI, EC 5.3.1.8; PGM, EC 5.4.2.2).

All genetic data were analyzed with FSTAT 2.9.3.2 updated from Goudet (1995) and global tests with 1000 permutations, as recommended when the number of loci is low. Due to the low number of clutches in some ponds, it was necessary to include more than one egg per clutch (*i.e.*, potential siblings) in some cases. Consequently, any difference observed may be due to increased sharing of alleles among related individuals in the fragmented populations. Therefore, a second test was conducted in order to compare the level of independent haploid genome genetic diversity between fragmented and non-fragmented populations. For this test, 10 datasets each composed of one randomly chosen individual per clutch were created, and the rarefaction method, which corrects for sample size, was used. Allelic richness was estimated following the rarefaction method (Hurlbert, 1971) implemented in FSTAT 2.9.3.2. As with the genetic diversity comparisons, potential sampling of siblings may upwardly bias genetic distance estimates due to the so-called Allendorf-Phelps effect (Waples, 1998). However, the level of potential bias of this effect can be estimated by accounting for intralocus sampling error (which has an average magnitude of  $1/[2S]$ , where S is the number of individuals sampled), and the occurrence of founder effect/drift associated with the Allendorf-Phelps effect can be estimated as follows (Waples, 1998):

$$E(F_{ST})(\text{adjusted null hypothesis}) \approx 1/(2S) + 1/(2N_b) \quad [1]$$

where the  $N_b$  term represents the effective number of breeders responsible for the juveniles that were sampled. In our case, these parameters can be estimated with high accuracy for each pairwise comparison. S values ranged from

24 to 36 (Table I), and  $N_b$  (estimated as twice the number of spawn clumps according to a 2:1 sex ratio in these ponds; Lodé *et al.*, 2004) ranged from 9 to 96. The statistical significance of differences in genetic diversity/divergence indices between fragmented and non-fragmented populations was assessed using the permutation scheme (1000 replicates) implemented in FSTAT. Components of genetic variance were also computed within populations, between populations within groups (fragmented and non-fragmented), and between groups to evaluate whether the presence of the highway has acted as a natural barrier against dispersal of agile frogs. The significance of the model was estimated with an AMOVA (Excoffier, Smouse & Quattro, 1992) by performing 992 permutations of multilocus haplotypes among individuals within populations, among populations within groups, and finally among groups in Arlequin version 2.2 (Schneider *et al.*, 1997). Additionally, as migration is an important characteristic of amphibian life cycles (Wilbur, 1980), we also estimated the number of migrants as  $N_m = (1 - F_{ST}) / (4 \times F_{ST})$  (Wright, 1969) to get estimates of gene flow (see Whitlock & McCauley, 1999 for limitations involved).

To test the isolation by distance hypothesis, we used two different methods. First, pairwise  $F_{ST}$  values were compared to geographic distance. Euclidean distance was used in preference to other types of measures because of the low profile of the Loire valley and the quasi linear movement of the agile frog (Lodé, 1993). On a National Geographic Institute map (precision = 10 m) we measured the distance between ponds in each of the two groups. Because pairwise elements of distance matrices are not independent and thus violate the basic assumptions associated with standard tests of significance,  $F_{ST}$ -geographic distances correlations were tested by Mantel test (Sokal & Rohlf, 1995). Secondly, in accordance with the low  $F_{ST}$  value observed in the non-fragmented populations, suggesting relatively large-scale movements, we regressed  $F_{ST} / (1 - F_{ST})$  estimates for pairs of populations against geographic distances separating

TABLE I. Observed ( $H_{\text{obs}}$ ) and expected ( $H_{\text{exp}}$ ) heterozygosity, as well as mean number of alleles per locus (A) in non-fragmented (1–7) and fragmented populations (A–D) of *Rana dalmatina*. NS = number of sampled clutches. Polymorphism was 100% in each population.

Population	NS	A	$H_{\text{obs}}$	$H_{\text{exp}}$
1	3	2.5	0.334	0.509
2	6	2.5	0.358	0.520
3	29	2.5	0.345	0.539
4	5	2.5	0.371	0.508
5	25	2.5	0.408	0.540
6	4	2.5	0.363	0.526
7	32	2.5	0.325	0.515
Non-fragmented average (SD)	14.86 (12.39)	2.5 (0.005)	0.358 (0.03)	0.522 (0.01)
A	5	2.32	0.176	0.347
B	3	2.00	0.137	0.264
C	3	2.32	0.158	0.386
D	8	2.12	0.131	0.322
Fragmented average (SD)	4.75 (2.36)	2.19 (0.15)	0.151 (0.02)	0.33 (0.05)

populations (Rousset, 1997). Finally, to check that the difference in  $F_{ST}$  estimates was not due to differences in the scale of the sampling between the two areas, we compared the populations separated by similar distances (populations A, B, C, and D and populations 1, 3, 5, and 7; Figure 1).

**Results**

All the ponds were of medium size (Mean  $\pm$  SD =  $952 \pm 346$  m<sup>2</sup>,  $n = 11$ ) with a relatively high sun exposure (Mean  $\pm$  SD =  $82 \pm 21\%$ ,  $n = 11$ ). They were permanent ponds and had similar habitat characteristics, with a low density of aquatic vegetation and a shallow water depth underneath the clutch. The main plant families found on the shoreline were Poaceae, Ranunculaceae, Asteraceae, Polygonaceae and Fabaceae. Juncaceae, Rosaceae, and Typhaceae were the main families found in the aquatic vegetation. Clutches were laid separately, attached to vegetation such as stalks or blades of reed. Landscapes around the ponds were similar, mainly covered by grassland vegetation, brambles, open fields, and bushes, with similar levels of nearby housing. None of the ecological variables (*viz.* pond size, maximum depth, pond cover, shoreline cover, and sun exposure) was significantly different between fragmented and non-fragmented populations (Mann–Whitney,  $U_{1,9} < 8.1$ ,  $P > 0.32$ ). Therefore, the presence of a highway was the only obvious potential dispersal barrier that differed between the two groups of populations. Similarly, the ecological characteristics of the five ponds where *R. dalmatina* was absent were not significantly different (Mann–Whitney,  $U_{1,7} < 6.1$ ,  $P > 0.51$ ).

The difference in female population size between the fragmented (Mean  $\pm$  SD =  $4.75 \pm 2.36$ ,  $n = 4$ ) and the non-fragmented (Mean  $\pm$  SD =  $14.86 \pm 12.39$ ,  $n = 7$ ) populations was not significant (Welch  $t_6 = 1.984$ ,  $P = 0.094$ ; Table I), but this non-significance was probably due to the small sample size. The mean number of alleles per locus was significantly lower in the fragmented populations than in the non-fragmented populations (2.2 and 2.5, respectively; FSTAT permutation test,  $P = 0.003$ ; Table I). However, as multiple individuals from single egg clutches were included in some cases, a second test was conducted (see Methods). Using the rarefaction method to correct for difference in sample size we observed that allelic richness was significantly lower in the fragmented populations than in the non-fragmented populations for all 10 comparisons ( $1.43 \pm 0.15$  and  $1.75 \pm 0.24$ , respectively; FSTAT permutation test,  $P < 0.009$  in all the cases). In the case of a particular locus, LDH-1, we observed the absence of the allele "c" in all fragmented populations, resulting in an estimated  $F_{ST}$  of 0.65 for that locus.

Observed heterozygosity levels did not differ among the seven non-fragmented populations (Wilcoxon,  $W = -2$ ,  $P = 0.94$ ) or among the fragmented populations (Wilcoxon,  $W = 0$ ,  $P > 0.99$ ). However, the observed heterozygosity was significantly lower in the fragmented as compared with the non-fragmented populations (Mann–Whitney,  $U_{1,9} = 28$ ,  $P = 0.006$ ). The difference remained significant when only one random individual per clutch was included (data not shown). No correlation was observed between genetic diversity and population size, either among all populations or within each of the two groups (all populations,  $r = 0.42$ ,

$P = 0.2$ ; non-fragmented populations,  $r = 0.06$ ,  $P = 0.89$ ; fragmented populations,  $r = 0.32$ ,  $P = 0.68$ ).

Mean pairwise  $F_{ST}$  was much higher among the fragmented populations than among the non-fragmented ones ( $0.238 \pm 0.07$  and  $0.022 \pm 0.008$ , respectively; FSTAT permutation test,  $P < 0.003$ ). The results of the AMOVA revealed that the fragmented *versus* non-fragmented dichotomy is supported by the data ( $P = 0.002$ , 5.7% of the variance explained). Additionally, the variance components due to the differences among populations within groups and due to differences among individuals within populations were also significant ( $P < 0.001$ , 9.6% of the variance explained and  $P < 0.001$ , 84.7% of the variance explained, respectively). All pairwise  $F_{ST}$  values were significantly different from zero among the fragmented populations, whereas only five out of 21 pairwise  $F_{ST}$  values were significantly different from zero among non-fragmented populations (Table II and III). Consequently, the estimate of relative migration per generation was  $N_m = 10.6$  migrants for the non-fragmented populations and  $N_m = 0.8$  migrants for the fragmented populations. Due to potential sampling of siblings, leading to a possible bias in genetic distance estimates (Waples, 1998), we corrected for the bias created by the intralocus sampling error (Mean  $\pm$  SD =  $0.016 \pm 0.0004$  and  $0.017 \pm 0.001$  for the fragmented and the non-fragmented populations, respectively) and the bias created by the Allendorf–Phelps effect (Mean  $\pm$  SD =  $0.041 \pm 0.009$  and  $0.025 \pm 0.012$  for the fragmented and the non-fragmented populations, respectively). However, the  $F_{ST}$  value for the fragmented populations ( $0.174 \pm 0.079$ ) was still significantly larger than the  $F_{ST}$  value for the non-fragmented populations ( $-0.02 \pm 0.005$ , fragmented *versus* non-fragmented, Welch  $t_5 = 5.53$ ,  $P = 0.003$ ).

$F_{ST}$  estimates were also compared among populations separated by similar distances (populations A, B, C, and D

TABLE II. Pairwise geographic distances (km, below diagonal) and  $F_{ST}$  estimates (above diagonal) among non-fragmented populations of *Rana dalmatina*.

Population	1	2	3	4	5	6	7
1		0.024	0.02	0.055*	0.029	0.013	0.061*
2	5.8		0.003	0.028	0.012	0.013	0.025
3	7.03	1.23		0.030*	0.001	0.019	0.035
4	25	19.7	18.47		0.008	0.032	0.034*
5	28.6	23.3	22.07	3.6		0.003	0.015
6	32.73	26.93	25.7	7.23	3.63		0.016*
7	34.53	28.73	27.5	9.03	5.43	1.8	

\* $P < 0.05$  after 1000 permutations and sequential Bonferroni correction.

TABLE III. Pairwise geographic distances (km, below diagonal) and  $F_{ST}$  estimates (above diagonal) among fragmented populations of *Rana dalmatina*.

Population	A	B	C	D
A		0.176*	0.187*	0.348*
B	22.3		0.286*	0.15*
C	29.7	7.4		0.241*
D	61	38.7	31.3	

\* $P < 0.05$  after 1000 permutations and sequential Bonferroni correction.

and populations 1, 3, 5, and 7; Figure 1) to check that the difference in  $F_{ST}$  estimates was not due to differences in the scale of the sampling between the two areas.  $F_{ST}$  estimates were still much higher among the fragmented populations than among the non-fragmented ones ( $0.238 \pm 0.07$  and  $0.021 \pm 0.017$ , respectively; FSTAT permutation test,  $P < 0.007$ ). As we observed a marginally non-significant population size difference, we also compared the populations presenting similar population size (populations A, B, C, and D and populations 1, 2, 4, and 6; Figure 1 and Table I) to check that the difference in  $F_{ST}$  estimates was not due to differences in sample size between the two areas.  $F_{ST}$  estimates were still much higher among the fragmented populations than among the non-fragmented ones ( $0.238 \pm 0.07$  and  $0.028 \pm 0.01$ , respectively; FSTAT permutation test,  $P < 0.012$ ).

Geographic distances between populations in the fragmented group were higher than between non-fragmented populations (Welch  $t_{25} = 2.45$ ,  $P = 0.022$ ). However, there was no detectable isolation by distance either in the fragmented populations (Mantel test,  $Z \pm SD = 19.6 \pm 1.34$ ,  $P = 0.99$ ) or the non-fragmented populations (Mantel test,  $Z \pm SD = 89.82 \pm 7.34$ ,  $P = 0.82$ ). Likewise, when we regressed  $F_{ST} / (1 - F_{ST})$  estimates for pairs of populations against geographic distance separating populations, no correlation was observed between the geographic and genetic distances for the fragmented populations ( $b \pm SE = -0.002 \pm 0.004$ ,  $F_{1,4} = 0.32$ ,  $P = 0.6$ ). For the non-fragmented populations, an almost significant isolation by distance was detected ( $b \pm SE = 0.0006 \pm 0.0003$ ,  $F_{1,19} = 4.27$ ,  $P = 0.06$ ).

## Discussion

The comparison of genetic diversity and differentiation in agile frog populations sampled from two areas with different degrees of fragmentation revealed that genetic variability was lower, and genetic differentiation higher, in the populations where habitat had been bisected by the presence of a highway. The differences in the degree of population substructuring suggest the influence of the road on the genetic structure of the populations. Similarly, allelic richness comparisons (which correct for differences in sample size; Hurlbert, 1971; Petit, el Mousadik & Pons, 1998) between fragmented and non-fragmented groups, where data from only one randomly chosen individual per clutch were included to correct for a potential bias (Castric *et al.*, 2002), also revealed that the level of genetic diversity per independent haploid genome was lower in fragmented populations.

The results also emphasize the importance of the “rescue effect” for maintenance of local genetic diversity (Brown & Kodric-Brown, 1977; Gilpin, 1991), whereby migration may re-introduce alleles to populations from which they were lost due to genetic drift. For example, allele “c” of LDH-1 was not observed in any of the fragmented populations. This allele may have never existed in the populations or may have completely disappeared as a result of fragmentation (Barton & Slatkin, 1986). While the LDH-1 genotype data support limited gene flow between the fragmented populations, the high locus specific  $F_{ST}$  value (0.65) could be interpreted as a potential sign of local

adaptation to some environmental factor through diversifying selection (Beaumont, 2005). Only by integrating surveys of geographic variation with detailed molecular-functional analyses can the study of allozyme variation provide much insight into genetic mechanisms of physiological adaptation (Storz & Nachman, 2003) and distinguish genetic drift from local adaptation.

The highway opened 21 years before these populations were sampled, suggesting that the genetic effect of highway development may be observed in as few as seven to ten anuran generations. A recent review showed that roads have mainly ecological effects on terrestrial and aquatic communities (Trombulak & Frissell, 2000). Our results suggest that the highways cause real migration barriers, preventing frog populations from exchanging a sufficient number of individuals to avoid substantial genetic drift (Kozakiewicz, 1993). Other factors, such as pond size and habitat characteristics, were comparable among all the ponds investigated and none of them experienced a shorter period of inundation that would potentially reduce recruitment. Additionally, while non-significant, the correlation between population size and genetic diversity might suffer from the small-sample effect; a larger number of populations would be needed to test this effect (Frankham, 1996). However, comparison of populations with little disparity in size also revealed greater genetic differentiation in fragmented populations. Therefore, the pattern of differentiation observed does not result solely from the smaller population size in fragmented populations. Finally, our results show that isolation by distance is not necessarily involved: there was no correlation between genetic distance and the geographical distances that separated the fragmented populations, and the interpopulation proximity and connectivity of the fragmented populations with other populations seem insufficient to compensate for the road-effect zone (Lesbarrères, Pagano & Lodé, 2003; Sjögren, 1991b). Therefore, reduced mobility of amphibians, which require an aquatic habitat for living and moving, and reduction of gene flow must be considered as factors potentially explaining the high  $F_{ST}$  values for amphibians in highly urbanized regions (Ward, Skibinski & Woodwark, 1992; Hitchings & Beebe, 1998; Vos *et al.*, 2001).

Extinction–recolonization dynamics are probably very important in this system, elevating the role of historical effects in determining genetic patterns. The dataset is insufficient to address such questions, but the fragmented populations are more concordant with an island model, where migrations, if they still occur, take place randomly (Schlueter & Guttman, 1998; Stewart *et al.*, 1999). Similarly, we cannot rule out the existence of local population structure within one large but sub-structured population. In such a scenario, the pattern of genetic structure observed may, for instance, result from fewer individuals migrating from a potential central wintering ground between the two main site areas to the fragmented ponds. However, the absence of a particular allele in the fragmented populations (see above) and the presence of a major river and the strong philopatry usually observed in amphibians (Berven & Grudzien, 1990) suggest that these two groups of populations are not exchanging individuals and should be seen as independent. More polymorphic markers, such as microsatellites, can offer a more sensitive measure of genetic variability, but

they are not yet available for *R. dalmatina* (Pidancier *et al.*, 2002).

With regard to the general debate on amphibian population declines (Houlahan *et al.*, 2000; Kiesecker, Blaustein & Belden, 2001), the strong level of genetic differentiation revealed here supports the notion that anthropogenic impact may be one of the main causes. Small populations are more prone to local extinctions than larger ones (Blaustein, Wake & Sousa, 1994; Hanski, 1998; Montgomery *et al.*, 2000), and our field data show that the species was only present in four out of the nine ponds near the highway that were investigated, despite the fact that all ponds presented the same ecological characteristics. Although the situation before the highway's construction is unknown and water chemistry has not been investigated here (Hecnar & M'Closkey, 1996), it is likely that this observation can be ascribed to the negative effects of fragmentation, together with more direct effects such as pollution and road mortality, because *R. dalmatina* is common in this area (Castanet & Guyétant, 1989). The majority of local extinction processes result from modification of the environment, and more specifically changes in farming methods (Beebee, 1997; Jonsson & Ebenman, 2001; Johansson *et al.*, 2005).

Habitat fragmentation is a burning issue in the twenty-first century (Reed, 2004). It is important to better understand the impact of road construction on the metapopulation dynamics of natural populations and on their long-term viability (Buckley & Beebee, 2004). This will aid in the development of methods aimed at minimizing the negative impacts of road construction on natural populations (Byron, 1999; Lesbarrères, Lodé, & Merilä, 2004; IUCN, Conservation International and NatureServe, 2004).

### Acknowledgements

We are grateful to the Autoroutes du Sud de la France for assistance with funding, to the site owners who allowed us access to their ponds, and to R. Alford, T. J. C. Beebee, E. Duffey, A. R. Hoelzel, G. Meffe, R. O'Hara, and R. S. Waples for constructive comments on earlier drafts.

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