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Genetic diversity, but not hatching success, is jointly affected by postglacial colonization and isolation in the threatened frog, *Rana latastei*

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Abstract

Both postglacial colonization and habitat fragmentation can reduce the genetic diversity of populations, which in turn can affect fitness. However, since these processes occur at different spatial and temporal scales, the consequences of either process may differ. To disentangle the relative role of isolation and postglacial colonization in determining genetic diversity and fitness, we studied microsatellite diversity of 295 individuals from 10 populations and measured the hatch rate of 218 clutches from eight populations of a threatened frog, *R. latastei*. The populations that were affected by fragmentation to a greater extent suffered higher embryo mortality and reduced hatch rate, while no effects of distance from glacial refugium on hatch rate were detected. Altogether, distance from glacial refugium and isolation explained > 90% of variation in genetic diversity. We found that the genetic diversity was lowest in populations both isolated and far from the glacial refugium, and that distance from refugium seems to have the primary role in determining genetic diversity. The relationship between genetic diversity and hatch rate was not significant. However, the proportion of genetic diversity lost through recent isolation had a significant, negative effect on fitness. It is possible that selection at least partially purged the negative effects of the ancestral loss of genetic diversity.

Keywords: embryo mortality, genetic diversity, hatch rate, inbreeding depression, landscape fragmentation, road density

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Introduction

During the quaternary glaciations, many terrestrial species were protected from climate-driven extinction by taking refuge in areas that were relatively free from glacial extremes. It is from these refugia, usually located in southern, warmer regions, that many species subsequently recolonized temperate regions by expanding their ranges along paths restricted by a combination of climate and geographical barriers (Hewitt 1996, 1999; Comes & Kadereit 1998). Because recolonization occurred over a prolonged time period and patterns of recolonization were proscribed by climatic and biogeographical barriers, forcing species to experience progressive founder events and population bottlenecks, colonization history may be currently represented in patterns of genetic diversity. Populations containing greater genetic variation are usually located more closely to refugia, and populations further from refugia exhibit less genetic variability (reviewed by Hewitt 1996, 1999, 2000; but see also Widmer & Lexer 2001; Petit *et al*. 2003; Brito 2005 for other patterns). By contrast, landscape fragmentation is a more recent process associated with habitat alteration due to increased land exploitation. Fragmentation reduces the availability of natural habitat by breaking it into discrete patches surrounded by a matrix made uninhabitable or impassable by anthropogenic processes (Wilcox & Murphy 1985; Bennett 1999). Patches are frequently small and isolated and therefore fragmentation can cause a loss of genetic diversity through the processes of genetic drift and

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population-level inbreeding (Hanski & Gilpin 1997; Hedrick 2001; Keller & Waller 2002). However, unlike patterns generated by recolonization, bottlenecks are not necessarily progressive and are unlikely to follow a geographical pattern, as evinced by recolonization routes. In temperate areas, both postglacial colonization and landscape fragmentation have the potential to influence the genetic diversity of natural populations.

Genetic diversity is known to relate positively to fitness, in that individuals from populations carrying reduced genetic variation are reduced in fitness when compared to those from populations with greater genetic variation (Reed & Frankham 2003; Reusch *et al*. 2005). It could therefore be expected that either the process of postglacial range expansion or fragmentation would influence fitness through the erosion of genetic variability. Several studies have detected reduced fitness of individuals from populations where genetic diversity has been negatively affected by fragmentation (e.g. Hitchings & Beebee 1998; Saccheri *et al*. 1998; Andersen *et al*. 2004; Edman *et al*. 2004; Galeuchet *et al*. 2005). Fewer studies have described a reduction of population-averaged performance caused by decreased genetic diversity, which in turn was eroded by colonization history (Schmitt & Hewitt 2004; Pearman & Garner 2005). For example, Schmitt & Hewitt (2004) showed that in several butterfly species, populations far from glacial refugia exhibit negative demographical trends when compared to populations located more closely to refugia. It is highly probable that in temperate zones many species suffer reduced genetic diversity caused by a combination of postglacial colonization and habitat fragmentation. Since these processes act at very different spatial and temporal scales they should have different and defined effects on genetic diversity and fitness (Swindell & Bouzat 2006). To date most studies have focused on one of these two processes (but see also Gullberg *et al*. 1998; Rowe *et al*. 1999; Desender & Verdyck 2001; Goodmnan *et al*. 2001; Petit *et al*. 2002; Wang *et al*. 2004; Johansson *et al*. 2005; Martìnez-Solano & Garcia-Paris 2005).

We examined the relationships between genetic diversity, landscape structure and average fitness in populations of the Italian agile frog, *Rana latastei*. *R. latastei* is a good model organism for unravelling the complex relationships between postglacial colonization history, habitat fragmentation, loss of genetic diversity and fitness. It is endemic to the lowlands of Northern Italy and adjacent countries, living in riparian woodlands and breeding in river washes and ponds, all of which have been strongly influenced by agriculture and urbanization (Pozzi 1980; Ficetola & De Bernardi 2004). In the north of Italy, *R. latastei* exists in small and isolated populations, which are known to exhibit strong variation in population genetic diversity (Ficetola & De Bernardi 2004; Garner *et al*. 2004). Patterns of genetic variability suggest a single glacial refugium, as genetic diversity decreases following a quadratic function along an east to west gradient. Because this pattern occurs across the entire species range, it is highly probable that postglacial colonization occurred from east to west and from a refugium located within the eastern part of the range (Garner *et al*. 2004). The overall range is limited; therefore it is reasonable to extend findings from studies performed at a landscape scale to the total range. Our goals were twofold. Specifically, we wished to determine the relative role of landscape structure (i.e. fragmentation and subsequent recent isolation) and geographical location along the east–west gradient (i.e. postglacial colonization history and ancestral isolation) in determining the genetic diversity of a set of populations. We also sought to test the relationship between genetic diversity and fitness in these populations, and evaluate the relative contributions of both processes to fitness. Amphibians frequently survive within metapopulations (Marsh & Trenham 2001) and the loss of fitness caused by isolation increases the risk of extinction of (sub)populations (Saccheri *et al*. 1998; Rowe & Beebee 2003); therefore the comparison of average fitness across populations can provide useful insights.

Methods

Study area

We studied 10 populations of *R. latastei* from the Lombardy in northern Italy (Fig. 1). The distribution of *R. latastei* in Lombardy is well known through a comprehensive 10-year census (Bernini *et al*. 2004a). The populations (ponds) were selected based on their different degrees of isolation.

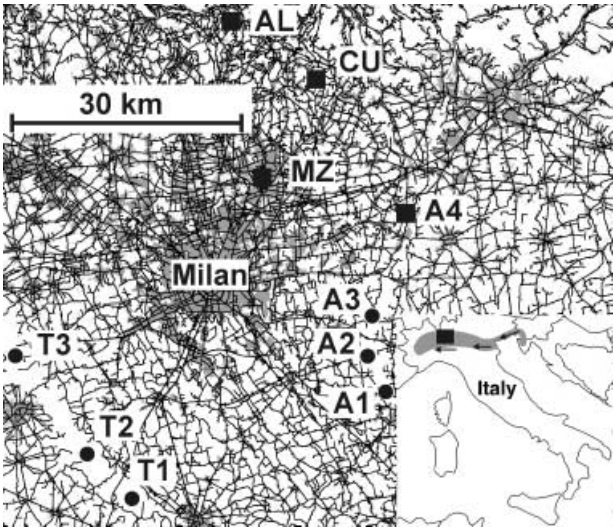

Fig. 1 Study area. Circles: non-isolated populations; squares: isolated populations; grey: urban areas; black lines: roads. In the insert it is also represented the range of *R. latastei* (grey) and the postglacial colonization route (arrows).

Table 1 Features and isolation of study populations; samples used for fitness assessment and genetic analysis. Populations are ordered following the east–west gradient

Population	Isolated	Road length within 1.5 km	Distance from the nearest *R. latastei* population known	No. of samples for fitness assessment	No. of samples for genetic analysis
A4	Y	19.74	6 km (Trezzo)	—	35
A1	N	7.78	< 0.5 km	26	24
A3	N	4.67	< 0.5 km	—	20
A2	N	6.74	< 0.5 km	30	35
CU	Y	12.90	4 km (Sartirana)	5	28*
MZ	Y	20.54	12 km (Lomagna)	32	35
AL	Y	13.20	3 km (Rogeno)	30	35
T1	N	0.05	< 0.5 km	31	30
T2	N	2.35	< 0.5 km	32	28
T3	N	1.42	< 0.5 km	32	25

*: for genetic analysis we used eight tadpoles hatched in 2003 and 20 tadpoles hatched in 2004.

Four study populations (Alserio Lake: AL; Curone park: CU; Monza Park: MZ; Adda River 4, A4) were 3–12 km distant from the closest conspecific population (Table 1), a distance far exceeding the known dispersal ability of *R. latastei* (see Smith & Green 2005 for data on dispersal in European brown frogs). These sites are located in an area that has been highly modified by human development and where the intervening spaces are composed of roads, fences and urbanized areas (Ficetola & Scali 2002; see also Fig. 1). The other six populations are located within two riparian woodlands that are part of natural parklands adjacent to two rivers (Ticino river: T1, T2, T3; Adda river: A1, A2, A3). The riparian areas are continuously wooded, and wetlands near to the river are washes that are frequently inundated during floods. Thus, populations are part of complex and interconnected networks of breeding sites (Bernini *et al*. 2004b; Ficetola & De Bernardi 2004); we considered them to be nonisolated. Further details on the study populations are reported elsewhere (Ficetola & De Bernardi 2005a, 2005b; population A3 corresponds to population CO in Garner *et al*. 2004). The longest axis of distance among the study populations along the east to west gradient (space between A4 and T3) was 54 km. Therefore, our sampling covered approximately 10% of the range of *R. latastei* (Fig. 1).

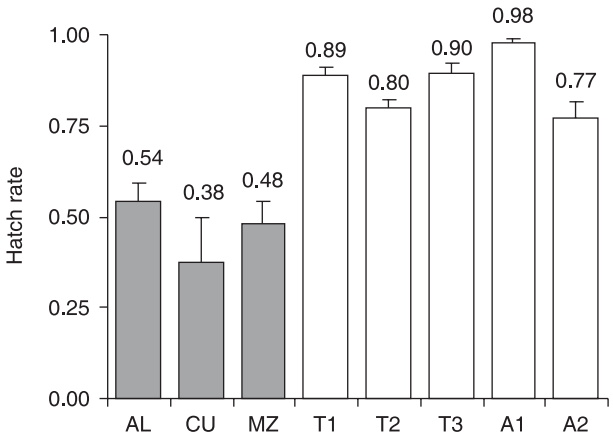

Fig. 2 Hatch rate of clutches collected from isolated (grey bars) and non-isolated (white bars) populations of *R. latastei*. Error bars are standard errors.

Geographical features and fitness assessment

The proposed glacial refugium of *R. latastei* is located in the eastern limit of the total species range and this region contains the greatest genetic diversity of the overall range (Garner *et al*. 2004; Fig. 2). We considered the distance from the glacial refugium to be equivalent to the straight-line distance of each individual population from the eastern limit of the species range. Throughout our analyses we used the squared distance, as the genetic diversity of *R. latastei* declines with distance from the estimated refugium following a quadratic relationship (Garner *et al*. 2004). Using a geographical information system, we calculated the summed road length (km) in a 1.5 km-radius area surrounding the wetland within which a given population was located. To do this we used the vector map of Lombardy. We used road length as an estimate of relative isolation because roads are strong barriers that are known to be impediments to gene flow in amphibians (e.g. Reh & Seitz 1990; Vos *et al*. 2001). We chose a 1.5 km radius because previous studies showed that isolation at this spatial scale influences the distribution of *R. latastei* (Ficetola & De Bernardi 2004).

In March 2003, we gently removed a small proportion (average ± SE: 33.65 ± 1.20 eggs) from 26–32 clutches from

eight of the 10 populations (Table 1, Fig. 1). For one population (CU), we only sampled five clutches due to the limited reproductive effort at that site in 2003. This method of sampling has previously been shown to have no influence on the mortality of embryos (Ficetola & De Bernardi 2005b). In total we collected 7335 eggs from 218 different clutches. We transferred the eggs, separated by clutch, into 200 mL plastic containers filled with aged tap water, which were brought to the laboratory the same day. All eggs were maintained in this manner under ambient outdoor conditions (outdoor temperature 8–20 °C; natural daylight photoperiod). We counted the number of eggs that hatched from each clutch sample. After the end of the experiment, tadpoles were released to their wetland of origin. We haphazardly collected a subsample of unhatched eggs from a subset of clutches, one week after the last observed hatching in a clutch had occurred. Here we sampled 590 eggs from 25 clutches (23.65 ± 3.07 eggs per clutch), 12 clutches from nonisolated and 13 from isolated populations. Eggs were examined under a stereomicroscope to determine if unsuccessful hatching could be attributed to mortality after the onset of development, or if development had not occurred.

DNA extraction and microsatellite analysis

For DNA extraction we collected a small portion from 20 to 35 clutches per populations (Table 1; total number of eggs = 295). In most of the populations eggs were from the same clutches as those used for the fitness assessment. Eggs from population A3 were collected in 2001 (Garner *et al*. 2004). Eggs were again reared to hatch and a single larva per egg mass was euthanized for DNA extraction. In *R. latastei*, each female lays a single clutch per year; therefore each clutch represents a unique full-sib family. Tadpoles from only eight egg masses were available from population CU in 2003, which we augmented by sampling a further 20 egg masses in 2004 (total sample size CU = 28). DNA was extracted from tail tips using a Chelex-based protocol (Rowe *et al*. 1998). Seven polymorphic microsatellite loci are currently available for *R. latastei*: RlatCa9, RlatCa17, RlatCa18, RlatCa21, TlatCa27, RlatCa41 and Rt2Ca9 (Garner & Tomio 2001). However, primer RlatCa18 was not used because of high rates of nonamplification and excessive homozygosity, which indicated the presence of one or more null alleles (Garner *et al*. 2004). We used primers for the six loci to perform polymerase chain reaction (PCR) amplification of extracts on a PTC-100 thermocycler (MJ Research Inc.), using the protocol of Moller *et al*. (2004). Amplification cycles followed Garner & Tomio (2001), but here we used [^{33}P]-labelled primers. Amplification products were electrophoresed through 6% polyacrylamide gels, autoradiographed, and alleles visually scored for each locus against a pGem®-3zf + (Promega) sequence reference marker (Rowe *et al*. 1998). We used amplification products run previously on other gels to ensure scoring consistency among gels. We determined the frequency of genotyping errors by re-amplifying 95 randomly selected locus-sample combinations. Only polymorphic loci were used in this test. After re-amplification we scored the new products blindly. These genotypes were compared to previous ones, and the number of allelic mismatches was counted (Bonin *et al*. 2004). Two genotyping errors were identified, indicating an error rate of 2.1%, comparable to those obtained by Bonin *et al*. (2004). Data for population A3 was generated previously (Garner *et al*. 2004).

Data analysis

Each microsatellite locus was tested in each population and across all populations for deviations from Hardy–Weinberg using the default settings of GENEPOP 3.3 (Raymond & Rousset 1995). Linkage disequilibrium was also tested using the default settings in GENEPOP 3.3. Measures of population genetic variability (percentage of polymorphic loci at the 0.99 criterion, allelic richness rarefied to 19 individuals, observed and expected heterozygosity) were calculated using FSTAT 2.9.3 (Goudet 2001) and GENETIX (Belkir 2004). We used BOTTLENECK 1.2 to test for heterozygote excess or deficiency using a Wilcoxon test, to detect recent population bottlenecks (Cornuet & Luikart 1996). We used a two-phase mutation model incorporating 5% of the infinite allele model and 95% of the stepwise mutation model, iterated 10 000 times. We used pairwise F_{ST} estimates (Weir & Cockerham 1984) to determine the degree of genetic differentiation among populations. The significance of pairwise F_{ST} was calculated using GENETIX and 10 000 iterations. Because we had only four isolated populations, all located within the northern portion of the study area, we also tested specifically to see if pairwise F_{ST} was reduced among road-isolated populations. We did this to ensure that our estimates of hatch rate and the genetic diversity of isolated locations were not pseudoreplicated. We used a permutation test with 10 000 replicates to first compare the pairwise F_{ST} among pairs of isolated populations with that of paired isolated and nonisolated populations. We then repeated the analysis using pairwise F_{ST} among pairs of isolated populations, and pairwise values generated among pairs of nonisolated populations located in different river systems (A and T populations).

We obtained an estimate of genetic diversity for each population from a principal component analysis (PCA) of allelic richness, percentage of polymorphic loci and observed heterozigosity. We did this to avoid the arbitrary use a single measure of genetic diversity (Garner *et al*. 2004). The first principal component explained 83.4% of the variation, and was strongly and positively related with all three measures of genetic diversity (all $r > 0.87$). This component

has previously been shown to be an effective summary statistic for genetic diversity and is also related to measures of fitness (Garner *et al*. 2004; Pearman & Garner 2005).

We used a mixed model ANOVA to evaluate whether hatch rate was different among the two categories of populations (isolated vs. nonisolated). We set isolation (yes vs. no) as a factor, and population identity as a random factor nested within isolation. We used multiple linear regressions to evaluate the relative role of distance from refugium and isolation (here measured as road length) in determining genetic diversity of populations. Distance from refugium and isolation explained > 90% of variation in genetic diversity (see Results), therefore we assumed these two factor to be the main drivers of genetic diversity in our system. We used Pearson's and partial correlations to unravel the relationship among estimated distance from glacial refugium, isolation, genetic diversity and hatching success. We used distance from refugium as a controlling factor in the first partial correlation to evaluate the relationship between hatching success and loss of genetic diversity attributable to isolation. Similarly, we used road density as a controlling factor to evaluate the relationship between hatching success and loss of genetic diversity loss due to postglacial recolonization. In all analyses proportion data were angularly transformed to better meet the parametric assumption of analysis (Sokal & Rohlf 1995). We did not find any violation of assumptions after transformation, and the residuals of parametric tests were normally distributed (Kolmogorov-Smirnov test, all $P > 0.2$). However, we present untransformed values in all figures for the purpose of clarity; all mean values are ± 1 SE.

Results

Geographical features and fitness assessment

Differences in the degree of isolation were related to differences in road density, confirming our a priori assumption (Fig. 1). Average (± SE) road length was 16.6 ± 2.1 km around isolated populations, and 4.3 ± 1.4 km around nonisolated wetlands (Table 1); these differences were significant ($F_{1,8} = 27.126$, $P < 0.001$). The average hatch rate of the three isolated populations ranged between 0.38 and 0.54, while in nonisolated populations the average hatch rate ranged between 0.77 and 0.98 (Fig. 2). Hatch rate was significantly higher in nonisolated than in isolated populations (mixed model ANOVA: $F_{1,7.351} = 19.618$, $P = 0.003$). Hatch rate was also significantly different among populations within a group ($F_{6,209} = 7.284$, $P < 0.001$).

Hatch rate decreased in populations surrounded by a high density of roads (linear regression: $F_{1,6} = 10.460$, $P = 0.018$, $r^2 = 0.635$; Fig. 3), while the relationship between hatch rate and estimated distance from glacial refugium was not significant ($F_{1,6} = 0.567$, $P = 0.480$, $r^2 = 0.086$). If both road length and distance from refugium were combined in the same multiple regression model, the effects remained the same (road density: $F_{1,5} = 8.272$, $P = 0.031$, distance from refugium: $F_{1,5} = 0.516$, $P = 0.505$). The reduced hatch rate of clutches collected from isolated populations was mainly caused by high mortality during early development. On average half (48.5 ± 7.9%) of unhatched eggs started larval development but died prior to hatching in egg masses from isolated populations, while 51.5% did not appear to begin development ($N = 13$ egg masses). In contrast, 26.4 ± 5.9% of unhatched eggs from egg masses collected from nonisolated populations started larval development but died prior to hatching, and 73.6% did not start embryo development ($N = 12$ egg masses). The proportion of eggs per clutch that started larval development but died prior to hatching was higher in isolated populations (ANOVA: $F_{1,23} = 4.906$, $P = 0.037$).

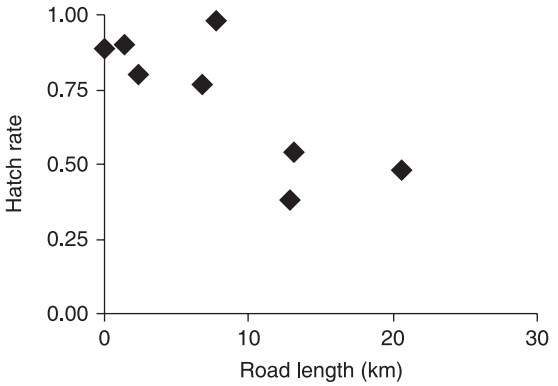

Fig. 3 Relationship between road length within a 1.5 km radius and hatch rate for eight populations of *R. latastei*.

Genetic features of populations

One locus (RlatCa21) was monomorphic for the same allele in all populations, a result consistent with those previously published for western populations (Garner *et al*. 2004; Table 2). Overall, genetic diversity in all populations was low. The number of polymorphic loci detected in a population ranged from two to five, and the total number of alleles across all loci per population ranged from nine to 17.

Twenty-five of a possible 60 locus–population combinations were monomorphic, leaving 35 possible comparisons testing for deviations from Hardy–Weinberg equilibrium, only one of which was significant after table-wide Bonferroni correction. Locus RlatCa27 was not in Hardy–Weinberg equilibrium in population AL ($P < 0.001$), and none of the 51 tests for linkage disequilibrium were significant after Bonferroni correction. We did not find evidence of heterozygote excess or deficiency in any of the populations (Wilcoxon test: all $P > 0.062$). However, it should be

Table 2 Measures of genetic variability. Populations are ordered following the east–west gradient

	K									
Population	RlatCa9	RlatCca17	RlatCa21	RlatCa27	RlatCa41	Rt2Ca9	Pl	Ar	H_E	H_O
A4	2	3	1	2	3	1	0.667	1.790	0.171	0.186
A1	2	3	1	4	3	3	0.833	2.461	0.232	0.229
A3	2	3	1	4	2	2	0.833	2.316	0.221	0.226
A2	3	3	1	4	3	4	0.833	2.655	0.242	0.209
CU	2	1	1	4	2	1	0.5	1.774	0.189	0.191
MZ	2	2	1	2	2	1	0.667	1.640	0.163	0.124
AL	4	1	1	4	2	1	0.5	2.050	0.237	0.194
T1	1	1	1	5	3	1	0.333	1.910	0.181	0.170
T2	1	1	1	4	2	1	0.333	1.607	0.166	0.167
T3	1	1	1	2	3	1	0.333	1.453	0.169	0.120

K: number of alleles observed at six microsatellite loci, Pl: percentage of polymorphic loci at the 99% criterion level; Ar: averaged allelic richness over all loci rarefied to 19 individuals; H_E and H_O: expected and observed heterozigosity, respectively

noted that the power of this test is low when less than 10 polymorphic loci are used (Cornuet & Luikart 1996).

Pairwise F_{ST} comparisons were significant in 35 of these tests after Bonferroni correction, but almost all comparisons were significant at a nominal value of 0.05 (see Appendix S1). Pairwise F_{ST} among pairs of isolated populations (average ±SD = 0.340 ± 0.156) were not significantly smaller than pairwise F_{ST} estimated among pairs of isolated and nonisolated populations (F_{ST} = 0.214 ± 0.157; permutation test: P = 0.516), or those for pairs of nonisolated populations from different river systems (F_{ST} = 0.178 ± 0.095, P = 0.824).

Relationships between distance from glacial refugium, road density and genetic diversity

Multiple regression analysis revealed how both estimated distance from glacial refugium and road density strongly influenced genetic diversity. Genetic diversity decreased at increasing distances from the estimated glacial refugium ($F_{1,7}$ = 66.703, P < 0.001, Fig. 4a). Moreover, when the east–west position was held constant, genetic diversity was lower in populations located in high road density areas ($F_{1,7}$ = 16.729, P = 0.005, Fig. 4b). This multiple regression model explained most of the among-population variation in genetic diversity (r^2 = 0.906). Despite both factors significantly affecting genetic diversity, the role of distance from glacial refugium seems to be the most important based on sum of squares values (SS) in the model (distance from glacial refugium SS = 8.056, road density SS = 2.020, error SS = 0.845). Moreover, if the effect of distance from glacial refugium was not considered, the relationship between road density and genetic diversity was not significant (univariate Pearson's correlation: r = 0.105, N = 10, P = 0.774). The position along the south-north gradient was not significantly related to the variation of genetic diversity ($F_{1,7}$ = 2.708, P = 0.144; effect of distance from glacial refugium as a covariate: $F_{1,7}$ = 23.459, P = 0.002).

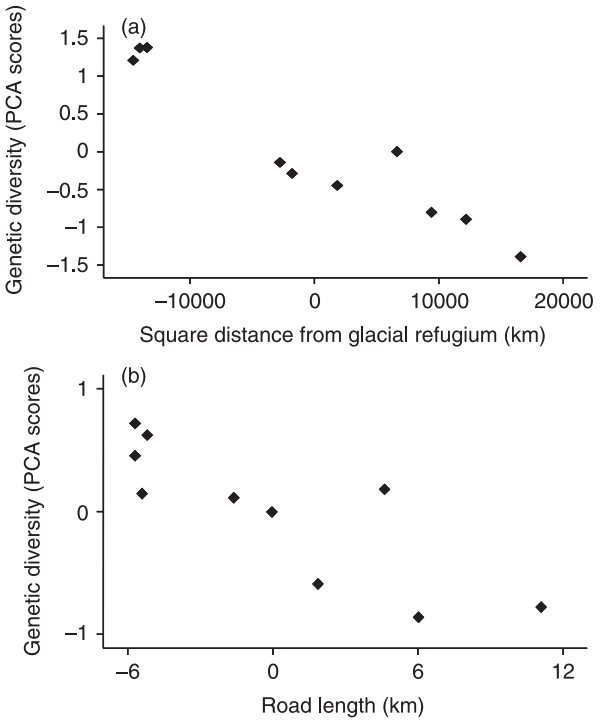

Fig. 4 Partial regression plots showing the relationship between (a) distance from glacial refugium and genetic diversity (PCA scores); (b) road length within a 1.5 km radius and genetic diversity (PCA scores) for 10 populations of *R. latastei*.

Relationship between genetic diversity and fitness

The relationship between genetic diversity and hatching success was not significant (Spearman's r = 0.137, N = 8,

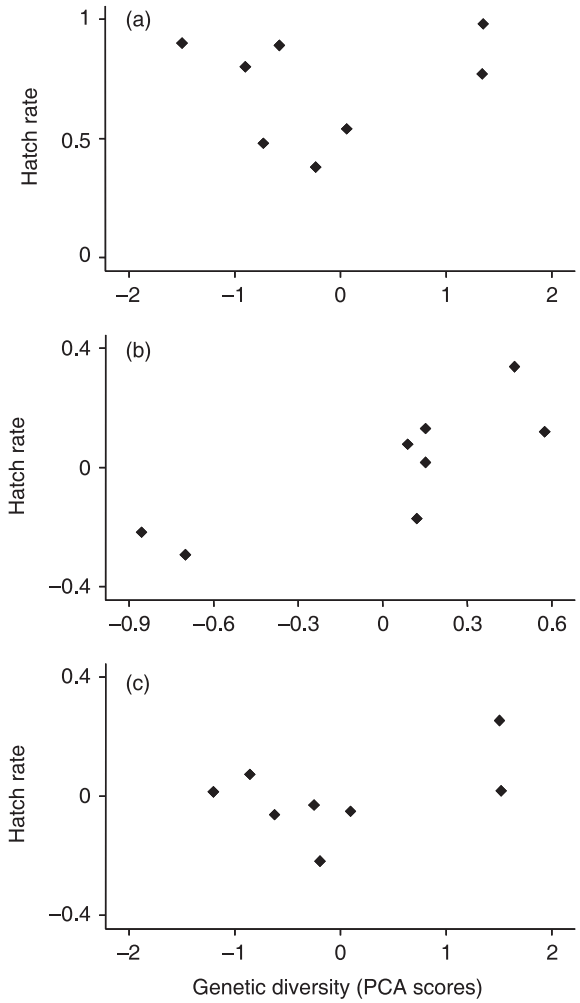

Fig. 5 Relationship between genetic diversity (PCA scores) and hatch rate in eight populations of *R. latastei*. a: simple bivariate plot; b: partial regression plot, controlling for distance from glacial refugium; c: partial regression plot, controlling for road density. The only significant relationship is those in (b).

$P = 0.746$, Fig. 5a). However, when the distance from glacial refugium was held constant, the remaining partial correlation between genetic diversity and hatching success was positive and significant (partial $r = 0.824$, d.f. = 5, $P = 0.023$, Fig. 5b). Conversely, when road density was held constant, the remaining partial correlation between genetic diversity and hatch proportion was not significant (partial $r = 0.432$, d.f. = 5, $P = 0.333$, Fig. 5c).

Discussion

Our study shows that both postglacial colonization history and isolation strongly affect genetic diversity in the frog *R. latastei*. Altogether, these processes explained > 90% of variation in genetic diversity, suggesting that they are the main drivers of genetic diversity in this species. Both increasing distance from the glacial refugium, as hypothesized by Garner *et al*. (2004), and increasing road density correlated negatively with genetic variation (Fig. 4). However, the loss of genetic diversity associated with either of these distinct events does not affect our measure of fitness equivalently. The reduced hatch rate exhibited in clutches collected from isolated populations (Figs 2 and 3) was not significantly related to distance from the proposed glacial refugium or with genetic diversity, *per se*. It is only when we controlled for the influence of distance from refugium that the loss of genetic diversity caused by isolation significantly affected our measure of average fitness (Fig. 5).

Recent isolation due to fragmentation does not play the primary role in determining overall genetic diversity. Instead, postglacial colonization is responsible for most of the genetic diversity detected in this and other studies. In our study, the number of polymorphic loci decreases from five in the easternmost and nonisolated populations (A1-A3) to two in the westernmost populations (T1-T3) (Table 2), which is in agreement with the results of a study examining patterns across the entire species range (Garner *et al*. 2004). Thus, the relationship between genetic variability and location along the east to west gradient is maintained over two spatial scales, which indicates that gene flow among populations after recolonization has been consistently low across time.

Neutral genetic diversity decreases with distance from glacial refugia in many species (see Hewitt 1996, 1999, 2000; Comes & Kadereit 1998 for references) but it is unclear whether this process and the concurrent loss of neutral genetic diversity associated with it causes the loss of adaptive genetic diversity in animals. Two studies have described how populations that are far from glacial refugia and with reduced diversity at neutral loci can be more sensitive to environmental alterations and, in the case of *R. latastei*, to novel pathogens (Schmitt & Hewitt 2004; Pearman & Garner 2005). However, we did not find any negative effect of an east–west gradient on the hatch rate of *R. latastei*. Postglacial recolonization is an ancient process that allows many generations, whereupon selection may operate, and loss of genetic diversity during this process may occur at a slow rate (Wang *et al*. 1999; Wang 2000). The number of individuals and the temporal scales at which genetic loss does occur can affect the efficiency of natural selection against deleterious alleles and therefore their consequences on fitness (Wang *et al*. 1999; Wang 2000; Keller & Waller 2002; Reed *et al*. 2002; Swindell & Bouzat 2006). Simulation studies have shown how natural selection can purge inbreeding depression from populations that are not too small, in species with large reproductive capabilities and if deleterious mutants have large effects (Wang *et al*. 1999; Wang 2000; Keller & Waller 2002). *R. latastei* females can lay more than 1000 eggs per year (Bernini *et al*. 2004a)

and our measure of fitness (hatch rate) is likely to be under strong selection. Mutation, on the other hand, is a slow process and may not be as effective at generating new alleles at neutral loci affected by the serial bottlenecks and founder effects associated with recolonization as selection is at purging deleterious mutations revealed by the same processes. The relationship between neutral and adaptive diversity could be expected to break down under such conditions (Reed & Frankham 2001; Zhang & Hill 2005).

Several studies showed that differences among populations for key traits, such as intrinsic growth rate, development rate and locomotor performance can evolve in response to environmental heterogeneity, even in populations that are far from glacial refugia and are suffering reduced genetic variability at neutral markers (e.g. Palo *et al*. 2004; Weitere *et al*. 2004; Ficetola & De Bernardi 2005a, 2006). For example, Weitere *et al*. (2004) demonstrated that populations of *Salamandra salamandra* originated from a single lineage that recently colonized western Germany. Nevertheless, populations exhibit genetic variation in growth rate in response to differences in food availability. This supports the idea that even genetically impoverished lineages can have the ability to adapt to local conditions, especially when exposed to strong selection.

Isolation is a more recent process and loss of genetic diversity and selection against deleterious mutants may instead be ongoing. This is suggested by the data from the three isolated populations where diversity was low, but not the lowest overall, and less than 60% of the eggs from clutches sampled at these sites hatched. Further, the reduced hatch rate in these clutches was more affected by death during development than by what appeared to be unsuccessful fertilization. Failure during embryonic development is a commonly cited situation where inbreeding is a factor (e.g. Daniels & Walters 2000; Richards 2000; Briskie & Mackintosh 2004; Edman *et al*. 2004; Jamieson *et al*. 2006; and references therein) and such effects can be rapidly removed from populations by selection (Wang *et al*. 1999; Wang 2000; Keller & Waller 2002; Reed *et al*. 2002). We have detected evidence of strong selection against the most inbred lines in one of the study populations (AL), providing more support that the observed relationship between loss of genetic variability due to recent fragmentation processes and fitness is an ongoing process that is not directly related to more ancient patterns of genetic diversity (G.F. Ficetola *et al*., unpublished data).

We do acknowledge that we are drawing conclusions from a study that involves a limited number of populations. It is possible that some of the non significant relationships we observed were not significant simply because of lack of statistical power. However, the sample size in our study was similar to or even larger than those of other studies investigating the relationships between genetic diversity and fitness in amphibians (e.g. Rowe *et al*. 1999; Zeisset & Beebee 2003; Andersen *et al*. 2004; Pearman & Garner 2005); therefore our results are comparable with the existing literature. All the relationships between genetic diversity and average fitness were positive (Table 3), and the effect size of non significant relationships (all $r > 0.15$) were well within the range of studies analysing the relationship between genetic diversity and fitness (Reed & Frankham 2003). Thus, our study does not rule out the possibility that genetic diversity *per se*, and the loss of genetic diversity caused by postglacial colonization, are still

Table 3 Comparison of anuran microsatellite studies

Species	NP	NL	K	Al	H_E	Source
Alytes muletensis	14	8	11–24	4.355	0.563	Kraaijeveld-Smit *et al*. (2005)
Litoria aurea	21	4	3–12	6.7	0.694	Burns *et al*. (2004)
Physalaemus pustolosus	17	7	12–42	13.6	0.820	Lampert *et al*. (2003)
Hyla arborea	12	12	—	—	0.447	Andersen *et al*. (2004)
Bufo bufo	8	8	—	5.1	0.579	Brede & Beebee (2004)
Bufo calamita	11	8	7–24	3.3	0.388	Beebee & Rowe (2000)
Rana arvalis	12	5	2–8	—	0.337	Vos *et al*. (2001)
Rana cascadae	19	7	3–20	4.5	0.73	Monsen & Blouin (2004)
Rana luteiventris	28	6	5–16	—	0.474	Funk *et al*. (2005)
Rana pipiens	10	8	—	3.6	0.459	Hoffman & Blouin (2004)
Rana ridibunda	5	5	1–6	2.7	0.428	Zeisset & Beebee (2003)
Rana sylvatica	12	5	2–18	4.1	0.474	Newman & Squire (2001)
Rana temporaria	8	8	—	7.9	0.669	Brede & Beebee (2004)
Rana temporaria	43	7	10–34	6.34	—	Johansson *et al*. (2006)
Rana latastei (full range)	19	6	2–13	3.1	0.310	Garner *et al*. (2004)
Rana latastei	10	6	1–5	2.1	0.197	This study

K: number of alleles observed per locus; Al: average alleles/locus/population; H_e: average expected heterozygosity.

influencing hatch rate, despite having a reduced magnitude of effect. We can thus only conclude that the effect size is smaller than that imposed by more recent fragmentation processes.

Conservation implications

A striking result of our study is that all study populations exhibit extremely low genetic diversity for an amphibian. Both the number of alleles per locus per population, and the expected heterozygosity values are considerably lower than in other microsatellite studies of anuran populations (Table 3), and are also lower when compared to studies of other vertebrates (e.g. birds: Jamieson *et al*. 2006). Our study populations are far from the glacial refugium but, perhaps more importantly, the north of Italy has been strongly modified by human activities (e.g. Ficetola & De Bernardi 2004). *R. latastei* is a small frog with a generally poor dispersal capability; a fact that would exacerbate the effects of both ancestral and recent restrictions on gene flow. The observed pattern of reduced genetic diversity associated with high embryo mortality, coupled with ongoing landscape modifications and intense agricultural land use, suggests that population management of this species is a conservation priority. One approach may be to translocate animals from the more genetically diverse eastern part of the species range. However, introducing individuals from populations with higher genetic diversity to restore the genetic diversity and fitness has its drawbacks (Tallmon *et al*. 2004). Local adaptation does exist among even genetically impoverished populations of *R. latastei* (Ficetola & De Bernardi 2005a, 2006), suggesting that they should be considered as evolutionary independent units, and hence should be independently managed wherever possible to avoid the break-up of coadapted gene complexes and other forms of outbreeding depression (Crandall *et al*. 2000; Tallmon *et al*. 2004; Ficetola & De Bernardi 2005a). For this reason, we believe that translocations should be performed only as a last option. Even with high embryo mortality, recently isolated populations do not seem to be on the verge of extinction, but the sizes of these populations are limited by the availability of suitable habitat. Habitat reclamation and management has been successful in the area of population AL, and the number of breeding females increased from 19 during 2001 to ~150 in 2005, after management (Gentilli *et al*. 2003; G.F. Ficetola, personal observation). Therefore, we call for a prioritization of habitat management as a conservation measure for *R. latastei*, with the goals of rapidly increasing census size by improving local habitat availability and landscape connectivity. This rapid increase in census size provides greater opportunity for natural selection, recombination and even mutation to improve fitness, while maintaining locally adapted traits (cf. Miller & Hedrick 2001). In the event that this approach fails, translocation could still be adopted as a conservation measure for this species.

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This study is part of the PhD thesis of Francesco Ficetola. Francesco Ficetola is interested in conservation and ecology of amphibians, and in the importance of recent evolutionary processes for conservation. Trent Garner is currently researching population genetic and ecological factors associated with disease emergence in amphibians. Fiorenza De Bernardi has a long standing experience in development biology of amphibians and other organisms, and in issues of conservation biology.
