Biological Journal of the Linnean Society, 2009, **98**, 661–676. With 3 figures

Genetic variation and its evolutionary implications in a Mediterranean island endemic lizard

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Received 24 February 2009; accepted for publication 4 June 2009

The peculiar bioclimatic and geographic features of Corso–Sardinian islands may provide an ideal scenario for investigating microevolutionary processes, given their large heterogeneity of environments, which could affect dispersal and gene flow among populations, as well as processes of local adaptation. The genetic variation and differentiation among populations of the endemic lizard *Archaeolacerta bedriagae* were studied by allozyme electrophoresis at 20 presumptive loci. The genetic structure of this species is characterized by relatively high levels of polymorphism and low differentiation among populations. The pattern of genetic differentiation cannot be explained by genetic drift as a function of geographic distance. Genetic distance data show that genetic variation is distributed into three geographically coherent population groups and suggest a recent (Late Pleistocene) origin for the observed geographic fragmentation. The analysis of environmental correlates of allozymic variation indicates a strong correlation of the *Idh-1* locus with climatic variables. The frequency of the *Idh-1*^{106} allele is negatively correlated with annual temperature, and positively correlated with annual precipitation. In addition, the observed heterozygosity at this locus decreases towards more arid climatic regimes. The results obtained support the assumption of differential selection acting on *Idh-1* allozymes under diverse climates. An association between *Idh-1* allozymes and local bioclimatic regimes was also observed for the sympatric lizard *Podarcis tiliguerta*, suggesting a key role for such selective agents on *Idh-1* polymorphism in these two Corso–Sardinian lacertids.

ADDITIONAL KEYWORDS: allozyme electrophoresis – *Archaeolacerta bedriagae* – Corsica – environmental correlates – Lacertidae – population differentiation – Sardinia.

INTRODUCTION

Subsequent to the origin of the evolutionary theory, islands have been considered as natural laboratories for the study of microevolutionary processes (Darwin, 1858, 1859; Wallace, 1858). Islands showing structural complexity force species to deal with increased heterogeneity of ecological conditions and with geographical barriers both on micro- and macrogeographical scales, and thus provide an ideal scenario to investigate colonization processes, adaptive radiations, allopatric fragmentation, and processes of genetic and morphological divergence (Soulé, 1976; Thorpe & Baez, 1987; Brown, Thorpe & Baez, 1991; Johnson, Adler & Cherry, 2000; Schluter, 2000; Hewitt, 2001; Thorpe, Reardon & Maalhotra, 2005). Various evolutionary factors may be more influential in populations from these islands than in mainland populations, including directional selection and genetic drift (Selander, 1976; Wright, 1978; Barton, 1996; Frankham, 1997; Clegg *et al.*, 2002). Indeed, species ranges might be fragmented and populations inhabiting ecologically diverse patches will be subject to diverse selective factors. Island populations are usually limited in size, enhancing the effect of stochastic processes. Studies that have focused on the evolutionary processes acting in insular ecosystems have provided useful insights into the comprehension of selection models and the evolutionary significance of selectively neutral molecular variation in various

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groups of organisms (Selander & Whittman, 1983; Barton & Charlesworth, 1984; Givnish *et al.*, 1995; Gavrilets & Hastings., 1996; Grant, 1996; Stuessy *et al.*, 2006; Mappes *et al.*, 2008).

The Mediterranean basin shows a wide variety of insular systems with different origins and large structural and environmental complexity. Some of these islands have been studied to test the biogeographical hypotheses and evolutionary processes underlying the pattern of species geographical variation (Gorman *et al.*, 1975; Capula, 1994a, b, 1996; Rosselló, Cebrián & Mayol, 2002; Gentile & Argano, 2005; Harris *et al.*, 2005; Brown *et al.*, 2008; Carranza *et al.*, 2008). The Corso–Sardinian system was used in an early application of the molecular clock hypothesis (Caccone *et al.*, 1997) because its endemic species have been isolated from the sister species on the continental landmasses from the early Miocene (Alvarez, 1972; Alvarez, Franks & Nairn, 1973; Casula *et al.*, 2001). Currently, Corsica and Sardinia are environmentally complex islands, highly heterogeneous, owing to the influence of climate, orography, and topography. This heterogeneity lead to the formation of natural barriers, which could affect dispersal and gene flow among populations, as well as favouring processes of local adaptation. Consequently patterns of intra-island geographical variation have been studied for some Corso–Sardinian species (Capula, 1996; Ketmaier *et al.*, 2003; Lecis and Norris, 2004; Harris *et al.*, 2005). Lacertid lizards have been shown to comprise excellent zoological material for studying evolutionary processes in island ecosystems and this is also true for the Corso–Sardinian region. For example, Capula (1996) found that the genetic structure of an endemic lizard, *Podarcis tiliguerta*, is moulded by the interplay of stochastic processes and local bioclimatic regimes selectively affecting allele frequency changes. The present study investigates the geographic variation in the rock lizard *Archaeolacerta bedriagae* (Camerano, 1885), a lacertid lizard endemic to Corsica and Sardinia. This species is polytypic and four subspecies have been described: *Archaeolacerta bedriagae bedriagae* (Camerano, 1885), *Archaeolacerta bedriagae sardoa* (Peracca, 1903), *Archaeolacerta bedriagae paessleri* (Mertens, 1927), and *Archaeolacerta bedriagae ferrerae* (Stemmler, 1962). Preliminary data on allozyme differentiation did not support this intra-specific taxonomy (Salvi *et al.*, 2009). *Archaeolacerta bedriagae* is distributed from northern Corsica to southern Sardinia, from sea level to the highest elevation of both islands (2700 m a.s.l. on M. Cinto in Corsica and 1932 m a.s.l. on Gennargentu Massif in Sardinia), and from interior to coastal and microinsular areas. Consequently, this lizard represents a vertebrate model species for assessing the geographical structuring of species diversity on Corsica and Sardinia. Moreover, this species shows a fragmented distribution, particularly in Sardinia (Delaugerre & Cheylan, 1992; Bombi & Vignoli, 2004; Bombi *et al.*, 2009b), most likely as a consequence of a high degree of ecological specialization to bare rocky habitats (Bombi *et al.*, 2009a). As shown for *P. tiliguerta*, the patchy occurrence of suitable habitats on both islands, together with the complexity of Corso–Sardinian islands environments, may facilitate the action of stochastic processes (geographic isolation, genetic drift) and adaptive phenomena promoted by local selective forces.

Analyses of several independent loci such as those gathered with allozyme electrophoresis enables the accurate definition of genetic structure within a species. The identification of single-locus idiosyncratic patterns equivalent to those expected under selection processes will help to explain how environmental heterogeneity plays a role on the structure of intraspecific diversity. The analysis of the geographic pattern of allozyme variation can make spatial patterns of genetic variation explicit (e.g. by testing 'isolation by distance' models and allele frequencies for clinal variation). Although most patterns can be interpreted as a result of either selective or stochastic processes, depending on the assumptions made about population size and migration rate (Lewontin, 1974), patterns of variation at a specific locus, evaluated in the appropriate functional, ecological, and/or historical contexts, can indeed reveal the occurrence of adaptive and neutral processes affecting the evolution of proteins (Feder & Watt, 1992; Carter, 1997). Indeed, although, on the one hand, allozymes are traditionally considered as neutral markers, on the other hand, the potential adaptive significance of single-locus allozyme variation has been demonstrated by a number of studies by estimation of correlation between allozyme genotypes and environmental variables (Clarke, 1975; Nevo *et al.*, 1979; Mitton, 1997; Eanes, 1999; Volis *et al.*, 2003; Del Lama *et al.*, 2004). In the present study, we analyse the genetic structure of *A. bedriagae*, and perform an explicit test for the association between allozyme frequency changes and local bioclimatic regimes.

The main goals of the present study are: (1) to estimate the distribution of genetic variation within and among populations of the species all over its range and (2) to test whether the observed genetic variation is explained by historical or recent adaptive processes.

MATERIAL AND METHODS

SAMPLING

Because *A. bedriagae* has a fragmented range, collecting localities were selected with the objective of

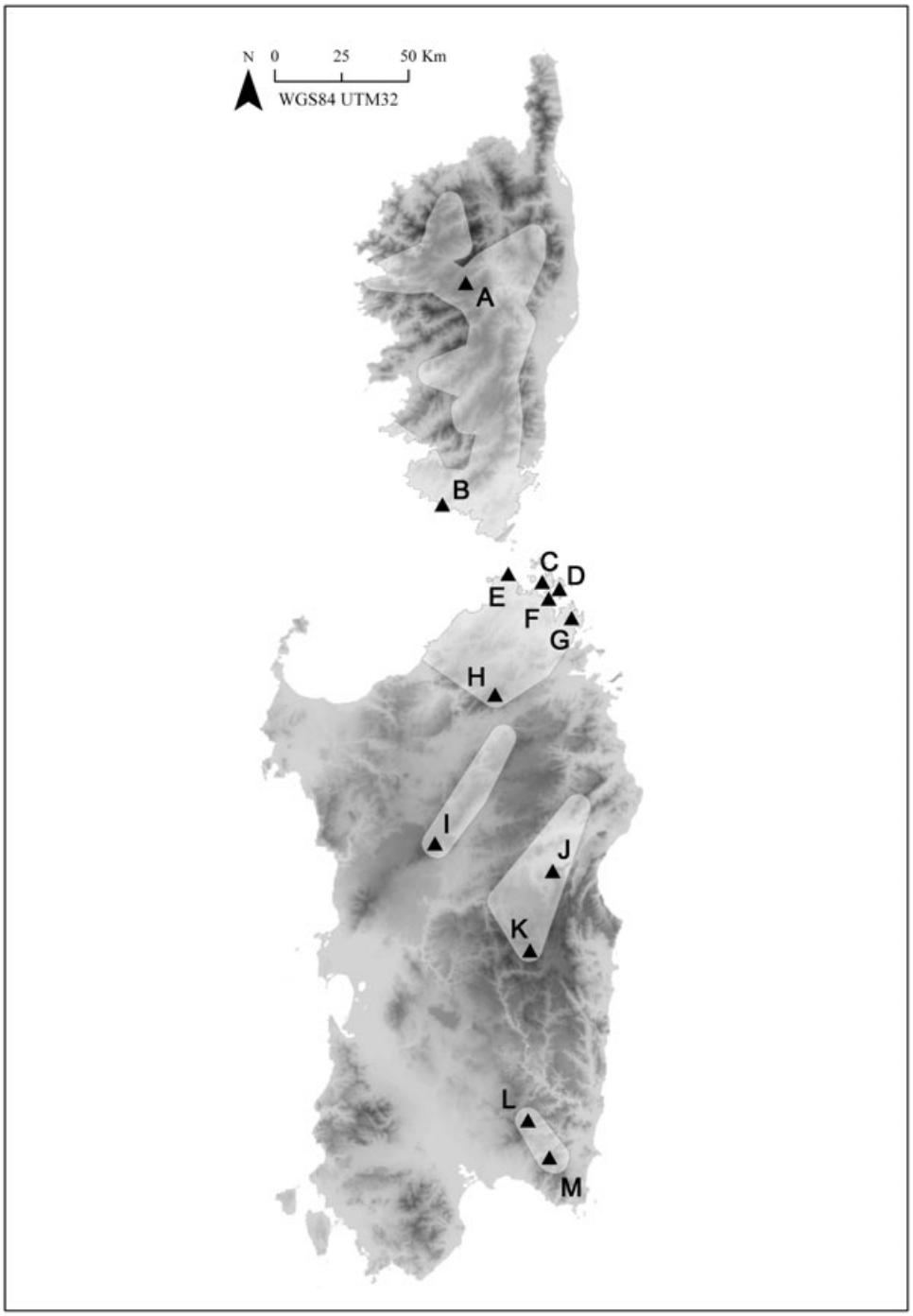

Figure 1. Sampling locations, sampling codes and distribution (light shaded area) of *Archaeolacerta bedriagae*. The range of the species is based on data points from Delaugerre & Cheylan (1992) and Bombi & Vignoli (2004), and defined applying buffers of three kilometres on subranges' local hulls. For geographical detail on sampling locations, see Table 1.

covering the whole range and obtaining adequate sampling to answer questions based on geographical, ecological, and taxonomical criteria. Therefore, a balanced number of individuals was included in the analysis from both Corsica and Sardinia, as well as from coastal, micro-insular, and mountain populations, and belonging to all the four described subspecies. A total of 122 individuals of *A. bedriagae* from 13 localities were studied. Details on sampling design are provided in Figure 1 and Table 1.

Table 1. Geographical location and sample size for the populations studied of *Archaeolacerta bedriagae*

Sample	Locality	Altitude (m a.s.l.)	Latitude	Longitude	Sample size
A	*Gorges de la Restonica* (Corte)	1255	9°02′	42°14′	12
B	*Roccapina* (Sartène)	83	8°56′	41°29′	16
C	*Punta dei Colmi* (La Maddalena Island)	106	9°23′	41°13′	6
D	*Petraiaccio* (Caprera Island)	72	9°27′	41°12′	8
E	*Punta Marmorata* (S. Teresa di Gallura)	3	9°14′	41°15′	5
F	*Capo d'Orso* (Palau)	113	9°24′	41°10′	5
G	*Monte Moro* (Cannigione)	419	9°31′	41°06′	10
H	*Monte Limbara* (Tempo Pausania)	1306	9°10′	40°51′	11
I	*Nuraghe Ortachis* (Bolotana)	1034	8°54′	40°21′	11
J	*Supramonte* (Oliena)	1233	9°25′	40°15′	7
K	*Gennargentu* (Fonni)	1783	9°19′	39°59′	12
L	*Monte Genis* (Villasalto)	776	9°18′	39°24′	4
M	*Monte Settefratelli* (Burcei)	483	9°24′	39°17′	15

The phylogenetic position of *A. bedriagae* within the Palaearctic lizard radiation is still not clear (Arnold, 1989; Arribas, 1999; Fu, 1998, 2000; Harris, Arnold & Thomas, 1998; Carranza, Arnold & Amat 2004; Arnold, Arribas & Carranza, 2007; Mayer & Pavličev, 2007). To obtain a scale reference from an interspecific comparison for the genetic differentiation observed within *A. bedriagae*, we analysed 11 specimens of *Iberolacerta cyreni* (Müller & Hellmich, 1937) from Central Spain (Sierra de Guadarrama). The geographic coordinates and altitude for each sampling site were recorded in the field using a GPS (Garmin; GPSmap 76S) and were used for environmental and geographical correlation analyses.

Allozyme electrophoresis

To avoid killing animals, approximately 1 cm of the tail of each lizard was taken, and stored below –70 °C prior to electrophoretic analyses. Standard horizontal starch gel electrophoresis was performed on muscle tissue, using the buffer systems and procedures detailed in Table 2. Gene products for the following 20 presumptive gene loci were analysed: *αGpd*, *Ldh-1*, *Ldh-2*, *Mdh-1*, *Mdh-2*, *Me-1*, *Me-2*, *Idh-1*, *Idh-2*, *6Pgd*, *Sod-1*, *Ak*, *Pgm-1*, *Pgm-2*, *Ck*, *Mpi*, *Gpi*, *Est-1*, *Gp-1*, and *Gp-2*. Another five loci, *Got-1*, *Got-2*, *Np*, *G3pdh*, and *Ada*, were studied, but they were not included in the analyses because of their low activity in muscle tissue, resulting in a weak banding patterns. Isozymes were numbered in decreasing mobility order from the most anodal; allozymes were designated numerically according to their mobility, relative to the commonest one, indicated as 100 (> 100 = faster mobility; < 100 = slower mobility).

Genetic structure

Genotypic and allele frequencies were estimated by direct count from allozyme phenotypes, and then analysed by independent statistical methods. To assess statistical significance of simultaneously multiple tests, Bonferroni's correction (Rice, 1989) was applied. Genotypic proportions expected on the basis of Hardy–Weinberg equilibrium (H–W) were calculated by Levene's (1949) formula for small samples. The statistical significance of departures from H–W was estimated using exact significance probabilities analogous to Fisher's exact test (Elston & Forthofer, 1977) adopting the pooling procedure of BIOSYS-2 (Sokal & Rohlf, 1969). To determine whether the heterogeneity in the genotypic distribution reflects differences in allele frequencies, the variation in genic proportions among populations was subjected to a contingency chi-square analysis (Workman & Niswander, 1970). The genetic variability of populations was estimated by means of the following parameters: mean number of alleles per locus (*A*); mean number of alleles independent of sample size per locus (*Ar*); proportion of polymorphic loci ($P_{99\%}$); observed mean heterozygosity per locus (H_o); Nei's (1978) unbiased estimate of expected mean heterozygosity per locus (H_e).

The distribution of genetic variability (H_o) and allele frequencies (log-transformed values) was tested for latitudinal and altitudinal patterns by means of Spearman rank correlation analysis in STATISTICA (StatSoft, Inc., 2004).

The distribution of genetic variation within and among populations was assessed calculating *F*-statistics (Wright, 1965) according to the method of Weir & Cockerham (1984) implemented in BIOSYS-2 (Swofford & Selander, 1999). Statistical significance

Table 2. Enzymatic and non-enzymatic proteins examined and electrophoretic condition employed

Protein	Migration*	Buffer system†	V cm^{-1}	Time (h)	Reference
Glycerol-phosphate dehydrogenase αGPDH (EC 1.1.1.8)	+	4	8	6	Ayala *et al.* (1972)
Lactate dehydrogenase LDH (EC 1.1.1.27)	+	5	7	6	Brewer & Sing (1970)
Malato dehydrogenase MDH (EC 1.1.1.37)	+	4	8	5	Shaw & Prased (1970)
Malic enzyme ME (EC 1.1.1.40)	+	2	8	6	Ayala *et al.* (1972)
Isocitrate dehydrogenase IDH (EC 1.1.1.42)	+	4	8	5	Shaw & Prased (1970)
6-Phosphogluconate dehydrogenase 6PGD (EC 1.1.1.44)	+	4	8	6	Shaw & Prased (1970)
Superoxide dismutase SOD (EC 1.15.1.1)	+	2	8	5	Selander *et al.* (1971)
Creatine kinase CK (EC 2.7.3.2)	+	2	8	4	Ayala *et al.* (1972)
Adenylate kinase AK (EC 2.7.4.3)	+	2	8	5	Ayala *et al.* (1972)
Mannose phosphate isomerase MPI (EC 5.3.1.8)	+	3	8	5	Harris & Hopkinson (1976)
Glucosio-phosphate isomerase GPI (EC 5.3.1.9)	–	4	8	6	Selander *et al.* (1971)
Phosphoglucomutase PGM (EC 2.7.5.1)	+	5	8	6	Brewer & Sing (1970)
Esterase EST (EC 3.1.1.1)	+	3	8	5	Harris & Hopkinson (1976)
General proteins GP	+	1	8	4	Scott & McClelland (1975)

*Migration: +, anodal; –, cathodal.

†Buffer system used: 1, discontinuous Tris/citrate (Poulik, 1957); 2, continuous Tris/citrate (Selander *et al.*, 1971); 3, Tris/versene borate (Brewer & Sing, 1970); 4, phosphate/citrate (Harris, 1966); 5, Tris/maleate (modified from Brewer & Sing, 1970).

was tested using a chi-square test *sensu* Workman & Niswander (1970).

An analysis of population differentiation was carried out by a statistical comparison between groups of populations identified on the basis of island origin (Corsica or Sardinia) and cluster analyses results. Tests for difference among groups of populations were computed for allelic richness (*Ar*), observed heterozygosity (H_o), and F_{ST}, in FSTAT, version 2.9 (Goudet, 2001) employing 10 000 permutations to assess their significance. A one-sided alternative was used to test the differences in genetic variability among populations at different latitude.

To investigate genotypic disequilibrium, the significance of association between genotypes at pairs of loci was tested after 10 000 permutations in FSTAT. This procedure, when compared with Fisher's test, has the advantage of taking into account the information content of each population by weighting *P*-values on the basis of polymorphism level at each locus.

The genetic distances between populations were calculated by Cavalli-Sforza & Edwards (1967) chord distances (D_{CSE}) and Nei's (1972) standard genetic distances (D_N). An approximate estimate of the evolutionary divergence times was computed using both Nei's (1975) formula and Sarich's (1977) calibration, corrected by Maxson & Maxson (1979).

A phenogram depicting the genetic relationships among populations was constructed by means of the Neighbour-joining (NJ) method based on the D_{CSE} values. Nodal support was assessed by 1000 bootstrap replicates with BOOTDIST option in BIOSYS-2 and the subroutines NEIGHBOUR and CONSENSE in PHYLIP, version 3.5c (Felsenstein, 1993).

The hypothesis of 'isolation by distance' pattern was tested by means of a Mantel test (10 000 permuta-

tions), comparing genetic (D_{CSE}, D_N, F_{ST} pairwise values) and geographical distance matrices, as implemented in the software IBDWS, version 3.15 (Jensen, Bohonak & Kelley, 2005). For all analyses, we used the log-transformed distances to reduce differences of scales in the measures.

Environmental correlates analysis

To explore how the environmental (bioclimatic) variables affect the distribution of genetic variability, we performed linear correlation analysis. Genetic variability parameters considered in the analysis were the allele frequency and the observed heterozygosity (H_o, at both single and overall loci level), employing log-transformed values. Bioclimatic parameters employed in the analysis were obtained: sampling sites were overlaid on climatic surfaces from WorldClim databanks, version 1.4 (Hijmans *et al.*, 2005) in DIVAGIS, version 5.4 (http://www.diva-gis.org) and climatic values were assigned to each site. The WorldClim databanks consist of climate surfaces with pixels of 30″ of geographic degree, corresponding to a resolution of approximately 1 km, for 19 bioclimatic variables with global coverage interpolated using data for the 1950–2000 period. We selected annual mean temperature (AMT) and annual precipitation (AP) as general descriptors of the bioclimatic regime. The association between bioclimatic variables and allele frequency/genetic variability data was tested by Spearman rank correlation analysis in STATISTICA (StatSoft, Inc., 2004).

We finally compared genetic variability parameters with those of a dataset not including loci affected by environmental variables, using a Mann–Whitney *U*-test.

RESULTS

Genetic structure

Nine out of 20 loci analysed were found to be monomorphic (i.e. fixed for the same allele, in all populations: *αGpd*, *Ldh-2*, *Mdh-1*, *Mdh-2*, *Sod-1*, *Ak*, *Pgm-1*, *Gp-1*, *Gp-2*). The allele frequencies at the polymorphic loci are presented in Table 3. Five out of 11 polymorphic loci were weakly polymorphic (*Ldh-1*, *Me-2*, *Idh-2*, *Mpi*, *Pgm-2*). Lizards from Corsica were characterized by two unique alleles (Mpi^{102} and $6Pgd^{95}$), and five were found in the samples from Sardinia ($Idh\text{-}2^{105}$, $Me\text{-}2^{95}$, $6Pgd^{105}$, $Pgm\text{-}2^{90}$, $Ldh\text{-}1^{95}$). Chi-square contingency analysis revealed that four loci (*Me-1*, *Pgm-2*, *Gpi*, *Est-1*) exhibit statistically significant heterogeneity in the allele frequencies.

At three loci (*Me-1*, *Gpi*, *Est-1*), seven alleles showed significant departures from a random distribution. At the *Me-1*, locus two alleles occur, $Me\text{-}1^{100}$ and $Me\text{-}1^{104}$. The first one is present at high frequencies in most of the Sardinian populations, whereas the second one occurs at high frequencies in the Corsican populations. At the *Gpi* locus, the Gpi^{100} allele is the dominant one, whereas, in the southernmost Sardinian populations, the Gpi^{105} allele occurs at higher frequencies. At the locus *Est-1*, three electrophoretic alleles occur: $Est\text{-}1^{100}$, $Est\text{-}1^{102}$, *and* $Est\text{-}1^{105}$. The $Est\text{-}1^{100}$ allele occurs at high frequencies all over Corsica and Sardinia; $Est\text{-}1^{102}$ occurs at lower frequencies in the Sardinian samples; and $Est\text{-}1^{105}$ occurs in the Corsican samples. For the *Me-1*, *Gpi*, *Est-1* loci, none of the above-mentioned patterns were congruent with latitudinal clines (Spearman: $-0.334 < r < 0.447$; $P > 0.05$). Moreover, the allele frequencies at the polymorphic loci did not show statistically significant altitudinal patterns of variation (Spearman: $-0.510 < r < 0.510$; $P > 0.05$).

Deviation from H–W equilibrium was observed in seven samples (A, C, G, H, I, J, and M). However, the deviation from H–W equilibrium was significant only in two cases once Bonferroni's correction was applied to the analyses. In the sample from M. Limbara (H), a heterozygote deficiency was detected at the locus *Gpi* ($P < 0.005$); in the sample from M. Settefratelli (M), a strongly significant departure from H–W expectations (heterozygote excess) was found at the locus *Idh-1* ($P < 0.001$).

The genetic variability measures are given in Table 4. The overall mean number of alleles per locus (*A*) in *A. bedriagae* was 1.28, in the range 1.10–1.40. A similar value was estimated for allelic richness ($Ar = 1.20$). As expected, the proportion of polymorphic loci ($P_{99\%}$) was positively correlated with the observed heterozygosity (Spearman: $r = 0.655$; $P < 0.05$). The distribution of the observed heterozygosity (H_o) is presented in Figure 2. The mean proportion of polymorphic loci ($P_{99\%}$) was 0.025, in the range 0.010 (sample C) to 0.035 (samples B and M), and the observed heterozygosity (H_o) was in the range 0.033 (sample C) to 0.142 (sample L), averaging 0.091. The observed heterozygosity did not show altitudinal or latitudinal pattern of variation across the species range (Spearman: altitude, $r = 0.203$, $P > 0.05$; latitude, $r = -0.451$, $P > 0.05$). However, in Sardinia, genetic variability was significantly higher in the southern populations (i.e. samples L and M; permutation test: $Ar_{L\text{-}M}$ versus $Ar_{C\text{-}K}$, one-sided *P*-value = 0.001; $H_{oL\text{-}M}$ versus $H_{oC\text{-}K}$, one-sided *P*-value < 0.001) and lower in the northern populations (i.e. samples C–G; permutation test: $Ar_{C\text{-}G}$ versus $Ar_{H\text{-}M}$, one-sided *P*-value < 0.01; $H_{oC\text{-}G}$ versus $H_{oH\text{-}M}$,one-sided *P*-value < 0.005). Within Corsica, no significant differences were found for *Ar* and H_o between northern and southern populations. No 'small island effect' (*sensu* Gorman *et al.*, 1975) was found in the populations

Table 3. Allele frequencies at polymorphic loci in populations of *Archaeolacerta bedriagae* (A–M) and *Iberolacerta cyreni* (O)

		Population													
Locus	Allele	A	B	C	D	E	F	G	H	I	J	K	L	M	O
Ldh-1	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.958	1.000
	95	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.000
Mdh-1	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
	90	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
Mdh-2	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
	80	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
Me-1	100	0.409	0.400	0.833	0.667	1.000	0.625	0.750	0.800	0.450	0.500	0.375	0.750	0.750	0.000
	104	0.591	0.600	0.167	0.333	0.000	0.375	0.250	0.200	0.550	0.500	0.625	0.250	0.250	0.000
	105	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.400
	111	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.600
Me-2	100	1.000	1.000	1.000	0.875	1.000	0.875	1.000	0.944	1.000	1.000	1.000	1.000	1.000	0.000
	95	0.000	0.000	0.000	0.125	0.000	0.125	0.000	0.056	0.000	0.000	0.000	0.000	0.000	0.000
	105	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.400
	110	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.600
Idh-1	100	0.500	0.667	1.000	0.929	0.700	1.000	0.700	0.800	0.591	0.500	0.643	0.625	0.500	0.000
	106	0.500	0.333	0.000	0.071	0.300	0.000	0.300	0.200	0.409	0.500	0.357	0.375	0.500	0.200
	104	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.300
	108	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.300
	110	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.200
Idh-2	100	1.000	1.000	1.000	1.000	1.000	1.000	0.950	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	105	0.000	0.000	0.000	0.000	0.000	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6Pgd	100	1.000	0.938	1.000	1.000	1.000	1.000	1.000	1.000	0.850	1.000	1.000	0.875	0.933	0.000
	105	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.150	0.000	0.000	0.125	0.067	0.000
	95	0.000	0.063	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	93	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
Ak	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
	105	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
Pgm-1	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
	105	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.571
	107	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.429
Pgm-2	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.375	1.000	1.000
	90	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.625	0.000	0.000
Ck	100	0.875	0.900	0.667	0.500	1.000	0.750	0.667	0.700	0.875	0.667	0.864	0.833	0.600	0.000
	95	0.125	0.100	0.333	0.500	0.000	0.250	0.333	0.300	0.125	0.333	0.136	0.167	0.400	0.000
	105	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
Mpi	100	0.917	0.938	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
	102	0.083	0.063	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	88	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
Gpi	100	0.875	0.594	1.000	1.000	1.000	1.000	1.000	0.727	1.000	0.857	1.000	0.375	0.300	1.000
	105	0.125	0.406	0.000	0.000	0.000	0.000	0.000	0.273	0.000	0.143	0.000	0.625	0.700	0.000
Est-1	100	0.611	0.786	0.333	0.375	0.750	0.800	0.800	0.389	0.556	0.500	0.750	1.000	0.643	0.000
	102	0.167	0.000	0.667	0.625	0.250	0.200	0.200	0.556	0.389	0.500	0.250	0.000	0.357	0.000
	105	0.222	0.214	0.000	0.000	0.000	0.000	0.000	0.056	0.056	0.000	0.000	0.000	0.000	0.000
	115	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.583
	118	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.417

For geographical designation, see Fig. 1 and Table 1.

Table 4. Genetic variability parameters in populations of *Archaeolacerta bedriagae*

Genetic parameters	Population												
	A	B	C	D	E	F	G	H	I	J	K	L	M
A	1.40	1.40	1.10	1.30	1.10	1.20	1.30	1.40	1.30	1.30	1.20	1.30	1.40
	(0.10)	(0.10)	(0.10)	(0.10)	(0.10)	(0.10)	(0.10)	(0.10)	(0.10)	(0.10)	(0.10)	(0.10)	(0.10)
Ar	1.11	1.11	1.07	1.10	1.04	1.08	1.09	1.11	1.11	1.12	1.09	1.13	1.13
$P_{99\%}$	30	35	15	25	10	20	25	30	25	25	20	30	35
H_o	0.118	0.102	0.033	0.120	0.035	0.070	0.073	0.057	0.094	0.123	0.087	0.142	0.127
	(0.053)	(0.037)	(0.033)	(0.055)	(0.026)	(0.034)	(0.038)	(0.032)	(0.046)	(0.057)	(0.044)	(0.056)	(0.055)
H_e	0.112	0.113	0.066	0.098	0.045	0.078	0.090	0.112	0.106	0.123	0.089	0.135	0.127
	(0.045)	(0.041)	(0.037)	(0.044)	(0.031)	(0.038)	(0.04)	(0.043)	(0.045)	(0.051)	(0.042)	(0.049)	(0.046)

A, mean number of alleles per locus; *Ar*, mean number of alleles independent of sample size per locus; $P_{99\%}$, proportion of polymorphic loci; H_o, observed mean heterozygosity per locus; H_e, Nei's (1978) unbiased estimate of expected mean heterozygosity per locus. Standard errors are given in parenthesis.

from La Maddalena and Caprera islands (permutation test: $Ar_{C\text{-}D}$ versus $Ar_{E\text{-}M}$, one-sided P-value > 0.05; $H_{oC\text{-}D}$ versus $H_{oE\text{-}M}$, one-sided P-value > 0.05).

Three (i.e. *Gpi*, *Pgm-2*, and *Est-1*) of the 11 variable loci showed a significant value for F_{ST}. The *Gpi* locus shows significant differences in allele frequencies when comparing populations from southern Sardinia (samples L and M) with the other populations (samples A–K). At the *Pgm-2* locus, the population from M. Genis (southern Sardinia, sample L) is characterized by a private allele ($Pgm\text{-}2^{95}$). The *Est-1* locus is characterized by alleles occurring at different frequencies in the Corsican and Sardinian populations. The mean F_{ST} value is 0.172, in the range 0.036 (*Ldh-1* locus) to 0.381 (*Gpi* locus). The average F_{IS} value is 0.033. There was no evidence of genotypic disequilibrium between any pair of loci.

The values of genetic distance between populations of *A. bedriagae* are given in Table 5. Low levels of genetic differentiation were found in *A. bedriagae*, with Nei's D in the range 0.003 (sample I versus K) to 0.082 (sample D versus L), with a mean ± SD genetic distance of 0.027 ± 0.017. The mean ± SD value of Nei's D between Corsican and Sardinian populations was 0.026 ± 0.013. The estimate of the evolutionary divergence time among *A. bedriagae* populations would place the origin of their geographical isolation in the Late Pleistocene [95 000–265 000 years ago, respectively, with Nei's (1975) and Sarich's (1977) calibration]. This estimate is based on neutral loci, and thus excluding the *Idh-1* locus from the dataset, becaase this locus would likely violate neutrality assumptions (see below).

The genetic relationships among populations are shown in the phenogram in Figure 3. The NJ tree showed that genetic variation is distributed into three geographically congruent groups. The first subcluster includes the populations from southern Sardinia (samples L and M), the second one the populations from Corsica (samples A and B) and the third one the populations from central and northern Sardinia (samples C–K) with the northern one (samples C–G) organized in a subgroup. The NJ tree depicted in Figure 3 has equivalent topology of those obtained from a distance matrix produced, excluding from the analysis (1) the *Idh-1* locus (likely not neutral, see below) or (2) *Iberolacerta* data. However, the nodes of the NJ trees showed a lack of bootstrap support after 1000 replicates. The differences among F_{ST} values found in these three groups (ranging from 0.029 for the Central Sardinian group to 0.112 for the North Sardinian group) were not significant after 10 000 permutations.

A slight but significant correlation was found between genetic distance measures (D_N, D_{CSE}, F_{ST} pairwise values) and geographical distance

Figure 2. Observed heterozygosity in populations of *Archaeolacerta bedriagae*; a complete black pie would represent $H_o = 15\%$; a complete white pie would represent $H_o = 0\%$.

($0.254 < r < 0.381$; $P < 0.05$). To take into account ancient differentiation as a result of long-term historical divergence, and to test whether the spatial structure in genetic distances is fully additive across populations, we applied the Mantel test without the most differentiated population groups from southern Sardinia (M. Genis and M. Settefratelli, samples L and M) *sensu* Telles & Diniz-Filho (2005). In this case, we did not find any significant association ($0.088 < r < 0.198$; $P > 0.05$).

Table 5. Genetic distances among populations of *Archaeolacerta bedriagae* (A–M) and *Iberolacerta cyreni* (O)

	A	B	C	D	E	F	G	H	I	J	K	L	M	O
A	–	0.088	0.174	0.159	0.165	0.158	0.122	0.118	0.099	0.098	0.098	0.194	0.147	0.660
B	0.008	–	0.212	0.203	0.196	0.179	0.160	0.152	0.156	0.149	0.146	0.171	0.155	0.667
C	0.038	0.048	–	0.074	0.146	0.093	0.113	0.113	0.140	0.135	0.133	0.246	0.186	0.668
D	0.033	0.045	0.004	–	0.164	0.083	0.102	0.098	0.126	0.111	0.116	0.241	0.174	0.664
E	0.025	0.032	0.021	0.031	–	0.152	0.118	0.148	0.145	0.157	0.137	0.220	0.190	0.660
F	0.022	0.023	0.015	0.014	0.016	–	0.104	0.126	0.136	0.142	0.112	0.224	0.191	0.668
G	0.015	0.022	0.017	0.016	0.009	0.007	–	0.114	0.100	0.091	0.073	0.201	0.152	0.661
H	0.024	0.028	0.007	0.009	0.017	0.016	0.013	–	0.125	0.085	0.127	0.197	0.109	0.666
I	0.005	0.017	0.024	0.021	0.020	0.016	0.012	0.018	–	0.095	0.071	0.209	0.158	0.659
J	0.008	0.020	0.022	0.016	0.026	0.022	0.012	0.012	0.006	–	0.079	0.195	0.106	0.660
K	0.004	0.013	0.030	0.025	0.022	0.012	0.010	0.023	0.003	0.009	–	0.206	0.162	0.660
L	0.053	0.036	0.080	0.082	0.052	0.057	0.049	0.053	0.061	0.058	0.057	–	0.163	0.683
M	0.033	0.025	0.047	0.045	0.042	0.046	0.032	0.020	0.039	0.023	0.042	0.035	–	0.672
O	0.673	0.708	0.697	0.679	0.700	0.691	0.682	0.697	0.665	0.673	0.679	0.806	0.736	–

Below the diagonal: Nei's (1972) standard genetic distance; above the diagonal: Cavalli-Sforza & Edwards (1967) chord distance.

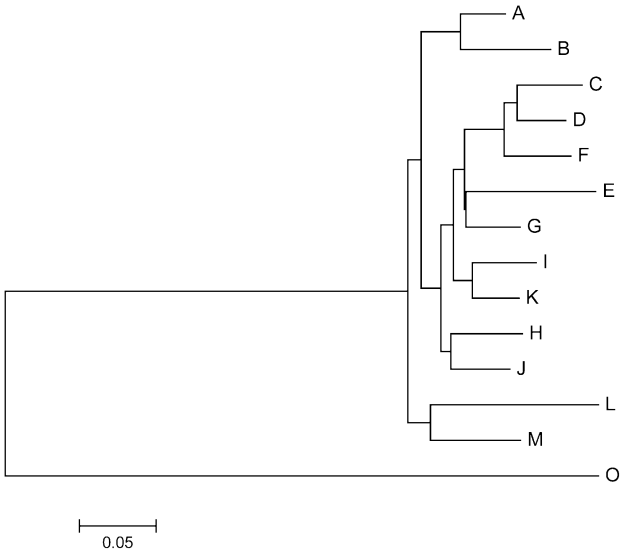

Figure 3. Neighbour-joining tree phenogram of 13 populations of *Archaeolacerta bedriagae*, based on Cavalli-Sforza & Edwards (1967) chord distances (for geographic origin of populations, see Fig. 1 and Table 1).

Environmental correlates of genic variation

Because AMT and AP values showed a strong negative autocorrelation (Spearman: $r = -0.900$; $P < 0.001$), we used a principal component analysis (PCA) to reduce them to a single 'bioclimatic' component, whose scores were used in correlation analyses. Variables were standardized to the same relative scales by calculating z-scores (Gotelli & Ellison, 2004). PCA extracted one component, which accounted for 96% of total variance, and was positively related to AMT (factor loading = 0.98) and negatively related to AP (factor loading = –0.98). A statistically highly significant correlation between bioclimatic scores and allele frequencies was found at the locus *Idh-1*. The frequencies of the $Idh\text{-}1^{106}$ allele were negatively related to 'bioclimatic' component and, conversely, the frequencies of the $Idh\text{-}1^{100}$ allele were positively related to 'bioclimatic' component (Spearman: $r = \pm 0.684$; $P < 0.01$). The distribution of single-locus heterozygosity in relation to bioclimatic factors was also tested. Also in this case, the observed heterozygosity at the *Idh-1* locus showed a distribution pattern clearly correlated to bioclimatic conditions, being negatively related to 'bioclimatic' component (Spearman: $r = -0.657$; $P < 0.05$). Moreover, the heterozygosity at this locus appears to reflect both altitudinal and latitudinal patterns of variation (Spearman: altitude, $r = 0.554$, $P < 0.05$; latitude, $r = -0.596$, $P < 0.05$).

The exclusion of this locus from the dataset does not affect the results of the genetic variability and the NJ tree topology. Indeed, the genetic variability estimates from the dataset without the *Idh-1* locus are not statistically different from those obtained with the original dataset (Mann-Whitney: A: $U = 0$, $P > 0.05$; $P_{99\%}$: $U = 70$, $P > 0.05$; H_o: $U = 0$, $P > 0.05$; H_e: $U = 0$, $P > 0.05$). Moreover the topology of the NJ tree produced with distance matrices computed from the dataset without the *Idh-1* locus reveals only one slight difference at a terminal branch with respect to the tree topology shown in Figure 2.

DISCUSSION

Archaeolacerta bedriagae shows a genetic structure characterized by relatively high level of polymorphism

and low differentiation among populations. These patterns are evident when analysing the genetic variability parameters. The observed heterozygosity found in *A. bedriagae* ($H_o = 0.092$) is higher than the average values (but included within the range) calculated for 17 species of lacertid lizards belonging to the same tribe of *A. bedriagae* (i.e. Lacertini Oppel, 1811) ($H_o = 0.059$; Gorman *et al.*, 1975; Cirer & Guillaume, 1986; Busack, 1987; Capula, 1994a, 1996, 1997, 2004; Fu *et al.*, 1995; MacCulloch *et al.*, 1995, 1997; Brehm *et al.*, 2001; Almeida *et al.*, 2002; Capula & Ceccarelli, 2003). The average observed heterozygosity of Corsican populations is similar to that of Sardinian populations. On the other hand, significant differences were found among Sardinian populations, with the southern populations being more variable than the northern ones. No 'small island effect' (*sensu* Gorman *et al.*, 1975) was found in the populations from Sardinian satellite islands (La Maddalena and Caprera, samples C and D, respectively). However, it should be noted that the populations from northern Sardinia showed the lowest levels of polymorphism and heterozygosity. This would indicate a severe reduction of genetic variability in the whole Gallura area (northern Sardinia), probably due to genetic drift phenomena.

Archaeolacerta bedriagae is characterized by low levels of genetic structuring. The estimated standardized variance in gene frequency for the total sample ($F_{ST} = 0.172$) indicates that gene diversity among populations accounts for less than 20% of the overall genetic variation observed in *A. bedriagae*. This value is lower than that estimated in another lacertid lizards endemic to Corsica and Sardinia ($F_{ST} = 0.460$ for *P. tiliguerta*; Capula (1996). F_{IS} values are low, presumably because H–W proportions are maintained within populations by random mating. Genetic distance estimates and cluster analysis show that *A. bedriagae* populations group into three geographically coherent assemblages: (1) southern Sardinia, (2) central and northern Sardinia, and (3) Corsica. The slight differentiation between the groups and the limited number of possible different resamples typically allowed by allozyme data (Van Dongen, 1995) may account for the lack of bootstrap support of these groups. However, this would suggest caution when drawing any conclusions based on these groups and their relationships.

Intraspecific genetic differentiation was relatively low compared with the sympatric lizard *P. tiliguerta* (Nei's *D* in the range 0.003–0.199, averaging 0.071; Capula, 1996) and similar to those found in other species inhabiting Mediterranean islands (Capula, 1994a, 2004). The low level of genetic differentiation found among populations indicates that there is no evidence of interruption or restriction of gene flow within *A. bedriagae*.

The distribution pattern of *A. bedriagae* is highly fragmented, especially on Sardinia (Delaugerre & Cheylan, 1992; Bombi & Vignoli, 2004) where four sub-ranges have been identified to date (Fig. 1). These sub-ranges are characterized by peculiar bioclimatic conditions (Bombi *et al.*, 2009b) and occurrence of suitable rocky habitats (Bombi *et al.*, 2009a). Genetic data would suggest a recent or very recent geographic fragmentation of populations. An approximate estimate of the evolutionary divergence time would place the origin of geographical isolation of populations in the Late Pleistocene. Palaeogeographic data are in accordance with this estimate, thus indicating that the observed genetic differentiation among population groups could be the result of range fragmentation produced by late Pleistocene geological events. Sardinia, Corsica, and other small satellite islands (e.g. La Maddalena) were linked to each other until the last phase of the Würmian glacial period (Lambeck *et al.*, 2004). Subsequently, they were physically separated by sea channels resulting from marine ingression. Late Pleistocene events were probably responsible for dramatic changes in bioclimatic regimes and vegetational conditions, and this most likely accounted for the current fragmented distribution pattern of *A. bedriagae* on Sardinia.

The allele frequencies do not show latitudinal or altitudinal clines, thus indicating that, in *A. bedriagae*, genetic diversity does not reflect any geographic pattern. By comparing genetic and geographic distance matrices, we observed a slight spatial structure in genetic distances. Nevertheless, before interpreting these results as an outcome of isolation-by distance processes (IBD), it is necessary to take into account the relative importance of several factors involved in the structuring of genetic distances. In particular, a correlation between genetic divergence and geographic distances could be produced by IBD process as a result of contemporary balance between local genetic drift and geographically mediated gene flow, but also by a long-term historical divergence among groups of populations (Telles & Diniz-Filho, 2005). The major differentiation found among *A. bedriagae* populations is structured in geographic space between the southernmost Sardinian populations (M. Genis and M. Settefratelli, samples L and M) and the others. The application of separate Mantel tests would suggest an ancient differentiation of the southernmost Sardinian populations as a result of long-term historical divergence, with a possible historical barrier, rather than a pattern produced by an IBD process.

The results obtained in the present study clearly show a correlation between allele frequencies at the *Idh-1* locus and some environmental variables. The frequency of the $Idh\text{-}1^{106}$ allele is negatively corre-

lated with annual mean temperature, and positively correlated with annual precipitation. The same pattern of correlation was found for the observed heterozygosity (H_o) at the *Idh-1* locus. These data clearly indicate that this locus is not neutral in *A. bedriagae*, as also suggested by the highly significant departure from H–W equilibrium at this locus. It must be noted that similar evidence was also reported in *P. tiliguerta*, which is another lacertid lizard endemic to Corsica and Sardinia. In this species, the frequency of the allele $Idh\text{-}1^{108}$ decreases towards the northern part of the species range in the direction of both increasing rainfall and decreasing temperature (Capula, 1996). Moreover, in *P. tiliguerta*, the observed heterozygosity (H_o) decreases in the direction of arid bioclimatic regimes (decreasing rainfall and increasing temperature). Climatic selection for allozyme loci was detected in other species (Hedrick, Ginevan & Ewing, 1976). Regarding lizards, Nevo (1978) found that allozymic variation at seven loci (including *Idh-1*) was significantly correlated with climatic variables (water availability and temperature). However, examining the association of genetic variation with discrete environmental heterogeneity, it is important to consider that, when populations are sampled over space, a clinal pattern in one or more loci is often recovered that correlates with some environmental parameters such as rainfall and temperature (Hedrick *et al.*, 1976; Hedrick, 1986). Although such an association may indicate adaptation to certain environment and would thus be the consequence of selection, the same pattern of variation could be moulded by stochastic processes producing an apparent clinal distribution of alleles or possibly be the by-product of species history and population structure. A single cline could rise from stochastic events, such as genetic drift. A clinal pattern common to several loci could result from secondary contact of two differentiated populations units (Nichols & Hewitt, 1994), so that the specific frequencies of both units would be maintained along the extreme edges of the distribution range, whereas, in the hybrid zone, an intermediary allele frequency would be found (Lobo, Del Lama & Mestriner, 1989). Although, in *P. tiliguerta*, Capula (1996) observed a clinal distribution of *Idh-1* alleles, we did not observe clinal variation at any locus in *A. bedriagae*. Thus, in this species, the association of *Idh-1* allele frequencies with bioclimatic parameters is not likely to be misled by stochastic or historical processes affecting the geographic pattern of variation of this locus.

The pattern of variation of the *Idh-1* alleles noted in *A. bedriagae* and *P. tiliguerta* is of course worthy of attention, and, besides other possible causes, would comprise evidence of natural selection processes in action, such as adaptation to local bioclimatic regimes. This hypothesis is supported by: (1) the strong correlation of allele frequency and heterozygosity at this locus with bioclimatic parameters (i.e. that is not hauled by a clinal pattern of variation); (2) the highly significant departure of genotypic frequency at the *Idh-1* locus from H–W equilibrium expectations; and (3) the correlated pattern of allozymic variation and bioclimatic correlates observed at the *Idh-1* locus in the lizard *P. tiliguerta* (co-occurring with *A. bedriagae* in Corsica and Sardinia). Selection could operate directly on the *Idh-1* locus or indirectly through selection for a linked locus (hitchhiking phenomenon). Discerning between these two processes would require the identification of functional differences among *Idh-1* allozymes and tests of individual fitness under different bioclimatic regimes (Feder & Watt, 1992). Although these studies could provide a compelling evidence of the occurrence of selection, such tests are difficult in that they require long-term studies (Watt, 1991) and it is not always achievable, as in this case, to set controlled environmental conditions. For this reason, although several studies have indicated the potential adaptive significance of allozyme variation through identification of selection of allozyme variants (Eanes, 1999), only a minority have attempted to demonstrate a cause–effect relationship between allozyme variants and natural selection by examining functional differences among allozymes and individual fitness in an appropriate ecological context (Mitton, 1997). Alternatively, the identification of correlated patterns of geographic allozyme variation in sympatric species could provide useful insights into their evolution. Correlated patterns of geographic change in closely-related species can result from the action of a common agent of natural selection. When parallel variation occurs at homologous enzyme loci, it has often been claimed as evidence for natural selection, even when a cause–effect relationship is not known (Clarke, 1975; Varvio-Aho & Pamilo, 1982). Several studies have adopted this approach to infer the action of selection on enzyme loci in sympatric species (Johnson, 1974; Harrison, 1977; Gill, 1981; Anderson & Oakeshott, 1984).

Although further investigations are needed to allow firm conclusions to be made, the evidence concerning the parallel variation at the *Idh-1* locus in *A. bedriagae* and *P. tiliguerta* suggests a key role of selective agents such as local bioclimatic regimes on allozyme polymorphism in these two Corso–Sardinian lacertids.

ACKNOWLEDGEMENTS

We are sincerely grateful to Valentina Bordoni, Manuela D'Amen, and Leonardo Vignoli for their assistance in the fieldwork; to Godfrey C. Akani for the improvement of the English; and to Miguel A.

Carretero, Mario Garcìa-Parìs, D. James Harris, Luca Luiselli and Paolo Mariottini and Marco Oliverio for helpful suggestions. We wish to thank Achille Casale (Sassari University) and the 'Ente Foreste della Sardegna' for logistic support in Sardinina and Marco A. Zuffi for carrying out sampling in Corsica. Lizards were captured under pemits from: the Italian Ministry of Environment DPN/2D/2003/2267, the Interreg II and III project – Corsica-Tuscany (province of Livorno and University of Pisa partnership), and the Consejeria de Medio Ambiente de la Comunidad de Madrid (BIOD-IBERIA project). This paper is part of the project 'Zoogeography of Mediterranean–southern African disjunct distributions by a multimethod approach' (grants from 'Ministero dell'Istruzione, dell'Università e della Ricerca', coordinator Marco A. Bologna, no. 2004057217).

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