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Genetic diversity and evolutionary history of the Tyrrhenian treefrog *Hyla sarda* (Anura: Hylidae): adding pieces to the puzzle of Corsica–Sardinia biota

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The Corsica–Sardinia archipelago is a hotspot of Mediterranean biodiversity. Although tempo and mode of arrival of species to this archipelago and phylogenetic relationships with continental species have been investigated in many taxa, very little is known about the current genetic structure and evolutionary history subsequent to arrival. In the present study, we investigated genetic variation within and among populations of the Tyrrhenian treefrog *Hyla sarda*, a species endemic to the Corsica–Sardinia microplate and the surrounding islands, by means of allozyme electrophoresis. Low genetic divergence (mean $D_{NEI} = 0.01$) and no appreciable differences in the levels and distribution of genetic variability (H_E: 0.06–0.09) were observed among all but one populations (Elba). Historical demographic and isolation-by-distance analyses indicated that this diffused genetic homogeneity could be the result of recent demographic expansion. Along with paleoenvironmental data, such expansion could have occurred during the last glacial phase, when wide and persistent land bridges connected the main islands and a widening of lowland areas occurred. This scenario is unprecedented among Corsica–Sardinia species. Together with the lack of concordance even among the few previously studied species, this suggests either that species had largely independent responses to paleoenvironmental changes, or that most of the history of assembly of the Corsica–Sardinia biota is yet to be written. *Biological Journal of the Linnean Society*, 2011, **103**, 159–167.

ADDITIONAL KEYWORDS: amphibians – allozymes – genetic differentiation – Mediterranean islands.

INTRODUCTION

Within the Mediterranean basin, the Corsica–Sardinia microplate is a well-known hotspot of biodiversity (Thompson, 2005; Blondel *et al*., 2010) and a major glacial refugium (Médail & Diadema, 2009). Furthermore, it is particularly rich in endemic species for many taxonomic groups (Thompson, 2005; Grill *et al*., 2007; Blondel *et al*., 2010).

Corsica–Sardinia endemics differ in their origin and in the way in which they populated the archipelago. Some were already present on islands at the time of the Corsica–Sardinia microplate's disjunction from the Iberian Peninsula (Lanza, 1983), or arrived during the mid-Miocene, when a land bridge connected the Sardo–Corsican block with the continent (Carranza & Amat, 2005). Other species originated during the Messinian crisis, when Mediterranean islands and the continent were largely connected for a long time (Carranza & Amat, 2005; Zangari, Cimmaruta & Nascetti, 2006; Carranza *et al*., 2008). Other endemics are instead the result of a relatively recent arrival, probably through land bridges caused by marine regression during the Plio-Pleistocene epoch (Lanza, 1983). Finally, for several of these species, more than one of the above historical scenarios could explain the available data equally well (Lanza, 1983; Carranza & Amat, 2005; Zangari *et al*., 2006; Carranza *et al*., 2008).

Although several studies, carried out with different kind of data (based on genetics and fossils), have been conducted on species of this archipelago with the aim

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of investigating tempo and mode of arrival and phylogenetic relationships with continental species, very little is known about population history subsequent to colonization, their current genetic structure, and how they coped with the Plio-Pleistocene paleoecological changes (but see Capula, 1996; Harris *et al*., 2005; Falchi *et al*., 2009; Salvi *et al*., 2009, 2010; Biollaz *et al*., 2010; Gentile *et al*., 2010; Ketmaier *et al*., 2010). This also applies to amphibian fauna. Indeed, there are twelve autochthonous amphibian species and, among these, eleven are endemic to the microplate and (in some cases) the surrounding islands (Lanza *et al*., 2008; see also Carranza *et al*., 2008). However, no studies have been carried out to date to infer the intraspecific evolutionary history following the microplate colonization.

In the present study, we analyse the genetic variation within and among populations of *Hyla sarda* (De Betta, 1853), sampled throughout the species range, by means of allozyme electrophoresis of 20 putative loci. *Hyla sarda* is a small, cryptically coloured treefrog, endemic to Corsica, Sardinia, and other neighbouring small islands, including Capraia and Elba, which belong to the Tuscan archipelago (Lanza *et al*., 2008). It breeds in ponds, pools, temporary waters, and other freshwater habitats (Lanza, 1983), and can be found at a wide range of altitudes but is particularly abundant in lowland areas (Vanni & Nistri, 2006; Lanza *et al*., 2008). Because of paucity of morphological and mating call differences, it has long been considered a subspecies of *Hyla arborea*. Only after allozyme data became available was it elevated to the species rank, based on a genetic distance (Nei, 1978) of 0.61 with nine diagnostic loci (Lanza, 1983; Nascetti *et al*., 1983, 1985).

The aims of the present study were to assess: (1) the current pattern of population genetic structure; (2) what it tells us about the recent evolutionary history of this species; and (3) to start delineating testable hypotheses about how the climate-driven paleoenvironmental changes which affected the Corsica–Sardinia microplate could have contributed to shaping intraspecific patterns of variation and the assembly of this microplate biota.

MATERIAL AND METHODS

We collected 111 individuals of *Hyla sarda* from ten localities distributed throughout the entire species range. The location of sampling sites and sample details are shown in Table 1 and Figure 1A. Each individual was anaesthetized in the field by submersion in a 0.02% solution of MS222 (3-aminobenzoic acid ethyl ester), allowing a tissue sample to be taken through a toe-clipping procedure, and was then

Table 1. Geographic location, sample size and estimates of genetic variability for the ten sampled populations of *Hyla sarda*

Islands	Samples		Latitude North	Longitude East	*N*	Variability			
						H_E	H_O	*A*	*P*
Elba	1	Elba	42°47′	10°15′	12	0.01 (0.01)	0.01 (0.01)	1.0 (0.1)	5
Capraia	2	Capraia	43°02′	9°50′	10	0.08 (0.03)	0.05 (0.02)	1.3 (0.1)	30
Corsica	3	Zente	42°43 ′	9°11′	6	0.08 (0.04)	0.10 (0.05)	1.3 (0.1)	20
	4	Avena	41°41′	9°20′	9	0.06 (0.03)	0.05 (0.03)	1.2 (0.1)	15
	5	L'Ospedale	41°39′	9°11′	10	0.06 (0.03)	0.06 (0.03)	1.3 (0.1)	25
Sardinia	6	Luogosanto	41°03′	9°12′	10	0.09 (0.04)	0.11 (0.05)	1.3 (0.1)	25
	7	L. Posada	40°38′	9°45′	10	0.06 (0.03)	0.07 (0.04)	1.3 (0.1)	20
	8	Oristano	39°54′	8°35′	33	0.08 (0.04)	0.08 (0.04)	1.5 (0.1)	40
	9	Giara di Gesturi	39°43′	9°02′	5	0.06 (0.04)	0.06 (0.04)	1.1 (0.1)	15
	10	Musei	39°18′	8°39′	6	0.08 (0.04)	0.07 (0.04)	1.2 (0.1)	20

SDs are given in parenthesis.
N, sample size; H_E, expected heterozygosity [Nei's (1978) unbiased estimate]; H_O, observed heterozygosity; *A*, mean number of alleles per locus; *P*, proportion of polymorphic loci (under the 99% criterion).

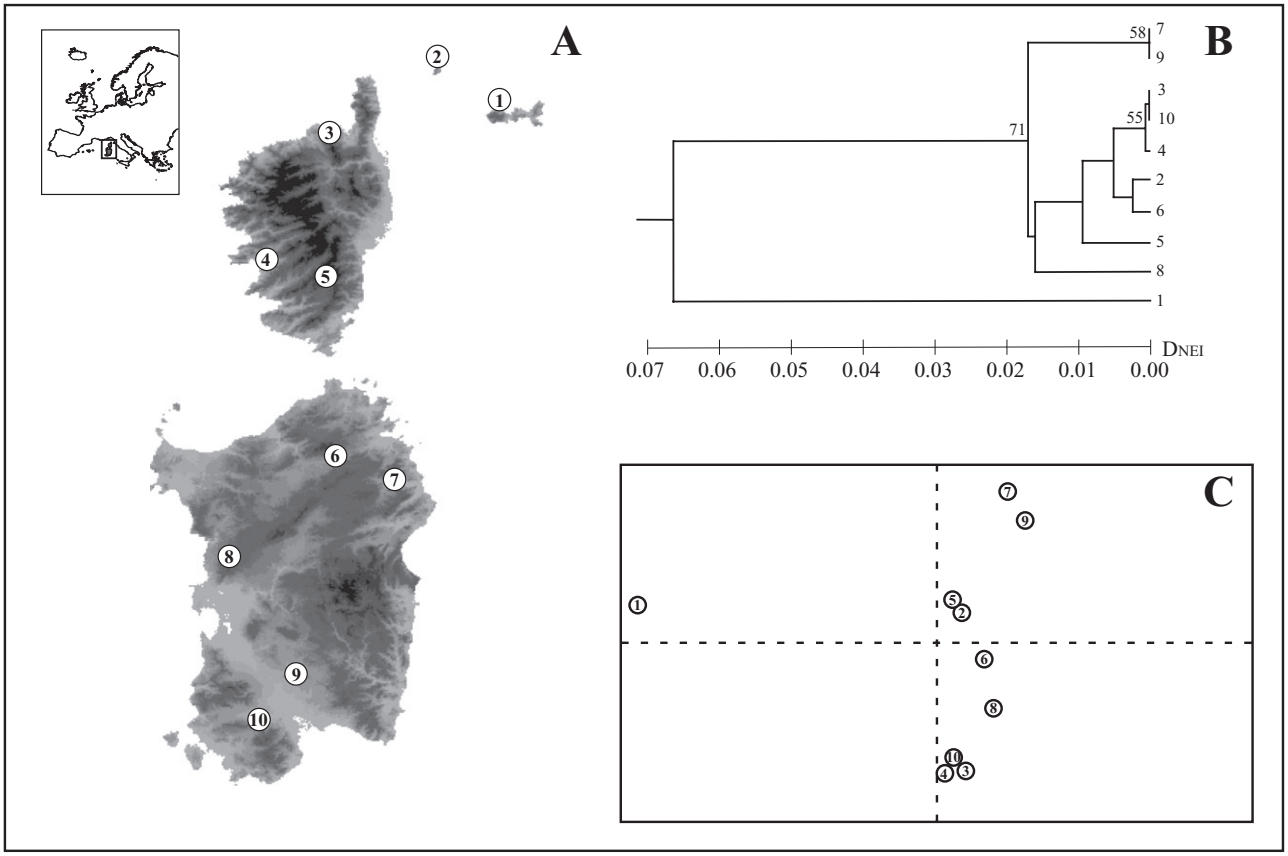

Figure 1. A, geographic distribution of *Hyla sarda* and geographic location of the ten populations sampled. Localities are numbered as in Table 1. B, unweighted pair group method with arithmetic mean phenogram showing genetic relationship among the ten populations sampled, based on Nei's (1978) unbiased genetic distance (D_{NEI}). Bootstrap values > 50% after 1000 pseudoreplicates are shown at the nodes. C, principal component analysis of allele frequencies among the studied samples of *H. sarda*. Only the first principal component (*x*-axis), explaining 61% of the total genetic variance, was significant ($P < 0.01$) over 10 000 randomizations. Samples are encoded as in Table 1.

released in the same sampling point. Collected tissues were carried to the laboratory in liquid nitrogen containers and stored at –80 °C.

Standard horizontal starch gel (10%) electrophoresis was performed to analyse the genetic variation of 20 putative allozyme loci, encoded by the following enzyme systems: glycerol-3-phosphate dehydrogenase (*G3pdh*; EC 1.1.1.8), lactate dehydrogenase (*Ldh-1* and *Ldh-2*; EC 1.1.1.27), malate dehydrogenase (*Mdh-1* and *Mdh-2*; EC 1.1.1.37), malate dehydrogenase NADP$^+$-dependent (*Mdhp-1* and *Mdhp-2*; EC 1.1.1.40), isocitrate dehydrogenase (*Icdh-1* and *Icdh-2*; EC 1.1.1.42), 6-phosphogluconate dehydrogenase (*6Pgdh*; 1.1.1.44), glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*; EC 1.2.1.12), superoxide dismutase (*Sod-1*; EC 1.15.1.1), aspartate transaminase (*Aat-1* and *Aat-2*; EC 2.6.1.1), adenosine kinase (*Adk*; EC 2.7.1.20), creatine kinase (*Ck*; EC 2.7.3.2), adenosine deaminase (*Ada*; EC 3.5.4.4), mannose phosphate isomerase (*Mpi*; EC 5.3.1.8), glucose phosphate isomerase (*Gpi*; EC 5.3.1.9), and phosphoglucomutase (*Pgm-2*; EC 5.4.2.2). Electrophoretic and staining procedures were performed following Canestrelli, Verardi & Nascetti (2007a). Alleles were designated by their mobility (in mm) relative to the most common one (=100) in a reference population.

Estimates of allele frequencies and genetic variability (as mean observed and expected heterozygosity, percentage of polymorphic loci and average number of alleles per locus) were obtained for each population using the software BIOSYS-2 (Swofford & Selander, 1999). The Hardy–Weinberg equilibrium was evaluated for each locus in each sample by exact tests as implemented in GENEPOP (Rousset, 2008). The same software was also used to compute Fisher's exact tests for deviation from the expected linkage equilibrium between each pair of loci in each sample.

Data were reduced and ordered using a principal component analysis (PCA) on allele frequencies of the polymorphic loci using the software PCAGEN 1.2

(Goudet, 1999). The statistical significance of each axis was evaluated over 10 000 randomizations.

Genetic distances between populations were estimated as Nei's (1978) unbiased genetic distance, and were then used to build a unweighted pair group method with arithmetic mean (UPGMA) phenogram by means of TFPGA (Miller, 1997). We ran 1000 bootstrap pseudoreplicates over loci to test the reliability of the UPGMA phenogram.

A hierarchical analysis of molecular variance (AMOVA; Excoffier, Smouse & Quattro, 1992) was carried out using ARLEQUIN, version 3.1 (Excoffier, Laval & Schneider, 2005; with significance assessed by 1023 permutations), with the aim of partitioning the total genetic variation into three hierarchical levels: among-groups, among-populations within-groups, and within-populations. We used results of previous PCA and UPGMA analyses to define a priori groups for the AMOVA analysis.

The occurrence of a significant correlation between genetic and geographic distances separating populations was assessed by means of a Mantel test. The geographic distance matrix was computed using GEOGRAPHIC DISTANCE MATRIX GENERATOR, version 1.2.3 (Ersts, 2010). To obtain estimates of the genetic differentiation between populations we first computed pairwise F_{ST} (Weir & Cockerham, 1984), using the FSTAT, version 2.9.3.2 (Goudet, 2001), and then converted these estimates into pairwise $F_{ST}/(1 - F_{ST})$, *sensu* Rousset (1997). The Mantel test was carried out using IBDW, version 3.16 (Jensen, Bohonak & Kelley, 2005), which was also used to assess the strength of the correlation between matrices through a reduced major axis regression.

Finally, the genetic signature of past population size changes (decline or expansion) was evaluated by means of Wilcoxon sign-ranks test (Cornuet & Luikart, 1996) as implemented in BOTTLENECK, version 1.2.02 (Piry, Luikart & Cornuet, 1999). We assumed an infinite-allele model of mutation, which has been shown to be the most appropriate for allozyme data (Chakraborty, Fuerst & Nei, 1980).

RESULTS

Nine out of the twenty loci analyzed (*Ldh-2*, *Mdh-1*, *Mdhp-2*, *Icdh-2*, *Gapdh*, *Sod-1*, *Adk*, *Ck*, *Gpi*) were found monomorphic for the same allele in all the samples studied. Two further loci (*Icdh-1*, *Aat-2*) were found to be polymorphic in a single sample (Luogosanto), with one allele not exceeding 5% in frequency. The allele frequencies at the remaining loci are shown in Table 2. No deviations from the expected Hardy–Weinberg and linkage equilibria were observed.

Estimates of population genetic variability are given in Table 1. For all the estimated indices, the population from Elba was the least variable. For example, the expected heterozygosity was 0.01 (±0.01) on Elba, whereas it ranged from 0.06 (±0.03) to 0.09 (±0.04) among the rest of samples.

Only the first principal component from the PCA analysis (Fig. 1C) was significant, and explained 61% of the total genetic variance. Along this principal component, two main groups of populations were clearly apparent; one including only the population from the Isle of Elba (sample 1), the other including all remaining populations (samples 2–10).

The UPGMA phenogram based on Nei's (1978) unbiased genetic distance is shown in Figure 1B. As for the PCA analysis, two main groups were apparent: one including the samples from Elba, the other clustering all the remaining samples. The mean ± SD genetic distance between these two groups was 0.07 ± 0.01, whereas, within the second group, it was 0.01 ± 0.01.

The hierarchical AMOVA (Table 3) was carried out by separating populations into the two groups indicated by both PCA and UPGMA analyses. This analysis revealed that the largest portion of the observed genetic variation (53%) is accounted for by the within-population level of variation. The remaining variation was almost entirely explained by the among-group level of variation (41%), whereas a minimal portion was explained by the among-population within-group level. All the variance components and the associated fixation indices were statistically significant ($P < 0.05$).

The possibility of a significant correlation between genetic and geographic distances between populations was investigated both including and excluding the sample from Elba. In both cases the correlation between matrices was very weak ($R^2 = 0.02$ and 0.01, respectively) and nonsignificant. However, the scatterplot of the genetic versus geographic distances (Fig. 2) appeared to be significantly different when including or excluding the sample from Elba. Indeed, in the latter case, a much lower degree of scattering was observed over the same range of geographic distances.

Inferences of population size changes are expected to become more reliable as more individuals and polymorphic loci are used (Cornuet & Luikart, 1996). Because previous analyses of population structure identified samples of 2–10 as a homogeneous group, BOTTLENECK analysis was carried out by grouping individuals from these samples into a single group. The Wilcoxon sign-ranks test indicated a statistically significant heterozygosity deficit (one-tailed test: $P < 0.05$).

Table 2. Allele frequencies of the allozyme loci found to be polymorphic among the ten sampled populations of *Hyla sarda*

	Population									
Locus/allele	1	2	3	4	5	6	7	8	9	10
G3pdh										
100	–	0.93	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
103	1.00	–	–	–	–	–	–	–	–	–
108	–	0.07	–	–	–	–	–	–	–	–
Ldh-1										
85	–	–	0.17	0.17	0.05	0.15	–	0.12	–	–
100	1.00	1.00	0.83	0.83	0.95	0.85	1.00	0.88	0.90	1.00
110	–	–	–	–	–	–	–	–	0.10	–
Mdh-2										
90	–	–	–	–	–	–	0.10	–	–	–
100	1.00	1.00	1.00	1.00	1.00	1.00	0.90	1.00	1.00	0.83
112	–	–	–	–	–	–	–	–	–	0.17
Mdhp-1										
96	0.04	0.05	0.17	0.21	0.20	0.15	–	0.15	–	0.33
100	0.96	0.95	0.83	0.79	0.80	0.85	1.00	0.85	1.00	0.67
6-Pgdh										
100	1.00	0.94	0.83	1.00	1.00	0.95	1.00	0.98	1.00	1.00
104	–	–	0.17	–	–	0.05	–	0.02	–	–
108	–	0.06	–	–	–	–	–	–	–	–
Aat-1										
100	1.00	0.80	1.00	1.00	0.90	1.00	1.00	0.97	1.00	1.00
105	–	0.20	–	–	0.10	–	–	0.03	–	–
Ada										
93	0.04	0.30	0.17	0.06	0.45	0.10	0.10	0.26	0.50	0.67
100	0.96	0.70	0.50	0.72	0.35	0.70	0.90	0.56	0.50	0.33
108	–	–	0.33	0.22	0.20	0.20	–	0.18	–	–
Mpi										
94	–	0.25	–	–	0.60	–	0.30	0.24	0.60	–
100	1.00	0.75	1.00	1.00	0.40	1.00	0.60	0.76	0.40	1.00
104	–	–	–	–	–	–	0.10	–	–	–
Pgm2										
95	–	–	–	–	–	–	0.10	–	–	0.17
100	1.00	1.00	1.00	1.00	1.00	1.00	0.90	1.00	1.00	0.83

Table 3. Results of the analysis of molecular variance for the ten sampled populations of *Hyla sarda*

Source of variation	Percentage of variation	Fixation indices
Among groups	41.23	$\Phi_{CT} = 0.41$
Among populations within groups	6.19	$\Phi_{SC} = 0.11$
Within populations	52.57	$\Phi_{ST} = 0.47$

Groups were defined as the two main clusters identified by the both principal component analyses and unweighted pair group method with arithmetic mean analyses (Fig. 1).

DISCUSSION

The population genetic structure and the evolutionary history of species with a Corsica–Sardinia distribution have been investigated in a limited number of studies (Capula, 1996; Harris *et al*., 2005; Falchi *et al*., 2009; Salvi *et al*., 2009, 2010; Biollaz *et al*., 2010; Gentile *et al*., 2010; Ketmaier *et al*., 2010), although none have involved amphibians. As also pointed out by Salvi *et al*. (2010), the degree of concordance between the geographic–genetic patterns observed in these studies is very limited, suggesting that we still have a scanty picture of the evolutionary history of the Corsica–Sardinia biota. The population

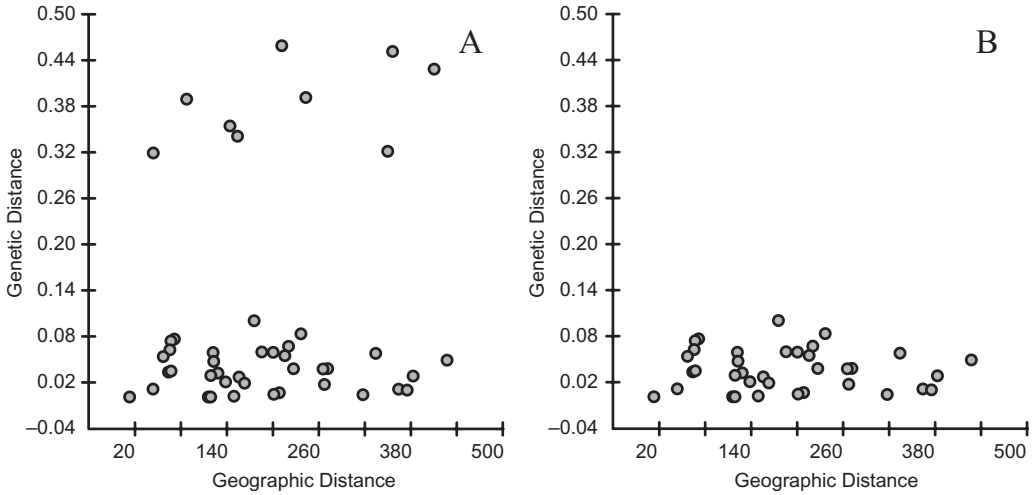

Figure 2. Scatterplots of the genetic $[F_{ST}/(1 - F_{ST})]$ versus log-geographic distances (km) among population pairs of *Hyla sarda*. A, all populations; B, the Isle of Elba excluded.

genetic structure observed in *H. sarda* adds a further piece to this puzzle.

The only discontinuity in the geographic distribution of genetic variation was observed between the Isle of Elba and the rest of the sampled populations. This discontinuity was shown by both the PCA and UPGMA analyses of population structure. The population from Elba was also the least variable at all the estimated indices. Taken together, the strong differentiation of this population, its low genetic diversity, and its geographic location at the northern edge of the species' range, suggest that it could have been founded through a recent founder event (Allendorf & Luikart, 2006), which probably occurred through rare overseas dispersals (Vences, Vieites & Glaw, 2003; Vences, Kosuch & Rödel, 2004). This scenario is also plausible in light of the paleogeographic knowledge of the island. Indeed, despite the close vicinity, it was not connected to the other islands by a Late Pleistocene land bridge. On the other hand, during this period, Elba was certainly connected to the Italian Peninsula (Tortora, Bellotti & Valeri, 2001). Thus, if we assume that colonization occurred not after but before the last insularization of the island (i.e. at the Holocene interglacial), a plausible expectation would be a species occurrence also somewhere on the Italian peninsula, given the large continuity of lowland environments (Porretta *et al*., 2011).

Interestingly, the same pattern of strong differentiation and low diversity was not observed for the population from Capraia island, which, similar to Elba, is located at the northern edge of the species' range and was connected neither to Corsica, nor to Elba islands (and thus not to the mainland; Dapporto, Palagi & Turillazzi, 2004) during the Late Pleistocene sea low-stand. This pattern does not fit strict founder-effect models of island colonization (Whittaker & Fernández-Palacios, 2007). However, recent findings in several species, including *Homo sapiens* (Clegg *et al*., 2002; Grant, 2002; Tabbada *et al*., 2010), suggest that single founder events could not affect levels of diversity, nor do they result in immediate genetic differentiation between populations. Instead, successive founder events (during island hopping) could be needed to yield such pattern.

Among populations from Corsica, Sardinia, and Capraia islands, neither evidence of a significant population structure, nor remarkable differences in levels of genetic variability were found. This pattern could at first appear to comprise evidence of long-term demographic stability and absence of significant structuring processes in the recent evolutionary history of *H. sarda* throughout much of its range. However, the shape of the scatterplot of geographic versus genetic distances, along with the BOTTLENECK analysis, suggest an alternative interpretation. When the population from Elba is removed from the analysis, the scatterplot of the geographic versus genetic distances (Fig. 2B) strictly matches case II reported in Hutchinson & Templeton (1999), whereby the observed pattern reflects the absence of a regional equilibrium between gene flow and genetic drift, with the former being more influential. This pattern is expected in cases of a recent range expansion from a relatively homogeneous source population, and when the time subsequent to expansion has not yet been sufficient for a new migration–drift equilibrium to be established (Hutchinson & Templeton, 1999). In the present case, this scenario would also be supported by the results of the BOTTLENECK analysis, which

indicated a significant heterozygosity deficit at a regional level, as expected if a recent population expansion has occurred (Cornuet & Luikart, 1996).

When the observed genetic pattern is seen in light of the Late Pleistocene paleogeographic history of the Corsica–Sardinia block, the scenario of a recent expansion appears especially plausible. At present, the two main areas of distribution of *H. sarda* (Sardinia and Corsica) are separated by a narrow seaway, the Bonifacio Strait, which was wider during the last interglacial, when the sea was, on average, 6 m above its current level. With the onset of the last glacial phase, the two areas became largely and persistently connected, until the sea-level rise at the beginning of the Holocene interglacial (Thiede, 1978). Thus, during the last glacial phase, there would have been many opportunities for significant dispersals between the two main islands. Accordingly, two main scenarios may be drawn: (1) during the last glacial phase, populations from both islands underwent a secondary contact somewhere around the Bonifacio strait and (2) during this phase, a population located in one of the two main islands expanded its range to the other parts of its current range. On the basis of the observed genetic pattern, we would tentatively favour the latter scenario over the former. Indeed, in the case of a secondary contact between two previously differentiated populations, we would expect a pattern of variation reflecting some degree of population structuring (e.g. in the form of clinal variation at some loci) and perhaps a significant correlation between geographic and genetic distances separating populations (Durrett *et al*., 2000). On the basis of the data obtained in the present study, although favouring the second scenario, we cannot indicate the likely distribution of *H. sarda* before the last glacial phase, and thus the routes followed during the inferred expansion. To fill in this gap, further investigation of the genetic variation will be carried out using sequence-based molecular markers (either mitochondrial DNA or nuclear DNA), aiming to assess the ancestral-derivative relationships between genetic variants found in different parts of the species range.

Although the scenario of an essentially thermophilic treefrog that underwent a demographic expansion during the glacial phase may initially appear to be counter-intuitive, at least two further lines of evidence lead us to favour it. First, lowland areas (i.e. those where the species currently reaches higher densities) actually underwent a large increase in size during the last glacial, and growing evidence suggests that lowland areas along the coasts of the Mediterranean basin could have provided suitable areas for the persistence of large populations of thermophilic species (Porretta *et al*., 2011). Second, demographic expansions linked to glacial oscillations of shorelines (and the consequent widening of lowland habitats) have recently been inferred for other amphibian species (Canestrelli, Cimmaruta & Nascetti, 2007b; Canestrelli & Nascetti, 2008), including the closely-related Italian treefrog *Hyla intermedia* (Canestrelli *et al*., 2007b).

The evolutionary history of the Tyrrhenian treefrog, as inferred in the present study, is unprecedented among species endemic to the Corsica–Sardinia microplate. Nevertheless, some aspects of this history are likely to be shared with other species, especially those linked to lowland freshwater environments. For example, the glacial widening of lowland habitats and opening of the Bonifacio Strait could have left detectable imprints in the genetic patterns of variation of other species. Further studies of these imprints are thus needed to test scenarios of common versus independent responses of species from this area to their shared paleoenvironmental vicissitudes (for an excellent discussion of this issue, see Sullivan, Arellano & Rogers, 2000).

It is worth noting that the study of genetic variation in more species from the Corsica–Sardinia microplate could also provide additional information. Over the last three decades or so, many phylogeographic and population genetic studies have been carried out for species from various parts of the Mediterranean basin, especially the Iberian and Italian peninsulas. Thus, more recently, sound and testable hypotheses have begun to be drawn about the general role of microevolutionary processes in shaping intraspecific patterns of variation and the assembly of biota (Hewitt, 2004; Hampe & Petit, 2005; Gómez & Lunt, 2007; Schmitt, 2007; Canestrelli *et al*., 2010). The Corsica–Sardinia microplate, with its complex paleogeographic evolution and its rich, unique and as yet understudied biota, appears to represent a unique opportunity to test many of these hypotheses in a geographically independent but (paleo-) climatically correlated context.

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