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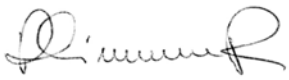
RUOLO DEI PROCESSI MICROEVOLUTIVI NELLA NASCITA DEGLI HOTSPOT INSULARI DI BIODIVERSITÀ: IL CASO DEL SISTEMA SARDO-CORSO

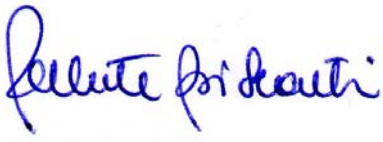
(BIO/07)

COORDINATORE
DOTT.SSA ROBERTA CIMMARUTA



TUTOR
DOTT. DANIELE CANESTRELLI

DOTTORANDA
DOTT.SSA ROBERTA BISCONTI



INDICE

Nothing in biology makes sense except in the light of Evolution

Theodosius Dobzhansky

Premessa

Questa tesi si basa sullo studio della storia evolutiva di due specie endemiche del complesso insulare Sardo-Corso. Nel capitolo 1 vengono presentate le tematiche e gli obiettivi generali che hanno ispirato lo studio. I risultati ottenuti sono stati elaborati, discussi ed inviati a riviste scientifiche peer-reviewed per la valutazione e l'eventuale pubblicazione. Ciascuno dei capitoli da 2 a 4 è in ogni sua parte conforme al manoscritto inviato ad una rivista, ad eccezione della letteratura citata che viene presentata in forma cumulativa nell'ultima sezione della tesi. Infine, nel capitolo 5 viene presentata una discussione generale dei risultati ottenuti ed una analisi della loro rilevanza ai fini degli obiettivi generali dello studio.

1. INTRODUZIONE

Le specie termofile che abitano il Paleartico occidentale sono state oggetto di una delle più intense indagini filogeografiche degli ultimi decenni (Feliner 2011, Hewitt 2011a). Ne è emerso come elemento comune tra tutti gli studi, il ruolo fondamentale che le oscillazioni climatiche Plio-Pleistoceniche hanno avuto nel plasmare gli attuali pattern filogeografici e la distribuzione della diversità genetica all'interno delle specie (Thompson 2005, Hewitt 2011b). Nell'ambito degli studi che hanno interessato specie temperate europee, sono state identificate vie preferenziali di ri-colonizzazione post-glaciale degli habitat settentrionali e numerose aree di rifugio glaciale, per lo più collocate nelle penisole a sud (Hewitt 1996, 1999, 2000, 2004, 2011a, 2011b, Taberlet *et al.* 1998, Schmitt 2007). Queste ultime aree, oggi particolarmente ricche di biodiversità, hanno svolto un ruolo fondamentale non solo nella persistenza a lungo termine delle specie durante i periodi glaciali, ma anche nella preservazione del loro potenziale evolutivo. Infatti, molti degli studi disponibili fin'ora individuano proprio in queste aree degli hotspots di diversità, anche a livello intraspecifico (per una rassegna vedi Hewitt 2011a, 2011b). Analogamente, l'indagine filogeografica sulle specie insulari è stata principalmente indirizzata all'identificazione delle aree di lunga persistenza, alle modalità con cui le specie hanno popolato le isole ed ai successivi percorsi di dispersione tra isole e con il continente (e.g. Thompson 2005, Stock *et al.* 2008, Papadopoulou *et al.* 2009, Lazàro *et al.* 2011).

Mentre i percorsi di dispersione post-glaciale e la collocazione geografica delle aree di rifugio sono stati così ampiamente indagati, ancora scarsa è la conoscenza su come i cambiamenti paleoclimatici e paleogeografici abbiano influenzato la struttura della diversità genetica delle specie all'interno delle aree di rifugio. Recentemente infatti, proprio in virtù della straordinaria rilevanza di queste aree come hotspot di biodiversità intraspecifica, molti autori hanno sottolineato la particolare importanza di comprendere come le popolazioni qui residenti abbiano sopravvissuto alle oscillazioni climatiche, quali processi microevolutivi siano stati indotti da tali oscillazioni e come questi processi abbiano contribuito a formare l'attuale struttura genetica e la diversità delle specie all'interno di esse (Hampe & Petit 2005, Gomez & Lunt 2006, Canestrelli *et al.* 2010, Feliner 2011). Capire i processi microevolutivi che hanno permesso alle popolazioni che abitano le aree rifugio di mantenere così elevati livelli di biodiversità, è dunque uno dei

quesiti centrali della attuale ricerca filogeografica, la cui risoluzione avrebbe implicazioni significative non solo in ambiti ecologici ed evolutivi ma anche conservazionistici (Hampe & Petit 2005), per poter gestire e preservare efficacemente questi serbatoi di diversità.

Il bacino del Mediterraneo è uno dei maggiori hotspot di biodiversità a livello mondiale (Myers *et al.* 2000). Su di esso si affacciano le principali penisole europee, sedi di numerose aree rifugio, ed inoltre contiene oltre 5000 isole, molte delle quali ricche di diversità e di antiche linee evolutive endemiche (Thompson 2005, Vogiatzakis *et al*. 2008). Tuttavia, in linea con quanto si osserva a scala globale, mentre la struttura filogeografica di specie continentali è stata largamente investigata, quella di specie insulari è rimasta fino a tempi recenti sostanzialmente inesplorata. Infatti, basandoci su una ricerca all' interno di un noto database scientifico, ISI Web of Knowledge (finestra temporale: 1985–February 2012; ricerca aggiornata al 2 febbraio 2012), usando il termine 'Phylogeography' abbiamo ottenuto 6719 articoli, mentre combinando 'Phylogeography AND Island' se ne ottengono 1140 (17%). Tuttavia, meno del 5% di tutti gli studi di filogeografia presenti analizzano pattern filogeografici intrainsulari, e meno dello 0,5% riguarda specie insulari del bacino del Mediterraneo. Questa scarsezza di studi apparirà ancora più significativa se si considera che il bacino del Mediterraneo – proprio in virtù delle sue oltre 5000 isole - è uno dei maggiori sistemi insulari al mondo (Vogiatzakis *et al*. 2008). Per di più, i recenti cambiamenti climatici e le alterazioni ambientali causate dall'uomo costituiscono una minaccia particolarmente marcata, proprio per quelle aree – peninsulari ed insulari – maggiormente ricche di diversità (Hampe & Petit 2005, Araujo *et al.* 2006, Kier *et al.* 2009). Ad esempio, Araujo e colleghi (2006) suggeriscono che per effetto dei cambiamenti climatici in corso, molte specie di anfibi presenti nel Paleartico occidentale subiranno un incremento particolarmente marcato della probabilità di estinzione, proprio in quelle aree del bacino del Mediterraneo che sono hotspot di diversità genetica per molte specie.

Per lo studio dei processi microevolutivi che hanno determinato la nascita di hotspot di diversità, le isole appaiono particolarmente attraenti. Innanzitutto perchè le isole hanno una lunga e fruttuosa storia come laboratori naturali per lo studio dei processi ecologici e sono state identificate come un eccellente teatro per lo studio dell'evoluzione (e.g. Darwin 1859, Wallace 1880, MacArthur & Wilson 1967, Grant 1998, Grant & Grant 2007, Whittaker & Fernandez-Palacios 2007). Molti processi microevolutivi risultano particolarmente complessi se studiati in ambito continentale, mentre in entità geografiche isolate, quantificabili nella dimensione e nella ricchezza di specie, come le isole, la

possibilità di formulare assunti semplificatori rende più agevole la verifica di ipotesi circa l'origine e lo sviluppo di questi processi (Whittaker & Fernandez-Palacios 2007).

Se le isole in generale possono considerarsi laboratori naturali per lo studio di processi microevolutivi, nel contesto del bacino del Mediterraneo il complesso sardo-corso è sicuramente un buon candidato "top-level-laboratory", a causa di alcune sue intrinseche proprietà. È certamente un hotspot di biodiversità mediterranea, caratterizzato da flora e fauna altamente endemiche (Thompson 2005), ed inoltre lo caratterizza una storia paleogeografica complessa (Alvarez 1972, Alvarez *et al.* 1974, Bellon *et al.* 1977, Bonin *et al.* 1979, Caccone *et al.* 1994, Orsini *et al.* 1980). In particolare, la paleogeografia di queste isole è stata fortemente influenzata dalle oscillazioni del livello marino. Per tutto il Quaternario infatti si sono verificate trasgressioni e regressioni del livello del mare, che hanno portato ad abbassamenti del livelli del mare anche fino a 120 m circa sotto l'attuale, ad esempio durante l'ultima epoca glaciale (Vogiatzakis *et al*. 2008). Queste variazioni hanno ripetutamente influenzato i collegamenti di terra tra la Corsica, la Sardegna e la penisola italiana. Infatti, le due isole venivano unite da un ponte di terra ampio e di persistente durata risultando di fatto un'unica massa di terre emerse (Van Andel & Shackleton 1982). In seguito alle regressioni marine pleistoceniche non solo le isole si univano tra loro, ma anche una notevole porzione di larghe piane costiere emergeva, aumentando quindi la superficie totale delle due isole (Van Andel & Shackleton 1982). Questo accadeva in maggior misura per le coste della Sardegna a causa della conformazione geomorfologica propria dell'area. Nonostante la loro affinità geologica e la storia paleoclimatica condivisa, la Sardegna e la Corsica differiscono notevolmente nella diversità di ambienti che le caratterizzano. La Sardegna è la seconda isola più grande del Mediterraneo e presenta una ampia varietà di ambienti, che comprendono aree salmastre, dune sabbiose e foreste su rilievi montuosi. Caratterizzata per la maggior parte da altipiani e da coste rocciose presenta un clima mite. La Corsica è l'isola più a nord, la più ricca di ambienti umidi e la più montuosa del bacino del Mediterraneo, avendo al suo interno diverse cime che superano i 2000 metri sul livello del mare separate da valli parallele e scoscese. Il clima è più umido e rigido rispetto alla Sardegna. In accordo con alcuni recenti studi sembra inoltre che durante l'ultima epoca glaciale, mentre la Sardegna sarebbe stata mitigata nel clima dalla sua posizione più meridionale e dall'influenza di correnti marine più miti, la Corsica avrebbe invece presentato un clima più rigido con abbondanti precipitazioni, con alcune formazioni glaciali interne e venti polari nelle zone più a nord

(Kulemhann *et al.* 2008). Durante il Pleistocene, quindi, la Sardegna e la Corsica sarebbero apparse come un'unica massa di terra con aree fredde e montuose al nord ed aree più miti e pianeggianti a sud, risultando quindi un sistema piuttosto simile geograficamente e climaticamente alle penisole europee. Nonostante quest'area sia particolarmente interessante e complessa sotto molti aspetti che riguardano le attuali caratteristiche e le passate condizioni geografiche e climatiche, pochissimi sono gli studi che hanno indagato le tracce che gli eventi sopra descritti hanno lasciato nel genoma delle specie che la abitano (Harris *et al*. 2005, Falchi *et al*. 2009, Biollaz *et al.* 2010, Gentile *et al*. 2010, Salvi *et al*. 2010, Ketmaier *et al*. 2010).

Per iniziare ad indagare queste tracce, in questa tesi è stata analizzata la struttura della variazione geografica e la storia evolutiva di due specie di anfibi: la raganella sarda (*Hyla sarda*) e l'euprotto corso (*Euproctus montanus*).

La raganella sarda, endemica di questo complesso di isole, appare un ottimo modello per capire quali processi microevolutivi possano essere stati innescati in questo complesso dai cambiamenti climatici Pleistocenici. È una specie ampiamente distribuita in entrambe le isole, con popolazioni soprattutto al di sotto dei 1000 metri sul livello del mare (Vanni & Nistri 2006, Lanza *et al*. 2007). Vive preferenzialmente in prossimità di zone umide con acque stagnanti, come pozze e piscine d'acqua dolce, dove si ritrova per riprodursi durante la stagione primaverile e estiva. Anche se non esistono dati precisi a riguardo, in analogia con quanto rilevato per la specie affine *Hyla arborea*, si ritiene che la raganella sarda abbia buone capacità dispersive. Per *H.arborea* infatti, si è stimata una capacità di dispersione di oltre 10 km in un anno (Marsh & Trenham 2000). *H.sarda* è stata ritenuta a lungo una sottospecie di *H. arborea* e alla luce di dati fossili, biogeografici e filogenetici si ritiene che la separazione tra le due specie sarebbe avvenuta durante la crisi di salinità del Messiniano (5.33 milioni di anni fa circa) (Kotsakis 1980, Stock *et al*. 2008). Nonostante vi siano studi che hanno indagato le relazioni filogenetiche e le ipotesi sui momenti di popolamento delle isole da parte di questa specie (Lanza 1983, Nascetti *et al.* 1985, Stock *et al.* 2008), ed altri che hanno indagato aspetti morfologici e bioacustici (Schneider 1974, Rosso *et al*. 2001, Castellano *et al*. 2002), mancano del tutto studi sulla sua struttura genetica e la storia evolutiva pleistocenica.

L'euprotto corso, endemico della sola Corsica, risulta anch'esso un buon modello per questo genere di studi. È una specie distribuita in gran parte dei complessi montani dell'isola sino al di sopra dei 2200 metri sul livello del mare, anche se lo si ritrova più

abbondante tra i 600 e i 1500 metri. Vive prevalentemente in torrenti e più raramente in laghi montani; inoltre nella fase terrestre del ciclo annuale di attività, può ritrovarsi in aree limitrofe a queste zone umide, purché presentino rocce o rifugi lungo le rive dei corsi d'acqua e vegetazione boscosa o a macchia. La distribuzione nell'isola dell'euprotto corso sembra essere limitata prevalentemente dalla selettività della specie per gli habitat acquatici, generalmente corsi d'acqua ben ossigenati e non inquinati, e per la temperatura ottimale, tende infatti a distribuirsi al di sopra dei 600 metri. Le capacità dispersive di questo tritone sono limitate, soprattutto a causa dall'elevata dipendenza dai corsi d'acqua dai quali non si allontana per più di pochi metri (Lanza *et al*. 2007 e riferimenti all'interno). Nella lista rossa IUCN delle specie minacciate l'*E. montanus* è attualmente considerata una specie a basso rischio d'estinzione perché, sebbene il suo areale di distribuzione risulti inferiore a 20.000 km², essa sarebbe distribuita con continuità sull'isola, con una sola grande popolazione, ed inoltre non mostrerebbe evidenze di declino tanto significative da giustificare l'assegnazione ad una categoria di rischio maggiore (IUCN 2011).

Obiettivo generale di questa tesi è stato quello di indagare se e come le oscillazioni climatiche Plio-Pleistoceniche abbiano influenzato la storia evolutiva di specie endemiche insulari, e di individuare quali processi microevolutivi siano stati implicati nel determinare gli attuali pattern di diversità e di struttura genetica. In particolare, si è voluto indagare se fosse verificabile la doppia predizione di prolungata stabilità per queste specie, predizione (come poc'anzi anticipato) derivabile sia dalla letteratura sulle popolazioni insulari, sia dalla letteratura sulle aree di rifugio. A tal fine sono stati indagati i pattern di struttura genetica delle popolazioni, la filogeografia e la storia demografica di due specie endemiche del complesso sardo-corso che come poc'anzi ricordato, sebbene entrambe anfibi, sono tuttavia ben diverse dal punto di vista ecologico: la raganella sarda, specie termofila, euriecia e con discrete capacità dispersive, e l'euprotto corso, specie montana, stenoecia e con capacità dispersive limitate. Infine i risultati sono stati comparati con quelli ottenuti per altre specie endemiche insulari, per popolazioni di aree hotspot, e con la scarsa ma crescente letteratura filogeografica sulla variazione intra-isola. Tutto ciò al fine di valutare quale contributo queste specie possano offrire alla risoluzione del quesito sulla nascita ed il mantenimento degli hotspot di biodiversità.

2. GENETIC DIVERSITY AND EVOLUTIONARY HISTORY OF THE TYRRENHIAN TREEFROG *HYLA SARDA* (ANURA: HYLIDAE): PIECING THE PUZZLE OF CORSICA-SARDINIAN BIOTA

Roberta Bisconti, Daniele Canestrelli & Giuseppe Nascetti

ABSTRACT

The Corsica-Sardinian archipelago is a hotspot of Mediterranean biodiversity. While 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 current population genetic structure and evolutionary history following arrival. We investigated the genetic variation within and among populations of the Tyrrhenian treefrog *Hyla sarda*, a species endemic to Corsica-Sardinian 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 due to a 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-Sardinian species. Together with the lack of concordance even among the few previously studied species, this suggests that either species had largely independent responses to paleoenvironmental changes, or the history of assembly of the Corsica-Sardinian biota has most chapters still to be read.

KEYWORDS: Amphibians, allozymes, *Hyla sarda,* Sardinia, Corsica, Mediterranean islands, genetic differentiation.

2.1 INTRODUCTION

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

Corsica-Sardinian endemic differ in their origin and in the ways in which they populated the archipelago. Some of them were already present on islands at the time of the Corsica-Sardinian microplate disjunction from the Iberian Penisula, during the Miocene. Other species colonized the region during the Messinian crisis, when Mediterranean islands and the continent were largely connected for long time. Others are instead of relatively recent arrival, probably through land bridges caused by marine regression during the Plio-Pleistocene epoch. Finally, for some of these species, more than one of the above scenarios could explain equally well the available data (e.g. Lanza 1983, Carranza *et al*. 2005, Zangari *et al*. 2006, Carranza *et al*. 2008, Van der Meijdenn *et al*. 2009)

While 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 of investigating tempo and mode of arrival and phylogenetic relationships with continental species, very little is known about population history of species following colonization, their current population genetic structure and how they coped with the Plio-Pleistocene paleoecological changes (but see e.g. Capula 1996, Harris *et al*. 2005, Biollaz *et al.* 2010, Falchi *et al*. 2009, Salvi *et al*. 2009, 2010, Gentile *et al*. 2010, Ketmaier *et al*. 2010). This also apply to what concerns the amphibian fauna. Indeed, twelve are the autochtonous amphibian species and, among them, eleven are endemic to the microplate and – in some case - the surrounding islands. Furthermore, no studies have been carried out to date to infer the intraspecific evolutionary history following the microplate colonization.

In this paper we analysed 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. *H. 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*. 2007). It breeds in ponds, pools,

temporary waters and other freshwater habitats (Lanza 1983), and can be found at a wide range of altitudes but it is particularly abundant in lowland areas (Lanza *et al*. 2007, Vanni & Nistri 2006). Owing to the paucity of morphological differences, it has long been considered as a subspecies of *Hyla arborea*. Only after allozyme data became available, it was elevated to the species rank (Nascetti *et al*. 1985).

Our aims were to assess: 1) the current pattern of population genetic structure, 2) what it tells us about the recent evolutionary history of this species, 3) to start delineating testable hypotheses about how the climate-driven paleoenvironmental changes which affected the Corsican-Sardinian microplate could have contributed to shape intraspecific patterns of variation and the assembly of this microplate biota.

2.2 MATERIALS AND METHODS

We collected 111 individuals of *Hyla sarda* from 10 localities distributed throughout the entire species range. The location of sampling sites and samples 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), in order to take a tissue sample through a toe-clipping procedure, and was then released in the same sampling point. Collected tissues were carried to the laboratory in liquid nitrogen containers and then stored at – 80 °C.

Standard horizontal starch gel (10%) electrophoresis was performed in order 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), Phosphoglucomutase (*Pgm-2;* EC 5.4.2.2). Electrophoretic and staining procedures followed Canestrelli *et al*. (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 ON THE WEB (Rousset 2008). The same software was also used to compute Fisher 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, by means of 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 an 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 *et al*. 1992) was carried out using ARLEQUIN 3.1 (Excoffier *et al*. 2005, significance assessed by 1023 permutation), with the aim of partition the total genetic variation into three hierarchical levels: among groups, among populations within groups, and within population. 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 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 software FSTAT 2.9.3.2 (Goudet 2001), and then converted these estimates into pairwise $F_{ST}/(1-F_{ST})$, following Rousset (1997). The Mantel test was carried out using IBDW 3.16 (Jensen 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) were evaluated by means of the Wilcoxon sign-ranks test (Cornuet & Luikart 1996) as implemented in the software BOTTLENECK 1.2.02 (Piry *et al.* 1999). We assumed an infinite-allele model of mutation, which has been shown to be the most appropriate for allozyme data (Chakraborty *et al.* 1980).

2.3 RESULTS

Nine out the twenty loci analysed (*Ldh-2, Mdh-2, Mdhp-2, Icdh-2, Gapdh, Sod-1, Adk, Ck, Gpi*) were found monomorphic for the same allele in all the samples studied. Further two loci (*Icdh-1, Aat-2*) were found polymorphic in a single sample (Siniscola), with one allele not exceeding 5% in frequency. The allele frequency at the remaining loci are shown in Table 2. No deviations from the expected HW 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 instance, the expected heterozygosity was 0.01 (±0.01) at 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 Elba island (sample 1), the other including all the remaining populations (samples 2-10).

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

The hierarchical analysis of the molecular variance AMOVA (Table 3) was carried out 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-populations level of variation. The remaining variation was instead almost entirely explained by the among-groups level of variation (41%), whereas a minimal portion was explained by the among- populations within groups level. However all the variance components and the associated fixation indices were statistically significant (all $P<0.05$).

The possible occurrence of a significant correlation between genetic and geographic distances between populations was investigated both including and excluding the sample from the Elba island. In both cases the correlation between matrices was very weak

(R^2=0.02 and 0.01 respectively) and non-significant. However the scatterplot of the genetic VS. geographic distances (Fig. 2) looked 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 be as much reliable as more individuals and polymorphic loci are used (Cornuet & Luikart 1996). Since previous analyses of population structure identified samples from 2 to 10 as a homogeneous group, in order to maximize power of the analysis, we carried out it by grouping individuals from these samples into a single group. The Wilcoxon sign-ranks test indicated a statistically significant heterozygosity deficit (one tail test: $P<0.05$).

2.4 DISCUSSIONS

The population genetic structure and the evolutionary history of species with a Corsica-Sardinian distribution have been investigated in a limited number of studies (e.g. 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), none involving 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-Sardinian biota. The population genetic structure observed in *Hyla sarda* adds a further piece to this building puzzle.

The only discontinuity in the geographic distribution of genetic variation was observed between the Elba island and the rest of the sampled populations. This discontinuity was supported 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 2005), likely occurred through rare overseas dispersals (Vences *et al*. 2003, 2004). This scenario is also plausible in light of the paleogeographic knowledges for Elba island. Indeed, in spite of the close vicinity, it was not connected to the other islands by a Late Pleistocene land-bridge. On the other hand, during this period it was certainly connected to the Italian peninsula (e.g. Tortora *et al*. 2001). Thus, in case colonization wouldn't have occurred after the last insularization of the Elba island (i.e. at the Holocene interglacial) but before, a plausible expectation would have been a species occurrence even somewhere on the Italian peninsula, given the large continuity of lowland environments (see Porretta *et al*. 2011).

Interestingly, the same pattern of strong differentiation and low diversity was not observed for the population from Capraia island, which – as Elba island – is located at the northern edge of the species' range and was not connected neither to Corsica nor to Elba islands (and thus to mainland) during the Late Pleistocene sea low-stand. This pattern do not fit strict founder-effect models of island colonization (Whittaker & Fernández-Palacios 2007). However, recent findings on several species including *Homo sapiens* (Cleggs *et al*. 2002, Grant 2002, Tabbada *et al*. 2010 and references therein) 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 could be needed - during island hopping - to yield such pattern.

Among populations from Corsica, Sardinia and Capraia islands neither evidences of a significant population structure, nor remarkable differences in the levels of genetic variability were found. At a first glance this pattern could be taken as 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 vs. genetic distances and the BOTTLENECK analyses suggest an alternative interpretation. When the population from Elba is removed from the analysis, the scatterplot of the geographic vs. genetic distances (see Fig. 2B) strictly match the case II of Hutchinson & Templeton (1999), whereby the observed pattern reflect the absence of a regional equilibrium between gene flow and genetic drift, with the former being relatively more influential. This pattern is expected in cases of a recent range expansion from a relatively homogeneous source population, and when the time since the expansion has not been already sufficient for a new migration-drift equilibrium to be achieved (Hutchinson & Templeton 1999). In the present case, this scenario would be also supported by the results of the BOTTLENECK analysis, which indicated a significant heterozygosity deficit at a regional level, as expected in case of a recent population expansion (Cornuet & Luikart 2006).

When the observed genetic pattern is seen in light of the Late Pleistocene paleogeographic history of the Corsica-Sardinian 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 even more extended during the last interglacial, when the sea was on everage 6 meters 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 large opportunities for wide migrations between the two main islands. Accordingly, two main scenarios could be drawn: 1) during the last glacial phase, populations from both islands underwent a secondary contact somewhere in the nearby of the Bonifacio strait; 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. Based on the observed genetic pattern we could tentatively

favour the latter scenario over the former. Indeed, in case of a secondary contact between two previously differentiated populations, we would expect a pattern of variation reflecting some degree of population structuring, even in the form of clinal variation at some loci, and eventually a significant correlation between geographic and genetic distances separating populations (e.g. Durret *et al*. 2000). Based on our data, 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 investigations of the genetic variation will be carried out using sequence-based molecular markers (either mtDNA or nuDNA), in order to assess the ancestral-derivative relationships between genetic variants found in different parts of the species range.

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

The evolutionary history of the Tyrrhenian treefrog, as we have inferred here, is unprecedented among species endemic to the Corsica-Sardinian microplate (see citations above). Nevertheless, some issues of this history are likely to be shared with other species, especially those linked to lowland freshwater environments. For instance, the glacial widening of lowland habitats and opening of the Bonifacio strait, could have left detectable imprints even in the genetic patterns of variation of other species. Further studies of these imprints are thus needed to test scenarios of common vs. independent response of species from this area to the shared paleoenvironmental vicissitudes (see Sullivan *et al*. 2000 for an excellent discussion to this issue).

Interestingly, the study of the genetic variation in more species from the Corsica-Sardinian microplate, could also tell us something more. In the last three decades and more, many phylogeographic and population genetic studies have been accumulated for

species from several parts of the Mediterranean basin, especially the Iberian and Italian peninsulas. Thus, more recently, sound and testable hypotheses have began to be drawn about the general role of several microevolutionary processes in shaping intraspecific patterns of variation and the assembly of biota (Hewitt 2004, Hampe & Petit 2005, Gomez & Lunt 2006, Schmitt 2007, Canestrelli *et al*. 2010). The Corsica-Sardinia microplate, with its complex paleogeographic evolution and its rich, unique and so understudied biota, appears as a unique opportunity to test many of these hypotheses in a geographically independent but (paleo-)climatically correlated context.

2.5 TABLES AND FIGURES

Table 1 Geographic location, sample size and estimates of genetic variability for the 10 sampled populations of *Hyla sarda*. Standard deviations are shown in brachets.

	Samples	n	Latitude N	Longitude E	Variability			
					H_e	H_o	A	P
1	Elba	12	42°47'	10°15'	0.01 (0.01)	0.01 (0.01)	1.0 (0.1)	5
2	Capraia	10	43° 2'	9°50'	0.08 (0.03)	0.05 (0.02)	1.3 (0.1)	30
3	Zente	6	42°43'	9°11'	0.08 (0.04)	0.10 (0.05)	1.3 (0.1)	20
4	Avena	9	41°41'	9°20'	0.06 (0.03)	0.05 (0.03)	1.2 (0.1)	15
5	L'Ospedale	10	41°39'	9°11'	0.06 (0.03)	0.06 (0.03)	1.3 (0.1)	25
6	Luogosanto	10	41° 3'	9° 12'	0.09 (0.04)	0.11 (0.05)	1.3 (0.1)	25
7	L. Posada	10	40°38'	9°45'	0.06 (0.03)	0.07 (0.04)	1.3 (0.1)	20
8	Oristano	33	39°54'	8°35'	0.08 (0.04)	0.08 (0.04)	1.5 (0.1)	40
9	GiaraGesturi	5	39°43'	9° 2'	0.06 (0.04)	0.06 (0.04)	1.1 (0.1)	15
10	Musei	6	39°18'	8°39'	0.08 (0.04)	0.07 (0.04)	1.2 (0.1)	20

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).

Table 2 Allele frequencies of the allozyme loci found polymorphic among the 10 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	0.850	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 AMOVA analysis for the eight sampled populations of *Hyla sarda*. Groups were defined as the two main clusters identified by the both PCA and UPGMA analyses (Fig. 1).

Source of variation	**Percentage of variation**	**Fixation indices**
Among groups	41.23	*Φct=0.41*
Among populations within groups	6.19	*Φsc=0.11*
Within populations	52.57	*Φst= 0.47*

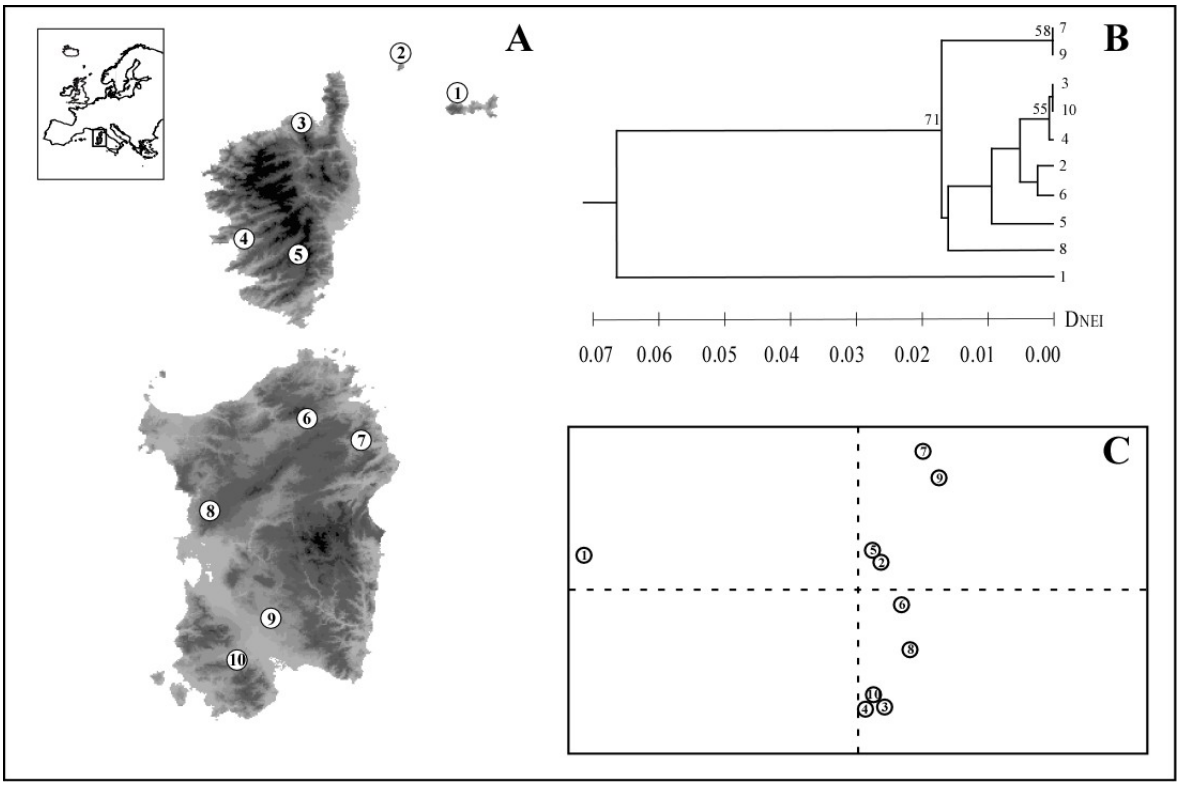


Figure 1 (A) Geographic distribution of *Hyla sarda* and geographic location of the 10 populations sampled. Localities are numbered as in Table 1. (B) UPGMA phenogram showing genetic relationship among the ten populations sampled, based on Nei's (1978) unbiased genetic distance (*D*NEI). Bootstrap values >50% after 1,000 pseudoreplicates are shown at the nodes. (C) Principal component analysis of allele frequencies among the studied samples of *Hyla sarda*. Only the first principal component (x-axis) was significant ($P < 0.01$) over 10 000 randomizations. Samples are encoded as in Table 1.

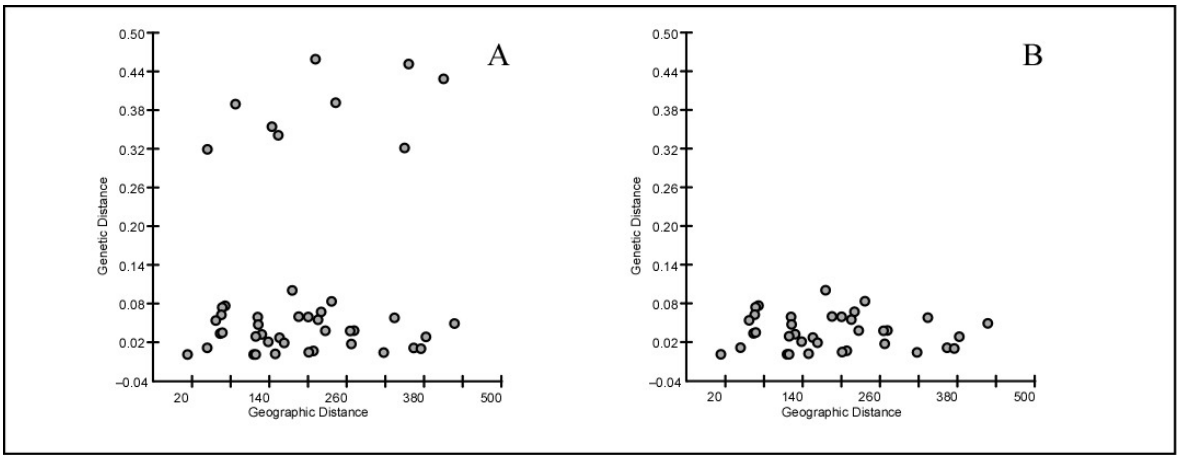


Figure 2 Scatterplots of the genetic [*F*ST/(1-*F*ST)] VS. log-geographic (in km) distances among population pairs of *Hyla sarda*. (A) all populations; (B) Elba island excluded.

3. MULTIPLE LINES OF EVIDENCE FOR DEMOGRAPHIC AND RANGE EXPANSION OF A TEMPERATE SPECIES (*HYLA SARDA*) DURING THE LAST GLACIATION

Roberta Bisconti, Daniele Canestrelli, Paolo Colangelo & Giuseppe Nascetti

ABSTRACT

Many temperate species experienced demographic and range contractions in response to climatic changes during Pleistocene glaciations. In this study we investigate the evolutionary history of the Tyrrhenian treefrog *Hyla sarda,* a species inhabiting the Corsica-Sardinia island system (Western Mediterranean basin). We used sequence analysis of two mitochondrial (overall 1229 bp) and three nuclear (overall 1692 bp) gene fragments to assess the phylogeography and demographic history of this species, and species distribution modelling (SDM) to predict its range variation over time. Phylogeographic, historical demographic and SDM analyses consistently indicate that *H. sarda* does not conform to the scenario generally expected for temperate species but rather underwent demographic and range expansion mostly during the last glacial phase. Paleogeographic data and SDM analyses suggest that such expansion was driven by the glaciation-induced increase in lowland areas during marine regression. This unusual scenario suggests that at least some temperate species may not have suffered the adverse effects of glacial climate on their population size and range extent, owing to the mitigating effects of other glaciations-induced paleoenvironmental changes. We discuss previous clues for the occurrence of such a scenario in other species, and some possible challenges with its identification. Early phylogeographic literature suggested that responses to the Pleistocene glacial-interglacial cycles were expected to vary among species and regions. Our results point out that such variation may have been greater than previously thought.

KEYWORDS: glacial expansion, Western Mediterranean, phylogeography, historical demography, ecological niche modelling, *Hyla sarda*.

3.1 INTRODUCTION

Past climate changes have contributed greatly in shaping the current geographic patterns of the distribution and genetic diversity of terrestrial species (Hewitt 2000, Hewitt 2004). Climate change, however, can influence the survival of a species, its distribution and its intra-specific patterns of variation in many ways, both directly and indirectly, as is well exemplified by the growing literature on the ecological and evolutionary consequences of the recent climate change (Davis *et al*. 2005, Parmesan 2006, Blaustein *et al.* 2010). While it is now well established that paleoclimate has influenced the evolutionary history of species, the importance of various climate-related processes in generating observed patterns of diversity has been less explored. Sea-level oscillation is one such process that has strongly affected the paleogeographic evolution of landscapes. Such oscillations have led to repeated cycles of land bridge formation, merging and separating islands among one another and the mainland, and there is ample biogeographic evidence supporting the relevance of these cycles in the assembly of the current biota (see Thompson 2005, Whittaker & Fernàndez-Palacios 2007, Cox & Moore 2010 and references therin). But sea-level oscillations also affect the shape and extent of coastal plains in many parts of the world, owing to the cycles of withdrawal and advancement of shorelines (Ray & Adams 2001). Arguably, the evolutionary history of species inhabiting these regions, and consequently their genetic structure, is also affected by these climate-related processes (e.g. Canestrelli *et al.* 2007b, Hofman *et al.* 2007, Canestrelli & Nascetti 2008, Marske *et al.* 2009, Sakaguchi *et al.* 2010).

The paleogeographic evolution of the Western Mediterranean basin has been particularly affected by sea-level oscillations. During the Messinian salinity crisis (5.96-5.33 million years ago) this basin was almost desiccated (Hsü *et al*. 1973, Krijsman *et al*. 1999), with major effects on the species distribution patterns in the region (Thompson 2005, Hewitt 2011a). Quaternary climate-driven sea-level oscillations were of lesser extent, but nonetheless, they also had major effects on the Western Mediterranean paleogeography. For instance, during the last glacial maximum (LGM; about 23 000 to 19 000 years ago), when the sea-level dropped to about 120 m below its current level, the islands of several archipelagos, such as Balearic, Dalmatian, Aegean and Corsica-Sardinia were merged, each one forming a single landmass and/or were connected to the continent

(Van Andel & Shackleton 1982, Shackleton *et al.* 1984). Furthermore, large coastal plains became exposed during this phase, such as those in the east of Spain and Provence (France), east of Tunisia and Libya, south of Sicily, north of the Adriatic sea, northwest of Italy, and those around some (current) archipelagos, such as the Tuscan, Corsica-Sardinia, Balearic and Aegean archipelagos (for descriptions and maps of the coastal paleogeography of the Mediterranean at the LGM, see Van Andel & Shackleton 1982 and Shackleton *et al.* 1984). Interestingly, although a comprehensive overview of the paleoecological conditions along the Mediterranean coasts during the LGM is still lacking, there is growing evidence for the occurrence of areas of relative ecological stability along the Mediterranean coastal plains, where climatic oscillations were attenuated, and where cold and wet, rather than dry, climatic conditions prevailed (Carrion *et al*. 2003, Beaudouin *et al* 2007, Ricci Lucchi 2008, Hughes & Woodward 2008).

The key role of sea-level lowstands during glacial stages has often been invoked to explain the dispersal patterns of the fauna and flora among the different areas of the Western Mediterranean (Hewitt 2011a). More recently, however, it has also been suggested that these lowstands may have been even more influential on the range dynamics of species, and in shaping the patterns of genetic diversity, than was previously thought. In fact to explain these patterns in some lowland-adapted species, it has been hypothesised that glaciation-induced increases in the extent of coastal plains may have counterbalanced the negative demographic consequences of climatic changes by providing new suitable habitats, and thus leading to net demographic stability or even expansions during these periods (Canestrelli *et al.* 2007b, Canestrelli & Nascetti 2008, Porretta *et al.* 2011).

Understanding the likely contributions of these processes to the current patterns of genetic diversity, may allow us to significantly improve our current knowledge regarding the location and extent of glacial refugia, historical demographic trends and the microevolutionary processes that drove them, and the tempo and mode of dispersal events between subregions. Furthermore, this improvement may be particularly important for the Mediterranean region, which is a major global hotspot of biodiversity, and has provided several areas for the long-term persistence of populations in the face of climatic instability (Myers *et al*. 2000, Medail & Diadema 2009, Blondel *et al*. 2010).

In this paper, to investigate the plausibility of a positive influence of glaciation-induced increases in the extension of coastal plains on population history trends, we analysed the pattern of genetic variation and carried out species distribution modelling of

the Tyrrhenian tree frog *Hyla sarda,* a species endemic to the Corsica-Sardinia island system. The Corsica-Sardinian block is in fact especially suitable for this purpose. Not only were these islands long connected throughout the glacial phase, but marine regressions also led to a significant increase of low-altitude areas. For instance, when considering the 0-500 m above sea level (a.s.l.) altitudinal range, the corresponding surface area was about 50% wider during the LGM than during the previous interglacial period (based on data from Jarvis *et al.* 2008), when the sea level reached about 6 m above its present level (Lambeck & Chappel 2001). Furthermore, the tree frog *H. sarda* also appears particularly appealing in the context provided above. *H.sarda* is widespread in the area but with more abundant populations at low and intermediate altitudes. It is mostly linked to a variety of lentic freshwater habitats including pools and temporary ponds. It breeds from spring to early summer, and remains near the breeding sites during most of the year (Lanza *et al.* 2007). It is generally considered as having good dispersal abilities, similar to its sister species *Hyla arborea* (which can disperse up to more than 10 km/year; see Stumpel & Hanekamp 1986), although these abilities have not been carefully evaluated for *H.sarda*.

A recent study analysing allozyme variation among 10 populations of this species (Bisconti *et al*. 2011), reported a genetic pattern including low divergence among populations, high variability within them, and lack of migration-drift equilibrium throughout the species range, which may be consistent with the above scenario. Here, we take advantage of a more extensive sampling throughout the whole species range, of sequence-based mitochondrial and nuclear markers, and of species distribution modelling. We carry out phylogeographic, historical demographic and paleodistribution reconstructions of this species. Our specific aim is to explore the plausibility of a scenario in which the temperate species *H. sarda* may have benefited from the glacial widening of lowlands areas, preventing or even reversing the demographic and range effects usually expected to be a consequence of the glacial climate.

3.2 MATERIALS AND METHODS

3.2.1 SAMPLING AND LABORATORY PROCEDURES

We sampled 174 individuals of *Hyla sarda* from 22 populations spanning its range. The geographical references and sample size of all sampled population are given in Table 1 and Figure1. Tissue samples were collected as toe clips and preserved in 95% ethanol until DNA extraction. All individuals were then released at the point of collection.

We extracted whole genomic DNA using proteinase K digestion followed by a standard phenol-chloroform protocol with RNase treatment (Sambrook *et al*. 1989). Polymerase chain reactions (PCR) were carried out to amplify fragments of two mitochondrial genes (cytochrome *b,* herein referred to as cyt *b*; NADH dehydrogenase subunit 1, herein referred to as ND1), and three nuclear genes (recombination-associated gene 1, RAG; ribosomal protein L9 intron 4, RPL; protein phosphatase 3 intron 4, PPP3). PCR cycling conditions were the same for all genes: 5 min at 92° C followed by 30 cycles of 1 min at 92° C, 1 min at the annealing temperature specific for each gene, and 90 s at 72° C, followed by a single final step of extension of 10 min at 72° C. Amplifications were conducted in 25 µL containing the following: $MgCl_2$ (2.5 mM), a reaction buffer (1X; Promega), 4 dNTPs (0.2mM each), 2 primers (0.2µM each), an enzyme *Taq* polymerase (1 unit; Promega) and 2 µl of DNA template. The annealing temperatures and primer sequences (taken from the literature or designed for this study) for each gene fragment are given in Table 2. PCR products were purified and sequenced by Macrogen Inc. (http://www.macrogen.com) using an ABI PRISM 3700 sequencing system. All sequences were deposited in GenBank (accession numbers: JN787963- JN88172).

3.2.2 GENETIC DATA ANALYSIS

We checked the electropherograms visually using the program CHROMAS 2.31 (Technelysium ltd.) and carried out sequence alignment using CLUSTALX (Thompson *et al.* 1997). Nuclear sequences that were heterozygous for more than 1 nucleotide position were phased using PHASE, whereas the possible occurrence of recombination was assessed for each nuclear gene using the pairwise homoplasy index (PHI statistic, Bruen *et al*. 2006)

implemented in SPLITSTREE V.4.11 (Huson & Bryant 2006). All subsequent analyses were carried out using phased nuclear data and indels treated as missing data.

To verify the existence of significant heterogeneity in the phylogenetic signal between the two mtDNA fragments we carried out a partition-homogeneity test (Farris *et al.* 1994) using the software PAUP * 4.0B10 (Swofford 2003). Since no significant differences were detected, we used the combined mtDNA dataset for all subsequent analyses.

Sequence variation was analysed using MEGA 5.1, whereas nucleotide (π) and haplotype (h) diversity (Nei 1987) were computed using the software DNASP 5 (Librado & Rozas 2009). Furthermore, in order to compare estimates of haplotype diversity between the two larger islands (i.e. Sardinia and Corsica) taking into account their different sample sizes, we applied a rarefaction procedure (El Mousadik & Petit 1996) and estimated the allelic richness using the software CONTRIB 1.02 (Petit *et al*. 1998).

The phylogenetic relationships among haplotypes were investigated using the statistical parsimony procedure for phylogenetic network estimations implemented in TCS 1.21 (Clement *et al*. 2000), with a 95% criterion for a parsimonious connection. We preferred using a network-building procedure over tree-building procedures, as it provides a more accurate estimate of intra-specific gene genealogies, especially in cases of shallow genetic divergence among haplotypes (Posada & Crandall 2001). To solve for ambiguities in haplotype networks (represented by loops) we applied empirical criteria derived from coalescent theory (see Pfenninger & Posada 2002 and references therein).

The best-fit model of sequence evolution was selected for each dataset among 88 models using the Akaike Information Criterion (AIC) as implemented in JMODELTEST (Posada 2008). This method suggested the TrN+Γ model (gamma distribution shape parameter=0.187) as the best fit for the mtDNA dataset, and HKY+I+ Γ (proportion of invariable sites=0.54; gamma distribution shape parameter =0.09), HKY and HKY+I+Γ (proportion of invariable sites=0.91; gamma distribution shape parameter =0.43) for the RPL, RAG and PPP3 genes respectively (Hasegawa *et al*. 1985, Tamura & Nei 1993). Nevertheless, since a gamma distribution already allows for sites with very low rates, we did not include a proportion of invariable sites during subsequent analyses (see Ren *et al*. 2005, Yang 2006).

A least-squares method (LS) recently described by Xia and Yang (2011) and implemented in the software DAMBE (Xia & Xie 2001) was used to estimate the time to the

most recent common ancestor (TMRCA), as well as the mean substitution rate for mtDNA data useed in subsequent analyses of historical demography. We chose to focus on mtDNA, as we had a larger data matrix for mtDNA than for individual nuclear genes, and because mtDNA evolves faster than nuclear genes do; thus mtDNA it is expected to contain more information to rely upon (see Heled 2010). A likelihood-ratio test was carried out with DAMBE to assess whether the sequences in our dataset can be reliably assumed to have diverged in a clock-like manner. Based on this teSt, the null hypothesis, which states that there are no differences in the evolutionary rates among lineages, cannot be rejected. To carry out the LS procedure, a phylogenetic tree topology and at least one calibration point are necessary. The phylogenetic tree topology was obtained using the maximum-likelihood method as implemented in PHYML (Guindon 2004), with the model of sequence evolution suggested by JMODELTEST, default options used for all other settings, and the closely related species *H. arborea* used as an outgroup. As a calibration point, we set the root of the tree to 5.33 million years, corresponding to the end of the Messinian salinity crisis and the consequent re-flooding of the Mediterranean basin. Based on fossil records (Kotsakis 1980) and the biogeographical and phylogenetic patterns for the Western Palearctic tree frogs (Stock *et al*. 2008), this historical event has been indicated as the most plausible cause of divergence between *H. sarda* and the continental species *H. arborea*. The analysis with DAMBE was run using the MLCompositeTN93 genetic distance (following suggestions in Xia & Yang 2011), a softbound option for the calibration point, along with 1000 bootstrap re-samplings to obtain the standard deviations of the estimates.

We used the simulated annealing procedure implemented in SAMOVA (Dupanloup *et al*. 2002) to define the groups of populations that are geographically homogeneous and maximally differentiated from each other. We carried out the analysis with the number of groups (K) ranging from 2 to 10, and assessed the optimal K as the one for which F_{CT} (i.e. the genetic variance due to divergences between groups) was the highest and significant. To verify its consistency, we ran the analysis five times for each K value with 1000 independent annealing processes. Afterward, analyses of the molecular variance (AMOVA; Excoffier *et al*. 1992) were carried out with the software ARLEQUIN 3.11 (Excoffier *et al.* 2005), using groupings suggested by SAMOVA and the best-fit models of sequence evolution as indicated by JMODELTEST.

The demographic history of the Tyrrhenian tree frog was explored by means of an Extended Bayesian Skyline Plot analysis (EBSP; Heled & Drummond 2008) using the

software BEAST 1.6.1 (Drummond & Rambaut 2007). The EBSP has an important advantage over previous skyline methods: it allows the simultaneous analysis of data from multiple unlinked loci, taking into account their specific mode of inheritance, thus significantly improving the reliability of demographic inferences (Heled & Drummond 2008, Ho & Shapiro 2011). The EBSP analysis was run using clock models, substitution models, and trees unlinked across all markers, with the substitution rate previously estimated for the mtDNA fragment used as a stable reference for all other markers (see Heled 2010). A strict molecular clock was enforced, as it is generally a good approximation for analyses at the intra-population level (Yang 2006), and since, by simplifying the coalescent model, it helps analyses to converge (Heled 2010). The analysis was run three times to check for convergence, with 1.5×10^8 generations sampled for every 5×10^3 generations in each run. Convergence, stationarity, effective sample size for each parameter of interest in the analysis, and the appropriate burn-in were evaluated using the software TRACER 1.41 (Rambaut & Drummond 2008). Moreover, we constructed a histogram describing the locations of the X-axis points of the demographic functions (i.e. with the bars' height proportional to the number of demographic functions having an X-point at each interval of time; see Heled 2010), and we considered the EBSP demographic reconstruction to be reliable, backward in time until the first time interval with fewer than two data points.

Finally, the occurrence of past demographic changes was also assessed for each dataset separately by computing the statistics F_S (Fu 1997) and D (Tajima 1989) using the software DNASP. The significance of the F_S and D values was assessed through 1000 coalescent simulations, carried out under the hypothesis of selective neutrality and population equilibrium. As suggested by Fu (1997), the 2% cut-off criterion was used to assess the 5% nominal level of significance of the estimated F_S values.

3.2.3 Species Distribution Modelling

Species distribution models for *H. sarda* were generated using MAXENT 3.3.3e (Phillips *et al.* 2006). MAXENT is a program for maximum entropy modelling of the geographic distributions of species; it combines presence-only data with ecological-climatic layers to predict species occurrence in areas where data points are unavailable. We used MAXENT to predict species occurrence under both present-day and LGM conditions.

Although potentially useful for this study, we avoided making predictions for the last interglacial phase (about 135 000-115 000 years before present; ybp), due to a lack of detailed bioclimatic data for this period. However it is worth mentioning that paleoclimatic conditions during this period were in many respects similar to current ones in the western Mediterranean basin (Bardaji *et al*. 2009 and references therein).

Since our specific aim was to predict the potential effect of sea-level drop during the last glacial phase, the models were built by considering only the Corsica-Sardinia microplate, i.e. an area that was largely and persistently connected into a single landmass during the Pleistocene glaciations.

A total of 190 localities were used to build the models. These localities included our own collection sites (20 localities), plus 170 additional sites drawn from the literature that we deemed reliable since detailed geographic data were provided by the authors. We built the models using the default parameters for convergence threshold (10^{-5}) and number of iterations (500). To ensure the consistency of the model predictions, 75% of the localities were used to train the model and 25% were used to test it.

The ecological layers for the present conditions (resolution 30 arc-seconds) and data for LGM (resolution 2.5 arc-minutes) were downloaded from the WorldClim database website (www.worldclim.org). For LGM prediction we used data from both the Community Climate System Model (CCSM) and the Model for Interdisciplinary Research on Climate (MIROC). The WorldClim database provides 19 bioclimatic variables. We built two models, one using the entire set of variables, the other using only those layers that we considered biologically significant for *H. sarda* and that were not strongly correlated to each other (Pearson correlation coefficient, r^2 <0.80). These layers included the following: BIO2, mean diurnal range in temperature; BIO3, isothermality (monthly/annual temperature range); BIO4, temperature seasonality (standard deviation *100); BIO7, annual range in temperature; BIO8, mean temperature of the wettest quarter of the year; BIO11, mean temperature of the coldest quarter; and BIO16, precipitation of the wettest quarter of the year.

The model performance was evaluated using the area under the Receiver Operating Characteristic (ROC) curve (AUC) calculated by MAXENT. Furthermore, to select the model that best-fit the data, we compared the two models using the Akaike Information Criterion (AIC) as implemented in the software ENMTOOLS (Warren *et al.* 2010, see Warren & Seifert 2011).

Finally, to allow a comparison of the area extensions of predicted presence between the present day and LGM, we also generated threshold models, using the ‘minimum training presence’ option for presence/absence prediction.

3.3 RESULTS

3.3.1 GENETIC DATA

The final mtDNA dataset included 531 bp of the cyt *b* gene (34 variable positions, 19 parsimony informative) and 698 bp of the ND1 gene (50 variable positions, 19 parsimony informative) sequenced across 169 individuals. The nuDNA dataset included 586 bp (29 variable positions, 17 parsimony informative) for the RPL gene, 480 bp (16 variable positions, 13 parsimony informative) for the PPP3 gene and 626 bp (10 variable positions, 7 parsimony informative) for the RAG gene, sequenced across 59, 81 and 62 individuals, respectively. No recombination events were indicated by the PHI tests carried out on the nuclear gene fragments. At the level of the entire dataset, 62 haplotypes were found in the mtDNA, 29 in the RPL, 25 in the PPP3 and 10 in the RAG.

Estimates of nucleotide (π) and haplotype (h) diversity for each marker in each sampled island are given in Table 3. For the mtDNA, the highest levels of both π and h were observed for the island of Sardinia. On the other hand, for the nuDNA, h was also, on an average, higher on Sardinia than elsewhere, while almost identical values of mean π were observed between Sardinia and the island of Corsica. At all the markers studied but the RAG, also the allelic richness was higher in Sardinia (mtDNA: 16.0; PPP3: 11.6; RPL: 5.8; RAG: 2.0) than in Corsica (mtDNA: 5.0; PPP3: 1.0; RPL: 2.0; RAG: 5.0).

The statistical parsimony network showing the phylogenetic relationships between mtDNA haplotypes is shown in Fig. 1B. All the haplotypes were connected into a single network. Two main group of haplotypes were identified, clade N and clade S, whose geographic distributions are shown in Fig. 1A. The most represented at the level of the entire dataset was clade N, with 53 haplotypes of the total 62 haplotypes found. This clade was geographically distributed throughout the range of the species. Four star-like structures (SLS) were observed within clade N, separated from one another by no more than 3 nucleotide positions. One of these SLS (yellow of Fig. 1B) included the only haplotypes observed on the islands of Corsica, Elba and Capraia, besides being the most abundant among the northern Sardinian samples. The other 3 SLS were restricted to Sardinia, with a mostly central and southern distribution. Clade S, separated by 12 inferred haplotypes from clade N, was represented by 9 haplotypes, and was geographically restricted to the three

southeastern Sardinian populations 2, 3 and 4, where it was also the most abundant. The three phylogenetic networks among the haplotypes found at each nuclear gene fragment are shown in Fig. 2. In no case was a clear phylogeographic structure observed.

The least-squares procedure implemented in DAMBE estimated the TMRCA of the mtDNA sequences at 550 000 years BP (±110 000 s.d.), and the average divergence rate at 2.74% (±0.20% s.d.).

The analysis carried out with SAMOVA on the mtDNA dataset (Fig.3) indicated 2 groups of populations (K = 2) as the best grouping option for our data, since higher values of K yielded lower values of F_{CT}. One group was assigned the three southeastern Sardinian samples (2, 3, 4) carrying clade S as the most abundant, while the other group was assigned all the remaining samples. The AMOVA analysis, carried out using this clustering option, revealed that the amount of variation explained by the among-group level of variation was 48.60 % (F_{CT}=0.49), the amount explained by the among-population within-group level of variation was 7.27% (F_{SC}=0.14) and the within-population level was 44.12% (F_{ST} = 0.56), with all variance components and fixation indexes being highly statistically significant (all P < 0.01). SAMOVA analyses based on nuDNA data (see Fig.3) do not allow identifying a population grouping best explaining the data. Indeed, F_{CT} values never exceeded 0.05, 0.09, and 0.22 for the RPL, PPP3 and RAG gene fragments, respectively, and in no case were the F_{CT} values statistically significant.

Historical demographic analyses were run after the exclusion from the dataset of populations 2, 3 and 4. In fact, according to the SAMOVA analysis, these populations appeared significantly differentiated with respect to mtDNA variation, while subsequent coalescent-based estimates of the historical demographic size changes assume the occurrence of panmixia (Ho & Shapiro 2011). In addition, a separate estimate was not carried out on this group of samples, due to the low overall sample size particularly at the nuclear loci. The EBSP showing the historical demographic trend is presented in Fig. 3B. Based on the histogram describing the locations of the X-axis points of the demographic functions, the historical demographic reconstruction can be considered reliable until about 150 000 ybp. According to the EBSP, after a phase of demographic stability lasting until about 75 000 ybp, a marked expansion event occurred, and it ended at approximately 10 000 ybp. The occurrence of a historical population expansion was also supported by the large negative and significant values of Fu's F_S statistic (see Table 3) observed for the mtDNA, PPP3, and RPL datasets, whereas the F_S value for RAG was negative but not

statistically significant. On the other hand, negative values of Tajima's *D* statistic were observed for all of the datasets, but they were statistically significant only for the mtDNA.

3.3.2 Species Distribution Modelling

Based on the Akaike Information Criterion, the SDM built using our selection of 7 bioclimatic variables outperformed the SDM based on the entire set of 19 variables in predicting the data (AIC scores= 3482.192 and 3533.310 respectively). Therefore, only the former model is presented here. This model yielded high AUC scores for both the training (0.77) and the test data (0.71), indicating a good performance of the trained model.

The projection of the model over present bioclimatic conditions (Fig. 5a) shows good habitat suitability in Sardinia, particularly along the coastal zone and low-altitude areas, although some high-altitude areas, such as the Gennargentu massif in central-eastern Sardinia, which is 1834 m a.s.l., also reached moderately high suitability scores. Areas of high suitability were inferred in the southwest and the northeast coastal areas. In Corsica, the most suitable areas were concentrated along the coastal zones and in valleys where rivers flow toward the coast.

The projection of the model over the LGM layers produced comparable suitability areas irrespective of the data used (i.e. CCSM vs. MIROC). Sardinia still presented large suitable areas, while in Corsica these were strongly reduced (Fig. 5B and 5C). In Sardinia, the coastal zone was by far the most suitable, with an area of very high suitability in the southwest. Interestingly, most of the areas of high suitability were located in lowlands made available by marine regression and are currently below sea level. On the other hand, in Corsica, suitable areas were strongly reduced and mostly limited to the southeastern coastlands. Moreover, the land bridge connecting Sardinia and Corsica during the entire glacial phase showed high suitability.

Finally, when the 'minimum training presence' option was used to predict the presence and absence (logistic threshold: 0.155) of the species , the present-day areas of predicted species presence extended 23 300 km^2 in Sardinia and 7700 km^2 in Corsica, whereas during the LGM the whole area of predicted presence was roughly 32 000 km^2 and 25 800 km^2 under the MIROC and CCSM models, respectively.

3.4 DISCUSSION

In recent decades, an extensive amount of research has ascertained that temperate species underwent demographic and range contractions in response to climate changes during the Pleistocene glacial phases (Hewitt 2011b and references therein). Our data do not conform to this scenario, indicating that the Tyrrhenian treefrog underwent a demographic and range expansion during the last glacial phase. Furthermore, they also imply that even such exception was driven by climatic influences on landscape features. In the subsequent sections, we will first discuss what our data can tell us about the Pleistocene evolutionary history of the Tyrrhenian tree frog *H. sarda*. We will then place these results in context, highlighting previous clues for such an unusual scenario in other temperate species, and discuss some possible issues toward assessing its relevance.

3.4.1 EVOLUTIONARY HISTORY OF *HYLA SARDA*

The phylogenetic network analysis of the mtDNA haplotypes showed two main clades (clades N and S of Fig. 1B), whose geographic distributions were clearly mirrored in the best population grouping suggested by the SAMOVA analysis. One group, N, was distributed within the entire range of the species, while the other, S, was geographically restricted to the southeastern part of Sardinian. The same pattern was not observed in the nuDNA data. This discordance is not surprising, however. It is well established that the usually slower divergence rates of the nuDNA markers; usually higher effective population size; and incomplete lineage sorting, can make the population genetic structure and imprints of the population history much less apparent with nuclear than with mitochondrial DNA data (see Zhang & Hewitt 2003, Brito & Edwards 2009 for reviews).

The split between the two mtDNA haplogroups was estimated to have occurred during the Middle Pleistocene age (550 000 ybp). Moreover, the geographic distribution of the mtDNA diversity suggests that these two groups originated from a fragmentation event that occurred within the island of Sardinia. In fact, for the clade S, a Sardinian origin is clearly indicated by its geographic distribution, which is restricted to the southeastern portion of the island. On the other hand, the occurrence of most of the haplotypes of clade N in Sardinia, including all those internal in the network structure, and thus supposedly

ancestral (Castelloe & Templeton 1994), suggests that this clade originated in Sardinia as well.

In light of the ubiquitous distribution of clade N in Sardinia, we could only postulate about the likely source of divergence between the two groups. Obvious geographic barriers to *H. sarda* dispersal (e.g. seaways or mountain chains) are not apparent in Sardinia, and to the best of our knowledge, there is no paleogeographic evidence in favour of their past existence (at least for the Middle and Late Pleistocene periods). On the other hand, when looking at the SDM reconstructions for the LGM (see Fig.5), the southeastern portion of Sardinia (i.e. the distribution range of clade S) appears separated from most of the neighbouring territories, by surrounding areas of largely unsuitable bioclimatic conditions (see in particular Fig. 5C). Therefore, it seems plausible that similar climatic conditions during a Middle Pleistocene glacial phase could have prompted the isolation between two population groups, priming the divergence between clades S and N. Finally, the geographic concordance between a plausible bioclimatic barrier to dispersal and the observed phylogeographic discontinuity, lead us to favour the above mentioned historical fragmentation rather than stochastic processes as the likely source of the phylogeographic discontinuity (see Irwin 2002).

The historical demographic reconstruction, based on multilocus EBSP analysis, showed a marked population growth starting at around 75 000 ybp and lasting until about 10 000 ybp. The occurrence of this demographic expansion was also supported by the analyses of single-locus-based F_S and (to a lesser extent) D statistics.

To fully appreciate the implications of the inferred expansion, the pre-expansion range of the species should first be inferred. Our data indicate that before the expansion the species' range was restricted to Sardinia. In fact, this island showed higher levels of haplotype diversity (see Table 3) and the occurrence of all haplotypes that were internal in the network structures (and therefore supposedly ancestral; see Fig. 1B and Fig. 2). Moreover, the mtDNA haplotypes found in the northern islands (Corsica, Capraia and Elba) were all closely related, i.e. not more than 2 base pairs divergent, to those found in northern Sardinia, suggesting that the northern islands were recently colonised from this area.

The SDM analysis suggested that, with respect to the hypothesised pre-expansion range (i.e. Sardinia), suitable habitats were displaced during the LGM, but were reduced neither in their overall extent nor in their degree of suitability, which in fact even increased

in certain areas (such as southwestern Sardinia). According to this analysis, during LGM, suitable areas spanned the lower altitudes, as well as the newly exposed lowlands around Sardinia, in particular. Interestingly, a major and rapid phase of sea-level fall, and a consequent large increase in coastal lowlands, began at about 80 000 ybp (see Fig.4A; see also Dawson 1992), roughly at the time of the inferred initiation of the expansion phase. A further indication that the demographic expansion was prominent in Sardinia comes from the observation that all the four star-like structures of the mtDNA clade N, expected just in the case of demographic expansion (Slatkin & Hudson 1991), were found almost exclusively on this island. Finally, according to Kuhlemann *et al*. (2008), during the last glaciations, milder climatic conditions, beyond those usually expected for coastal areas, occurred in the centre of the Western Mediterranean, including Sardinia, relative to areas of the basin just to the north.

The colonisation of Corsica likely occurred later, and we suggest during the post-LGM phase of the inferred expansion. Indeed, not only did our SDM analyses indicate that this island had largely unfavourable bioclimatic conditions during the LGM, but according to Kuhlemann *et al*. (2008), most of Corsica island (especially its central and northern portions) was substantially invaded by polar air during the LGM. Consequently, glacial worsening of climatic conditions was more pronounced in this area compared to the south. Furthermore, in addition to the differences in climatic change, the widening of the lowland areas caused by marine regression was not equal around both Corsica and Sardinia (see Fig. 5), due to the steeper topography of most of the Corsican coasts. Therefore, while wider lowland areas and milder climatic conditions in Sardinia during the last glaciations provide a plausible paleoenvironmental context for the inferred phase of demographic expansion before the LGM, it seems equally plausible that the northern territories were colonised later, with climatic amelioration occurring after the LGM. Moreover, according to SDM, the land bridge connecting northern Sardinia with southern Corsica was not only wide and persistent during the glacial phase (Thiede 1978), but it also provided wide and highly suitable areas to *H. sarda* even during the LGM. In turn, this suggests that the expansion front during the northward re-colonisation could also have been accordingly wide. Such a wide expansion front, together with a relatively short colonisation distance and substantial gene flow (as suggested by the lack of population genetic structure in the area; see also Bisconti *et al*. 2011), may suitably explain why the almost entire reduction in genetic diversity expected during post-glacial re-colonisations (Hewitt 1996, 1999) was

instead not observed here, neither through sequence data (this study) nor with allozymes (Bisconti *et al*. 2011). Finally, the presence of a Middle Pleistocene fossil record of *H. sarda* in northern Corsica (at Castiglione; Martín & Sanchiz 2011) indicates that at least one extinction event occurred on this island, and therefore, the recent colonization was rather a re-colonisation.

3.4.2 PERSPECTIVES AND CONCLUSIONS

In light of the extensive research on demographic and range dynamics of temperate species in response to Pleistocene climatic oscillations, the scenario of glacial expansion, as we have reported here for *H. sarda*, must certainly be regarded as an exception to the general trend. Nevertheless, certain clues from previous literature suggest that this exception may not be limited to this species. For instance, within the Mediterranean basin, a similar scenario was hypothesised for other two species, *H. intermedia* (Canestrelli *et al*. 2007b) and *Rana (Pelophylax) lessonae* (Canestrelli & Nascetti 2008), in the Pò plain (northern Italy). Furthermore, growing data indicate a role for glacial lowlands in providing (cryptic) refugia for temperate species in several parts of the world (e.g. Burns *et al.* 2007, Buckley *et al.* 2009, King *et al.* 2009, Marske *et al.* 2009, Marko *et al.* 2010, Porretta *et al.* 2011). In some of these cases, historical demographic analyses based on genetic data also suggest a possible pre-LGM initiation of the expansion phase (e.g. Buckley *et al.* 2009, Marske *et al.* 2009). Finally, geographical settings making glacial expansions particularly plausible for temperate species are those areas that were fragmented by seaways during interglacial sea-level highstands, and were re-joined into a single landmass during subsequent lowstands. In these areas, the species ranges became continuous during glacial periods. In the Western Mediterranean, such a scenario was repeatedly inferred for southern Italy, where it was also linked to the rising of hotspots of intra-specific diversity (Santucci *et al.* 1996, Podnar *et al.* 2005, Canestrelli *et al.* 2006, 2007b, 2008, 2010, 2012, Canestrelli & Nascetti 2008, Barbanera *et al*. 2009, Vega *et al.* 2010), and similar scenarios have also been suggested for other geographic regions, such as the Aegean, Balearic Islands, Baja California, Philippines, Japan, and south-East Asia (Hall & Holloway 1998, Riddle *et al*. 2000, Martinez-Solano *et al*. 2009, Papadopoulou *et al*. 2009, Jones *et al*. 2008, Hurtado *et al*. 2010, Kyoda *et al*. 2010, Ravago-Gotanco & Juinio-Meñez 2010, Bauzà-Ribot *et al*. 2011).

Several potential challenges may complicate the assessment of a scenario such as the one presented here in other species or regions. Among these challenges, whose careful examination goes beyond the scope of our work, at least two deserve special attention: the extinctions of refugial haplotypes or entire populations, and the need for an appropriate sampling strategy. In fact, both have already posed challenges in the assessment of other now well-established scenarios, such as the refugia-within-refugia, nunataks, and peri-glacial refugia scenarios (Gomez & Lunt 2006, Maggs *et al*. 2008, Schneeweiss & Schönswetter 2011 and references therein). In certain circumstances, the former may completely erase the imprints of previous patterns, leading to erroneous inferences regarding population history and past demographic trends in particular. This seems particularly plausible when the whole refugial area was re-flooded by a subsequent marine transgression, and for those species whose low dispersal abilities prevented newly formed genetic variants to spread in neighbouring areas. In such cases, a careful integration of phylogeographic, historical demographic and SDM approaches will be particularly crucial (Waltari *et al*. 2007). Finally, in those cases in which such a scenario can be a plausible hypothesis based on species ecology and the paleogeographic evolution of the study area, special attention should be given to sampling coastal lowlands and neighbouring areas. Indeed, just these areas would be expected to harbour higher diversity and ancestral haplotypes, whose lack in the data set would completely misguide inferences of population history.

In conclusion, early phylogeographic literature suggested that responses to Pleistocene glacial-interglacial cycles were expected to vary among species and regions (Hewitt 1996). Subsequent studies largely met this expectation, indicating, among others, a variety of post-glacial re-colonisation routes (Hewitt 2011b and references therein), and refugial range locations and structures, including the cryptic northern refugia (Stewart & Lister 2001), as well as the aforementioned nunataks, refugia-within-refugia, and peri-glacial refugia scenarios. Our results, which indicate the possibility of glacial expansion for temperate species, contribute to point out that such variation may have been even wider than previously thought.

3.5 TABLES AND FIGURES

Table 1 Geographic location of the 20 sampled populations of *Hyla sarda,* and number of individuals analysed for both mitochondrial (mtDNA) and nuclear (nuDNA) markers.

Island	Sample	Latitude N	Longitude E	mtDNA	nuDNA		
					PP3	RAG	RPL
Sardinia	1 Monte Arcosu	39°11'	08°51'	14	4	3	3
	2 Villasimius	39° 08'	09° 31'	3	-	-	1
	3 Is Piscinas	39°43'	09°39'	23	8	6	5
	4 Villagrande	39°58'	09°31'	18	6	4	-
	5 Musei	39°18'	08°39'	1	1	2	2
	6 Isola di San Pietro	39°09'	08°17'	1	3	1	1
	7 Domusnovas	39°19'	08°39'	6	4	2	1
	8 Giara Gesturi	39°43'	09°02'	4	3	3	1
	9 Oristano	39° 54'	08° 35'	8	5	1	4
	10 Monteferro	40°12'	08°35'	14	4	3	2
	11 Siniscola	40°34'	09°46'	20	6	2	8
	12 Posada	40°38'	09°45'	6	4	5	5
	13 Vallicciola	40°51'	09°09'	5	2	2	3
	14 Asinara	41°03'	08°14'	2	1	2	1
	15 Luogosanto	41°03'	09°12'	9	2	3	5
Corsica	16 L'Ospedale	41°39'	09°11'	12	10	11	3
	17 Curzo	42°19'	08°40'	9	6	1	2
	18 Biguglia	42°38'	09°26'	3	1	1	-
Elba	19 Elba	42°47'	10°15'	6	4	6	6
Capraia	20 Capraia	43°02'	09°50'	5	7	4	6

Table 2 PCR primers and annealing temperatures used to amplify the two mitochondrial and three nuclear DNA fragments used in this study.

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Gene	Primer name	Primer sequence 5'-3'	Annealing (°C)	Reference
mtDNA				
cyt *b*	HSVITF1	ACCTCAATAGCCTTCTCATCTGTAG	58	This study
	HSVUTR1	GGGTTAGCGGGAGTGAAATTAT		
ND1	ND1vitF1	TATTAGGCTACATACAACATCGAA	48	This study
	ND1vitR2	AAGTGTATAAGTTGGTCATACCG		
nuDNA				
RPL	RPL94F	CGTGTKGACAAATGGTGGGGTAA	52	Pinho *et al.* 2009
	RPL95R	ATGGGAAAGTGAGCRTACACAGA		
PPP3	PPP3CA4F1	CTGTAYTTGTGGGCCTTGAAAATTC	50	Pinho *et al.* 2009
	PPP3CA5R2	GGCAGTCAAAGGCATCCATGCAGGC		
RAG	RAGIF	CCAATGTCGCAGTGCAARGCRTC	52	Biju & Bossuyt 2003
	MARTFL1	AGCTGGAGYCARTAYCAYAARATG		Chiari *et al.* 2004

Table 3 Estimates of the haplotype (h) and nucleotide (π) diversities (Nei 1987; standard deviations in brackets) for the four islands sampled across the Tyrrhenian tree frog's range, and estimates of Fu's (1997) F_S and Tajima's (1989) D statistics for the concatenated mtDNA and the three nuDNA markers analysed.

Island	**mtDNA**		**RPL**		**PPP3**		**RAG1**		**Nuclear average**	
	h	**π**	**h**	**π**	**h**	**π**	**h**	**π**	**h**	**π**
Sardinia	0.96	0.0068	0.89	0.0043	0.76	0.0048	0.51	0.0009	0.72	0.0033
	(0.01)	(0.0005)	(0.02)	(0.0005)	(0.04)	(0.0004)	(0.04)	(0.0001)	(0.20)	(0.0021)
Corsica	0.80	0.0011	0.62	0.0052	0.31	0.0019	0.77	0.0033	0.57	0.0035
	(0.06)	(0.0002)	(0.14)	(0.0010)	(0.09)	(0.0005)	(0.04)	(0.0003)	(0.23)	(0.0016)
Elba	0.53	0.0004	0.62	0.0012	0.54	0.0033	0.60	0.0033	0.59	0.0026
	(0.17)	(0.0001)	(0.09)	(0.0002)	(0.12)	(0.0008)	(0.09)	(0.0007)	(0.04)	(0.0012)
Capraia	0.40	0.0003	0.68	0.0016	0.32	0.0013	0.71	0.0041	0.57	0.0024
	(0.02)	(0.0002)	(0.10)	(0.0004)	(0.16)	(0.0007)	(0.12)	(0.0013)	(0.22)	(0.0015)
F_S	-47,77**		-14.27**		-11.77**		-1.73^{NS}			
D	-2.31**		-1.26^{NS}		-0.39^{NS}		-0.48^{NS}			

* P<0.001; NS= not significant

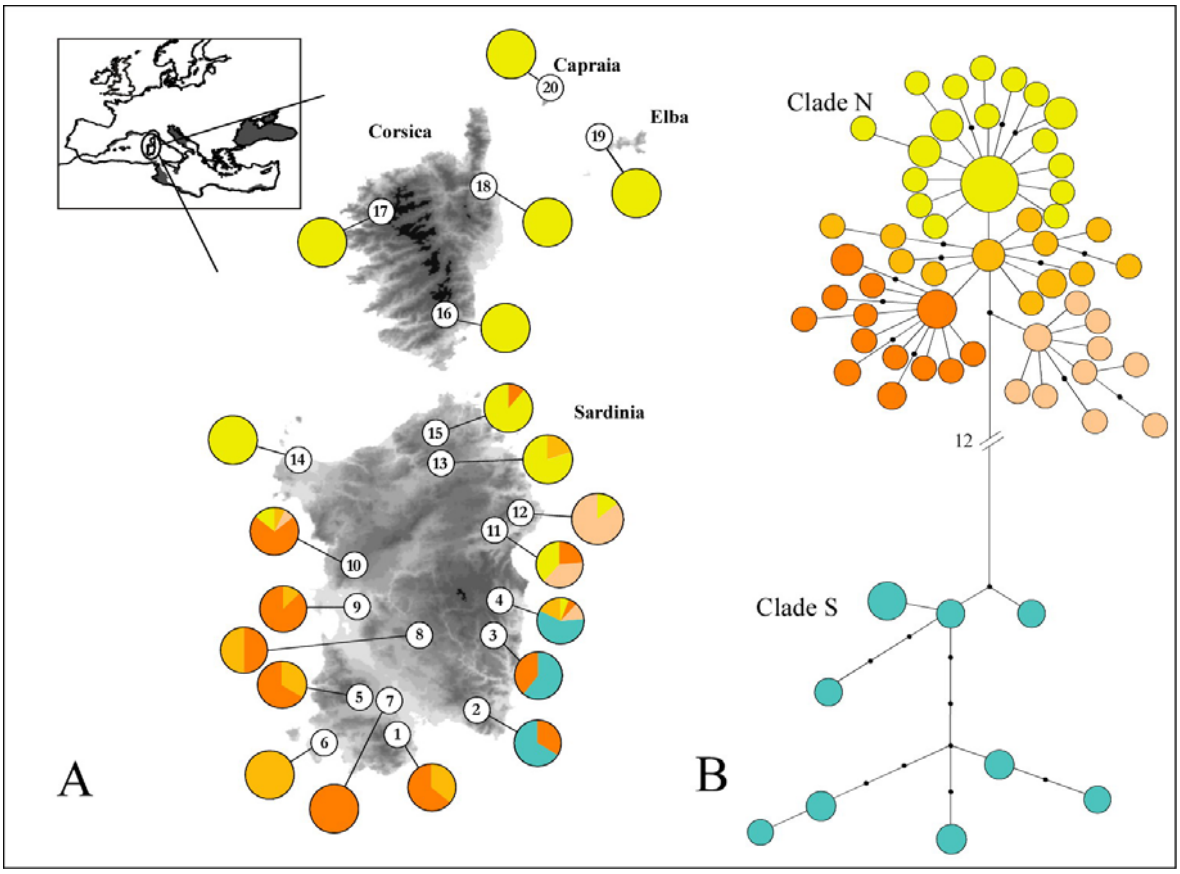

Figure 1 A) Geographic distribution of the 20 sampled populations of *Hyla sarda*. Pie diagrams show the frequency distribution of the main haplotype groups among the populations. The inset shows the geographic location of the study area within the Western Palearctic region, with areas that were emerged during the Last Glacial Maximum shown as gray shading. B) Statistical parsimony network showing genealogical relationships among the 62 mtDNA haplotypes found

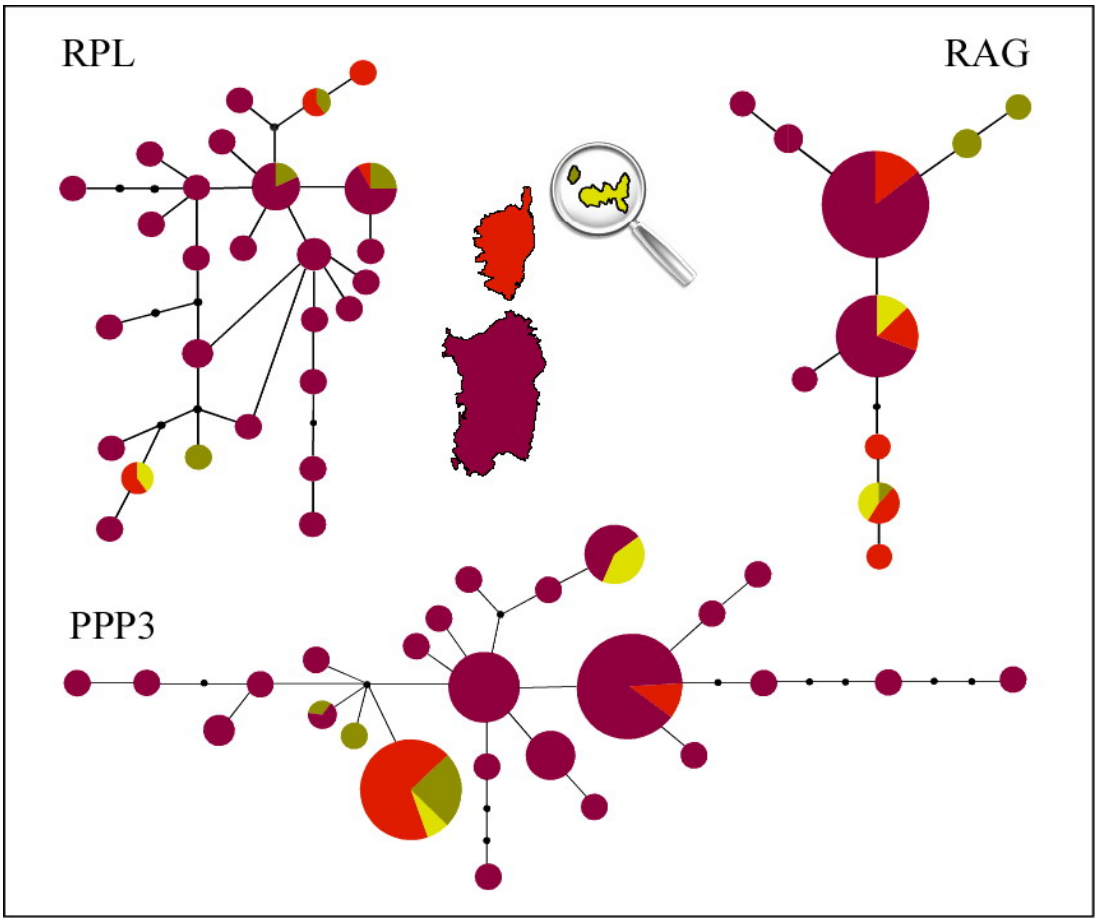

Figure 2 Statistical parsimony network showing the genealogical relationships among the haplotypes found at the three nuclear gene fragments analysed. Haplotypes are presented as pie diagrams, with slices proportional to the haplotype frequency in each sampled island.

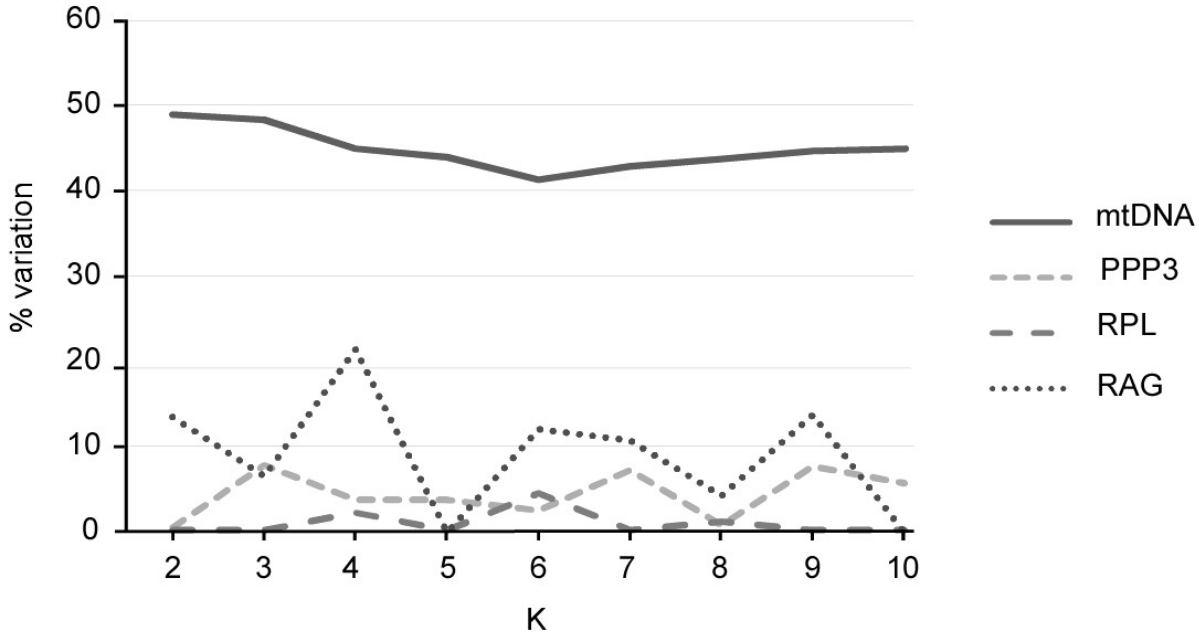

Figure 3 Spatial analysis of the molecular variance (SAMOVA) of the 20 populations sampled of *Hyla sarda*. The percentage of variation explained by the among-group level of variation is reported for the best-clustering option obtained for each pre-specified value of K (the number of groups), with K ranging from 2 to 10.

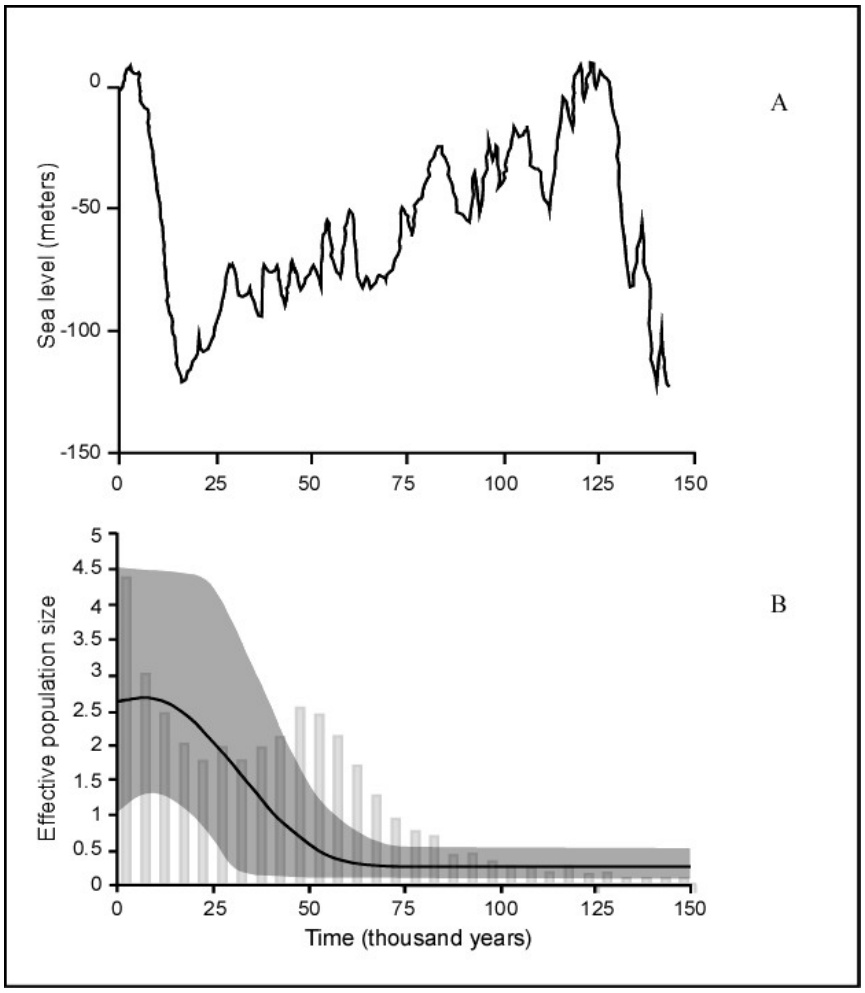

Figure 4 A) Variation of sea-level (relative to the current level) during the last 140 000 years (redrawn from Dawson 1992). B) Extended Bayesian Skyline plot showing historical demographic trend of *Hyla sarda*. The background histogram shows the number of demographic functions (proportional to the bar height) having an X-point at each time interval.

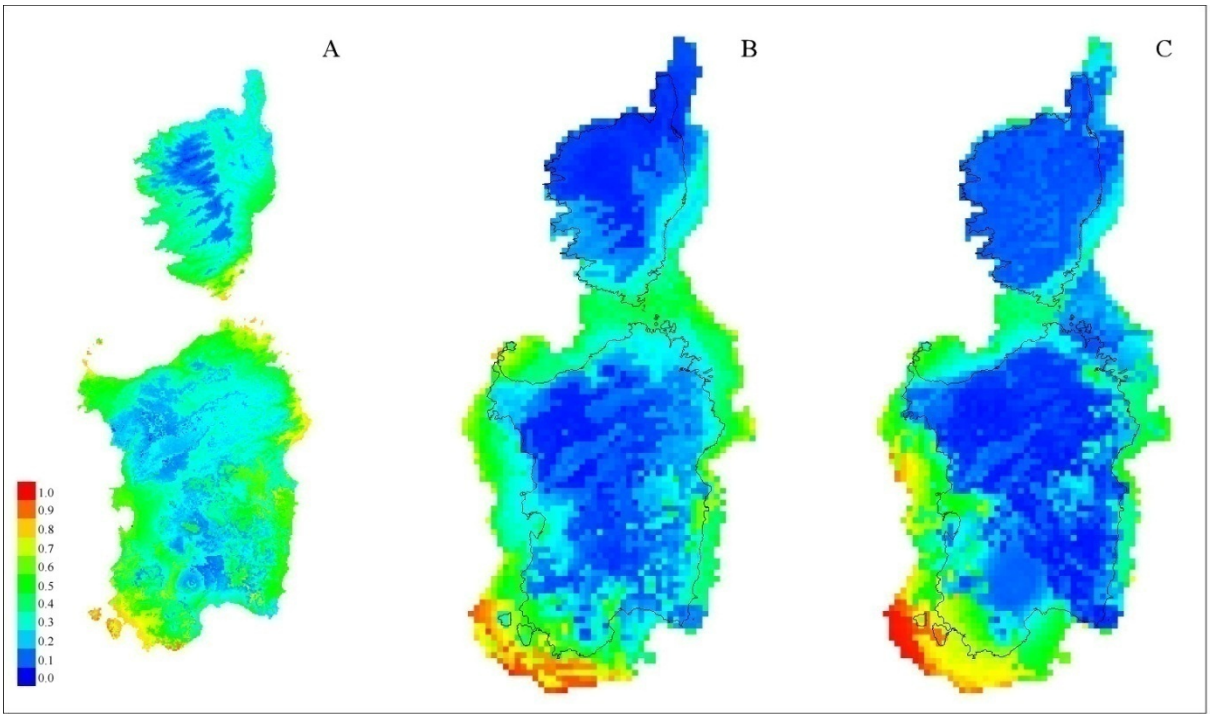

Figure 5 Bioclimatic models for *Hyla sarda* on the Corsica-Sardinia microplate, estimated for present-day conditions (A) and for the Last Glacial Maximum, based on both MIROC (B) and CCSM (C) paleoclimatic models. Warmer colours show areas with better predicted conditions. The logistic threshold under the 'minimum training presence' criterion for presence-absence prediction is 0.155.

4. A GEOGRAPHIC MOSAIC OF EVOLUTIONARY LINEAGES WITHIN THE INSULAR ENDEMIC NEWT *EUPROCTUS MONTANUS*

Roberta Bisconti, Daniele Canestrelli, Daniele Salvi, & Giuseppe Nascetti

ABSTRACT

Islands are hotspots of biodiversity, with a disproportionately high fraction of endemic lineages, often of ancient origin. Nevertheless, intra-island phylogeographies are surprisingly scarce, leading to a scanty knowledge about the microevolutionary processes induced on island populations by Plio-Pleistocene climatic oscillations, and the manner in which these processes contributed to shape their current genetic diversity. We investigated the phylogeography, historical demography, and species distribution models of the Corsican endemic newt *Euproctus montanus* (north-western Mediterranean). As for many island endemics, the continuous distribution of *E. montanus* throughout its range has hitherto been considered as evidence for a single large population, a belief that also guided the species' categorisation for conservation purposes. Instead, we found a geographic mosaic of ancient evolutionary lineages, with five main clades of likely Pliocene origin (2.6-5.8 My), all but one restricted to northern Corsica. Moreover, the co-presence between two of these main lineages in the same population was limited to a single case. In light of the lack of significant discontinuities in the geographic distribution of populations, as well as geographic, geological, or bioclimatic barriers to dispersal, the most plausible explanation for such pattern appears to be the occurrence of some intrinsic barriers to gene exchange among populations. As also suggested by growing literature on intra-island phylogeographic variation, it seems that the extensive use of simplifying assumption on the population structure and historical demography of island populations—both in theoretical and applicative studies—should be carefully reconsidered, a claim which is well exemplified by the case presented here.

KEYWORDS: Corsica, *Euproctus montanus*, Intra-island phylogeography, Mediterranean basin, Diversification.

4.1 INTRODUCTION

In the last three decades, the temperate species of the Mediterranean region have been the subject of one of the most intensive phylogeographic surveys (Feliner 2011; Hewitt 2011a). As a consequence, there is now ample evidence that Plio-Pleistocene climatic oscillations and climate-induced paleoenvironmental changes have had a prominent role in promoting species range dynamics and in shaping current patterns of intra-specific diversity of species inhabiting this region (Thompson 2005, Hewitt 2011b). The main focuses of phylogeographic studies on mainland species have been the identification of major glacial refugia, of hotspots of genetic diversity, as well as of post-glacial re-colonisation routes (Taberlet *et al.* 1998, Schmitt 2007, Hewitt 1996, 2000, 2004, 2011a, 2011b). Studies on island species have been mostly aimed at assessing how they occupied their current range and the subsequent patterns of gene exchange among islands and with the continent (e.g. Thompson 2005, Nieberding *et al.* 2006, Stock *et al.* 2008, Papadopoulou *et al.* 2009, Lazàro *et al.* 2011, Stroscio *et al.* 2011). On the whole, these studies have allowed the identification of both shared and unique patterns, and the realisation that the diversity of species' responses to Plio-Pleistocene climatic changes may have been wider than initially thought (Hewitt 2011a).

Nevertheless, in recent years, growing emphasis has also been directed towards the need to move forward phylogeographic investigations from being mostly focused on locating areas of long-term persistence during unfavourable climatic phases, or source areas and routes for range expansions during favourable climatic phases, to taking a closer look at how populations survived climatic oscillations within these areas, what microevolutionary processes were induced by these oscillations, and how these processes have shaped the current genetic structure and diversity within these areas (Hampe & Petit 2005, Gomez & Lunt 2007, Canestrelli *et al.* 2010, Feliner 2011). In fact, these areas have been crucial for the long-term persistence of species, of their intra-specific diversity, and thus of their evolutionary potential, and they are particularly threatened by the recent climate changes and other human-induced environmental alterations (Hampe & Petit 2005, Araujo *et al.* 2006, Kier *et al.* 2009). Therefore, a thorough appreciation of how diversity has been moulded within these areas is of primary interest under evolutionary, ecological,

and conservation perspectives (see Hampe & Petit 2005, Canestrelli *et al.* 2010, Feliner 2011 for lengthier discussions of these issues).

To move in this direction, islands appear particularly appealing (see also Thorpe & Malhorta 1998, Emerson *et al.* 2006). First, islands have a longstanding and undoubtedly fruitful history as natural laboratories for the study of evolutionary processes (e.g. Darwin 1859, Wallace 1892, MacArthur & Wilson 1967, Grant 1998, Grant & Grant 2007, Whittaker & Fernandez-Palacios 2007). In fact, several features of islands, particularly that they are discrete and isolated spatial entities, allow making a number of simplifying assumptions that are useful for both experimental designs and data interpretation (e.g. Frankham 1996a, 1996b, Woolfit & Bromham 2005, Whittaker & Fernandez-Palacios 2007, Vellend & Orrock 2010). Second, both at the global level and in the Mediterranean basin, islands are hotspots of biodiversity, with a disproportionately high fraction of this diversity being endemic, a feature that is consistent across taxa and geographic regions and that makes islands a conservation priority (Thompson 2005, Whittaker & Fernandez-Palacios 2007, Vogiatzakis *et al.* 2008, Kier *et al.* 2009, Medail & Diadema 2009, Blondel *et al.* 2010). Third, in spite of their usefulness as natural laboratories and their disproportionate importance as biodiversity hotspots both at global and regional levels, only a minority of the phylogeographic studies conducted thus far have investigated intra-island patterns of variation of endemic species. A screening of the ISI Web of Science database (temporal window: 1985–February 2012; search updated to 2nd February 2012) with the term 'Phylogeography' yielded 6719 hits, whereas the terms 'Phylogeography AND Island' retrieved 1140 hits (17%). However, <5% of the overall phylogeographic studies presented and discussed intra-island phylogeographic patterns (Canestrelli *et al.* in prep.). Furthermore, among these, there was a strong bias towards a few well-studied islands; for example, New Zealand, Japan, Taiwan, and the Canary Islands account for more than a half of these studies (see also Juan *et al.* 2000, Wallis & Trewick 2009). This paucity of phylogeographic studies is also well apparent for the Mediterranean basin, for which only a handful of intra-island phylogeographies are available, even though it is a major world hotspot of biodiversity (Myer *et al.* 2000) and counts >5000 islands (thus forming one of the world largest island systems; Vogiatzakis *et al.* 2008).

If islands can be used as natural laboratories for the study of microevolutionary processes, then Corsica Island is certainly a good candidate top-level laboratory within the Mediterranean basin. It is a hotspot of Mediterranean biodiversity, with both flora and

fauna that are rich of endemic species (Thompson 2005, Mouillot *et al.* 2008). It is the northernmost, the wettest, and the most mountainous island of the Mediterranean basin, with a central mountain chain having many summits exceeding 2000 m in altitude. Consequently, it has a particularly complex array of landscapes and microclimatic regions, spanning from Mediterranean climate at low altitudes, to temperate montane climate at intermediate altitudes, to alpine climate at higher altitudes (see Mouillot *et al.* 2008 for an extensive description of the Corsican environments). Furthermore—and interestingly—the marine buffering of climatic oscillations, which is thought to contribute to the high endemism richness and to the persistence of old lineages on islands (Cronk 1997), has not been a prominent feature of this island. Indeed, during the last glaciation, northern Corsica was substantially invaded by polar air (Kuhlemann *et al.* 2008), and thus climatic oscillations were more pronounced here than in neighbouring areas. Moreover, extensive glaciers formed on the central mountain chain during Pleistocene glacial phases (Kuhlemann *et al.* 2005). Finally, since few phylogeographic studies have been conducted thus far at the intra-island level on Corsican species (see Discussion), the genetic imprints of Plio-Pleistocene climatic oscillations on this island biota are still largely unknown.

The Corsican endemic *Euproctus montanus* seems an outstanding model organism to start looking at these imprints. It is a lung-less newt, widely and continuously distributed on the island, particularly—albeit not exclusively—at intermediate and high altitudes (between 600 and 1500 m above sea level; Gasc 1997). It has primarily aquatic habits, living within mountain streams, brooks (which are abundant on the island), or ponds during the aquatic period, or close to them during the terrestrial period (Gasc 1997). Interestingly, as no study has yet investigated the population structure of *E. montanus*, it has not been given a high conservation priority in the International Union for Conservation of Nature (IUCN) Red List in spite of its restricted range, because '…although its Extent of Occurrence might be less than 20 000 km^2, it is common with a presumed large population…' (IUCN 2011).

Previous studies of the phylogenetic relationships and divergence timing of *E. montanus* indicated that this species started diverging from the closely related Sardinian brook newt *Euproctus platycephalus* in the Miocene (Caccone *et al.* 1997, Carranza & Amat 2005, Veith *et al.* 2005, see also below). In this paper, we analyse the phylogeographic and historical demographic patterns of *E. montanus*. Our aim is to assess if and how the Plio-Pleistocene climatic oscillations influenced the evolutionary history of

this island endemic species and, more specifically, what microevolutionary processes they primed, and how these processes contributed shaping its current patterns of population genetic structure and diversity. Finally, we will also evaluate the conservation implications of our results.

4.2 MATERIALS AND METHODS

4.2.1 SAMPLING AND LABORATORY PROCEDURES

We sampled 193 individuals of *E. montanus* from 15 localities spanning the whole species range. Geographical references for sampling localities and sample sizes are shown in Table 1 and Fig. 1A. Tissue samples were collected as tail tips after anaesthesia in a 0.1% solution of MS222 (3-aminobenzoic acid ethyl ester) and were stored in 96% ethanol. All the individuals were then released in the collection place. DNA extractions were performed using the standard CTAB (cetyltrimethylammonium bromide) protocol (Doyle & Doyle 1987). Two mitochondrial DNA (mtDNA) fragments were amplified and sequenced: one from the cytochrome *b* gene (*cytb*) and one from the cytochrome oxidase I gene (*cox*). The following specific primers were designed to amplify and sequence all the individuals: 494SALAMOD (CATCAAACATCTCCTACTGATGAAA) and Mvz16mod (AAATAGGAARTATCAYTCTGGTTTRAT) for the *cytb* fragment and VF1deupr (TTCTCYACRAATCAYAAAGACATTGG) and VR1deupr (TATACTTCAGGGTGRCCAAAAAATCA) for the *cox* fragment.

Amplifications were performed in a 25 µl volume containing $MgCl_2$ (2.5 mM), the reaction buffer (5×, Promega), the four dNTPs (0.2 mM each), the two primers (0.2 µM each), the enzyme Taq polymerase (1 U, Promega), and 2 µl of DNA template. PCR cycling started with a step at 94°C for 4 min followed by 33 (*cytb*) or 35 (*cox*) cycles at 94°C for 1 min, 48°C (*cytb*) or 53°C (*cox*) for 1 min, 72°C for 1 min, and a single final step at 72°C for 10 min. Purification and sequencing of PCR products were conducted by Macrogen Inc. (http://www.macrogen.com). All sequences were deposited in GenBank (accession numbers: XXXX-XXXX).

4.2.2 PHYLOGENETIC ANALYSES AND MOLECULAR DATING

The electrophoregrams were checked using CHROMAS 2.31 (Technelysium Ltd.), and consensus sequences were aligned with CLUSTALX (Thompson *et al.* 1997). Nucleotide variation was assessed using MEGA 5 (Tamura *et al.* 2011).

Maximum likelihood (ML) and maximum parsimony (MP) methods were used to investigate phylogenetic relationships among haplotypes. A partition-homogeneity test (Farris *et al.* 1994) with 100 replicates was performed in PAUP* 4.0b10 (Swofford 2003) to test the homogeneity of the phylogenetic signal between the two mtDNA fragments. This test did not reject the null hypothesis of the homogeneity of the phylogenetic signal. Thus, subsequent analyses were conducted on the combined dataset. The best-fit model of nucleotide substitution for our dataset was selected using the Bayesian information criterion (Akaike 1973), as implemented in JMODELTEST 0.1.1 (Posada 2008). The TrN+Γ (Tamura & Nei 1993) model was indicated as the best-fit one, with a gamma distribution shape parameter of 0.13.

ML analyses were performed using PHYML 3.0 (Guindon *et al.* 2010). The tree topology was estimated using the SPR&NNI option, and the best-fit model as suggested by JMODELTEST. MP analysis was conducted in PAUP, with all characters equally weighted and unordered. A heuristic search was performed, with tree-bisection reconnection branch swapping and ten rounds of random sequence addition. The robustness of both ML and MP tree topologies was assessed by the non-parametric bootstrap method with 1000 replicates. Finally, the statistical parsimony procedure implemented in the software TCS 1.2.1 (Clement, Posada & Crandall 2000) was used to infer phylogenetic networks among the haplotypes found (Templeton, Crandall & Sing 1992).

Times to the most recent common ancestor (TMRCA) of the main haplogroups and divergence time among them were estimated through the distance-based least squares (LS) method recently described by Xia & Yang (2011) and implemented in the software DAMBE (Xia & Xie 2001). The hypothesis that our sequences evolved in a clock-like manner was tested by means of a likelihood ratio test as implemented in DAMBE. This test did not reject the molecular clock hypothesis. The ML tree as previously estimated by PHYML was used to specify a tree topology, and the divergence between *E. montanus* and its sister species *E. platycephalus* was used to set a calibration point. Caccone *et al.* (1994) suggested that *E. montanus* and *E. platycephalus* began diverging after the separation between Corsica and Sardinia islands was completed (9 My). However, Carranza & Amat (2005) suggested that this divergence would date back to the onset of the Messinian salinity crisis (MSC), 5.5 My. The estimation by Carranza & Amat was calibrated using a hypothesised split time between *Pleurodeles waltl* and *P. poiretii* of 5.33 My, corresponding to the end of the MSC. Nevertheless, an explicit test of this hypothesis against its alternatives showed that

the scenario that would best fit molecular, fossil, and paleogeographic data would involve a much older split among *Pleurodeles* lineages, approximately 14 My (Veith *et al.* 2003). This would suggest an accordingly older split between *E. montanus* and *E. platycephalus*, approximately at the time when Sardinia and Corsica islands began separating, 15 My (see Caccone *et al.* 1994). Furthermore, the use of the MSC as a calibration point has been recently criticised on several grounds (Hewitt 2011b). Therefore, we performed TMRCA estimates setting the split between *E. montanus* and *E. platycephalus* alternatively to 9 My (hereon referred to as calibration I) and 15 My (hereon referred to as calibration II). Finally, the LS analyses in DAMBE were run using the 'softbound' option and the 'MLCompositeTN93' genetic distance, as suggested by Xia & Yang (2011), with 1000 bootstrap re-samplings to obtain standard deviations (s.d.) of the estimates.

4.2.3 POPULATION GENETIC STRUCTURE AND HISTORICAL DEMOGRAPHY

To investigate the geographic pattern of genetic differentiation among populations, we used a modified version of the genetic landscape shape (GLS) interpolation analysis implemented in AIS (Miller 2005). Modifications included the use of the best-fit model of sequence evolution for our data, and a two-dimensional (2D) representation of the GLS, instead of the less easily interpretable 3D version yielded by AIS. A population network was built using the Delaunay triangulation by means of QUANTUM GIS (Quantum GIS Development Team 2012). The net between-population genetic divergences were computed with MEGA 5 (Tamura *et al.* 2011), using the TrN+Γ model of sequence evolution. Divergence values among population pairs connected with edges by the Delaunay triangulation were imported in QUANTUM GIS and georeferenced to the midpoint of the corresponding edges. Finally, an inverse-distance-weighting interpolation procedure was performed to obtain the 2D representation of the GLS.

The amount of genetic variation that can be accounted for by differences among groups of population, among populations within groups, and within populations was assessed by carrying out an analysis of molecular variance (AMOVA) with ARLEQUIN 3.5.1.2 (Excoffier *et al.* 2005). Groups of populations were defined according to the main phylogeographic discontinuities as suggested by previous phylogenetic analyses. The analysis was run using the TrN+Γ model of sequence evolution. The statistical significance of the variance components and fixation indices was tested using 1092 permutations.

Historical demographic trends were investigated through a mismatch distribution analysis (Rogers & Harpending 1992) with the software ARLEQUIN. This distribution is expected to be unimodal and bell-shaped in populations that underwent a sudden demographic expansion, whereas a prolonged demographic stability is expected to lead to a multimodal distribution. The observed distribution and the one expected under a sudden expansion model of population growth were compared (Rogers & Harpending 1992, Excoffier 2004). The sum of square deviations among the observed and estimated mismatch distributions was used as a goodness-of-fit statistic, and its significance was evaluated by means of 1000 parametric bootstrap replicates. From the mismatch distribution analysis, we also estimated the parameter τ, the mutational time since the expansion ($\tau = 2ut$, where u is the mutation rate per sequence and per generation, and t is time in generations). Finally, for each mtDNA lineage, we also computed the R_2 (Ramos-Onsins & Rozas 2002) and the F_S (Fu 1997) statistics by using the software DNASP 5 (Librado & Rozas 2009).

4.2.4 SPATIAL DISTRIBUTION MODELLING

To predict the potential distribution of *E. montanus* under both current and glacial bioclimatic conditions, species distribution models (SDMs) were generated using MAXENT 3.3.3e (Phillips *et al.* 2006), a program for maximum entropy modelling of the geographic distributions of species, based on presence-only data. A total of 104 data points were used to build the models. These included our own 15 collection sites, plus 89 sites drawn from previous literature or from museum collections. We built the models by using the default parameters for convergence threshold (10^{-5}) and number of iterations (500). To ensure the consistency of the model predictions, 75% of the localities were used to train the model and 25% were used to test it.

An SDM was generated under present-day bioclimatic conditions and then projected into last glacial maximum (LGM) conditions. The bioclimatic layers were downloaded from the WorldClim database website (http://www.worldclim.org). For the LGM prediction, we used data from both the Community Climate System Model (CCSM) and the Model for Interdisciplinary Research on Climate (MIROC). Layers for the present conditions are available at a resolution of 30 arc-seconds, while those for the LGM conditions are available at a resolution of 2.5 arc-minutes, which was too coarse-grained

for the geographic scale relevant in this study. Thus, we rescaled these layers at a resolution of 30 arc-seconds, using the following procedure: i) we downloaded the layers at a 2.5 arc-minute resolution also for the present conditions; ii) the difference between present and LGM conditions was computed for each variable with DIVA-GIS 7.5 (Hijmans *et al.* 2001); iii) the obtained differences were then subtracted to the 30 arc-second resolution layers for the present conditions. This procedure has the dual advantage of yielding data at a spatial resolution relevant to the geographic scale of this study and at the same resolution of current data (Rodder *et al.* 2010).

The WorldClim database provides 19 bioclimatic variables. We built two series of models, one with the entire set of variables and the other with only variables that were not strongly correlated to each other (Pearson correlation coefficient, $r^2 < 0.80$), choosing among correlated variables those that we considered more biologically significant for *E. montanus*. These variables included the following: BIO2, mean diurnal range in temperature; BIO4, temperature seasonality (s.d. × 100); BIO5, maximum temperature of warmest month; BIO12, mean temperature of the coldest quarter; BIO14, precipitation of driest month; and BIO16, precipitation of the wettest quarter of the year.

Warren & Seifert (2011) recently showed that the default settings in MAXENT have inferior performances compared with tuned settings, particularly the regularisation multiplier beta (Warren & Seifert 2011). Therefore, as suggested by these authors, we built models with a range of beta values (from 1 to 15) for both the whole set of 19 variables and the reduced set of six variables. Finally, to select the model that best fit the data, we used the Akaike Information Criterion (AIC) as implemented in the software ENMTOOLS 1.3 (Warren *et al.* 2010).

4.3 RESULTS

For the 193 individuals analysed, we obtained a fragment of 643 bp from both the *cytb* gene and the *cox* gene (overall 1286 bp). In the combined dataset, 211 variable positions were found, 172 being parsimony informative. No indels, stop codons, or non-sense codons were observed. A comprehensive number of 97 haplotypes were found, whose distribution among sampled populations is reported in Table 1.

4.3.1 PHYLOGENETIC ANALYSES AND MOLECULAR DATING

The ML and MP algorithms yielded phylogenetic trees with essentially identical topologies, with minor differences limited to terminal nodes. The MP tree length was 452 steps (consistency index = 0.68; retention index = 0.93). The log-likelihood score for the ML tree was -4421.33. The ML tree is shown in Fig. 1B. Five main clades were found (clades A, B, C, D, and E), whose geographic distribution is shown in Fig. 1A. Four of these clades (clades B, C, D, and E) were geographically restricted to no more than two populations in the northern portion of the island, whereas a single clade (clade A) was widespread throughout central and southern Corsican populations. The average genetic divergence (ML corrected) among these five clades ranged between 0.049 (0.009 s.d.; clades B and A) and 0.082 (0.014 s.d.; clades B and C; see Table S2 in Appendix). Within clade A, three sub-clades were found, each supported by high bootstrap values. Throughout the south-central and southern samples, sub-clade AI was either fixed (samples 1–5) or the most abundant, while sub-clades AII and AIII were restricted to the central Corsican populations (samples 6–9). The average genetic divergence (ML corrected) among these sub-clades ranged between 0.011 (0.003 s.d.; clades AIII–AII) and 0.015 (0.004 s.d.; clades AI–AIII). Also within clades C and E, two well-differentiated sub-clades were observed, showing genetic divergences of 0.013 (0.004 s.d.) and 0.021 (0.005 s.d.), respectively.

The phylogenetic networks among haplotypes are shown in Fig. 1C. On the basis of the 95% criterion for a parsimonious connection, it was not possible to connect all the haplotypes into a single network. Instead, six separate networks were built, corresponding

to the four main clades A, B, C, and D, and the two sub-networks EI and EII. A single star-like structure was observed, within the sub-clade AI.

Estimates of the TMRCAs for the main clades and sub-clades are shown in Fig. 2 and presented in detail in Table S1 (see Appendix). As expected, the two alternative calibrations led to substantially different estimates, although some common patterns were clearly apparent. TMRCAs for nodes I, G, and H were very close in time to one another, indicating that the divergence between clades C, D, E, and B + A occurred within a very short time lap, in the late Miocene according calibration II, or in the Pliocene according to calibration I. Also of Pliocene origin would had been the divergence between clades A and B according to both calibrations I and II. Finally, according to both the alternative calibrations, the divergences within each of these clades (i.e. AI – AII - AIII, CI - CII, and EI - EII) were dated to the Early Pleistocene, whereas most of the TMRCAs of the terminal haplogroups fell well within the Middle Pleistocene.

4.3.2 Population genetic structure and historical demography

The genetic landscape shape interpolation analysis showing the geographic pattern of distribution of genetic differentiation among populations is presented in Fig. 3. Two main areas are clearly present: one area of extensive homogeneity, encompassing the southern and central-western portions of the island, and one area of high differentiation in the northern and central-eastern portions. Within the latter, spotted areas of low differentiation are also apparent.

AMOVA analysis was performed by separating populations according to the geographic distribution of the nine terminal haplogroups. Since haplogroups CI and CII showed an identical distribution, eight population groups were defined: [1-6], [7, 9], [8], [10, 11], [12], [13], [14], and [15]. With this grouping option, the AMOVA analysis indicated that 77.3% of the overall genetic variation can be attributed to the among-group level of variation (F_{CT} = 0.77), 4.5% to the among-population within-group level (F_{SC} = 0.20), and 18.2% to the within-population level (F_{ST} = 0.82), all variance components and fixation indices being highly statistically significant ($P < 0.001$).

Mismatch distribution analyses and values of the demographic test statistics for the main terminal haplogroups are presented in Fig. 4. We limited the analyses to those haplogroups having a sample size of not lower than 10 individuals. The only haplogroups

for which all analyses converged in indicating a recent demographic expansion were AI, CII, and D, whereas for haplogroup B all analyses rejected this scenario. Finally, a demographic growth was suggested for haplogroups AII and AIII just by the test statistics R_2 and F_S, respectively.

4.3.3 SPATIAL DISTRIBUTION MODELLING

The model comparison conducted in ENMTOOLS indicated that our selection of six bioclimatic variables fit the data better than the entire set of 19 variables, and that the model receiving the lowest AIC was the one built using the value 3 for the regularisation multiplier beta parameter. This model yielded AUC scores of 0.73 and 0.67 for the training and the test data respectively, indicating a good performance of the model. Among the selected variables, the one giving the highest percentage contribution to the model (57%) was BIO 14, i.e. the precipitation during the driest month.

The SDM under the present bioclimatic conditions (Fig. 5A) indicated high suitability for most of the island area (as expected on the basis of the known species distribution), with exceptions for mountain tops and coastal areas, especially southern and eastern lowlands.

Projections of the model over LGM bioclimatic conditions were performed using both the MIROC and CCSM databases. Nevertheless, several variables were outside the training range when the CCSM database was used, indicating poor predictive value of this projection (see Elith *et al*. 2010). Therefore, only the model built using the MIROC database was considered further (Fig. 5B). This model suggested that during the LGM, areas of very high suitability for the species were restricted to the southern and eastern portions of the island. southern and eastern lowlands appeared more suitable for the species than under present-day conditions, while moderate to high suitability was still present in the northern lowlands. The least suitable areas were those located at higher altitudes, throughout the central mountain chain. On the whole, although bioclimatic suitability appeared reduced during the LGM with respect to present conditions in the northern and western portions of the island, in the southern and the eastern portions wider areas of high suitability seems to have been present for the species during the LGM than at present.

4.4 DISCUSSION

The continuous geographic distribution of *E. montanus* populations throughout its restricted range would have plausibly predicted a phylogeographic pattern without substantial discontinuities, suggestive of a single large population (e.g. IUCN 2011). Instead, we found evidence of a geographic mosaic of deeply divergent and ancient evolutionary lineages, a pattern which also has major implications for the species' conservation. In the subsequent sections, we will first discuss these issues, together with a cautionary note on the possible taxonomic implications of our results. Then, we will evaluate our data in the context of the intra-island patterns of genetic diversity, to highlight some key issues arising.

4.4.1 EVOLUTIONARY HISTORY OF *EUPROCTUS MONTANUS*

Five main lineages (clades A, B, C, D, and E) were clearly apparent by phylogenetic analyses. Their genetic divergence (shown in Table S2) is conspicuous, largely exceeding the one observed among several species pairs of European newts, such as *Calotriton asper–C. arnoldii* (Carranza & Amat 2005), *Triturus marmoratus–T. pygmaeus* (Carranza & Amat 2005), and *T. carnifex–T. macedonicus* (Arntzen *et al.* 2007). Furthermore, the geographic scale at which these divergences were observed is surprisingly small. Four lineages were restricted to one or two populations each, all located in the northern portion of the Corsica Island, whereas the fifth lineage (A) was the only one extending its distribution also to the central and southern portions of the island. Furthermore, even within lineage A, two sub-clades were geographically restricted to northern Corsica, whereas only sub-clade AI was found in the southern portion of the island. Thus, almost the entire genetic differentiation observed within *E. montanus* was found restricted to northern Corsica (see Fig. 3).

Irrespective of the calibration used to date the divergence events, as shown in Fig. 2 and Table S1, the three basal splits (leading to lineages A + B, C, D, and E) are likely to have occurred at the same time or at least within a very short time lapse, between 3.6 and 5.8 My depending on the calibration used, whereas the split between lineages A and B

likely occurred somewhat later (2.6–4.1 My). Interestingly, an estimate of the divergence time very close to those we found among the four basal lineages of *E. montanus* was obtained for two lineages of the lizard *Archeolacerta bedriagae* which are parapatric in northern Corsica (3.7–5.9 My; Salvi *et al.* 2010), and also the land snail *Solatopupa guidoni*, the isopod *Helleria brevicornis*, and the shrubby plant *Cystus creticus* showed main phylogeographic breaks among divergent lineages in northern Corsica (Falchi *et al.* 2009, Gentile *et al*. 2010, Ketmaier *et al.* 2010).

As pointed out in previous studies (Salvi *et al.* 2010), there are no obvious geographic barriers to dispersal in northern Corsica that could be indicated as plausible causal factors for the observed divergences, and there are no evidence that they could have been active in the past. Furthermore, the clustering of divergence times among several lineages of *E. montanus* discount the role of geographic factors acting at a local scale in explaining the pattern, and point to processes acting at a range-wide scale. Among these factors, those that could best accommodate both the ancient and more recent divergences found within *E. montanus* seem to be the Plio-Pleistocene climatic changes.

According to our modelling of the bioclimatic niche (Fig. 5), the precipitation of the driest month would account for most of the pattern of distribution of *E. montanus* (57%). Interestingly, as suggested by Suc (1984) on the basis of palynological and macroflora analyses, during the Pliocene and Early Pleistocene, the paleoenvironmental evolution of north-western Mediterranean was characterised by a progressive decrease in moisture, with two main events: the installation of summer dryness, favouring forest clearing, approximately 3.2 My ago, and the first dry period with development of stepping associations, at approximately 2.3 My. These estimates would fit particularly well with our divergence estimates for the four main lineages and for A vs. B (see calibration I in Table S1), particularly when the expected time lag between genetic and population divergences is considered (Hein *et al.* 2005; Edwards & Beerli 2000).

A close link between moisture regimes and the demographic history of *E. montanus* would also comfortably explain the time estimates of subsequent splits and TMRCAs within each main clade to the Early and Middle Pleistocene, respectively, when cycles of humid-dry climate would have followed the initiation of the glacial-interglacial oscillations (see Thompson 2005 for an account of climatic oscillations and the consequent vegetational changes). Indeed, the SDM indicated that habitat suitability decreased in northern Corsica during phases of increased drought, as were the Pleistocene glacial

phases. On the other hand, the long-term persistence in northern Corsica of *E. montanus* populations is testified just by the occurrence in this area of most of the lineages found. Thus, it can be argued that during these phases, *E. montanus* populations did not disappear but underwent fragmentations and demographic contractions into small areas of more suitable microclimatic conditions. This scenario would be reminiscent of the so-called microrefugia scenario (see Rull 2009, Mosblech *et al.* 2011 and references therein). Since *E. montanus* is an essentially montane species living close to streams and brooks, and considering the topographic features of Corsica, we speculate that such small areas of microrefugia could have been provided by some mountain river valleys, which are so abundant on the island, have been already reported for several continental species, and are more likely to present significant decoupling between local and regional climate trends (Dobrowski 2010).

Contrary to northern Corsica, in the central and southern portions of the island, we observed a substantial genetic homogeneity (Figs. 1A and 3), with the single clade A spanning the entire area. The TMRCAs of clade A and its sub-clades suggest the persistence of *E. montanus* populations in this area over multiple glacial/interglacial cycles. Bioclimatic data are not available for Early and Middle Pleistocene glaciations; however, the SDM analysis for the LGM suggests that highly suitable bioclimatic conditions occurred in this area also during the glaciation. This is consistent with recent data from Kulheman *et al.* (2008) suggesting that while northern Corsica was substantially invaded by polar air during the LGM, milder climatic conditions were present more to the south. Thus, genetic data, SDM analysis, and paleoclimatic reconstructions concordantly support the possible occurrence of a wider refugium to the south than to the north. Furthermore, when the SDMs for present day and the last glaciation are compared, it seems that while highly suitable areas were much less extended during the LGM than at present in northern Corsica, more to the south they were even wider during the LGM. Thus, populations of southern Corsica could have expanded rather than contracted during the LGM, as already suggested for several montane species (Hewitt 2011a). In this respect, it is also worth nothing that among the sub-clades showing concordant signs of recent demographic expansion (clades AI, CII, and D) across multiple tests (F_S, R_2, mismatch distribution analysis, see Fig. 4), the higher value of the parameter τ (i.e. the time since the expansion in generations, scaled by the mutation rate) was obtained for clade AI (and very similar values were obtained for AII and AIII, also showing some evidence of recent

expansion), suggesting that its expansion phase could in fact have predated those of the northern sub-clades.

Finally, throughout the above discussion, we considered the phylogeographic pattern as the likely outcome of historical processes. Nevertheless, several authors pointed out that phylogeographic breaks could arise not only in response to historical events but also as a result of stochastic processes (Irwin 2002, Kuo & Avise 2005). Discriminating between the two cases can be difficult when evidence from multiple kinds of markers (e.g. mitochondrial and nuclear data) are not available. Nevertheless, in the present case, two main lines of evidence converge in indicating that the observed pattern is not spurious. First, stochastic processes are not expected to yield a pattern of multiple divergences that are both spatially or temporally clustered, as we found in northern Corsica and for the TMRCAs of both the main lineages and several sub-clades (Avise 2008). Second, stochastic processes are not expected to yield patterns of concordance (either spatial or temporal) among multiple co-distributed taxa, as is the case here (see Gentile *et al*. 2010, Ketmeier *et al.* 2010, Salvi *et al.* 2010).

4.4.2 Implications for taxonomy and conservation

Although most of the genetic variation of *E. montanus* was found among northern Corsican populations (i.e. within an area of limited extent), the only evidence of syntopy among main clades was observed within sample 14 for clades EI and B. This lack of evidence for a more diffuse syntopy among main clades is quite surprising. Indeed, as mentioned above, there are neither obvious geographic barriers to dispersal for *E. montanus* in northern Corsica nor evident bioclimatic barriers (see Fig. 5), as also witnessed by its rather continuous distribution. The genetic imprints of recent range expansions for several sub-clades would also dismiss the hypothesis that the species failed to expand after the last or previous glaciations. Furthermore, this hypothesis would also appear implausible when considering that the species can be actually found on several mountain tops in northern Corsica (Delaugerre & Cheyla 1992), i.e. in areas that were covered by glaciers throughout the LGM (Kulheman 2005), which implies post-glacial altitudinal migrations. Finally, a more pronounced female phylopatry—which would lead to a more structured phylogeography of the maternally inherited mtDNA with respect to most of the biparentally inherited nuclear genes—seems rather improbable as well. Indeed,

first, we should assume a substantial lack of female dispersal for not less than 3 My; second, this does not fit with the already mentioned continuous distribution of breeding sites in the area, which obviously implies a continuous distribution of females. Thus, the most plausible explanation for the observed pattern of distribution of genetic variants seems some form of intrinsic barrier to gene exchange among distinct evolutionary lineages. However, this inference will need to be confirmed by a more extensive sampling in northern Corsica. Moreover, the exact nature of this barrier will need to be further investigated and cannot be indicated here. The close geographic proximity among reciprocally monophyletic lineages that are so genetically differentiated and whose estimated times of divergences are so ancient would suggest the possibility that these lineages are in fact distinct species, both under the biological species concept (Mayr 1942) and in comparison with that observed among closely related European newts (see citations above). Nevertheless, two features of our data do not allow solving this issue: first, in light of the continuous geographic distribution, contact populations among main lineages should exist, and thus the study of their genetic structure will be mandatory; second, being haploid, the mtDNA is inappropriate to reveal the occurrence and extent of admixture among lineages, and thus the use of nuclear markers will be mandatory as well. Moreover, both the way of inheritance (uniparental vs. biparental) and the selective regimes differ among mitochondrial and nuclear genomes, and thus comparisons among the respective patterns of geographic variation will help obtain deeper insights into the interactions among lineages.

The overall pattern of genetic variation that we have found in *E. montanus* has at least two main implications for the species' conservation. First, northern Corsica has emerged as the core area for its conservation. This is clearly indicated by the occurrence in this area of most of the genetic variation found in this species, and of all the most deeply divergent evolutionary lineages. Furthermore, a special focus of conservation practices on this area would benefit other species as well, which also have their most differentiated lineages found in close contiguity here (Gentile *et al*. 2010, Ketmeier *et al.* 2010, Salvi *et al.* 2010). Second, and most importantly, this species can no longer be considered as if it were formed by a single panmictic population ranging the whole island (IUCN 2011). Our data clearly show that it is strongly fragmented into several reciprocally monophyletic lineages of ancient origin, which thus form distinct evolutionary significant units (ESUs sensu Moritz 1994), deserving separate conservation efforts. In this respect, it is worth

recalling that even if future studies based on nuclear markers would show a less structured, more admixed pattern of population genetic structure, as pointed out by Avise (2008), the pattern shown by the maternally inherited mtDNA would in any case be evidence of demographic independence of each evolutionary lineage, which would thus deserve specific conservation efforts.

4.5 PERSPECTIVES AND CONCLUSIONS

Species or populations with a continuous geographic distribution across restricted ranges have long been thought as single homogeneous entities, a prediction particularly pervasive in studies involving island endemics (e.g. Frankham 1996a, 1996b). Notably, it is a necessary premise in order to consider island size as a correlate of effective population size, for instance in comparisons of diversity patterns among islands of different sizes and with the continent (Frankham 1996a, 1996b, Burton 1998, Woolfit & Bromham 2005). Such comparisons have been extensively used in the study of ecological and evolutionary processes (see e.g. Grant 1998, Whittaker & Fernandez-Palacios 2007). Furthermore, this prediction has also been commonly used in assessments of biodiversity conservation priorities. For instance, not only *E. montanus* but also the other two Corsican endemic amphibians, *Salamandra corsica* and *Discoglossus montalenti*, were not assigned to higher categories of the IUCN Red List of Threatened Species, mostly based only on this prediction (IUCN 2011). As discussed above, our results concerning the genetic structure of *E. montanus* do not fit this prediction at all, as we found a pattern of strong phylogeographic structure and ancient divergences at different time scales among several evolutionary lineages in this species. This pattern appears somewhat extreme—particularly for a vertebrate species—and unlikely to be a general rule; however, according to the limited but growing literature about intra-island phylogeographic variation of island endemics, similar patterns of strong intra-island differentiations and ancient lineages are increasingly emerging in many world regions for a wide range of taxonomic groups (see, e.g. Thorpe & Malhotra 1998, Holland & Hadfield 2002, Wallos & Trewick 2009). On the whole, in light of these findings, an expectation of panmixia in species with a restricted range, especially island endemics, could in fact be unwarranted or at least far from being of universal value.

Finally, the geographic coincidence of areas of long-term species persistence—as are glacial refugia and islands—and hotspots of genetic diversity has been long explained as the outcome of higher climatic stability of these areas (Hewitt 1996, Whittaker & Fernandez-Palacios 2007, Blondel *et al*. 2010), which would have favoured the maintenance of large, stable, and thus genetically diverse populations. In recent years,

however, phylogeographic data about populations from glacial refugia are increasingly showing that this is not always the case. Rather, hotspots of intraspecific biodiversity are often the outcome of a complex array of microevolutionary processes, both directly or indirectly driven by paleoenvironmental changes that occurred within the refugia (Gomez & Lunt 2006, Canestrelli *et al*. 2006, 2010). Interestingly, the growing phylogeographic literature on island endemics parallels these recent findings (e.g. Thorpe & Malhotra 1998, Emerson *et al.* 2006, Villacorta *et al.* 2008, Wallos & Trewick 2009, Bisconti *et al.* 2011), and the evolutionary history of the Corsica endemic newt *E. montanus* well exemplifies this. Indeed, these data are increasingly showing that a plethora of microevolutionary processes that often occurred within islands have played the major role in shaping insular patterns of genetic diversity. Consequently, the extensive use of several simplifying assumptions usually made on island populations, including range-wide panmixia, an effective population size correlated to island size, and long-term stability, will deserve to be deeply reconsidered in the future.

4.6 TABLES AND FIGURES

Table 1 Geographic location, sample size (n) and haplotypes found, for each of the 15 sampling sites of *Euproctus montanus*.

	Locality	Latitude N	Longitude E	n	Haplotypes (n)
1	Naseo	41°34’	9°05’	11	AI19(4);AI20(1); AI22(1); AI23(5)
2	Agnarone	41°40’	9°11’	15	AI1(4); AI4(1); AI5(1); AI9(2); AI14(3); AI21(2); AI25(1); AI32(1)
3	Spartu	41°46’	9°13’	17	AI2(1); AI3(1); AI6(1); AI7(1); AI8(1); AI9(5); AI13(1); AI15(1); AI16(1); AI17(1); AI33(2); AI37(1)
4	Col de Bavella	41°48’	9°14’	19	AI9(8); AI16(1); AI18(2); AI27(3); AI31(1); AI34(1); AI35(1); AI36(1); AI37(1)
5	Scrivano	41°57’	9°11’	1	AI9(1)
6	Col de Verde	42°02’	9°11’	18	AI10(2); AI11(1); AI12(1); AI24(1); AI28(1); AI29(4); AI30(7); AII43(1)
7	Vizzavona	42°07’	9°08’	18	AI26(2); AI 30(2); AII44(1); AIII54(3); AIII55(4); AIII56(2); AIII58(1); AIII59(2); AIII60(1)
8	Gorges de la Restonica	42°16’	9°05’	13	AII39(2); AII40(1); AII41(3); AII42(1); AII45(1); AIII57(5)
9	Aitone	42°15’	8°50’	13	AII38(1); AIII46(1); AIII47(2); AIII AIII48(2); AIII49(1); AIII50(1); AIII51(3); AIII52(1); AIII53(1)
10	Parata	42°22’	9°24’	9	CI71(1); CI73(1); CII78(6); CII80(1)
11	San-Gavino-d'Ampugnan	42°24’	9°25’	13	CI70(1); CI72(3); CII74(1); CII75(1); CII76(1); CII77(1); CII79(1); CII81(1); CII82(3)
12	Forêt Communale d'Asco	42°25’	8°57’	3	EII95(1); EII96(1); EII97(1)
13	Campitello	42°31’	9°20’	21	B63(1); B64(2); B65(5); B67(2); B68(8); B69(3)
14	Bocca Capanna	42°33’	9°03’	6	B61(1); B62(1); B66(1); EI93(2); EI94(1)
15	Vignale	42°57’	9°23’	16	D83(1); D84(2); D85(2); D86(1); D87(1); D88(2); D89(4); h90(1); D91(1); D92(1)

APPENDIX

Table S1 TMRCAs for the main clades and subclades identified by the phylogenetic analyses, based on two alternative estimates of the split time between *E. montanus* and *E. platycephalus*. Times are given in million years, with standard deviations in brackets.

Clade	Calibration I	Calibration II
AI	0.439 (0.085)	0.656 (0.130)
AII	0.338 (0.095)	0.547 (0.168)
AIII	0.263 (0.083)	0.419 (0.130)
B	0.573 (0.132)	0.989 (0.245)
CI	0.183 (0.088)	0.295 (0.139)
CII	0.145 (0.070)	0.228 (0.092)
D	0.253 (0.073)	0.389 (0.126)
EI	0.050 (0.044)	0.089 (0.072)
EII	0.238 (0.085)	0.346 (0.149)
AII+AIII	0.727 (0.180)	1.159 (0.321)
C	0.788 (0.209)	1.263 (0.270)
A	0.940 (0.197)	1.462 (0.290)
E	1.380 (0.283)	2.086 (0.393)
F	2.640 (0.380)	4.082 (0.491)
G	3.636 (0.390)	5.586 (0.820)
H	3.725 (0.362)	5.715 (0.713)
I	3.790 (0.363)	5.833 (0.676)

Table S2 Genetic divergence (TrN+Γ) among the five main clades of *E. montanus* identified by phylogenetic analyses. Average estimate and their standard deviations are given below and above the diagonal respectively.

	A	**D**	**E**	**B**	**C**
A	-	0.012	0.011	0.009	0.013
D	0.066	-	0.013	0.014	0.014
E	0.066	0.075	-	0.014	0.014
B	0.049	0.081	0.079	-	0.014
C	0.072	0.074	0.081	0.082	-

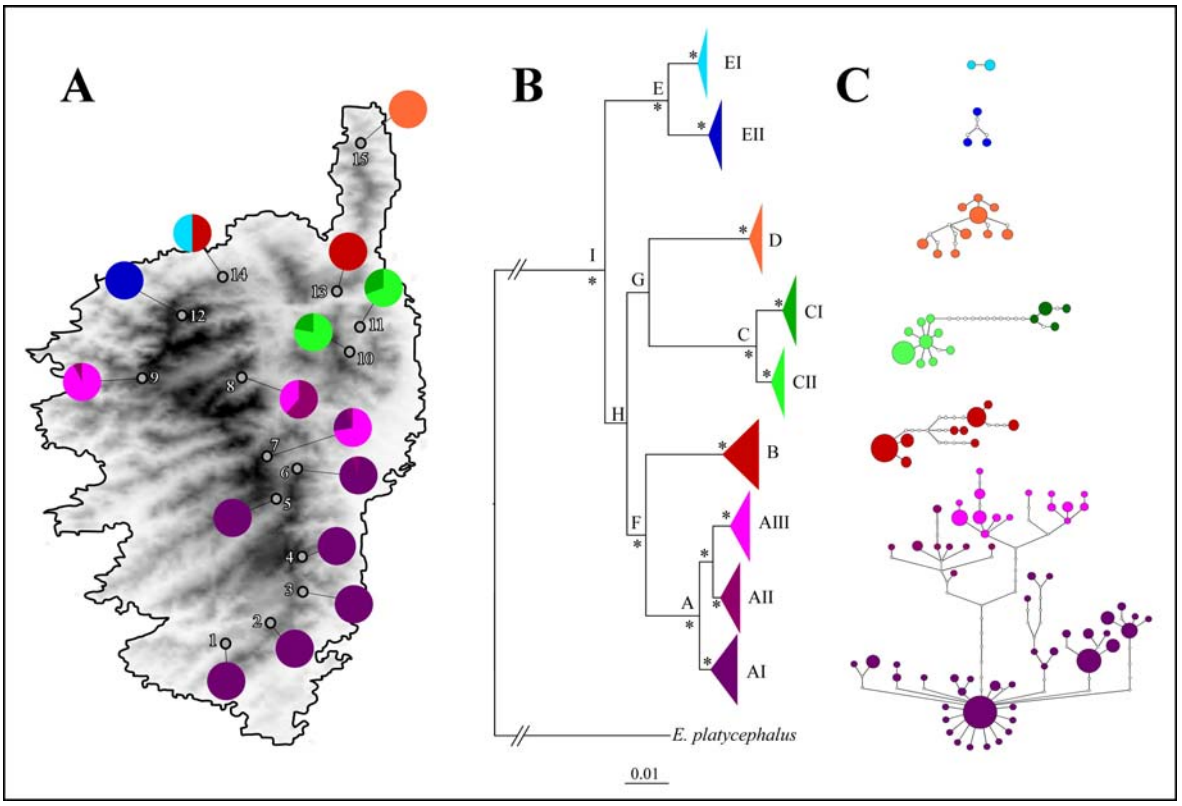

Figure 1 (A) Geographic location of the 15 sampled populations of *Euproctus montanus*, and frequency distribution of the main haplogroups, shown as pie diagrams. Populations are numbered as in Table 1. (B) Maximum likelihood (ML) phylogenetic tree based on the TrN+Γ model of sequence evolution. Nodes receiving bootstrap supports >70% with both ML and MP tree-building methods are marked by an asterisk; terminal haplogroups were collapsed. (C) Phylogenetic networks based on the statistical parsimony procedure. Circle sizes are proportional to haplotype frequency; missing intermediate haplotypes are shown as open dots.

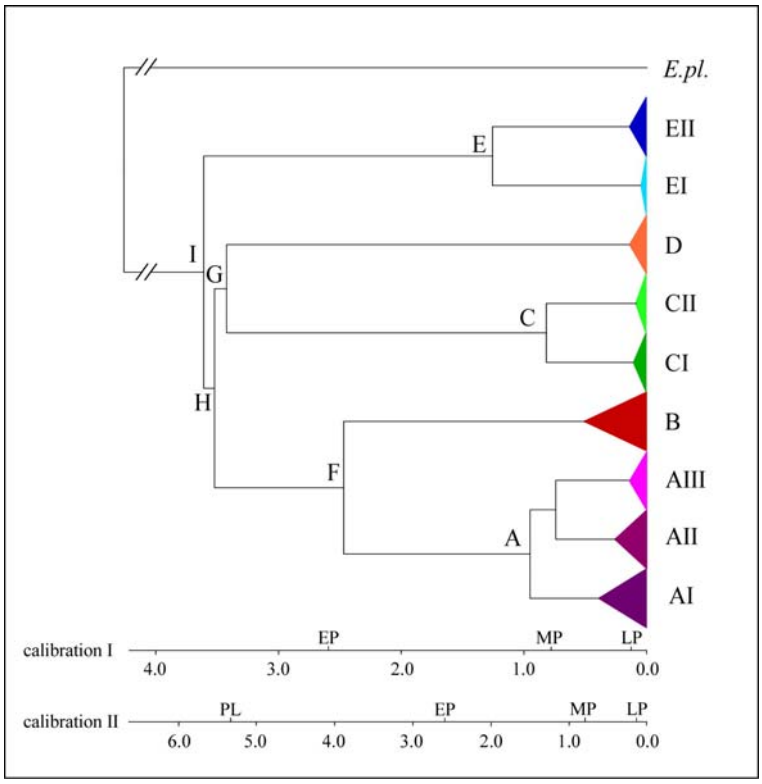

Figure 2 Chronogram based on the ML phylogeny of the haplotypes found in *E. montanus*, estimated using the least-square procedure of Xia & Yang (2011). Scale axes are in million years. Above the axes, the beginning of the main historical epochs mentioned in the main text is reported: PL=Pliocene; EP=Early Pleistocene; MP=Middle Pleistocene; LP=Late Pleistocene. See Table S1 for estimated values and relative standard deviations.

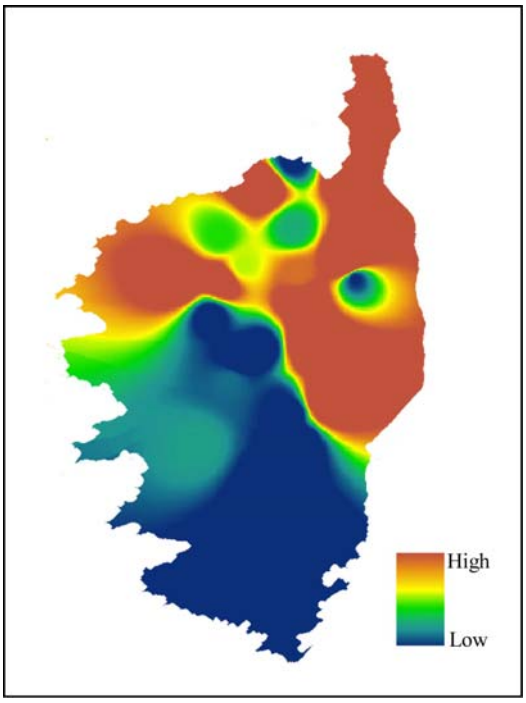

Figure 3 Genetic landscape shape showing genetic differentiation among populations, based on an inverse distance weighted interpolation of the estimated Nei's (1987) net between-population genetic divergence (TrN+Γ corrected). Warmer colours represent higher levels of genetic differentiation.

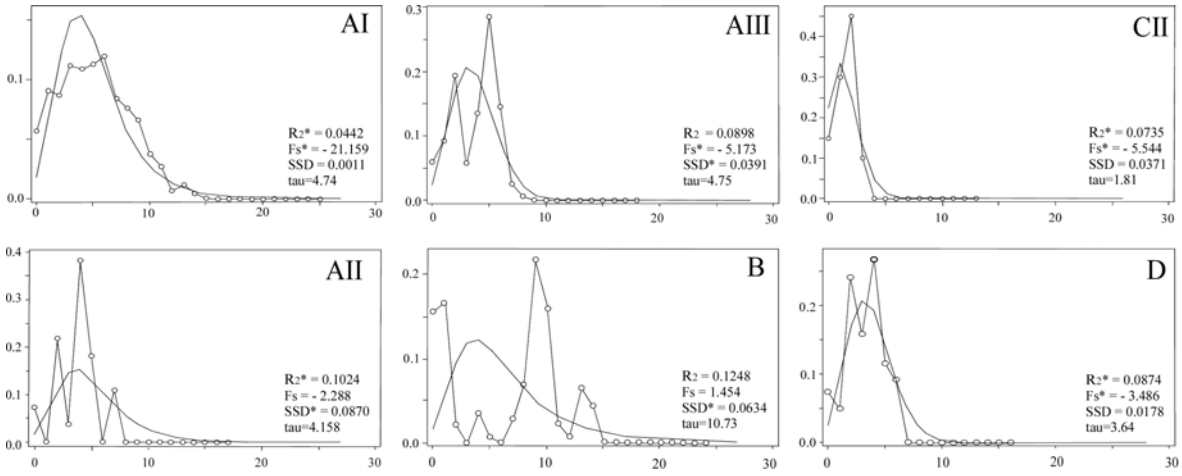

Figure 4 Mismatch distributions and values of the demographic test statistics for six terminal haplogroups identified with phylogenetic analyses. Only haplogroups having sample size >9 were considered. r: raggedness statistic (Harpending, 1994). F_S: Fu's F_S statistic (Fu 1997). R_2: Ramos-Onsins & Rozas' (2002) R_2 statistic. *P < 0.05. Dotted line: observed mismatch distribution; continuous line: mismatch distribution expected under a pure demographic expansion model.

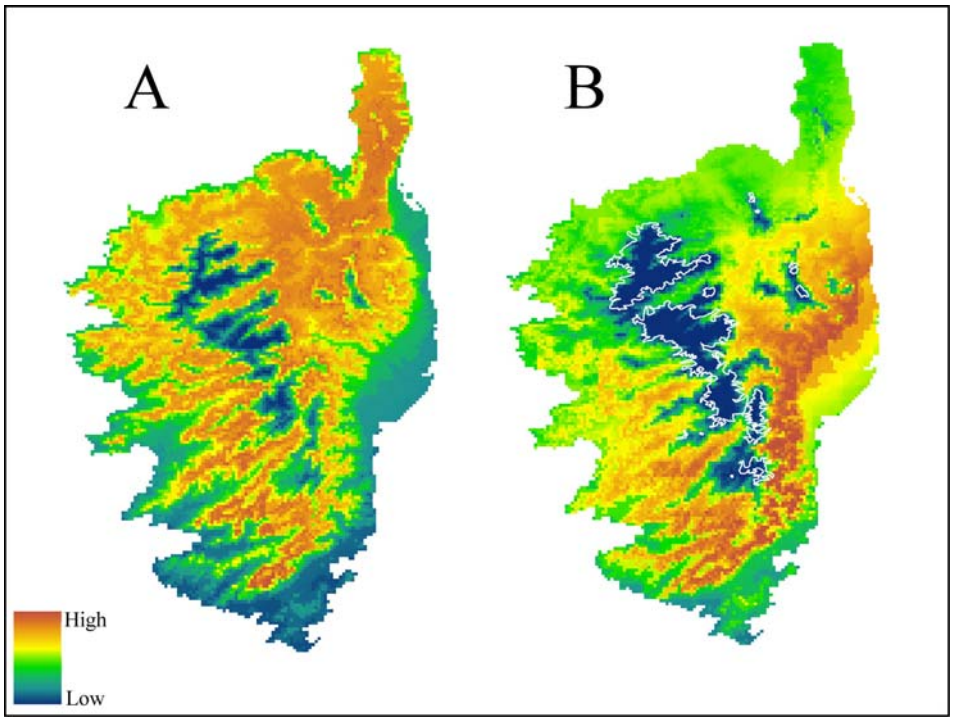

Figure 5 Species distribution models for *E. montanus*, estimated for present-day conditions (A) and for the last glacial maximum (LGM), based on the Model for Interdisciplinary Research on Climate (MIROC) paleoclimatic model (B). Warmer colours represent higher predicted suitability. White line shows the maximum extent of glaciers during the LGM (according to Kulhemann 2005).

5. DISCUSSIONE

I sistemi insulari sono stati a lungo ritenuti, e lo sono tutt'oggi, eccellenti laboratori naturali, modelli per lo studio dei processi ecologici ed evolutivi (Darwin 1859, Wallace 1880, MacArthur & Wilson 1967, Grant 1998, Grant & Grant 2007, Whittaker & Fernandez-Palacios 2007). Infatti, le popolazioni e le comunità insulari sono considerate sistemi ecologicamente semplici, se comparate a sistemi continentali. Dal punto di vista della diversità genetica, i fattori principali che si ritiene regolino le dinamiche ed i livelli di diversità in contesti insulari sono: 1) la dimensione dell'isola; 2) il grado di isolamento (i.e. i livelli di scambio genico con altre isole e con il continente); 3) le dimensioni demografiche dell'evento di fondazione, per quelle specie che hanno colonizzato l'isola dopo la sua formazione (Frankham 1996a, 1996b). Inoltre, si è anche ritenuto che l'effetto tampone esercitato dalle aree circostanti marine sulle oscillazioni del clima, determinando condizioni ambientali più stabili di quelle continentali, potesse determinare una maggiore stabilità demografica delle popolazioni insulari rispetto a quelle di aree continentali (per una estesa discussione sulla diversità in sistemi insulari si veda Whittaker & Fernandez-Palacios 2007, Cronk 1997). In termini di genetica delle popolazioni questi assunti si traducono nell'idea che i processi principalmente imputati nel determinare i livelli di diversità di popolazioni insulari siano mutazione e deriva genetica (Frankham 1996a, White & Searle 2007 e riferimenti all'interno), con una proporzione fra le due dipesa dalla dimensione dell'isola. Infine, alla base di questi assunti vi è l'idea che le popolazioni insulari siano ben approssimate da singole unità panmittiche (e.g. Frankham 1996a, Woolfit & Bromham 2005).

Sulla base di queste premesse, il pattern di diversità genetica atteso per specie quali quelle qui studiate avrebbe dovuto prevedere: 1) una certa omogeneità genetica all'interno delle singole isole; 2) limitatamente alla raganella, un certo grado di differenziamento fra popolazioni delle diverse isole (peraltro atteso anche sulla base di dati preliminari sul differenziamento morfologico; Rosso *et al*. 2001); 3) livelli di diversità genetica inferiori a quelli delle affini specie continentali, ad areale ben più ampio e meno frammentato. I risultati ottenuti in questa tesi mostrano, tanto per la raganella sarda quanto per l'euprotto corso, un quadro del tutto difforme da quello atteso.

Per quanto riguarda la raganella sarda, la principale discontinuità filogeografica è stata osservata non fra popolazioni di isole diverse, ma fra popolazioni diverse all'interno della Sardegna, in particolare fra i campioni dell'area sud-orientale e quelli di tutto il resto dell'areale. Per contro il differenziamento fra popolazioni delle diverse isole è risultato nullo a tutti i marcatori. Inoltre, a livello mitocondriale, anche i differenziamenti di minore entità (quelli fra subcladi del clade N) sono stati osservati tutti all'interno della Sardegna, mentre la porzione settentrionale di quest'isola, la Corsica e le altre isole minori sono risultate del tutto omogenee. Infine, le popolazioni di *H.sarda* hanno mostrato livelli di diversità genetica pari o superiori, e non inferiori, a quelli delle specie affini continentali. Infatti, per un area geografica che complessivamente abbraccia l'intero Paleartico occidentale, studi precedenti sulla diversità allozimica di popolazioni delle specie continentali *H.arborea*, *H.savigny*, *H.intermedia* e *H.meridionalis* (Busack 1986, Nevo & Yang 1979, Canestrelli *et al.* 2007a), hanno mostrato livelli superiori di variabilità solo nei campioni della ristretta zona di contatto fra *H.intermedia* e *H.arborea* (situata nell'area del carso triestino), interessati da ibridazione introgressiva interspecifica (Verardi *et al.* 2010). Inoltre, anche a livello mitocondriale, sia la ricchezza di aplotipi complessivamente trovati, sia la diversità intrapopolazionale sono risultati del tutto comparabili con le specie sopra citate (v.d. Canestrelli *et al.* 2007b, Recuero *et al*. 2007, Stock *et al.* 2008, Gvozdik *et al*. 2010).

Anche la storia evolutiva tardo Pleistocenica della raganella sarda, come qui ricostruita, è del tutto difforme da quella attesa sulla base degli assunti poc'anzi citati. Infatti, ci saremmo aspettati, se non un calo demografico delle popolazioni di *H.sarda*, almeno un certo grado di stabilità demografica all'interno delle isole durante la passata epoca glaciale. Le ipotesi fino ad oggi formulate suggeriscono infatti che il mare avrebbe avuto un effetto tampone sulle oscillazioni climatiche, facendo risultare le isole ambienti climaticamente stabili (Cronk 1997). I repentini e drastici cambiamenti climatici del Pleistocene che hanno afflitto gli ambienti continentali, causando contrazioni ed espansioni dell'areale delle specie temperate europee, sarebbero dovuti essere pressoché privi di effetti in ambiente insulare, traducendosi nella demografia delle specie come stabilità delle popolazioni. Invece quello che mostrano le analisi sulla demografia storica della raganella sarda è una marcata espansione demografica proprio durante l'ultima importante fase glaciale. L'evento di espansione sarebbe avvenuto in Sardegna, mentre in Corsica la specie si sarebbe estinta nel glaciale e, in una fase tardiva dell'espansione, avrebbe ri-colonizzato

quest'isola e le altre dell'arcipelago Toscano a partire dalla vicina Sardegna. La Corsica, vista la sua posizione più settentrionale e le condizioni climatiche più rigide, sarebbe stata verosimilmente inospitale per la specie durante le ultime glaciazioni; mentre in Sardegna, oltre alle condizioni climatiche più favorevoli, si sarebbero rese disponibili nuove aree a seguito delle regressioni marine, aree ecologicamente idonee ad ospitare popolazioni consistenti della specie (Kuhlemann *et al*. 2008, Vogiatzakis *et al*. 2008). Questo scenario risulta decisamente insolito per una specie insulare. Tuttavia, è interessante notare come sia stato invocato in modo invece piuttosto ricorrente per le specie temperate continentali. Queste subivano contrazioni dell'areale nelle aree settentrionali europee durante la fase glaciale, proprio come è successo nell'isola a nord, la Corsica, seguite da ri-colonizzazioni a partire dalle aree rifugio meridionali durante le fasi intergraciali, in questo caso dall'isola meridionale, la Sardegna.

A questo punto vale la pena ricordare che non solo la letteratura sulla diversità in specie insulari, ma anche quella sulla diversità in aree di rifugio glaciale avrebbe suggerito un pattern atteso ben diverso da quello osservato per la raganella sarda. Come poc'anzi ricordato, la letteratura che descrive i pattern di diversità genetica in specie continentali europee ha individuato aree di rifugio ed hotspot di diversità principalmente nelle penisole mediterranee. Secondo lo scenario più spesso invocato, all'interno di queste penisole le popolazioni sarebbero state stabili durante le fasi glaciali e, non subendo oscillazioni demografiche, avrebbero mantenuto dimensioni tali da preservare il loro patrimonio genetico (Hewitt 1996). Nel caso della raganella sarda, le dinamiche microevolutive innescate dalle oscillazioni pleistoceniche del clima sarebbero state del tutto diverse e piuttosto inaspettate. Ora, la discordanza principale coi pattern inferiti per la gran parte delle specie europee sta nel fatto che, all'interno dell'area rifugio (la Sardegna) la raganella non sarebbe rimasta demograficamente stabile, piuttosto avrebbe subito una forte espansione demografica proprio durante la glaciazione. Questo evento, decisamente plausibile a fronte di un fattore principale, ovvero l'aumento sostanziale delle piane costiere, avrebbe impedito la perdita di diversità genetica attesa per effetto della riduzione di habitat, e quindi delle dimensioni delle popolazioni.

Analogamente a quanto detto finora per la raganella sarda, anche i risultati ottenuti nello studio dell'euprotto corso si discostano sonoramente da quanto atteso sia per una specie endemica insulare, sia per una specie endemica di un'area hotspot. Peraltro, nel caso dell'euprotto, queste attese sono già state tradotte in una declassificazione del rischio

potenziale per la specie (IUCN 2011). Ben lungi da un pattern di omogeneità genetica, la struttura della variazione osservata in questa specie unitamente alle stime dei tempi di divergenza, hanno suggerito l'esistenza di ben cinque linee di probabile derivazione Pliocenica, e un totale di nove unità evolutive distinte di derivazione almeno Medio Pleistocenica. Un simile pattern non trova peraltro analoghi nelle specie continentali ad esso affini (generi *Calotriton* e *Triturus;* Carranza & Amat 2005*,* Arntzen *et al.* 2007), rivela chiaramente il ruolo della frammentazione allopatrica (e dunque non della stabilità demografica) nel determinare la diversità e la divergenza osservate (Canestrelli *et al.* 2010, Canestrelli *et al.* 2012), ed indica come l'euprotto corso non sia affatto costituito da una singola entità panmittica, e non lo sia da almeno 3 milioni di anni a questa parte.

I pattern emersi dalle nostre analisi risultano certamente peculiari ed in disaccordo con i pattern generali attesi, sia per la diversità genetica di specie insulari sia per quel che riguarda i processi microevolutivi in atto nei rifugi durante le glaciazioni. Tale singolarità dei casi presentati suggerisce una certa cautela nella valutazione sulla generalizzabilità delle inferenze da essi derivabili. Tuttavia, tre importanti implicazioni generali emergono con assoluta chiarezza. Primo, la possibilità di espansione glaciale per specie temperate. Tale scenario è un'eccezione ai pattern generali, era stato ipotizzato raramente per altre specie temperate (*Hyla intermedia e Rana Lessonae*; Canestrelli *et al*. 2007b, Canestrelli & Nascetti 2008), e nessun percorso d'indagine prima d'ora aveva portato a validare questa ipotesi. Mentre qui, partendo proprio da questa ipotesi (v.d. capitolo 2) si è cercato un percorso sperimentale per poterla verificare (v.d. capitolo 3). La struttura ed il dettaglio del campionamento, tale da ricoprire per intero l'areale di distribuzione della raganella sarda a scala geografica fine, la scelta delle analisi su un pannello diversificato di marcatori molecolari, la ricostruzione della nicchia ecologica durante la recente glaciazione, e soprattutto la concordanza fra tutte le linee di inferenza, ci hanno permesso di validare questa ipotesi con un certo grado di accuratezza. Quello che ne emerge è un nuovo ed alternativo scenario per spiegare l'elevata diversità genetica oggi riscontrabile all'interno delle aree rifugio, ed è questa la seconda importante implicazione di questa ricerca. Fino ad oggi, infatti, gli scenari proposti nelle indagini filogeografiche sulle aree hotspot di diversità intraspecifica, coincidenti spesso con i rifugi glaciali, indicavano due pattern alternativi e ricorrenti. Uno di questi pattern è la stabilità demografica delle specie all'interno delle aree rifugio, la quale avrebbe impedito colli di bottiglia e impoverimento del corredo genetico delle specie qui residenti (Hewitt 1996). L'altro pattern, invece,

descriverebbe fenomeni di frammentazione delle popolazioni all'interno delle aree rifugio durante le fasi glaciali e individuerebbe negli eventi di rimescolamento e contatto secondario, nelle fasi interglaciali, la causa degli attuali livelli di diversità genetica elevati per le popolazioni di queste aree (Gomez & Lunt 2006, Canestrelli *et al.* 2006). Nel caso della raganella sarda, le oscillazioni della dimensione dell'areale causate dall'aumento delle coste a seguito delle regressioni marine pleistoceniche avrebbero messo a disposizione nuove aree che hanno avuto un impatto demografico rilevante. La crescita demografica che ne è risultata ha permesso alla specie non soltanto di potersi espandere nonostante la fase glaciale, ma di farlo all'interno dell'area rifugio, mantenendo così alta la propria diversità genetica. Questo scenario alternativo potrebbe essersi verificato altre volte in altre aree del pianeta che hanno avuto condizioni climatiche e geografiche simili a quelle sarde (Burns *et al.* 2007, Buckley *et al.* 2009, King *et al.* 2009, Marske *et al.* 2009, Marko *et al.* 2010, Porretta *et al.* 2011). In ultimo, l'esistenza e l'importanza dei processi microevolutivi intraisola è la terza implicazione di questo lavoro. Tali processi avrebbero agito su scale temporali diverse nella raganella e nell'euprotto, e tuttavia in entrambi i casi sono certamente questi i primi responsabili dei pattern e dei livelli di diversità osservata. Dal momento che, come indicato nel capitolo introduttivo, i lavori che hanno indagato i processi evolutivi all'interno delle isole sono meno del 5% della letteratura globale sulla filogeografia, possiamo certamente dire che il contributo di tali processi sia stato fino ad oggi sottovalutato, ed anche alla luce dei risultati qui presentati, meriti ben più attenta considerazione. Sotto questa luce il complesso sardo-corso emerge certamente come un "top-level-laboratory" per future ricerche.

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