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PLEISTOCENE EVOLUTIONARY HISTORY AND CONSERVATION OF MOUNTAIN SPECIES WITHIN MEDITERRANEAN PENINSULAS: INSIGHT FROM THE ALPINE NEWT IN THE APENNINE CHAIN

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1. INTRODUCTION

1.1 BACKGROUND AND AIMS OF THE RESEARCH

Investigations of species genetic structure have shown that genetic diversity is usually distributed unevenly among populations (Hewitt, 2004). Historical processes linked to palaeoecological changes and on-going processes linked to population responses at the strong human impact on the environment must both be considered among the main causes of this unevenness (Hewitt, 2004; Yannic *et al*, 2014).

Among historical processes, a leading role was played by the Pliocene climatic revolutions and above all, by the Pleistocene glacial cycles (Hewitt, 1996, 1999, 2000, 2011a,b). Such climatic variations have deeply affected species distribution, repeatedly fragmenting the populations during climatically unsuitable phases, and triggering demographic and adaptive responses, which resulted in genetic population divergence (Hewitt, 1996, 2000; Dynesius & Jansson, 2000; Davis & Shaw, 2001). Many species have survived the adverse conditions of Pleistocene glacials in relatively suitable areas named refugia (Bennet & Provan, 2008; Stewart *et al*., 2010). Several studies have highlighted that pleistocenic refugial areas were not only the richest in species biodiversity, but are often areas that retain the most intraspecific biodiversity (Petit *et al*, 2003). Some researchers have suggested that long-term stability within refugia may have promoted the accumulation of genetic diversity over time (Carnaval *et al*., 2009; Abellan & Svennig, 2014). However, the main assumption of this hypothesis is the maintaining over time, a population size large enough to avoid the effects of genetic drift and inbreeding, which negatively affect the levels of genetic diversity (Keller & Waller, 2002; Charlesworth, 2003). Nevertheless, such a condition is difficult to realize in geographically heterogeneous refugia such as mountain refugia (Knowles & Richards, 2005). Moreover, the numerous cases where many differentiated lineages were found within a single refugial area suggest the engagement of more complex microevolutionary processes, possibly linked to a putative fragmentation within the refugium (Stewart *et al*., 2010).

Within fragmented areas, secondary contact among previously isolated populations may have carried out a leading role in triggering the microevolutionary processes that affect the genetic diversity of populations (Taberlet, 1998; Hewitt, 2011b). Secondary contact promotes different mechanisms according to the duration of contact and genetic

differentiation previously gained by populations (Barton & Gale, 1993; Burke & Arnolds, 2001). On one hand, if genetic differentiation is high, secondary contact may promote the evolution of reproductive isolation, and then speciation (Coyne & Orr, 1997); on the other hand, if genetic differentiation is low or moderate, secondary contact allows the admixing of gene pools, causing reinvigoration of populations (Crow, 1948; Barton, 2001). In the latter case, secondary contact drives populations to gain size and heterozygosis, thus improving their health status (Ingvarsoon & Whitlock, 2000; Whitlock *et al*, 2000; Chapman *et al*, 2009). Secondary contact of short duration, which is not long enough to completely admix the gene pools of populations, may even allow introgression of private genes from one population to another (Hewitt, 2011b). Traces of palaeo-introgression may provide useful information about past gene flow between two genetic lineages (McKinnon *et al*, 2004). Such mechanisms can be considered not only as an explanation for high levels of genetic diversity, but also as responsible for long-term survival of populations.

Several evidences regarding the role of fragmentation and secondary contact in increasing genetic variations within refugia arise from phylogeographic studies in Southern European peninsulas (Taberlet, 1998; Hewitt, 2011a,b). These peninsulas are hotspots of biodiversity at both the inter- and intra-specific level, and were refugia for several Palaearctic species during the Pleistocene glacial phases, when the rest of Europe was covered by ice and tundra (Hewitt, 2004, 2011a; Weiss & Ferrand, 2007; Feliner, 2011). Many of these studies have identified several genetic lineages strongly differentiated within a single refugium, in some cases sufficient to be split in two separate species (e.g. *Salamandrina terdigitata* e *S. persicillata*, Canestrelli *et al*., 2006c). A variation so high, has suggested that these areas could be composed of more sub-refugia, and the high genetic diversity might be a result of repeated contraction and expansion of populations, which resulted in repeated isolation and secondary contact among their gene pools (Gomez & Lunt, 2007). Three evidences could confirm that this mechanism, and not stability, should be responsible for the observed pattern of genetic variation: areas with the highest diversity are also most affected by palaeoclimatic or palaeogeographic changes; the highest levels of heterozygosis are not within refugia, but in the contact area among the expanding lineages; the areas with the most healthy populations are the ones affected by this mechanism. Some case studies on the Apennine peninsula can be considered to summarise these evidences: *Talpa romana* (Canestrelli et. al., 2010), *Rana italica* (Canestrelli *et al*., 2008), and *Bombina pachypus* (Canestrelli *et al*., 2006b). All three cases highlight the leading role of admixture among genetic lineages differentiated inside different sub-

refugia, in realizing the hotspot of intraspecific genetic diversity. Of particular relevance is the case of *B. pachypus*: this species shows strong decline across all Apennine peninsula, caused by a synergistic effect of on-going climate change and infection from the chytrid fungus *Batrachochytrium dendrobatidis*. The only populations that do not show any sign of decline are the calabrian populations, which have the highest level of genetic diversity and which resulted from the admixing of more lineages from different sub-refugia (Canestrelli *et al*., 2013). This case again confirms the leading role of genetic diversity in conferring the evolutionary potential indispensable to survive in natural stress. Such evidences support the hypothesis that genetic diversity inside refugia is closely linked to the dynamics of fragmentation, differentiation, and secondary contact, and that genetic diversity may have played an active role in the long-term survival of populations, rather than being a by-product of persistence.

Most evidences on evolutionary dynamics involved in southern European peninsular refugia come from studies on temperate species (Hewitt, 2011a). However, many mountain chains that run across these peninsulas have also provided refugia to a number of cold-adapted species, which represent a large portion of the biodiversity in these areas (Schmitt, 2009; Medail & Diadema, 2009; Vogiatzakis, 2012). Mountain refugia have a strongly fragmented distribution, and they were highly instable, since many of them were covered with ice during the glacial phases, forcing the cold-adapted populations to move away to the peripheral areas (Schönswetter *et al*., 2005; Schmitt *et al*., 2006; Varga & Schmitt, 2008; Holderegger & Thiel-Egenter, 2009; Schmitt, 2009; Garrick, 2011). Both habitat discontinuity and instability of their Pleisocene refugia are useful prerequisites of mountain species to study the role of fragmentation and secondary contact in shaping the distribution of genetic diversity among the populations inside refugia.

Understanding the distribution of genetic diversity among populations of cold-adapted species could be fundamental to direct conservation planning for species that are threatened by on-going climate changes (Parmesan, 2006). It has been observed that climate change is altering mountain habitats, and severe population decline of many cold-adapted species caused by loss of habitat suitability have been reported in the last few years (Diaz *et al*., 2006). Moreover, climate changes may disrupt the equilibrium of some ecosystem interactions such as host-pathogen interactions, resulting in increased risk of extinction for several species (Harvell *et al*., 2002). Association among amphibian decline, climate changes, and the outbreaks of an infectious disease, chytridiomycosis, was reported in the last decade (Pounds *et al*., 2006). Chytridiomycosis, caused by the chytrid fungus *B.*

dendrobatidis, is an infectious disease affecting the keratinized epidermal cells of its amphibian hosts, and is currently among the main causes of amphibian decline (Berger *et al*., 1998; Daszak *et al*., 1999; 2003; Fisher *et al*., 2009; Kilpatrick *et al*., 2010). In the above-mentioned decline of *B. pachypus* in the Apennine peninsula, different responses of populations to the pathogen have been observed, along with a delay between the presence of the pathogen in the populations and the signs of decline. Such a temporally and spatially heterogeneous pattern was attributed to the differing capacities of populations to respond to stress, linked to their differing levels of genetic variability and evolutionary potential. Moreover, altitude seems to be positively related to the severity of this disease, since the most severe mass mortalities have been observed in mountain species (Berger *et al*., 1998; Fellers *et al*., 2001; Bosch *et al*., 2001). In Europe, a strong association was found between altitude and the mass mortality of three common species: *Alytes obstetricans*, *Salamandra salamandra*, and *Bufo bufo* (Bosch *et al*., 2001; Bosch & Martínez-Solano, 2006, Walker *et al*., 2010). This stronger vulnerability of high-altitude populations to both climate changes and chytrid disease, and the possible association between this vulnerability and genetic diversity suggest that cold-adapted amphibians could be used to study the relationship between evolutionary history, genetic diversity, and the evolutionary potential of populations.

In the present study, we evaluate the role of Pleistocene glaciations in moulding the genetic structure of a cold-adapted amphibian living in the Apennine peninsula, *Ichthyosaura alpestris*. The main aim is to evaluate the hypothesis that even in cold-adapted species, fragmentation of refugia and secondary contact among differentiated populations could have played a leading role in shaping the genetic variation within the peninsular refugia. This is the first study on Apennine cold-adapted species with exhaustive sampling across the peninsular range, using a multi-locus dataset. We assess the genetic variation in both mitochondrial and nuclear markers with the following objectives: to analyse the genetic structure of Apennine populations; identify the evolutionary and biogeographic processes involved; to evaluate the role of fragmentation and secondary contact in distribution of genetic diversity; and to evaluate if differences in genetic diversity among populations may be related to population health and the capacity to respond to stress factors. Moreover, we use molecular diagnostic assays to test for the occurrence of chytrid fungus among the populations of *I. alpestris* and two other sympatric

mountain species, to assess any relationship between pathogen occurrence, altitude, and population decline.

1.2 THE STUDY SPECIES *ICHTHYOSAURA ALPESTRIS*

The study species is the Alpine newt *Ichthyosaura alpestris* (Laurenti, 1768), a cold-adapted amphibian of the family Salamandridae. This species is widespread throughout much of Europe, ranging from the French Atlantic coastline to the north of Denmark and eastwards to the Ukrainian Carpathians, Romania, and Bulgaria; it is widely distributed in the Balkans and in the northern Apennines, whereas isolated populations are present in southern Italy and northern Spain (Sillero *et al*., 2014). Within the Apennine peninsula, there are two geographically, morphologically, and genetically differentiated subspecies: *I. alpestris apuana* (Bonaparte, 1839), ranging from the north-western to central Apennines and *I. alpestris inexpectata* (Dubois e Breuil, 1983), which is present as a few isolated populations in the southern Apennine (Sindaco *et al*., 2006). Apennine populations have shown to be a distinct genetic lineage with respect to the Alps and European populations, and their isolation was estimated to be of the lower Pliocene origin (Recuero *et al*., 2014). The habitats of the Alpine newt in the Apennine peninsula include permanent and semi-permanent ponds as well as small lakes between 800 and 1800 m s.l.m., even though several populations can be found in the wet hills of the Ligurian Apennines below 500 m s.l.m (Ambrogio & Gilli, 1998).

Among European newts, *I. alpestris* is the most linked to water, and several adults can be found within ponds all around the year. Most of the Apennine populations show a high percentage of pedomorphic individuals, which are sexually reproductive individuals that maintain morphological and physiological features of larva. The relative roles of genes and the environment in determining pedomorphism in *I. alpestris* is unknown but it has been suggested that pedomorphism could be an adaptation to the unstable climatic conditions of Apennine mountains habitats. Pedomorphism negatively affects dispersal, by reducing the number of individuals that leave native ponds (Denoel *et al*., 2001, Denoel, 2003). The breeding season is between March and July, according to altitude and latitude of the reproductive sites, but a second breeding season can be observed in autumn; metamorphosis typically occurs before winter or in the subsequent spring, and sexual maturity is achieved around the second and fourth year. The Alpine newt feeds on little

invertebrates, and it is preyed upon by insects (Odonate, Ditiscidae and aquatic Eteroptera), as well as by bigger newts, fish, or aquatic birds when present (Ambrogio & Gilli, 1998).

Apennine populations of *I. alpestris* are considered as "Near Threatened" by the IUCN, except for the Southernmost isolated populations, which are considered as "Endangered" (Rondinini *et al*., 2013). The main causes of extinction include climate changes, pollution, habitat degradation or loss, habitat fragmentation and population isolation, and introduction of fishes. Evidence of individual mortality or population decline attributable to chytridiomycosis has not been reported for the Apennine peninsula until now.

2. GENETIC STRUCTURE AND EVOLUTIONARY HISTORY OF *ICHTHYOSAURA ALPESTRIS* WITHIN APENNINE PENINSULA

ABSTRACT

Southern European peninsulas provided refugia for several species during Quaternary climatic oscillations, and several phylogeographic studies have investigated the evolutionary processes involved. The view of these refugia as static and stable has been changing since even more studies are revealing many intraspecific genetic lineages within them, highlighting the role of refugia sub-structuring in enhancing genetic diversity. Although mountains harbour a great part of biodiversity in these peninsulas, most of the study was focalized on temperate species, whereas the evolutionary dynamics involving the cold-adapted species are underinvestigated. Here, we use a bayesian phylogeographic approach, coupled with a species distribution model to investigate the genetic structure and the evolutionary history of the cold-adapted amphibian, *Ichthyosaura alpestris* within the Apennine peninsula. Both mitochondrial and nuclear markers identified three distinct lineages, whose divergence dates back to the Early and Middle Pleistocene. Two of these lineages form a secondary contact zone in the Ligurian Apennine, which corresponds to one of the main biogeographic discontinuities of the peninsula. Our spatiotemporal phylogeographic reconstruction identified the Riss glacial as the period of expansion and contact for these lineages. Nevertheless, SDM analysis indicated the contact zone as a suitable region during both the last interglacial and the last glacial, conditions that may have prolonged lineage admixture until now. Our results confirm the role of southern European peninsulas as multiple refugia during the Pleistocene for cold-adapted species as well, and suggest that repeated cycles of allopatric fragmentation followed by secondary admixture may have been a common process in forming hotspots of intraspecific diversity within these peninsulas.

KEY WORDS: genetic diversity, bayesian phylogeography, Pleistocene refugia, Apennine peninsula, cold-adapted species, *Icthyosaura alpestris*

2.1 INTRODUCTION

Southern European peninsulas are hotspots of biodiversity at both the species and genetic levels, and the processes generating this richness have always attracted the interest of biogeographers and evolutionary biologists (de Lattin, 1967; Hewitt, 1996, 2011; Taberlet *et al*., 1998; Weiss & Ferrand, 2007). It is now established that Pleistocene climatic oscillations have influenced the distribution of biodiversity in these areas, causing shifts in species ranges and triggering evolutionary processes that have moulded species' genetic structures (Comes & Kadereit, 1998; Hewitt, 2000; Hofreiter & Stewart, 2009; Feliner, 2011). During glacial maxima, several species took refuge in southern European peninsulas, and vicariance events among isolated populations promoted differentiation and speciation (Taberlet *et al*., 1998; Hewitt, 2001; Schmitt, 2007; Bennett & Provan, 2008). Genetic diversity was often increased by further sub-structuring within these refugia, where populations were repeatedly fragmented and reshuffled by cyclic range contractions and expansions (Petit *et al*.,2003; Hampe e Petit, 2005; Gomez & Lunt, 2007; Canestrelli *et al*., 2010; Stewart *et al*., 2010). The strong habitat heterogeneity within these peninsulas, where lowland, hilly and mountain habitats contain refugia for temperate as well as cold-adapted species, made them suitable as both glacial and interglacial refugia (Stewart *et al*., 2010). However, differences between the processes involved in glacial and interglacial refugia are not yet fully understood, and the phylogeographical investigation of these areas continues to be of great interest (Hewitt, 2011a).

Mountains play a key role in understanding species' responses to past climate change in southern European peninsulas. They have had a strong impact on species genetic structure by acting as ecological barriers to temperate species during glacial periods as well as refugia for cold-adapted species during interglacial phases (Schmitt, 2009). Despite the fact that several Mediterranean refugia are located in mountainous areas (Medail & Diadema, 2009; Vogiatzakis, 2012), most attention has been paid to temperate species, and few phylogeographic studies have been conducted on cold-adapted species (Schönswetter *et al*., 2005; Schmitt, 2009). However, because climatic events had different consequences on cold-adapted species, investigating their evolutionary history is crucial in understanding the dynamics involved in species evolution during the Pleistocene (Louy *et al*., 2014). Indeed, mountain refugia are structurally highly discontinuous (Varga & Schmitt, 2008; Holderegger & Thiel-Egenter, 2009; Garrick, 2011), and cold-adapted populations

probably encountered stronger fragmentation during interglacial periods. However, because interglacial phases were shorter than glacial phases, populations isolated during interglagials should be less genetically differentiated than population isolated during glacial (Stewart *et al*., 2010; Garcia *et al*., 2011). Moreover, many interglacial refugia were unstable environments because they were covered by ice or were too dry for cold-adapted species during glacial culminations, forcing populations to move to peripheral areas (Schönswetter *et al*., 2005; Schmitt *et al*., 2006; Varga & Schmitt, 2008; Holderegger & Thiel-Egenter, 2009; Schmitt, 2009). Therefore, populations of cold-adapted species could have experienced strong environmental constraints, both during interglacial and glacial periods, with different time frames, refugial sites and recolonization routes that could have produced several unique evolutionary pathways.

Despite the Italian Peninsula was intensively studied phylogeographically, few studies have investigated the role of interglacial refugia in Pleistocene evolutionary history of cold-adapted species. Italian Peninsula is rich of mountainous areas, thanks to the presence of Apennines, a mid-to-high-altitude mountain chain, which span more than 1200 km in a NW-SE direction, surrounded by hilly and lowland regions, and climatically very heterogeneous. Their role as refugia has been recognized for both temperate and cold-adapted species (Hewitt, 2011a, and references therein). Nonetheless, phylogeographic studies on the Italian Peninsula have almost exclusively examined temperate species, investigating their dynamics within glacial refugia and the role that major mountain blocks played on shaping their genetic variation (eg. Fritz *et al*., 2005 ; Boheme *et al*., 2006; Canestrelli *et al*., 2006, 2008, 2010, 2012, 2014; Heuertz *et al*., 2006; Ursembacher *et al*., 2006; Maura *et al*., 2014). In contrast, the few phylogeographic studies on cold-adapted species that have been conducted have not investigated the fine-scale genetic structure of Apennine populations, making it difficult to reconstruct their Pleistocenic evolutionary history (eg. Castiglia *et al*., 2009; Stefani *et al*., 2012).

In this study, we investigated the genetic structure and evolutionary history of an Apennine cold-adapted amphibian, the Alpine newt *Ichthyosaura alpestris* (Laurenti, 1768). This species is widely distributed in central and Eastern Europe where it breeds in small lakes and semi-permanent ponds, while in the Apennines its distribution is fragmented and closely linked to mid- and high-altitude ponds (Sillero *et al*., 2014). Morphological, ecological and genetic evidences suggest that the Apennine populations of this species should be considered two distinct subspecies: *I. a. apuana* for northern and central Apennine populations, and *I. a. inexpectata* for the southernmost Calabrian

populations (Breuil, 1986; Ambrogio & Gilli, 1998; Lanza *et al*., 2007). Interestingly, the separation of the Apennine clade from other European clades (including the Alps) has been estimated to be of pre-Pleistocene origin, but very little information is available about the genetic structure of the Apennine clades, making the Alpine newt an excellent model with which to study the impact of Quaternary glaciations on the evolution of cold-adapted species.

Using information from mitochondrial and nuclear DNA sequences in combination with Bayesian phylogeographic analysis and ecological niche modelling, we (1) assessed how, and how much, Quaternary climatic oscillations influenced the evolutionary history of these populations; (2) localised putative Pleistocenic refugia in the Apennines for this cold-adapted species; and (3) evaluated biogeographical and microevolutionary pathways that drove their evolution during the Pleistocene.

2.2 MATERIALS AND METHODS

Sampling and laboratory procedures

We collected 149 individuals of *I. alpestris* from 15 localities spanning its distribution in the Apennines; geographical references for sampling locations and sample sizes are shown in Table 1 and Fig. 1. Tissue samples were collected from tail tips after the newts had been anaesthetized by submersion in a 0.1% solution of MS222 (3-aminobenzoic acid ethyl ester). All of the individuals were then released at the respective collection site. Samples were stored in 95% ethanol until subsequent analyses.

DNA extractions were performed by following the standard cetyltrimethyl-ammonium bromide (CTAB) protocol (Doyle & Doyle 1987). Two mitochondrial DNA (mtDNA) and three nuclear DNA (nDNA) fragments were amplified and sequenced. The mtDNA fragments consisted of one from the cytochrome B gene (*CytB*) and one from NADH dehydrogenase subunit 2 gene (*ND2*); the three nDNA fragments consisted of one from the fourth intron of the growth hormone gene (*GH*), one from the seventh intron of the β-fibrinogen gene (*β-FIB*) and one from the eleventh intron of the platelet-derived growth-factor receptor gene (*PDGFR*). The primers used and their sequences are presented in Table 2. Amplifications were performed in a 10-μL reaction volume containing MgCl2 (2 mM), four dNTPs (0.2 mM each), two primers (0.2 μM each), the enzyme *Taq* polymerase (0.5 U, Promega), its reaction buffer (1X, Promega) and 40–200 ng of DNA template. The polymerase chain reactions (PCRs) were conducted with an initial step at 95°C for 5 min, 32 cycles at 94°C for 1 min, annealing temperature and duration depending on fragment (see Table 2), 72°C for 1 min, followed by a single final step at 72°C for 5 min. To increase the specificity and yield of the *β-FIB* amplification, we used a nested PCR as in Sequeira *at al*. (2006), with slight modifications in the cycling conditions of the second PCR (26 cycles of 94°x30'', 59°x30'', 72°x45''). Purification and sequencing of the PCR products were conducted on both strands by Macrogen Inc. (http://www.macrogen.com) using an ABI PRISM® 3730 sequencing system (Applied Biosystems). All of the sequences were deposited in GenBank (accession numbers: XXXX)

We also analysed variation at nine microsatellite loci (*Copta1*, *Copta3*, *Copta8*, *Copta13*, *Copta9*, *TaCa1*, *Ta3Ca8*, *Ta3Caga2* and *Ta2Caga3*) following previously

published protocols (Prunier *et al*., 2012, 2014). We chose a subset of available loci after the exclusion of those that exhibited reaction inconsistency in over 30% of samples. Forward primers were fluorescently labelled and PCR products were electrophoresed by Macrogen Inc. on an ABI 3730xl genetic analyser (Applied Biosystems) with a 400-HD size standard. The DNA fragments were analysed, and genotypic data were generated, using GeneMapper® 4.1. Micro-Checker 2.2.3 (Van Oosterhout *et al*., 2004) was used to test for null alleles and large-allele dropout influences. Because the tetranucleotide locus *Copta9* exhibits a dinucleotide variation in some populations, each allele was sequenced as above; extraordinary allele sequences revealed a two-base-pair insertion in the flanking region of the simple sequence repeat (SSR). To use only true SSR polymorphisms in our analyses, the fragment length values of these alleles were corrected by subtracting 2.

Phylogenetic data analysis

Electropherograms were visually checked using FinchTV 1.4.0 (Geospiza Inc.) and sequences were aligned using Clustal X 2.0 (Larkin *et al*., 2007). Heterozygous nuclear sequences were phased using the PHASE method implemented in DnaSP 5.10 (Librado & Rozas, 2009), maintaining the default features; superimposed traces, produced by the direct sequencing of heterozygous indels, were resolved as suggested by Flot *et al*.(2006). For each nuclear gene, the probability of recombination was evaluated using the pairwise homoplasy index (PHI statistics, Bruen *et al*., 2006) in SplitsTree 4.13.1 (Huson & Bryant, 2006). All of the subsequent analyses were conducted using phased nuclear data, and indels were treated as missing data. Nucleotide variation was assessed using MEGA 6.0 (Tamura *et al*., 2013); haplotype and nucleotide diversity (Nei, 1987) were estimated using DnaSP 5.10.

Because no differences had been detected by a partition-homogeneity test (Farris *et al*., 1994) implemented in PAUP* 4.0B10 (Swofford, 2003), the two mtDNA fragments were combined into a unique haplotype using Concatenator 1.1.0 (Pina-Martins & Paulo, 2008). All of the subsequent analyses were conducted on the combined dataset.

Phylogenetic relationships between the mtDNA haplotypes were inferred using a maximum likelihood algorithm in PhyML 3.10 (Guindon *et al*., 2010). We used the default settings in PhyML for all of the parameters except for the type of tree improvement, choosing the SPR & NNI algorithm, and for the substitution model, using the Bayesian information criterion (BIC) best-fit model of evolution (HKY) selected by jModelTest

2.1.3 (Darriba *et al*., 2012); the robustness of the inferred tree was assessed using the non-parametric bootstrap method with 1000 replicates. The estimated tree topology was then converted into haplotype genealogy using Haplotype Viewer software (Salzburger *et al*., 2011). Genealogical relationships between the mtDNA haplotypes were investigated using a statistical parsimony approach in TCS 1.21 (Clement *et al*., 2000), with a 95% cut-off criterion for a parsimonious connection. The statistical parsimony approach was also used to investigate genealogical relationships between haplotypes for each nuclear fragment. MEGA 6.0 was used to compute the net sequence divergence among the haplogroups.

Divergence time estimations

The Bayesian procedure implemented in BEAST 1.8.1 (Drummond *et al*., 2012) was conducted in order to estimate the divergence time across lineages and the pattern of spatial diffusion. For these analyses we only used the mtDNA dataset, because it had exhibited much more variation than the nuclear sequence dataset. PartitionFinder 1.1 (Lanfear *et al*., 2012) was used to select the optimal partitioning strategy and substitution models for each partition, using linked branch length options and forcing the software to choose only among the models implemented in BEAST. Using BIC, the best scheme was the same for both genes: HKY for the first position, HKY for the second position and TrN93 for the third position. This partition scheme was used for all subsequent analyses.

We first estimated the divergence time between the haplotypes. A Bayesian skyline plot was chosen as a coalescent tree prior (Drummond *et al*., 2005). The molecular clock was calibrated by setting a normal prior for the treeModel.rootHeight parameter with a mean of 2.1 million years (standard deviation of 0.45), following the estimate of the most common ancestor of *I. a. apuana* in Recuero *et al*. (2014). This estimate was generated by a Bayesian analysis of divergence time among the main *I. alpestris* lineages using datation of the first *Triturus* fossil to calibrate the molecular clock. Tuning analyses ran with an uncorrelated relaxed lognormal molecular clock model (Drummond *et al*., 2006). However, because they had shown a standard deviation (ucdl.stdev) close to zero for the relaxed clock, subsequent analyses were conducted with a strict clock model. Two definitive runs were performed, each with a Markov chain Monte Carlo (MCMC) length of 10 million generations, sampling every 1000 generations. Traces were inspected using Tracer 1.6 (Rambaut *et al*., 2014) to evaluate the effective sample size (ESS) of the parameters estimated (above 200 was satisfactory, as recommended by the authors) and the

convergence across the runs, after removing the first 10% of samples as burn-in. The two runs were combined using LogCombiner 1.8.1 in the BEAST package, and a maximum clade credibility tree was extracted by TreeAnnotator 1.8.1 (in the same package) after removing the first 10% of trees as burn-in.

Bayesian spatial diffusion analysis

To estimate the spatial diffusion patterns of the main *I. a. apuana* lineages we conducted a Bayesian phylogeographic analysis in continuous space as implemented in BEAST (Lemey *et al*., 2010). Because we were interested in the patterns and timelines of the diffusion processes of each of the main lineages resulted by precedent analyses, we performed a separate analysis with the same settings for each of them. Geographical coordinates were provided for each individual, and a slight perturbation of ±0.001 was applied to duplicates to avoid confounding the analysis. We applied the strict molecular clock model with the mutation rate estimated by the divergence time analysis, the Bayesian skyline plot as a coalescent tree prior, a MCMC length of 90 million generations and sampling every 9000 generations.

A run was generated for each spatial diffusion model (Brownian, Cauchy and Gamma), and the marginal likelihood (ML) of each model was estimated (MCMC length of 500 000, 100 path steps). Traces of the runs were inspected using Tracer, as above. The best model was evaluated using a Bayes factor, which was generated by confronting the path sampling and stepping-stone sampling estimates of the ML of each (Baele *et al*., 2012; Baele *et al*., 2013).

The best models for each lineage were visualized by projecting the maximum clade credibility (MCC) tree (selected by TreeAnnotator) and locations on a map using SPREAD 1.0.7 (Bielejec *et al*., 2011), and opening the ".kml" output file with Google Earth. The Time Slicer option in SPREAD was used to conduct an estimate of the 80% highest posterior density (HPD) of the spatial locations of the populations during their diffusion at four dates selected before the present [250 000 years ago (kya), 140 kya, 100 kya and the present] based on all of the trees obtained (the first 10% were removed as burn-in).

Microsatellite data analysis

Descriptive statistics were obtained for the microsatellite dataset using FSTAT 2.9.3 (Goudet, 1995) in order to test for deviations from the expected Hardy-Weinberg and linkage equilibria, using the Bonferroni procedure to correct the significance level for multiple comparisons. GENETIX 4.05 (Belkhir *et al*., 1996) was used to obtain estimates of genetic diversity for each population, based on the mean observed and expected heterozygosity and the mean number of alleles.

Analysis of genetic structure

The population genetic structure across the study area was evaluated using the microsatellite and nuclear sequence datasets separately. We used a Bayesian clustering algorithm implemented in TESS 2.3.1 (Chen *et al*., 2007), with the spatial distribution of individuals as a prior, since it performs better than other Bayesian methods when the structure is not deep and/or there are not many loci (Chen *et al*., 2007; Francois & Durand, 2010). For the nuclear sequences dataset we compiled a genotype multilocus matrix using phased haplotypes as alleles. For both datasets, analyses were conducted by modelling admixture using a conditional autoregressive model (CAR). Preliminary analyses were conducted to assess model performance, with 20 000 runs (the first 5000 were discarded as burn-in) and 10 replicates for each K value (i.e. the number of clusters) between 2 and 10. The final analysis contained 100 replicates with K = 2–10, each with 50 000 runs (the first 20 000 were discarded as burn-in). The spatial interaction parameter was kept at the default value (0.6), and the option to update this parameter was activated. For both datasets, the model that best fitted the data was selected using the deviance information criterion (DIC). DIC values were averaged over the 100 replicates for each K value, and the most likely K value was selected as the one at which the average DIC reached a plateau. The estimated admixture proportions of the 10 runs with the lowest DIC values were averaged using CLUMPP 1.1.2 (Jakobsson & Rosemberg, 2007). The contribution of each obtained cluster in each population was represented in a pie chart. We obtained two separate graphs, one for the microsatellite dataset and one for the nuclear sequences dataset.

Species distribution model

In an attempt to locate hypothetical glacial and interglacial refugia for *I. a. apuana*, Species Distribution Models (SDMs) were used to forecast the bioclimatic suitability for

this species during the last glacial cycle. A maximum entropy modelling approach implemented in MAXENT 3.3.3k (Philips *et al*., 2006) was used to build a model of ecological niches of the species based on presence-only data. The model was built under current bioclimatic conditions and then projected onto reconstructions of past climatic conditions during the last interglacial phase (LIG, 120–140 kya) and last glacial maximum (LGM, 22 kya).

A total of 160 points of *I. alpestris* presence from all over its peninsular distribution (including the 15 sampling sites) were obtained from the literature and museum collections (Appendix I). The layers of bioclimatic data were downloaded from the WorldClim database (http:\\www.worldclim.org; Hijmans *et al*., 2005), with a 30-arc-second resolution for the current and the LIG and a 2.5-arc-minute resolution for the LGM; for the LGM we used predictions from both the Community Climate System Model (CCSM) and the Model for Interdisciplinary Research on Climate (MIROC). From 19 variables, we first excluded highly correlated variables (Pearson correlation coefficient r^2>0,8) and then selected a subset of seven variables of major biological significance for *I. alpestris*, inferred from ecological studies (e.g. Fasola & Canova, 1992; Ambrogio & Gilli. 1998; Denoel *et al*., 2001; Kopecky *et al*, 2012) and SDM studies (Girardello *et al*., 2010) on this species. The variables were: the mean temperature of the wettest quarter (BIO8), the mean temperature of the driest quarter (BIO9), the mean temperature of the warmest quarter (BIO10), precipitation seasonality (BIO12), precipitation in the driest quarter (BIO17), precipitation in the warmest quarter (BIO18) and precipitation in the coldest quarter (BIO19). The layers were cropped to span from 4°1'30"E to 18°46'30"E and from 37°31'60"N to 48°40'30"N using DIVA-GIS 7.5 (Hijmans *et al*., 2001).

The model was built using MAXENT's default parameters, with a convergence threshold of 10^{-5}, 500 iterations and 10 000 random background points. Several tuning runs were conducted with different values (0.25 to 15) of the regularization multiplier parameter (Warren & Seifert, 2011; Radosavljevic & Anderson, 2014). To select the model that best fitted the data, we used the corrected Akaike Information Criterion (AICc) implemented in ENMTools (Warren *et al*., 2010). The definitive runs were performed in 10 replicates, with a sub-sample replicated run type and 25% of the data as a random test percentage. The mean value of the area under the curve (AUC) of a receiver-operating characteristic (ROC) plot was used to evaluate model performance (Araujo *et al*., 2005). The results of the two LGM projections were first evaluated individually and then averaged (Araujo *et al*., 2007).

2.3 RESULTS

For all individuals analysed we obtained a 399-bp fragment from *CytB* gene and an 878-bp fragment from *ND2* gene (1277 bp in total). No indels, stop codons or non-sense codons were observed on either gene. In the combined mtDNA dataset, we found 13 haplotypes that were identified by 35 variable positions, 31 of which were parsimony informative. Mean haplotype diversity (*h*) and nucleotide diversity (π) values for this dataset were 0.803 (±0.019 SD) and 0.0077 (±0.0002 SD), respectively.

The nDNA dataset included a 328-bp fragment of *β-FIB* from 104 individuals, a 565-bp fragment of *PDGFR* from 111 individuals, and a 670-bp fragment of *GH* from 103 individuals. In the *β-FIB* alignments, we found six haplotypes that were identified by eight variable sites, seven of which were parsimony informative; $h = 0.365$ (±0.041 SD) and $\pi = 0.0041$ (±0.001 SD). In the *PDGFR* alignments, we found five haplotypes that were identified by four variable sites, four of which were parsimony informative; $h = 0.413$ (±0.038 SD) and $\pi = 0.0018$ (±0.0002 SD). In the *GH* alignments, we found eight haplotypes that were identified by 17 variable sites, 15 of which were parsimony informative; $h = 0.376$ (±0.039 SD) and $\pi = 0.0017$ (±0.0004 SD). No recombination events were indicated by the PHI tests conducted on the nuclear gene fragments (all $P > 0.05$). Using phased haplotypes as alleles, a multilocus genotype matrix was available from 121 individuals for the three nuclear loci, with 8% of the data missing. A full list of all the haplotypes found for each gene is presented in Table 3.

Phylogenetic data analysis

The phylogenetic network of the mtDNA haplotypes yielded by HaplotypeViewer is shown in Fig. 2. The log-likelihood score for the ML tree was -1963.08. We found three geographically distinct clades: a north-western Apennine clade (hereafter the Ligurian clade) that was poorly differentiated in the Piedmont population (sample 15), in all central and western Ligurian populations (samples 11–14) and at low frequency in an eastern Ligurian population (sample 10); a north-central Apennine clade that was more differentiated and found from eastern Ligurian to the central Apennine populations (samples 2–10); and the last clade consisted of a single haplotype, related to the north-central Apennine clade, and found exclusively in the Calabrian Apennine population

(sample 1). Interestingly, only within the geographically intermediate population of Mount Penna (sample 10) we found both the Ligurian and north-central Apennine clades. The statistical parsimony analysis showed the same clustering and topologies as the ML analysis, but two distinct networks were generated: one for the Ligurian clade and one for the north-central Apennine clade and the Calabrian clade (not shown). The mean Kimura 2-parameter sequence divergence value between the N-W and S-E clades was 0.012 (±0.003 SEM), between the S-E clade and the Calabrian haplotype was 0.007 (±0.002 SEM), and between the N-W and Calabrian clades was 0.015 (±0.003 SEM).

The phylogenetic networks among the haplotypes for each nuclear gene are shown in Fig. 2. In all cases, one single network connected all of the haplotypes, except for one haplotype in *GH* that did not connect with the main network. Despite all three genes showed low levels of variation, a geographical structure could be observed. In the *β-FIB* fragment, the most common haplotype was found spanning the whole range; it was the unique haplotype in central Italy, whereas it was found together with two different haplotypes in the Ligurian populations. A very similar pattern was found in the *GH* fragment; the differentiated haplotype was found at low frequencies, and exclusively in sample 9. In contrast, the opposite pattern was observed in the *PDGFR* fragment: the most common haplotype was the unique one found in the Ligurian populations, and its frequency gradually decreased towards the central Apennines, where three slightly differentiated haplotypes were found with growing frequency; in the Calabrian population, only one exclusive haplotype was found.

Divergence time estimation

Both BEAST runs for the divergence time estimation converged to a stationary distribution, and had satisfactory ESS values that were largely above 200 for all the estimated parameters. After the two runs had been combined, the molecular substitution rate was 4.586×10^{-9} (95% HPD interval, 1.766×10^{-9}, 8.08×10^{-9}). The chronogram based on the MCC tree is shown in Fig. 3. The topology of the tree was in accord with the other analyses: a deep and ancient split was estimated between the N-W clade and the others (mean 1.86 million years ago (mya); 95% HPD interval, 0.89–2.86], whereas the separation between the Calabrian and north-central Apennine clade was more recent (mean 0.82 mya; 95% HPD interval, 0.25–1.57).

Bayesian spatial diffusion analysis

Spatial diffusion pattern analyses were performed on the Ligurian and north-central Apennine clades separately; the Calabrian clade was excluded from this analysis because it did not exhibit enough variation. All of the runs converged to a stationary distribution and showed satisfactory ESS values. The comparison of the different spatial diffusion models based on the ML estimates suggested that the Brownian model fitted the data better than Cauchy or Gamma models for Ligurian clade, whereas Gamma model fitted the data better than Brownian and Gamma for north-central Apennine clade. The $\log_e$ ML values are shown in Table 4. Therefore, since there were only slight differences in ML, the simplest Brownian model was selected to reconstruct phylogeographic diffusion patterns for both clades. The diffusion patterns during the time slices considered are shown in Fig. 4. This analysis localized the ancestral areas of the two lineages: one was in western Ligurian and the other in the Tuscan-Emilian Apennines. The first spread of both lineages occurred during the penultimate glacial phase, and before the beginning of the last interglacial phase, the two lineages had already realized a secondary contact in eastern Liguria. Any significant spatial expansion was not observed during the last interglacial, whereas a further spread was observed during the last glacial, during which *I. alpestris* probably colonized all of the areas that are currently occupied.

Microsatellite data analysis

A total of 185 individuals from 15 populations were genotyped at nine microsatellite loci. *TaCa1* exhibited no variation across the samples and was excluded from further analyses. *Ta2Caga3* was also removed, because it tested positive for null alleles in four populations. The final dataset consisted of a multilocus genotype for 185 individuals at seven microsatellite loci, with 2.8% of the data missing. We found significant deviation from the Hardy-Weinberg equilibrium in population 1 for *TaCa1* and in population 13 for *Copta9*; no linkage disequilibrium was found. Across all populations, the number of alleles at each locus ranged from 2 (*Copta 13* and *Copta 1*) to 19 (*Copta 9*). The mean allelic richness and the mean expected heterozygosity for each population are presented in Table 1, and allelic frequencies for each locus and population are presented in Table 5.

Analysis of genetic structure

The analysis performed on the population genetic structure by TESS on the two datasets gave the same results and the same patterns of genetic variation across the populations. K = 3 was the best grouping option for the nuclear sequences dataset, because only a minor decrease in DIC values was observed at higher K values (Fig. 5a). The DIC curve in the microsatellite dataset analysis reached a plateau at K = 4 (Fig. 5b). However, an inspection of the plotted membership coefficients revealed that only three genetic clusters were present. It is not unusual for DIC to select models in which K_{MAX} is greater than the actual number of clusters (Durand *et al*, 2009; Temunovič *et al*., 2013). Bar plots showing the admixture proportion of each individual and pie charts showing the proportion of each group within each population are presented in Fig. 5. The spatial distribution of the genetic groups revealed a clear geographical structure that followed the one observed in the mtDNA data: one group consisted of the Ligurian populations, a second group included the north-central Apennine populations, and the third group contained only the Calabrian population. However, the level of population admixture was higher than that showed by the mtDNA; the geographically intermediate samples 9 and 10 were the most admixed populations, in which all of the individuals were admixed; admixture gradually decreased away from these populations. The nuclear sequences dataset had a higher proportion of admixture in the Ligurian populations, because all but one had other than 25% of other-group contributions, whereas only two populations in the north-central Apennine group were slightly admixed (samples 7 and 8).

Species distribution model

The model built with the value "1" as the regularization multiplier parameter was the one that best fitted our data (lowest AICc value). The average test AUC for the 10 replicate runs of the model was 0.966 (±0.010 SD), indicating a high and consistent performance (Araujo *et al*., 2005). Precipitation in the warmest quarter (BIO18) was the environmental variable with the highest percentage contribution and the highest gain when used in isolation; precipitation seasonality (BIO12) decreased the gain the most when omitted. MAXENT logistic output maps for the current model and for both past projections are shown in Fig. 6.

Under the present bioclimatic conditions, the SDM indicated that the Ligurian and Apuan Apennines are the most suitable areas, and the Tusco-Emilian Apennines are also suitable. These areas correspond to a great part of the *I. a. apuana* range. A slightly disjunct, suitable area was found in the central Apennines, from Gran Sasso and the Laga Mountains to the Majella Massif, where only one population has been recorded to date. The area where the relict Calabrian population survives was indicated as unsuitable by the current model. This pattern fits quite well with our current knowledge of the biology and distribution of *I. alpestris* on the Apennine Peninsula.

Both the CCSM and MIROC projections from the LGM predicted an expansion of suitable conditions for *I. alpestris*, but with a slightly different pattern between the two databases (data not shown). After the two projections were averaged, we observed three areas of major suitability: one in western Ligurian with the best values, a small, slightly separate one corresponding to the Apuan Apennines, and one that corresponded to the Latium and Abrutian Apennines. However, these areas were linked by large corridors of medium suitability in lowland areas of Latium and Tuscany. Projections from the LIG showed a marked reduction in suitable areas for this species. Good conditions were restricted to a narrow area in western Liguria and the northernmost Tusco-Emilian Apennines, and to small fragmented areas of the Latium and Abrutian Apennines. Overall, the SDM data indicated that climatic conditions for *I. a. apuana* were better during the LGM than at present, and even more so than during the LIG.

2.4 DISCUSSION

The genetic structure shown by *I. alpestris* on the Apennine Peninsula clearly reflects its ancient Pleistocene history, which was spent in at least three separate refugia where populations underwent strong genetic differentiation. This pattern is in agreement with the precedent evidences of multiple genetic lineages within the "*apuana* clade", as suggested by the species phylogenies of Sotiropoulus *et al*., (2007) and Recuero *et al*., (2014). Nevertheless, our denser sampling scheme, coupled with the use of more markers, has clarified the geographical structure and the amount of such genetic variation.

Both the mitochondrial and nuclear markers identified three main genetic lineages that were strongly differentiated, one restricted to the southernmost populations in Calabria, one ranging from the northern to the central Apennines, and one ranging from the western to the eastern Ligurian Apennines. The Calabrian populations are more than 500 km from the nearest population in the central Apennines, and show genetic divergence of a mid-Pleistocene origin, confirming that it is a biogeographical relict. The low level of genetic diversity found might be attributable to significant inbreeding, since the population size was estimated to be only around 300 individuals (Dubois & Ohler, 2009). However, despite a greater divergence, the other two lineages have actually a contiguous distribution and co-occur in a contact zone in the eastern Ligurian Apennines, where the populations are strongly admixed. Interestingly, the contact zone is wider for nuclear genes than mitochondrial genes, because only one population shares the mitochondrial haplotypes of both lineages. This type of mito-nuclear discordance can be explained by male-biased dispersal (Toews & Brelsford, 2012), which might result in faster movement of nuclear genes (biparental) than mitochondrial genes (only matrilineal). Male-biased dispersal has been reported in juvenile *I. alpestris* (Joly & Grolet, 1996), and was previously invoked to explain mito-nuclear discordance in this species (Recuero *et al*., 2014). In addition, male-biased dispersal could have been increased by the high prevalence of paedomorphic females in the Apennine populations, because paedomorphic individuals do not disperse (Denoel *et al*, 2001).

The contact zone falls in an area of great biogeographical interest, since other taxa have shown traces of secondary contacts among closely related lineages, particularly among mountain species (e.g. Porter *et al*, 1997; Cimmaruta *et al*, 2015). Moreover this area marks the range boundary of several taxa, including amphibians (*Hyla meridionalis*,

Pelodytes punctatus and *Rana italica*), suggesting that it might exhibit, or have exhibited, peculiar climatic or geographical characteristics. Nevertheless, since strong geographical discontinuities in this area were hitherto not reported as a possible causal factor, the hypothesis that peculiar climatic conditions could have affected the evolutionary histories of these species remains the most probable explanation. The Apennines are particularly close to the sea in this area, a situation that strongly affects the climate (Rapetti & Vittorini, 2013) and might have affected the climate during past glacial cycles. In addition, the Ligurian Apennines was already indicated as a crossway during recolonization pathway of several taxa, which have differentiated inside and outside the peninsula in Western Europe, including the Alps (Taberlet, 1998; Hewitt, 1999). All of these factors make this area an interesting hotspot of biodiversity, as well as a hotspot of evolutionary and biogeographical processes, increasing the interest on dynamics involved in the evolutionary history of inhabiting species.

Evolutionary history of **I. alpestris** *on the Apennine Peninsula*

The strong genetic differentiation found among the Apennine lineages of *I. alpestris*, and even more so among these lineages and other European ones, suggests an evolutionary history of ancient origin. The estimated time to the most recent common ancestor (TMRCA) among Apennine and other European lineages is 9.2 ± 2.1 mya (Recuero *et al.* 2014), suggesting that *I. alpestris* may have colonized the Apennine Peninsula during the Late Miocene. This period coincided with the establishment of the first connection between the Apennine Peninsula and Europe (Popov *et al.*, 2004), which resulted in the immigration of several faunal elements from Europe (Rook *et al.*, 2006). However, the biogeographical connection was repeatedly interrupted from the Late Miocene until the Middle Pliocene by the long submersion of the Northern Apennines, which was caused by eustatic variations in sea level (Ghibaudo *et al.*, 1985; Yilmaz, *et al.*, 1996; Rook *et al.*, 2006). These long interruptions probably caused the isolation and differentiation of peninsular populations of *I. alpestris* from European populations, as has been observed for several other taxa that exhibit a coeval split (Cimmaruta *et al.*, 2015).

Thereafter, the evolutionary history of *I. alpestris* on the Apennine Peninsula seems to have been strongly linked to the large climatic revolutions of the Pleistocene, since splits between the main genetic lineages are estimated to have occurred in the Early Pleistocene and in the beginning of the Middle Pleistocene. The split between the Ligurian and central

Apennine lineages was estimated to have occurred during the Early Pleistocene (1.86 mya ± 1.0), which coincided with a general cooling of the Northern Hemisphere that was caused by the effects of the first major glacial cycles (Kahlke *et al*., 2011). Because of this cooling, frigophilous species such as *I. alpestris* might have initially benefited from the spread of suitable habitats at lower altitudes and latitudes, and might have expanded their ranges along the peninsula. A general spread of other cold-adapted taxa during the Early Pleistocene has been reported by Bertini (2003, 2010), Masini & Sala, (2007) and Kahlke *et al*. (2011). Nevertheless, the effects of climatic instability, presumably the effects of the warm interglacials, might have fragmented the distributions of these species after the early expansion, trapping populations within small areas. *I. alpestris* is a short range disperser (Joly & Miaud, 1989), and populations might not have had sufficient time to return in contact during favourable phases, since cold phases in the Early Pleistocene were shorter than the last glacial phases (Head & Gibbard, 2005). In addition, population isolation may have been increased by paedomorphism, which probably evolved as an adaptation to Quaternary climatic instability by not encouraging dispersal from relatively stable habitats and refugia. Moreover, other species inhabiting mountain habitats have exhibited vicariance in the Ligurian Apennines from the Early Pleistocene (eg. *Hydromantes*, Cimmaruta *et al*., 2015), suggesting that they had common biogeographical and evolutionary pathways.

The TMRCA of the Calabrian and central Apennine lineages was estimated to be around 800 kya, which coincided with the Mid-Pleistocene revolution. This period was characterized by an increase in the amplitude and length of glacial cycles (from 40 to 100 kya) that had major biogeographical effects (Head & Gibbard, 2005; Hewitt, 2011a). The Italian Peninsula saw large changes in community assemblages (Capraro *et al*., 2005; Palombo *et al*, 2005), and several species' ranges underwent severe fragmentation that was followed by genetic differentiation (Canestrelli *et al*., 2012b; Maura *et al*, 2014). However, two particularly humid glacial cycles [corresponding to Marine Isotope Stage (MIS) 20 and MIS 18, which were 750–850 kya] caused the expansion of alpine forests in Calabria (Capraro *et al*., 2005), and might have favoured a southward expansion of *I. alpestris* that was closely linked with mountain forest habitats. Such a great expansion may never have been repeated, because we did not find any traces of successive contacts between the two lineages, which have accumulated large genetic differences.

In contrast, the secondary contact between the Ligurian and central Apennine lineages was estimated to be during the Late Pleistocene. Our spatiotemporal

phylogeographical reconstruction of the last phases of the Pleistocene showed that most of the expansion from the Ligurian and central Apennine refugia had already occurred at the end of the penultimate glacial period, which supports the hypothesis that *I. alpestris* populations underwent major expansions during the cold, glacial phases. Nevertheless, the penultimate glacial (MIS 6) was particularly cold and humid, and was interspersed by several interstadials with intermediate climate (Roucoux *et al*, 2011; Wainer *et al*., 2012; Litt *et al*., 2014). Such an unusual condition for a glacial stage favoured the expansion of montane tree forests in southern Europe (Roucoux *et al*, 2011), and may have favoured a greater expansion of *I. alpestris* from its refugia. However, at the beginning of the last interglacial, the two lineages had already established a secondary contact in eastern Liguria. The contact probably persisted during the last interglacial, because the SDM projection indicated that the contact zone was suitable for this species during the interglacial, suggesting that it was an interglacial refugium. Nevertheless, the SDM supported the hypothesis that there was a reduction in suitable bioclimatic conditions for this species during the interglacial and it expanded during glacial periods, despite the fact that the suitable area was more fragmented during the LGM. Despite the fragmentation and the fact that some putative, suitable areas were covered by ice, e.g. the Apuan and central Apennines (Ehlers & Gibbard, 2004), the Bayesian phylogeographical reconstruction indicated a further expansion of *I. alpestris* during the last glacial phase.

The genetic diversity distribution analysis of the *I. alpestris* populations revealed some interesting patterns. Haplotype diversity within each group, and within each population, was low compared to that of other peninsular amphibians (*Lissotriton italicus*, Canestrelli *et al*, 2012a; *Triturus carnifex*, Canestrelli *et al*, 2012b; *L. vulgaris*, Maura *et al*, 2014), but was similar to that of other cold-adapted amphibians, such as *Rana temporaria* (Stefani *et al*., 2012). The population genetic diversity as revealed by the microsatellite markers was low compared to that of other European populations of the same species (Pabijan & Babik, 2006; Prunier *et al*., 2014; Emaresi *et al*, 2011). These low levels of genetic diversity could have been caused by repeated bottlenecks of the Apennine populations during the Pleistocene, as well as the fragmentation and isolation of the peninsular populations. The lowest values of genetic diversity were exhibited by the southernmost and northernmost populations (samples 1 and 15). Nevertheless genetic diversity increased towards the core of the species's range. We found the highest levels of genetic diversity within the populations inside the contact zone, where all individuals were highly admixed with genetic components from the different lineages. Other peninsular taxa

have shown similar patterns, with the highest genetic diversity within the contact zones of different intraspecific lineages, rather than inside the refugia of such lineages (Canestrelli *et al*., 2008, 2010). This does not support the hypothesis that high levels of biodiversity within southern European peninsulas were caused by the relative stability of refugia, but highlights the importance of sub-refugia in shaping genetic variation and of melting-pot areas in reshuffling such variation and increasing biodiversity.

2.5 TABLES AND FIGURES

Table 1 Geographic location, and estimates of genetic variability at microsatellite loci for each of the 15 sampling sites of *Ichthyosaura alpestris*. n, sample size; Ar, Allelic richness; He, expected heterozygosity.

	Sample	Lat. N	Long. E	Microsatellites		
				n	***Ar***	***He***
1	**Montalto Uffugo**	39° 33'	16° 01'	**14**	1.99	0.22
2	**Monti della Laga**	42° 42'	13° 19'	**14**	2.13	0.25
3	**Iesa**	43° 05'	11° 14'	**7**	2.17	0.28
4	**Torsoli**	43° 33'	11° 23'	**12**	1.97	0.19
5	**Camaldoli**	43° 48'	11° 49'	**13**	2.38	0.30
6	**Monghidoro**	44° 13'	11° 17'	**10**	2.27	0.28
7	**Lago del Greppo**	44° 07'	10° 40'	**12**	2.36	0.24
8	**Minucciano**	44° 09'	10° 14'	**13**	1.87	0.23
9	**Stagno Bargone**	44° 19'	09° 29'	**16**	2.51	0.40
10	**Monte Penna**	44° 29'	09° 29'	**14**	2.44	0.38
11	**Capanne di Marcarolo**	44° 33'	08° 46'	**14**	2.47	0.37
12	**Rossiglione**	44° 32'	08° 37'	**10**	2.05	0.26
13	**Piampaludo**	44° 26'	08° 35'	**13**	2.50	0.32
14	**Madonna del Lago**	44° 07'	07° 59'	**13**	1.75	0.21
15	**Pecetto Torinese**	45° 02'	07° 43'	**10**	1.43	0.15

Table 2 PCR primers and annealing temperatures used to amplify the two mitochondrial and three nuclear DNA fragments used in this study. References: (1) Canestrelli *et al*., 2006a; (2) Nadachowska & Babik, 2009; (3) Sequeira *et al*., 2006.

Marker	Primers (5' -> 3')			annealing T (°C)	References
CytB	CytBtrit-F	F:	ACGCAAYATRCACATCAACGG		1
	CytBtrit-R	R:	GGAGTGACTATAGARTTTGCTGGG	53	1
NADH2	L3780mod2	F:	GGAGAAACCCCTTCTTTTGC		This study
	H5018mod1	R:	TGAAGGCCTTTGGTCTTGTTAT	59	This study
GH	GH-f	F:	TCTCATCAAGGTGAGTTTGAACA		2
	GH-r	R:	CCTTCTTGTGTCAGAGGTGCTAT	58	2
PDGF-R	Pdgfr-F	F:	TGCAGCTGCCATATGACTCTA		2
	Pdgfr-R	R:	TACGCTGTTCCTTCAACCACT	60	2
β-FIB	FibX7	F:	GGAGANAACAGNACNATGACAATNCAC		3
	FibX8	R:	ATCTNCCATTAGGNTTGGCTGCATGGC	50	3
	BFXF	F:	CAGYACTTTYGAYAGAGACAAYGATGG		3
	BFXR	R:	TTGTACCACCAKCCACCRTCTTC	59	3

Table 3 List of haplotypes found for each studied gene in each studied population. n, sample size; among brackets the number of haplotypes found.

Sample	Haplotypes							
	n	**mtDNA**	**n**	**β - Fibrinogen**	**n**	**PDGFR**	**n**	**GH**
1	**11**	AVIII	**6**	AI(6), AII(6)	**9**	AII	**9**	AI(18)
2	**10**	AI	**7**	AI	**7**	AI(1), AIV(4), AV(9)	**7**	AI(14)
3	**4**	AI(3), AIII(1)	**4**	AI	**4**	AI(6), AIV(2)	**4**	AI(8)
4	**9**	AI(9)	**9**	AI	**8**	AI(9), AIV(7)	**7**	AI(12), AII(2)
5	**11**	AI(4), AII(7)	**6**	AI	**6**	AI	**6**	AI(12)
6	**7**	AI	**8**	AI	**5**	AI(5), AIV(5)	**7**	AI(10), AII(3), AVI(1)
7	**9**	AI(8), AV(1)	**11**	AI	**10**	AI(15), AIII(1), AIV(4)	**10**	AI(19), AII(1)
8	**10**	AI	**8**	AI	**8**	AI(11), AIV(5)	**8**	AI(16)
9	**14**	AVI(13), AVII(1)	**11**	AI(13), AIV(2), B1(5), BII(2)	**14**	AI	**8**	AI(12), C(4)
10	**14**	AVI(12), BII(2)	**8**	AI(13), AIII(1), BII(2)	**9**	AI	**7**	AI(9), BI(5)
11	**9**	BI(8), BVI(1)	**5**	AI(4), BI(4), BII(2)	**6**	AI	**5**	AI(6), BI(4)
12	**9**	BI	**5**	AI(4), BI(4), BII(2)	**5**	AI	**4**	AI(5), AIII(2), BI(1)
13	**12**	BI(3), BIV(2), BV(1), BIII(6)	**6**	AI(6), BI(6)	**8**	AI	**7**	AI(11), BI(3)
14	**10**	BII	**5**	BII(8), AI(2)	**5**	AI	**6**	AI(3), AV(1), BI(7), BII(1)
15	**10**	BI	**5**	AI	**7**	AI	**8**	AI(5), BI(10), BII(1)

Table 4 Results of the spatial diffusion models selection showing marginal likelihood under path sampling (PS) and stepping stone sampling (SS) procedures for the two studied lineages (details within text).

Clade	model	$\log_e$ Marginal Likelihood	
		PS	**SS**
Ligurian clade	Brownian	-1490.1	-1491
	Cauchy	-1497.6	-1498.5
	Gamma	-1492.4	-1493.3
north- central Apennine clade	Brownian	-1335.8	-1338.1
	Cauchy	-1347.1	-1350.1
	Gamma	-1330.5	-1333.1

Table 5 Allele frequencies of the microsatellites loci found polymorphic among the 15 sampled populations of *Ichthyosaura alpestris*.

LOCUS/ ALLELE	POPULATIONS														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Copta1															
229	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.63	0.86	0.57	0.28	0.23	---	0.33
231	---	---	---	---	---	---	---	---	0.37	0.14	0.43	0.72	0.77	1.00	0.67
Copta3															
162	---	---	---	---	---	---	---	---	0.03	---	---	---	---	---	---
168	---	---	---	---	---	---	---	0.04	---	---	---	---	---	---	---
170	---	---	---	---	---	---	0.04	---	0.06	---	---	---	---	---	---
172	1.00	1.00	1.00	1.00	1.00	1.00	0.96	0.96	0.91	1.00	1.00	1.00	0.92	1.00	1.00
174	---	---	---	---	---	---	---	---	---	---	---	---	0.08	---	---
Copta8															
165	---	0.71	0.70	0.79	0.81	0.72	0.92	0.62	0.56	0.75	1.00	1.00	1.00	1.00	1.00
167	---	0.29	0.30	0.21	0.19	0.28	0.04	0.35	0.34	0.25	---	---	---	---	---
169	0.68	---	---	---	---	---	0.04	0.04	0.09	---	---	---	---	---	---
171	0.32	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Copta9															
218	---	---	---	---	---	---	---	---	---	---	0.04	0.05	0.04	---	---
222	---	---	---	0.17	---	---	---	---	---	---	0.08	---	---	0.12	---
226	0.04	0.04	---	0.04	---	---	---	0.04	---	0.11	0.19	0.25	0.08	0.23	---
230	0.04	0.11	---	0.04	0.15	---	0.22	0.04	---	0.21	0.23	0.20	0.08	0.31	0.50
234	0.19	---	0.17	0.08	0.04	---	0.06	---	0.30	0.21	0.08	0.35	0.33	0.35	---
238	0.08	0.14	---	0.04	0.15	0.15	---	0.27	0.20	---	0.19	0.10	0.17	---	0.05
242	---	0.04	0.25	0.33	0.04	0.05	0.11	---	0.10	0.25	0.04	0.05	0.13	---	0.40
246	0.23	---	---	0.04	---	0.10	0.11	---	0.10	---	---	---	0.08	---	---
250	---	---	---	---	0.08	0.35	0.11	0.19	0.07	---	0.15	---	0.04	---	0.05
254	---	0.21	0.33	---	0.12	0.10	0.06	0.35	---	---	---	---	---	---	---
258	0.04	---	0.08	---	0.04	---	0.17	0.12	---	0.07	---	---	---	---	---
262	0.19	0.04	---	0.08	0.23	0.10	0.06	---	---	0.11	---	---	0.04	---	---
266	0.12	---	---	0.04	0.04	---	---	---	---	---	---	---	---	---	---
270	---	0.07	---	---	---	---	0.06	---	0.23	---	---	---	---	---	---
274	---	0.04	---	0.13	---	0.15	---	---	---	0.04	---	---	---	---	---
278	---	0.11	0.08	---	---	---	---	---	---	---	---	---	---	---	---
282	0.08	0.14	---	---	0.12	---	---	---	---	---	---	---	---	---	---
286	---	0.04	0.08	---	---	---	0.06	---	---	---	---	---	---	---	---
294	---	0.04	---	---	---	---	---	---	---	---	---	---	---	---	---

follows

following

Table 5 Allele frequencies.

LOCUS/ ALLELE	POPULATION														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Copta13															
199	---	0.63	---	---	0.77	0.06	0.04	---	---	0.04	---	---	---	---	---
203	1.00	0.38	1.00	1.00	0.23	0.94	0.96	1.00	1.00	0.96	1.00	1.00	1.00	1.00	1.00
Ta3Ca8															
125	---	---	---	---	---	---	---	---	---	---	0.19	---	0.08	---	---
131	---	---	---	---	0.17	0.05	---	---	0.09	0.08	0.35	0.11	0.25	0.05	---
135	---	---	---	---	---	---	---	---	---	---	---	---	0.08	---	---
141	---	---	0.25	---	0.04	0.10	0.09	0.15	0.38	0.42	0.15	0.17	0.33	0.85	1.00
145	---	---	---	---	---	0.05	---	---	---	---	---	---	---	---	---
147	---	---	0.25	0.09	0.21	0.30	0.68	---	0.22	0.17	0.19	0.72	0.25	0.05	---
151	0.15	1.00	0.50	0.91	0.58	0.50	0.23	0.85	0.31	0.33	0.12	---	---	0.05	---
153	0.85	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Ta3Caga2															
132	---	---	0.07	---	---	---	---	---	---	---	---	---	---	---	---
136	1.00	1.00	0.93	1.00	1.00	1.00	1.00	1.00	0.94	0.54	0.54	0.85	0.92	0.46	1.00
160	---	---	---	---	---	---	---	---	0.06	0.46	0.46	0.15	0.08	0.54	---

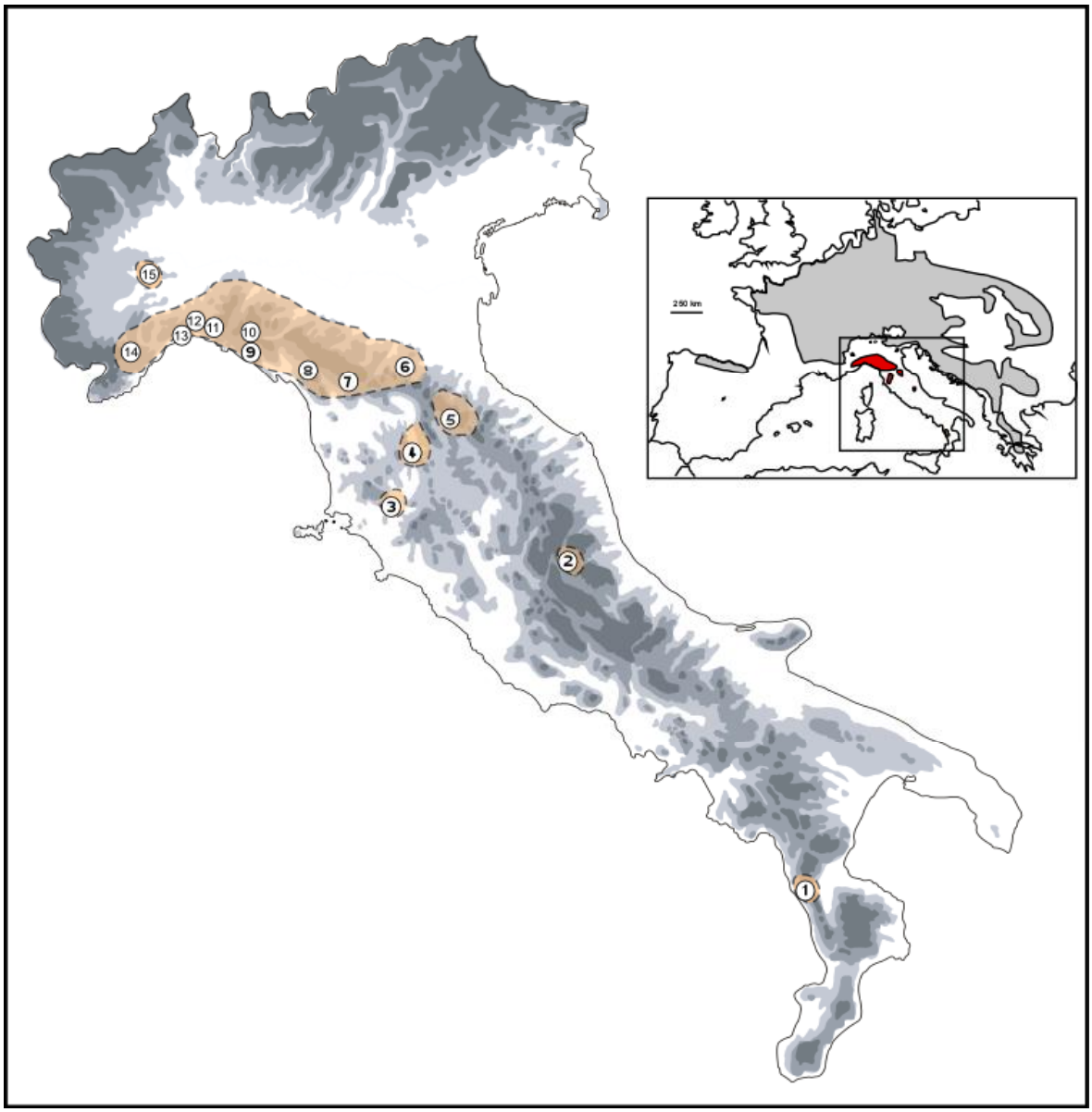

Figure 1 Geographic distribution of *Ichthyosaura alpestris* within Apennine peninsula and geographic location of the 15 sampled populations. Localities are numbered as in Table 1. The inset shows distribution of *I. alpestris* within the Western Palearctic region.

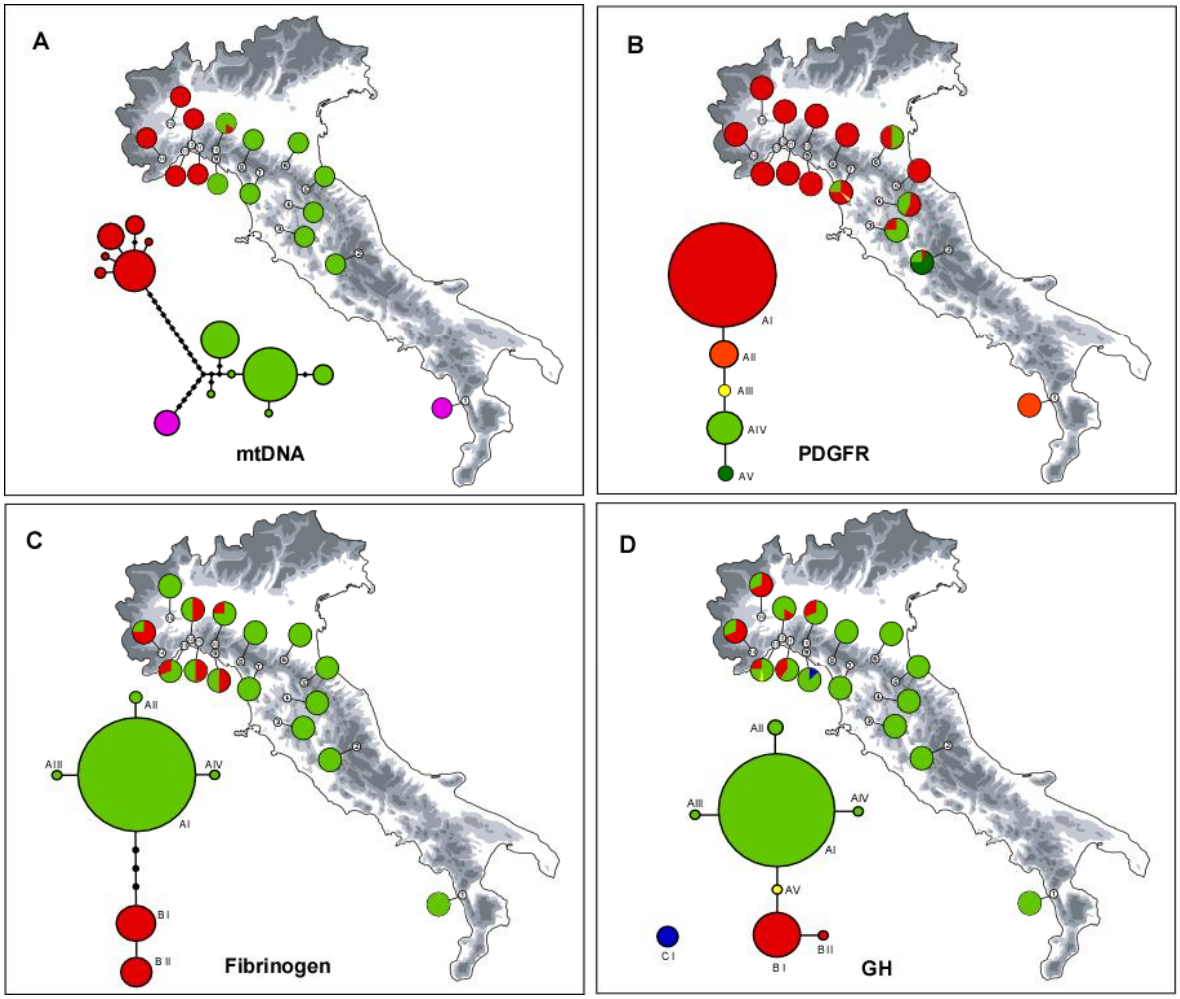

Figure 2 A) Haplotype genealogy yielded using HAPLOTYPEVIEWER, based on the ML phylogenetic tree of the *Ichthyosaura alpestris* mtDNA haplotypes found. B-D) Statistical parsimony network showing genealogical relationships among haplotypes found in PDGFR gene, Fibrinogen gene, GH gene, respectively. Circle sizes are proportional to haplotype frequency; missing intermediate haplotypes are shown as black dots. Pie diagrams show the frequency distribution of the haplogroups among studied populations.

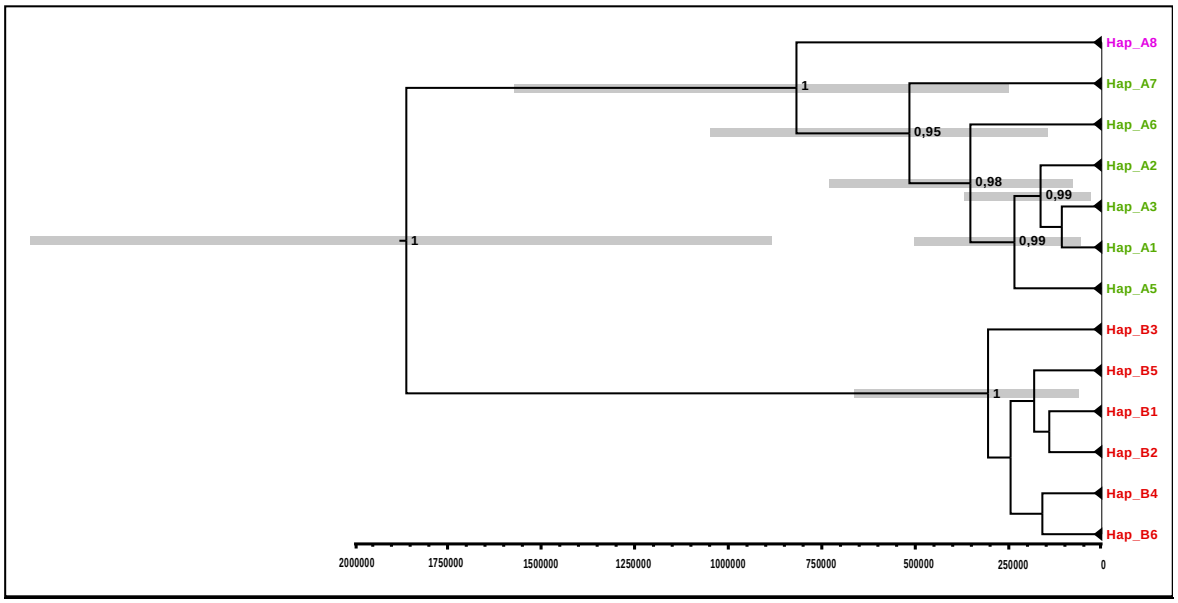

Figure 3 Maximum clade credibility tree based on *Ichthyosaura alpestris* mtDNA dataset recovered by Bayesian analysis implemented in BEAST, showing TMRCA for major clades. Node bars (grey) represent 95% HPD intervals for node ages. Also shown the posterior probabilities for each node (only values > 0.9 are shown). The scale is in years before present.

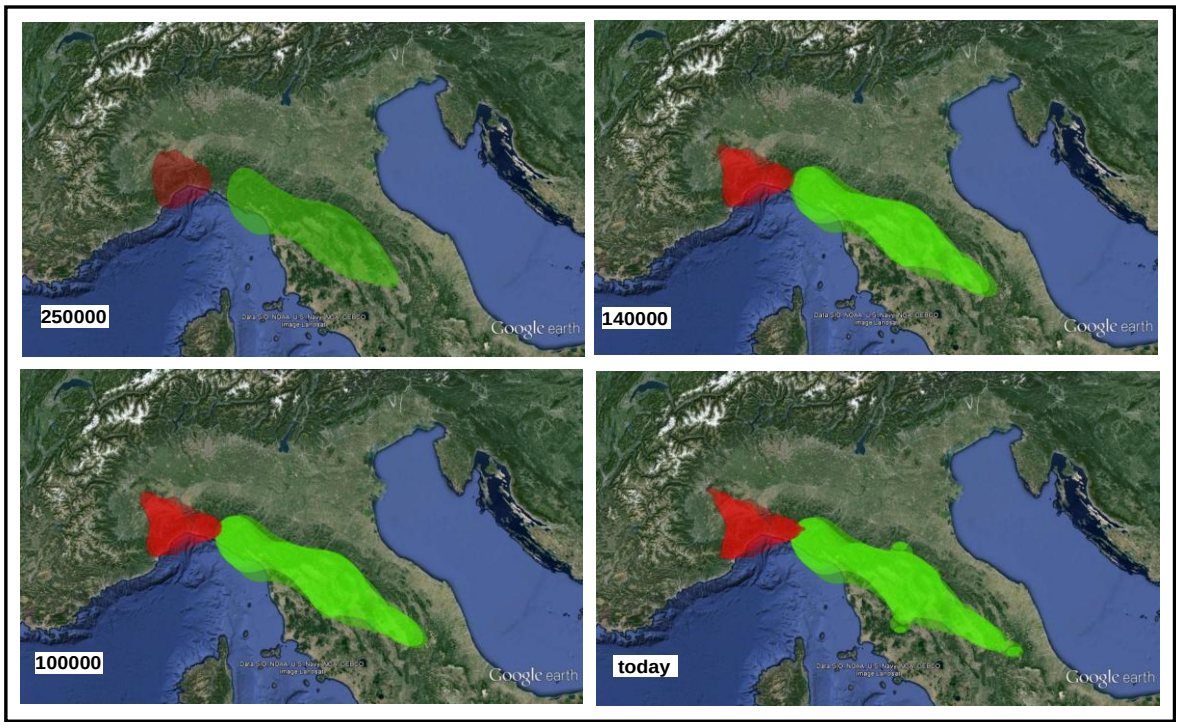

Figure 4 Spatial diffusion of the two main genetic lineages of *Ichthyosaura alpestris* through time, based on the Bayesian phylogeographical analyses implemented in BEAST and carried out on the mtDNA data sets independently for both lineages. Four time intervals are shown. Polygons represent 80% HPD uncertainty for the spatial location of ancestral populations; colours follow Fig. 1A. Time is in years before present.

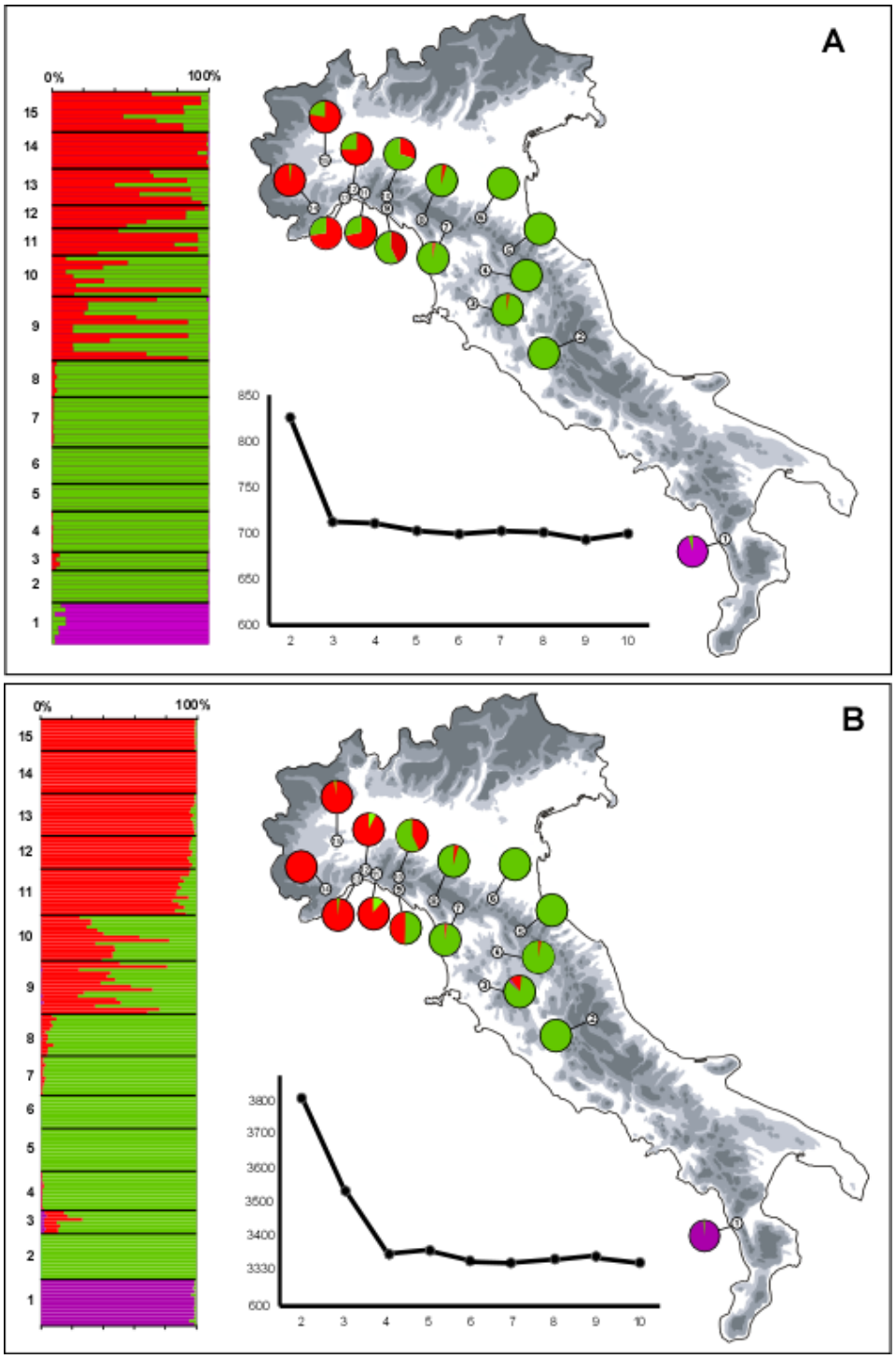

Figure 5 Genetic structure of *Ichthyosaura alpestris* populations in Italy, estimated using TESS, based on (A) nuclear sequence dataset and (B) microsatellite dataset. The bar plots show the admixture proportions of each individual for the 3 recovered genetic clusters. Pie diagrams on maps show the frequency distribution of each cluster among the populations. Line charts show mean values of the DIC statistics (averaged over 100 runs) estimated for models with the number of genetic clusters (K) ranging from 2 to 10.

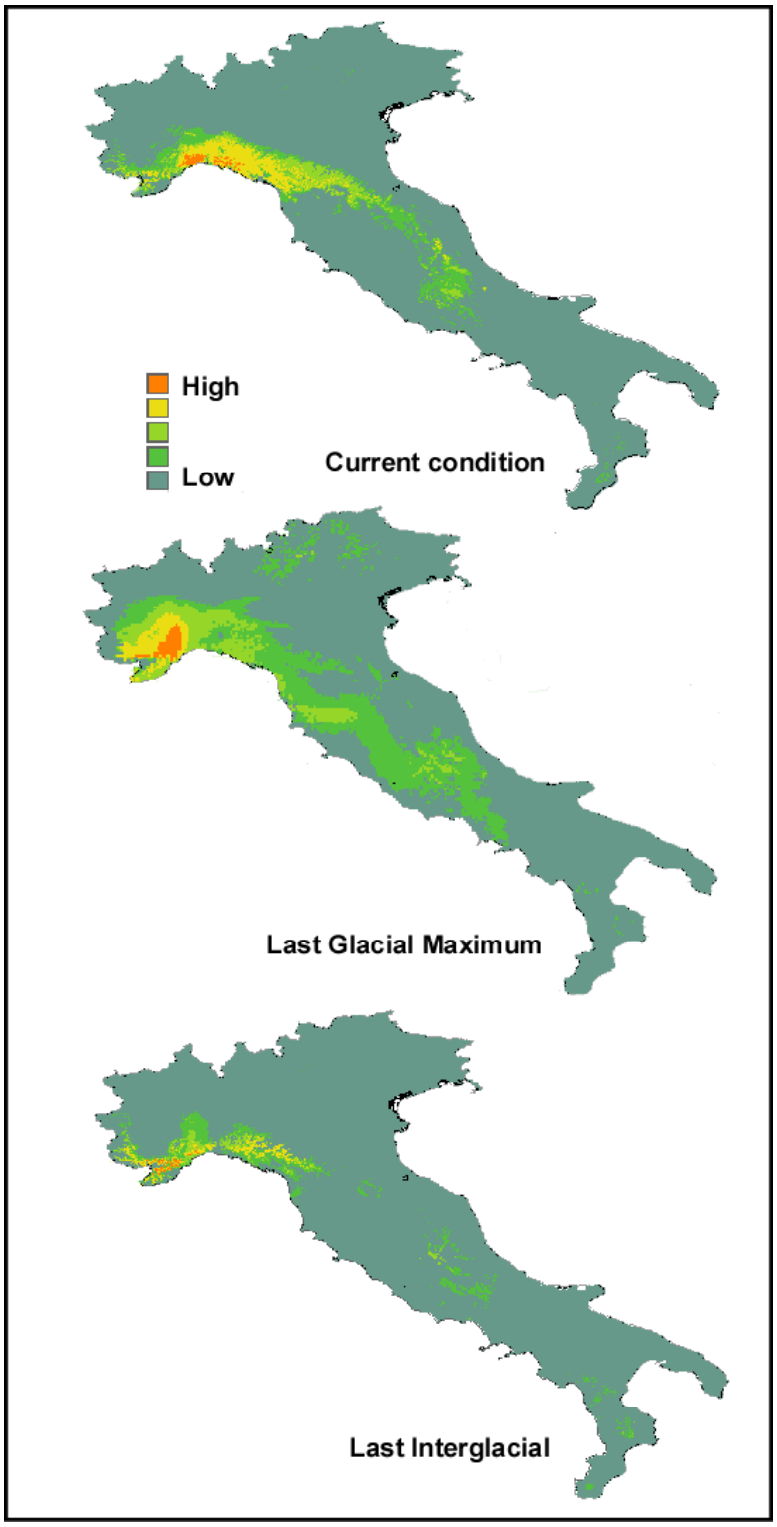

Figure 6 Species distribution models for *Ichthyosaura alpestris* on the Apennine peninsula, estimated by MAXENT algorithm for current conditions (A), for the Last Glacial Maximum, mean between MIROC and CCSM (B), and for Last Interglacial. Warmer colours show areas with better predicted conditions.

3. GEOGRAPHIC DISTRIBUTION OF THE CHYTRID PATHOGEN *BATRACHOCHYTRIUM DENDROBATIDIS* AMONG MOUNTAIN AMPHIBIANS ALONG THE ITALIAN PENINSULA

Diseases of Aquatic Organisms 107: 61-68.

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ABSTRACT

The amphibian chytrid pathogen *Batrachochytrium dendrobatidis (Bd)* is considered a major cause of amphibian population declines, particularly in montane areas. Here, we investigated the presence and distribution of *Bd* among populations of 3 mid- to high-altitude species spanning the entire Italian peninsula (486 individuals from 39 sites overall): the stream frog *Rana italica*, the fire salamander *Salamandra salamandra gigliolii*, and the alpine newt *Mesotriton alpestris apuanus*. We found *Bd* in all of the analyzed species. Despite the widespread distribution of the pathogen, its overall prevalence (6, 9 and 19%, respectively) was lower than previously reported for the endangered Apennine yellow-bellied toad *Bombina pachypus* (62.5%). Moreover, several populations of the species studied here were not infected, even at sites where *Bd* has been detected in other host species. When coupled with the lack of evidence for *Bd*-related mortalities in these species in peninsular Italy, these results suggest that mechanisms of resistance and/or tolerance are protecting populations of these species from the pathogenic activity of *Bd*. Nevertheless, in light of the dynamic pattern of *Bd*−host interactions reported in other studies, of *Bd*-related mortalities in at least 1 study species *(S. s. salamandra)* in other areas, and the ongoing climate changes in montane environments, we suggest that the occurrence of *Bd* should be considered a potential threat to the long-term persistence of these species, and urge the implementation of monitoring and conservation plans.

KEY WORDS: *Batrachochytrium dendrobatidis, Bd,* Italian peninsula, *Salamandra salamandra, Rana italica, Mesotriton alpestris,* Amphibian conservation

3.1 INTRODUCTION

Amphibians are declining worldwide, and chytridiomycosis is among the main causes (Berger *et al*. 1998, Daszak *et al*. 1999, 2003, Fisher *et al*. 2009, Kilpatrick *et al*. 2010). This emerging infectious disease is caused by the chytrid fungus *Batrachochytrium dendrobatidis (Bd)*, which affects the keratinized epidermal cells of its amphibian hosts (Longcore *et al*. 1999, Pessier *et al*. 1999). In recent years, many studies have been devoted to assessing the global distribution of *Bd* and the susceptibility of different amphibian species and populations to decline following infection (e.g. Berger *et al*. 1998, Bosch *et al*. 2001, Muths *et al*. 2003, Schloegel *et al*. 2006, Goka *et al*. 2009). Despite these efforts, many aspects of the epidemiology of *Bd* are still poorly understood (McCallum 2005, Rachowicz *et al*. 2005, Fisher *et al*. 2009). The geographic origin of *Bd,* its spread dynamics, interactions with other factors, and the variability in the outcome of infection are among the main issues still unclear (Weldon *et al*. 2004, Rachowicz *et al*. 2005, Pounds *et al*. 2006, Blaustein *et al*. 2011). Indeed, field observations show that, despite its wide distribution and the many species it infects, the outcome of the infection varies not only between different species, but also between populations within species and between geographic areas (e.g. Tobler & Schmidt 2010, Walker *et al*. 2010).

Altitude is one of the geographic factors apparently related to the severity of disease; the most severe mass mortalities have been observed in mountain species (Berger *et al*. 1998, Bosch *et al*. 2001, Fellers *et al*. 2001). Early observations of chytridiomycosis have been reported from montane rain forests in Australia and Central America (Berger *et al*. 1998). Severe die-offs at high altitudes have also been observed in temperate regions in North America and Europe (Bosch *et al*. 2001, Fellers *et al*. 2001). In Europe, mass mortalities of 3 common species *(Alytes obstetricans*, *Salamandra salamandra* and *Bufo bufo)* have been observed at a high altitude in a protected area (Bosch *et al*. 2001, Bosch & Martínez-Solano 2006), and a strong association between altitude and fatal chytridiomycosis has been detected (Walker *et al*. 2010).

In a previous assessment of *Bd* occurrence along the Apennine Mountains (Italy), we found evidence of this pathogen's widespread presence in the endangered yellow-bellied toad *Bombina pachypus*, a species with a mid-to-high altitude range (Canestrelli *et al*. 2013). This Italian endemic species has experienced severe population declines in most of its range, with the exception of the southernmost area, over the last 15 yr (Barbieri *et al*.

2004, Andreone *et al*. 2009), and it has been proposed that *Bd*-related mortality has played a role in this (Stagni *et al*. 2004). Interestingly, we found a delay of at least 1 decade between the first evidence of *Bd* occurrence and the beginning of the *B. pachypus* decline. Moreover, we observed heterogeneity in the spatial patterns; while *Bd* was widespread throughout the study area, *B. pachypus* population declines varied by geographic region (Canestrelli *et al*. 2013). On the whole, these observations suggests that (1) *Bd* did not act as a 'lone killer', (2) *Bd* can occur in susceptible host species without signs of decline until triggered by other factors, and (3) other factors can influence the outcome of the host−pathogen interaction, resulting in a temporally and spatially heterogeneous pattern (see Rosenblum *et al*. 2013). Since this pattern could apply to other amphibian species, we stress the importance of assessing the infection status of potential host species in areas of *Bd* occurrence, even in the absence of pathological signs or evidence of population declines.

In this study, we extended the assessment of *Bd* occurrence along the Italian peninsula to 3 species with a mid-to-high altitude range, which are sympatric and frequently syntopic with *Bombina pachypus* along the Apennine Mountains. We carried out diagnostic tests using a nested PCR approach to check for the presence of *Bd* on individuals of the Italian fire salamander *Salamandra salamandra gigliolii*, the Italian endemic stream frog *Rana italica* and the Italian alpine newt *Mesotriton alpestris apuanus*. These species are not considered to be as severely endangered as *B. pachypus*, although population declines have been recently reported (e.g. Ambrogio & Gilli 1998, Bologna *et al*. 2000, Canestrelli *et al*. 2006, Lanza *et al*. 2007). In light of the occurrence of the pathogen along their range and their frequent syntopy with a highly infected species, our aim was to assess the *Bd* infection status for these species in the wild and so, to evaluate if *Bd* could be considered a potential risk factor for these species from a conservation perspective.

3.2 MATERIALS AND METHODS

We analyzed a total of 486 individuals: 77 *Salamandra salamandra gigliolii*, 136 *Rana italica* and 273 *Mesotriton alpestris apuanus*. Sampling locations, as well as the number of individuals sampled in each site and year are presented in Table 1 and Fig. 1. All of the samples were from adult individuals, collected from April to June. *S. s. gigliolii* individuals were captured on the ground, within 50 m from the nearest stream; *R. italica* individuals were captured near the banks of streams; and *M. a. apuanus* were captured in a variety of standing-water habitats, during their aquatic phase. Sampling was carried out using finetipped swabs (Medical Wire & Equipment Co. MW 113) for samples collected from 2010 onwards and toe clipping for samples collected before 2010.

Genomic DNA was extracted from swabs following the protocol of Boyle *et al*. (2004), with some modifications. We placed a single swab in a 2 ml tube, adding 30 to 40 mg of glass beads measuring 0.4 to 0.6 mm in diameter (Sartorius) and 70 µl of PrepMan Ultra (Applied Biosystems). Tubes were vortexed for 1 min at 2400 rpm in a vortex and centrifuged for 30 s at 13 000 × *g* in a microfuge (Eppendorf Centrifuge 5415 D). The vortex and centrifugation procedures were done twice. Next, samples were boiled for 10 min, cooled at room temperature for 2 min and centrifuged at 13 000 × *g* for 3 min; 20 µl of supernatant were retained and stored at −20°C until further analysis.

Genomic DNA was extracted from tissue samples following the standard cetyltrimethyl ammonium bromide (CTAB) protocol (Sambrook *et al*. 1989) with final elution in 30 µl in order to gain a concentrated DNA solution.

The molecular diagnostic screening to test for the presence of *Bd* DNA within the pool of extracted DNA was conducted using the nested-PCR protocol developed by Goka *et al*. (2009), with some modifications. The target DNA was amplified twice using 2 different pairs of primers.

The first PCR was performed using the primer pair Bd18SF1 (5'-TTT GTA CAC ACC GCC CGT CGC-3') and Bd28SR1 (5'-ATA TGC TTA AGT TCA GCG GG-3'). The reaction mix (25 µl) contained: 2 µl of template DNA (DNA extracted from swabs using PrepMan Ultra was diluted 1:10 with distilled water), 0.5 µM of each primer, 2 mM MgCl2, 0.2 mM of each dNTP, Colorless GoTaq reaction buffer 1× and 1 U of GoTaq Polymerase (Promega). The PCR cycling process was as follows: an initial denaturation

step for 9 min at 95°C, 30 cycles of 30 s at 94°C, 30 s at 50°C and 2 min at 72°C, and a final extension step of 7 min at 72°C (Goka *et al*. 2009).

The second PCR was performed using the primer pair Bd1a (5'-CAG TGT GCC ATA TGT CAC G-3') and Bd2a (5'-CAT GGT TCA TAT CTG TCC AG-3') (Annis *et al*. 2004). The reaction mix (25 μl) contained: 2 μl of the first PCR product used as template, 0.5 μM of each primer, 2 mM MgCl2, 0.2 mM of each dNTP, Colorless GoTaq reaction buffer 1× and 1 U of GoTaq Polymerase (Promega). The PCR cycling was conducted with a touchdown program. After an initial denaturation for 5 min at 95°C, 35 cycles were performed as follows: 30 s at 94°C, 30 s at 65°C with a decrement of 0.2°C in each cycle and 30 s at 72°C. These cycles were followed by a final extension of 7 min at 72°C. Each assay included a positive control with DNA extracted from *Bd* zoospores (JEL423 kindly provided by Prof. Joyce Longcore, University of Maine) and a negative control of DNA-free distilled water.

PCR products (if any) were separated and visualized on a 1% agarose gel. We considered a positive result to occur if a sample returned an amplification product of the correct size (approximately 300 bp). To confirm that the amplification products were from the *Bd* genome, a randomly selected 18% of the PCR products were double-sequenced (n = 12) and compared with reference sequences in GenBank. Sequencing were carried out by Macrogen Inc. (www.macrogen.com) using an automated capillary electrophoresis system (ABI 3730XL).

3.3 RESULTS

From 486 total screened samples the diagnostic nested PCR assay yielded 66 positive results, i.e. showing a PCR band of the expected size (approximately 300 bp). Among these, the highest *Bd* prevalence was observed in *Mesotriton alpestris apuanus*, in which 51 out of 273 samples (19%; 95% confidence interval: 15–24%) tested positive, while the observed prevalence in other analyzed species was 7 out of 77 (9%; 95% confidence interval: 5–18%) in *Salamandra salamandra gigliolii* and 8 out of 136 (6%; 95% confidence interval: 3–11%) in *Rana italica*. All the sequenced PCR products showed 100% identity with the *Bd* sequences available from GenBank.

Numbers of positive and tested individuals in each population, along with geographic information on the sampling site and year are reported in Table 1. The pathogen was widespread geographically among the analyzed species (Fig. 1). Among them, *Mesotriton alpestris apuanus* showed a wider *Bd* distribution, with positive individuals detected in 50% of the sampling sites, distributed throughout its entire range. *Salamandra salamandra gigliolii* and *Rana italica* showed *Bd* occurrence in 36 and 30% of the sampling sites, respectively.

A significant increase in *Bd* prevalence with increasing altitude was observed in *Mesotriton alpestris apuanus*, in which 22% individuals tested positive from populations sampled at sites above 800 m a.s.l., while 5% individuals tested positive from populations sampled at sites below 800 m a.s.l. (Fisher exact test, $p = 0.002$). In *Salamandra salamandra gigliolii* and *Rana italica* we did not observed significant differences in *Bd* prevalence with altitude. The prevalence of *Bd* occurrence in individuals from *S. s. gigliolii* populations sampled above and below 800 m a.s.l. was 8 and 15%, respectively (Fisher exact test, $p = 0.336$), and 6% of individuals tested positive from *R. italica* populations sampled both above and below 800 m a.s.l. (Fisher exact test, $p = 1$).

Finally, contrary to what was previously observed for *Bombina pachypus* (Canestrelli *et al*. 2013), we did not find significant differences in *Bd* prevalence according to latitude. The prevalence of *Bd* in *Salamandra salamandra gigliolii* was 9% among both northern and central populations and populations from Calabria (Fisher exact test, $p = 1$), while its prevalence in *Rana italica* was 11% among northern and central populations and 3% in populations from Calabria (Fisher exact test, $p = 0.139$).

3.4 DISCUSSION

Our results are in agreement with previous findings in showing a wide distribution of *Bd* along the Apennine Mountains (see Canestrelli *et al*. 2013). Nevertheless, both the infection prevalence and the proportion of the affected populations in each species were lower in the 3 species analyzed here than in *Bombina pachypus*. Moreover, several of the populations testing negative were sampled at sites where individuals from other species tested positive (Sites 13, 29, 32, 37 and 38) or at sites where *B. pachypus* populations were previously observed to be highly infected (Sites 14, 29, 35, 38 and 39). Furthermore, despite some reports of population declines (e.g. for *Salamandra salamandra gigliolii* in the north-central Apennines and for *Mesotriton alpestris apuanus* in the southern areas; see Ambrogio & Gilli 1998, Bologna *et al*. 2000, Canestrelli *et al*. 2006, Lanza *et al*. 2007), no evidence of *Bd*-related die-offs have been reported for these species in Italy, and other factors have been indicated as the main drivers, such as the introduction of fishes, eutrophication due to intensive farming, water catchment, climate changes, geographic isolation and reduced genetic variability. On the other hand, some populations testing positive did not appear de - mographically impoverished or unstable over multiple years of observation (e.g. *M. a. apuanus* at Site 13; D. Canestrelli pers. obs.).

On the whole, contrary to what has previously been suggested for *Bombina pachypus*, and even if we cannot exclude a role for *Bd* in past population disappearances on the basis of our data, it does not seem that *Bd* is currently threatening populations of *Salamandra salamandra gigliolii*, *Rana italica*, or *Mesotriton alpestris apuanus* in Italy. In turn, this suggests that mechanisms of resistance or tolerance are protecting populations of these species from the pathogenic activity of *Bd*.

Nevertheless, we suggest that the widespread distribution of *Bd* along the Italian peninsula and its occurrence in the studied populations should be regarded as a serious threat for the long-term survival of *Salamandra salamandra gigliolii*, *Rana italica*, and *Mesotriton alpestris apuanus* in the area, for at least 2 reasons. First, a time lag between the earliest evidence of *Bd* occurrence and host population declines has been observed in other studies (Puschendorf *et al*. 2006, Canestrelli *et al*. 2013), indicating that currently resistant/tolerant populations can eventually become threatened in the future, when altered environmental conditions shift host− pathogen interactions toward the pathogen's optimum or increase host susceptibility. For instance, within Peñalara Natural Park in central Spain

the fire salamander *Salamandra salamandra* and the common toad *Bufo bufo* underwent mass mortalities 4 yr after chytridiomycosis almost extirpated the midwife toad *Alytes obstetricans* from the same area (Bosch *et al*. 2001, Bosch & Martínez- Solano 2006). In addition, in the case of *Bombina pachypus*, population declines were observed more than a decade after the first evidence of *Bd* occurrence in the area (Canestrelli *et al*. 2013). Second, most of the reported events of *Bd*−related mass mortality are from montane areas, both in tropical and temperate regions, suggesting that the outcome of host−pathogen interactions could be more affected by environmental changes at high altitude (Berger *et al*. 1998, Bosch *et al*. 2001, Fellers *et al*. 2001). On the one hand, most montane amphibians show prolonged aquatic life stages, giving them a prolonged exposure to *Bd* zoospores (Catenazzi *et al*. 2013). On the other hand, the particularly severe impact of ongoing climate change on montane biodiversity (Diaz *et al*. 2003, Nogués-Bravo *et al*. 2007; Raxworthy *et al*. 2008) could lead to a combination of suboptimal environmental conditions for host species due to the upward shift of the climatic niche and increased pathogenic activity as the 'chytrid thermal optimum' is approached (Pounds *et al*. 2006). Interestingly, we found a significantly higher prevalence of *Bd* among high-altitude populations of *M. a. apuanus*, the species most strictly confined to montane habitats. Moreover, as mentioned above, at least one study species *(S. salamandra)* has already been observed to undergo *Bd*−related mortalities in montane areas in other geographic regions (Bosch & Martínez-Solano 2006).

Unravelling the regional distribution of *Bd* is a first, highly necessary but insufficient step toward the appreciation of disease dynamics in the area and the implementation of management and conservation plans for its amphibian hosts (Woodhams *et al*. 2011). Here we have shown that this pathogen is widespread among montane amphibians throughout the Italian peninsula. Monitoring plans to assess *Bd* prevalence and virulence and their variation over time among multiple host populations should be carried out in the future, in order to identify possible shifts in host−pathogen interactions toward increased pathogenicity. Moreover, it will be of the utmost importance to extend the study to the whole amphibian fauna in the area, to better understand disease ecology and dynamics among different species and populations, and to fully appreciate the potential threats posed by *Bd* to the amphibians in the area. Finally, in light of recent advances in the development of strategies to mitigate chytridiomycosis (Woodhams *et al*. 2011, Bletz *et al*. 2013), it now appears increasingly reasonable to develop management plans that include *ex situ* conservation programs for host populations.

3.5 TABLES AND FIGURES

Table 1 Geographic location, altitude and sampling year of the populations of *S. s. gigliolii*, *R. italica*, and *M. a. apuanus*, with number of individuals analysed (n), and number of individuals testing positive for the presence of *B. dendrobatidis* (p) are also reported.

	Site	Latitude N	Longitude E	Altitude (m a.s.l.)	*S. s. gigliolii*			*R. italica*			*M. a. apuanus*		
					Year	N_{tot}	p	year	N_{tot}	p	Year	N_{tot}	p
1	Pianpaludo	44°26’	8°35’	800							2010	10	0
2	Rossiglione	44°32’	8°37’	480							2010	16	1
3	Capanne di Marcarolo	44°33’	8°46’	780							2009	18	0
4	Vallecalda	44° 32’	8° 57’	580	2007	6	0						
5	Monte Penna	44°29’	9°29’	1460							2007	16	0
6	Lago di Bargone	44°19’	9°29’	850							2009	18	0
7	Minucciano	44°09’	10°14’	800							2009	20	5
8	Cipollaio	44° 03’	10° 15’	820	1981	3	0						
9	Stazzema	44°01’	10°18’	890							1984	5	0
10	Abetone	44°07’	10°37’	1800							2009	24	0
11	Monghidoro	44°13’	11°17’	830							2009	16	4
12	Greve in Chianti	43°33’	11°23’	500							1983	4	0
											2009	14	2
13	Camaldoli	43° 48’	11° 49’	1000							2001	5	0
											2004	12	12
					2007	10	0						
					2012	4	0	2012	1	0	2012	18	17
14	Bagno di Romagna	43° 50’	11° 57’	460				2007	7	0			
								2012	5	0			
15	Lama	43° 05’	11° 14’	260							2009	7	0
16	Monte Rufeno	42° 48’	11° 53’	500				2007	1	0			
17	Blera	42° 15’	12° 03’	270				2007	2	0			
18	Tolfa	42°07’	11°56’	400				2007	7	0			
19	Monti della Laga	42°42’	13°19’	1500							2000	3	0
											2003	23	5
											2008	18	1
											2012	2	0
20	Percile	42° 04’	12° 54’	580				2003	6	1			
21	Patrica	41°35’	13°14’	400				2007	2	0			
22	Palena	41° 59’	14° 08’	770				2003	1	0			
23	Pescolanciano	41° 43’	14° 20’	945	2003	2	1						
24	Mercogliano	40°54’	14°44’	600	2004	1	0						
25	Serino	40° 50’	15° 51’	420				2005	5	0			
26	Oppido	40°51’	15°10’	600				2009	2	1			
27	S. Angelo a Fasanella	40° 27’	15° 20’	520				2003	10	2			
28	Laurino	40°19’	15°20’	900				2006	3	1			
29	S. Severino Lucano	40° 03’	16° 07’	680	2003	6	2						
								2012	5	0			
30	Viggianello	39° 58’	16° 05’	550				2003	4	1			
31	S. Lorenzo Bellizzi	39° 53’	16° 20’	830				2003	6	0			
32	Fagnano Castello	39°33’	16°01’	1090	2012	6	0	2012	2	0	2010	19	2
											2012	5	2
33	Villaggio Mancuso	39° 04’	16° 33’	1200	2003	10	2	2003	2	1			
34	Serra S. Bruno	38° 35’	16° 20’	805	1998	5	0						
								2003	7	0			
					2010	3	0	2010	10	0			
					2012	1	0	2012	1	0			
35	Stilo	38° 29’	16° 28’	290				2003	4	0			
								2010	12	0			
								2012	9	0			
36	Zomaro	38° 20’	16° 09’	960				2006	9	0			
37	Carmelia	38° 14’	15° 55’	1220	2006	6	2	2006	3	0			
38	Gambarie	38° 10’	15° 50’	1310	2003	14	0	2003	1	1			
								2012	7	0			
39	Cardeto	38° 05’	15° 46’	680				2006	2	0			

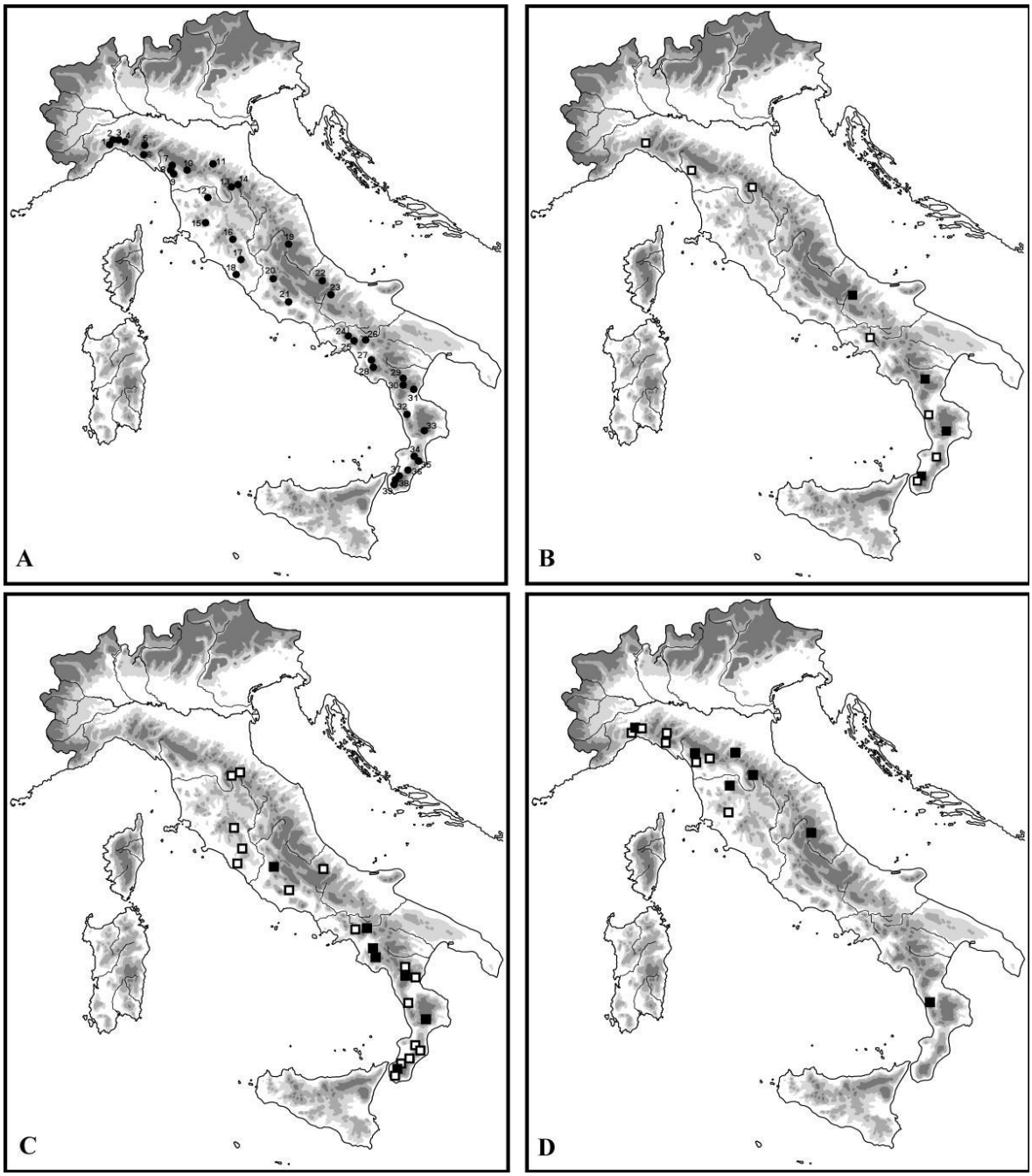

Figure 1 Geographic distribution of the 39 sampling sites (A) and *B. dendrobatidis* occurrence on *S. s. gigliolii* (B), *R. italica* (C) and *M. a. apuanus* (D). Full squares indicate chytrid positive populations, empty squares indicate chytrid negative populations.

4.DISCUSSION

As hotspots of biodiversity, southern European peninsulas are also the hotspots of biogeographic and evolutionary processes (Weiss & Ferrand, 2007; Feliner, 2011). Studies on these processes have proven Pleistocene climate changes as the main factor responsible for the current pattern of biodiversity within these peninsulas, having strongly influenced species distribution, and hence, their evolutionary history (Hewitt, 2011a). Despite the highly individualistic species responses to such an influence (Stewart *et al*., 2010), it was possible to identify some common patterns: genetic diversity is often higher within peninsular refugia than across areas of post-glacial recolonization; refugia were often highly fragmented, resulting in strong genetic differentiation within peninsular lineages; and within peninsular refugia, areas with higher genetic diversity are the least stable areas, where fragmentation and recolonization have promoted genetic differentiation of populations and subsequent reshuffling. However, such evidences are obtained from studies on temperate species, while relatively fewer studies have investigated the evolutionary history of mountain species within southern European peninsulas to the same detail. This research tried to fill this gap, by analysing the genetic structure of an Apennine cold-adapted species with a panel of markers that provides information about its evolutionary history and genetic diversity distribution, along with interesting biogeographic, evolutionary, and conservationist implications.

Firstly, more genetic lineages of ancient differentiation were revealed within the Apennine populations of *I. alpestris*. The amount of genetic differentiation is higher than that revealed within the rest of Europe, except for the Balkan peninsula, where genetic differentiation is even greater (Recuero *et al*., 2014). This pattern reflects the pattern shown by other temperate species, including newts, where most of the genetic lineages were found within the peninsulas (e.g. Canestrelli *et al*, 2012b; Pabjan *et al*, 2015). This evidence suggests that the mountain chains of Mediterranean peninsulas may have provided multiples refugia during the Pleistocene, even to mountains species.

The observed phylogeographic discontinuities among Apennine populations of *I. alpestris* correspond to the discontinuities already observed in other species. The southern genetic lineage results isolated from Middle Pleistocene within the Calabrian Apennine. Calabrian region promoted the isolation of genetic lineages in several other species,

mostly among temperate species (e.g. Canestrelli *et al*., 2006b, 2010, 2011; Vega *et al*., 2010). The presence of refugia for both mountain and temperate species suggests the presence of microclimatic peculiarity, maybe linked to its position near the sea, as well as the action of paleo-biogeographic processes influencing species with different ecological requirements. The insularization of this area, due to marine ingression within the surrounding valleys during the interglacial phases (Colella *et al*, 1987; Bonfiglio *et al*., 2002), is one of the most invoked processes to explain the genetic differentiation among populations of different Calabrian mountain massifs (Canestrelli *et al*., 2010). The insular condition, if on one hand could have caused the isolation of *I. alpestris* populations, could also have caused climatic mitigation during such warmer phases, allowing the persistence of cold-adapted species.

The second phylogeographic discontinuity was identified within the Ligurian Apennine, and it corresponds with one of the main biogeographic discontinuities of the peninsula. Curiously, this area also corresponds to one of the strongest geological discontinuities within the peninsula: it represents the transition between the Alps and Apennines. Nevertheless, as outlined in Chapter 2, during the Pleistocene, this area did not undergo strong geographic revolutions as the southern Apennine, and so we have to shift our focus to the palaeoclimatic distinctiveness, which could have been acted on the fragmentation of both mountain and temperate species (Canestrelli *et al*, 2012b; Maura *et al*, 2014; Cimmaruta *et al*, 2015). Some of these species such as *Hydromantes* have shown coeval vicariant event during the Early Pleistocene, which could have been caused by the same event. However, this area actually represents the contact zone among *I. alpestris* lineages differentiated within separate sub-refugia, and it retains most of the intraspecific biodiversity. As observed for many other peninsular taxa, the area with the highest level of genetic diversity corresponds to the one most affected by palaeoclimatic or palaeogeographic changes and the highest levels of heterozygosis are not found within refugia, but in the contact area among the expanding lineages. This evidence highlights the leading role of hybridization and admixture among genetic lineages differentiated within different sub-refugia, in realizing the hotspot of intraspecific genetic diversity in mountain species as well.

Our results suggest that refugia-within-refugia may have been a common pattern in Pleistocene evolution of mountain species within the southern European peninsulas. Nevertheless, we cannot detect the same pattern within the genetic structure of other mountain species, since the few phylogeographic studies did not show enough resolution in

their data. The study carried out on *Rana temporaria* within the Apennine (Stefani *et al*., 2012) did not reveal much genetic variation within the Apennine lineage since only six Apennine populations with one mitochondrial gene were analysed; the European phylogeography of the snow mole *Chionomys nivalis* examined only three Apennine populations (Castiglia *et al*., 2009); the study on *Parnassius apollo* (Todisco *et al*., 2010) revealed recent colonisation of the Italian peninsula that occurred during the last glacial, and so we cannot find any traces of relevant substructure within the Apennines. This lack of studies with which to compare our evidences, necessitates implementing the knowledge on mountain species evolution within the southern European Peninsulas.

Nevertheless, despite some similarity between mountain and temperate species in the processes involved in genetic differentiation, we have found several differences in how these processes affect the genetic diversity of populations. Indeed, despite the presence of three strongly differentiated lineages, the levels of genetic diversity within lineages and within populations of *I. alpestris* are quite low when compared to other European populations of the same species (Pabijan & Babik, 2006; Prunier *et al*, 2012, 2014; Emaresi *et al*, 2011), or Apennine populations of closely related species (eg. Canestrelli *et al*, 2012a,b; Maura *et al*, 2014). Low levels of genetic diversity in both mitochondrial and nuclear markers might have been caused by other different processes. One of these processes may have been the recent origin of these lineages, which did not allow internal differentiation. Effectively, despite an ancient separation, each single lineage has shown a relatively recent common ancestor and a relatively recent demographic/spatial expansion (as inferred by spatial diffusion models). Nevertheless, several species have shown more recent demographic expansion but higher population genetic diversity (e.g. Maura *et al*., 2014), suggesting that other factors may have been involved, for example the strong instability of refugia for cold-adapted species. As already outlined, the populations of cold-adapted species are expected to have undergone strong contractions during the warm interglacial phases, during which they stayed in high altitude habitats (Schmitt, 2009). Nevertheless, during cold glacials, the populations could not persist on the high altitude habitat, since most of them were covered by ice, and they had to colonize new lowland areas. This indicates an almost continuous process of local extinction and recolonisation, which resulted in strong impoverishment effect on genetic richness (Greenbaum *et al*, 2014).

Low level of genetic diversity can also be caused by on-going processes that affect population decline, as in disease or in habitat degradation (e.g. Zellmer & Knowles, 2005).

We tested for the presence of chytrid fungus within *I. alpestris* populations to assess the extent of pathogen spread among the mountain species. However, comparing prevalence data with the knowledge on species genetic structure, we found a significant association between pathogen presence within the population and the level of genetic diversity of that population (Fisher exact test < 0.05 for populations grouped by expected heterozygosity > or ≤ to 0.3; not shown). One question could be whether chytrid fungus is affecting the genetic diversity of these populations or is the level of genetic diversity responsible for population health? In our case, chytrid positive populations do not show any evidence of decline attributable to Chytridiomycosis. The observed declines in the Calabrian and central Apennine populations seem linked to other factors such as isolation and climate changes, which alters ecological and hydrological equilibriums of these areas. Therefore, we can apparently exclude the recent effect of chytrid on the genetic structure of *I. alpestris* populations.

Nevertheless, we cannot exclude the role of genetic diversity in protecting the populations. We cannot overlook the observation that populations with higher genetic diversity, inside or close to the contact zone, tested negative for chytrid, and the population with lower genetic diversity tested positive. Such evidence fits our hypothesis that areas with the most healthy populations are in the contact zone among the expanding lineages, where populations have the highest levels of heterozygosis. The role of genetic diversity in providing evolutionary potential to respond to stress factors is one of the main findings in conservation biology (Frankam, 2005; Dufresness & Perrin, 2015), and high levels of genetic variation have often been correlated with lesser susceptibility for emergence (Spielman *et al*., 2004a; Acevedo-Whitehouse *et al*., 2003; Hughes & Boomsma, 2004; Pearman & Garner, 2005). These observations should be used in conservation planning by prioritizing the preservation of population genetic diversity, which means to preserve the evolutionary potential of species, which is the natural weapon for species survival. Even though decline was not observed in chytrid positive populations, population die-off can occur with time lag from early observations of chytrid presence (Canestrelli *et al*., 2013, and references therein). As observed above, changes in habitat suitability such as habitat degradation or climate changes may alter ecosystem equilibriums such as host-pathogen equilibriums by triggering the weakening of the host immune system or by increasing chytrid pathogenicity (Pounds *et al*., 2006; Raffel *et al*., 2006; Altizer *et al*, 2013; Rohr & Raffel, 2010), a mechanism already observed worldwide, also in Apennine amphibians (Canestrelli *et al*., 2013). The current tolerant populations could lose their tolerance and

low diversity populations are more threatened, having lesser evolutionary potential to respond to such stress (Louquet *et al.*, 2012). Being a mountain species, *I. alpestris* is especially threatened, since mountains species are the most affected by both climate changes and chytrid infection and, as per the effect of its evolutionary history, by low levels of genetic diversity.

CONCLUDING REMARKS

This study has analyzed the genetic structure and evolutionary history of a mountain species inhabiting the Italian peninsula, a well recognized hotspot of biodiversity. We found a strong and ancient genetic structure within peninsula, which supports the recent observations that Mediterranean hotspot may be more hot than we believed. Nevertheless, we highlight the necessity to increase the knowledge about the Pleistocene evolution of mountain species within Mediterranean peninsular refugia, in order to better understand the evolutionary processes involved. A better knowledge about the genetic structure of mountain species could help to identify common hotspot areas of intraspecific diversity. Such areas should be selected as priority conservation areas since they retain the evolutionary potential of species to respond to the stress which have been threatening species in the last century.

APPENDIX

Points of presence of *I. alpestris* used in Species Distribution Models analyses.

Site	Longitude	Latitude
1	7.477434	45.119675
2	7.733483	45.027336
3	7.779823	44.293203
4	7.805084	45.100857
5	7.833894	44.332212
6	7.839857	44.165907
7	7.867999	44.128901
8	7.892052	44.402450
9	7.992967	44.118782
10	8.014715	44.477308
11	8.072320	44.302732
12	8.142397	44.421692
13	8.153149	44.284021
14	8.184816	44.612454
15	8.230862	44.265034
16	8.343371	44.242828
17	8.357699	44.380726
18	8.360083	44.439291
19	8.457469	44.423725
20	8.462347	44.506328
21	8.531354	44.417066
22	8.575379	44.423549
23	8.585396	44.596902
24	8.586614	44.476361
25	8.604081	44.683419
26	8.630426	44.549827
27	8.644880	44.582162
28	8.676688	44.438335
29	8.722465	44.666638
30	8.734371	44.442886
31	8.777042	44.555993
32	8.798320	44.613162
33	8.823204	44.506524
34	8.840178	44.652797
35	8.868076	44.538538
36	8.871606	44.590669
37	8.877944	44.779682
38	8.886757	44.821667
39	8.911406	44.739127
40	8.953050	44.452134
41	8.977642	44.660802
42	9.044474	44.504934
43	9.067042	44.634319
44	9.084228	44.815566
45	9.101136	44.551434
46	9.116364	44.757407
47	9.171951	44.666929
48	9.179777	44.719425
49	9.210139	44.569490
50	9.214337	44.933395
51	9.232523	44.853409
52	9.236786	44.483648
53	9.252423	44.630913
54	9.288341	44.752630
55	9.303157	44.514291
56	9.322523	44.720149
57	9.357448	44.824754
58	9.378237	44.309799
59	9.432948	44.311218
60	9.435915	44.535910
61	9.448828	44.747223
62	9.487190	44.849279
63	9.488611	44.321010
64	9.492112	44.484157
65	9.502725	44.561865
66	9.509377	44.423293
67	9.510956	44.234583
68	9.532651	44.644804
69	9.577086	44.204574
70	9.587496	44.277308
71	9.604217	44.724261
72	9.615329	44.464266
73	9.629341	44.868599
74	9.653928	44.642342
75	9.727204	44.472618
76	9.774412	44.379996
77	9.807755	44.613675
78	9.809239	44.265913
79	9.842303	44.191986

follows

following

Site	Longitude	Latitude
80	9.844717	44.557568
81	9.860173	44.653901
82	9.864610	44.478859
83	9.918231	44.473601
84	9.945822	44.399916
85	9.954578	44.172110
86	10.033551	44.104461
87	10.041821	44.138413
88	10.073107	44.512436
89	10.078698	44.362363
90	10.099427	44.089877
91	10.208358	44.105487
92	10.208641	44.731571
93	10.228201	44.217333
94	10.232946	44.152734
95	10.233713	44.319986
96	10.244601	44.005527
97	10.270303	44.040653
98	10.282123	44.379435
99	10.291835	44.109510
100	10.327781	44.163028
101	10.334768	44.313722
102	10.342285	44.002003
103	10.361326	44.208708
104	10.370735	44.151479
105	10.405097	44.390289
106	10.440423	43.905836
107	10.446354	44.140571
108	10.466732	44.245780
109	10.509900	44.061169
110	10.547812	44.376889
111	10.588869	44.129870
112	10.601213	43.966832
113	10.609087	44.197576
114	10.622584	43.908316
115	10.623227	44.296817
116	10.672912	44.119870
117	10.710045	44.007431
118	10.714772	44.205013
119	10.756368	44.045726
120	10.762933	43.937320
121	10.772508	44.120343
122	10.808169	43.943590
123	10.817812	44.172520
124	10.841881	44.044561
125	10.868016	44.307186
126	10.901903	44.010531
127	10.912637	44.062895
128	10.915956	44.293629
129	10.950740	44.420387
130	11.031019	44.242622
131	11.070692	44.376944
132	11.075323	43.095980
133	11.086330	44.157304
134	11.105127	44.121831
135	11.137914	44.243777
136	11.152869	43.058377
137	11.175533	44.387134
138	11.233504	43.085736
139	11.262614	44.160489
140	11.276459	44.072177
141	11.285211	44.054772
142	11.294116	44.283943
143	11.299732	44.218045
144	11.313600	43.797183
145	11.321434	44.148410
146	11.332761	44.186733
147	11.343895	43.527081
148	11.348255	44.080776
149	11.382801	44.205529
150	11.420822	43.571741
151	11.438660	43.501812
152	11.819474	43.809221
153	11.937605	43.702816
154	12.076657	43.784809
155	12.117621	43.684114
156	12.258781	43.675309
157	13.319043	42.703248
158	16.007030	39.578912
159	16.023058	39.552734
160	16.085179	39.425534

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