



Population structure, genetic diversity and evolutionary history of the narrow endemic squirrel *Sciurus meridionalis*

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ABSTRACT

Endemic species and their genetic diversity are critical components of global biodiversity, yet they usually face a higher risk of decline and extinction than species with broader distributions. Loss of genetic diversity within geographically restricted and fragmented ranges plays a significant role in driving endemic species to extinction, threatening their ability to adapt to environmental challenges. Hence, considering genetic diversity and its spatial distribution within populations is mandatory in planning conservation actions for narrow endemic species. Here, we investigated the population genetic diversity, structure and evolutionary history of the Calabrian black squirrel *Sciurus meridionalis*, a species endemic to the Calabrian region in the south of the Italian peninsula. A fine-scale sampling procedure was carried out throughout the whole species range, and genetic variation was assessed from multiple mitochondrial and nuclear markers. Phylogeographic and population structure analyses showed three evolutionary significant units within the current species range, each characterised by extremely low levels of genetic diversity. Bayesian phylogeographic analyses suggested that Calabrian black squirrel populations experienced a range fragmentation during the Last Glacial Maximum (~20 ka), which geographically mirrors the three main mountain massifs within the species range. Historical demographic reconstructions revealed a recent and still ongoing demographic expansion for Calabrian black squirrel populations, likely promoted by a concurrent expansion of the Calabrian black pine in this area. Overall, our findings clarify the designation of the evolutionary significant units for a narrow endemic squirrel and underscore the importance of analysing range-wide population genetic data and considering species interactions when defining management strategies for narrow endemic species' conservation.

1. Introduction

Endemic species and their genetic diversity are essential components of the world's biodiversity (Malcolm et al., 2006; Frankham et al., 2017; Manes et al., 2021; Kraus et al., 2023). These species frequently show unique genetic variation and adaptations to their local environments, and play a vital role in ecosystem function, providing important and often irreplaceable ecosystem services, such as pollination, seed dispersal, or habitat provision (Lyons et al., 2005; McConkey et al., 2006; Gaston, 2012; Mouillot et al., 2013; Dee

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et al., 2019). Not last, many endemic species are iconic to local human communities, and their conservation can help to preserve entire regional biotas (Cardinale et al., 2012; Dee et al., 2019; Cardoso et al., 2020). At the same time, given their narrow geographic distribution, endemic species are also especially threatened of extinction by various factors, including habitat loss, fragmentation and degradation, climate change, alien species, overexploitation, and disease (Soulé and Wilcox, 1991; Fontaine et al., 2007; Burlakova et al., 2011; Manes et al., 2021; Mayani-Parás et al., 2021; Kraus et al., 2023). Hence, endemic species are especially vulnerable to the direct consequences of these threats, including rapid population declines, loss of genetic diversity, inbreeding depression, mutational meltdown, maladaptation to changing environments and, ultimately, species extinction (Frankham, 2010; Allendorf et al., 2012; Manes et al., 2021; Kraus et al., 2023).

The usefulness of population genetic tools in biodiversity conservation is undisputed since decades, it was already emphasized by the seminal work of Soulé and Wilcox (1991). For rare, relict, and endemic species, key contributions to the identification of the most effective conservation strategies have come from the coupling of phylogeographic and population genetic perspectives to species' conservation (Rosauer et al., 2016; Quiroga et al., 2019; Bobo-Pinilla et al., 2022). This integrated approach has substantially improved our understanding of the eco-evolutionary processes shaping patterns of genetic diversity, biogeographic distribution, and historical demography in narrowly distributed species (Crandall et al., 2000; Moritz and Potter, 2013; Médail and Baumel, 2018), and it has been particularly fruitful in some contexts, such as the study of insular and montane species (Bisconti et al., 2016; Salvi et al., 2016; Chiocchio et al., 2017). In these contexts, the integration of phylogeographic and population genetic toolboxes has revolutionized our view of entire faunas and floras and their evolution through space and time (Bloomquist et al., 2010; Zachos and Habel, 2011), revealing hidden, fine-scale population structures, their historical origin, patterns of colonization and gene exchange between geographical regions, as well as identifying hotspots and cold spots of genetic diversity over minimal spatial scales (Hewitt, 2011; Melián et al., 2015). By focusing on well-characterized population structures, unique genetic lineages, and areas of high genetic diversity, conservation efforts for endemic species can now be devoted on preserving potential sources of adaptive potential, which may be critical for the long-term survival of populations under changing environmental conditions (DeWoody et al., 2021).

The Calabrian black squirrel *Sciurus meridionalis* is an endemic mammal of the Italian peninsula, with narrow distribution in the montane regions of southern Italy (Amori and Castiglia, 2018). Once deserved the southern fringe of the broader European red

Table 1

Geographic location of the Calabrian black squirrel samples, *Sciurus meridionalis*, analysed in this study.

	Mountain massif	Locality	Latitude N	Longitude E	mtDNA haplotype
1	Pollino	Castelluccio	40.012	15.975	H5
2		Bosco Difesa	40.016	15.985	H5
3		Castelluccio	39.994	15.989	-
4		Rotonda	39.950	16.044	H7
5		Rotonda	39.952	16.046	H5
6		P. della Contessa	39.575	15.989	H8
7	Sila	S.fili	39.325	16.119	H11
8		Sila grande	39.366	16.500	H2
9		Sila grande	39.366	16.500	H2
10		Sila grande	39.366	16.500	H2
11		Sila grande	39.366	16.499	H2
12		Camigliatello	39.337	16.438	H6
13		Sila	39.366	16.500	H9
14		Sila	39.366	16.500	H2
15		Serra Pedace	39.306	16.586	H12
16		Spezzano	39.323	16.494	H2
17		Spezzano	39.337	16.438	H2
18		Spezzano	39.327	16.499	H13
19		Serra Pedace	39.297	16.525	H13
20		Spezzano	39.337	16.438	H14
21		Spezzano	39.337	16.438	H15
22		Spezzano	39.337	16.438	H2
23		Spezzano	39.337	16.438	H13
24		Parenti	39.166	16.399	H13
25		M.Gariglione	39.101	16.614	
26		Lorica	39.282	16.624	H6
27		Lago del Passante	39.099	16.532	
28		Malarotta	39.361	16.486	-
29		Longo Bucco	39.388	16.570	H1
30		LeMacchie	39.272	16.537	H2
31	Aspromonte	S. Eufemia	38.249	15.854	H3
32		S. Eufemia	38.249	15.854	H3
33		Gambarie	38.157	15.833	H10
34		Gambarie	38.175	15.809	H10
35		Delianuova	38.215	15.916	H3
36		Delianuova	38.215	15.916	H3
37		MteBasilicò	38.157	15.833	H4
38		S.Stefano	38.170	15.828	-

squirrel, *Sciurus vulgaris*, this Calabrian black squirrel has been recently recognized as a distinct, geographically and genetically isolated species (Wauters et al., 2017). Calabrian black squirrels are mostly diurnal and active throughout the year, with seasonal variation in activity patterns. Populations occur at middle-high altitudes, with a spatial distribution substantially overlapping the distribution of mixed forests dominated by the endemic Calabrian pine (*Pinus nigra laricio*), since these habitats provide high quality stands for feeding and drey buildings (Cagnin et al., 2000; Rima et al., 2010; Amori et al., 2014). Like other tree squirrels, *S. meridionalis* plays a key role in many ecological processes, such as seed dispersal, nutrient cycling, forest regeneration, and even ecosystem restoration (Steele and Yi, 2020). For example, by burying (and even forgetting) seeds in the ground, it helps restoring forests that have been impacted by fires or other disturbances (Lambert et al., 2005). Not last, as well testified by a number of myths and popular stories, squirrels are iconic organisms, whose conservation may help protecting other species and their habitat. Besides having a narrow geographic distribution, Calabrian black squirrel populations are also spatially fragmented, being mostly restricted to the forested areas of the three well-separated mountain systems of the Calabrian region (i.e. the Aspromonte, Pollino, and Sila massifs Fig. 1), although recent evidence suggests some spatial expansion of northern populations (Rima et al., 2010). Because of its narrow range and the rate at which its habitat is being fragmented and degraded, this species has been listed as Near Threatened (NT) in the last regional IUCN assessment (Rondinini et al., 2022), and is currently subjected to monitoring plans. Previous phylogenetic investigations assigned Calabrian black squirrel populations to a single evolutionary unit, likely deriving by past geographic isolation from the northern red squirrel populations during glacial epochs (Grill et al., 2009; Wauters et al., 2017). Nevertheless, these studies analyzed multiple samples from a single locality, thus preventing a thorough evaluation of spatial patterns of population structure and diversity, that instead would be useful for conservation purposes.

With this study, we aim at filling these gaps of knowledge, by analyzing the fine-scale genetic pattern of Calabrian black squirrel populations sampled throughout the whole species range. To this end, we investigated levels of genetic diversity at both mitochondrial and microsatellite markers to infer patterns of population structure in space and time, adding insights into the evolutionary history of *S. meridionalis*. Results from this study will therefore provide the spatial context for drawing a genetically informed conservation strategy for this narrow endemic and threatened species.

2. Material and methods

2.1. Sample collection and DNA extraction

Tissue samples were collected from roadkills across the entire distribution area of *S. meridionalis*. We obtained good quality sample from 38 individuals (see Fig. 1 and Table 1). Genomic DNA was extracted using ZR Universal Kit (Zymo Research), following manufacturer instructions. Extracted DNA of each sample was quantified with a Qubit 2.0 fluorometer (Invitrogen), using the Qubit dsDNA BR Assay kit, then each sample was standardized to a concentration of 10 ng/μL for all the subsequent analyses.

2.2. Mitochondrial DNA analysis

Genomic DNA was used to amplify 4 distinct mitochondrial (mtDNA) gene fragments: a portion of the protein encoding cytochrome c oxidase I (COX1), cytochrome c oxidase II (COX2), and cytochrome b (CYTB), and the non-coding D-loop region (Dloop). Primers pairs were eventually re-drawn to improve the amplification success on our samples (see Table S1 for primer sequence details). Amplifications were run in a 15-μl reaction volume containing MgCl₂ (2 mM), dNTPs (0.2 mM each), two fragment-specific primers (0.2 μM each, Table S1), the enzyme HotStartTaq polymerase (0.5 U, Promega), the reaction buffer (1X, Promega), and 10 ng of DNA template. The PCR cycling conditions were composed of an initial denaturation step of 5 min at 95 °C, 35 cycles of 1 min at 94 °C, 45 s at the specific annealing temperature of each primer pairs (see Table S1), and 90 s at 72 °C, with a final extension step of 10 min at 72 °C. PCR products were PCR-purified and sequenced by Macrogen Inc. (<http://macrogen.com>). Obtained sequences were deposited in GenBank (Accession numbers: PP587771-PP587805; PP584206-PP584312).

Sequences obtained for each gene fragment were checked by eye using FinchTV 1.5 (Geospiza Inc.) and aligned using ClustalX 2.0 (Larkin et al., 2007). Nucleotide variation was evaluated using MEGA 11 (Tamura et al., 2021). The partition-homogeneity test (Farris et al., 1994) implemented in the software Paup* 4.0b10 (Swofford and Sullivan, 2003) was used to test for discordant phylogenetic signals among the 4 gene fragments. Since the test indicated the absence of discordance, the 4 fragments were concatenated using Concatenator 1.1.0 (Pina-Martins and Paulo, 2008) and used as a single mtDNA fragment in all downstream analyses.

Phylogenetic relationships among the mtDNA haplotypes were inferred using the maximum-likelihood algorithm implemented in PhyML 3.0 (Guindon et al., 2009). We set TrN+I as substitution models, as addressed by Jmodeltest 2.1.10 (Darriba and Posada, 2016), and standard bootstrap replicates to assess node support. The software HaplotypeViewer (Ewing, 2012) was used to visualize the estimated phylogenetic tree as a haplotype genealogy. Haplotype (*h*) and nucleotide (π) diversity (and their associated standard deviations; Nei, 1987) were estimated using DnaSP 6 (Rozas et al., 2017).

To evaluate the spatial diffusion pattern for *S. meridionalis* throughout its range during the past, we used the Bayesian Phylogeographic (BP) analysis in continuous space implemented in Beast 1.10.4 (Suchard et al., 2018). The analysis was run setting: different substitution models for each mtDNA fragment, as indicated by Jmodeltest (Darriba and Posada, 2016) (the HKY model for COX2, CYTB and Dloop, and the TN93 model for COX1); the Bayesian skyline as coalescent tree prior; Cauchy as spatial diffusion model (Lemey et al., 2010). Preliminary analyses were runs using two alternative clock rates: a strict clock with fixed rate at 1.0 E-8, commonly used for mitochondrial genes in mammal species (Irwin et al., 1991); normal distribution of clock rate with mean 9.4E-9 ± 5.0E-9 (Pesole, 1999; Madsen et al., 2015). The MCMC was run for 10 million generations, sampling every 1000th generation. The

software Tracer 1.7.1 (Rambaut et al., 2018) was used to evaluate the effective sample size (ESS) values of the estimated parameters and the convergence between runs, after removing the first 10% of samples as burn-in. The two runs lead to very similar results for all of the parameters, in particular for the tree root height, i.e. the parameter that mostly affects the timing of diffusion pattern (median age: 404190 vs 435170 years before present). Thus, the definitive analysis was run using the fixed rate at 1.0 E-8, which ensured more stable estimates for most of the parameters (better convergence of the run, higher ESSs and less uncertainty for most of the estimated parameters). Then, we generated an annotated MCC tree using TreeAnnotator and projected it on a geographic map using Spread 1.0.7 (Bielejec et al., 2015) to visualize the spatial and temporal diffusion patterns of lineages. We estimated the geographical location of the most recent common ancestor (MRCA) at predefined time slices, by examining the full posterior of trees with the TimeSlicer options implemented in Spread 1.0.7 at the root age and at three points in time encompassing last glacial phase (in thousand years before the present, ka): 125 ka, 90 ka, and 20 ka.

Historical variation in effective population size for *S. meridionalis* was investigated using the Bayesian Skyline Plot (BSP) coalescent method implemented in Beast 1.10.4. The runs were performed with the same substitution models as above, a strict clock with fixed rate at 1.0 E-8, a Bayesian skyline as coalescent tree prior, and MCMC of 10 million generations, sampling every 1000th generation. The software Tracer was used to evaluate the ESS values and to remove the first 10% of samples as burn-in.

2.3. Microsatellite genotyping and data analysis

We genotyped 10 microsatellite loci previously developed for *Sciurus vulgaris*: Rsu-3, Rsu-5, and Rsu-6 (Todd, 2000) and Scv-1, Scv-3, Scv-4, Scv-9, Scv-13, Scv-19, and Scv3-1 (Hale et al., 2001). PCR reactions were performed using the Multiplex PCR Kit (Qiagen), following previously published protocols (Todd, 2000; Hale et al., 2001; Hale et al., 2004). The PCR products were genotyped by Macrogen Inc. with a 400-HD-size standard. Alleles were analysed and called using GeneMapper 4.1 (Applied Biosystems).

We used Microchecker software to check for the presence of null alleles and large allele dropout in our dataset. Deviations from the expected Hardy–Weinberg and linkage equilibria was valued using FSTAT 2.9.4 (Goudet, 2002), and the same software was also used to evaluate the allelic richness (A_R) over all loci. Genetic diversity estimates, as the observed (H_o) and the expected (H_e) heterozygosities, was computed using the software Genetix 4.05.2 (Belkhir et al., 2004).

The population genetic structure of *S. meridionalis* was investigated using the Bayesian clustering method implemented in Tess 2.3.1 (Durand et al., 2009). This software allows to use information on the spatial distribution of the individual genotypes in the analysis, and it is particularly effective when shallow population genetic structure is expected. The analysis was performed with admixture and conditional autoregressive (CAR) model. An exploratory analysis was carried out, with 20,000 runs (2000 rejected as burn-in) and 5 replicates for each grouping option (K) from 2 to 5. The final analysis was carried out with 100 replicates for each K value, with K ranging from 2 to 5, each with 100,000 sweeps and 20,000 sweeps discarded as burn-in, and the default spatial parameter option. The deviance information criterion (DIC) was used to select the model best fitting the data. For each K value, DIC estimates were averaged over the 100 replicates, and the best K value was selected as the one where the average of DIC estimate reached the plateau.

3. Results

3.1. Mitochondrial DNA

We obtained a concatenated alignment of 2068 bp including four mtDNA gene fragments: 510 bp of COX1, 508 bp of COX2, 555 bp of CYTB and 492 bp of Dloop. No indels, non-sense or stop codons were found in the protein-encoding gene fragments. The combined dataset revealed 15 unique haplotypes, defined by 28 variable positions, 19 being parsimony informative (Pi). We observed low values of nucleotide diversity for all the three mountain chain areas (i.e. Pollino, Sila, and Aspromonte), with the lowest found in Aspromonte massif (0.0009 s.d. 0.00045; Table 2).

The mtDNA haplotype network resulting from the Maximum Likelihood tree (log-likelihood score: −3076.76327) clearly identify three main lineages mirroring the three mountain massifs (Fig. 1B). The most diversified haplogroup occurred in Sila, with 9 out of the 15 haplotypes found, that was separated from the Aspromonte haplogroup by 5 nucleotide positions. Both the Aspromonte and Pollino lineages contained three haplotypes each and were separated from one another by a minimum of 6 nucleotide positions. None of the haplotypes were found to be shared among the three mountain massifs. Haplotype diversity (h) and nucleotide diversity (π) were calculated overall and according to geographic distribution of sampled squirrels, i.e. by clustering individuals by the three main mountain massifs present within the species' range (i.e. Pollino, Sila, and Aspromonte), and shown in Table 2.

Both the BP and BSP analysis implemented in Beast yielded effective sample size (ESS) values that largely exceeded the threshold of

Table 2

Estimates of genetic diversity for both mtDNA and microsatellite markers. h =haplotype diversity; π = nucleotide diversity; A_R =allelic richness; H_o =observed heterozygosity; H_e =expected heterozygosity.

Mountain massif	mtDNA		Microsatellite		
	h	π	A_R	H_e	H_o
Pollino	0.7 (s.d. 0.1)	0.0015 (s.d. 0.0008)	2.75	0.34 (s.d. 0.29)	0.28 (s.d. 0.24)
Sila	0.8 (s.d. 0.1)	0.0011 (s.d. 0.0003)	2.51	0.34 (s.d. 0.29)	0.27(s.d. 0.26)
Aspromonte	0.7 (s.d. 0.1)	0.0009 (s.d. 0.0004)	1.44	0.07 (s.d. 0.13)	0.06 (s.d. 0.1)

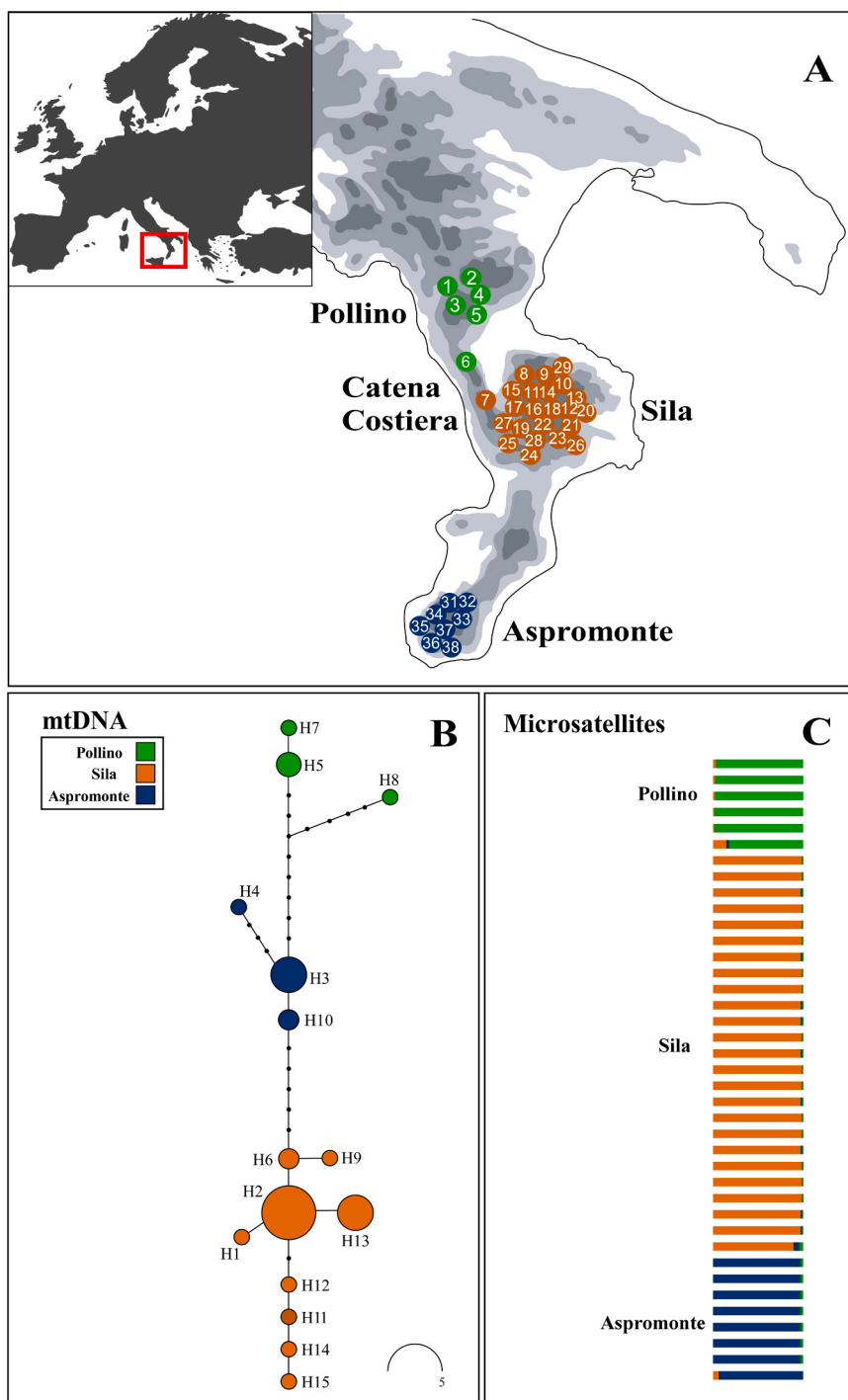


Fig. 1. A) Geographic location of the 38 samples of *Sciurus meridionalis* analysed in this study. B) Haplotype genealogy yielded using HaplotypeViewer, based on the ML phylogenetic tree of the 15 haplotypes found. Circle sizes are proportional to haplotype frequency (see inset, lower right); missing intermediate haplotypes are shown as open dots. C) Genetic structure of *S. meridionalis* populations as inferred with the Bayesian clustering method implemented in TESS, based on the microsatellite dataset. The bar plots show the admixture proportions of each sampled individual for the 3 genetic clusters recovered.

200 for all parameters of interest, and obtained full convergence for parameter estimates between runs. The BP analysis estimated that the MRCA (Most Recent Common Ancestor) of *S. meridionalis* lineages about 400 ka, located between the Catena Costiera Mountain chain and the Sila massifs (Fig. 2A). This analysis indicated that the distribution of Calabrian black squirrel populations was

continuously connected throughout the Calabrian Mountain chains at least until the end of the Last Interglacial period (LIG; ca 125 ka; Fig. 2B). During the Last Glacial Period (LGP; ca 115–12 ka) Calabrian black squirrel populations experienced a strong fragmentation of their habitat, which reached its extreme at approximately 20 ka, during the Last Glacial Maximum (LGM; Fig. 2B). The demographic trend showed by the BSP analysis inferred a substantial demographic stability, lasting until the end of the LGM, at about 20 ka, when the species underwent a sudden demographic expansion (Fig. 2C).

3.2. Microsatellites

All the samples were genotyped at the ten microsatellites loci. Loci Scv9 and Scv13 showed no variation across samples and therefore were excluded from downstream analysis. The final dataset of the remaining eight loci returned an overall proportion of missing data of 2.4%. None of them showed evidence of null alleles or large allele dropout, and no evidence of significant deviation from the Hardy-Weinberg equilibrium and of linkage disequilibria were observed. The number of alleles for each locus ranged from

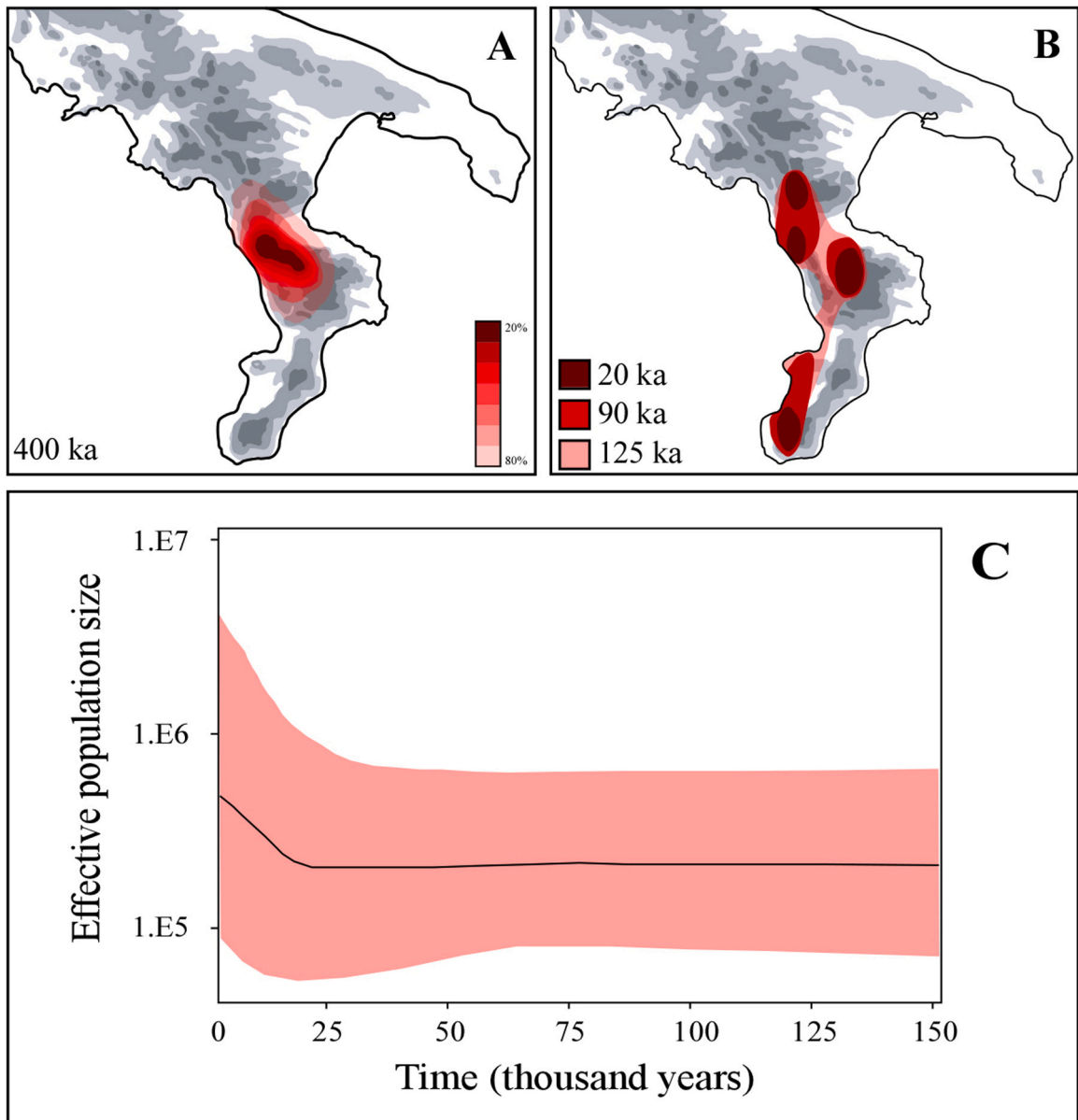


Fig. 2. A) Highest posterior density (HPD) regions of the geographical location of the most recent common ancestor of *Sciurus meridionalis* based on mtDNA data. B) Spatial diffusion of *S. meridionalis* populations through time, based on the Bayesian phylogeographical analyses implemented in Beast. Three time intervals are shown. Polygons represent 90% HPD uncertainty for the spatial location of ancestral populations. Time is in thousand years before present (ka). C) Bayesian Skyline Plot showing historical demographic trend of *S. meridionalis*.

two (Rsu5-Rsu6-Scv19) to ten (Scv3). Genetic diversity values were estimated by grouping individuals according to the mountain massif of origin (Table 2). Allelic richness ranged from 1.44 to 2.75 for the Aspromonte and Pollino populations, respectively. The same geographic pattern was found for the heterozygosity values, with the Pollino and the Aspromonte populations being the most and the least variable, respectively (see Table 2).

The Bayesian clustering method used to study the population genetic structure selected $K = 3$ as the best clustering option. The deviance information criterion (DIC) value showed minimal decrease at K values higher than 3. The three clusters clearly mirrored the mtDNA geographic pattern of variation: a northern group clustered all the Calabrian black squirrels sampled in the Pollino mountain massif, the central group those from the Sila massif, and the southern group those from the Aspromonte massif (Fig. 1C).

4. Discussion

Given its limited range distribution and the recent literature that described the Calabrian black squirrel *S. meridionalis* populations as a single evolutionary unit (Grill et al., 2009), we expected to find a shallow structure of genetic diversity among populations. Instead, three well-defined population groups emerged from the analyses performed on both mitochondrial and nuclear genetic variation (Fig. 1B-C). The geographical distribution of these three groups clearly mirrors those of the three main mountain blocks of Pollino, Sila, and Aspromonte along the Calabria region (Fig. 1B-C). The evolutionary history of the Calabrian black squirrel populations, as reconstructed here, showed a scenario of fragmentations and expansions that contribute to explain such differences. The most recent ancestral population of the Calabrian black squirrel was estimated in the Middle Pleistocene (ca 400 ka; Fig. 2A), and it was spatially located in the centre of the Calabrian region. During this epoch, the Italian peninsula experienced glacial phases characterized by harsh climate conditions that generally triggered the reductions of suitable habitats for temperate species, including mixed forests that currently characterize the Calabrian black squirrel habitats (Hewitt, 2000, 2004; Svenning et al., 2008). After the amelioration of the climatic conditions during the LIG period (ca 125 ka; Kukla et al., 2002; Felis et al., 2004), the Calabrian black squirrel would have expanded its range throughout the whole forested area of the Calabrian region (Fig. 2B) till the subsequent LGM phase (ca 20 ka). During this last glacial epoch, squirrel populations would be fragmented within the main mountain massifs (Fig. 2B). Finally, our historical demographic reconstructions clearly suggest the occurrence of demographic expansions for populations at the end of LGM (Fig. 2C).

This spatial pattern of population diversification is not unexpected among species inhabiting this geographical area of the Italian peninsula (Canestrelli et al., 2010; Bisconti et al., 2018). Environmental changes consequent to glacial-interglacial cycles of the Pleistocene period promoted range dynamics that have been repeatedly invoked as one of the major factors in explaining current spatial patterns of intraspecific genetic diversity in this geographical area (Canestrelli et al., 2010, 2014, 2018; Chiocchio et al., 2019, 2022). Indeed, most non-volant vertebrates of the Italian peninsula showed well-structured patterns of population genetic diversity along the Calabrian region, well explained by past climate-driven biogeographic processes of fragmentations and (re)connections (Bisconti et al., 2018; Chiocchio et al., 2019). However, we observed an important difference between patterns of genetic diversity of *S. meridionalis* populations and most of the co-distributed species studied to date. In fact, the Calabrian region is an undisputed hotspot of intraspecific biodiversity along the Italian peninsula, where populations often showed the top-ranking values of genetic diversity indexes within this area (Canestrelli et al., 2010, 2014). On the contrary, genetic diversity values of all three groups of Calabrian black squirrels were unexpectedly low compared to other vertebrates inhabiting this area (Table 2; Canestrelli et al., 2010; Slimen et al., 2012; Liu et al., 2014; Koshev et al., 2023). Moreover, these estimates appear especially low when compared to other squirrel species, including those species described as endangered, suggesting the need to carefully re-assess the conservation status of this species (Lampila et al., 2009; Slimen et al., 2012; Liu et al., 2014; Koshev et al., 2023).

Our results indicate that historical, climate-driven events of population fragmentation and contraction of suitable habitats would have drastically impacted population distribution, size and, consequently, current levels of genetic diversity of Calabrian black squirrel populations. At the same time, the tight association of the Calabrian black squirrel with some forest tree species, such as the Calabrian pine *P. n. laricio* (Cagnin et al., 2000), suggests that demographic size and genetic diversity of Calabrian black squirrel populations might also have been impacted by the intensification of human activities, such as agriculture, grazing, and deforestation, that have reduced the extent *P. n. laricio* forests in recent times (Nicolaci et al., 2014). Support to this hypothesis also comes from the recently observed expansion of squirrel populations within the Catena Costiera massif, which would have strictly followed the recent replanting activities of the Calabrian pine within this area (Rima et al., 2007).

In conclusion, the study of population genetic structure, diversity and evolutionary history of the Calabrian black squirrel populations in southern Italy revealed at least three main issues to deal with from a conservation perspective. First, three divergent and geographically well-defined lineages emerged for Calabrian black squirrel populations within the Calabrian region, indicating that populations from all three different mountain areas must be considered to conserve the whole evolutionary potential of the species. Second, although populations of the Calabrian black squirrel have been reported as abundant (Wauters et al., 2017), and a recent expansion of populations has been reported (Rima et al., 2007), the levels of genetic diversity estimated throughout its range are extremely low, pointing out the need to re-evaluate its conservation status. Such re-evaluation is compelling on the light of the severe pressures that biological communities are currently undergoing in this region, as a consequence of fires (Coscarelli et al., 2021), habitat degradation, and alien species introduction (Bisconti et al., 2019; Laface et al., 2020; Martino et al., 2022; Scaravelli and Aloise, 2023). Accordingly, we suggest to re-consider *S. meridionalis* as Vulnerable in the IUCN Red List. Third, a tight association between Calabrian black squirrel populations and the endemic Calabrian tree pine (*P. n. laricio*) appears well supported by both past and more recent range dynamics (see also Afzal-Rafii and Dodd, 2007; Rima et al., 2007; Nicolaci et al., 2014). Together with the evidence that pine seeds mostly compose the diet of squirrel species even when other food sources are available (Lurz et al., 2005; Molinari et al., 2006;

Steele and Yi, 2020), these observations suggest that mutual interaction between squirrels and pines could be so exclusive to determine the distribution range of both species (Zong et al., 2010; Steele and Yi, 2020). While the evolutionary underpinnings of this tight association need to be further enlightened, it clearly highlights the importance of preserving pine tree forests, as a key management strategy to conserve Calabrian black squirrel populations in the long-term.

CRediT authorship contribution statement

Adriana Bellati: Writing – review & editing, Investigation. **Gaetano Aloise:** Writing – review & editing, Investigation, Data curation. **Daniele Canestrelli:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Conceptualization. **Roberta Bisconti:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Andrea Chiochio:** Writing – review & editing, Investigation, Formal analysis.

Declaration of Competing Interest

The authors do not declare any conflicts of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.gecco.2024.e02919](https://doi.org/10.1016/j.gecco.2024.e02919).

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