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# Quaternary Climate-Driven Diversification of Alpine Grasshoppers in the Central Apennines: Insights Into Mediterranean Sky Islands as Centers of Microendemism

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## ABSTRACT

**Aim:** Pleistocene climatic oscillations profoundly shaped Mediterranean mountain biotas through recurrent cycles of isolation and connectivity. Yet, despite the Central Apennines being a major centre of local endemism, this region has received limited attention in genomic studies addressing biogeographic processes. Here, we examine how climatically driven distributional shifts have promoted microgeographic diversification and endemism in sky-island systems of this mountain region.

**Location:** Central Apennines, Italy.

**Taxon:** *Italohippus* (Orthoptera: Acrididae).

**Methods:** We used genome-wide ddRAD-seq data to infer phylogenetic relationships, divergence times, species limits, population structure, demographic history and introgression. Morphological differentiation was quantified using geometric and linear morphometrics of forewings. Past range dynamics and spatial patterns of climatic suitability were reconstructed by projecting species-specific environmental niche models from the Last Glacial Maximum to the present.

**Results:** Phylogenomic analyses reveal a Middle–Late Pleistocene diversification of *Italohippus* structured along a continuum of evolutionary divergence across the Central Apennines. Northern populations, corresponding to the taxon *I. modestus*, form a distinct allopatric lineage likely originating under geographic isolation and the evolution of partial reproductive barriers. In contrast, populations at the southern rear edge of the genus range, corresponding to *I. albicornis,* show weak genomic and morphological differentiation from *I. monticola*, consistent with incipient but incomplete speciation. Paleodistribution models and demographic reconstructions indicate marked latitudinal contrasts in glacial impacts, with higher availability and continuity of climatically suitable habitats and introgression in northern and central sectors and more persistent isolation in the south.

**Main Conclusions:** Our results support a biogeographic model of intermittently connected sky-island refugia in the Central Apennines, generating spatially heterogeneous opportunities for divergence and persistence across latitudes. By integrating different sources of data, we show how climatically driven distributional shifts have shaped the emergence and maintenance of geographically structured evolutionary lineages in Mediterranean mountain systems, highlighting key processes underlying the origin and persistence of endemic alpine biodiversity in the face of rapid climate warming.

Francesco Forte and Alessandra Riccieri equally contributed to the paper.

## 1 | Introduction

Quaternary climate oscillations have left profound imprints on the biodiversity of temperate regions, forcing many species into latitudinal and/or elevational range shifts and shaping their spatial patterns of genetic variation (G. Hewitt 1999, 2000; Sandel et al. 2011). During periods of harsh environmental conditions, species persisted in glacial or interglacial refugia, which later served as centers of expansion once climate became favourable again (Taberlet et al. 1998; Binney et al. 2009). In Europe, the three major Mediterranean peninsulas (Iberia, Italy and the Balkans) acted as pivotal refugia primarily during glacial periods but also during interglacial stages, while serving as cradles of diversification for numerous groups of organisms (Taberlet et al. 1998). The evolutionary outcomes of isolation in different refugia varied depending on the geographic and ecological context of each region (G. M. Hewitt 1996) as well as on the biological traits of the species involved (Stewart et al. 2010; Massatti and Knowles 2016). For instance, during interglacial periods, Mediterranean mountain ranges provided suitable conditions for the persistence of alpine species, which survive in highly isolated populations distributed across 'sky islands' embedded within a matrix of unsuitable habitat (Love et al. 2023). Such isolation in high-altitude open habitats promoted divergence and often speciation (Stewart et al. 2010; Ortego and Knowles 2022). By contrast, colder phases promoted downslope shifts that facilitated secondary contact among lineages that had remained isolated over extended periods of time (Knowles and Massatti 2017; Maier et al. 2019; Tonzo and Ortego 2021). If reproductive barriers were incomplete, secondary contact could lead to gene flow, either reversing divergence and incipient speciation (Maier et al. 2019) or resulting in varying levels of genetic introgression without compromising the evolutionary distinctiveness of the lineages involved (Ortego and Knowles 2022). These recurrent processes of contraction-expansion and fragmentation-isolation shaped the biodiversity of Mediterranean sky islands, making these mountain systems important hotspots of local endemism for alpine biotas (Kenyeres et al. 2009; G. M. Hewitt 2011; Smyčka et al. 2017; Amori et al. 2019).

Among European mountain systems, the Apennines host one of the most distinctive Pleistocene-derived biota (Ruffo 1971; La Greca 2002; Stoch 2006). These mountains are one of the youngest ranges in the Holarctic, having formed between the Neogene and the Quaternary (Cavazza et al. 2004) as a result of the collision between the African and Eurasian plates (Patacca et al. 1991) and the migration and rotation of the Sardinian-Corsican block from the Iberian plate (see Schmitt et al. 2021 for a synthesis). Their southern location, north–south orientation and the sharp climatic contrast between the Tyrrhenian and Adriatic sides generated glacial dynamics that differed markedly from those of the Alps and other European ranges (Jaurand 1999). On the Alps, Quaternary glaciations formed a vast continuous ice cap, forcing alpine species to mainly persist in peripheral refugia at the margins of the range, where isolation by large glacial streams promoted divergence and speciation (Schönswetter et al. 2005; Seguinot et al. 2018; Rota et al. 2024). On the contrary, in the Apennines ice caps were greatly fragmented and restricted to some massifs of Central Apennines (Giraudi and Frezzotti 1997; Giraudi and Giaccio 2017), which include the highest peaks of the chain, arranged into three subparallel ridges separated by valleys (Vittorj 1989). The pronounced alpine bioclimatic and ecological conditions of this sector made it a focal area of the Italian Peninsula–Sicily refugium during the Pleistocene, particularly for cold-adapted species (Menchetti et al. 2021). Mountain summits served as isolated refugia during interglacial periods, triggering microgeographic diversification of alpine biotas (Musher et al. 2022) and leading to the emergence of the numerous neo-endemic elements in both plants and animals that characterise the region (e.g., Stanisci et al. 2005; Fattorini 2010; Riccieri et al. 2024; Freda et al. 2025).

Orthoptera stands out as one of the insect groups with the highest level of endemism in the Apennines, with ca. 25 taxa exclusive to the Central Apennines, most of them associated with subalpine and alpine habitats (Massa and Fontana 2020). Their limited dispersal ability, often resulting from wing reduction or loss, has likely promoted diversification, leading to the formation of numerous microendemic species confined to a single or a few mountain peaks (Hochkirch 1998). These characteristics make them particularly well suited for understanding the evolutionary consequences of Pleistocene climatic oscillations (e.g., G. M. Hewitt 1996; Tonzo and Ortego 2021; Ortego and Knowles 2022). At the same time, these very traits that foster diversification may increase their vulnerability to ongoing climate warming. In alpine ecosystems, rising temperatures are expected to force species to shift their elevational ranges upwards; however, many of these species already occupy the highest elevations, leaving little opportunity for further upslope migration. This situation is further exacerbated by their low dispersal capacity, which may limit their ability to track suitable climatic conditions or colonise newly available habitats, making them extremely vulnerable to extinction and potentially valuable indicators of the biological impacts of climate change in alpine ecosystems (Hochkirch et al. 2016; Urbani et al. 2017; e.g., Ortego 2025; Stefanidis et al. 2025).

Against this background, a robust taxonomic framework becomes essential for accurately assessing patterns of diversity, endemism and species responses to environmental change. However, species boundaries in Orthoptera are often poorly defined and taxonomic uncertainties persist, partly because different diagnostic traits are rarely evaluated within a holistic framework. In Gomphocerinae grasshoppers, for example, acoustic communication plays a central role in mate recognition, as males produce species-specific songs by stridulating the hind femora against the forewings (e.g., Sevastianov et al. 2023, 2026). Because these acoustic signals are often involved in courtship and mate recognition, they have traditionally played a prominent role in species diagnosis and systematics within the group. Morphological traits associated with sound production, particularly the structure and length of the tegmina, are also frequently used as taxonomic characters in Gomphocerinae (e.g., Noguerales et al. 2018; Neumeister et al. 2025). However, studies in this subfamily have shown that divergence in song patterns and morphology does not always provide sufficient resolution for delimiting closely related species (Vedenina and Mugue 2011; Sevastianov et al. 2023, 2026). This taxonomic complexity is further compounded by the limited resolution often provided by mitochondrial DNA barcoding in Orthoptera (e.g., Kock et al. 2024; Hawlitschek et al. 2022). Consequently, integrative approaches combining genomic data with detailed phenotypic analyses are increasingly required to clarify species boundaries in this group (e.g., Noguerales et al. 2018).

Here, we focus on the acridid genus *Italohippus* Fontana and La Greca 1999, which currently comprises three allopatric species restricted to one or a few mountain ranges: *Italohippus modestus* (Ebner 1915), confined to the Monte Terminillo; *I. monticola* (Ebner 1915), found on several mountains of the Central Apennines; and *I. albicornis* (La Greca 1949), limited to the Matese massif (Cigliano et al. 2025; Figure 1). All three putative species occur between 1500 and 2200 m a.s.l. and are primarily associated with semi-arid alpine and subalpine open grasslands with limestone outcrops and interspersed formations of creeping junipers (*Juniperus communis* Linnaeus, 1753). They are univoltine, with adults active in summer and early autumn (Baccetti 1956, 1958, 1971; La Greca and Messina 1982) and brachypterous (Fontana and La Greca 1999). Phenotypic divergence among the three species is subtle (Ebner 1915; La Greca 1949). The most prominent morphological difference concerns wing length, with relatively longer tegmina in *I. modestus* (Massa et al. 2012). Bioacoustically, the songs of *I. albicornis* and *I. monticola* share a similar overall structure characterized by repeated echemes that differ only slightly in duration and inter-echeme intervals, whereas *I. modestus* exhibits a distinct pattern in which two to four echemes are grouped into short sequences (Fontana and La Greca 1999; Massa et al. 2012; Figure 1). Nevertheless, the internal syllable structure remains highly similar across the three taxa (Fontana and La Greca 1999; Massa et al. 2012; Figure 1). The diversification of the genus *Italohippus* is hypothesized to have taken place during the Pleistocene as a result of repeated isolation events during interglacial periods (La Greca 1996; Fontana and La Greca 1999). Due to their narrow ranges and the increasing vulnerability of their habitat to human activities and climate change, all three species are currently listed on the IUCN Red List, with *I. modestus* categorized as Vulnerable and *I. monticola* and *I. albicornis* as Endangered (Hochkirch et al. 2016).

Despite the high relevance of the Central Apennines as a major center of local endemism, this region has received limited attention in studies applying genomic data to address biogeographic questions at fine spatial and temporal resolutions. By integrating genomic, morphological and paleoclimatic data, we aim to provide novel insights into how Pleistocene climatic oscillations may have triggered micro-speciation processes and contributed to the remarkable levels of endemism characterizing the Central Apennines, focusing on the genus *Italohippus*. Studying the distributional and demographic impacts of past climatic fluctuations on this genus will also help to understand how sky-island populations may respond to ongoing climate change, with important conservation implications. Specifically, we: (i) reconstruct phylogenetic relationships and estimate divergence times among populations, assess the phenotypic and genetic cohesiveness of putative taxa and delineate independently evolving lineages (i.e., species) within an integrative, speciation-based taxonomic delimitation framework; (ii) quantify patterns of genetic differentiation and population structure and perform demographic reconstructions to test whether present-day isolation in sky islands and limited dispersal capacity have led to pronounced genetic fragmentation, as well as to evaluate whether inferred changes in effective population size ($N_e$) through time are consistent with range dynamics derived from paleodistribution modelling; and (iii) test for signals of genetic admixture and introgression among delineated species and assess whether these patterns reflect historical range overlap and secondary contact driven by species-specific distributional shifts.

## 2 | Materials and Methods

### 2.1 | Populations Sampling

During the summer of 2023, we sampled 13 populations spanning the entire distribution range of the genus *Italohippus* (Iorio et al. 2019; Cigliano et al. 2025; Figure 1). We aimed to sample 4–5 adult males and 4–5 adult females per population, but these numbers could not be reached in some small-size populations. For phylogenomic analyses, we also included five individuals of *Chorthippus crassiceps* Ramme (1926) as an outgroup. We recorded spatial coordinates with GPS and specimens were preserved in 96% ethanol at −20°C until needed for genomic and morphometric analyses. Further details on sampled populations are provided in Table 1.

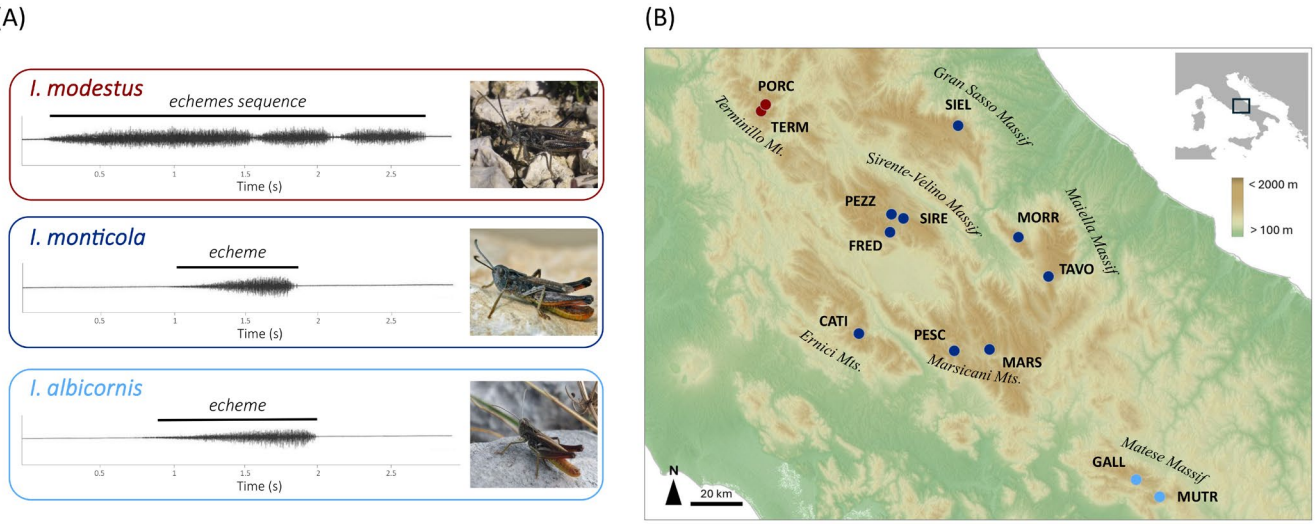


**FIGURE 1** | (A) Oscillograms of the male calling song of the three putative species of the genus *Italohippus* (time in seconds), as reported by Fontana and La Greca (1999), with photographs of adult males for each species (photos by P. Fontana, R. Scherini and J. Ortego). (B) Map showing the sampling localities along the Central Apennines. Population codes are given in Table 1.

**TABLE 1** | Geographical location of sampled populations for the genus *Italohippus*, including species assignment before and after species delimitation analysis (see Section 3.3) and values of haplotype diversity (Hd) and nucleotide diversity ($\pi$) of only populations with seven or more genotyped individuals.

| Species (putative) | Species (assigned) | Locality | Code | *n* | Latitude, longitude | Elevation (m a.s.l.) | Hd | $\pi$ |
|---|---|---|---|---|---|---|---|---|
| *Italohippus modestus* (Ebner 1915) | *I. modestus* | Mt. Porcini | PORC | 5 ♂, 4 ♀ | 42.4915, 13.0121 | 1920 | 0.1631 | 0.0018 |
| | | Mt. Terminillo | TERM | 5 ♂, 1 ♀ | 42.4717, 12.9989 | 2090 | | |
| *Italohippus monticola* (Ebner 1915) | *I. monticola* | Campocatino | CATI | 4 ♂, 4 ♀ | 41.8460, 13.3669 | 1870 | 0.0937 | 0.0009 |
| | | Pesco di Iorio | PESC | 4 ♂, 3 ♀ | 41.7970, 13.7296 | 1800 | 0.1437 | 0.0014 |
| | | Mt. Freddo | FRED | 6 ♂ | 42.1325, 13.4850 | 1810 | | |
| | | Vado di Pezza | PEZZ | 4 ♂, 4 ♀ | 42.1831, 13.4906 | 1480 | 0.2461 | 0.0025 |
| | | Mt. Sirente | SIRE | 5 ♂ | 42.1697, 13.5475 | 1830 | | |
| | | Mt. Siella | SIEL | 4 ♂, 4 ♀ | 42.4322, 13.7441 | 1910 | 0.1070 | 0.0011 |
| | | Mt. Marsicano | MARS | 4 ♂, 4 ♀ | 41.8010, 13.8636 | 2150 | 0.1467 | 0.0017 |
| | | Mt. Morrone | MORR | 2 ♂, 3 ♀ | 42.1184, 13.9733 | 1810 | | |
| | | Mt. Tavola Rotonda | TAVO | 4 ♂, 4 ♀ | 42.0077, 14.0880 | 1840 | 0.1485 | 0.0016 |
| *Italohippus albicornis* (La Greca 1949) | | Mt. La Gallinola | GALL | 4 ♂, 4 ♀ | 41.4311, 14.4214 | 1860 | 0.0653 | 0.0007 |
| | | Mt. Mutria | MUTR | 5 ♂, 4 ♀ | 41.3819, 14.5105 | 1690 | 0.0639 | 0.0007 |

## 2.2 | Genomic Library Preparation and Processing

We extracted and purified DNA from each individual using NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). Two genomics libraries were prepared following a double-digest restriction-site associated DNA sequencing (ddRAD-seq) protocol, as described by Peterson et al. (2012) and detailed in Methods S1. Raw reads were demultiplexed and preprocessed with STACKS v. 2.66 (Rochette et al. 2019) and assembled using IPYRAD v. 0.9.93 (Eaton and Overcast 2020). Methods S2 provides all details on sequence data filtering and assembling.

## 2.3 | Phylogenomic Inference and Divergence Time Estimation

First, we used the coalescent-based method implemented in SVDQUARTETS (Chifman and Kubatko 2014) to reconstruct the phylogenetic relationships among populations of *Italohippus*. This method infers lineage relationships directly from SNP data by evaluating quartets of taxa under the multispecies coalescent model. Five individuals of *Chortippus crassiceps* Ramme (1926) were used as an outgroup. We ran SVDQUARTETS as implemented in PAUP* v.4.0a169 (Swofford 2002). The input dataset contained 3001 unlinked SNPs (*.usnp* file from IPYRAD). We exhaustively evaluated all possible quartets and performed non-parametric bootstrapping with 1000 replicates to quantify topological uncertainty. Second, we used the A00 analysis implemented in BPP v.4.7.0 (Flouri et al. 2018) to estimate the posterior distribution of divergence times ($\tau$), using the phylogenetic tree inferred with SVDQUARTETS as the fixed topology. The *.loci* file from IPYRAD was edited and converted into a BPP input file using a custom R script (J. Ortego, https://github.com/OrtegoLab/ipyrad2bpp). Due to the high computational demands of the analyses, we included only three representative individuals per population. We set a uniform prior on rooted species trees, applied the automatic adjustment of fine-tune parameters, selected the diploid option to indicate that the input sequences were unphased and specified inverse-gamma priors for $\theta$ ($\alpha=3$, $\beta=0.04$) and $\tau$ ($\alpha=3$, $\beta=0.07$) based on empirical estimates of the number of segregating sites per site (Huang et al. 2020). Note that BPP analyses do not require defining outgroup, as the program samples the

root position along with the other nodes of the tree. To ensure convergence (ESS > 200), we ran two independent replicate analyses for 2,000,000 generations, sampling every two generations after a burn-in of 100,000 generations. Divergence times were calculated using the equation $\tau = 2\mu t$, where $\tau$ represents the divergence in substitutions per site estimated by BPP, $\mu$ is the per-site mutation rate per generation and $t$ is the absolute divergence time in years. We used a mutation rate of $2.8 \times 10^{-9}$ per site per generation, estimated for *Drosophila melanogaster* Meigen, 1830 (Keightley et al. 2014), which is similar to the spontaneous mutation rate reported for *Heliconius melpomene* Linnaeus, 1758 ($2.9 \times 10^{-9}$; Keightley et al. 2015).

### 2.4 | Species Delimitation Analysis

We performed constrained, partitioned species delimitation analysis using DELINEATE v. 1.2.3, an approach that integrates an explicit model of protracted speciation into the multispecies coalescent framework to identify sets of population lineages that represent distinct species (Sukumaran et al. 2021). Based on a population-level ultrametric phylogeny and prior knowledge of species assignment for a subset of populations in the dataset, DELINEATE evaluates whether the remaining populations should be assigned to a previously described species or constitute a new one (Sukumaran et al. 2021). DELINEATE analyses were performed using the ultrametric tree obtained from A00 analyses in BPP (Flouri et al. 2018) together with a species assignment reference file, which indicated whether each population was assigned to a nominal species (status = 1) or considered of unknown identity (status = 0). For details on phylogenomic analyses, see Section 2.3. Status assignments were made based on current taxonomic knowledge of the system and the evolutionary relationships among population lineages as inferred from the phylogeny (Sukumaran et al. 2021). In practice, only populations with well-established taxonomic identity (e.g., those sampled at type localities or clustering within the same well-supported clades in the phylogeny) were assigned to nominal species, whereas populations with uncertain taxonomic placement were coded with an unknown status and evaluated by DELINEATE. Populations previously described as *I. albicornis* (MUTR, GALL) were assigned to an unknown taxonomic status because they were phylogenetically nested within *I. monticola*, the latter having nomenclatural priority (Cigliano et al. 2025; see Section 3.2). For the two remaining taxa, populations sampled at type localities (Fontana and La Greca 1999), as well as those clustering within the same clades in the phylogeny, were assigned to their corresponding nominal species, whereas the remaining populations were assigned an unknown taxonomic status (Table S1).

### 2.5 | Population Genetic Structure

We used STRUCTURE v. 2.3.3 (Pritchard et al. 2000) to quantify genetic structure and admixture across populations. STRUCTURE analyses were run for 200,000 MCMC cycles, following a burn-in step of 100,000 iterations, assuming correlated allele frequencies and admixture and without using prior population information. For each value of $K$ (ranging from $K = 1$ to $K = 10$), we performed 15 independent runs and retained the 10 replicates with the highest likelihood. Following the recommendations of Gilbert et al. (2012) and Janes et al. (2017), we used two statistics to interpret the number of genetic clusters ($K$) that best describes our data: log probabilities of the data (LnPr(X|$K$); Pritchard et al. 2000) and $\Delta K$ (Evanno et al. 2005), calculated as implemented in STRUCTURE HARVESTER (Earl and vonHoldt 2012). We used CLUMPP v. 1.1.2 and the Greedy algorithm to align multiple runs of STRUCTURE for the same $K$ value (Jakobsson and Rosenberg 2007) and DISTRUCT v. 1.1 (Rosenberg 2004) to visualise the individuals' probabilities of population membership in bar plots. As a complementary approach, we performed a principal component analysis (PCA) of genetic variation using the package 'adegenet' (Jombart 2008) in R v. 4.4.0 (R Core Team 2024). Before running PCAs, we replaced missing data by the mean allele frequency of the corresponding locus estimated across all samples (Jombart 2008). We also estimated genetic differentiation between populations calculating the Weir & Cockerham weighted fixation index ($F_{ST}$), as implemented in ARLEQUIN v. 3.5 (Excoffier and Lischer 2010). We determined statistical significance with Fisher's exact tests after 10,000 permutations, applying a false discovery rate (FDR) adjustment (5%, $q < 0.05$) to control for multiple tests.

### 2.6 | Analysis of Introgression

Bayesian clustering analyses suggested genetic introgression from *I. modestus* into different populations of *I. monticola* from the Central Apennines (see Section 3.4). For this reason, we first used four-taxon ABBA/BABA analyses based on the $D$-statistic to test for signals of genetic introgression from *I. modestus* into the different populations of *I. monticola* (Durand et al. 2011). Briefly, for the sister species P1 and P2, which diverged from a common ancestor with P3 and the outgroup O, the $D$-statistic is used to test the null hypothesis of no introgression ($D = 0$) between P3 and P1 or P2. $D$-values significantly different from zero indicate gene flow between P1 and P3 ($D < 0$) or between P2 and P3 ($D > 0$). We set independent ABBA/BABA tests including as P1 each population assigned to *I. monticola*, as P2 populations previously attributed to *I. albicornis* (MUTR, GALL) and as P3 populations assigned to *I. modestus* (PORC, TERM). Five individuals of *C. crassiceps* were used as an outgroup (O). Tests were performed in IPYRAD, with 1000 bootstrap replicates used to estimate standard deviations of the $D$-statistic and assess significance levels (Eaton and Overcast 2020).

Second, we analysed whether the degree of genetic differentiation between each population of *I. monticola* and one reference population of *I. modestus*, calculated as pairwise $F_{ST}$ values (see Section 2.5), was explained by (i) latitude, (ii) geographical distance and/or resistance distances defined by (iii) climatically suitable habitats at present, (iv) climatically suitable habitats during the time period when the ranges of the two taxa reached their maximum extent (17 ka BP in both taxa; see Section 3) and (v) habitat suitability stability from the LGM to present. Owing to its larger sample size, we used population PORC of *I. modestus* as the reference population for analyses (Table 1). To obtain resistance distances, we first used the R package '*raster*' to transform suitability maps obtained from species-specific environmental niche models (ENM; see Section 2.8) for each time period into binary layers (presence = 1; absence = 0) using the maximum

training sensitivity plus specificity (MTSS) logistic threshold of MAXENT (Liu et al. 2005). Climatically suitable habitats, both at present and during the time of maximum range extent, were defined as areas predicted to be suitable for either *I. modestus* or *I. monticola*. Climatic suitability stability was calculated as the proportion of time intervals during which a given pixel was predicted to be suitable for either species from the LGM to the present. Resistance distances under each isolation-by-resistance scenario were calculated using an eight-neighbour cell connection scheme in CIRCUITSCAPE v. 4.0.5 (McRae 2006; McRae and Beier 2007). Because resistance distances cannot be calculated between completely isolated populations (i.e., conductance = 0), we transformed zero-pixel values to very small conductance values (= 0.0001) before running CIRCUITSCAPE. We analysed the data using generalized linear models (GLMs), as implemented in SPSS v. 29 (IBM, NY, USA). Because the precision of gene flow estimates may vary among populations owing to differences in sample sizes, we applied a weighted least-squares (WLS) method, where the weight corresponded to the number of genotyped individuals for each population of *I. monticola* (Table 1). Except for resistance distances based on suitable habitats at the time of maximum range extent, all other independent variables were highly intercorrelated ($r > 0.85$). Therefore, we identified the variable providing the best model fit using the adjusted coefficient of determination ($R^2$) from univariate models.

## 2.7 | Genetic Diversity and Past Demographic History

We calculated haplotype diversity (Hd) and nucleotide diversity ($\pi$) in DNASP v. 6.12.03 (Rozas et al. 2017) and reconstructed the demographic history of each population using the program STAIRWAY PLOT v. 2.1.2 (Liu and Fu 2020). Only populations with $n \geq 7$ genotyped individuals were considered for these analyses (Table 1). To maximise the number of retained SNPs for the calculation of the SFS, we ran the *step 7* from IPYRAD separately for each specific population and retained loci that were represented in at least 50% of the individuals of the focal population (*min_samples_locus* = 50% of samples in the focal dataset). To remove all missing data for the calculation of the SFS and minimise errors in allele frequency estimates, each population was projected down to $n-2$ diploids for each dataset of $n$ genotyped individuals using the *easySFS.py* script (I. Overcast, https://github.com/isaacovercast/easySFS). We ran STAIRWAY PLOT assuming a mutation rate of $2.8 \times 10^{-9}$ per site per generation (Keightley et al. 2014) and performing 200 bootstrap replicates to estimate 95% confidence intervals. We considered a one-year generation time, consistent with the univoltine life cycle of *Italohippus* (Baccetti 1956, 1958, 1971; La Greca and Messina 1982). Finally, we used estimates of effective population size ($N_e$) over time, inferred by STAIRWAY PLOT, to calculate demographic instability and long-term $N_e$ for each population and to analyse whether these parameters are associated with latitude. Demographic instability and long-term $N_e$ were calculated as the coefficient of variation and the harmonic mean of $N_e$ over time, respectively. As for analyses of genetic differentiation (Section 2.6), we analysed the data using generalized linear models (GLMs) and applied a weighted least-squares (WLS) method to take into account differences among populations in sample sizes used to estimate demographic parameters (Table 1).

## 2.8 | Environmental Niche Modelling

To reconstruct the geographic distribution of climatically suitable habitats for *Italohippus* from the LGM (ca. 22 ka) to present, we built two independent environmental niche models (ENMs), one for *I. modestus* and another for *I. monticola*, in accordance with the results of the species delimitation analyses (see Section 3.3). We used this information to infer distributional shifts in response to Quaternary climatic oscillations and to assess range size dynamics and the spatial distribution and continuity of climatically suitable habitats across different timeperiods, as well as the extent of overlap between the ranges of *I. modestus* and *I. monticola* (i.e., secondary contact). To build the ENM, we used the maximum entropy algorithm implemented in MAXENT v.3.4.1 (Phillips et al. 2006; Phillips and Dudík 2008), occurrence records for *I. monticola* ($n = 44$) and *I. modestus* ($n = 12$) and the 19 bioclimatic layers (30-arcsec resolution; for variable description, see Table S2) from the CHELSA database (https://chelsa-climate.org/bioclim/; Karger et al. 2017). Occurrence records historically attributed to *I. albicornis* were included under *I. monticola* (see Section 3.3). To estimate environmental suitability from the LGM to present and dynamics of range overlap between the two taxa, we projected the ENM to bioclimatic conditions during the last 22,000 years at 100-year time intervals (i.e., from 1990 CE to the LGM, for a total of 220 snapshots) using bioclimatic layers available at a high resolution (30-arcsec) from the CHELSA-TraCE21k v. 1.0 database (https://chelsa-climate.org/; Karger et al. 2023). Further details on ENM are presented in Methods S3.

## 2.9 | Geometric and Linear Morphometric Analyses

We used both geometric and linear morphometric approaches to characterise variation in the forewing, the only morphological trait reported as variable among the three historically described species (Massa et al. 2012) and a structure associated with male song in Gomphocerini grasshoppers (Nattier et al. 2011; Neumeister et al. 2025) and other Orthoptera (Bennet-Clark and Bailey 2002; Montealegre-z 2009). All specimens included in the genomic dataset were analysed and divided into two subsets (males and females) to account for the sexual dimorphism characteristic of this genus (Fontana and La Greca 1999). Forewings and hind femora were photographed using a Leica Flexacam C3 camera mounted on a Zeiss Stemi 2000 stereomicroscope. We used TPSDIG v. 2.32 (Rohlf 2016) to digitise 12 homologous landmarks (Figure S1) on each tegmen photograph; all subsequent analyses were conducted in the R package '*geomorph*' (Adams and Otárola-Castillo 2013), separately for males and females. Landmark coordinates were subjected to Generalized Procrustes Analysis (GPA) to remove the effects of scale, rotation and translation. To assess shape variation, we performed a PCA and tested the effects of centroid size (as a proxy for wing size) and taxon identity using Procrustes ANOVAs (function *procD.lm* in *geomorph*) with 999 permutations under the randomized residual permutation procedure (RRPP). Post hoc pairwise tests were used to evaluate differences among species. In this model, species effects were tested while accounting for size effect, allowing the assessment of shape differences independent of allometry (*see*

Klingenberg 2016). A second PCA was performed on the residuals from the regression of shape on centroid size to visualise size-independent variation. Finally, to test whether interspecific differences are primarily related to forewing length, as pointed out by Fontana and La Greca (1999), we applied a linear morphometric approach. We assessed wing size variation by calculating the ratio between forewing length and hind femur length, a metric widely used in taxonomic and ecological studies as a proxy for relative wing development (e.g., Noguerales and Ortego 2022). Hind femur length was used as a proxy for overall body size (e.g., Ortego et al. 2012). Forewing and hind femur lengths were extracted using the *interlmkdist* function from the R package '*geomorph*'. All landmarks and measurements were taken by the same observer (FF). We calculated the ratio between forewing length and hind femur length and, after verifying model assumptions, performed non-parametric Kruskal–Wallis tests followed by post hoc Dunn's tests for pairwise comparisons. Variation in the forewing-to-femur length ratio among populations with at least three individuals was visualized using density plots generated with the R package '*ggplot2*' (Wickham et al. 2016).

## 3 | Results

### 3.1 | Genomic Datasets

After all quality filtering steps, we retained an average of 3,961,198 reads per specimen (range: 1,585,505–8,375,891). The mean proportion of missing data was 14% and ranged from 6% to 45% (median = 11%). All genotyped specimens were retained for downstream analyses. Details of the genomic datasets used for the different analyses are provided in Table S3.

### 3.2 | Phylogenomic Inference and Divergence Time Estimation

Phylogenomic analyses with SVDQUARTETS revealed a split into two main clades, one comprising populations assigned to *I. modestus* from Mt. Terminillo and the other including the populations of *I. monticola* and *I. albicornis* (Figure 2). While *I. modestus* appeared clearly monophyletic, *I. monticola* was paraphyletic, with populations historically attributed to *I. albicornis* (GALL, MUTR) fully nested within the clade including the southernmost populations of *I. monticola*. The node with the lowest support separated the population CATI from the remaining populations within the same clade (Figure 2). Divergence time estimates from BPP (A00) place the split between the two main clades in the Middle Pleistocene (ca. 0.3 Mya; Chibanian), whereas diversification within *I. monticola-I. albicornis* clade occurred more recently, during the Late Pleistocene (ca. 0.22–0.08 Mya; Figure 2).

### 3.3 | Species Delimitation Analysis

Species delimitation analysis in DELINEATE did not support *I. albicornis* as a distinct species, assigning GALL and MUTR to *I. monticola*. All other populations of uncertain taxonomic status were also confirmed as belonging to *I. monticola* (Figure 2).

### 3.4 | Population Genetic Structure

Results from STRUCTURE revealed a genetic structure congruent with the main lineages and clades inferred from phylogenomic analyses (Figure 2, Figure S2). In STRUCTURE analyses, $\Delta K$ peaked at $K=3$, whereas $\ln Pr(X|K)$ increased gradually and reached a plateau at $K=8$ (Figure S3). At $K=3$, populations of *I. modestus* separated from those within the *I. monticola—I. albicornis* clade, which in turn split into two clusters corresponding to the two main subclades of this group; only the population CATI showed marked genetic admixture between the clusters corresponding to *I. modestus* and the northern lineage of *I. monticola* (Figure 2). At $K=4$, the southernmost populations, originally attributed to *I. albicornis* (GALL, MUTR), formed a distinct genetic cluster. From $K=5$ to $K=8$, populations progressively split into hierarchical genetic clusters with very limited signatures of admixture (Figure S2). Principal component analyses (PCA) showed a clustering pattern fully consistent with the STRUCTURE results and the main phylogenomic lineages (Figure S2). Pairwise $F_{ST}$ values ranged between 0.086 and 0.634 and all were significantly different from zero (Table S4).

### 3.5 | Analysis of Introgression

The *D*-statistic was negative and statistically significant in all ABBA/BABA tests, revealing an excess of the BABA pattern. This indicates a significantly higher overall allele sharing between populations assigned to *I. monticola* (P1) and *I. modestus* (P3) compared with the reference group used as P2 (GALL+MUTR) (Table S5). The strongest signal of introgression was observed in the comparison involving the population CATI of *I. monticola* ($D=-0.363$, $Z=14.44$), indicating substantial gene flow between this population and *I. modestus*. Although genetic differentiation ($F_{ST}$) between *I. modestus* and each population of *I. monticola* was positively associated with both geographical distance ($t=4.22$, $p=0.002$, $n=11$, $R^2=0.628$) and resistance distances defined by climatically suitable habitats at present ($t=3.49$, $p=0.007$, $n=11$, $R^2=0.529$), resistance distances defined by habitat suitability stability from the LGM to present provided the best model fit ($t=4.48$, $p=0.002$, $n=11$, $R^2=0.691$) (Figure 3). Genetic differentiation was not significantly associated with latitude ($t=-1.96$, $p=0.082$, $n=11$, $R^2=0.298$) or with resistance distances based on climatically suitable habitats during the period of maximum range extent for both species ($t=-1.58$, $p=0.148$, $n=11$, $R^2=0.130$).

### 3.6 | Genetic Diversity and Past Demographic History

Haplotype diversity (Hd; range: 0.0639–0.2461) and levels of nucleotide diversity ($\pi$; range: 0.0007–0.0025) for each population are presented in Table 1. STAIRWAY PLOT analyses revealed a shared demographic history across all analysed populations. All populations experienced demographic expansions during glacial periods, with effective population sizes ($N_e$) peaking around the Last Glacial Maximum (ca. 21 kya), followed by a steady decline after the onset of the Holocene. Remarkably, the southernmost populations GALL and MUTR exhibited the smallest fluctuations in $N_e$ through time (Figure 4). Demographic instability increased

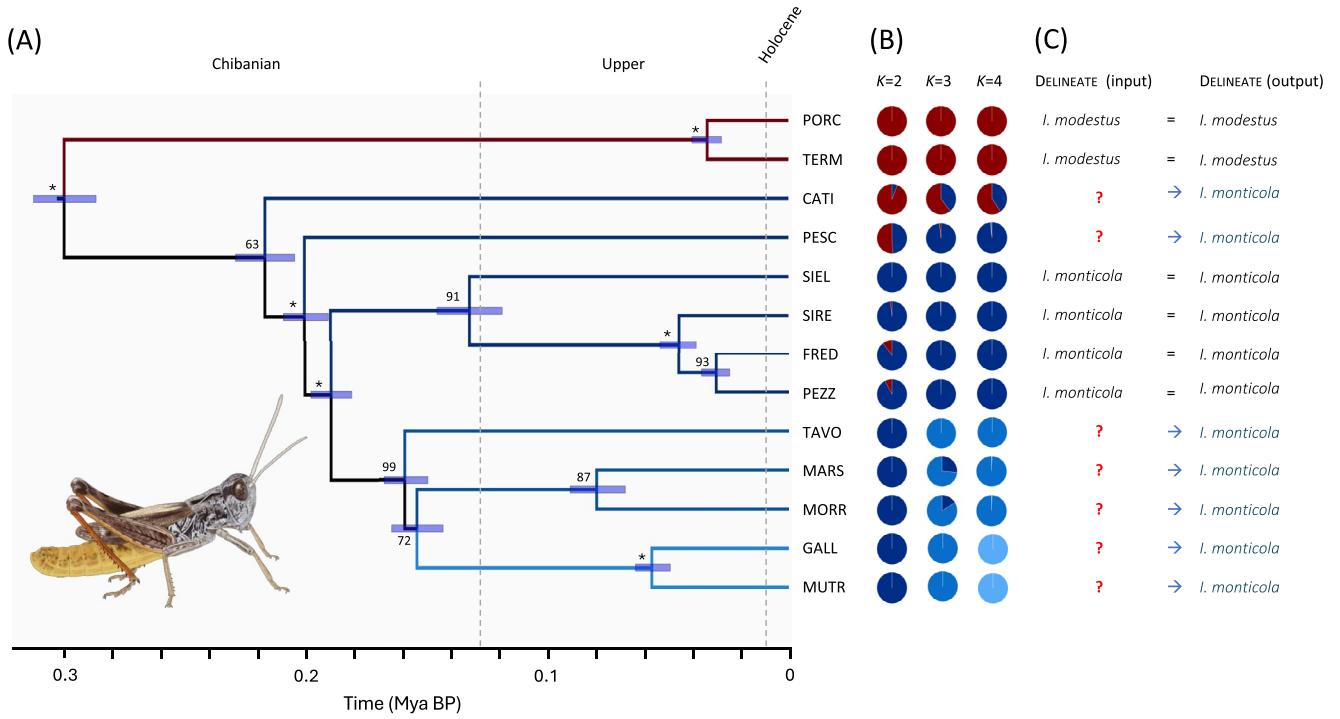


**FIGURE 2** | (A) Phylogenetic tree obtained using SVDQUARTETS (3001 SNPs) and dated with the A00 analysis implemented in BPP. Bars on nodes indicate 95% highest posterior densities (HPD) of divergence times estimated considering a genomic mutation rate of $2.8 \times 10^{-9}$ per site per generation and a one-year generation time. Asterisks on nodes indicate bootstrap support values of 100%. (B) Results of the genetic assignment for each population from $K=2$ to $K=4$ based on the Bayesian method implemented in the program STRUCTURE; additional assignments from $K=5$ to $K=8$ are shown in Figure S2. (C) Species delimitation results obtained with DELINEATE, which uses the ultrametric phylogeny from BPP together with prior species assignments for a subset of populations to evaluate whether the remaining populations belong to previously described species or represent distinct lineages; further details on species delimitation are provided in Table S1. Population codes as described in Table 1. Inset image shows a male of *Italohippus monticola* (illustration by Marina Trillo).

with latitude ($t=4.66$, $p=0.002$, $n=9$, $R^2=0.721$), whereas long-term $N_e$ decreased with latitude ($t=-2.47$, $p=0.043$, $n=9$, $R^2=0.389$) (Figure 5).

### 3.7 | Environmental Niche Modelling

The selected ENM for *I. monticola* (lowest AICc) was built using a regularisation multiplier of 1 and the hinge feature class (H). After variable selection, eight bioclimatic layers were retained and used to build the final model (BIO1 = 84.98%, BIO2 = 8.60%, BIO8 = 4.63%, BIO9 = 0.03%, BIO12 = 0.72%, BIO14 = 0.14%, BIO15 = 0.80% and BIO19 = 0.09%). The model showed high discriminatory power, with an area under the receiver operating characteristic curve for the test data ($AUC_{TEST}$) of 0.983. The minimum training presence omission rate estimate was moderate ($OR_{MTP}=0.227$), pointing to some degree of model overfitting. For *I. modestus*, the best model was obtained with a regularisation multiplier of 3 and linear, quadratic and hinge feature classes (LQH). Five predictors were retained (BIO1 = 62.41%, BIO2 = 24.50%, BIO9 = 2.38%, BIO15 = 0.17%, BIO18 = 10.54%). This model also had a high discriminatory power ($AUC_{TEST}=0.998$) and presented a moderate minimum training presence omission rate ($OR_{MTP}=0.167$), also indicating some degree of model overfitting.

Climatic habitat suitability predicted by ENMs was largely consistent with the current distribution of the two species, showing fragmented populations restricted to high-elevation areas of the Central Apennines (Figure 6). Reconstruction of range dynamics from the LGM to the present, at 100-year intervals, showed that the extent of habitat suitability for the two species peaked at the end of the Last Glacial Maximum (ca. 17 ka) and has declined since then (Figure 6). Focusing on the ENM of *I. monticola*, the southernmost populations from the Matese massifs appear to have remained relatively isolated over time compared with the rest of the range. During periods of maximum expansion, a partial overlap between the ranges of the two species was inferred, primarily driven by the southward expansion of *I. modestus* (Figure 6).

### 3.8 | Geometric and Linear Morphometrics Analyses

Procrustes ANOVAs on forewing shape revealed a statistically significant allometric effect in both sexes, confirming a strong relationship between shape and size, which was particularly pronounced in females (36% of the variation). After accounting for size, a significant effect of species identity was detected only in males (Table S6) and post hoc pairwise comparisons indicated differences among all three putative species (Table S6). PCA of Procrustes coordinates showed that the first two components explained most of the shape variation (76.7% in males, 81.3% in females), but the three species overlapped broadly in morphospace (Figure 7a,d). This overlap was even more pronounced in

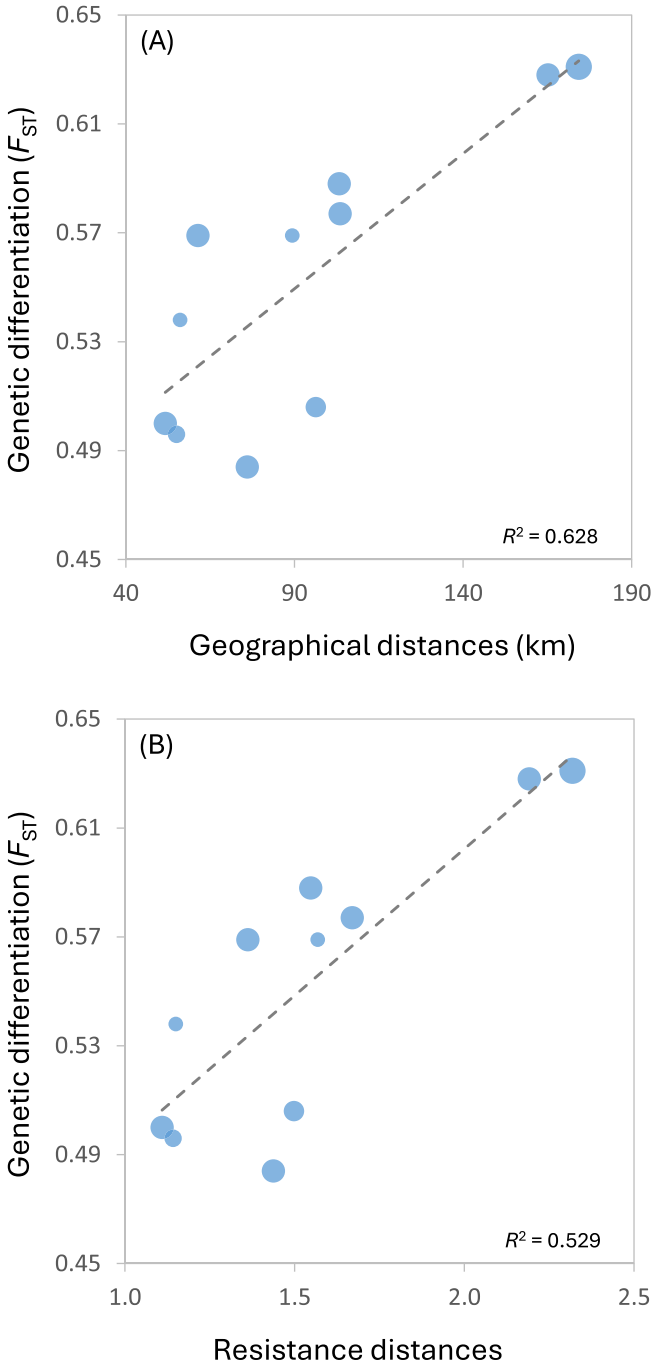


**FIGURE 3** | Relationship between (A) geographical distances and (B) resistance distances derived from habitat suitability stability between the last glacial maximum (LGM) and the present and genetic differentiation ($F_{ST}$) between *I. modestus* (using population PORC as the reference; Table 1) and each population of *I. monticola*. Regression lines are shown and dot size is proportional to the sample size of each population of *I. monticola*.

the PCA of allometric residuals (first two components ~73% in both sexes), highlighting the key role of size in shaping interspecific variation (Figure 7b,e). Complementarily, nonparametric Kruskal–Wallis tests on forewing length showed significant interspecific differences in both sexes. Dunn's post hoc tests showed that *I. modestus* had significantly longer forewings than both *I. monticola* and *I. albicornis*, while no differences were found between the latter two (Table S7). Density plots further illustrated these results, highlighting that CATI and PESC populations displayed intermediate forewing lengths between *I. modestus* and *I. monticola* in both sexes (Figure 7c,f).

## 4 | Discussion

Understanding evolutionary processes in regions characterized by high micro-endemism, such as Mediterranean mountains, requires integration of multiple lines of evidence. Here, we use the grasshopper genus *Italohippus* as a model system to investigate how the interplay between Pleistocene glaciations and complex topography shaped diversification in an understudied but biogeographically significant region: the Central Apennines. Our results indicate recent diversification (< 1 Ma) and an evolutionary history characterized by cycles of isolation and secondary contact, leading to the emergence of reproductive barriers of varying permeability.

### 4.1 | Integrative Species Delimitation

Although divergence time estimates should be interpreted with caution given uncertainty surrounding genomic mutation rates, our dating analyses suggest that diversification within *Italohippus* probably began during the Middle Pleistocene, around 0.3 Mya and continued through the Late Pleistocene (Figure 2a). This temporal framework is consistent with the Pleistocene-driven speciation scenario previously proposed for *Italohippus* (Fontana and La Greca 1999) and with patterns documented in several other radiations of alpine and montane grasshoppers (e.g., Ortego et al. 2021, 2024). The first split within the genus separates *I. modestus* from *I. monticola* and *I. albicornis*, supporting the distinctiveness of the former species (Ebner 1915; Fontana and La Greca 1999; Massa et al. 2012) (Figure 2a,b). The two analysed populations of *I. modestus* (TERM, PORC; Figure 2b) form a well-supported monophyletic clade and a distinct genetic cluster in STRUCTURE analyses, consistent with both phylogenetic and genotypic cluster species concepts (De Queiroz 2007). In contrast, populations traditionally assigned to *I. albicornis* (GALL, MUTR) are monophyletic but nested within *I. monticola*, rendering the latter paraphyletic (Figure 2a). Accordingly, species delimitation analyses assigned the Matese populations to *I. monticola* (GALL, MUTR; Figure 2c), providing no support for the species-level status of *I. albicornis*. Nonetheless, Bayesian clustering analyses revealed the genotypic distinctiveness of populations from the Matese (Figure 2b; Figure S2), consistent with their strong isolation at the southernmost edge of the genus range (Figure 1b).

Geometric morphometric data complement the genomic results and provide additional insights into species delimitation and the taxonomic status of the Matese populations. Forewing shape variation showed that individuals tend to cluster according to the three putative taxa in both sexes, albeit with extensive overlap. However, after removing allometric effects, these shape differences remained significant only in males (Figure 7, Table S6). This subtle morphological differentiation aligns with the genotypic distinctiveness found for the Matese populations in Bayesian clustering analyses (Figure 2b), but the substantial overlap suggests it is insufficient to justify species-level status (Figure 7; Table S6). In contrast, linear morphometric

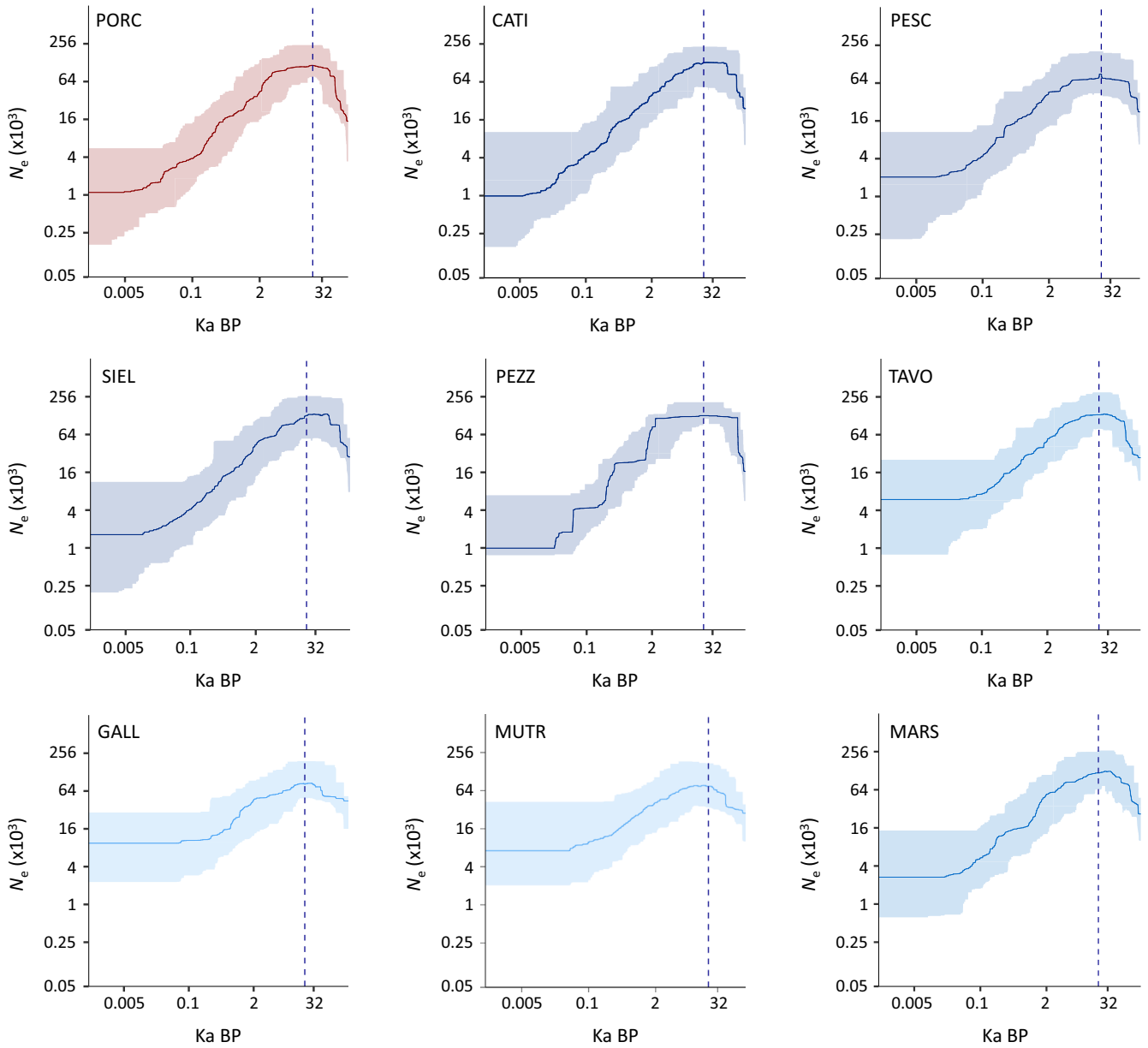


**FIGURE 4** | Demographic history of the studied populations of *Italohippus* inferred using STAIRWAY PLOT. Panels show the median of effective population size ($N_e$) over time, estimated assuming a mutation rate of $2.8 \times 10^{-9}$ and a one-year generation time (both axes on a logarithmic scale). Vertical dashed line indicates the Last Glacial Maximum (LGM; ~21,000 years ago). Only populations with seven or more genotyped individuals were analysed. Population codes as described in Table 1.

analyses align with the phylogenetic and species delimitation results: forewing length, previously identified as a diagnostic trait (Massa et al. 2012), clearly separates *I. modestus*, whereas *I. albicornis* and *I. monticola* exhibit nearly identical values (Figure 7; Table S6). These morphological patterns also parallel the reported bioacoustics of the genus: *I. modestus* produces a markedly distinct calling song structure, with sequences of 2–4 echemes, whereas the song of *I. albicornis* closely resembles that of *I. monticola* (Fontana and La Greca 1999; Massa et al. 2012; Figure 1a). This pattern suggests that the acoustic divergence observed in the Matese Massif populations may represent geographic variation rather than species-level differentiation. Overall, our results support the idea that although some phenotypic, genotypic and acoustic traits show subtle differentiation at the southernmost edge of the range, these differences are not substantial enough to justify maintaining *I. monticola* and *I. albicornis* as separate species.

### 4.2 | Allopatric Speciation With Incomplete Reproductive Isolation

Species-specific distribution models revealed strong isolation between the range of *I. modestus* in the Terminillo system and that of *I. monticola* across the rest of the Central Apennines, providing an ideal setting for allopatric divergence and speciation (Figure 6). Such isolation may have facilitated the gradual evolution of reproductive barriers, which in Gomphocerini are primarily mediated by divergence in calling songs, traits that can evolve rapidly under allopatry through the combined effects

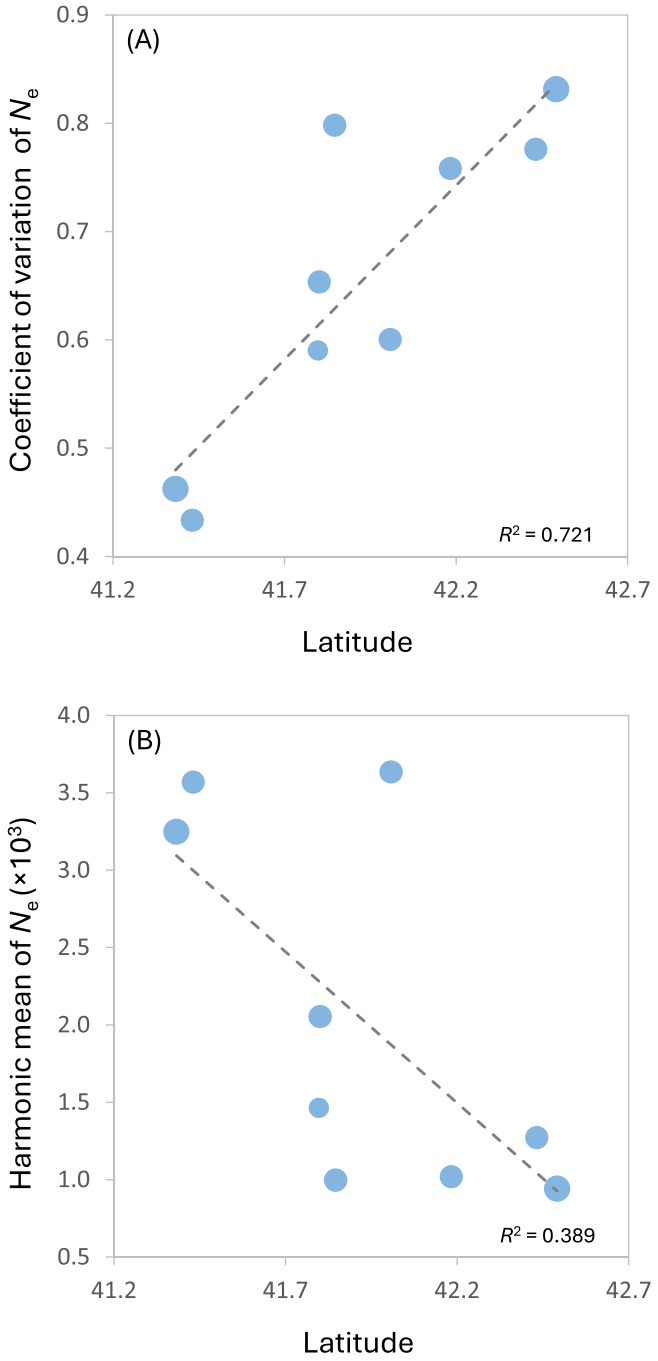


**FIGURE 5** | Relationships between latitude and (A) the coefficient of variation of effective population size ($N_e$) (i.e., demographic instability) and (B) the harmonic mean of $N_e$ (i.e., long-term $N_e$). Demographic instability and long-term $N_e$ were estimated from demographic reconstructions generated with STAIRWAY PLOT. Regression lines are shown and dot size is proportional to sample size (i.e., number of individuals used to run STAIRWAY PLOT).

of genetic drift and natural and sexual selection (Servedio and Boughman 2017; Nolen et al. 2020; Sevastianov et al. 2023; Sevastianov et al. 2026).

Although *I. modestus* and *I. monticola* currently exhibit allopatric distributions, paleodistribution reconstructions revealed extensive range overlap during glacial periods. Bearing in mind the intrinsic limitations of environmental niche models, particularly when they are projected to climatic conditions different from those used to calibrate the model (Hampe 2004; Wiens et al. 2009; Zurell et al. 2020), our results suggest a predominant southward expansion of *I. modestus* during glacial stages, leading to incursions into the range of *I. monticola*. This created broad zones of overlap between the two species, suggesting that the two taxa likely came into secondary contact and had opportunities to hybridise if reproductive isolation was incomplete (Figure 6). Consistently, analyses of introgression and admixture revealed a reticulate evolutionary history, with most populations assigned to *I. monticola* showing genomic signatures of introgression from *I. modestus*. Notably, genetic differentiation between *I. modestus* and *I. monticola* was best explained by resistance distances based on habitat suitability stability from the LGM to the present, supporting an important role of the spatial distribution of suitable habitats for both species in shaping the observed patterns of genetic introgression (Figure 3). These results indicate that, despite clear phylogenetic, behavioural and morphological differentiation, secondary contacts have led to hybridisation and different levels of genetic introgression. Among these, it is remarkable the case of the population from Monti Ernici (CATI), which received the weakest node support in phylogenetic reconstructions and represents the earliest split within the *I. monticola* clade (ca. 0.22 Mya; Figure 2a). Although this population was assigned to *I. monticola* in species delimitation analyses (Figure 2c), *D*-statistic tests and Bayesian clustering analyses revealed clear evidence of historical hybridisation, with a large fraction of its ancestry derived from *I. modestus* (Figure 2b; Figure S2; Table S5). In line with genetic clustering analyses, introgression tests showed that only populations from the Matese mountains exhibit no evidence of introgression with *I. modestus*, further supporting their long-term isolation.

Taken together, divergence dating and introgression analyses support a scenario of recent allopatric speciation and indicate that *I. monticola* and *I. modestus* have not yet achieved complete reproductive isolation, consistent with previous findings from other Pleistocene radiations of grasshoppers (Ortego and Noguerales 2025; Ortego and Knowles 2022; Tonzo et al. 2020). These results suggest that calling song acted as a partially effective but porous reproductive barrier, allowing occasional gene flow along the speciation continuum. Thus, the evolutionary history of *Italohippus* illustrates how geographic isolation and secondary contact can generate contrasting outcomes, from well-defined species to hybridisation and incipient speciation.

### 4.3 | Demographic and Range Dynamics

The present distribution of *Italohippus*, with isolated populations confined to different peaks of the Central Apennines and exhibiting a marked genetic structure and differentiation, exemplifies the sky island distribution characteristic of cold-adapted species with narrow ecological requirements (Knowles and Massatti 2017; Flantua et al. 2020; Love et al. 2023) (Figure 2; Table S4). However, paleodistribution modelling suggests that the evolutionary history of these taxa was not shaped exclusively by isolation, supporting range expansions and latitudinal and elevational shifts during

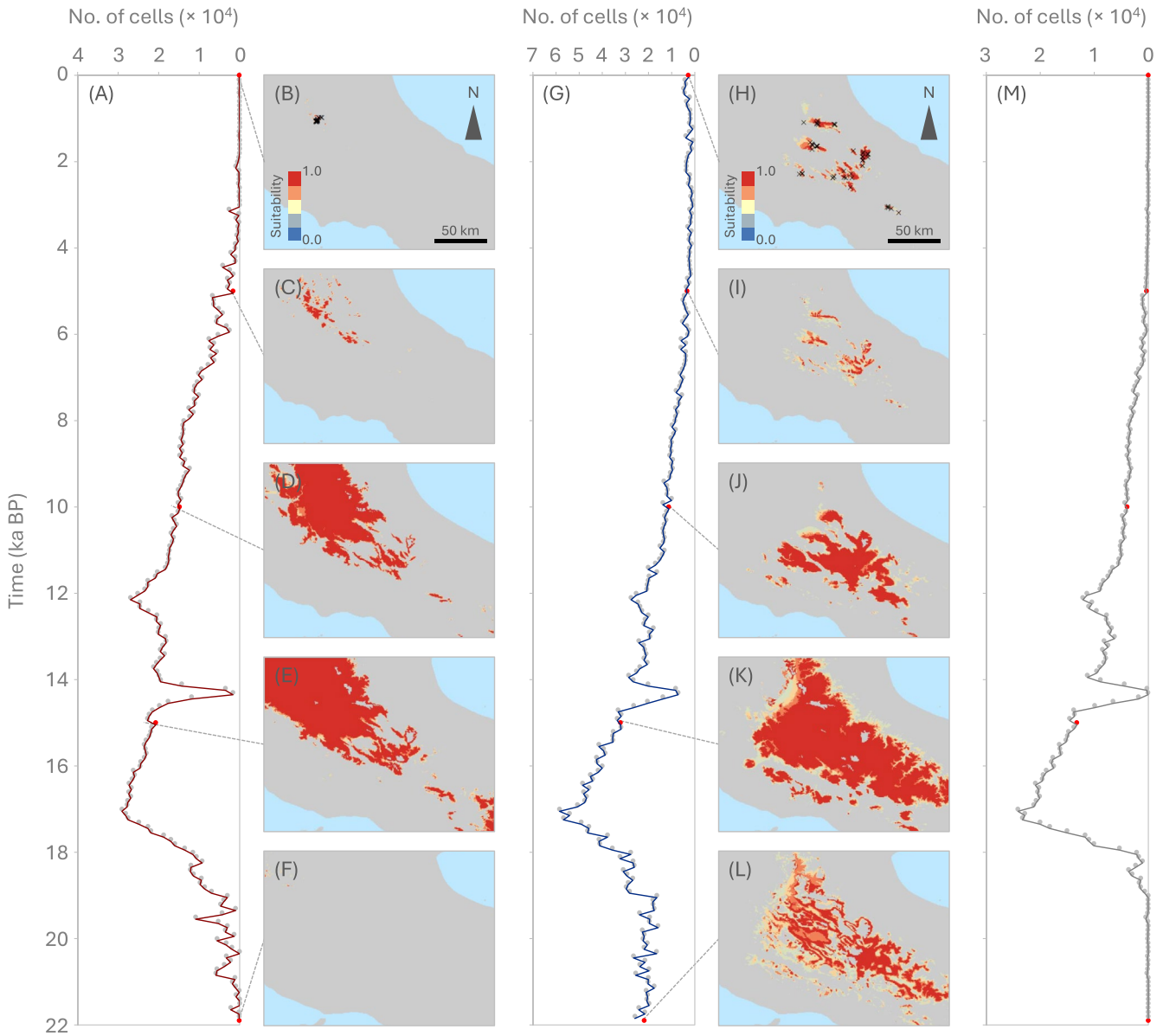


**FIGURE 6** | Climatically suitable habitats for *Italohippus modestus* (A–F) and *Italohippus monticola* (G–L), as well as the extent of range overlap between the two taxa (M), as inferred from projections of species-specific environmental niche models (ENMs) onto bioclimatic conditions spanning the last 22,000 years (from 1990 CE to the Last Glacial Maximum, LGM), at 100-year intervals. (A, G) Suitable habitat availability through time, expressed as the number of grid cells where the predicted probability of presence exceeded the maximum training sensitivity plus specificity (MTSS) logistic threshold. (B–F, H–L) Maps of climatically suitable habitats for *I. modestus* and *I. monticola* at five temporal snapshots (red dots in panels A and G), including (B, H) the present (0 ka; crosses mark occurrence records used for ENMs), (D, J) the Holocene Climate Optimum (ca. 10 ka) and (F, L) the LGM (ca. 22 ka). Maps display only areas with predicted suitability values above the MTSS logistic threshold; grey areas indicate regions predicted as unsuitable. (M) Temporal variation in the extent of overlap between the distributions of the two taxa, calculated as the number of grid cells where both species are predicted to occur according to the MTSS logistic threshold of their respective ENMs.

colder periods followed by fragmentation during interglacials (Figure 6). Although demographic reconstructions for some populations may be influenced by relatively small sample sizes, the inferred trajectories are broadly consistent with these range dynamics, showing a peak in effective population size ($N_e$) during the Last Glacial Maximum (LGM), followed by a sharp decline with rising temperatures at the onset of the Holocene (Figure 4). Southward range shifts suggest that glaciations had a stronger impact on the northern sector of the Central Apennines, a pattern supported by weaker demographic fluctuations in southern populations (e.g., GALL, MUTR from Matese) compared to the northern ones (e.g., PORC from Terminillo) (Figure 5a).

## 5 | Conclusion

Our study provides an integrative framework for investigating how Pleistocene climate fluctuations shaped the diversification of microendemic alpine species inhabiting Mediterranean sky islands. Our results support a biogeographic model of intermittently connected refugia, where isolation in interglacial periods

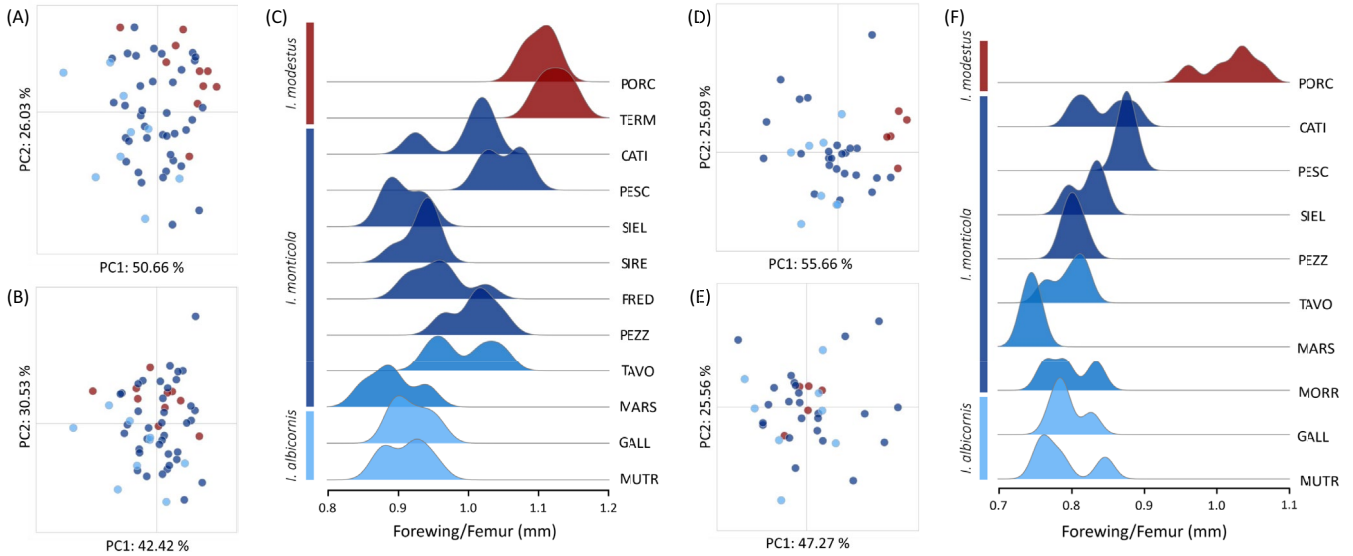


**FIGURE 7** | Principal component analyses (PCA) and density plots of forewing variation in *Italohippus*. Panel (A) shows the PCA of Procrustes coordinates for male tegmina, while panel (D) reports the same analysis for females. Panels (B) and (E) illustrate the PCA performed on allometric residuals for males and females, respectively. In PCA plots, the percentages shown next to each principal component indicate the proportion of total variance explained by that axis. Panels (C) and (F) display density plots of the ratio between forewing and femur length across populations for males and females, respectively. Population codes as described in Table 1.

and secondary contact during cooler phases generated different outcomes along the speciation continuum, a pattern also documented in other organisms in the Apennines (Canestrelli et al. 2008, 2012; Salvi et al. 2013; Chiocchio et al. 2021; Berrilli et al. 2025). Populations from the Matese exhibit evolutionary traits relevant to conservation. However, our integrative analyses indicate that *I. albicornis* is phylogenetically nested within *I. monticola* and both taxa exhibit nearly identical wing morphology and calling songs. Accordingly, we propose the formal inclusion of *I. albicornis* within *I. monticola* as a subspecies: *Italohippus monticola albicornis* **stat. nov**. Recognizing subspecies offers a clear advantage for conservation planning (Dufresnes et al. 2023), preserving the legal status of this 'Endangered' lineage (Hochkirch et al. 2016). However, the current European Red List primarily restricts assessments to the species level (Hochkirch et al. 2016), highlighting the urgent need for a National Red List. Such a tool is essential under ongoing climate warming, as these alpine populations face severe risks of range contraction and genetic diversity loss (Urbani et al. 2017; e.g., Ortego 2025). Their marked genetic structure reveals a very low dispersal capacity, suggesting that local extinctions are unlikely to be compensated by immigration, making these isolated mountain lineages particularly vulnerable to the rapid loss of suitable thermal habitats.

## Author Contributions

Francesco Forte, Alessandra Riccieri, Paolo Fontana and Joaquín Ortego conceived and designed the study. Francesco Forte, Fabrizio Freda and Joaquín Ortego collected the samples. Francesco Forte prepared the genomic libraries with the assistance of Marina Trillo. Francesco Forte obtained and analysed the data, guided by Joaquín Ortego and with help from Marina Trillo. Francesco Forte led the writing of the manuscript, with contributions from Alessandra Riccieri, Joaquín Ortego and Marco A. Bologna. All authors read and approved the final version of the manuscript.


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## Conflicts of Interest

The authors declare no conflicts of interest.

## Data Availability Statement

Raw Illumina reads have been deposited at the NCBI Sequence Read Archive (SRA) under BioProjects PRJNA1450054 and PRJNA702631. Input files for all analyses are available for download on Figshare (https://doi.org/10.6084/m9.figshare.31864996).

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## Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Studied populations of the different species within the genus *Italohippus*, including their putative taxonomic status and status assignments used in the species delimitation analyses. **Table S2:** Bioclimatic variables from the CHELSA dataset used to build the environmental niche model. **Table S3:** Attributes of the genomic datasets used for the different analyses, including number of individuals and retained reads and loci. **Table S4:** Pairwise $F_{ST}$ values and their corresponding $q$ values for populations of *Italohippus*. **Table S5:** Analyses of introgression using four-taxon $D$-statistic (ABBA/BABA) tests. **Table S6:** Results of Procrustes ANOVA testing the effects of centroid size and species identity on forewing shape variation. **Table S7:** Results of post hoc pairwise comparisons testing differences in the ratio between forewing length and hind femur length among *Italohippus* species. **Figure S1:** Location and numbering of landmarks used for geometric morphometric characterisation of forewings. **Figure S2:** Genetic clustering results from STRUCTURE and principal component analysis (PCA) of genetic variation for *Italohippus*. **Figure S3:** Results of STRUCTURE HARVESTER showing the mean log probability of the data and the magnitude of $\Delta K$ for each value of $K$. **Methods S1**. Genomic library preparation. **Methods S2**. Genomic data filtering and assembling. **Methods S3**. Environmental niche modelling.