

# Apennine–Pyrenees disjunct distribution: an unusual biogeographic pattern revealed in flea beetles of the *Longitarsus candidulus* species-group (Coleoptera, Chrysomelidae)

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## Abstract

European mountain systems have played a crucial role in shaping the distribution of species and of their genetic diversity during the Quaternary climatic changes, with the establishment of allopatric patterns across main mountain ranges. Here we investigated the evolutionary history of flea beetles of the *Longitarsus candidulus* species-group showing an uncommon disjunct biogeographic pattern across the Apennine and the Pyrenees. We applied a multilocus molecular approach and multispecies coalescent models to establish a phylogenetic and systematic framework for this morphologically homogeneous species-group and to estimate the time of main cladogenetic events underlying the origin of the Apennine–Pyrenees pattern. We found strong support for the monophyly of the *candidulus* group with a sister relationship between *Longitarsus laureolae* and *L. leonardii* endemic to the Apennine and the Pyrenees mountains respectively. The timing of speciation events in the *candidulus* species-group coincides with 2 major climatic transitions during the Early and Middle Pleistocene which resulted in significant environmental changes in Europe and suggest a scenario of allopatric isolation and divergence on distinct mountain ranges. The split between the thermophilic species *L. candidulus* and the ancestor of the temperate species *L. laureolae* and *L. leonardii* is estimated at ~3 Ma during the transition from Pliocene to Pleistocene and was probably triggered by their segregation in xerophilous and temperate habitats. The speciation between *L. laureolae* and *L. leonardii*, estimated at ~1 Ma during the Mid-Pleistocene Transition, can be explained by the establishment of unfavorable conditions in West Alps and Central Massif underlying the onset of the Apennine–Pyrenees disjunct pattern. Finally, the strict association between members of the *candidulus* group and distinct Thymelaeaceae plants suggests further studies to address the hypothesis that speciation in these flea beetles might have been also associated with Pleistocene range changes of their host plants.

**Key words:** Alticini, biogeography, Mid-Pleistocene transition, mountain systems, temperate species, West Palearctic region.

Quaternary climatic changes determined large-scale range shift in many species (Avisé 2000; Hewitt 2000, 2003). These species distribution shifts have been strongly influenced by mountain areas that had served either as dispersal barriers or environmentally suitable areas, depending on their geographical location, spatial orientation, local biotic context, and species’ ecological requirements (Schmitt 2009; Schmitt et al. 2016; Rahbek et al. 2019). The role of major European mountain ranges, such as the Alps, Pyrenees, and Balkans, in structuring the distribution and genetic diversity of fauna and flora is well known (Hewitt 1999, 2004; Schmitt 2009, 2017).

Many phylogeographic studies have highlighted how mountain systems have acted as important dispersion barriers for temperate species for postglacial expansions both northward, from the 3 Mediterranean peninsulas (Iberian, Italian, and Balkan Peninsulas) and southward, from extra-Mediterranean refuges (Taberlet et al. 1998; Hewitt 2001, 2004, 2011; Stewart et al. 2010; Schmitt and Varga 2012). However, several studies have demonstrated a wide plethora of species-specific responses of temperate species to

Pleistocene climate oscillations explained by differences in ecological requirements, dispersal capacity, and their interplay with a complexity of additional factors operating at regional scale and shaping population demography and species ranges (Stewart and Lister 2001; Deffontaine et al. 2005; Pauls et al. 2006; Bisconti et al. 2011; Schmitt and Varga 2012; Maura et al. 2014; Salvi et al. 2014, 2016; Senczuk et al. 2019). During glacial periods, many temperate species lived at lower altitudes in periglacial areas, and moved at higher altitudes during dry interglacial periods (Pauls et al. 2006; Zeisset and Beebe 2008; Maura et al. 2014; Morales-Barbero et al. 2018). Therefore, during interglacial periods mountain systems have provided a sort of island archipelago refugium for these species rather than a barrier to dispersal (Mardulyn et al. 2009; Schmitt 2009; Martinet et al. 2018). Such range fragmentation determined gene flow disruption among populations isolated on distinct mountains, causing allopatric divergence and possibly speciation (Schmitt et al. 2016; Schmitt 2017). Traces of such processes are today visible as shared biogeographic patterns for mountain species

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with allopatric distributions of genetic diversity across major mountain ranges of Pyrenees, Alps, Apennines, Balkans, and Caucasus (Pauls et al. 2006; Varga and Schmitt 2008; Valtueña et al. 2012; Štundlová et al. 2019; Lucati et al. 2020; Ehl et al. 2021; Sistri et al. 2022).

Among these biogeographic patterns, the Apennine–Pyrenees disjunct is very uncommon in literature. These 2 mountain systems have very different geological histories. The Pyrenees evolved since the early Mesozoic by the collision of the Iberian and Eurasian plates, and they extend nearly 500 kilometers from the Bay of Biscay to the Gulf of Lion on the Mediterranean coast (Vergés et al. 2002). Instead, the Apennines, extending north to south along the Italian Peninsula for 1,200 kilometers, evolved over several tectonic phases, most of which occurred during the Cenozoic Era, and culminated in the Miocene and Pliocene epochs (about 23–2.6 million years ago) by the collision of the African and Eurasian plates and the rotation of the Corsican-Sardinian block (Corrado et al. 1997; Bosellini 2005). Nowadays, Pyrenees and Apennines are about 500 kilometers apart, with the Massif Central and the western Alps providing some environmental connectivity between them. Indeed, many plant and animal species associated with medium-high mountain environments have a range which extends across these 4 major mountain ranges, demonstrating the historical biogeographic connection between western European mountains (Dixon et al. 2009; Todisco et al. 2010; Dillenberger and Kadereit 2013; Sanz et al. 2014; Feuda et al. 2015; Schmitt et al. 2016; Zlatkov and Huemer 2017; Hinojosa et al. 2019). However, cases of species or closely related taxa with a disjunct Apennine–Pyrenean distribution are uncommon, and are found only in some plants, such as *Adenostyles alpina* (Dillenberger and Kadereit, 2013), *Androsace vitaliana* (Dixon et al. 2009), or *Saponaria bellidifolia* (Csörgö et al. 2009), and in a few animals such as the ground beetles *Nebria orsinii* (Ruffo 1971) or the iconic case of the Pyrenean chamois *Rupicapra pyrenaica* (Salari et al. 2014; Masseti and Salari 2017; Fioravanti et al. 2019).

To investigate the Apennine–Pyrenees biogeographic pattern, the flea beetles *Longitarsus laureolae* Biondi and *L. leonardii* Doguet (Coleoptera, Chrysomelidae) offer a suitable study system. These 2 species, together with *Longitarsus candidulus* (Foudras), constitute a homogeneous group (species-group *sensu*, Bergeal and Doguet 1991) sharing similar external, aedeagial and spermathecal morphology (Figure 1), as well as being mainly associated with host plants of the family Thymelaeaceae (Biondi 1988). This species-group probably also includes *Longitarsus arnoldi* described from Algeria, for which (Bergeal and Doguet 1991), however, the host plant is not yet known. *Longitarsus laureolae* and *L. leonardii* occur in medium-mountain environments, the former in the central-southern Apennines, including the Sicilian range, and the latter along the Cantabrian–Pyrenean chain (Figure 2). Beside living in similar environments, these 2 species also share the same host plant, *Daphne laureola* (Biondi 1988, 1991). Differently, *L. candidulus* is a thermophilus element with a Holo-Mediterranean distribution. It occurs mainly on coastal and sub-coastal environments (only extends into inland continental areas in the Iberian Peninsula and in the Maghreb) and is associated with the host plants *Thymelaea hirsuta* and *Daphne gnidium* (Biondi 1988).

Here we investigated the evolutionary history of the *Longitarsus candidulus* species-group, with a focus on the

origin of the Apennine–Pyrenees biogeographic pattern of *L. laureolae* and *L. leonardii*. First, we assessed the monophyly of the *candidulus* species-group within a phylogenetic framework including other Western Palaearctic *Longitarsus* species. Second, we estimated the divergence time of cladogenetic events within the *candidulus* species-group using a multispecies coalescent model to determine whether speciation events might have been associated to isolation on distinct mountain ranges throughout the Pleistocene glacial cycles. The main aim of this study is to identify the biogeographic and evolutionary processes underlying the diversification within the *candidulus* species-group and the origin of the Apennine–Pyrenees pattern.

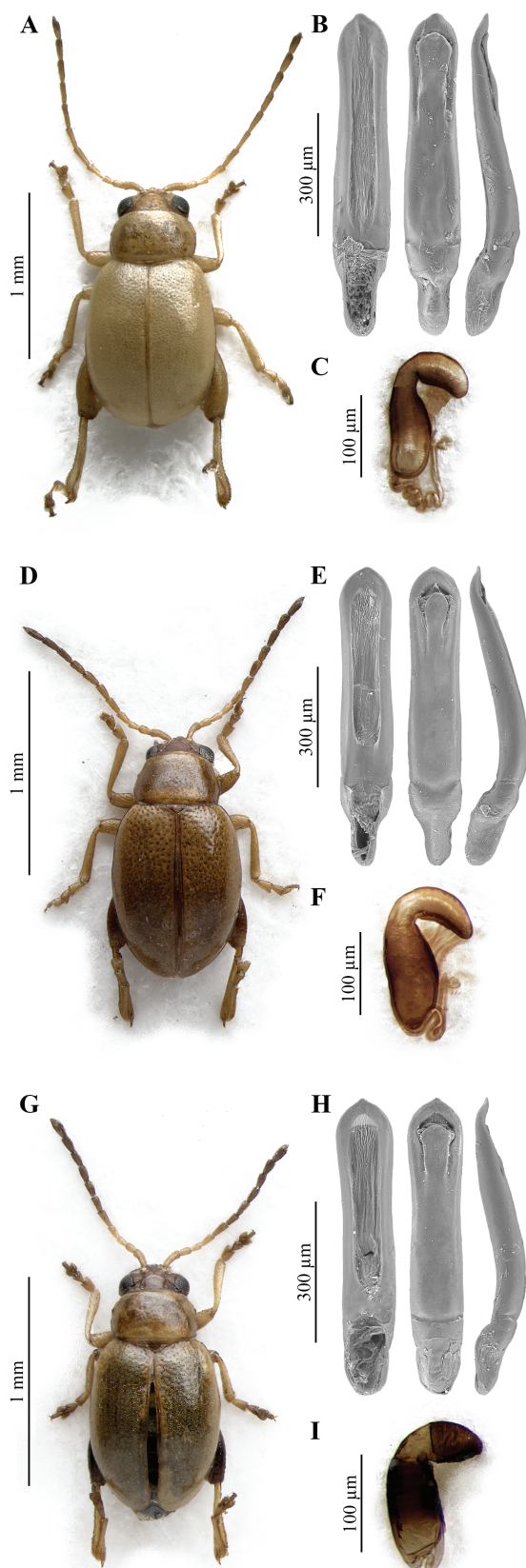
## Materials and Methods

### Sampling and morphological identification

For this study, 27 specimens of *L. candidulus*, *L. laureolae*, and *L. leonardii* were collected from 8 localities in central and southern Italy, Sicily, Sardinia, Spain, and Portugal (Table 1). Specimens were collected on their respective host plants by sweep net and the aid of aspirator and then stored in 95% ethanol. Morphological identification at the species level was performed by Maurizio Biondi through the dissection and study of the genitalia, median lobe of the aedeagus for males and spermatheca for females. Dissection of the specimens were carried out using a Leica M205C binocular microscope. Photographs of the habitus and spermatheca were taken employing a Leica DMC5400 camera and composed using the Zerene Stacker software, version 1.04. Scanning electron micrographs of the median lobe of the aedeagus were taken using a Hitachi TM-1000.

### DNA extraction, amplification, and sequencing

Total genomic DNA was extracted using a standard high-salt protocol (Sanbrook et al. 1989) with 2 different methods to allow subsequent morphological analysis: (i) using the 3 left legs of each specimen, and (ii) using the non-invasive method proposed in Salvi et al. (2020). This method involves the separation of the head–prothorax portion of the animal from the rest of the body with the use of an entomological pin and the immersion of the 2 parts directly in lysis buffer and proteinase K, allowing the subsequent reassembly of the animal on an entomological card point. We amplified the standard barcode region of the mitochondrial *cytochrome c oxidase I* gene (*cox1*; primer pairs: LCO1490Lon-F/HCO2198Lon-R; Salvi et al. 2019). For selected individuals of the 3 species, we amplified 1 additional mitochondrial gene fragment, the 16S ribosomal DNA (16S rDNA; primer pairs: 16Sdir-Lon CACCTGTTTAAWTA AAAACAT/16Srev; this study and Palumbi et al. 1991), and 3 protein-coding single copy nuclear genes: Carbamoylphosphate synthase (CAD; primer pairs: CAD1bFw\_mod/CD-668Rlon; Berrilli et al. 2023), Crossveinless 2 (*Cv2*; primer pairs: Cv2-F/Cv2-R; Gikonyo et al. 2024), and Wingless (*Wg*; primer pairs: Wg550F/WgAbRZ-R; Wild and Maddison 2008). Successful amplification was determined by gel electrophoresis and PCR products were purified and sequenced by an external service (Genewitz, UK). The obtained chromatograms of each sequence were manually edited and assembled into a consensus sequence using Geneious Prime 2021 (Biomatters Ltd., Auckland, New Zealand). Heterozygous positions for the nuclear coding gene fragments were identified based on the



**Figure 1.** Habitus (A, D, G), median lobe of the aedeagus (B, E, H) in ventral, dorsal, and lateral view (from left to right), and spermatheca (C, F, I), of members of the *Longitarsus candidulus* species-group: *L. candidulus* (A–C), *L. laureolae* (D–F), and *L. leonardii* (G–I).

presence of 2 peaks at a single site in the chromatograms and were coded in the alignment using IUPAC ambiguity codes.

Consensus sequences were deposited in GenBank (GenBank accession: OR373124-OR373138; OR378514-OR378524; OR397789- OR397817).

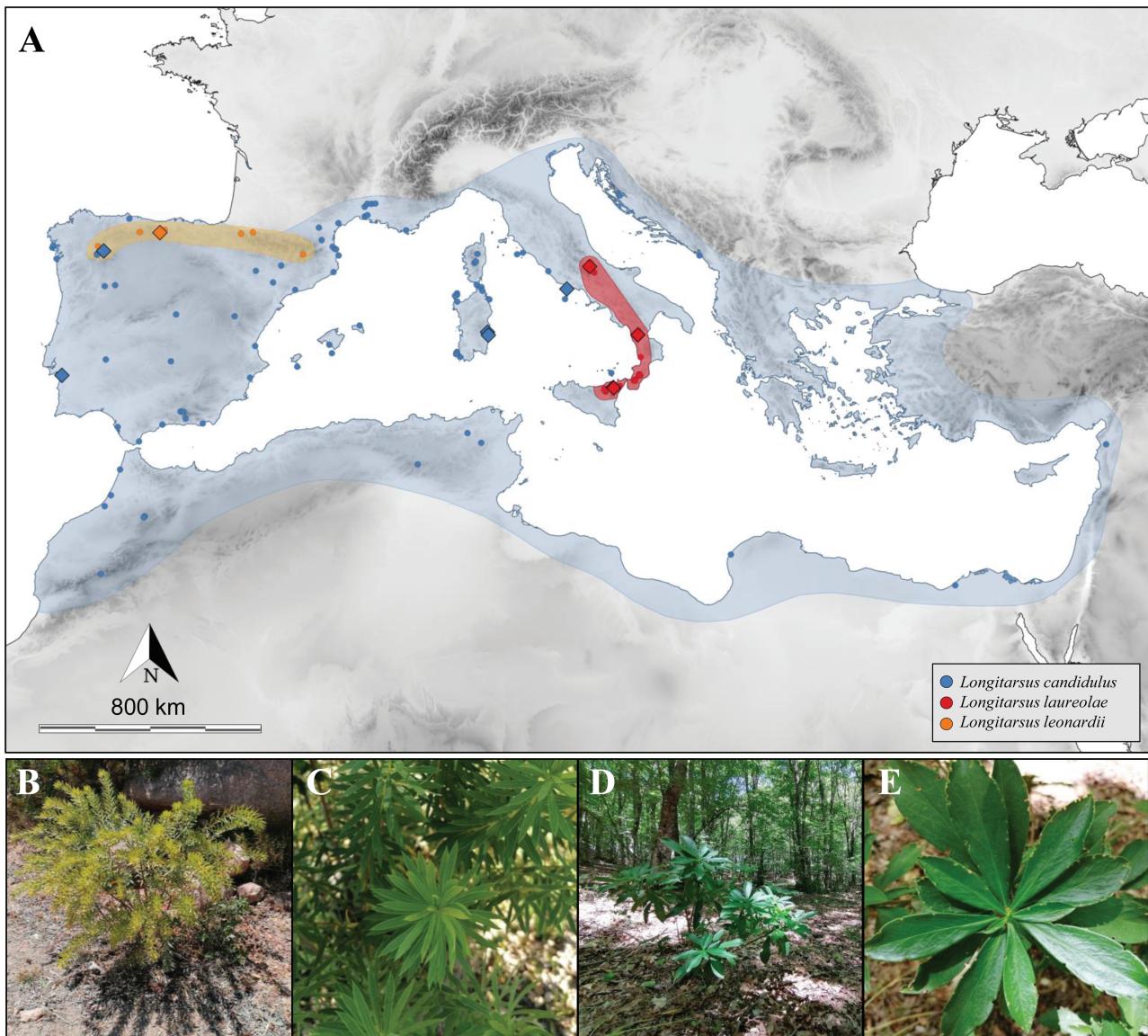
### Phylogenetic analyses

Prior to phylogenetic analyses we performed species delimitation analysis to infer putative species boundaries within the *candidulus* species-group. We implemented the multirate Poisson Tree Processes model (mPTP; Kapli et al. 2017) on a Maximum likelihood (ML) tree based on *cox1* sequence data of *L. candidulus*, *L. laureolae*, and *L. leonardii* (*cox1* dataset; see below). Analyses were performed using mPTP v. 0.2.4 with 10 runs of 100 million MCMC generations each, sampling every 10,000 (burn-in = 10%). The convergence of the independent runs was assessed through the average standard deviation of delimitation support values (ASDDSV) and the overall support for the ML estimate calculated computing the mean of the average support values (ASV) over the 10 runs.

Phylogenetic analyses were performed in 2 steps. To determine whether the *candidulus* species-group formed a monophyletic clade, consistently with morphological characters, we built a dataset (mitochondrial dataset) including sequences of the 2 mitochondrial markers *cox1* and 16S of 52 *Longitarsus* species and the outgroup *Batophila aurata* (Marsham) obtained from Salvi et al. (2019), which currently provides the most comprehensive datasets for Western Palaearctic *Longitarsus* species. Once the species-group's monophyly within the genus *Longitarsus* was established, we used the sequences of both mitochondrial and nuclear markers (mito-nuclear dataset) to assess the phylogenetic relationship within the *candidulus* species-group, using *Longitarsus pellucidus* (Foudras) as an outgroup based on the results of the first analysis.

Multiple sequences alignment was performed separately for each gene with MAFFT v7.450 using the G-INS-I progressive method algorithm (Katoh et al. 2002). The *cox1* alignment (*cox1* dataset) and concatenated sequence alignments (mitochondrial dataset and mito-nuclear dataset) were used to infer ML trees in IQ-TREE 1.6.12 (Nguyen et al. 2015) using the W-IQ-TREE webserver (Trifinopoulos et al. 2016). The best substitution models of each gene partition were determined by the ModelFinder module, including flexible rate heterogeneity across site models (Kalyaanamoorthy et al. 2017), based on the Bayesian Information Criterion. We used the Edge Linked partition model to allow each partition to have its own evolutionary rate. Branch support was assessed by 1,000 replicates of ultrafast bootstrapping (uBS) (Minh et al. 2013; Hoang et al. 2018) and SH-like approximate likelihood ratio test (SH-aLRT) (Guindon et al. 2010). FigTree v1.3.1 (Rambaut and Drummond, 2009) was used to depict the trees.

The close phylogenetic relationship between *L. laureolae* and *L. leonardii* recovered in ML analyses was further investigated using a phylogenetic network approach based on sequence data of the 5 markers (Supplementary Table S1). Haplotype phase of nuclear genes was determined using the algorithm PHASE (Stephens et al. 2001; Stephens and Donnelly, 2003) as implemented in DnaSP v5 (Librado and Rozas, 2009) with the initial 1,000 iterations discarded as burn-in, 1 as thinning interval and 1,000 post-burnin iterations. Phylogenetic relationships among haplotypes were inferred through the median-joining distance method (Bandelt et al. 1999) using PopArt 1.7 (Leigh and Bryant 2015).



**Figure 2.** Map with geographical range of *L. candidulus* (light-blue), *L. laureolae* (red) and *L. leonardii* (orange) along with historical records for the 3 species (small circles) and sampling localities (squares) (A). Host plants of *L. candidulus*: *Thymelaea hirsuta* from Sardinia (B) and *Daphne gnidium* from Portugal (C). Host plant of *L. laureolae* and *L. leonardii*: *Daphne laureola* from Sicily (D) with the detail of foliar damage on the leaf margin caused by feeding of *L. laureolae* (E).

To estimate the species tree and divergence times of the *candidulus* species-group we used the multispecies coalescent method implemented in the StarBeast2 packages of BEAST2 v.2.7.1 (Ogilvie et al. 2017; Bouckaert et al. 2019). For this analysis, we used alignments of the 2 mitochondrial genes and phased alignments of the 3 nuclear genes. We unlinked substitution models and clock models of gene partitions, with the exception of the trees of the mitochondrial genes *cox1* and 16S. We used a relaxed clock model (uncorrelated lognormal clock) calibrated using the available 16S substitution rate (clock.rate = 0.0054, SD = 0.0009) estimated for beetles by Papadopoulou et al. (2010). The remaining settings were as follows: (unlinked) models of nucleotide substitution for each gene partition, we set HKY as substitution model and frequencies to empirical for all the gene partitions; we unchecked the estimate box of substitution rate and checked the estimate box of clock rate of each partition, except for the 16S; we set a constant population model and a Yule process as species

tree prior. StarBeast2 was run 2 times, with 300 million generations, sampling every 30,000 generations. We used Tracer v.1.7.1 (Rambaut et al. 2018) to check the runs for convergence (burn-in = 25%), LogCombiner and TreeAnnotator to combine runs and summarize the trees in a Maximum Clade Credibility Tree representing the posterior distribution. We used DensiTree and FigTree to visualize the results (Rambaut and Drummond, 2009; Bouckaert 2010).

## Results

### Phylogenetic relationships and molecular dating

Species delimitation analyses based on mPTP retrieved convergence between runs (ASDDSV < 0.01) and support the 3 species of the *candidulus* species-group as distinct species clusters (ASV median: 80%).

ML analyses based on *cox1* and 16S resolved the phylogenetic position of *L. candidulus*, *L. laureolae*, and

**Table 1.** Details on sampling localities for each studied species (see also Figure 2)

| Species              | Locality  | Number of specimens | Coordinates  | Elevation (m) |
|----------------------|---|---------------------|--------------|---------------|
| <i>L. candidulus</i> | Italy, Lazio, Sabaudia (LT)                               | 3                   | 41.25, 13.04 | 8             |
|                      | Italy, Sardegna, Gairo (NU)                               | 2                   | 39.74, 9.67  | 67            |
|                      | Italy, Sardegna, Bari sardo (NU)                          | 2                   | 39.84, 9.63  | 255           |
|                      | Portugal, Monte Novo do Sul, Alcácer do Sal               | 4                   | 38.40, -8.67 | 6             |
|                      | Spain, Parque Natural Serra da Enciña da Lastra, Ourense* | 8                   | 42.47, -6.87 | 1,095         |
| <i>L. laureolae</i>  | Italy, Sicily, Malabotta (ME)                             | 5                   | 37.97, 15.05 | 1,230         |
|                      | Italy, Calabria, Lungro (CS)                              | 2                   | 39.75, 16.09 | 1,241         |
|                      | Italy, Abruzzo, Bosco di Sant'Antonio (AQ)                | 2                   | 41.97, 14.02 | 1,164         |
| <i>L. leonardii</i>  | Spain, Piedrasluengas, Palencia                           | 2                   | 43.04, -4.45 | 1,340         |

\*Sequence data from Baselga et al. (2015).

*L. leonardii* in a monophyletic group within the genus *Longitarsus* (SH-aLRT = 89, uBS = 85; Figure 3). The phylogenetic tree of the *candidulus* species-group based on mitochondrial and nuclear marker shows a sister relationship of *L. candidulus* to the clade composed of *L. laureolae* and *L. leonardii* (SH-aLRT = 100, uBS = 100; Figure 3). The 3 species were recovered as reciprocally monophyletic (SH-aLRT = 100, uBS = 100; Figure 3).

The median-joining network based on 30 sequences of *cox1* data shows 3 distinct haplogroups corresponding to the species *L. candidulus*, *L. laureolae*, and *L. leonardii*, with a closer relationship between the latter (Figure 4). A similar pattern is observed in the 16S network based on 9 sequences, although with a lower number of mutational steps. The 3 species are reciprocally monophyletic also in the network based on the 12 sequences of nuclear marker *CAD*, whereas a lack of sorting between *L. laureolae* and *L. leonardii* is apparent in the *Cv2* and *Wg* networks, based on 16 and 12 sequences respectively (Figure 4).

Also in the coalescent species tree, relationships within the *candidulus* species-group are well resolved (Figure 5), with a high level of nodal support and a topology consistent with the ML analysis. *L. candidulus* is supported as a sister to the clade composed by *L. laureolae* and *L. leonardii* (Bayesian posterior probabilities, BPP = 0.97). The split between *L. candidulus* and the *L. laureolae/L. leonardii* clade is estimated to have occurred during the Late Pliocene (3.08 Mya, million years ago; 95% high posterior density interval, HPD95: 3.98–2.20 Mya). The phylogenetic relationship between *L. laureolae* and *L. leonardii* received the maximum support (BPP = 1), with the separation between these two species estimated to have occurred in the late Middle Pleistocene (0.86 Mya; HPD95: 1.29–0.46 Mya).

## Discussion

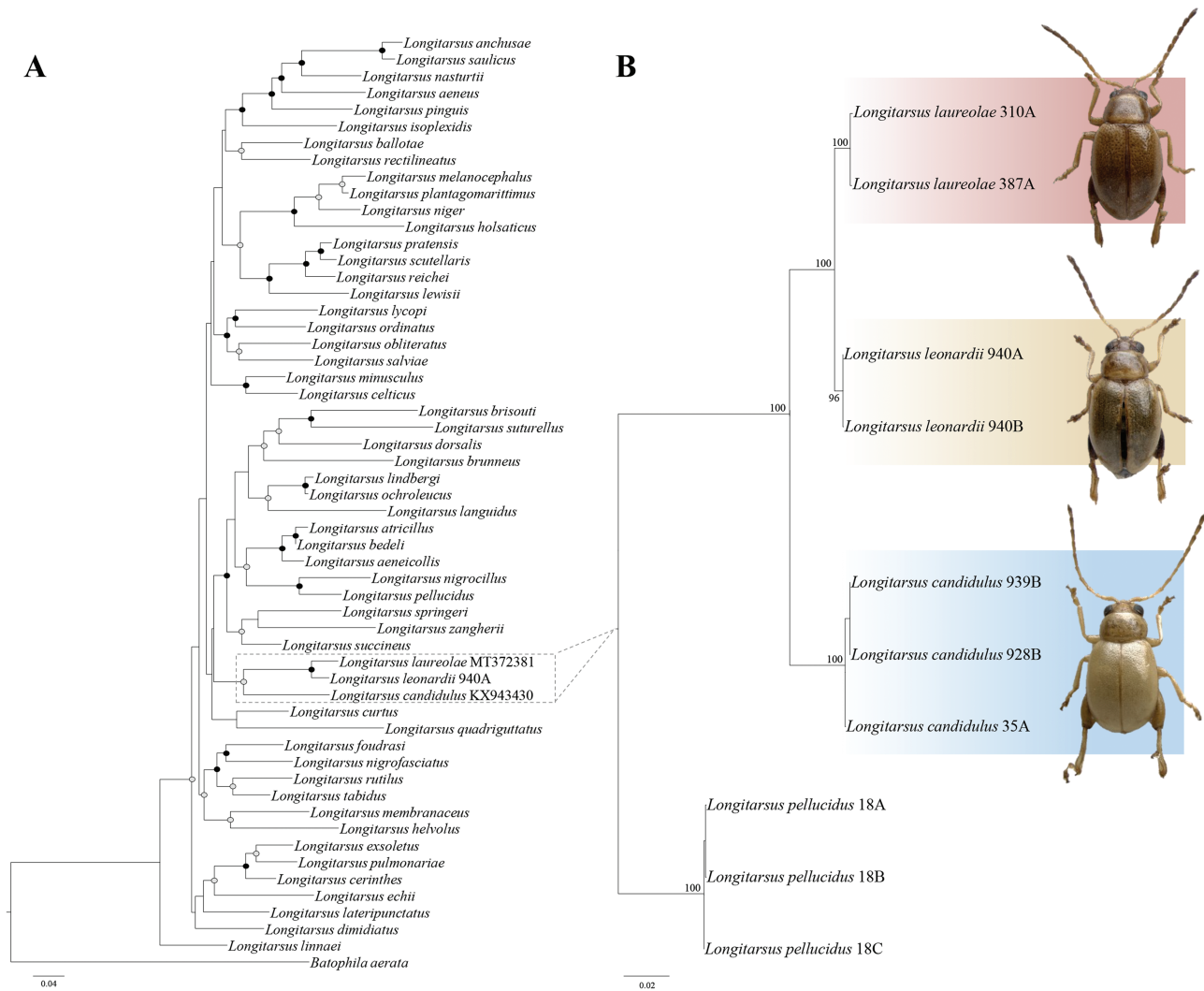
The phylogenetic analysis of *candidulus* species-group allowed clarifying the systematics and the evolutionary history of these species and provided insights into the main palaeoclimatic events that have triggered the diversification of this group in the Mediterranean region and the origin of the uncommon Apennine–Pyrenees biogeographic pattern.

Phylogenetic analyses recovered the *candidulus* species-group as a distinct lineage within the genus *Longitarsus* with a closer relationship between *L. laureolae* and *L. leonardii* endemic to Apennines and Pyrenees, respectively (Figure

3). This result is consistent with the shared morphological features among species in this group, especially between the Apennine–Pyrenees species (Biondi 1988, 1991), and with their association with host plants of the family Thymelaeaceae (Biondi 1988). Such association is unique among *Longitarsus* flea beetles and further corroborates the finding that host-use patterns are phylogenetically constrained in *Longitarsus*, with a clear association between closely related species and specific plant families (Salvi et al. 2019).

The timing of speciation events in the *candidulus* species-group seems associated with major Plio-Pleistocene climatic events which resulted in significant environmental changes in Europe. The split between *L. candidulus* and the ancestor of *L. laureolae* and *L. leonardii* is estimated around 3 million years ago (HPD95: 3.98–2.20 Mya), during the transition from the Pliocene to the Pleistocene (Piacenzian age). During this period, a change from warmer and humid to colder and dry climates took place in Europe, culminating in the establishment of the Quaternary glacial–interglacial climatic cycles (Bertini and Combourieu-Nebout 2023). This climatic transition resulted in a significant change in the distribution of ecosystems across Europe, with the partial disappearance of the subtropical vegetation present during the Zanclean age (5.3–3.6 Myr), the expansion of the temperate “mixed mesophytic” forest (with *Picea* and *Fagus*) and the concomitant southward shift of more thermophilic species (Bertoldi et al. 1989; Bertini 2010; Birks and Tinney 2016). These major climatic and environmental changes have resulted in the emergence of new niches, ecological barriers, and increased habitat complexity, providing occasion for dispersal and isolation processes which ultimately lead to speciation (Hewitt 2000; Schluter 2009). According to this scenario, the divergence between the thermophilic species *L. candidulus* and the ancestor of the temperate species *L. laureolae* and *L. leonardii* would have been triggered by habitat segregation, with the former associated to xerophilous vegetation in peri-Mediterranean coastal and southern areas, and the latter tracking temperate environments. A similar case of ecological speciation associated with the environmental changes taking place during the Pliocene-Pleistocene transition was suggested for the Mediterranean endemic land snail *Murella muralis* (Fiorentino et al. 2013).

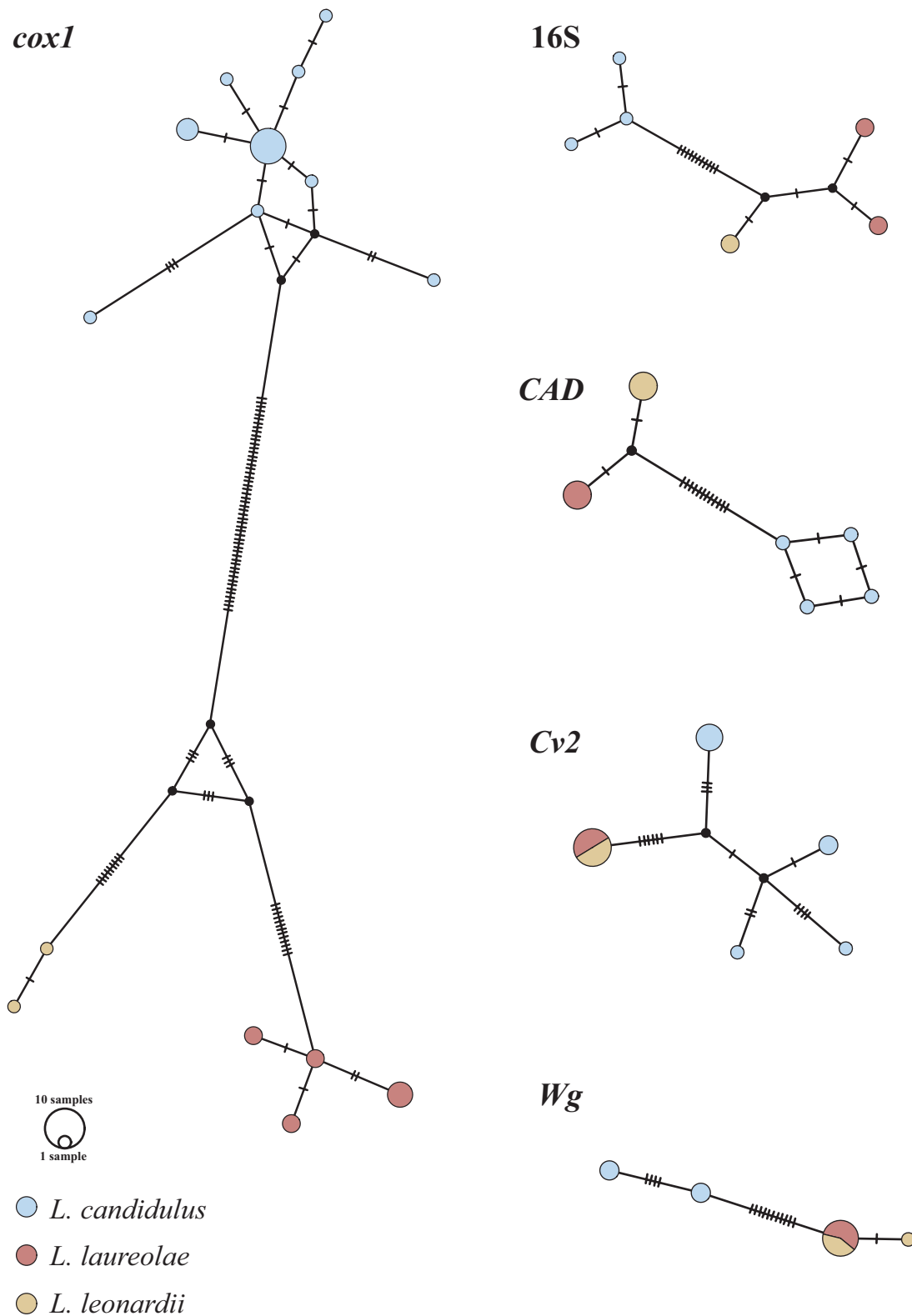
The diversification between *L. laureolae* and *L. leonardii* is estimated at 0.86 Mya (HPD95: 1.29–0.46 Mya), during the Mid-Pleistocene Transition (1.25–0.7 Mya). The recent speciation between these species is further corroborated by their



**Figure 3.** Maximum likelihood phylogenetic tree of Western Palaearctic *Longitarsus* species based on the *cox1* and 16S gene fragments (A). Circles in correspondence of nodes represent SH-aLRT support (SH-aLRT, left half) and ultrafast bootstrapping support (uBS, right half): black for uBS > 90 and SH-aLRT > 90; gray for uBS of 70–90 and SH-aLRT of 70–90. Half white circles or no circles represent nodes with <70 uBS and <70 SH-aLRT values. The *candidulus* species-group is highlighted by a dashed rectangle. Maximum likelihood phylogenetic tree of the *candidulus* species-group based on 2 mitochondrial (*cox1* and 16S) and 3 nuclear (*CAD*, *Cv2*, and *Wg*) gene fragments (B). The SH-aLRT support and uBS support is reported on the nodes. For each sequence the voucher code is reported.

reciprocally monophyly observed in fast-evolving genes (i.e., genes with short coalescent time) as opposed to the lack of sorting observed at slow-evolving loci (Figure 4). During this period, the intensification of glacial cycles from a 41,000-year periodicity to a strongly asymmetric 100,000-year cycle, with long-duration cooling of the climate and build-up of thick ice sheets, caused significant environmental changes (Head et al. 2008). In Alpine regions, the enhanced glacier development, with an average depression of the snowline to 1200 m, disrupted the continuity of temperate forest cover and of associated biotas (Nagl 1972; Blondel et al. 2010; Muttoni et al. 2010; Thompson 2020). These factors would explain how the establishment of unfavorable conditions for the persistence of temperate flea beetles in western Alps and the Massif Central, have led to the isolation of distinct lineages in the Pyrenees and Apennines, respectively. This scenario is consistent with that proposed for *Rupicapra pyrenaica*, whose Pyrenean and

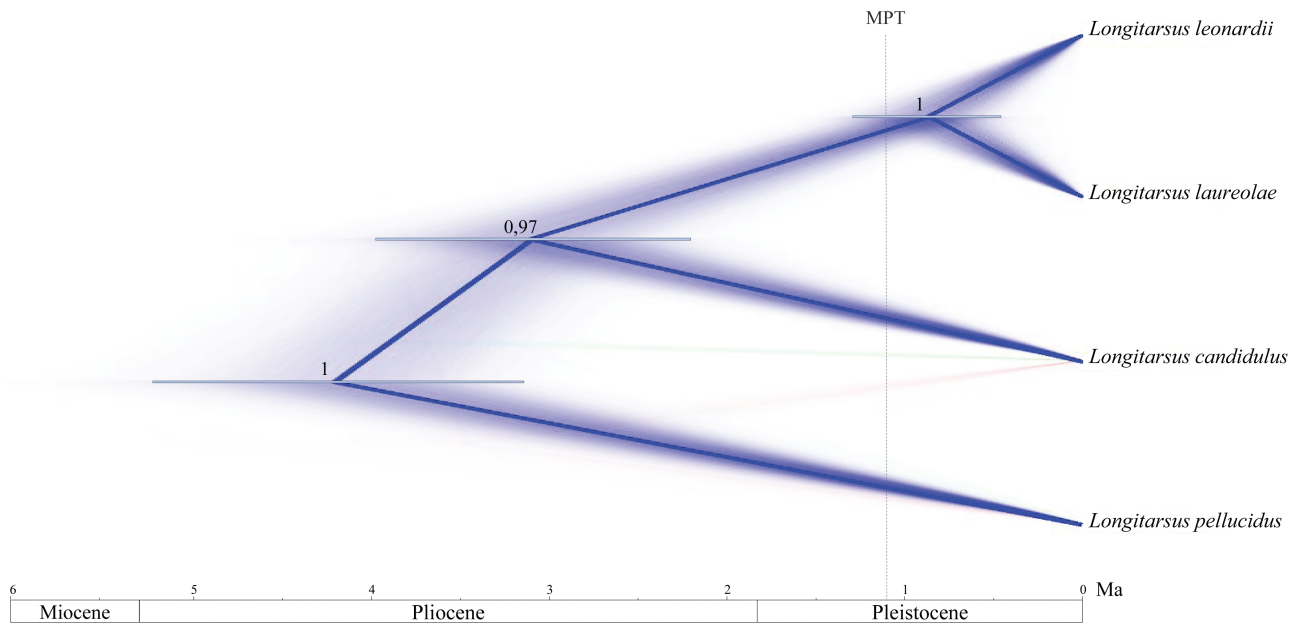
Apennine populations were probably separated during the latest middle Pleistocene due to environmental changes and competition with the northern chamois *Rupicapra rupicapra* (Masetti and Salari 2017; Fioravanti et al. 2019). Indeed, for several other species, the current disjunct distribution on different mountain ranges has been explained as the result of allopatric divergence associated with the environmental changes occurring during the Middle-Pleistocene transition, such as in the case of butterflies of the genera *Zerynthia* (Dapporto 2010) and *Erebia* (Hinojosa et al. 2019) or in the case of the leaf beetles of the *Cryptocephalus flavipes* species complex (Montagna et al. 2016) and the genera *Oreina* (Borer et al. 2010). This pattern of allopatric divergence can also be found in species related to mountain deciduous forests, such as in the case of Fire-colored beetles of the genus *Pyrochroa* (Molfini, Mancini, and Bologna 2023). On the other hand, the origin of the Apennine–Pyrenees



**Figure 4.** Haplotype networks showing the phylogenetic relationships within the *candidulus* species-group based on two mitochondrial (*cox1* and 16S) and 3 nuclear (*CAD*, *Cv2* and *Wg*) markers. Haplotypes are represented by circles colored according to the 3 species and with a size proportional to their frequency (see the size reference inset in the bottom left corner). Vertical bars represent mutational steps (nucleotide substitutions).

disjunct pattern observed in the perennial herbaceous plant *Adenostyles alpina* has been explained by a long-distance dispersion event from southern Italy (subsp. *macrocephala*) to the Pyrenees (subsp. *pyrenaica*) (Dillenberger and Kadereit

2013; see also the case of *Androsace vitaliana* in Dixon et al. 2009). Long-distance dispersal is unlikely in the case of *L. laureolae* and *L. leonardii* because flea beetles have subapter wings and extremely low dispersal capacity. These findings



**Figure 5.** Time-calibrated species tree of the *candidulus* species-group estimated in BEAST showing the consensus tree topology (dark blue line) and trees from the posterior distribution visualized using the software DensiTree. Bayesian posterior probability values are reported on each node along with light blue bars representing the 95% high posterior density interval (HPD95) of node age. Time scale is in millions of years (Ma). The dashed line highlights the onset of the Mid-Pleistocene transition (MPT, ~ 1.25–0.7 Mya).

corroborate the idiosyncratic nature of species response to Pleistocene climatic oscillation and reinforce the notion that shared biogeographic patterns do not always equate shared evolutionary histories (Provan and Bennett 2008; Stewart et al. 2010; Canestrelli et al. 2012; Salvi et al. 2016).

Finally, the strict association between flea beetles in the *candidulus* species-group and distinct host plants raises the question of whether diversification processes in this group were associated with the historical availability of host plants. Although today the distribution of *Daphne laureola*, *D. gnidium*, and *Thymelaea hirsuta* is continuous across the West Palearctic, past fragmentation of their ranges might have triggered the allopatric divergence between flea beetles. According to this hypothesis speciation in flea beetles might have been associated to the historical range changes of their host plants throughout the Pleistocene climatic cycles. Further study using climatic suitability reconstructions for both *Longitarsus* flea beetles and Thymelaeaceae host plants applied to current and past bioclimatic envelopes would allow testing this hypothesis.

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## Supplementary Material

Supplementary material can be found at <https://academic.oup.com/cz>.

## Disclosures

The authors have no conflict of interests to declare.

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