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RESEARCH ARTICLE

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Abstract

Aim: Discerning the relative role of geographical and ecological factors in promoting diversification is central to our understanding of the origin and maintenance of biodiversity. We explore the roles of geology and ecological tolerance in the diversification of a group of wingless beetles with low dispersal potential.

Location: Western Mediterranean (Iberian Peninsula and North Africa).

Taxon: Darkling beetles (Tenebrionidae: *Misolampus*).

Methods: We sequenced nine gene fragments from the mitochondrial and nuclear genomes in all extant *Misolampus* species to reconstruct their phylogeny, evaluate species boundaries and potential contact zones and estimate divergence times. We modelled species distributions for different time periods to infer ecological preferences and assess the effects of climatic changes since the last interglacial. We used a time-stratified process-based biogeographical model to estimate ancestral areas of origin and the evolution of geographical ranges.

Results: The palaeoclimatic model projections show contractions of favourable areas during the last interglacial period and mid-Holocene, and wide stretches of suitable areas during the last glacial maximum. Analyses of ancestral bioclimatic preferences reveal ecological adaptations in isolated lineages within three species. The phylogeny of *Misolampus* is strongly supported and unveils deep divergences within the six species. Two well-supported clades were recovered, one distributed in North Africa-Balearic Islands and another in the Iberian Peninsula. The divergence between the North African and Iberian clades occurred during the early Miocene. Biogeographical

analyses infer an ancestral range including the Iberian, Betic and Rifean Plates, with subsequent splits followed by dispersal events.

Main conclusions: Our results favour a dual role of vicariance and dispersal in driving the historical biogeography and diversification of *Misolampus* since the early Miocene. We also found evidence for incipient speciation events, underscoring the role of tectonic events and adaptation to local climatic conditions in the diversification of the group.

KEYWORDS

ecological niche modelling, historical biogeography, *Misolampus*, palaeogeography, phylogeny, speciation, Tenebrionidae, Western Mediterranean

1 | INTRODUCTION

The geographical ranges of species are dynamic, shaped by biotic (e.g. life history, demography, species interactions) and abiotic (e.g. tectonic events, climatic variables) factors that vary through space and time. This variation ultimately dictates differential patterns of population viability, connectivity and growth through time in different sections of species ranges, with important consequences for species diversification processes (Bellard et al., 2012; Gouveia et al., 2014; Thuiller et al., 2005; Wiens, 2011). Following climatic changes, species can respond by contracting their ranges into climatic refugia and/or dispersing to areas where their environmental preferences are maintained (niche tracking), by adapting to new environmental conditions in situ (niche evolution), and often by undergoing extinction in environmentally unsuitable areas (Kozak & Wiens, 2006; Yesson & Culham, 2006a). The fragmentation of ancestral distributions because of tectonic or climatic changes promotes allopatric speciation, sometimes associated with niche evolution, whereas demographic expansion of populations tracking climatic changes can lead to range fusion and admixture between formerly isolated population groups. Discerning the relative role of biotic and abiotic factors in promoting diversification is central to our understanding of the origin and maintenance of biodiversity (Wiens & Graham, 2005).

The ability of different taxa to track climatic conditions or evolve new adaptations depends on life-history traits like dispersal. Dispersal limitation can result in geographical isolation and ecological specialization, by promoting species persistence in small areas, which could lead to some resilience to changing climatic conditions and might accelerate the consolidation of a species' ecological niche even at the population level (Baselga et al., 2011). Usually, local adaptation to extrinsic ecological factors is initiated by the presence of a barrier to gene flow. Additionally, dispersal limitation may lead to ecological specialization and reinforce restrictions to gene flow, promoting speciation even in the face of future demographic processes such as population contractions, fragmentations or expansions (Wiens, 2004).

Addressing the question of how much lineage diversification is affected by range expansions/contractions through historical biogeography is often tackled by reconstructing ancestral range shifts using phylogenetic analyses (Ree & Smith, 2008; Ronquist & Sanmartín, 2011). However, the question often remains partially answered because of the lack of information on the timing of range shifts regarding the speciation process itself (Recuero et al., 2012; Recuero & García-París, 2011). Robust, accurate estimates of divergence times between species and intraspecific lineages are key to consider evolutionary processes at different temporal scales that lead to diversification, which is controlled by multiple extrinsic ecological factors and intrinsic organismal traits (Wiens, 2004). Therefore, assessing the temporal scale of demographic processes such as range contractions, fragmentations and expansions and their impact on lineage divergence provides decisive evidence to reconstruct the evolutionary history of species.

Within a temperate region such as the Western Mediterranean, the influence of demographic processes under climatic oscillations has been well studied, providing evidence for two major evolutionary patterns. On the one hand, allopatric fragmentation, or demographic contraction into refugial areas during glacial periods have allowed species to persist as isolated populations over long periods of time (Gutiérrez-Rodríguez et al., 2017; Hewitt, 2004, 2011; Martínez-Freiría et al., 2020; Martínez-Solano et al., 2006; Recuero & García-París, 2011). On the other hand, during interglacial periods many species have expanded their ranges through newly available colonization routes, sometimes forming secondary contact areas with related species or lineages (Branco et al., 2000; Gómez & Lunt, 2007; Gonçalves et al., 2009; Miraldo et al., 2011). These two scenarios involve periods without effective contact or gene flow between isolated populations, which has consequences on the process of lineage divergence. For instance, intraspecific divergence could be either indicative of allopatric cladogenetic events (cladogenesis), or ecological specialization (niche differentiation) (Hewitt, 2011).

The Western Mediterranean hunchback darkling beetles *Misolampus* (Tenebrionidae: Stenochiinae: Misolampinii) are well suited for studies on the contribution of geology-driven processes and ecological divergence to diversification in species groups with limited dispersal capacity. These flightless beetles are ecologically linked to woodlands and live mainly beneath the bark or inside dead logs of *Pinus*, *Quercus* and other tree species along a broad altitudinal

range (from sea level to >2000 m a.s.l.). The genus includes five species endemic to the Iberian Peninsula (*M. gibbulus*, *M. lusitanicus*, *M. ramburii*, *M. scabricollis* and *M. subglaber*) with a patchy distribution and few cases of sympatry, and one species distributed throughout Morocco, Algeria and the Balearic Islands (*M. goudotii*). Based on this distribution, we use molecular and climatic data to propose and test three hypothetical biogeographical scenarios that could explain current patterns of distribution and diversity in the genus.

On the one hand, *Misolampus* could have an ancient origin (Paleogene) and given sufficient evolutionary time, geological events in the Western Mediterranean Basin (Payros et al., 2016) could have led to geology-based vicariance of ancestral populations (Figure 1a). Subsequently, hybridization of these relict populations/new species in secondary contact areas would be prevented by the evolution of pre- and post-zygotic isolation mechanisms (including ecological barriers). This scenario could be expected given the poor dispersal ability of these animals and their geographical distribution, which suggests a substantial role of allopatry as

a main driver of diversification. Previous studies have suggested that the diversification of Iberian *Misolampus* could have entailed two vicariance events leading to speciation (Palmer, 1998; Palmer & Cambefort, 2000). On the other hand, *Misolampus* could be of recent origin (Pliocene), with less time to experience geological events, and diversification could be then largely attributed to dispersal events from their ancestral area to other areas (Figure 1b). This scenario might be facilitated by the broad climatic tolerance of *Misolampus*, which are also associated with tree species that are widespread throughout the Mediterranean Basin (Rosas-Ramos et al., 2020). Finally, while it has been previously proposed that the ancestor of *Misolampus* originated in North Africa and subsequently dispersed towards the Iberian Peninsula (Palmer, 1998; Palmer & Cambefort, 2000), an alternative scenario could invoke an intermediate origin (Miocene) and a dual role of vicariance and dispersal in driving the biogeographical pattern observed today (Figure 1c). In the Miocene, tectonic activity shaped the Western Mediterranean Basin, in particular the Iberian Peninsula, and
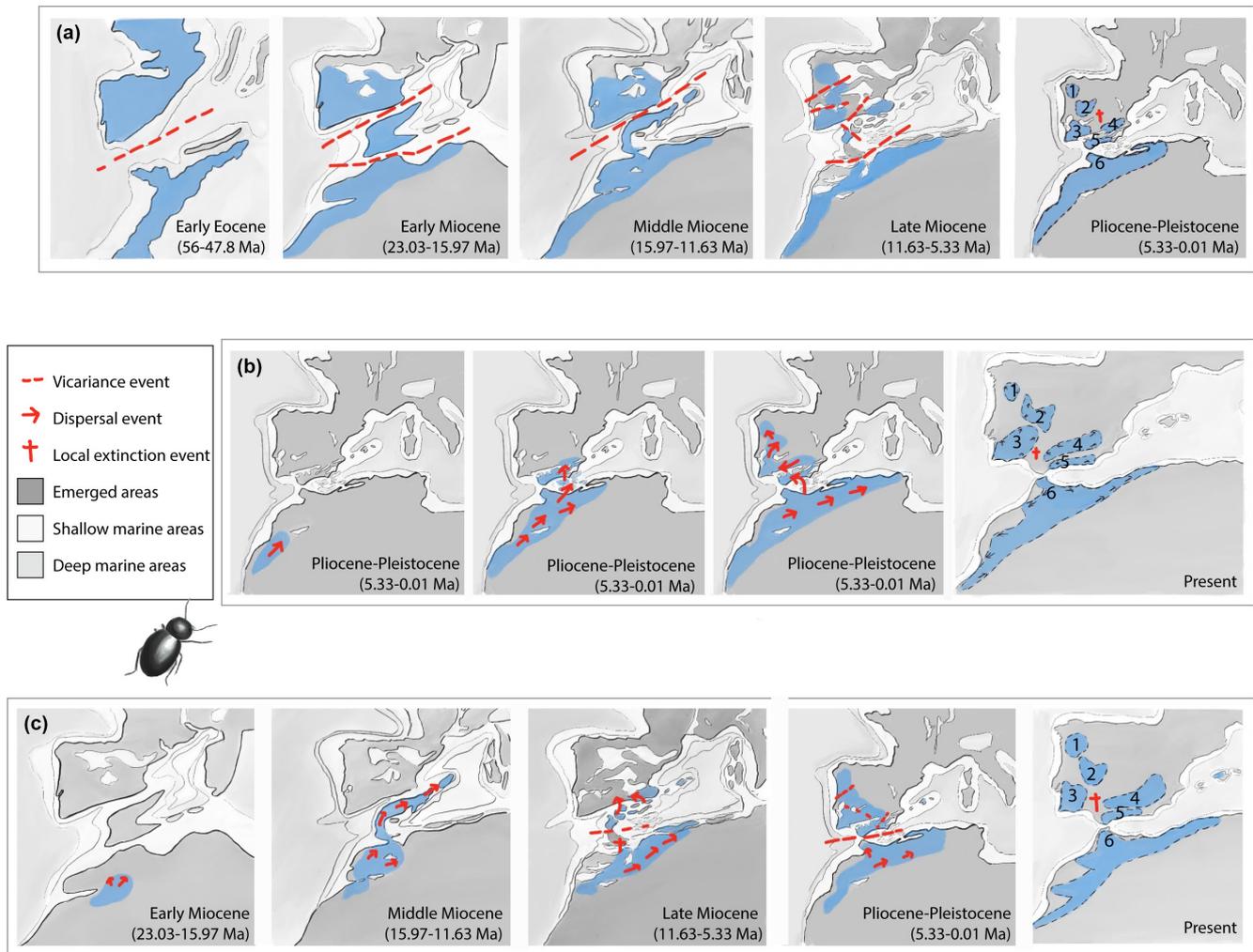
FIGURE 1 Three hypothetical biogeographical scenarios explaining the current diversity pattern in *Misolampus*. (a) Vicariant scenario with an ancient origin (Paleogene) and a widespread ancestral range splitting into smaller ones. (b) Dispersal scenario with a recent origin (Pliocene) in a smaller ancestral area that has fostered its current distribution through range expansion. (c) Intermediate origin (Miocene) in a smaller ancestral area with a dual role of vicariance and dispersal events in shaping current species ranges.

created temporary land bridges between Iberia and North Africa (Booth-Rea et al., 2018). Such a dynamic geological history could have split ancestral populations but also allowed range expansions through dispersal, eventually followed by range fragmentation. For instance, current patterns of sympatry between Iberian *Misolampus* have been hypothesized to result from recent contact between taxa expanding from their respective glacial refugia (Palmer & Cambefort, 2000; Rosas-Ramos et al., 2020). Glacial cycles in the Pleistocene may have also influenced the diversification of *Misolampus*, as documented for many taxa (Gómez & Lunt, 2007). Under favourable climatic conditions, barriers separating allopatric groups of populations could vanish, potentially leading to demographic expansions, and eventually leading to sympatry and hybridization.

Here, we used molecular sequences, species records and climatic data to reconstruct the phylogeny of the group, test biogeographic hypotheses and explore the roles of geology and ecological tolerance in the diversification of *Misolampus.*

2 | MATERIALS AND METHODS

2.1 | Taxon sampling and DNA sequencing

We sampled all species of *Misolampus* across their ranges, including 108 individuals from 60 populations in the Iberian Peninsula, the Balearic Islands and Morocco (Appendix S1, Table S1). We also included four species from the tribe Cnodalonini (Stenochiinae) and one species of *Nalassus* (subfamily Tenebrioninae) as outgroups.

Most specimens were captured by hand and preserved in absolute ethanol at −20°C. Genomic DNA was extracted from leg tissue using commercial kits (Qiagen DNeasy). Polymerase chain reaction (PCR) was used to amplify fragments of three mtDNA genes: *cytochrome b* (*cytb*, 705 bp); *cytochrome oxidase 1* (*cox1*, 805 bp); and *16S rRNA* (*16S*, 801 bp). We also amplified fragments of six nuclear genes: *wingless* (*Wg*, 456 bp), *histone 3* (*H3*, 327 bp), *ribosomal 18S* and *28S* (941 and 713 bp, respectively), and the internal transcribed spacers *ITS1* and *ITS2* (1265 and 574 bp) (Appendix S1, Table S2). Products were purified and directly sequenced at Macrogen Inc. (Macrogen).

All sequences were compiled, assembled and edited using Sequencher 5.4.1 (Gene Codes Corporation), and then aligned automatically in MAFFT 7.0 (Katoh & Toh, 2008). The final alignment was manually revised and edited in Mesquite 3.04 (Maddison & Maddison, 2015).

2.2 | Phylogenetic analyses and divergence time estimates

Phylogenetic analyses were performed with multiple specimens per species, to assess the possible presence of cryptic or incipient species. We define incipient species as a group of individuals

(populations) that are about to become genetically isolated from the rest of the species, for instance because of a geographical barrier (as inferred from biogeographical analyses), though at this time can still reproduce with other populations of the same species. Phylogenetic analyses were carried out under both maximum likelihood (ML) and Bayesian inference (BI) (Appendix S2, Table S4). Species delimitation analyses were initially performed but resulted in a non-realistic number of species. We therefore relied on our expertise to assess species boundaries.

We estimated divergence times between lineages and their credibility intervals using relaxed molecular clock analyses implemented in BEAST 1.8.4 (Drummond et al., 2012). Given the absence of fossil record for *Misolampus*, we relied on the fossil record of the subfamily Stenochiinae, for which the most ancient and non-ambiguous fossil species †*Pseudohelops groenlandicus* provides a minimum age of 58.7 Ma for the crown group of the subfamily Stenochiinae (Nabozhenko, 2019, Appendix S2, Table S4). Sensitivity analyses on the prior distribution for the fossil calibration were performed to evaluate the robustness of the time estimations.

All analyses were run in the CIPRES Science Gateway cluster using BEAGLE (Ayres et al., 2012; Miller et al., 2010).

2.3 | Current climatic suitability and ancestral climatic preferences

We inferred climatically suitable areas for all *Misolampus* species under current and past climatic conditions using species distribution models (SDMs). Based on current occurrence data of *Misolampus* (Rosas-Ramos et al., 2020), we built SDMs with a total of 509 presence records (56 of *M. subglaber*, 133 of *M. scabricollis*, 30 of *M ramburii*, 13 of *M. lusitanicus*, 137 of *M. goudotii*, and 140 of *M. gibbulus*). The 19 bioclimatic variables available in the WorldClim 2.0 dataset (Fick & Hijmans, 2017) were used as predictor variables to build models of environmental suitability under current and past climate conditions. We calculated the correlation coefficients among all the 19 climatic variables for the present time and the variance inflation factor (VIF) to reduce any potential multicollinearity using R package *HH* (Heiberger, 2016). We selected those variables with an appropriate temporal and spatial scale for our study, with direct or indirect influence with the occurrence of *Misolampus* and with values of poor spatial correlation with each other: $R < 0.7$ and VIF < 3 (Zuur et al., 2010) (Appendix S2, Table S3). We first built SDMs for the present and then projected these models towards different periods in the past to predict suitable areas based on palaeoclimatic simulations including the Last Interglacial period (LIG, 120,000–140,000 years ago [ya]) in the Late Pleistocene, and three different models (CCSM4, MIROC-ESM, MPI-ESM) for the Last Glacial Maximum (LGM, 19,000 ya) in the Holocene.

We used Maxent 3.3.3 k software (Phillips et al., 2006; Phillips & Dudík, 2008) to create species range maps with binary presence/absence data, including 10,000 random pseudo-absences.

We built 10 replicate SDMs for each species, of which 20% corresponded to testing and 80% were used as training. Model performance was evaluated based on the receiver operating characteristic (ROC) and its area under the curve (AUC), with AUC scores >0.7 indicating good performance (Fielding & Bell, 1997). Data preparation, modelling and calculations were performed using R packages *dismo* and *raster*.

We also inferred ancestral climatic preferences by analysing the uncorrelated climatic variables. Given all values for each species throughout their geographical distributions, we computed the mean and median values for each variable and for each species. Across the time-calibrated phylogeny of *Misolampus*, we inferred the ancestral state using R package *phytools* 0.7–70 (Revell, 2012). We computed variances and 95% confidence intervals for each node ('*fastAnc*' function) and plotted the ancestral state of each key variable onto the tree ('*contMap*' function). We replicated this in two different analyses, one including the six *Misolampus* species and another including incipient species recovered in phylogenetic analyses (see Section 3).

2.4 | Biogeographical analyses

We estimated the ancestral area of origin of *Misolampus* and its geographical range evolution using the dispersal-extinction-cladogenesis (DEC, Ree & Smith, 2008) model as implemented in the C^{++} version (Beeravolu & Condamine, 2016). To infer the biogeographical history of the genus, we incorporated the time-calibrated tree generated, a set of geographical areas according to the current distribution, and a time-stratified geographical model with the connectivity and the dispersal matrices for each time interval. The following areas were selected: [A] Iberian Plate, [B] Betic, [C] Rifean, [D] Atlas, [E] North Sahara, [F] Balearic Islands and [G] North Kabylia. We categorized the geographical distribution of *Misolampus* species as present or absent in each of these areas. The connectivity and dispersal changed between the following time bins: [I] 0 to 5.3 Ma (Pliocene to Present), [II] 5.3 to 11.6 Ma (late Miocene), [III] 11.6 to 16 Ma (middle Miocene) and [IV] 16 to 23 Ma (early Miocene). Based on the literature, we mapped the geological evolution of the predefined areas since the early Miocene including major tectonic changes, to represent them in a time-stratified geographical model defining the connectivity and dispersal matrices for each considered time bin. For the connectivity matrices, we coded 0 if any of two areas were not connected (non-adjacent) and 1 if there was a connection in each time interval. For the dispersal matrices, we set up simple rules to define a dispersal scalar from area X to area Y as follows: $d = 0.5$ when the areas are adjacent and connected (no barrier), $d = 0.25$ when the areas are adjacent but separated by a barrier, $d = 0.125$ or 0.0625 when the areas are not connected and separated by another area Z ($dXZ \times dZY$), and $d = 0.01$ for long-distance dispersal (more than two areas) (Figure 2). We reconstructed the biogeographical history of *Misolampus* in two different analyses: one including the six species of *Misolampus*

described, and another including the cryptic or incipient species detected in phylogenetic analyses (see Section 3).

3 | RESULTS

3.1 | Phylogenetic relationships and incipient speciation

The final matrix comprised 6277 bp, divided in seven partitions according to ModelFinder (Appendix S1, Table S1.5). Our analyses under ML and BI yielded identical topologies, supporting the monophyly of *Misolampus* and of each of the six species with maximal node support (posterior probability, PP = 1; bootstrap support, BS = 100; Figure 3). *Misolampus* species are grouped into two well-supported clades, one that corresponds to *M. goudotii* (PP = 1, BS = 100) distributed in North Africa-Balearic Islands, and the other clade containing the Iberian *Misolampus* species (PP = 1, BS = 100).

Misolampus goudotii includes four well supported lineages distributed as follows: along the Rifean region reaching the Larache area (*goudotii1*, PP = 1, BS = 99), throughout the High Atlas (*goudotii2*, PP = 1, BS = 100), in the Balearic Islands (*goudotii3*, PP = 1, BS = 100), and between the Southwestern Anti-Atlas region and the Western Sahara (*goudotii4*, PP = 1, BS = 100; Figure 3). Within Iberian *Misolampus*, the first split involved *M. lusitanicus* and the remaining taxa (PP = 1, BS = 100). *Misolampus lusitanicus* is currently restricted to the Northwest of the Iberian Peninsula. This split was followed by the split of *M. subglaber* from the other lineages (PP = 1, BS = 100). *Misolampus subglaber* includes two supported lineages distributed: in the South-East of the Iberian Peninsula (*subglaber*1, PP = 1, BS = 100), and restricted in the Genal Valley in Málaga (*subglaber*2, PP = 1, BS = 100) (Figure 3). *Misolampus scabricollis* split from the clade formed by *M. ramburii* and *M. gibbulus* (PP = 1, BS = 100), and diversified throughout the Central South-West of the Iberian Peninsula. Finally, *M. ramburii* and *M. gibbulus* are sister species (PP = 1, BS = 100). Currently *M. ramburii* is restricted to the South-East of the Iberian Peninsula and the Balearic Islands, and *M. gibbulus* is distributed in Central and South-Western Iberia. *Misolampus gibbulus* contains three lineages: *gibbulus1* (PP = 1, BS = 100) in central Iberia, *gibbulus*2 (PP = 0.97, BS = 96) in Sierras de Montánchez and Aracena, and *gibbulus3* (PP = 1, BS = 100) in the southwest margin of its distribution in Portugal and Sierra de Aracena.

3.2 | Current and Late Pleistocene climatic favourable areas

The four uncorrelated variables used in the SDMs were isothermality (BIO3, mean diurnal range/temperature annual range), temperature annual range (BIO7, max temperature of warmest month-min temperature of coldest month), mean temperature of wettest quarter (BIO8, mean temperature of wettest quarter), and precipitation of driest quarter (BIO17). Model predictions for the six species had good overall evaluation scores, with
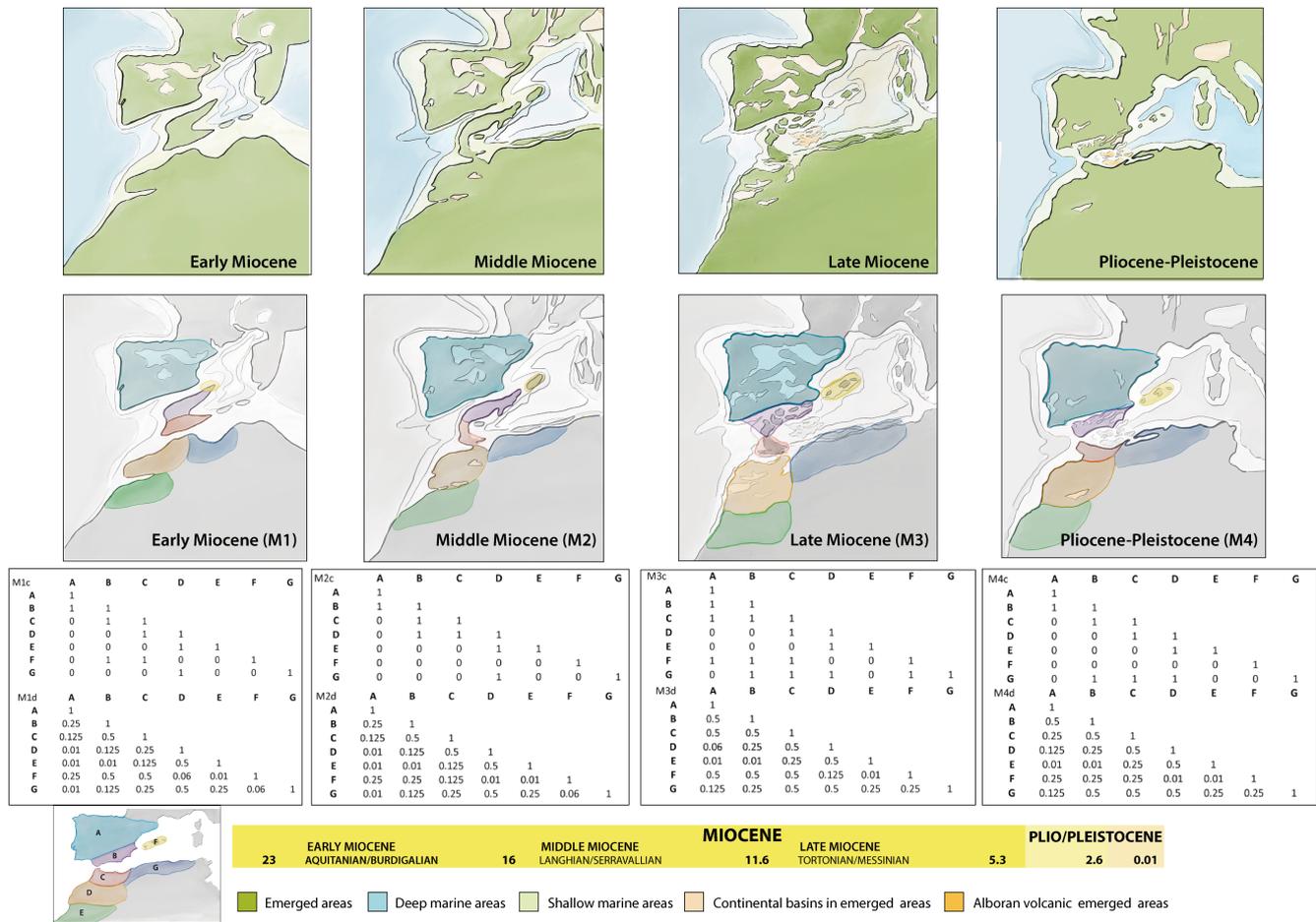
FIGURE 2 Palaeogeographical evolution of the Western Mediterranean Basin since the early Miocene. A time-stratified geographical model represents the connectivity matrices (M1c, M2c, M3c and M4c) and dispersal matrices (M1d, M2d, M3d and M4d). In connectivity matrices, 0 indicates that any two areas were not connected (adjacent), and 1 indicates a past connection in the time interval. In dispersal matrices, dispersal rate from one area to another area is defined as follows: $d = 0.5$ for adjacent and connected areas (i.e. no barrier); $d = 0.25$ for adjacent areas but separated by a barrier; $d = 0.125$ or 0.0625 for unconnected areas and separated by another area ($dXZ = dXY \times dYZ$); and $d = 0.01$ for long-distance dispersal (dispersal separated by more than two areas). Bottom right: The following biogeographical areas were selected: [A] Iberian Plate, [B] Betic, [C] Rifean, [D] Atlas, [E] North Sahara, [F] Balearic Islands, and [G] North Kabylia. Connectivity and dispersal changed between the following time bins: [I] 0 to 5.3 ma (Pliocene to present), [II] 5.3 to 11.6 ma (late Miocene), [III] 11.6 to 16 ma (middle Miocene), and [IV] 16 to 23 ma (early Miocene).

AUC ≥ 0.96 in all cases (*M. lusitanicus*: 0.98; *M. gibbulus*: 0.97; *M. goudotii*: 0.97; *M. ramburii*: 0.96; *M. scabricollis*: 0.98 and *M. subglaber*: 0.96).

The potential distribution maps for the six species located high suitability areas largely matching their current ranges. In addition, other areas where *Misolampus* species are absent, including the Cantabrian Mountains, the Pyrenees and Sardinia, were recovered as potential suitable environments for some species (Appendix S2, Figures S2.1–S2.6). The general pattern inferred from palaeoclimatic model projections shows contractions of favourable areas during the LIG and mid-Holocene, and wide expansions during the LGM (Appendix S2, Figure S1.6). Both projections similar climatic refugia during climate fluctuations: the Galician Massif in the northwest of Spain for *M. lusitanicus*, Serra da Estrela Mountains for *M. scabricollis*, and SW Iberia for *M. gibbulus*. The analyses also recovered a large ecologically suitable area for *M. goudotii* throughout the

North-Western African coast, Balearic Islands and Sardinia. Areas with unequivocal high suitability in the past in the Iberian Peninsula were not recovered for *M. subglaber*, whereas for *M. ramburii* a small suitable area in the Penibetic Mountain ranges was recovered, but with low probabilities (0.38–0.52). Species that could have been highly favoured during glacial periods (Appendix S2, Figures S2.3, S2.4 and S2.6), at the present time have restricted distributions especially in the case of *M. lusitanicus* or *M. ramburii*.

3.3 | Ancestral climatic preferences

Ancestral state estimation based on mean values of the uncorrelated variables applied in the SDMs (BIO3, BIO7, BIO8 and BIO17) showed phylogenetic signal in two cases: BIO17 and BIO8, except in *M. ramburii* and *M. gibbulus* (Figure 4, Appendix S2, Figures S13–S20). BIO17

FIGURE 3 Molecular phylogenetic tree for *Misolampus*, based on the concatenated matrix. Support for each node is provided by posterior probabilities (PP) and bootstrap support values (BS), with outgroups removed. Right: Distribution maps for each species based on occurrence records compiled in Rosas-Ramos et al. (2020).
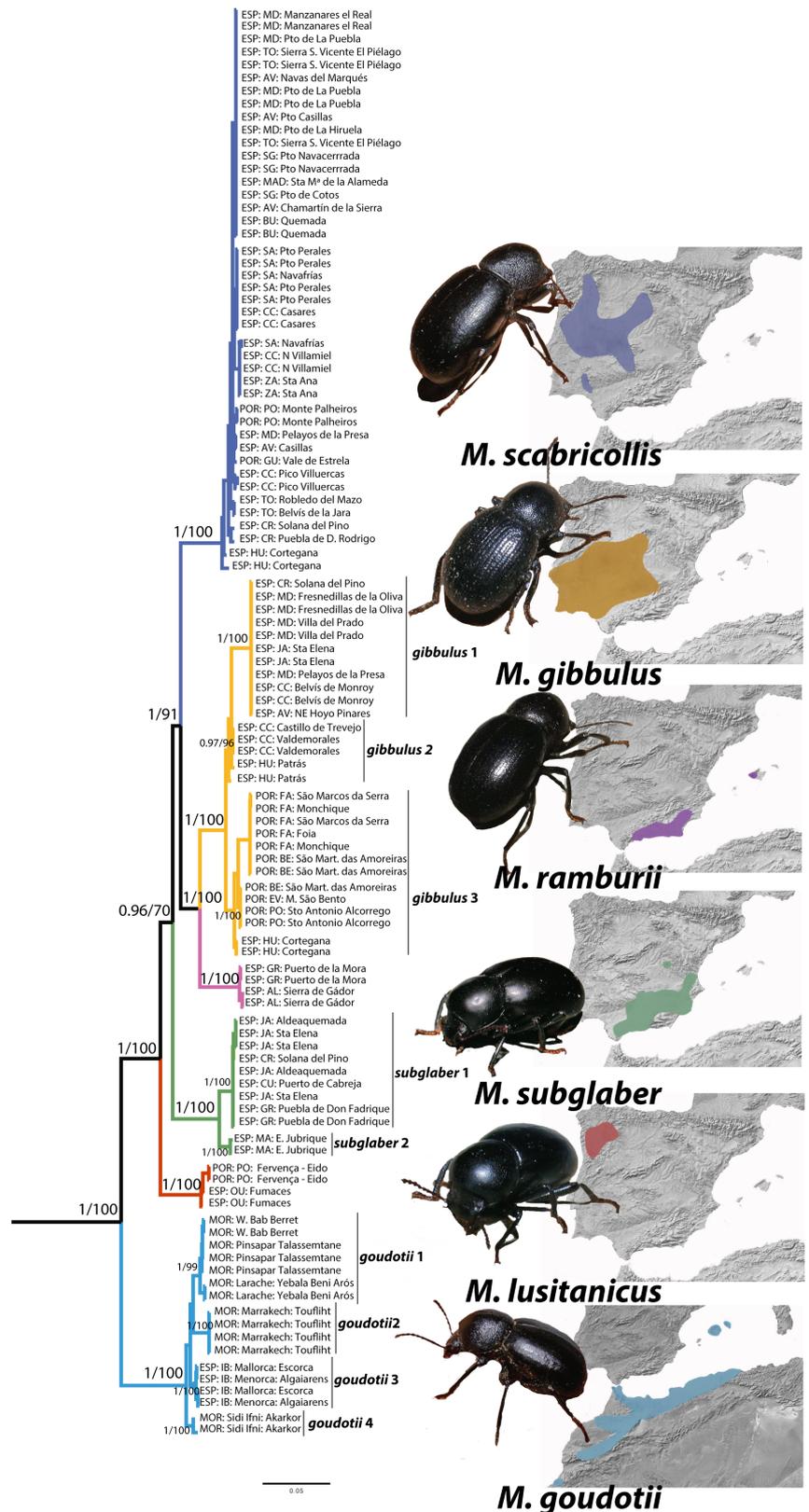
showed values ranging from 120 mm in the case of *M. lusitanicus* to 27 mm in *M. goudotii*, and BIO8 values ranged from 5.1°C in *M. lusitanicus* to 11.12°C in *M. ramburii* (Table 1; Appendix S3, Tables S1).

Based on these results, analyses of the distribution of the variables (Appendix S2, Figures S7–S12) reveal differences among isolated lineages within the species *M. gibbulus*, *M. goudotii* and *M. subglaber* (Figure 4). We conducted the same analysis for the twelve cryptic or incipient species within *Misolampus* following our phylogenetic results. This allowed us to recover phylogenetic signals that were previously masked for the four variables within
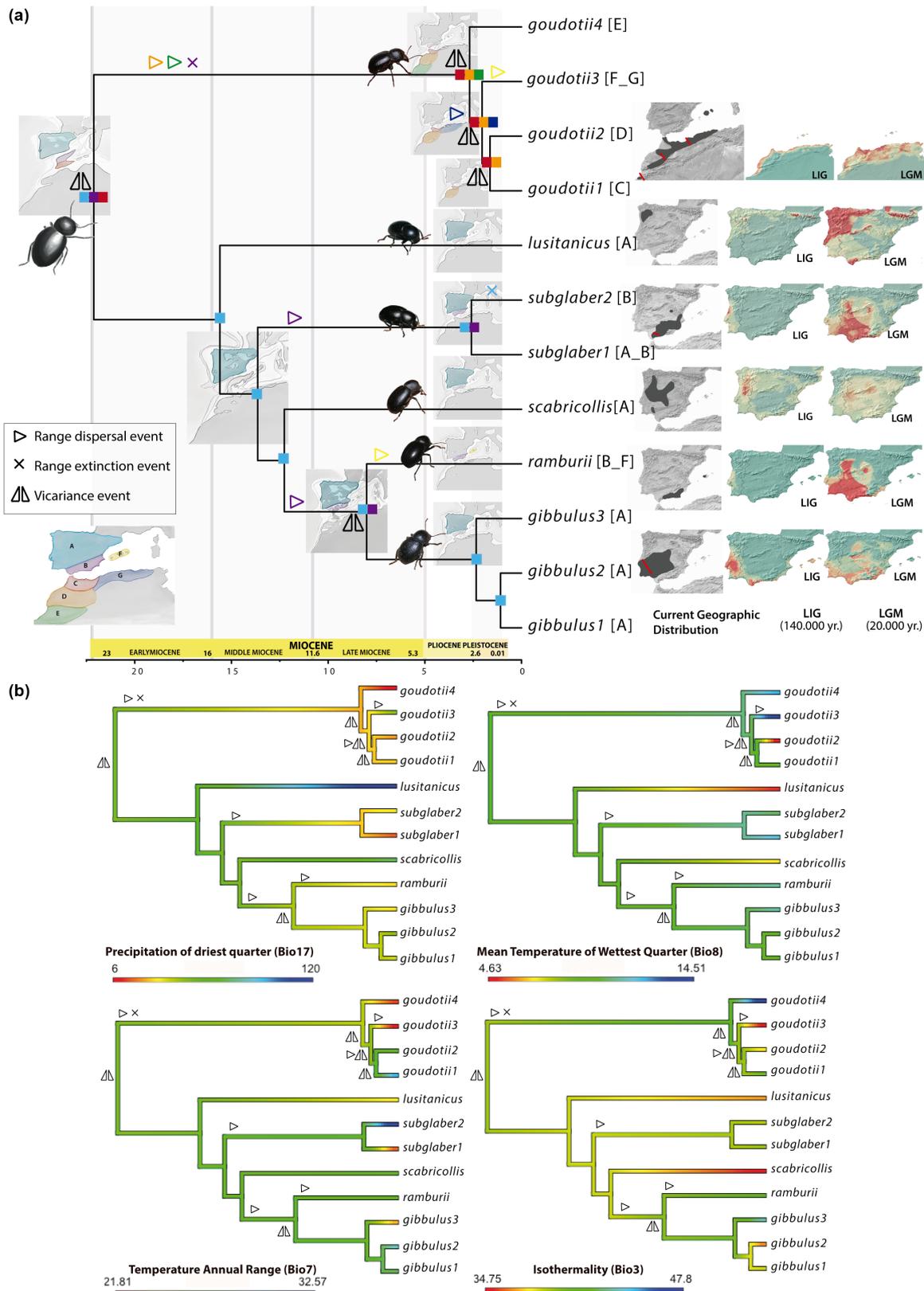
FIGURE 4 Origin, biogeographical and ecological processes explaining diversification in *Misolampus*. (a) Historical biogeography as inferred with DEC and the time-stratified model. Coloured squares at nodes represent the ancestral ranges, and symbols along branches represent biogeographical processes. In the right are depicted, for each species, the current geographical distribution with a red line indicating possible barriers to gene flow linked with incipient species, and the inferred past distributions for the last inter-glacial (LIG) and for the Last Glacial Maximum (LGM) based on MPI-ESM-P simulations. (b) Ancestral state reconstructions of key climatic variables (BIO17 = precipitation of driest quarter, BIO8 = mean temperature of wettest quarter, BIO7 = temperature annual range, and BIO3 = isothermality) showing the niche conservatism and niche evolution in *Misolampus*.

TABLE 1 Results of historical biogeography with the 12-species tree

Speciation event or branch	Node number in the R tree	Ancestral and current estimates for climatic variables				Ancestral and current biogeographic estimates		
		bio17 (precipitation of driest quarter)	bio8 (mean temperature of wettest quarter)	bio3 (isothermality)	bio7 (precipitation of coldest quarter)	DECX M0 (unconstrained)	DECX M1 (adjacency)	DECX M2 (adjacency) & dispersal)
Root of Misolampus	13	49.44	9.28	39.01	25.86	ACE	ABC	ABC
Divergence of goudotii4	14	25.36	10.70	40.83	25.02	CDE	CDE	CDE
goudotii4	–	6	12.38	47.80	22.44	E	E	E
Divergence of goudotii3	15	29.21	10.34	39.22	25.61	CDF	CDG	CDG
goudotii3	–	48	14.51	34.75	21.81	FG	FG	FG
Split between goudotii1 and 2	16	27.89	9.27	39.10	26.75	CD	CD	CD
goudotii1	–	22.5	4.63	37.51	27.78	C	C	C
goudotii2	–	28	9.69	40.18	30.24	D	D	D
Divergence of lusitanicus	17	57.53	8.81	38.39	26.14	A	A	A
lusitanicus	–	120	5.1	36.61	24.30	A	A	A
Divergence of subglaber	18	52.13	9.13	38.43	26.46	A	A	A
Split between subglaber1 and subglaber2	19	27.85	10.96	38.95	27.47	AB	AB	AB
subglaber1	–	34	10.42	38.82	32.57	AB	AB	AB
subglaber2	–	16	11.92	39.21	22.61	B	B	B
Divergence of scabricollis	20	51.34	9.13	38.40	26.55	A	A	A
scabricollis	–	72	6.75	34.95	27.10	A	A	A
Divergence of ramburii	21	41.76	9.96	39.48	26.67	AB	AB	AB
ramburii	–	30	11.12	40.87	27.20	BF	BF	BF
Divergence of gibbulus3	22	37.30	10.24	39.94	26.44	A	A	A
gibbulus3	–	30	11.17	43.50	23.54	A	A	A
Split between gibbulus1 and gibbulus2	23	40.23	9.81	38.14	27.94	A	A	A
gibbulus1	–	46	9.80	36.33	29.24	A	A	A
gibbulus2	–	37	9.44	38.38	27.95	A	A	A

M. goudotii, *M. subglaber* and less markedly within *M. gibbulus* (Figure 4, Table 1, Appendix S3, Tables S1).

3.4 | Biogeographical history and divergence times

Including adjacency and dispersal matrices, the biogeographical analyses for the twelve incipient species recovered an ancestral range of *Misolampus* composed by the Iberian Plate [A], Betic [B], and Rifean [C] (relative probability = 0.461 for the 6-species tree; = 0.514 for the 12-incipient-species tree) as the most likely ancestral range in the early Miocene (dispersal = 0.045, extinction = 0.012 for the 6-species tree; dispersal = 0.074, extinction = 0.012 for the 12-incipient-species tree), splitting early into two lineages (one in North Africa and the other in Iberia) during the early Miocene ~22.2 Ma (95% HPD: 16.4–29.6) (Figure 4, Table 1, Appendix S3, Table S1).

DEC results support the dispersal of the ancestor of *M. goudotii* along the Atlas [D] and North Sahara [E], followed by local extinction events in the Betic area. According to the 12 incipient-species DEC analysis, the most likely ancestral area of *M. goudotii* occupied a range composed of the Rifean [C], the Atlas [D] and the North Sahara [E] (relative probability = 0.802), where a vicariant event during Pliocene-early Pleistocene ~2.8 Ma (95% HPD: 1.5–4.1) may have promoted the isolation and divergence of the *goudotii4* lineage in the North Sahara region. This was followed by dispersal along North Kabylia [G]. Subsequently, the split of three lineages during the Pleistocene was fostered by two vicariant events: *goudotii3* in the North Kabylia also currently present in Balearic Islands [F] (relative probability = 0.907); *goudotii2* in the High Atlas [D]; and *goudotii1* along the Rifean region reaching the Larache area (relative probability = 0.964) (Figure 4).

The MRCA for the Iberian *Misolampus* species dates to the middle Miocene ~15.7 Ma (95% HPD: 11.8–19.9) in the Iberian Plate [A] (relative probability = 0.5072), followed by divergence between *M. subglaber* and the rest of the Iberian species 13.7 Ma (95% HPD: 9.6–17.9), and its dispersal along the Betic Region [B] (relative probability = 0.552). Later, during the late Pliocene-early Pleistocene, it split into two lineages (*subglaber*1 and *subglaber*2) from the Iberian Plate [A] and Betic regions [B] (relative probability = 0.801). *Misolampus scabricollis* differentiated in the Iberian Plate around the Middle Miocene 12.3 Ma (95% HPD: 8.2–16.4), [A] (relative probability = 0.815), followed by the dispersal of the ancestor of *M. ramburii* and *M. gibbulus* throughout the Betic Region [B]. A vicariant event split these two species around the late Miocene ~8.1 Ma (95% HPD: 4.6–17.9) from the Iberian Plate [A] and the Betic region [B] (relative probability = 0.363), where *M. ramburii* would be restricted to the Betic region [B] and dispersed along the Balearic Islands [F], and *M. gibbulus* remained in the Iberian Plate [A] (relative probability = 0.992). Since the late Pliocene–early Pleistocene, *M. gibbulus* split into three lineages distributed in the Iberian Plate (*M. gibbulus1*, *M. gibbulus*2 and *M. gibbulus* 3; Figure 4).

4 | DISCUSSION

4.1 | Combining ecological niche modelling and historical biogeography

Understanding the evolution of a clade implies discerning the role of ecological factors and biogeographical processes in species diversification. The combination of phylogenetic analyses, divergence times estimation, ancestral ecological niche modelling and inference of ancestral areas provides a robust framework to reconstruct the evolutionary history of *Misolampus*. Our methodological approach, making use of within- and between-species molecular and ecological data, allowed us to infer their macroevolutionary history and identify putative cases of incipient speciation. This combination also provides a track of the biogeographical history and ecological tolerances of species and lineages, enabling the identification of convergent or divergent evolution associated with climate niche variables (Smith & Donoghue, 2010). Ancestral climate reconstructions can also provide testable hypotheses about the origin of lineages (Rivera et al., 2020; Yesson & Culham, 2006a). In fact, repeated phylogenetic patterns of climate characteristics across taxonomic groups have entailed the identification of shared environmentally favourable areas, for instance regarding the formation of Mediterranean climate zones (Yesson & Culham, 2006b).

4.2 | A dynamic biogeographical scenario in Western Mediterranean during the Miocene drove early speciation

Biogeographical analyses favour the hypothesis invoking a dual role of vicariance and dispersal driving the biogeography of *Misolampus* since the early Miocene (Figure 4; Appendix S3, Figure S3.2). Interestingly, there is mixed evidence for each biogeographical scenario in previous works on beetle historical biogeography (Condamine et al., 2013; García-Vázquez et al., 2017; Mas-Peinado et al., 2018; Micó et al., 2009; Riccieri et al., 2017; Ruiz et al., 2012; Trichas et al., 2020; Trizzino et al., 2011). Our results reveal an ancient vicariance between the proto-Iberian Peninsula and North Africa that has led to an old divergence within the Western Mediterranean region of two isolated lineages.

Discrepancies with alternative hypotheses mainly involve the timing of speciation events. Our results contradict the idea that speciation occurred mainly during the Pleistocene and cast doubts about the origin and dispersal of *Misolampus* from the Maghreb. Further, we did not recover a monophyletic clade comprising *M. lusitanicus*, *M. subglaber* and *M. scabricollis*, in contrast with previous studies (Palmer & Cambefort, 2000). Regarding the hypothesis of current sympatry because of recent secondary contact (Rosas-Ramos et al., 2020), our ancestral ecological estimates discard the possible existence of niche overlap among species pairs of *Misolampus* found in sympatry (Figure 4). The TMRCAs between sympatric species like *M. gibbulus*–*M. scabricollis* or *M. subglaber*–*M. ramburii* date back to the middle Miocene, in line with results in other tenebrionid beetles in the

Palearctic (Condamine et al., 2013; Mas-Peinado et al., 2018; Stroscio et al., 2011) and Nearctic Regions (Johnston, 2019), where sympatry usually involves highly divergent taxa within the same genus.

The dynamic palaeogeographical evolution of the Western Mediterranean, especially since the late Oligocene (Andeweg, 2002; Carminati et al., 2012; Mas-Peinado et al., 2018; Rosenbaum et al., 2002; Appendix S1), involved the emergence of land bridges in the middle Miocene and volcanic archipelagos in the Pliocene that probably had a decisive role in the evolutionary history of many flightless species (Condamine et al., 2013; Hidalgo-Galiana & Ribera, 2011; Mas-Peinado et al., 2018; Micó et al., 2009; Riccieri et al., 2017). The subsequent disappearance of these land bridges later contributed to the disjunct distribution of a variety of taxa, including beetles (Faille et al., 2014; García-Reina et al., 2014; Palmer & Cambefort, 2000).

The biogeographical history of hunchback darkling beetles displays four main stages: initial split in the early Miocene; speciation events within the Iberian Plate in the middle Miocene; speciation events within the Iberian Peninsula in the late Miocene; and lineage isolation driving incipient speciation events in the early Pleistocene. The *Misolampus* ancestor settled on the Iberian Plate-Betic-Rifean as the most probable ancestral region, with a vicariant event during the early Miocene triggering the divergence of the two main lineages (Iberian and Moroccan) with no evidence of transcontinental dispersal since then.

Throughout the early Miocene, along the East of Iberia, the back-arc stretched continental lithosphere in the Alpine-Betic foreland and in the South the back-arc stretched areas previously belonging to the Alpine-Betic belt. This extensional geodynamic framework has favoured the development of a wide Mediterranean Basin bordering Iberia to the East and South, and separated it from Africa (Carminati et al., 2012). Within this basin there were islands, some of them relatively large, but which unlikely acted as bridges between the two continental masses (Andeweg, 2002; Rosenbaum et al., 2002). However, the Moroccan *Misolampus* lineage stayed since then isolated in North Africa. Within the Iberian Plate, because of the convergence of Africa and Eurasia, the development and lifting of two mountain ranges progressed in a compressive regime: the Iberian Chain (Cadena Ibérica) and Sistema Central (De Vicente et al., 2011; Guimerà et al., 2004). Simultaneously, the formation of continental basins continued dominated by the development of extensive lake systems: the Duero Basin (between the Cantabrian Mountains through the West Pyrenees in the north and the mountainous group Iberian Chain-Sistema Central in the south); the Tagus Basin (between the Iberian Chain and the Sistema Central), and the Ebro Basin (between the Pyrenees and the Iberian Chain) (Alonso-Zarza et al., 2002; Calvo et al., 1993; De Vicente et al., 2011). This geological framework with a system of wide lakes and mountain ranges occupying the interior of the Iberian Peninsula entailed physical barriers to the displacement of flightless species as *Misolampus*. During the early Miocene, areas north of the Mediterranean Sea were mainly covered by forests, whereas southern areas were occupied mainly by open vegetation (Suc et al., 2018). Within the Iberian Peninsula, the abundance of thermophilous species during the early Miocene suggests a main subtropical climate. Indeed, the north-east

of the peninsula would have been dominated by herbaceous vegetation, with small-isolated areas within the regional steppic feature with local riparian forest (Jiménez-Moreno et al., 2010). These past conditions and vegetation composition, together with the formation of the Ebro and Tagus Basins, provide clues for the current absence of *Misolampus* in the region, yet with favourable areas like Iberian Chain (Rosas-Ramos et al., 2020). The southern area of the Iberian Peninsula was also characterized by herbaceous vegetation, even subdesertic flora implying dry and warm conditions, where mesothermic trees were probably in mid-or high altitudes. High altitude, characterized by an evergreen and deciduous mixed forest including *Quercus*, *Fagus*, *Ilex* and *Acer*, would remain the only suitable area for *Misolampus*, being more abundant in the North and West of the Iberian Peninsula (Jiménez-Moreno et al., 2010). At the end of the Burdigalian, from 18 to 15 Ma, Iberia and Africa were connected while Kabylia was colliding with Africa (Frizon de Lamotte et al., 2000). However, this does not seem to have entailed new contact or gene flow between the two main *Misolampus* lineages.

During the middle Miocene, speciation events within the Iberian Peninsula may have occurred via peripatry in a relatively tectonically stable period, while the climate was warm and shrublands were more developed. Within the Iberian Plate, the compression regime of the Iberian Chain and the Sistema Central continued together with the Duero, Ebro and Tagus Basins (De Vicente et al., 2011; Perea et al., 2021). This warm climate scenario could lead to the disappearance of many forest areas in the Iberian Plate, isolating lineages like the ancestor of *M. lusitanicus* in the North-West of the Iberian Peninsula around 15.7 Ma, the ancestor of *M. subglaber* in the South-East dating from 13.7 Ma, or the ancestor of *M. scabricollis* in the West around 12.3 Ma. The ancestors of *M. gibbulus* and *M. ramburii* dispersed along the Betic Region, while the Betic/Rif tectonic uplift occurred. Subsequently, the formation of the Guadalquivir Foreland Basin in the South of the Peninsula bordered by the Iberian Massif in the North and the Betic Orogenic Belt in the South (Garcia-Castellanos et al., 2002; Sierro et al., 1996) likely constituted a major vicariant event for flightless beetles (Mas-Peinado et al., 2018). During the early Tortonian, the Guadalquivir Basin reached its maximum development and was connected towards the West/South-West with the Atlantic Ocean and towards the East/North-East with the Mediterranean Sea (Capella et al., 2020; Krijgsman et al., 2018). Therefore, this geographical barrier could entail the allopatric speciation of these two species around 8.1 Ma (Appendix S3, Figure S3.1). The Tortonian stage was characterized by a generally humid and wooded biotope, with reduced presence of thermophilous plants. Species of sclerophyllous woods currently present in the South-Western Mediterranean Region, for example *Pinus* and *Quercus* associated with open vegetation, were located in the northern part of the Sistema Central. Progressively, the connection between the Mediterranean and Atlantic became limited, culminating with the Messinian Salinity Crisis (MSC, 5.96–5.33 Ma) (Krijgsman et al., 2018). The MSC could have promoted the last dispersal event within Iberian *Misolampus*, when *M. ramburii* reached the Balearic Islands from the Betic Mountain Ranges (Dumitru et al., 2021).

4.3 | Ecological and geological factors involved in incipient speciation

We detected three possible cases of incipient speciation, underscoring the role of both geology-based vicariance and potential adaptations to local climatic conditions. These results lend support to a scenario of multiple refugia for *Misolampus* in the Western Mediterranean Area, with range expansions–contractions since the end of the Pliocene potentially linked with speciation.

Palaeoclimatic projections display a general pattern of expansion of environmentally favourable areas during the LGM and suggest range contractions during the LIG and mid-Holocene. This scenario implies a different temporal perspective in analyses of diversification in Tenebrionidae (Condamine et al., 2013; Mas-Peinado et al., 2018; Trichas et al., 2020), which considers the possible role of glaciations in driving incipient speciation. Under this perspective, allopatric isolation may be followed by adaptation to new environmental conditions in relatively short periods of time, counteracting phylogenetic conservatism, which tends to counter ecological niche divergence (Pyron et al., 2015).

Our results support ecological adaptation in the incipient species (Figure 4; Table 2). The four selected bioclimatic variables for the ancestral state estimations showed a strong phylogenetic signal, particularly marked in the case of precipitation of driest quarter (BIO17) and mean temperature of wettest quarter (BIO8, Figure 4; Table 1). The recent history of isolation across populations with obvious patterns of morphological differentiation within *M. gibbulus* and *M. goudotii* (Rosas-Ramos et al., 2020) were confirmed by our analyses. These species, characterized by high levels of intraspecific diversity, are distributed in the southwest of the Iberian Peninsula and in Morocco, regions previously identified as climatic refugia across a variety of taxa (Abellán & Svenning, 2014; Gutiérrez-Rodríguez et al., 2017; Martínez-Freiría et al., 2020; Miraldo et al., 2011; Sánchez-Montes et al., 2019).

Mountain massifs in Northwest Africa have been documented as refugia for Mediterranean plants during interglacial periods, subsequently dispersing to neighbouring regions in cooler periods (García-Aloy et al., 2017), a pattern shared with *M. goudotii*, where allopatric incipient speciation was likely driven by geological factors (three successive vicariant events) and ecological adaptations linked to Pleistocene climate oscillations (Figure 4). Since the late Miocene, the ancestor of *M. goudotii* dispersed along the Atlas and North Sahara and underwent local extinction in the Betic. A vicariant event during the late Pliocene-early Pleistocene involved the divergence of *goudotii*4 from the other three lineages as an isolated lineage in the Northern Sahara, North-East of Drâa River, another previously described climatically stable area (Martínez-Freiría et al., 2017). Following this, the lineage that reached North Kabylia (*goudotii*3) split from the rest, and the isolation between the Rif and Atlas Mountains promoted the differentiation of *goudotii*2 and *goudotii*1.

During the late Pliocene, the Western Mediterranean was already similar to the current Mediterranean (Andeweg, 2002; Rosenbaum et al., 2002; van Hinsbergen et al., 2014), with the Betic orogenic belt ("Alpine-Betic belt") in the South of Iberia and the Rif-Tell-Kabylides belt ("Apennines-Maghrebides Belt") in the North of Africa, separated both by the Sea of Alboran and linked to the West by the Arch of Gibraltar (formed since the Tortonian). The Alboran volcanic archipelago vanished (Booth-Rea et al., 2018; Capella et al., 2020; Krijgsman et al., 2018), and possible land bridges between Iberia and Africa disappeared. The distribution of *M. subglaber* throughout the Betic area, suggesting the isolation of *subglaber*2 in the Penibetic with a subsequent ecological adaptation to warmer conditions, and the "long-range" dispersal of the Cuenca population from the main range of the species (*subglaber*1) (ca. 280km) suggests a recent population expansion (Figure 4). Recent studies of flightless saproxylic beetles alludes that distributions were shaped by habitat limitation instead of dispersal limitation, and were able to recolonize recent forests (Cateau et al., 2018; Janssen et al., 2016). Within the Guadalquivir Basin, between the Iberian Massif and the Betic Orogenic belt, marine influence remained limited to a small Western area, considered as a physical barrier to dispersal. This barrier did not allow a new contact between *M. ramburii* and *M. gibbulus*, and currently represents the southeast boundary of the *M. gibbulus* range. Both *M. ramburii* and *M. gibbulus* retained quite similar ancestral ecological characteristics (niche tracking). Within *M. gibbulus*, these similarities seem to be circumscribed to *gibbulus*3 (the most divergent lineage) distributed in the Southwest of the Iberian Peninsula. Recent range shifts may have favoured secondary contact between *gibbulus*1 and *gibbulus*2, as suggested by phylogenetic analyses of nDNA sequences. Indeed, both *M. scabricollis* and *M. gibbulus* display a star-haplotype network within the Sistema Central, suggesting a recent population expansion event to the Sierra de Aracena and Picos de Aroche (Western Sierra Morena), which could have functioned as refugia for both species. The population of *M. scabricollis* from Huelva remains isolated, while waiting to confirm if recent distribution data in Badajoz (Barreda et al., 2021) could be assigned to the isolated lineage or there is gene flow.

Our results support the dual role of geological and ecological factors driving speciation in *Misolampus*, and extinction could have played a role that is not studied here in detail. However, it remains unknown how *Misolampus* was resilient to climatic fluctuations since the early Miocene, and how such an ancient origin coupled to a long-term isolation that hinder gene flow have led to a few extant species of *Misolampus* today. We hypothesize that their microhabitats, beneath barks or inside dead logs, could have favoured tolerance for dry and warm conditions during climatic fluctuations of these flightless species. Due to the dead-wood association, although the exact food source remains unknown (Rosas-Ramos et al., 2020), mycobiota association may influence its preferences. Recent global analysis alludes to the main role of precipitation determining the fungi community (Tedersoo et al., 2014), and new evaluation of beetle and fungus assemblages confirmed that beetle host identity may affect the fungal communities between trees beyond environmental variables (Miller et al., 2019). We believe it is necessary to deepen into microhabitat conditions and perhaps symbiotic relationships that have entailed its resilience linked to woodlands against climatic fluctuations for long periods of time.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

DNA sequences used in this work have been deposited in GenBank, and accession numbers are included in Appendix S1, Table S1.1. Detailed information about the data and molecular analyses, ecological niche modelling and historical biogeography analyses is presented in the Appendices.

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BIOSKETCH

Paloma Mas-Peinado is broadly interested in the evolutionary history of darkling beetles and biogeography of the Western Mediterranean region. This work represents the last chapter of her PhD thesis titled "Evolutionary processes and speciation patterns in the Mediterranean Basin darkling beetles" realized at MNCN-CSIC/UAM, completed by a collaboration with the CNRS (Institut des Sciences de l'Evolution de Montpellier).

Author Contributions: PM-P, MG-P and FLC conceived the ideas; MGP and ER conducted the fieldwork; PM-P together with MG-P, YJ-R, AV, DB and FLC have developed the methodology; PM-P along with MG-P, ER, IM-S, DB and FLC analysed the results; PMP led the writing with assistance from all coauthors.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.