



Integrative analysis reveals the divergence and speciation between sister Sooty Copper butterflies *Lycaena bleusei* and *L. tityrus*

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ABSTRACT

The comparison of closely related taxa is cornerstone in biology, as understanding mechanisms leading up to differentiation in relation to extant shared characters are powerful tools in interpreting the evolutionary process. Hotspots of biodiversity such as the west-Mediterranean, where many lineages meet are ideal grounds to study these processes. We set to explore the interesting example of Sooty Copper butterflies: widespread Eurasian *Lycaena tityrus* (Poda, 1761) comes into contact in Iberia with closely related and local endemic, *L. bleusei* (Oberthür, 1884), which hasn't always been considered a distinct species. An integrative analysis was designed, combining the use of extensive molecular data (five genes), geometric morphometrics analyses, verified and up-to-date distribution data, and environmental niche modelling, aimed at deciphering their true relationship, their placement within European *Lycaena* and trace their evolutionary history. We revealed several levels of differentiation: *L. bleusei* and *L. tityrus* appear to be reciprocally monophyletic independent gene-pools, distinct in all genes analysed, having mutually diverged 4.8 Ma ago. *L. tityrus* but not *L. bleusei*, further displays a genetic structure compatible with several glacial refugia, where populations assignable to infraspecific taxa surface. Conversely, *L. bleusei* shows a loss in mtDNA diversity in relation to nuDNA. Morphological analyses differentiate both species according to size and shape but also discriminate strong seasonal and sexual traits and a geographical phenotype segregation in *L. tityrus*. Finally, updated distribution and its modelling for current and glacial timeframes reveal both species respond differently to environmental variables, defining a mostly parapatric distribution and an overlapping belt where sympatry was recovered. During the last glacial maximum, a wider expansion in *L. bleusei* distribution explains current isolated populations. Our study highlights the importance of gathering several lines of evidence when deciphering the relationships between closely related populations in the fringe of cryptic species realm.

1. Introduction

Understanding the processes underlying the current biodiversity patterns and how, why and when lineages differentiate and ultimately become species, is at the core of evolutionary biology. The comparison of closely related taxa allows inferring critical drivers and events leading up to their isolation and differentiation. Recently diverged populations, species or subspecies are still expected to show similarities in their

genetic structure, ecology and morphology as a result of their recently shared past. Even so, the evolutionary forces behind their differentiation may be strong enough to continuously reinforce the ecological and life-history traits responsible or leading to their isolation (Descimon & Mallet 2009).

In the western Mediterranean region, Plio-Pleistocene cyclic climatic changes have had a pronounced effect on communities and species, affecting both their distribution and their genetic structure (Hewitt,

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2001, 2004). In this context, the Iberian Peninsula (Southern Europe) has acted both as a refugium for many taxa and a diversity generator by providing conditions for their genetic isolation (Gómez & Lunt 2007). Here, contrasting geological and floristic diversity within a relatively wide area have provided species with a range of habitats, from alpine meadows to warm semi-deserts, to colonise, survive and adapt to (e.g. Henriques et al. 2018). As such, the Iberian Peninsula has become a well-known dynamic hotspot and repository of genetic diversity and a source-area for species to recolonise other regions following climate amelioration (Hewitt, 1996; Taberlet et al. 1998).

Mountain areas are hotspots of diversity and among the major promoters of biodiversity through time (Garrick, 2010). They typically offer habitat heterogeneity and an increase of available area, where species move along altitude gradients according to their tolerance to climate changes (Gutiérrez-Illán et al. 2010; Hewitt, 2011a). The Central Iberian Mountain System (CIMS) is among the widest and most biodiverse mountain ranges within the Iberian Peninsula (López-López et al. 2011). It bisects ENE-WSW Iberia, along a 550 km corridor comprising several smaller ranges, from Sierra de Pela (Guadalajara, Spain) to Serra da Lousã (Coimbra, Portugal). Structurally and geologically heterogeneous, it forms an island of water availability within a Mediterranean matrix and is both a stronghold for newly arrived temperate and cold-adapted taxa (López-Sáez et al. 2020), and a keeper of previous arrivals which may have since differentiated into endemic forms (Martin-Piera et al. 1992; Paulo et al. 2001; Jiménez-Valverde et al., 2008; Hewitt, 2011a). It is also one of the hypothesized major glacial/interglacial refugial areas for biodiversity within Iberia itself (Gómez & Lunt 2007; Hewitt, 2011b). Numerous CIMS endemism examples are found in animals and plants whose sister taxa are Euro-Siberian, temperate or mesophilic, distributed over the Cantabrian mountains or beyond the Pyrenees. Examples of such cases are lizards in the *Iberolacerta monticola* complex (Carranza et al. 2004; Crochet et al. 2004) and *Lacerta schreiberi* (Paulo et al. 2002; Godinho et al. 2008), the Spanish birthwort *Aristolochia castellana* (Costa, 2008), the Iberian shrew, *Sorex granarius* (García-Perea et al. 1997) and the Iberian Sooty Copper butterfly *Lycaena bleusei* (García-Barros et al. 2004).

The genus *Lycaena* (Papilionoidea, Lycaeninae) comprises more than 60 butterfly species of mostly Holarctic distribution, although some are also found in south Africa and New Zealand (Klots 1936; Bozano & Weidenhoffer 2001). Currently, the western Nearctic and Central Palaearctic regions are hotspots for *Lycaena* diversity, although the majority of Euro-Mediterranean species are widespread to central Asia and some reach Japan (Bozano & Weidenhoffer 2001). The only Western-European endemic species is the Iberian Sooty Copper, *Lycaena bleusei*.

Lycaena bleusei is a narrow endemic but still taxonomically contentious species characteristic of the CIMS and surrounding Iberian mountain ranges (García-Barros et al. 2004), where it replaces its putative sister species, the Sooty Copper, *Lycaena tityrus*. The latter is a widespread Palaearctic species recorded from northern Portugal and Spain to the Altai in Central Asia. *Polyommatus bleusei* Oberthür 1884 was originally described as a form of *Papilio tityrus* Poda 1761, upon specimens collected in the Sierra de Guadarrama near Madrid (TL: route to El Pardo and Escorial) (Oberthür 1884). Since its discovery, its status has shifted from race or subspecies of *L. tityrus* (Higgins & Riley 1970; Tolman & Lewington 2001; Lafranchis 2004; Asal 2008; Leraut 2016), to fully recognised species (Bozano & Weidenhoffer 2001; García-Barros et al. 2004; Kudrna et al. 2011; Wiemers et al. 2018). Despite an absence of in-depth studies focused on this species-pair, the morphological cladistic analysis of Cassulo et al. (1989) first hinted at their distinction at the species level through slight wing-pattern and genitalic differences, which later led (Bozano & Weidenhoffer 2001) to suggest raising this taxon to species rank. Only more recently, while carrying out a barcode meta-analysis of Iberian butterflies, have Dincă et al. (2015) provided the first genetic evidence of the two Sooty-Copper distinction.

Throughout its wide range, *L. tityrus* s.l. is a variable species with

many named forms (Oberthür 1910; Courvoisier, 1921; Koçak & Kemal 2007), of which the most differentiated is the Alpine endemic *L. t. sub-alpinus* (Speyer, 1851), often considered a species on its own or approaching reproductive isolation (Descimon, 1980; Parenzan & Porcelli 2006; Balletto et al. 2014; Litman et al. 2018). In parallel, *L. bleusei* displays an array of consistent morphological differences from all populations of *L. tityrus*: 1) relaxed sexual dimorphism, where both males and females possess a bright golden-copper forewing upper-side; 2) underside of male forewing with an orange hue, instead of uniform yellow/white background colour; 3) seasonal dimorphism, where springtime adults are lighter-coloured than summer ones, which bear a prominent hindwing tail-like projection following vein Cu2 (Fig. 1A). Particularly, the last character is nearly always absent in *L. tityrus* although this has never been properly addressed statistically. Both Sooty Coppers have largely overlapping ecological needs, occupying flower-rich meadows, light scrubland and fallow land by streams and water beds, with up to 4 annual generations between April and November (Verity 1948; García-Barros et al. 2014). Nevertheless, empirical evidence about *L. bleusei* (Vicente Arranz et al. 2015) suggests it to be more tolerant of strongly Mediterranean conditions, i. e. summer-dry meadows and more exposed situations, whereas *L. tityrus* is apparently displaced to areas only mildly suffering from summer drought.

Despite their ecological and phenotypical differences, both species' distributions have not yet been consistently addressed because of overall wing-pattern similarities and unresolved taxonomic status leading to non-informative literature in this matter. For instance, many Portuguese records of *L. tityrus* (Maravalhas 2003; García-Barros et al. 2004) prior to the first recognition of *L. bleusei* in the country (Marabuto et al. 2004) should be revised for correct species assignment. The same happens in Spain where only recently has the research on the latter species gained traction (Asal, 2008; Muñoz Sariot 2011), but still mostly at a provincial scale (Pérez De-Gregorio et al. 2010; Vicente Arranz & Arjona 2010; De Arce Crespo & Sánchez-Fernández 2013; Vicente Arranz et al. 2015, 2016; Hernández Martín & Vicente Arranz 2016; Pérez Fernández 2018; Vázquez-Maza 2018). What most of these works however reveal is an important knowledge-gap regarding eventual contact zones, and the extremely local approaches taken. There is a need for a broader, integrative analysis covering their full Iberian distribution and the exploration of several aspects concerning their possible interaction in the Iberian Peninsula: from the genetic, morphological and ecological points of view.

In this study, we aim at deciphering how closely related are in fact *Lycaena tityrus* and *Lycaena bleusei*, focusing on the region where they come into contact, the Iberian Peninsula. We start off by genetically framing both species among their European *Lycaena* relatives. Then, we define how differentiated and diverse are both taxa at the genetic level, and when did differentiation occur, using a combination of molecular markers, both nuclear and mitochondrial. We further aim at substantiate that comparison including other lines of evidence and tools such as geometric morphometrics and species distribution modelling, to comprehend their morphological, geographical and ecological space. Through these, we aim at deciphering their evolutionary histories, and how they have coped with climate-change in relation to past refugial hypothesis. Further important questions to address are to what extent do these two Sooty Coppers actually interact and if there is ongoing gene-flow. Does *L. bleusei* merit species status? What causes may be behind their distribution and differentiation? These questions may only be properly tackled through the multi approach integrative study we followed here.

2. Material and methods

2.1. Sampling, genetic markers and sequencing

To frame *Lycaena bleusei* and *L. tityrus* within the European *Lycaena* species, our dataset also includes genetic samples of: *Lycaena helle*,

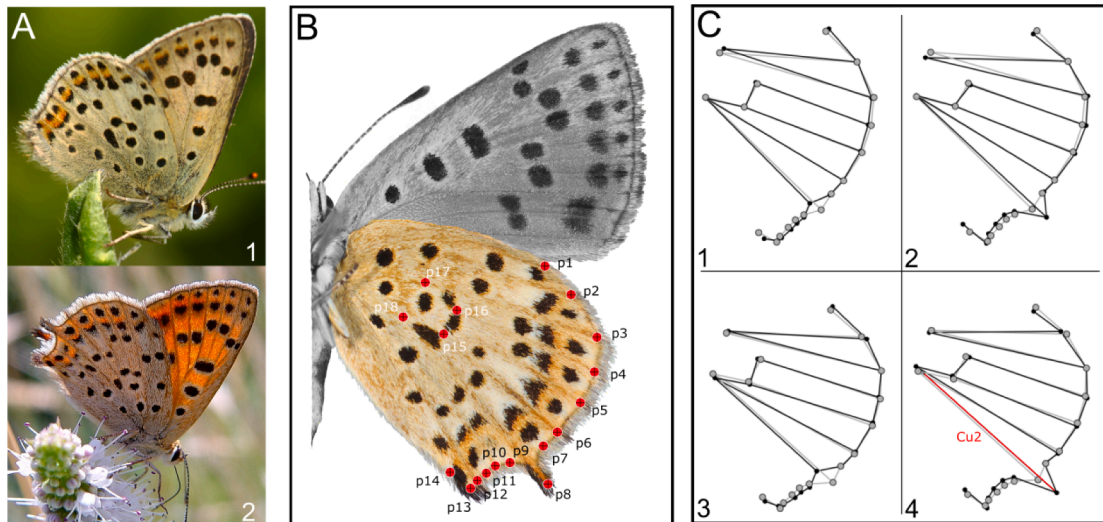


Fig. 1. Phenotype analysis. (A): habitus of (A1) *Lycaena tityrus* and (A2) *Lycaena bleusei*, (B) Landmark distribution; (C) Obtained shape for each category: (C1) - *L. tityrus* males, (C2) - *L. bleusei* males, (C3) - *L. tityrus* females and (C4) - *L. bleusei* females, superimposed to average shape of respective gender. Cu2 vein is highlighted in red in figure C4.

L. phlaeas, *L. dispar*, *L. ottomanus*, *L. virgaureae*, *L. alciphron*, *L. hippothoe*, *L. candens*, *L. thersamon* and the North African *L. phoebus*. Both focal species were collected throughout their distribution range, but most systematically in the Iberian Peninsula (Fig. 2). Specimens were collected alive using an entomological net and kept at -20°C until DNA extraction. In each sampling site, specimens were assigned to species, either *L. bleusei* or *L. tityrus* according to their phenotype and above mentioned main distinguishing features. Sampling sites span not only previously known areas of occurrence, but also potential contact zones and gaps between published distribution. Most DNA sequences have been directly obtained for this study upon newly collected samples, but these were complemented by publicly available datasets from NCBI Genbank, Barcode of Life (BOLD) or Wiemers et al. (2019). Overall, we

used genetic data of 60 *L. bleusei* specimens, of which 11 sequences were taken from Genbank/ BOLD, and 106 *L. tityrus* (52 from Genbank/ BOLD).

DNA extraction was performed on two legs per specimen with an E.Z. N.A® Tissue DNA Kit (Omega, Biotek), following manufacturer's protocol. Amplification of five genes was carried out: 5' cytochrome c oxidase subunit I, barcode fragment (COI), 16S rRNA (16S), carbamoylphosphate synthase domain protein (CAD), elongation factor-1 α (EF-1 α) and wingless (wg) with already published primers (Table S1).

For COI and 16S we used a standard Polymerase Chain Reaction (PCR) protocol: initial denaturation step of 5 min at 94°C , followed by 35 cycles at 94°C for 30 s, 53°C for 45 s, 72°C for 1 min with a final extension step of 72°C for 7 min. Final volume was of 20 μL , comprising

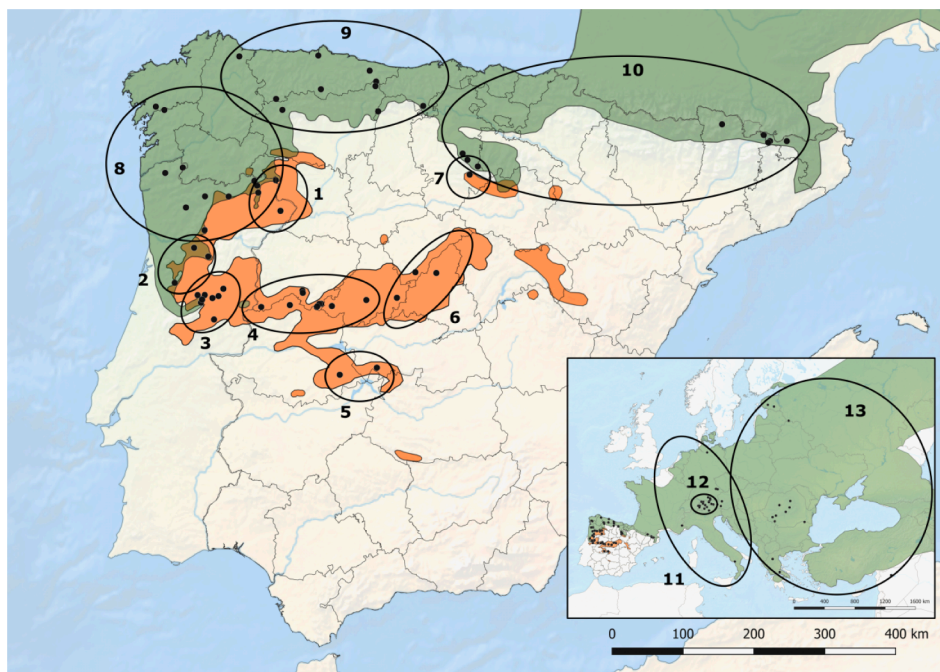


Fig. 2. Sampling localities (black dots) and AMOVA groups: Western Iberia (WB): 1) Bragança, 2) Beira, 3) Estrela, 4) Gredos, 5) Toledo, 6) Guadarrama, 7) Burgos, 8) Douro-Galicia, 9) Cantabrian. 10) Eastern Iberia (EB); 11) Western Europe (WE); 12) *L. t. subalpinus*; 13) Eastern Europe (EE). Populations 3, 4 and 6 are within the Central Iberian Mountain System (CIMS). Distribution: Green - *L. tityrus*, Orange: *L. bleusei*.

2.5 μ L DNA (10–50 ng/ μ L), 4 μ L Colorless GoTaq® Flexi Buffer, 0.16 μ L GoTaq® DNA Polymerase, 1.44 μ L 1.8 mM MgCl₂, 0.8 μ L 0.1 mM of dNTPs and 0.8 μ L 4 μ M of each primer. Nuclear genes EF-1 α , wg and CAD were amplified according to the following protocol: an initial denaturation step of 94° C for 7 min, followed by 35 cycles of 94° C for 20 s, 56° C for 30 s, 72° C for 40 s with a final extension step of 72° C for 7 min. PCR products were checked on 1 % agarose gel, stained with 2x Red Safe™ Nucleic Acid Staining Solution (iNtRON Biotechnology, Inc), and fragments were then purified using SureClean (Bioline), following manufacturer's protocol. Samples were sequenced by Macrogen Inc. Europe (Amsterdam, the Netherlands), and chromatograms were checked and corrected in Sequencher v.4.0.5 (Gene Codes Co.). Nucleotide ambiguities, considered as heterozygous sites in nuclear genes, were classified according to IUPAC ambiguity codes. All sequences were aligned using MAFFT online (Katoh et al. 2019) and trimmed in BioEdit (Hall, 1999). For the nuclear sequences, the phase of heterozygous ambiguities was determined using SeqPhase online and PHASE v.2.1.1 (Stephens et al. 2001). A full list of specimens used is available in Table S2, alongside relevant associated data and Genbank accession numbers referring to sequences.

2.2. Phylogenetic reconstructions and divergence-time estimates

We constructed a comprehensive phylogeny of Euro-Mediterranean *Lycaena* in order to frame *L. bleusei* and *L. tityrus*. An alignment of 13 *Lycaena* species, two outgroups (*Lampides boeticus* and *Hamearis lucina*) and five genes (16S, COI, CAD, EF-1 α , wg – 3061 bp.) was produced using Concatenator v.1.1.0 (Pina-Martins & Paulo 2008) (Table 1). Maximum likelihood (ML) and Bayesian inference (BI) methods were used independently: ML was accomplished using RAxML v4.0b10 (Stamatakis 2014) implemented in the raxmlGUI1.5b2 (Silvestro & Michalak 2012) under a GTR Gamma model. BI was implemented in MrBayes v.3.2.6 (Ronquist et al. 2012), where the best fitting nucleotide substitution models were selected according to jModelTest v.2.1.7 (Darriba et al. 2012), under the corrected Akaike's Information Criterion (AIC): GTR + G + I for COI & 16S, SYM + G + I for EF-1 α , TIM for CAD, and SYM + G for wg. Both analyses were performed with multiple replicates: 10,000 bootstrap for ML and two runs of four Monte Carlo Markov Chain (MCMC), iterated for 5 M generations for BI. Bayesian posterior probabilities (bpp) were calculated using the 'sumt' command of MrBayes. The five-gene combined dataset was also subjected to a partitioned Bayesian analysis implemented in BEAST v2.6.2 (Bouckaert et al. 2014)

to infer divergence times among taxa. As evolutionary models were already attributed by jModeltest (COI & 16S: GTR + G + I; EF-1 α : TIM2 + G + I, CAD: TIM2, wg: TIM2 + G), these were implemented here as well. A relaxed clock, log normal model, was assumed to every partition, where we applied different clock rates for each gene: COI – 0.0115; 16S – 0.0086; EF-1 α – 0.001277; Wingless – 0.007044 and an estimated 0.00253 for CAD. Partitions were linked to share the same tree topology. Tree prior was set to the Birth Death Model, while all the remaining parameters were left as default. Although there are no known fossil records of *Lycaena*, recent phylogenomic studies in butterflies estimate a MRCA of ~ 90 Ma between the Riodinidae (*Hamearis lucina*) and Lycaenidae, and of ~ 60 Ma between *Lampides boeticus* and *Lycaena* (Espeland et al. 2018; Kawahara et al. 2019). These calibration points were used, with monophyly, and uniform distributions between [92–88] and [63–57] Mya. The analysis was run six times for 20 million generations of MCMC each, sampling every 1 k iterations. Validation of each run quality and the concatenation of the six runs with 10 % discarded as burn-in was obtained with Tracer 1.7 (Rambaut et al. 2018). Tree annotation was carried out in TreeAnnotator of the BEAST software package, and finally edited in FigTree. *Hamearis lucina* (Riodinidae) and *Lampides boeticus* (Lycaenidae, Polyommatainae) were used as outgroups for all phylogenetic analyses.

Haplotype networks under the TCS method were constructed in PopArt v.1.7 (Leigh & Bryant 2015) for *L. tityrus* and *L. bleusei* using COI (N = 166) and EF-1 α (N = 102) datasets and including both species in the same graphics. The COI dataset was further employed in population genetic differentiation analyses of molecular variance (AMOVA), nucleotide diversity (π), haplotypic diversity (h) and F_{ST} pairwise distances in ARLEQUIN v.3.5 (Excoffier et al. 2005). In AMOVA and pairwise F_{ST} analyses, population groups were assembled based on geographical proximity of sampling sites or species, according to perceived geographical structure (Fig. 2). Population groups of *L. tityrus*, *L. tityrus subalpinus* and *L. bleusei* were aggregated in different hierarchical combinations to infer the effect of their shifting ranking, for instance, considering *L. bleusei* as a population of *L. tityrus*. Finally, we also performed a pairwise F_{ST} considering *L. bleusei* as having the same rank as *L. t. subalpinus* and *L. t. tityrus*.

2.3. Morphological analysis

A total of 182 specimens from Iberia and other European locations were chosen for geometric morphometrics analysis: n = 82 *L. bleusei* and

Table 1

Gene coverage within the ingroup and outgroup taxa used in the present study, with Genbank accession numbers and source.

Gene positions	COI	16S	EF-1 α	wg	CAD
<i>Lycaena helle</i>	1–658 JF847995 Dincă et al. 2015	659–1243	1244–1834	1835–2211	2212–3061
<i>L. phlaeas</i>	HQ004663 Dincă et al. 2011	JX262887 Zhang et al. 2013	OP622814 this study FJ490517 Oliver & Stein 2011	OP622835 this study	OP622841 this study
<i>L. dispar</i>	GU372564 (Kim et al., 2010)	GU372473 (Kim et al., 2010)	GU372655 (Kim et al., 2010)		
<i>L. tityrus</i>	(See Table S2) this study	(See Table S2) this study	(See Table S2) this study	OP622831 this study	OP622838 this study
<i>L. bleusei</i>	(See Table S2) this study	(See Table S2) this study	(See Table S2) this study	(See Table S2) this study	(See Table S2) this study
<i>L. ottomanus</i>	OP601562 this study				
<i>L. virgaureae</i>	FJ490477 Oliver & Stein 2011	OP601628 this study	OP622815 this study	OP622832 this study	
<i>L. alciphron</i>	(See Table S2) this study	(See Table S2) this study	(See Table S2) this study	OP622833 this study	OP622839 this study
<i>L. hippothoe</i>	GU688462 Hausmann et al. 2011	OP601631 this study	OP622818 this study	OP622834 this study	OP622840 this study
<i>L. candens</i>	AY556890 (Wiemers & Fiedler 2007)		KJ671879 (Vodolazhsky & Stradomsky 2012)	Wiemers et al 2019	
<i>L. thersamon</i>	HQ004672 Dinca et al 2011		OP622819 this study		
<i>L. thetis</i>	AY557116 (Wiemers 2003)		OP622820 this study	Wiemers et al 2019	
<i>L. phoebus</i>	OP601565 this study		OP622821 this study		
<i>Lampides boeticus</i>	GQ129023 Vila et al. 2011	GU372493 (Kim et al., 2010)	GQ128712 Vila et al 2011	GQ128924 Vila et al 2011	Wiemers et al 2019
<i>Hamearis lucina</i>	DQ018953 Wahlberg et al 2005		DQ018920 Wahlberg et al 2005	KT285949 Espeland et al. 2015	KT286300 Espeland et al. 2015

$n = 100$ *L. tityrus*. We focused on identifying differences in butterfly hindwing shape using landmark-based geometric morphometric procedures. We used a combination of 18 type I landmarks (Bookstein, 1991) applied to both vein intersections with the wing margin and around the cell of the left hindwing on each specimen (Fig. 1B). The hindwing underside was chosen for its clear pattern and discriminant power in differentiating both species, where a small tail-like projection is present along the Cu2 of most *L. bleusei* individuals. Chosen landmarks follow Zelditch et al. (2004) criteria of independence, homology among specimens and ease of identification. Similar landmarks have previously been used in butterflies (Breuker et al. 2010).

For each specimen, a picture was taken on a fixed set and the acquisition of two-dimensional coordinates of these landmarks was accomplished using FIJI (Schindelin et al., 2012). To reduce measurement error, specimens were digitised and processed always by the same user. Analysis of shape and size were implemented with the R package geomorph v.3.2.1 (Adams & Otárola-Castillo 2013). Raw coordinates were superimposed using a Generalized Procrustes Analysis (GPA) to standardise the size and to translate and rotate the configurations of landmark coordinates (Rohlf & Slice 1990; Goodall, 1991; Klingenberg & McIntyre 1998). The resulting superimposed shape coordinates are size free but not allometry free. We used centroid size (CS), an isometric size estimator defined as the square root of the sum of the squared distances between the center of the configuration of landmarks and each separate landmark (Bookstein, 1991), to analyse wing size variation. Centroid sizes of different groups were compared by means of ANOVA and pairwise t-tests. The relationship between wing shape and size (allometry) was examined through multivariate analysis of covariance (MANCOVA) using log-transformed centroid size, species, sex and their interactions as full model, with randomised residual permutation procedures (1000 permutations) and post-hoc pairwise slope comparisons. To test for shape differences between groups, we used MANOVA with pairwise tests, using centroid size as a factor to account for the allometric effect. Principal Component Analysis (PCA) were also conducted for a better visualization of data.

2.4. Distribution records and modelling

To evaluate differences in the distribution of both *L. tityrus* and *L. bleusei* in Iberia alongside changes in their Iberian distribution ranges in relation to Quaternary climatic fluctuations, we performed species distribution modelling for both species independently, using maximum entropy analysis in MAXENT v.3.4.1 (Phillips et al. 2006), at two timeframes: Present day and during the Last Glacial Maximum (LGM).

Because of the caveats associated with both taxa not always having been consistently considered independent, published occurrence data had to be thoroughly scrutinised and filtered beforehand. On one hand, the most informative published sources are García-Barros et al. (2004) and its update in García-Barros et al. (2014) but uncertainty is cast upon many of the records because of no discrimination between species. From this study, *L. bleusei* was considered for data-points in the Spanish CIMS from Ayllón to Gata ranges, Moncayo range, and Castilla la Mancha and Cáceres provinces, but not from Portugal and Badajoz. *L. tityrus* was considered for data coming from Catalonia to Galicia, Zamora, Burgos, and La Rioja provinces but not for Portugal. We also considered many of the source references of García-Barros et al. (2004) and other of later publication, especially if relating explicitly to *L. bleusei* (Jiménez-Valverde et al. 2004; Marabuto et al. 2004; Monteiro 2006; Vicente Arranz & Arjona 2010; Díaz-Regañón, 2012; De Arce Crespo & Sánchez-Fernández 2013; Blázquez-Caselles, 2014; Vicente Arranz et al. 2015, 2016; Hernández Martín & Vicente Arranz 2016; Fernández Vidal, 2017; Martínez-Pérez et al. 2022). In fact, as there was an uncertainty to the species assignment of previously published Portuguese records, all presence points for Portugal before (Marabuto et al. 2004) were omitted.

Next, we aimed at gathering all possible unpublished evidence of both *L. tityrus* and *L. bleusei* in Portugal and Spain. This was

accomplished in a three-phase process: 1) through the access to online databases (GBIF, [Observation.org](https://www.gbif.org)), and new field-work by 2) the authors and 3) colleagues (see acknowledgements). Throughout the data acquisition process, each potential record was scored to species by the first author. Point distribution data was then converted to 10 km² quadrats fitting to the MGRS grid to be plotted into a distribution map.

Finally, we used 19 bioclimatic variables from WORLDCLIM (<https://www.worldclim.org>) (Fick & Hijmans 2017), clipped to the western Mediterranean area. For the estimation of current potential distribution of species, we ran 5 bootstrap replicates using 75 % of species localities as training data, and the remaining 25 % to test the model. Then, assuming niche conservatism through time we projected the resulting distribution models to past climatic conditions, namely the last climatic cycle (Nogués-Bravo 2009). Projections to the LGM (ca. 21 kyr before present) were performed using paleoclimate data obtained from the Community Climate System Model (CCSM4; Gent et al. 2011) and the MaxPlanck-Institut Earth System Model (MPI-ESM-P; Giorgetta et al. 2013) of atmospheric circulation, as available in WORLDCLIM.

3. Results

3.1. Molecular data

The five-gene phylograms obtained for European *Lycaena* species placed *Lycaena tityrus* and *Lycaena bleusei* as clearly independent sister clades with good support (Fig. 3, Figure S1 A and B). *L. bleusei* and *L. tityrus* show no genetic overlap in any of the studied markers, displaying pairwise distances of 2.3 – 3.1 % for COI, 1.6 – 2.4 % for 16S, 0.9 – 1.9 % for EF-1 α , 2.1 % for wg, and 0.9 % for CAD. COI (Barcode) data's p-distance to other species is much superior, > 3.3 % (Table S3). The most closely related taxon to the focal pair is consistently *L. virgaureae* (5 genes, nuDNA), although this relationship was not recovered in the COI-only tree (Fig. S1C), which delivers a poorly supported *L. virgaureae* + (*L. hippothoe* + *L. candens*) clade, sister to *L. tityrus*, whereas *L. bleusei* is here placed as a poorly supported sister to *L. ottomanus*.

The Bayesian phylogenetic tree obtained with BEAST (Fig. 3) delivers a primary divergence among European Copper butterflies at around 14 mya (95 % HPD: 16.51 – 31.65 mya). The divergence between the ancestor of the Sooty Coppers and its putative sister taxon is timed at having occurred around 9.9 mya (95 % HPD: 6.37 – 13.85 mya) while that between *L. tityrus* and *L. bleusei* is estimated to have occurred around 4.8 Mya (95 % HPD: 3.14 – 10.32 mya). Intraspecific divergence in both taxa is much more recent, as all extant *L. tityrus* haplotypes coalesce to <1 My ago, and the same applies to *L. bleusei*.

3.1.1. Genetic diversity and structure in the *Lycaena tityrus* group

COI and EF-1 α haplotype networks and diversity indices for both *L. tityrus* and *L. bleusei* delivered a concise picture of the diversity within each taxon (Fig. 3B). This method also highlights a clear separation of both gene pools by seventeen mutational steps between the two coppers in COI, and six in EF-1 α . *Lycaena tityrus* samples from across Europe ($n = 106$ on COI; $n = 52$ on EF-1 α), coalesce into ten mtDNA COI, and seven nuDNA EF-1 α haplotypes. In Iberian endemic *L. bleusei* ($n = 60$ on COI; $n = 51$ on EF-1 α), we found only two mtDNA COI haplotypes, while it also has seven haplotypes in EF-1 α but in neither case do they present a geographic structure.

L. tityrus shows geographic structure for both COI and EF-1 α haplotypes (Fig. 4). COI haplotypes T1 and T2 are exclusive to NW Iberia where the first is found in higher frequency; T6 and T7 are associated with *L. t. subalpinus* and limited to the Alps at high altitudes; T3 is widespread in Europe, extending from Estonia and Russia to eastern Iberia. Haplotype T8 is confined to south-eastern Europe, and the remaining T4, T5, T9 and T10 are represented by singletons from Germany, Greece and Romania, respectively. EF-1 α geographic structure is less defined but five EF-1 α haplotypes were found restricted to the Iberian Peninsula, either widespread (T1 and T2) or confined to the NW

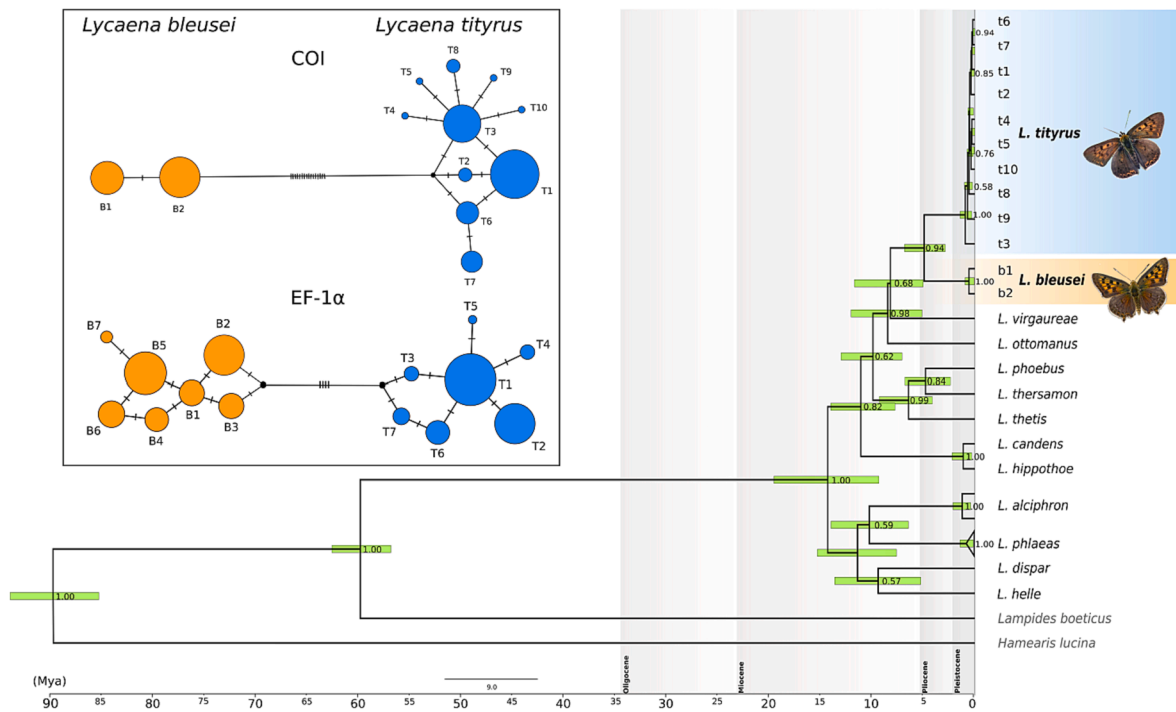


Fig. 3. Bayesian inference chronogram of the concatenated 5 gene dataset (COI, 16S, EF-1 α , wg, CAD). Posterior probabilities > 0.60 indicated. Green bars over nodes indicate the 95 % HPD range for the posterior distribution of node ages. Inset: Maximum parsimony haplotype networks based on COI and EF-1 α markers of both species. Mutations are scored as hatch-marks. Circle size is proportional to the representation of each haplotype in the genetic pool sampled.

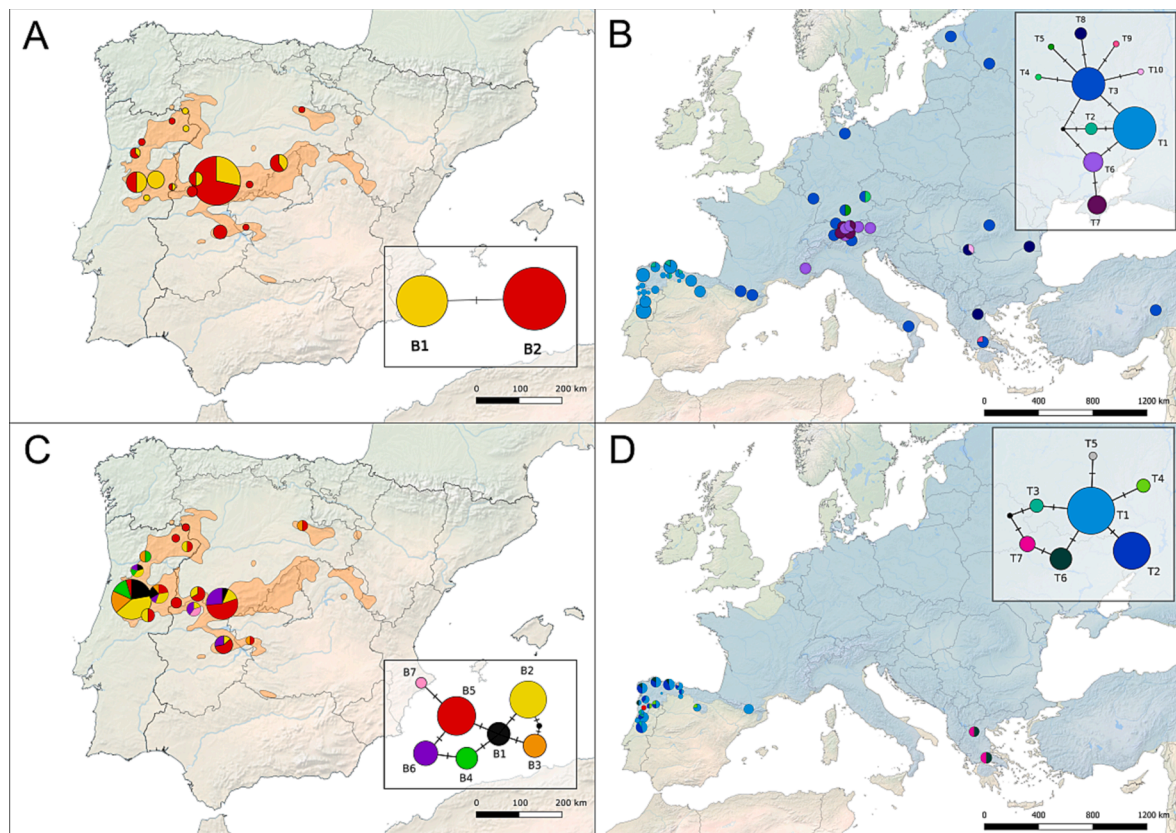


Fig. 4. Geographic distribution of COI and EF-1 α haplotypes. (A) *L. bleusei*, COI; (B) *L. tityrus* COI; (C) *L. bleusei*, EF-1 α ; (D) *L. tityrus* EF-1 α : Note red samples representing introgressed *L. tityrus* individuals with *L. bleusei* haplotype B5.

(T3-T5) while one of the haplotypes is shared with Greek samples (T6). In *L. bleusei*, neither COI nor in EF-1 α show a geographical genetic structure, precluding a sole panmictic population.

Haplotype networks show high congruence among species, marker and geographical location of samples except for two individual butterflies (LTI32, LTI33; Table S2), which present a mito-nuclear discordance. These correspond to two male specimens morphologically and barcode determined as *L. tityrus*, but bearing EF-1 α haplotypes B2/B4 (after phase determination) of *L. bleusei*. These specimens were collected on the same occasion and meadow within a *L. tityrus* exclusive area (Fig. 4D), although not far from known *L. bleusei* range. No other samples display such marker mismatch, not even the many sampled within sympatric populations. We thus consider these specimens as likely naturally admixed/ hybrids of both species. Haplotype and geographical assignment of samples is detailed in Supplementary Material (Table S2).

3.1.2. Population genetic differentiation

We defined groups of sampling sites into discrete populations, according to their geographical distance (Fig. 2, Table S2). Within the *L. tityrus* population groups, the highest haplotype (h) and nucleotide (π) diversity values were found in “Eastern Europe”, “Eastern Iberia” and “*L. t. subalpinus*”. In *L. bleusei*, the highest values were recovered for populations “Bragança” and “Guadarrama” (Table S5).

Next, all COI sequences of *L. tityrus* and *L. bleusei* were considered for analysis of molecular variance (AMOVA). We defined nine AMOVA iterations varying in the grouping of populations, aiming at inferring the changing importance of populations or taxa for the whole gene-pools. Three *a priori* taxonomic entities were considered: *L. tityrus tityrus*, *L. t. subalpinus* and *L. bleusei*, and these were either given the same rank or sunk as populations of *L. tityrus*, so that the results would reflect the effect of these changes on the whole AMOVA (Table 2). Among all hierarchical arrangements, the highest percentage of variation was achieved in AMOVA 2 (slightly above AMOVA 3 and 4), i. e. when considering four main units (*L. tityrus* Iberia, *L. tityrus* Non Iberia, *L. bleusei* and *L. t. subalpinus*), while the lowest variation among groups was obtained with AMOVA 5, i. e. considering *L. bleusei* as a mere population of *L. tityrus*. Finally, individual AMOVA for each species are shown in AMOVA 6–9: Within-species AMOVA for *L. tityrus* shows higher percentage of explained variance for the grouping [*L. tityrus* Iberia; *L. tityrus* (WE + Catalonia, EE)]; *L. t. subalpinus* (Table 2). F_{ST} , as a measure of population differentiation, shows a convergent result to the AMOVA set: greatest differentiation between *L. bleusei* and *L. tityrus* s.l. (0.965 and 0.970) (Table S4) than between any conspecific populations, independent of species. In fact, pairwise population differentiation (Table S4) shows high F_{ST} values (>0.6) between populations from these three groups. “Western Europe” and “Eastern Europe” are genetically very similar ($F_{ST} = 0.05$), while Iberian Peninsula populations are highly differentiated from other European populations ($0.6 < F_{ST} < 0.8$). “Eastern Iberia” stands at an intermediate (genetic and geographical) position between west Iberian populations ($0.4 < F_{ST} < 0.5$) and the rest of Europe ($F_{ST} \sim 0.2$). Western Iberian populations (Estrela, Beira and Douro-Galicia) are genetically very similar ($F_{ST} < 0.02$). *L. t. subalpinus* always stands out for its high differentiation against all other populations (>0.6). No such results could be obtained for *L. bleusei*, as the species is poorly variable in its mtDNA and not geographically structured.

3.2. Morphological data

3.2.1. Adult morphology

After a literature review, new field-work data and graphical evidence available on online databases GBIF.org and Observation.org, we noted that besides the existence of a well-marked, discrete character allowing for easy discrimination between *L. bleusei* and *L. tityrus*: the presence of a hindwing tail-like projection along the Cu2 vein, *L. tityrus* males of Iberian populations are also dimorphic. Although subject to variability,

Table 2

Analysis of Molecular Variance. (A) Multispecies approach used to ascertain the effect of *L. bleusei* and *L. t. subalpinus* positioning on population structure, whether as a mere *L. tityrus* population (AMOVA 1, 4) or as major groups (AMOVA 2, 3). (B) Each species AMOVA (6–9) made under different grouping to ascertain best population clustering. Key: DoF: Degrees of Freedom; SS: Sum of Squares; Var: Variance components; % Var: Variation explained. Fct: Variation among groups; Fsc: Variation among populations within groups; Fst: Within populations. WB: Western Iberia, EB: Eastern Iberia, WE: Western Europe, EE: Eastern Europe.

A	DoF	SS	Var.	% Var.	
AMOVA 1					
	1	1,32,259	−0.22925	−4.02	Fct
● Iberia (WB, EB, <i>L. bleusei</i>)	4	5,62,937	5.71293	100.25	Fsc
● Non Iberia (WE, EE, <i>subalpinus</i>)	160	34,418	0.21511 Vc	3.77	Fst
AMOVA 2					
● <i>L. tityrus</i> Iberia (WB, EB)	3	6,91,956	5.90637	95.03	Fct
● <i>L. tityrus</i> Non Iberia (WE, EE)	2	3,240	0.09357	1.51	Fsc
● <i>L. bleusei</i>	160	34,418	0.21511 Vc	3.46	Fst
● <i>subalpinus</i>					
AMOVA 3					
● <i>L. tityrus</i> (WB, EB, WE, EE)	2	6,77,416	6.83778	92.75	Fct
● <i>L. bleusei</i>	3	17,780	0.31959	4.33	Fsc
● <i>subalpinus</i>	160	34,418	0.21511 Vc	2.92	Fst
AMOVA 4					
	1	6,60,988	8.33235	92.63	Fct
● <i>L. tityrus</i> (WB, EB, WE, EE, <i>subalpinus</i>)	4	34,208	0.44824	4.98	Fsc
● <i>L. bleusei</i>	160	34,418	0.21511 Vc	2.39	Fst
AMOVA 5					
	1	54,644	−2.33984	−57.46	Fct
● <i>L. tityrus</i> (WB, EB, WE, EE, <i>L. bleusei</i>)	4	6,40,552	6.19677	152.18	Fsc
● <i>subalpinus</i>	160	34,418	0.21511 Vc	5.28	Fst
B					
AMOVA 6					
	1	16,428	0.32017	38.16	Fct
● <i>L. tityrus</i> (WB, EB, WE, EE)	3	17,780	0.32053	38.21	Fsc
● <i>subalpinus</i>	101	20,018	0.19820 Vc	23.63	Fst
AMOVA 7					
● <i>L. tityrus</i> (WB, EB, WE, EE, <i>subalpinus</i>)	4	34,208	0.44915	69.38	Fsc
	101	20,018	0.19820	30.62	Fst
			Vb		
AMOVA 8					
● <i>L. tityrus</i> Iberia (WB, EB)	2	36,265	0.54091	73.60	Fct
● <i>L. tityrus</i> (WE + Catalonia, EE)	1	0.553	0.02334	3.18	Fsc
● <i>subalpinus</i>	102	17,409	0.17068 Vc	23.22	Fst
AMOVA 9					
● <i>L. bleusei</i> (Estrela, Beira, Bragança, Gredos, Guadarrama, Toledo, Burgos)	6	2,775	0.03336	13.20	Fsc
	53	11,625	0.21934	86.80	Fst
			Vb		

these two phenotypes can be ultimately distinguished by the presence of copper-coloured scales on the cell-area of the male forewing upper-side. A review of this character throughout the available dataset (see distribution data below) reveals a geographical structure to it (Figure S2):

- 1) Phenotype 1, shows no copper-scales on the cell-area of the male forewing upper-side. It occurs from the eastern Pyrenees westwards to Sierra de la Demanda and Picos de Europa and is predominant or exclusive in Catalonia and the Pyrenean mountain range but decreases its frequency westwards. It can be further characterised by a strong sexual dimorphism, where males are of a uniform dark upper-side, at best with a grey sheen on the central part of the forewing, and where the black spots stand out. Both wings tend to have a row of submarginal orange markings of variable extent. Females are of the usual copper phenotype (Bozano & Weidenhoffer 2001). The earliest available name to this population is *Palaeoloweia tityrus pallidepicta* Verity, 1934, described from southern France (TL: Mont Ventoux), but also *Heodes tityrus catherinei* Verity 1948 (TL: Cauterets, Pyrenees). However, the characters used to distinguish both these populations from nominal *L. t. tityrus* (TL: Vienna, Austria) (Verity 1934, 1948) are subject to much variation and are not geographically or ecologically structured, thus we suggest both to be junior synonyms of *L. t. tityrus*.
- 2) Phenotype 2 is characterised by the presence of light-copper / rust coloured scales on the central part of the upper-side male forewing. These may be restricted to the cell or fill the entire forewing. Like the previous phenotype and unlike *L. bleusei*, this form also shows a strong sexual dimorphism, where males are still predominantly dark brown on the upper-side and females are nearly always light coloured orange. It was recorded as the only phenotype in Portugal, Galicia, Asturias and León. It further extends in sympatry with phenotype 1 eastwards to Sierra de la Demanda and into western France. The oldest available name and only one unequivocally attributed to these populations is that of *Palaeoloweia tityrus praebleusei* Verity 1934 (TL: Puerto de Pajares, Asturias, Spain). At the time of description it was considered phenotypically intermediate between the southern French populations (*L. t. pallidepicta*) and *Lycaena bleusei*, then considered a subspecies of *L. tityrus* (Verity 1934). We consider this form as a valid morphological entity at the subspecies level.

García-Barros et al. (2014), considered *L. t. subalpinus* to be the only *L. tityrus* taxon to occur in Iberia. *L. t. subalpinus* is found in the Alpine chain (TL: Patscherkofel, Austria) and is characterised by an univoltine life-cycle with adults appearing in high summer, occurrence only above 1400 m and a characteristic phenotype with a deep dark-brown upper-side on all wings of both sexes. In this taxon, only the cell black spots stand out over the background, and no marginal orange markings are present, while the underside is grey and almost deprived of orange submarginal spots (Descimon, 1980; Bozano & Weidenhoffer 2001; Litman et al. 2018). We found no morphological (photographic) or genetic evidence of the presence of this subspecies within the studied area, although some high altitude specimens (>2000 m a.s.l.) in the Pyrenees do appear concolourous dark brown with no trace of orange on the upper-side. However, females of corresponding populations bear orange markings, and both sexes' undersides are coloured as other populations from lowland areas. Such phenotype corresponds to the description of *L. t. catherinei* (Verity, 1948) but there are no clear morphological or distributional boundaries between these populations and lowland ones (Descimon, 1980).

3.2.2. Geometric morphometrics

L. bleusei and *L. tityrus*' morphological differentiation was assessed through geometric morphometrics applied to the hindwing shape and size. The results of the MANCOVA for allometry revealed a group-specific allometric pattern (unique allometry) ($F = 2.27$, $df = 1$, $P =$

0.006), however the effect size ($Z = 2.45$) and variation ($R^2 = 0.028$) explained by the interaction of size and group is small and negligible. Post-hoc pairwise comparisons only showed a marginal difference between the females of both species in slope direction ($Z = 1.9$; $p = 0.03$). For this reason, we chose to work with a common allometry model in the remaining analysis. Our results suggest that all a priori groups differ from each other in wing shape ($F = 7.41$; $df = 4$; $p = 0.001$, $R^2 = 0.27$; $Z = 7.41$).

Pairwise comparison of wing-shape shows the greatest differences between *L. tityrus* males and *L. bleusei* females, with a Procrustes distance (Pd) of 0.08, while that between males of different taxa is lowest (Pd = 0.04). A graphical comparison between the two coppers' wing shapes reveals the major differences in wing silhouettes and tail projection (Fig. 1C).

The ANOVA analysis on centroid size shows significant differences between groups ($F = 36.24$; $df = 3$; $P < 0.001$), corroborated by pairwise *t*-test with Bonferroni correction, except for males and females of *L. tityrus* ($p = 0.9$). Females of *L. bleusei* display the largest wing size (average centroid size of 1871), followed by conspecific males (avg. centroid = 1684) and both are significantly different from both male and female of *L. tityrus*: smaller winged with centroid sizes of 1511 and 1561, respectively (Fig. 5A). The analysis also delivers a seasonal influence on overall centroid size, with a stronger effect on *L. tityrus* (spring generation significantly larger), than for *L. bleusei* (no significantly different males, slightly larger spring females). A PCA of size and shape delivered by landmarks shows slightly different morphological spaces occupied by both species, where PC1 (50 % variability) and PC2 (12 %) show a major overlap of both taxa, while PC3 (11 %) reasonably separates them and reveals *L. bleusei* as the most variable taxon (Fig. 5B).

3.3. Distribution data

3.3.1. Current Iberian distribution of Sooty Coppers

GBIF database at the time of data acquisition included 37,428 geo-referenced records of *Lycaena tityrus* and 338 of *L. bleusei*, of which 685 of the former and all of the latter are of Iberian origin (Portugal, Spain, Andorra) (GBIF.org 2020b; a). However, visual inspection of all *L. tityrus* records with associated pictures revealed 83 records classified as *L. tityrus* to correspond in fact to *L. bleusei*. Therefore, the final dataset from this source encompasses 602 *L. tityrus* and 421 *L. bleusei* records. Literature review (see Materials and Methods) and new field-work yielded a further 1094 records (547 *L. tityrus*, 547 *L. bleusei*) from the Iberian Peninsula. As to account for the absence of resolution for many of the available GBIF / literature records we standardised the 2094 usable records into 10 km MGRS quadrats. The final dataset encompasses 429 such 10 km presence quadrats for *L. bleusei* and 489 quadrats for *L. tityrus*. These were used in the production of the distribution map (Fig. 6) and in the species distribution modelling.

L. bleusei is widespread in the CIMS of both Portugal and Spain, but also along its satellite ranges of Guadalupe and Toledo mountains to the south and the Iberian System to the east, reaching Albarracín and Sierra de la Demanda in Burgos (Figs. 2-5). To the west, it reaches the sub-coastal ranges of central Portugal (Caramulo, Arada), and to the north, extends to the southern slopes of the Montes de León range and penetrating into south-eastern Galicia. Isolated population pockets exist away from the core of its distribution, the furthest away in Sierra Madrona (Ciudad Real, Spain). *L. tityrus* has a continuous distribution in temperate Iberia, from the Pyrenees in Catalonia to Galicia, Sierra de la Demanda and the northern third of Portugal, south to the western CIMS. Its southernmost isolated occurrences are population pockets within the western CIMS itself: in western Serra da Estrela and in Serra da Malcata. Otherwise, the Sooty Copper is absent from the CIMS and elsewhere in the Peninsula. We obtained a large overlap belt and broad sympatry (47 quadrats) on the distribution of both species in central-northern Portugal, and into the Galicia- Castilla y León region boundary area, as well as in the southern slopes of Sierra de la Demanda (Burgos). Strict

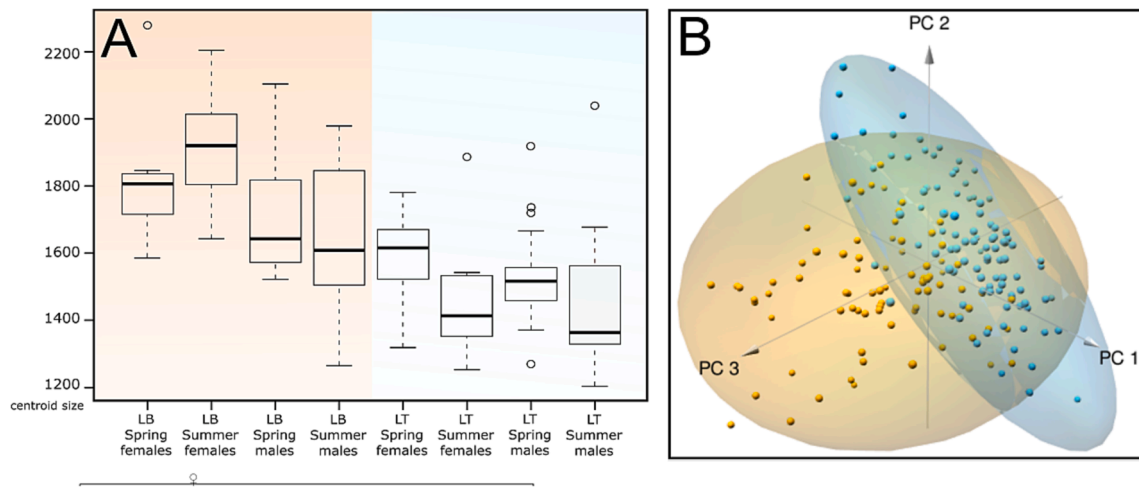


Fig. 5. Geometric Morphometric results from Iberian Sooty Coppers. (A) Hindwing centroid size variation across species and season. It compares taxa (*L. bleusei*, *L. tityrus*), sexes and season. LB - *Lycaena bleusei*, LT - *Lycaena tityrus*. (B) Principal Components Analysis of centroid size variation across species. Blue: *L. tityrus*; Orange: *L. bleusei*.

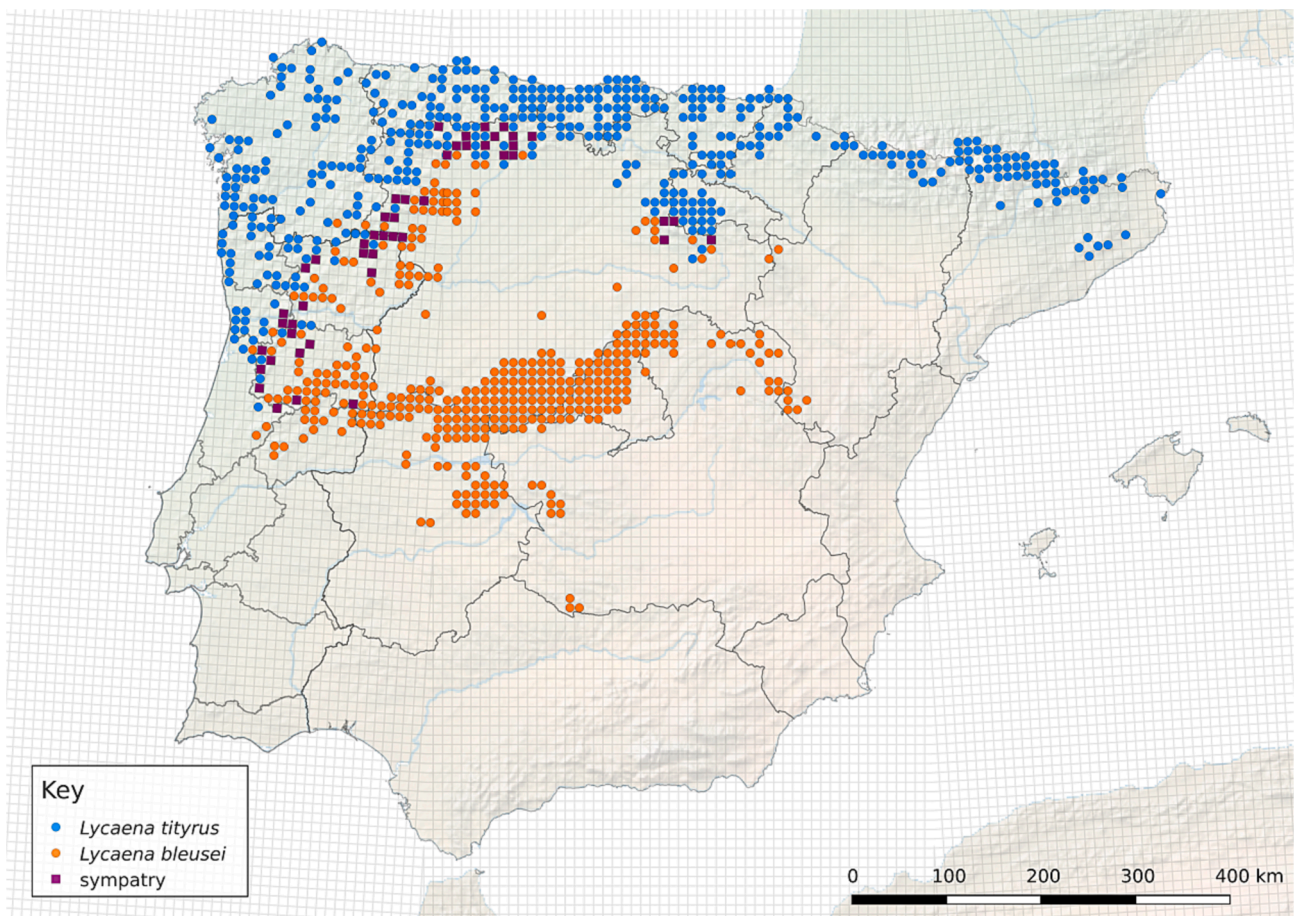


Fig. 6. Distribution of Sooty Coppers in the Iberian Peninsula, represented at 10 km quadrat resolution corresponding to MGRS 10 km quadrats. *Lycaena tityrus* (blue dots), *Lycaena bleusei* (orange dots) and sympatry areas (purple).

sympatry was observed on 26 instances during field-work in central and northern Portugal and NW Spain (Castilla-y-León).

3.3.2. Species distribution modelling

Species distribution models for Iberian populations of both Sooty Coppers were implemented in Maxent using the previously obtained

distribution data (Fig. 6). These models resulted in a good fit, with AUC of 0.977 and 0.978 for *L. tityrus* and *L. bleusei*, respectively. Present modelled distributions correlate moderately with the currently known range of both species, performing better for *L. tityrus* than *L. bleusei* (Fig. 7):

L. tityrus model does not predict its presence much further away from

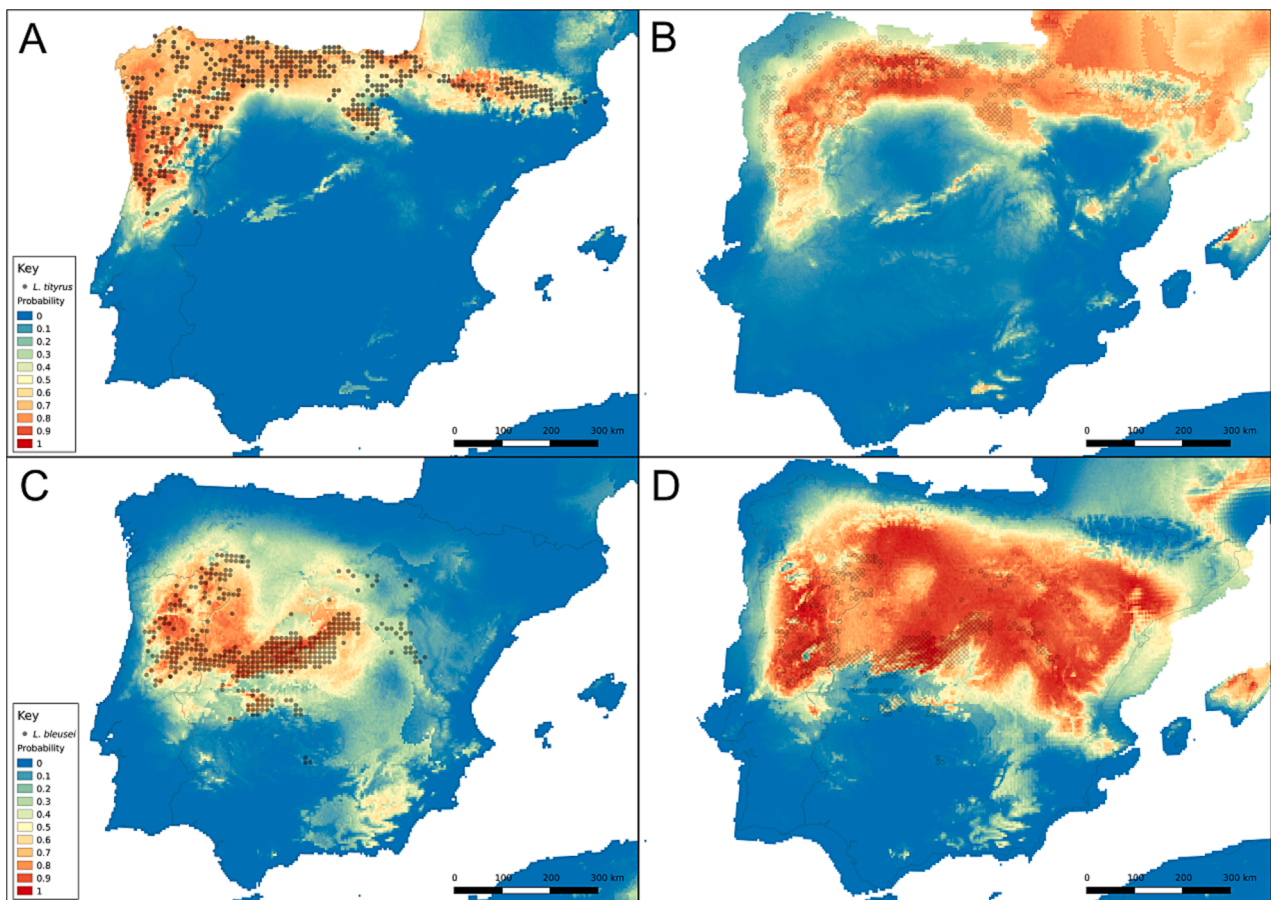


Fig. 7. Species distribution modelling maps for the distribution of *L. tityrus* during (A) Present and (B) LGM. *L. bleusei* during (C) Present and (D) LGM.

the northern third of the Peninsula, not missing currently occupied areas by this species. Jackknife evaluation of variable importance revealed that this species' range depends more on temperature than precipitation variables: BIO 4 (temperature seasonality), BIO 7 (temperature annual range) and BIO 9 (mean temperature of driest quarter) explain this distribution better than the next variable (BIO 12 - annual precipitation), and the remaining ones (Table S6). The absence of BIO 4 disrupts the model the most.

For *L. bleusei*, the model fitting to distribution was not so precise. Despite this species' current distribution along the CIMS and northern Portugal is widely predicted and slightly extended - possibly indicating insufficient distribution knowledge in the north of Spain, i. e. in Galicia and León, or climate-unrelated absence of the species - many of the small isolated populations known to occur away from this area (such as Cuenca, Burgos and Sierra Madrona) are not identified as important or probable by the model (Fig. 7C). Unlike *L. tityrus*, Jackknife evaluation revealed that most relevant variables for this species are related to precipitation: BIO 18 - precipitation of the warmest quarter, BIO 17 precipitation of driest quarter and BIO 14 - precipitation of driest month (Table S6). BIO 2 - mean diurnal range, is the variable which omission affects the model the most. Modelled distributions for both species suggest a large overlapping area in central and northern Portugal, bounded to the east by western Castilla y León, to the north in southern Galicia, and to the south, meeting in the CIMS range of Serra da Estrela. To a much lesser extent, a contact zone is also suggested in the Burgos area of Castilla y León (Fig. 7).

Last Glacial Maximum (LGM) projections reveal contrasting factors affecting both species differentially, even under a changing climate. The climate envelope of *L. tityrus* suggests that this species has not significantly changed its potential occurrence area since the LGM, although we

note a more generalised presence in the Catalan Transversal and Coastal ranges (Fig. 7B). On the other hand, the LGM model for *L. bleusei* greatly expands its currently known area of occurrence to encompass almost the whole of inland northern half of Iberia, south of the Cantabrian belt, eastwards to the foothills of the Pyrenees and reaching the then further away Mediterranean coast (Fig. 7D). This potentially wide LGM area also includes the currently isolated population remnants of the Iberian System, Burgos, and other smaller areas.

4. Discussion

Our integrative analysis aimed at comparing distinct populations in the *Lycaena tityrus* species complex, focusing on Iberian populations of *L. tityrus* and *L. bleusei*, as an attempt to resolve their status. Two independent genetic markers (COI and EF-1 α) and two phenotypic analyses (upper-side pattern and hindwing shape), coupled with environmental niche modelling were employed, while the use of three additional genes (16S, CAD, wg) allowed for assessing their positioning among the European *Lycaena*.

Previous to this study, the status of Iberian endemic *L. bleusei* had been a disputed one. However, all of our studied genetic markers and methods revealed a strong differentiation in relation to its putative sister species, *L. tityrus*. We clearly identified both to be sister taxa among their relatives, and their ancestor probably split circa 8 Ma ago from the nearest extant taxon, *Lycaena virgaureae*. Our focal species split occurred circa 4.8 Ma ago and since then, have evolved independently from one another. Both Sooty coppers do occupy similar habitats but we have also shown they have responded differently to environmental conditions across time and exhibit markedly different levels of genetic and phylogeographic structure.

While attempting at clarifying the spatial distribution of *L. bleusei* and *L. tityrus* in Iberia, we demonstrated that the latter is not morphologically homogeneous and instead shows a degree of geographical structure. Our findings are relevant to decipher how lineages diverge and follow different paths, depending on the changing environment they evolve in. An in depth integrative discussion of current findings can be further addressed in the following topics.

4.1. Origins and relationships

The Sooty Coppers were both placed deeply nested among European *Lycaena* species by ML, BI and BEAST analyses (Fig. 3, Figure S1), which also identified *Lycaena virgaureae* and *L. ottomanus* as their putative sister species, with the former as the most likely extant sister species. This association among the *L. tityrus* group and *virgaureae* + *ottomanus* is one of the oldest among the seminal works providing order to the Lycaeninae (Klots 1936; Eliot, 1973; Sibatani 1974; Higgins 1975; Cassulo et al. 1989; Bozano & Weidenhoffer 2001), supported on the morphology of male genitalia and wing-pattern. Early on, these species were all included in genus/ subgenus *Heodes* Dalman 1816 (TS: *Papilio virgaureae*), whose members may be distinguished from other *Lycaena* by the male genitalia bearing oval or rectangular, cup-shaped valves with a prominent densely chitinised costal tooth, connected to the vinculum by a fibrous articulation (Klots 1936; Higgins 1975). The original concept of *Heodes* (Klots 1936, Sibatani 1974) included only *L. virgaureae*, *L. tityrus* and *L. ottomanus* but Higgins (1975) also added *L. alciphron* to the group. Our genetic analyses support the original circumscription of *Heodes*, adding *L. bleusei*, and following Klots (1936)' thesis that

potential genital similarities between *L. alciphron* and *Heodes* are homoplastic. Our placement of *Heodes* alongside two other known species-groups/ subgenera, *Thersamonia* (*L. thetis*, *L. thersamon* and *L. phoebus*) and *Palaeochrysophanus* (*L. hippothoe* and *L. candens*), corroborates some results of van Dorp (2004) and Jong & van Dorp (2006) using COI + COII but not the latest phylogeny of European butterflies (Wiemers et al. 2019), which places *L. bleusei* as sister species to all other *Heodes*, in a clade which also includes *L. phlaeas*. We believe our greater gene coverage of core species is responsible for these differences, revealing a more accurate lineage relationship.

Our divergence-time estimates place the differentiation of *Heodes* from remaining European *Lycaena* at circa 10 Mya and that of its most basal species, *L. ottomanus* and *L. virgaureae* at 8.3–8.1 Ma (Fig. 3), during the upper Miocene (Tortonian). Understanding the Tortonian (11.62–7.245 Mya) climate and biotic setting is thus essential to comprehend the early evolution of the *Heodes* coppers. This period sets a major transition in Europe marked by the onset of the first Arctic glaciations, producing an increase in latitudinal and seasonal temperature gradients across the continent and a progressive global cooling, although most of Europe was still subject to subtropical/ warm-temperate conditions with humid to subhumid summers (Prista et al. 2015). Warm-temperate evergreen forests were progressively replaced by mixed mesophytic forests, and finally by deciduous broad-leaved forests interspersed with wide open steppe-like areas expanding from the east to the west and north of the Palaearctic (Casanovas-Vilar et al., 2010; Kovar-Eder, 2003; Pound et al., 2011; Rögl and Daxner-Höck, 1996; Steininger and Wessely, 1999; Suc et al., 2018). Most major European *Lycaena* lineages, and especially *Heodes* coalesce at this point and

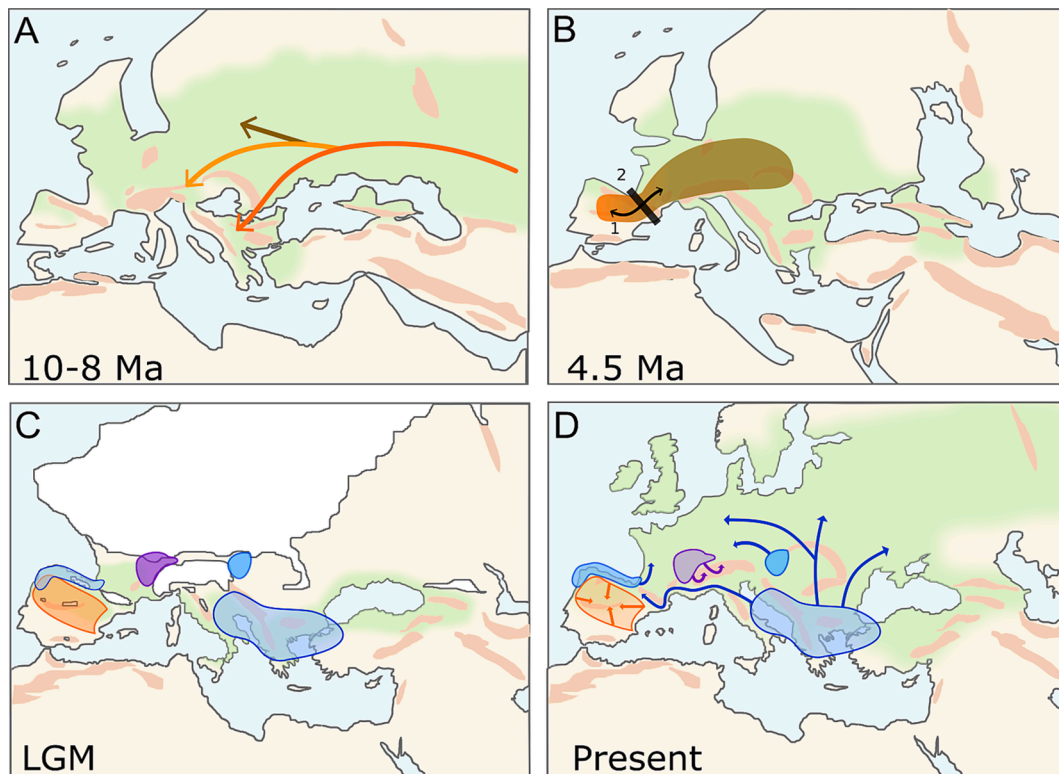


Fig. 8. Hypothesized biogeographical scenarios and colonisation routes taken by *Lycaena* lineages in the western Palaearctic from the Tortonian to right after the LGM. A) Tortonian arrival of *Heodes* into Europe from central Asia and the almost simultaneous divergence of *L. ottomanus* into the Balkans, *L. virgaureae* to the mountains of Europe and proto *L. tityrus/ bleusei* into temperate areas. B) Early Pliocene (Zanclean) and the (1) colonisation of either Europe or Iberia favoured by a continuous habitat availability followed by a (2) interruption of gene flow along the Pyrenees or the Ebro basin; C) LGM 21 ka ago with coloured areas representing putative *L. bleusei* refugium in central Iberia, *L. t. praebleusei* in NW Iberia, *L. t. subalpinus* around the W Alps and C/ E lineages of *L. tityrus* near the eastern Alps and the Tyrrhenian area, respectively; D) Potential colonisation routes following deglaciation of central and northern Europe 11 ka ago: *L. bleusei* retreating into the CIMS, *L. t. praebleusei* extending eastwards, *L. t. tityrus* extending from C and E Europe into the whole continent, penetrating into E Iberia, and *L. t. subalpinus* displaced altitudinally into the high Alps. Green shading corresponds to suitable habitat according to François et al. (2011) (A-warm temperate mixed forests); (Salzmann et al. 2011) (B- temperate mixed forest) and our modelling in (C) and (D).

their original differentiation and divergence may have been triggered by these climate and habitat changes which made available a wide area from central Europe to Japan. *Heodes* in relation to its sister lineage *Thersamonia*, may have taken advantage of this habitat availability and colonised most of the northern area while the latter took the drier, southwestern Palaearctic as its stronghold (Bozano & Weidenhoffer, 2001). Assuming an origin in western Asia or SE Europe south of the Paratethys as well, *Heodes* may have expanded westwards and northwards alongside several mammals of Asian descent (Koufos et al. 2005) (Fig. 8A). The earliest derived lineage, nowadays represented by *L. ottomanus*, is eastern-Mediterranean and inhabit the Balkanic Peninsula and western Anatolia. Its niche is limited to wet, hot grasslands and gullies at low altitudes not tolerating summer drought (Settele et al. 2008; Koren et al. 2012), which may be interpreted as a relictual ecology most similar to the original *Heodes* niche, before the Tortonian climate cooling. *L. virgaureae* then probably colonised the boreal eco-zone or the growing Alpine area, while the Sooty Copper ancestor occupied lowland areas north of the Paratethys sea.

L. tityrus and *L. bleusei* would only diverge much later, circa 4.8 (3.14 – 10.32) Mya, during the early Pliocene (Zanclean: 5.33 – 3.6 Ma) (Fig. 8B). At this point, the Sahara became drier and there was a generalised cooling of the northern hemisphere (Prista et al. 2015) although still 4 °C warmer than today (Brierley & Fedorov 2010). Temperate deciduous and mixed forests expanded at the expense of formerly widespread evergreen subtropical forests (Favre et al. 2007) and formed an almost continuous belt from Russia to Iberia (Salzmann et al. 2011). These conditions may have allowed for an ecological corridor to form between central Europe and the CIMS, and for the ancestor of *L. bleusei* to reach this area (Fig. 8B-1), later to become isolated during the Plio-Pleistocene climatic pulses (Fig. 8B-2). These included aridification peaks (Agustí et al. 2001), the cyclical expansion of subtropical communities (Suc et al. 1995) and intense summer drought (Mijarra et al. 2005). *L. bleusei* has thus likely differentiated and diverged this way from *L. tityrus*, in Iberia, becoming a temperate (see below) element cast-away in a habitat island represented by the CIMS.

4.2. Sooty coppers' diverging genetics, morphology and ecology

Our genetic analyses recovered *L. tityrus* and *L. bleusei* as two genetic units with no haplotype overlap and showing a higher than expected genetic divergence, given their controversial taxonomic history. We found no general mito-nuclear discordance, with the exception of two individuals we consider infrequent hybrids, but noted a higher than expected diversity in the nuclear EF-1 α gene in respect to COI, evident in *L. bleusei*, but also in the Western Iberian populations of *L. tityrus* (Fig. 3). Despite being uncommon, such mismatch has however also been reported elsewhere, being attributed to the occurrence of bottlenecks (Fay & Wu 1999; Ji et al. 2011), because the relative differences in effective population size in mtDNA may translate into more dramatic effects than on nuDNA. Nonetheless, alternative explanations for this pattern may be equally valid; the first being direct positive selection of certain mtDNA lineages as may happen in birds (Hung & Zink 2014). A second potential source of mitochondrial selective sweeps could be through hitch-hiking either by maternally inherited symbiotic microbes such as *Wolbachia*, or a linkage effect with the sexual chromosome as happening with the Hill-Robertson effect in birds (Berlin et al. 2007), which share the same ZW sex-determination system.

L. tityrus and *L. bleusei* also differ in their structure and molecular variance. While we found no nuDNA or mtDNA structure in *L. bleusei* (Fig. 4, Table 2, Table S4), probably related to an often small but generally panmictic population subject to severe fluctuations, in *L. tityrus* multiple patterns emerge. The latter exhibits a genetic structure compatible with both glacial-induced allopatric isolation with later contact between lineages, and an ecological population differentiation, today expressed along an altitude gradient. The first phenomenon occurs in the geographic segregation of mtDNA haplotypes T1 + T2 in Iberia,

T3, T4 and T5 in central Europe, and T8-10 alongside the widespread T3 in SE Europe. This segregation is probably of glacial origin (Fig. 8C) and the wide distribution of T3 indicates a barrier-less recent range expansion from the S or SE of Europe, to occupy most of the continent as happened to many other eastern animal populations since the last glaciation (Hewitt, 1996, 1999; Schmitt & Seitz 2001; Schmitt 2007). Only lately has it reached the Iberian Peninsula through the eastern Pyrenees (Fig. 8D), producing a secondary contact zone with the N Iberian resident populations belonging to T1 and T2 haplotypes, and which we generally associate with *L. t. praeblyseus* phenotype (Figure S2). *Melitaea cinxia* and *Aricia* spp. butterflies also broadly comply to this biogeographic pattern and suture zone (Wahlberg & Saccheri 2007; Sañudo-Restrepo et al. 2013). The phenotypic and genetic independence of NW Iberian *L. tityrus* respective to Eastern Iberian and other European populations are further supported by long-term local habitat suitability spanning both glacials and interglacials (Fig. 7), and the AMOVA/ F_{ST} analyses, which preferentially groups eastern Iberian populations among populations outside the Peninsula (Table 2, S4). Indeed, E Iberian *L. tityrus* butterflies belong to the widespread European phenotype *L. t. tityrus* (= *pallidepicta*) (Figure S2), but the precise extent of its Iberian penetration is still to be properly assessed. Currently, morphological evidence puts the contact zone in the Burgos area whilst the genetic evidence places this somewhere between Burgos and Catalonia.

As an example of ecological differentiation within the *L. tityrus* gene-pool, sister haplotypes T6 and T7 correspond to the ecologically differentiated and phenotypically distinct *L. t. subalpinus* from the Alpine chain above 1500 m a.s.l. (Descimon, 1980). This population may have evolved locally during the Pleistocene Würm stadial phases, characterised by widespread open biotopes under a cold, seasonal regime (Starnberger 2012). With post LGM climate amelioration, this cold-adapted butterfly retreated to the higher altitudes it occupies today. The poor genetic divergence between *L. t. subalpinus* and the rest of *L. tityrus* s.l. populations (Tables 2, S3, S4) contrasts with that of *L. bleusei*, and reinforces the different timing and factors at play in each event.

Finally, AMOVA (Table 2) and F_{ST} (Table S4) analyses specifically targeted at testing the influence of *L. bleusei* within the Sooty Copper complex as a whole and using *L. t. subalpinus* as a measure scale of differentiation, returned revealing results. Treating both *L. bleusei* and *L. t. subalpinus* as merely subsets of Iberian or Extra-Iberian *L. tityrus* revealed extremely low values of between-group differentiation, whereas considering them as belonging to different groups from *L. tityrus* increased enormously that differentiation. AMOVA 3–5 identified the main differentiation not to be between Iberian and extra-Iberian samples but rather *L. bleusei* caused such high differentiation values, not *L. t. subalpinus*. In view of this, the most parsimonious way to accommodate a balance between intra and inter population differentiation is that *L. t. subalpinus* should be considered as another population within the *L. tityrus* group, while *L. bleusei* exerts a major influence on the percentage of variation. Their (genetic) rank should therefore be deemed different: *L. t. subalpinus* as a differentiated population of *L. tityrus* and *L. bleusei* as an independent species. An analysis of pairwise F_{ST} differences supports these conclusions, with *L. bleusei* displaying the highest differentiation of all Sooty Coppers, turning that of *L. t. subalpinus* vs *L. tityrus* s.str. negligible (Table S4). Although the fixation index F_{ST} has been mostly used to assess differentiation levels among populations, our evidence agrees with Hey & Pinho (2012) in that it could be applied to find a threshold for species and subspecies delimitation as well.

Morphological data also reveal diverging trends in wing shape and pattern between *L. tityrus* and *L. bleusei*. Beforehand, the two taxa can readily be distinguished by discrete characters such as the hindwing tails, but we found a more complex pattern of sexual and seasonal variation when applying Geometric Morphometric Analysis. All the four considered groups show differences in relation to species (2 species) and sex (males and females), but also a great overlap in shape and size

(Fig. 1, Fig. 5A). Males of both species show similarly shaped hindwings, considerably different in relation to females, but also specifically distinct at the place (landmark p8) where the hindwing-tails develop in *L. bleusei* but not in *L. tityrus* (Fig. 1). The same specific pattern emerges for females. Wing-size in *L. bleusei* is on overall larger than in *L. tityrus* (Fig. 5), especially so during summer generations but less evidently in the spring. A sexual trend was also observed, where females of both species scored consistently larger centroid size values than respective males. In all groups, all butterflies are larger in the spring than in the summer, with the notable exception of *L. bleusei* females. We can relate this trend with the Temperature-Size Rule (TSR), which suggests ectotherms become larger when developing under cold temperatures rather than higher ones (Angilletta & Dunham 2003). Whereas *L. tityrus* was already known to conform to TSR, both in its lowland *L. t. tityrus* (Fischer & Fiedler 2000), and Alpine *L. t. subalpinus* (Karl 2008; Karl et al. 2009), we have recovered the same pattern for the Iberian populations. It has been argued that such smaller summer size and shortened generation-time allows for the development of further generations and the earlier access to breeding grounds, thus overcoming intra-specific competition. On the other hand, *L. bleusei* appears to respond differently. A likely explanation may rely on its 4.8 Ma metabolic adjustment towards particular Mediterranean conditions without an extreme summer drought, there existing an advantage in producing more eggs laid during this dry season, as to increase survival chances. Moreover, this adaptation may also be observed in its diverging phenotype involving the development of a hindwing tail, a feature to disrupt shape, and of a darker-yellow wing background that better matches the summer dried meadows it inhabits. Similar cases of butterfly seasonal size variation with inverted TSR have been discovered, involving other factors than temperature (Mega 2014).

Finally, the divergence between *L. bleusei* and Iberian *L. tityrus* may also be addressed from distribution data. We found that different environmental variables are behind their mostly parapatric distribution in Iberia: *L. tityrus* responds more to (high) temperatures, and is excluded from too thermic areas while *L. bleusei* is mostly influenced by precipitation, being also more widespread than expected (compare to García-Barros et al. 2004). Mixed environmental conditions in a broad area spanning central and northern Portugal predicted an ultimately confirmed overlapping distribution belt, and where both taxa often occur in strict syntopy. For *L. tityrus*, the current and LGM potential ranges do not differ substantially, except in the very East of the Iberian Peninsula, where current projections do not include the widely LGM projected but recently extinct Montseny populations (Parmesan et al. 1999). A likely explanation for the recent disappearance of these *L. tityrus* Montseny populations may be that they were already of a relictual character when recent post-Industrial climate-change and changing land-uses hit the area during the 20th century.

Among the most interesting results given by species distribution models under both present and LGM situations, was the finding that despite the current presence of *L. bleusei* in some small patches in S and E Iberia was not recovered in the Present scenario, its potentially much wider LGM distribution over most of northern Iberia (Fig. 7) does include these areas. While we attribute the present mismatch to the microclimatic conditions being favourable for the species but are not represented at the scale of bioclimatic data, we further argue that these isolated population pockets are better explained after an LGM range expansion. In fact, the difference between LGM and present potential ranges configures two interesting points: 1) a counter-intuitive LGM range expansion pattern more usual in Arctic-Alpine taxa (Schmitt & Hewitt 2004; Muster & Berendonk 2006), which has nevertheless also been recovered for other NW Iberian taxa such as the mesophilic viper *Vipera seoanei* (Martínez-Freiría et al. 2015); 2) an extensive range-contraction after the LGM: the species is nowadays retreated to its near-optimal refugium along the CIMS and peripheral ranges. The latter point is in agreement with the reported hypothesis of genetic bottlenecks, attributed to the mtDNA diversity loss in *L. bleusei*, explaining the

large differences in the species range in Iberia during LGM and the current interglacial (Fig. 7), situations which probably also occurred during previous climatic oscillations. However, these do not match so well with the practically immutable western Iberian distribution of *L. tityrus praebleasei*, whose lower mtDNA diversity is better explained by the hypotheses associated with active selection of certain mtDNA haplotypes, either through *Wolbachia*-associated sweeps or the Hill-Robertson effect.

5. Final considerations

Our study-design was aimed at specifically deciphering the differentiation among the several components of the Sooty Copper species complex, while looking at their origin and evolution. Starting off with an unclear situation relating to the status of *L. bleusei*, we further uncovered the relationships among *L. tityrus* populations. Our multiple lines of evidence revealed several levels of differentiation among Sooty Coppers. Fundamentally, we provide an unambiguous result as to Iberian endemic *L. bleusei* and Palearctic *L. tityrus* being independent species under the most important criteria, namely phylogenetic, morphological, reproductive and geographic (Descimon & Mallet 2009):

L. bleusei and *L. tityrus* form reciprocally monophyletic, independent gene-pools, having diverged around 4.8 Ma. They belong to a group of species (*Heodes*) also including *L. virgaureae* and *L. ottomanus*. *L. tityrus* shows some genetic structure compatible with multiple glacial refugia in Europe: one in Iberia, two in S and E Europe and the Alpine endemic lineage *L. t. subalpinus*: an ecologically and morphologically yet genetically poorly differentiated taxon. In *L. bleusei*, allied to an absence of genetic structure, we unveiled a loss in mtDNA diversity when compared to its nuclear one, likely explained by past demographic contractions. When focusing on the contact zone between both species, and taking into consideration both nuDNA and mtDNA evidence analysed over an extensive dataset, only two introgressed individuals were found, likely the result of a sporadic interspecific mating. The morphology-based analyses also recovered important size and shape differences between both species, where *L. bleusei* does not follow the usual Temperature-Size Rule, likely to cope with habitat shrinkage during Mediterranean summer. In Iberian *L. tityrus*, we additionally uncovered the existence of two geographically segregated phenotypes: one attributable to *L. t. praebleasei* (NW Iberia), and the other to *L. t. tityrus* (NE Iberia into the rest of Europe), distinguishable on forewing colouration. Finally, distribution data and SDM's projections for current and LGM distribution of both species, allowed to reveal their niches to be dependent upon different environmental variables, yet still overlapping on a wide geographic area. During the LGM, *L. tityrus* may have occupied a NW range in Iberia, whereas *L. bleusei* is projected to have expanded into most of Iberia's northern half during the same period. *L. bleusei*'s wider LGM distribution area and recent interglacial contraction explain current population pockets away from the CIMS area of influence: a present-day stronghold for the species. Presently, a wide area in Central Portugal-NW Spain holds sympatric and syntopic populations of both species, whose long-term interaction under a changing climate should be addressed in further studies involving genomic approaches, and common-garden raising to decipher the pre- and post-mating barriers at play.

Our study highlights the importance of gathering several lines of evidence when deciphering the relationships between closely related yet differentiated populations likely to correspond to different species. However, it also reveals that not always such perceived differences between taxa are of equal magnitude and the whole differentiation process is fluid and subject to environmental and ecological constraints, as well as isolation. In this respect, the CIMS appears to have acted as a vast habitat-island allowing for an ancient lineage of Sooty Coppers to survive and adopt different strategies for survival once gene-flow with extra-Iberian populations was hampered. We look forward to follow the results of other studies on species also sharing the CIMS mountain range as a stronghold, for they may show a similar biogeographic pattern as

Lycaena bleusei, a remarkable temperate-evolved, turned Mediterranean butterfly.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ympcv.2022.107699>.

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