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Rapid colour shift by reproductive character displacement in *Cupido* butterflies

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Abstract

Reproductive character displacement occurs when competition for successful breeding imposes a divergent selection on the interacting species, causing a divergence of reproductive traits. Here, we show that a disputed butterfly taxon is actually a case of male wing colour shift, apparently produced by reproductive character displacement. Using double digest restriction-site associated DNA sequencing and mitochondrial DNA sequencing we studied four butterfly taxa of the subgenus *Cupido* (Lepidoptera: Lycaenidae): *Cupido minimus* and the taxon *carswelli*, both characterized by brown males and females, plus *C. lorquinii* and *C. osiris*, both with blue males and brown females. Unexpectedly, taxa *carswelli* and *C. lorquinii* were close to indistinguishable based on our genomic and mitochondrial data, despite displaying strikingly different male coloration. In addition, we report and analysed a brown male within the *C. lorquinii* range, which demonstrates that the brown morph occurs at very low frequency in *C. lorquinii*. Such evidence strongly suggests that *carswelli* is conspecific with *C. lorquinii* and represents populations with a fixed male brown colour morph. Considering that these brown populations occur in sympatry with or very close to the blue *C. osiris*, and that the blue *C. lorquinii* populations never do, we propose that the taxon *carswelli* could have lost the blue colour due to reproductive character displacement with *C. osiris*. Since male colour is important for conspecific recognition during courtship, we hypothesize that the observed colour shift may eventually trigger incipient speciation between blue and brown populations. Male colour seems to be an evolutionarily labile character in the Polyommatinae, and the mechanism described here might be at work in the wide diversification of this subfamily of butterflies.

KEYWORDS
lepidoptera, RAD sequencing, reinforcement, reproductive character displacement, speciation

1 | INTRODUCTION

The combination of morphological analyses and molecular techniques provides a powerful tool to tackle species delimitation issues (Padial et al., 2010; Pante et al., 2014), although these two approaches may produce apparently contradictory results. Several studies have shown that lineages demonstrating considerable genetic divergence may not necessarily have diagnostic morphological characters, an extreme case being that of cryptic species (reviewed in Chenuil et al., 2019). On the other hand, other studies have found faint genetic differentiation but pronounced morphological divergence (Hu et al., 2019). If genetic

but not morphological differentiation is present, processes at work might imply morphological stasis due to stabilizing selection (Davis et al., 2014) or developmental constraints (Donoghue & Ree, 2000; Smith et al., 1985). Such cryptic species are generally incapable of interbreeding with success, but conserve most of the ancestral external traits or, alternatively, their traits could have initially diverged but converged a posteriori (Struck et al., 2018). Cryptic taxa are not always sibling species and can display remarkable levels of genetic divergence (Dincă et al., 2011; Vodă et al., 2015; Vrijenhoek, 2009).

When morphological variation is high but it is not reflected in genetic structuring, one could invoke a wide set of processes, such as neutral (Bernatchez et al., 1995) or adaptive introgression (Clarkson et al., 2014), hybrid speciation (Amaral et al., 2014), incomplete lineage sorting (McGuire et al., 2007) or phenotypic plasticity linked to local adaptation (Antoniazza et al., 2010; Brakefield, 1997). In the case of local adaptation, distinct external traits prevail depending on the population as a result of the combination between selection to optimize fitness in specific environments and the available genetic repository (Kawecki & Ebert, 2004). Local adaptation can be driven by the presence in sympatry of another species, whose interaction causes a phenotypic change mainly due to ecological character displacement (Lamichhaney et al., 2016; Schluter & McPhail, 1992) or reproductive character displacement (Fishman & Wyatt, 1999; Lemmon, 2009). In the case of ecological character displacement, a phenotypic change is selected in order to avoid competition for the same resources (Garner et al., 2018; Losos, 2000), while reproductive character displacement takes place to avoid interbreeding or costly wrong courtships between two lineages (Pfennig, 2016). The consequence of both processes is phenotypic divergence between the populations in traits either related to the use of the resource or to mating. In these circumstances, phenotypic change is the product of natural selection mediated by a biotic interaction, and it might evolve extremely fast and imply changes in only a small set of genes (Lamichhaney et al., 2016).

A large number of Lepidoptera lineages are relevant to study evolutionary processes associated with a marked contrast in genetic versus morphological differentiation. These commonly exhibit a wide set of phenotypic variations that are easy to observe and measure. For instance, some intraspecific morphs are so different that they have been classified as distinct species, as happened in the iconic Palearctic butterfly *Araschnia levana*, in which its two seasonal morphs were originally described as *Papilio levana* and *P. prorsa* (Goldschmidt, 1982). The interaction of the butterflies with a usually restricted set of larval host plants drives their confinement and specialization in particular habitats, and most species adopt a metapopulation system where populations are partially isolated but maintain a certain degree of gene flow (Thomas & Hanski, 1997). The metapopulation network changes with time and can be altered by geography (emergence of new geographic barriers) or ecology (irruption of novel species, climatic changes, etc), hence enhancing differentiation of the populations and producing local adaptations. For example, the interaction between *Heliconius erato* and *H. melpomene* resulted in a set of wing morphs that imitate each other in sympatry, a case of Müllerian mimicry (Merrill et al., 2015; Meyer, 2006).

The subgenus *Cupido*, represented in the western Palearctic by *Cupido osiris* (Meigen, 1829), *Cupido lorquinii* (Herrich-Schäffer, 1851), *Cupido minimus* (Fuessly, 1775) and the taxon *carswelli* (Stempffer, 1927) (Figure 1 and Figure S1), includes two notorious examples of genotype-phenotype "discordance": two cryptic entities, with diverged mitochondrial DNA (mtDNA) but almost identical in morphology (*C. minimus* and the taxon *carswelli*), and a pair markedly different in morphology but without differences in the mtDNA (*C. lorquinii* and the taxon *carswelli*). The taxon *carswelli* is brown-winged and occurs locally in mountain ranges of the south-eastern Iberian Peninsula (Gil-T, 2017). It is considered a valid species by several authors (Gil-T, 2017; Obregón et al., 2016; Tolman & Lewington, 2008), but, because of morphological similarity, it has also been treated as a subspecies of *C. minimus* (de Jong et al., 2014; García-Barros et al., 2013; Prieto et al., 2009), a widespread and common species found across the Palearctic. Rather surprisingly, the barcode fragment of the cytochrome c oxidase I (*COI*) showed *carswelli* was not monophyletic, but was included in the same clade as the Ibero-African *C. lorquinii* with even some shared haplotypes between them (Dincă et al., 2015). Unlike the brown *carswelli*, males of *C. lorquinii* are blue. Finally, *C. osiris* demonstrates the most diverging mtDNA lineage across the quartet (Dincă et al., 2015). It spans the Mediterranean Europe and has blue males.

With the aim of understanding the underlying biological causes behind the patterns explained above and of clarifying the taxonomic status of the taxon *carswelli*, we examined the genetic structure of these taxa by using double digest restriction-site associated DNA sequencing (ddRADseq; Peterson et al., 2012) and mtDNA (*COI*) sequencing. We envisaged and tested three main hypotheses, with different taxonomic implications (Figure 1):

1. The taxon *carswelli* is a distinct species. In this case, we would expect a monophyletic lineage in the ddRADseq data, well differentiated from *C. minimus* and *C. lorquinii*. Complementarily, we evaluated through introgression analyses if *carswelli* is of hybrid origin. Because recurrent cases of hybrid taxa were documented in butterflies (e.g., Capblancq et al., 2015; Gompert et al., 2008; Kunte et al., 2011), *carswelli* exhibits characteristics of both *C. lorquinii* (closely related in terms of mtDNA) and *C. minimus* (morphologically extremely similar), and its geographical range lies between both, the possibility of a hybrid origin seemed plausible.
2. The taxon *carswelli* is a subspecies of *C. minimus*, as it has been traditionally considered given their morphological similarity.
3. The taxon *carswelli* is a morphologically different subspecies of *C. lorquinii*, a scenario that would agree with the mtDNA patterns, but that to our knowledge has never been suggested.

For the last two hypotheses we expected low differentiation in the ddRADseq data between *carswelli* and *C. minimus* or *C. lorquinii*, respectively. After identifying the correct scenario, we inferred a

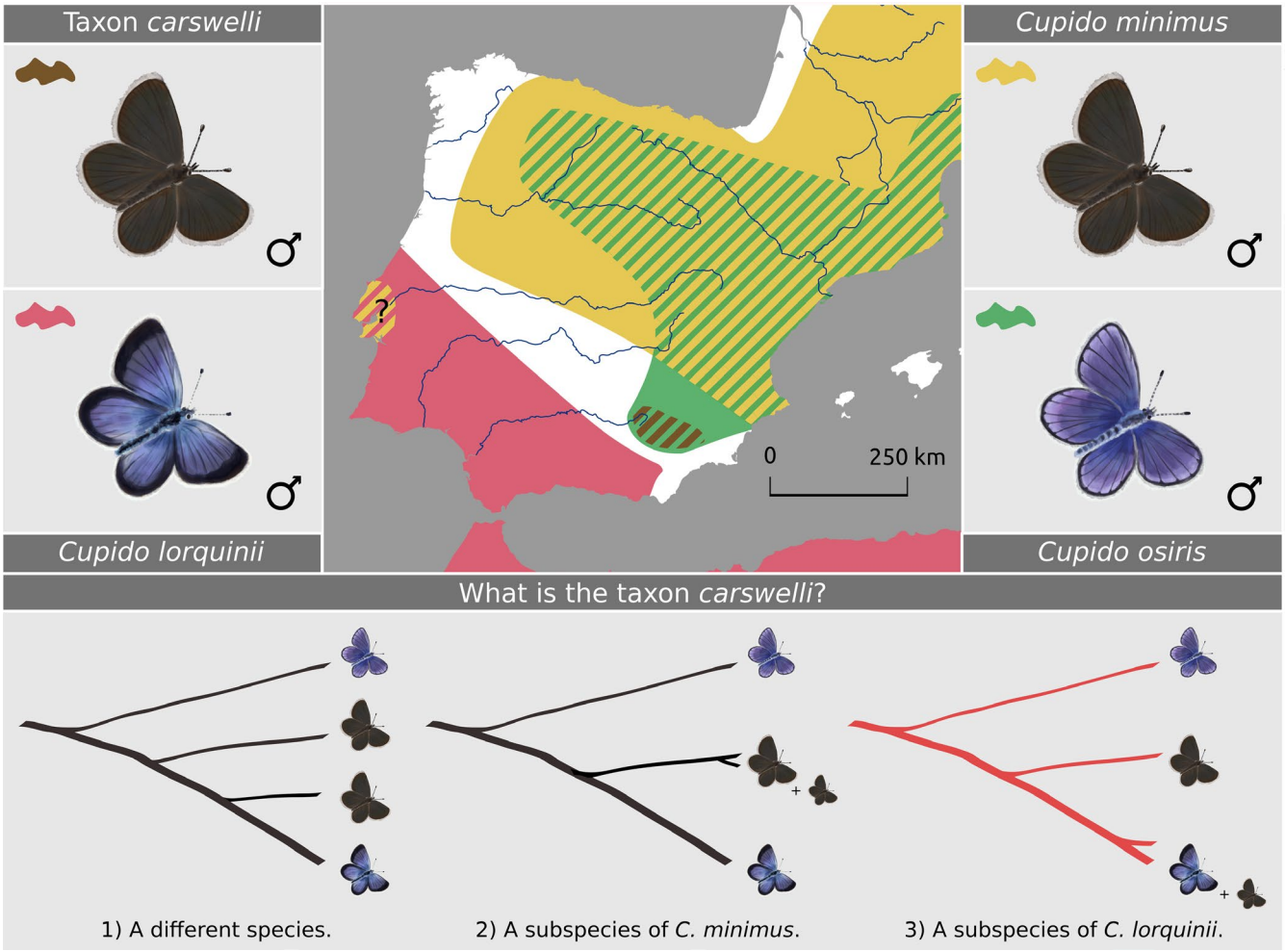

FIGURE 1 Summary of the study system. The male typical forms and the approximate distribution ranges in the Iberian Peninsula of the four European taxa of the subgenus *Cupido* are illustrated. Sympatry is represented by diagonal lines. Sympatry of *C. minimus* and *C. lorquinii* in western Portugal is unclear. The three main hypotheses tested are shown. Hypothesis 3 (highlighted in red) is the one best supported by our results. Drawings: Nàdia Sentís

suite of mechanisms related to reproductive character displacement as the evolutionary processes by which the taxon *carswelli* has probably evolved its idiosyncratic male wing colour.

2 | MATERIALS AND METHODS

2.1 | Sampling

We analysed 45 samples including 20 *C. lorquinii* (17 males), 10 *carswelli* (nine males), 13 *C. minimus* and two *C. osiris* (Table S1), from which we retrieved both *COI* and ddRADseq data. We covered the full known distribution area of the taxon *carswelli*, most of the range of *C. lorquinii* and the European range of *C. minimus* (Figure S1). *Cupido osiris* is the sister to the rest of the taxa and the two specimens analysed were used to root the phylogenetic trees and as outgroup in the introgression analyses. Butterflies collected from the field were dried as soon as possible and wings were kept separately as vouchers; bodies were stored in 99% ethanol at −20°C.

2.2 | Mitochondrial DNA sequencing

Total genomic DNA was extracted using Chelex 100 resin, 100–200 mesh, sodium form (Biorad), under the following protocol: one leg was removed and put into 100 µl of Chelex 10% and 5 µl of Proteinase K (20 mg/ml) were added. The samples were incubated overnight at 55°C in the shaker VorTemp 56 (Labnet International). Subsequently, they were incubated at 100°C for 15 min.

PCR amplification of a 658 bp barcoding fragment of the *COI* was done with the primers (Sigma) LepF1 (5′-ATTCAACCAATCATAAAGATATTG-3′) and LepR1 (5′-TAAACTTCTGGATGTCCAAAAAATC-3′). Double-stranded DNA was amplified in 25 µl volume reactions: 13.2 µl ultra-pure (HPLC quality) water, 5 µl 5X Green GoTaq Flexi Buffer (Promega), 3.2 µl 25 mM $MgCl_2$, 0.5 µl 10 mM dNTP, 0.5 µl of each primer (10 mM), 0.1 µl GoTaq G2 Flexi Polymerase (Promega) and 2 µl of extracted DNA. Reaction conditions were as follows: 92°C for 60 s, then 92°C for 15 s, 48°C for 45 s and 62°C for 150 s in five cycles and other 30 cycles changing the annealing temperature to 52°C with the final

extension step at 62°C for 7 min. PCR products were purified and Sanger sequenced by Macrogen Inc. Europe (Amsterdam, North Holland, the Netherlands). All sequences have been deposited in GenBank (Table S1). *Cupido osiris* specimen RVcoll17B693 was sequenced twice from two independent DNA extractions for confirmation, as it clustered within the *C. minimus* clade.

2.3 | Analyses of mitochondrial DNA sequences

DNA sequences were manipulated with GENEIOUS v11.0.5 (Kearse et al., 2012) and aligned with the Geneious Alignment method. The best fitting model was found using JMODELTEST v2.1.7 (Darriba et al., 2012) under the Akaike information criterion and a Bayesian phylogeny was reconstructed in BEAST v2.5.0 (Bouckaert et al., 2014). Base frequencies were estimated, four gamma rate categories were selected, and a randomly generated initial tree was used. Estimates of node ages were obtained by applying a strict clock and a normal prior distribution centred on the mean between two substitution rates for invertebrates: 1.5% and 2.3% uncorrected pairwise distance per million years (Brower, 1994; Quek et al., 2004, respectively). Albeit these substitution rates provide very rough divergence estimates, better calibrations are unavailable for this taxon group due to the absence of fossils and of alternative phylogenetically-close calibration points. The standard deviation was modified so that the 95% confidence interval of the posterior density coincided with the 1.5% and 2.3% rates. Parameters were estimated using two independent runs of 20 million generations each, and convergence was checked using TRACER 1.7.1 (Rambaut et al., 2018) with a 10% burnin applied. Genetic distances (d_{XY}) were calculated in MEGA v10.0.4 (Kumar et al., 2018) using the bootstrap method and uncorrected p-distances.

2.4 | ddRADseq library preparation

For the ddRADseq library preparation, genomic DNA (gDNA) was extracted from half of the thorax using the DNeasy Blood & Tissue Kit (Qiagen). The quantity of gDNA extracts was checked using PicoGreen kit (Molecular Probes). To increase gDNA quantity, whole genome amplification was performed using REPLI-g Mini Kit (Qiagen). Concentration of the amplified gDNA was estimated with the PicoGreen kit (Molecular Probes) according to the kit instructions. For every sample, we digested 500 ng of DNA with 1 μl PstI, 2 μl MseI and 5 μl of CutSmart Buffer (New England Biolabs) and we added water as needed to bring total volume to 50 μl. It was then incubated for 2 hr at 37°C and enzymes were deactivated by freezing. A purification step with AMPure XP magnetic beads (Agencourt) was done in a Biomek automated liquid handler (Beckman Coulter) with a final elution in 40 μl. DNA concentration was measured with PicoGreen; this value was used for the pooling step. For ligation we added in every sample: 5 μl T4 DNA Ligase Buffer (New England Biolabs), 1 μl T4 DNA Ligase (New Englad Biolabs), 0.6 μl rATP (Promega), 5 μl P1 adapter (50 nM), 5 μl P2 adapter (50 nM) and 2.4 μl water. The P1 adapter included 45 unique Illumina sequencing primer sequences, 5 bp barcodes, and a TGCA overhang on the top strand to match the sticky end left by PstI. The P2 adapter included the Illumina sequencing primer sequences, and AT overhangs on the top strand to match the sticky end left by MseI. It also incorporated a "divergent-Y" to prevent amplification of fragments with MseI cut sites on both ends. We extended the ligation process for 1 hr at 22°C and enzymes were deactivated at 65°C for 20 min. 200 ng of each individual where pooled in tubes making three pools in three different tubes with a final volume of ~450 μl each. Every pool was purified with AMPure XP magnetic beads. We size selected the pools at 300 bp with BluePippin (Sage Science). Finally, we performed PCR amplification with primers RAD1.F (5′-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG-3′) and RAD2.R (5′-CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGC-3′). DNA was amplified in 60 μl volume reactions: 9 μl water, 30 μl Phusion High-Fidelity PCR Master Mix (Finnzymes), 3 μl of each primer (10 mM) and 15 μl of DNA. Reaction conditions comprised a first denature at 98°C for 30 s, then 98°C for 10 s, 60°C for 30 s and 72°C for 40 s in 16 cycles with the final extension step at 72°C for 5 min. PCR products were purified with AMPure XP magnetic beads and DNA concentration was measured with PicoGreen. The size distribution and concentration of the pools were measured with a Bioanalyzer (Agilent Technologies). Libraries were finally pooled in equimolar amounts and sequenced on an Illumina HiSeq 2500 PE 100 in FIMM Technology Center. The demultiplexed fastq data are archived in the NCBI: SRR11918995-SRR11919039.

2.5 | ddRADseq data set processing

Initial filtering steps, single nucleotide polymorphism (SNP) calling and alignment were carried out using IPYRAD v.0.7.23 (Eaton & Overcast, 2016) pipeline. Two data sets were created: one including all the samples and another with only *C. lorquinii* and *carswelli*. The following parameters were changed from the default settings: datatype was set to ddradseq, restriction overhang to *TGCAG*, *TAA*, maximum low quality bases to three, minimum depth (statistical) to eight, clustering threshold to 0.9, minimum trimmed length to 70, maximum Ns to two, maximum heterozygous bases to five, minimum number of samples with a given locus to six (five in the data set with only *C. lorquinii* and *carswelli*), maximum SNPs per locus to 14, and maximum indels per locus to five.

We identified and removed potential contaminant loci from raw IPYRAD data sets, i.e. all loci that were classified as being of a non-insect origin, with CENTRIFUGE v1.0.4 (Kim et al., 2016). The resulting loci were concatenated in GENEIOUS v11.0.5. A rare allele filtering step excluding alleles with a frequency lower than 5% was performed with VCFTOOLS v0.1.13 (Banks et al., 2011). This step

helped to improve the data quality. The number of loci and SNPs of each data set and the analyses where they have been used are indicated in Table S2.

2.6 | Phylogenetic analysis of ddRADseq data

A phylogeny based on ddRADseq data was used to infer the relationships between *carswelli* and the other taxa, hence testing the three hypotheses presented in the introduction. We ran a maximum likelihood inference with RAXML v8.2.4 (Stamatakis, 2014) using the alignment of loci without contaminants (16,312 loci). The GTRGAMMA model and 1,000 bootstrap replicates were selected. We visualized the resulting phylogeny and assessed bootstrap support using FIGTREE v.1.4.2 (Rambaut, 2015).

2.7 | Genetic structuring

We inferred a coancestry matrix and a tree (based on the algorithm described in Lawson et al., 2012) through the FINERADSTRUCTURE pipeline (Malinsky et al., 2018) with the IPYRAD raw output (17,825 loci). Coancestry matrices illustrate the pairwise similarities between samples translated into a colour scale. Here, this method was used to obtain a snapshot of the potential relationships among the studied taxa. *Cupido osiris* was removed for a better visualization of the colour scale matrix because its high level of divergence masked details for the rest of the taxa. In order to have more detailed information of the genetic structure of the target species, principal component analysis (PCA) and STRUCTURE analyses were performed. In STRUCTURE v2.3.4 (Pritchard et al., 2000), we tested values of *K* from 1 to 5. The unlinked SNPs data set (16,562 SNPs) and the rare allele filtered data set without contaminants (62,533 SNPs for the data set of all the samples and 45,753 SNPs for the data set with only *C. lorquinii* and *carswelli*) were used. The selected burnin was 75,000, followed by 250,000 MCMC replicates run to obtain the cluster data. Ten runs were done for each *K* and afterwards combined in one per group with CLUMPAK v1.1 (Kopelman et al., 2015). The best *K* under the Evanno method was calculated using STRUCTURE HARVESTER v0.6.94 (Earl & vonHoldt, 2012). A plot was constructed with DISTRUCT v1.1 (Rosenberg, 2004). We performed a PCA using the R software package adegenet 1.4-1 (Jombart et al., 2010) with the rare allele filtered data set without contaminants (62,533 SNPs for the data set with all the samples and 45,753 SNPs for the data set with only *C. lorquinii* and *carswelli*). The 3D view was plotted with the R package scatterplot3d (Ligges & Mächler, 2002).

2.8 | Analysis of introgression

We performed an ABBA-BABA analysis, also known as *D*-statistics (Durand et al., 2011), to test for differential introgression between *C. minimus* and either *C. lorquinii* or the taxon *carswelli*. The variant calling file (109,221 SNPs) of the data set without contaminants was used. Calculations were done with the software DTRIOS, included in DSUITE (Malinsky, 2019). DTRIOS uses a standard block-jackknife procedure to assess whether the *D* statistic is significantly different from zero (the null hypothesis). *Cupido osiris* was selected as outgroup and *C. minimus* as P3. Results are always positive since P1 and P2 are ordered so that nABBA ≥ nBABA. Thus, the species selected as P2 by the analysis would be the potentially introgressed if the results are significant. We selected 10,000, 3,000 and six blocks, which correspond approximately to 250 bp, 1 kb and 0.5 Mb and to 9, 35 and 18,269 SNPs per block. The mean size of the loci was 186 bp, with a mean of 7.3 SNPs per locus. In order to have the most comparable set of specimens for *C. lorquinii* and *carswelli*, individuals of *C. lorquinii* from Portugal and Africa were removed because, based on the phylogenies (Figures 2 and 3) and on their location, we suspected they have been isolated for some time and should not be treated as the same gene pool.

2.9 | Detecting loci related to wing colour

From the IPYRAD raw output with only *C. lorquinii* and *carswelli* (17,522 loci), we searched for fragments that may be related to the wing colour differences in males. These samples were examined under a stereomicroscope and sexed (Table S1). Two sets of two groups were defined. In the first analysis, the partition was related to phenotype: blue males (16 individuals) and brown males (10 individuals, including one brown *C. lorquinii* male). In the second analysis the same groups were selected but without the brown *C. lorquinii* individual, hence the groups corresponded to taxa: the males of *C. lorquinii* and the males of the taxon *carswelli*. The brown male of *C. lorquinii* was found flying in sympatry with blue males in Sierra de Huétor; pictures of the wings and the genitalia are shown in Figure S2. These two groups were compared in BAYESCAN v2.1 (Foll & Gaggiotti, 2008), using the default parameters. The aim was to detect which loci had the highest q-values and to subsequently revise them manually in order to find out if they displayed alleles exclusive for each colour morph. The sequences of these candidate loci were compared to published Lepidoptera genomes using BLAST Lepbase (Challi et al., 2016).

2.10 | *Wolbachia* infection analysis

Wolbachia bacteria are maternally inherited and may cause male-killing or cytoplasmic incompatibility (Hurst & Jiggins, 2005; Jiggins, 2003; Ritter et al., 2013; Werren et al., 2008). As a result, infection by these bacteria may trigger selective sweeps, where a particular mitochondrial genome is associated with the expansion of a *Wolbachia* strain. In swallowtail butterflies, *Iphiclides podalirius* got infected by *Wolbachia* and, at some point, introgressed to *I. feisthamelii*, which acquired the mtDNA of *I. podalirius* and its associated *Wolbachia* strain. Infected females kept spreading this specific mtDNA and *Wolbachia* strain throughout Iberia until all populations

of *I. feisthamelii* acquired them (Gaunet et al., 2019). Thus, a similar event could explain mtDNA similarities between *C. lorquinii* and *carswelli*. *Wolbachia* sequences were identified in the ddRADseq data set using CENTRIFUGE v1.0.4, as it has been demonstrated to be a quick and efficient method to detect these bacteria (Hinojosa et al., 2019). Genetic distances (d_{XY}) were calculated using MEGA v10.0.4 (Kumar et al., 2018) with the bootstrap method and using uncorrected p-distances; here, invariant loci and individuals with only one or two loci were removed.

3 | RESULTS

3.1 | ddRADseq data sets

The number of loci and SNPs of each data set and the analyses in which they have been used are indicated in Table S2. Missing data values in the raw IPYRAD output are provided in Table S1. All the data sets were deposited in Dryad (https://doi.org/10.5061/dryad.wm37pvmhm).

3.2 | Mitochondrial and ddRADseq phylogenies

The Bayesian mitochondrial phylogeny based on the barcode region of the *COI* gene (658 bp) did not recover any taxon as monophyletic (Figure 2). *Cupido lorquinii* and *carswelli* were polyphyletic and grouped in the same clade (posterior probability, PP = 1); some haplotypes were shared and maximum genetic distance between them was 1%. Two main clades were found inside this group, although they did not follow any consistent geographic pattern and each included both taxa. The African *C. lorquinii* grouped together with PP = 0.93. *Cupido minimus* was monophyletic (PP = 1). The *C. lorquinii* + *carswelli* clade split from the *C. minimus* ca. 0.97 (±0.45) million years ago (Ma). The *COI* sequence of one specimen of *C. osiris* (a male from southern Iberia) fell within the *C. minimus* group, suggesting an ancestral introgression event (they are not currently in sympatry in this locality), although incomplete lineage sorting cannot be discarded. The node representing the most recent common ancestor of the European subgenus *Cupido* in the mitochondrial chronogram was dated to ca. 1.52 (±0.7) Ma. Minimum genetic distances between the taxa (d_{XY}) are provided in Table S3.

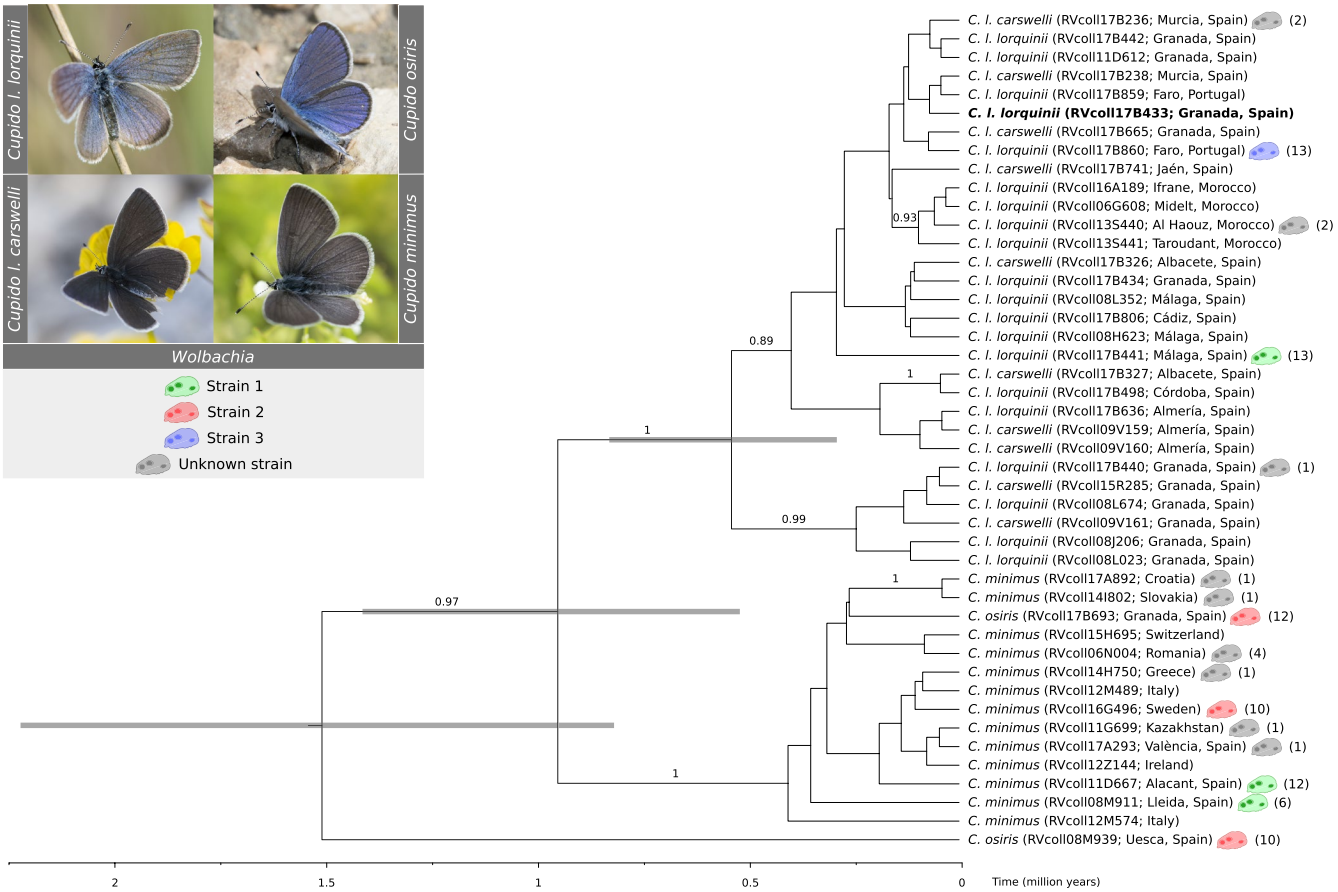

FIGURE 2 Bayesian inference chronogram based on the *COI* mitochondrial marker, with posterior probabilities >0.70 indicated. The x-axis indicates time in million years and the grey bars show the 95% HPD range for the posterior distribution of node ages. The brown *C. l. lorquinii* male is highlighted in bold. The *Wolbachia* strain type and the number of *Wolbachia* loci found in every sample (including invariable loci) are indicated next to the labels. The typical male morphology of each taxon is shown. Photographs: Vlad Dincă

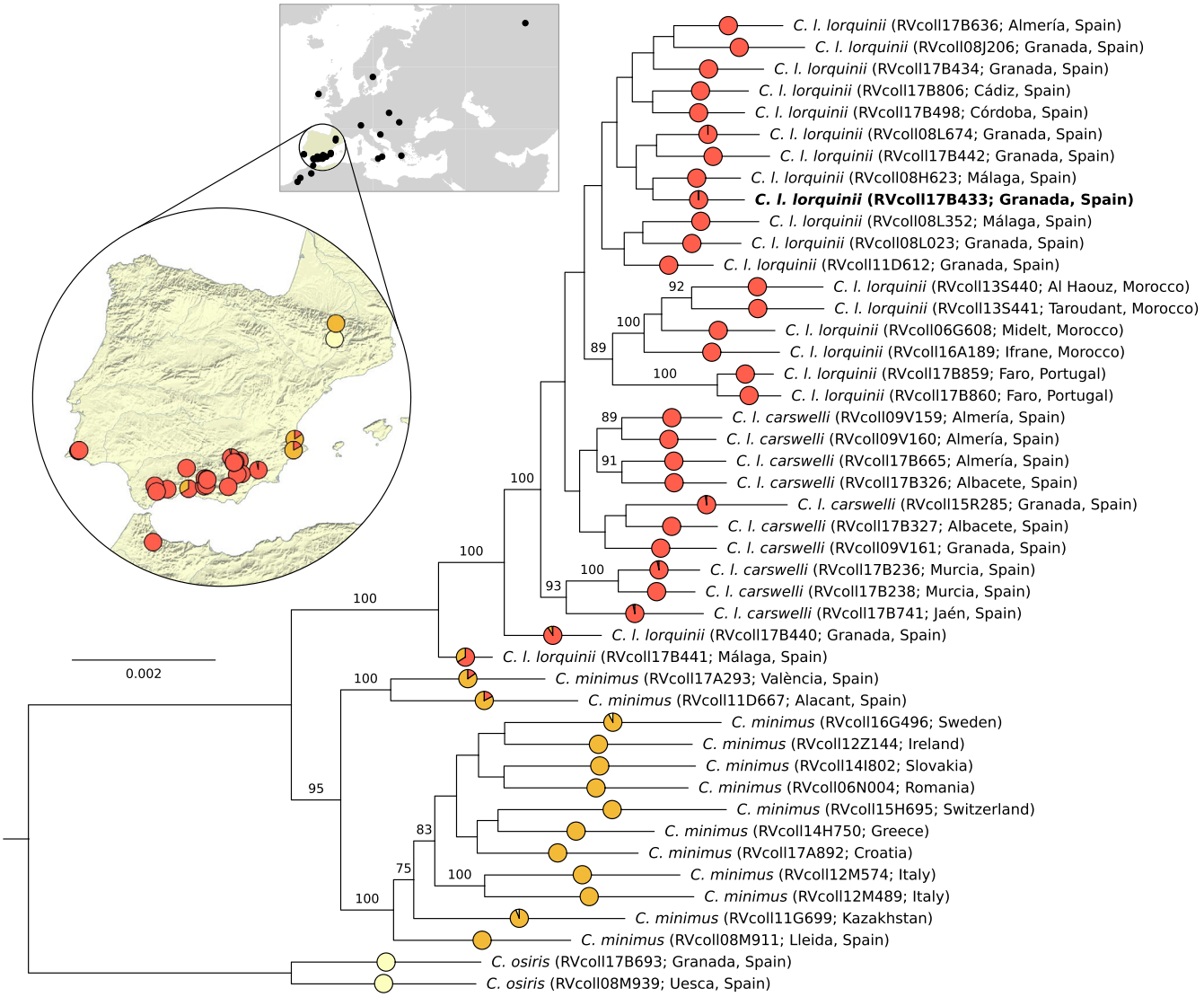

FIGURE 3 Maximum Likelihood inference tree based on 16,312 ddRADseq loci. Node bootstrap supports >70 are indicated. Scale represents 0.002 substitutions per site. The brown *C. l. lorquinii* male is highlighted in bold. STRUCTURE results ($K = 3$), represented as pie charts (colours match those of Figure S4), are indicated on the branches and on the sampling sites in the Iberian Peninsula; they are based on 62,533 SNPs. Sampling sites are indicated with black dots in the map of the western Palearctic

In the ddRADseq phylogeny (Figure 3) based on 16,312 loci *carswelli* formed two clades that were paraphyletic (albeit without support) and nested within a *C. lorquinii* clade (bootstrap support = 100). The *C. lorquinii* + *carswelli* clade showed poor structuring and well-supported relationships were obtained only among individuals from the same mountain range and among the African specimens. The brown *C. lorquinii* male (RVcoll17B433) was placed among the other *C. lorquinii* from the same and neighbouring populations. *Cupido minimus* displayed two distinct clades (bootstrap support = 100), one consisting of two individuals from south-eastern Iberia, and another one including the rest of Eurasian samples.

3.3 | Three genetic clusters with nuances

The coancestry matrix obtained with FINERADSTRUCTURE (Figure S3) did not show visible differences between *C. lorquinii* and *carswelli*. Interestingly, the latter was recovered as monophyletic in the tree. In the same line, $K = 3$ had the highest ΔK with the Evanno method in STRUCTURE HARVESTER, where the three clusters corresponded to the three main clades found in the RADseq phylogeny (Figure 3 and Figure S4): *C. osiris* (yellow), *C. minimus* (orange), and *C. lorquinii* + *carswelli* (red). *Cupido minimus* from south-eastern Iberia showed signs of introgression from *C. lorquinii*. The *C. lorquinii* specimen RVcoll17B441, and possibly RVcoll17B440, exhibit cluster

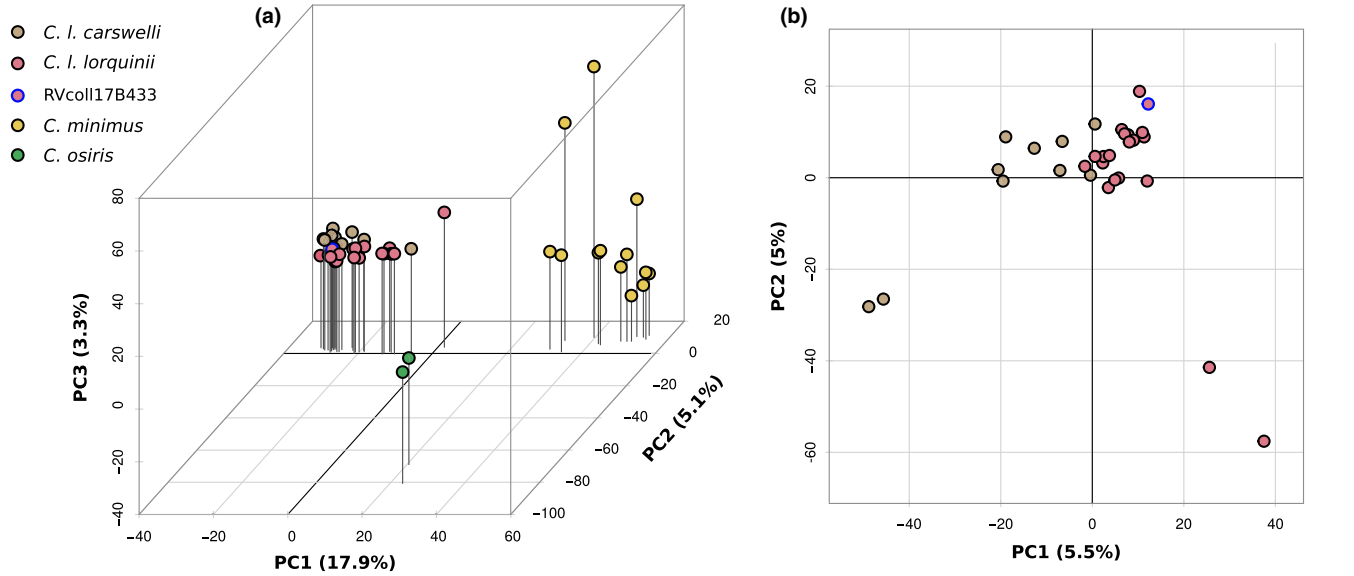

FIGURE 4 Principal component analysis (PCA) based on ddRADseq data; (a) 3D PCA including all the samples (62,533 SNPs); and (b) 2D PCA including only *C. l. lorquinii* and *C. l. carswelli* (45,753 SNPs)

admixture with *C. minimus*. In Figure S5 ($K = 3$) the same two individuals had cluster admixture with *carswelli*. This could indicate a certain degree of introgression between *C. lorquinii* and *C. minimus* or the taxon *carswelli*. However, admixed clusters in STRUCTURE do not always mean hybridization (Lawson et al., 2018). The rest of traces of cluster admixture (including the brown *C. lorquinii* male) were below 10%. The test of admixture using *D*-statistics failed to detect significant introgression (p-value = 0.30–0.42) between *C. minimus* and *C. lorquinii* or *carswelli* (*carswelli* selected as P2; $D = 0.0067$ and $f_G = 0.0041$). Similar STRUCTURE results were obtained with the unlinked data set (Figure S4). The only difference was that a small percentage (up to 14%) of cluster admixture from *C. osiris* was found in all Moroccan *C. lorquinii*, which is possibly an artefact because *C. osiris* is absent in Africa.

The separation in three groups (*C. osiris*, *C. minimus*, and *C. lorquinii* + *carswelli*) was clearly reflected in the PCA (Figure 4a). The most divergent individuals of *C. lorquinii* (RVcoll17B441) and *C. minimus* (RVcoll17A293 and RVcoll11D667) corresponded to the ones with the highest level of admixture showed by STRUCTRUE (Figure 3 and Figure S3). In the data set with only *C. lorquinii* and *carswelli* specimens, the PCA (Figure 4b) separated most of the individuals of both taxa. A similar result was obtained in STRUCTURE (Figure S5), where none of the clusters was exclusive to *carswelli*. In this analysis, STRUCTURE HARVESTER selected $K = 3$ as the best K (highest ΔK).

3.4 | Loci covarying with male wing colour

BAYESCAN retrieved 30 outlier loci when using males of *C. lorquinii* and males of *carswelli* as distinct groups, excluding the brown male of *C. lorquinii* (sample RVcoll17B433). When this individual was included within the brown male group (hence together with *carswelli*) we obtained 23 outliers, 19 of them also found in the previous analysis. Considering that *carswelli* and *C. lorquinii* have allopatric distributions, the effect of geographic differentiation was minimized by including the *C. lorquinii* the specimen RVcoll17B433, collected ca. 100 km far from the nearest populations of *carswelli* and in sympatry with blue males of *C. lorquinii*.

Surprisingly, while frequencies were sometimes markedly different, none of the outlier loci showed fixed haplotypes in *C. lorquinii* or *carswelli*. One locus had an exclusive haplotype for all the brown males, including *carswelli* and the brown *C. lorquinii*. The locus has a reading frame without stop codons, according to which one of the SNPs involved is nonsynonymous and translates to an amino acid change from T (found in *C. lorquinii*) to S (in *carswelli*). This locus is therefore a candidate to be, or linked to, a locus that is responsible for the brown coloration. Blasting in Lepbase database was unsuccessful for the amino acid sequence, but hits were retrieved from the nucleotide sequence (Table S4). With a score of 152.77 and an E value of 4.31×10^{-34}, a fragment of the gene (with unknown function) cce37.3 of the lycaenid *Calycopis cecrops* was the best match. The alignment of this locus and all the outliers were deposited in Dryad https://doi.org/10.5061/dryad.wm37pvmhm).

3.5 | *Wolbachia* infection

A total of 16 *Wolbachia* loci (including three invariable ones) were found in 16 individuals, including one *carswelli*, four *C. lorquinii*, nine *C. minimus* and two *C. osiris* (Figure 2). The final alignment without invariable loci had 2,435 bp and seven samples. Three main strains were found (Figure 2 and Table S5). Individuals RVcoll11D667 (*C. minimus* from south-eastern Iberia) and RVcoll17B441 (*C. lorquinii* from south-eastern Iberia) shared the same *Wolbachia* strain with *C. minimus* RVcoll08M911 from the Pyrenees. Both *C. osiris* shared a second strain, the same found in RVcoll17G496 (*C. minimus* from

Sweden), an individual that displayed a small degree of admixture with *C. osiris* in the STRUCTURE analysis (Figure 3 and Figure S4). *Cupido lorquinii* RVcoll17B860 had its own *Wolbachia* strain. Nonetheless, these results should be interpreted with caution because identity percentages of our loci compared to public *Wolbachia* sequences never reached 100%. *Wolbachia* loci and the alignment used can be retrieved in Dryad (https://doi.org/10.5061/dryad.wm37pvmhm).

4 | DISCUSSION

4.1 | The taxon *carswelli* is not a distinct species or a subspecies of *C. minimus*

The taxon *carswelli* has been treated as a species in numerous publications (e.g., Gil-T, 2017; Obregón et al., 2016; Tolman & Lewington, 2008). This treatment seemed logical because it is morphologically distinct from *C. lorquinii* and genetically (mtDNA) different from *C. minimus* (Dincă et al., 2015). *Cupido lorquinii* males have blue wing upperside while *carswelli* males are totally brown, and this character has been the strongest point used by several authors to treat them as distinct species. Surprisingly, we captured a brown male within the range of *C. lorquinii* (RVcoll17B433, from Sierra de Huétor, south-eastern Iberia), ca. 100 km far from the closest known *carswelli* populations; in the same place blue males were found (RVcoll17B440 & RVcoll17B442), as well as a female (RVcoll17B434). Photographs of the wings and genitalia of these three individuals are shown in Figure S2. To our knowledge, this is the first published case of a brown male present within the range of *C. lorquinii*. This specimen was recovered among blue *C. lorquinii* in the ddRADseq phylogeny (Figure 3) and in the PCA (Figure 4). Given the genetic results and geographic distance, we discard the possibility that this specimen is the product of dispersal. These findings suggest that male wing colour is not a fully diagnostic character and that the brown morph is present in *C. lorquinii* at low frequency. Neither other morphological characters nor ecological preferences seem to support the specific status of *carswelli* (see "Notes on morphology" and "Notes on ecology" in Supporting Information).

Genetically, both taxa are extremely close and polyphyletic in the *COI* phylogeny (Figure 2). Their mtDNA similarities are not caused by *Wolbachia*-mediated sweeps because most of the individuals were uninfected. The ddRADseq loci phylogeny (Figure 3) showed a similar pattern. In contrast, they are well differentiated with respect to *C. minim*us, which had a minimum p-distance of 1.3% respect to *C. lorquinii* + *carswelli* (Table S3). The coancestry matrix and the STRUCTURE analyses also grouped *C. lorquinii* and *carswelli* (Figure 3, Figures S3 and S4) while *C. minimus* formed its own cluster. The PCA placed *C. lorquinii* and *carswelli* in a compact group, distant from *C. minimus* (Figure 4a). Overall, *carswelli* cannot be considered a subspecies of *C. minimus*, but a taxon tightly related to *C. lorquinii*.

Due to the extreme genetic similarity of the taxa *carswelli* and *C. lorquinii*, only the presence of genomic islands of speciation could be invoked to support their specific differentiation. Genomic islands of speciation/differentiation are parts of the genome responsible of reproductive isolation or adaptation. They are capable to maintain genetically close species as units even in the presence of high levels of gene flow (Marques et al., 2016; Poelstra et al., 2014; Turner et al., 2005). Here, this possibility is especially difficult to test because of allopatry. If genomic islands of speciation existed they would involve an extremely low number of loci because, with BAYESCAN and out of 17,825, we failed to identify any locus with haplotypes exclusive to *C. lorquinii* or *carswelli*.

The fact that *carswelli* shares traits with both *C. lorquinii* (same mtDNA) and *C. minimus* (extremely similar morphology) and that it is geographically distributed between *C. lorquinii* and *C. minimus* (Figure 1), suggested the possibility of a hybrid origin. Nonetheless, STRUCTURE results (Figure 3 and Figure S4) did not show substantial cluster admixture from *C. minimus* into *carswelli*. The *D*-statistics analysis selected *carswelli* as P2 but retrieved a nonsignificant value ($D = 0.0067$, p-value = 0.30–0.42), which does not support the hypothesis of differential introgression between *C. minimus* and either *C. lorquinii* or *carswelli*.

4.2 | One species, two colours

Taking into account all available information, we conclude that the taxon *carswelli* should be considered north-eastern populations of *C. lorquinii*. The brown populations have three key characteristics: they are allopatric with respect to the blue populations (Figure 1), they have shallow genetic differences (Figure 4b and Figure S5) and they exhibit a noticeably distinctive character, the brown male coloration. These attributes exceed the ecotype definition (in fact there is no clear ecological differentiation) and agree with widely accepted criteria for subspecies (James, 2010; O'Brien & Mayr, 1991). Even if the brown morph is present within the blue populations of *C. lorquinii*, it seems to represent a notably rare form, a fact that is compatible with the concept of subspecies. Other cases where populations exhibited different wing colour morphs with almost identical genotypes (produced in a context of mimicry) were solved by describing subspecies (Arias et al., 2017; Zhang et al., 2016). Thus, we propose to treat this taxon as a subspecies: *Cupido lorquinii carswelli* stat. nov.

4.3 | The origin of the diverging coloration

Despite extensive research by lepidopterists, there are no records of blue individuals within the range of *C. l. carswelli*, and in that of *C. l. lorquinii* only one brown male specimen has been documented (this study, shown in Figure S2). Given these observations, it seems unlikely that a founder effect produced the entirely brown populations. Moreover, the action of genetic drift is hindered by the generally high effective population sizes of butterflies, and the taxa addressed in this study are locally abundant, including *C. l. carswelli*.

Consequently, it seems improbable that the colour shift has been produced by a neutral process.

Variability in wing colour due to plasticity is widespread in butterflies, and in many studies it has been linked to climatic and seasonal factors (Daniels et al., 2012; Koch & Bückmann, 1987; Otaki et al., 2010). In our case, *C. l. lorquinii* inhabits a wide range of habitats, from low altitude forest glades to rocky environments at the top of mountains at more than 2,000 m (Gil-T, 2017; Tennent, 1993). Despite the habitat variety, *C. l. lorquinii* does not show substantial colour variation. No intermediate forms between *C. l. lorquinii* and *C. l. carswelli* have been found. The only populations with brown males are those of *C. l. carswelli*, but virtually identical habitats are occupied by blue males in other parts of the *C. lorquinii* range (see "Notes on ecology" in Supporting Information). Thus, the hypothesis of colour shift due to plasticity or due to local adaptation to abiotic conditions appears unlikely and another type of selective pressure must be invoked.

In Andalusia (southern Spain), out of a total of 988 UTM squares of 100 km^2, the three *Cupido* taxa are distributed as follows: *C. l. lorquinii* = 95 squares, *C. osiris* = 8, *C. l. carswelli* = 10 (Obregón et al., 2016 and R. Vila personal observations). There are zero shared squares between *C. l. lorquinii* and *C. osiris*, but four between *C. l. carswelli* and *C. osiris* (and the rest are always at a distance ≤10 km, one empty square). The probability that *C. l. carswelli* overlaps with *C. osiris* in at least four squares by chance is <0.05. Additionally, Obregón et al. (2016) predicted that the current range of *C. l. carswelli* overlaps with *C. osiris* using altitude, climatic variables and larval host plant presence and, interestingly, the predicted distribution of both taxa matched perfectly. Besides Andalusia, *C. osiris* is present in mountain systems of southern Albacete province and the region of Murcia (south-eastern Spain), where the rest of populations of *C. l. carswelli* occur (García-Barros et al., 2004). Importantly, flight times of both species coincide. These aspects taken together suggest a connection between the presence of the blue *C. osiris* and the brown colour form of *C. l. carswelli*. More precisely, the interaction between *C. lorquinii* and *C. osiris* would have led to character displacement in the males of the first taxon, in this case a colour shift from blue to brown. Since the current distribution pattern of the three taxa involved is postglacial, the colour shift has probably happened postglacially (<12,000 years). The recent temporal scenario is supported by the fact that the two proposed subspecies of *C. lorquinii* were only very slightly differentiated based on genomic data of thousands of loci (lack of substantial genetic drift in allopatry), genitalia morphology, preimaginal morphology or ecology (see Supporting Information for more details).

It is worth noting that we cannot entirely discard the possibility of a shift from brown to blue, but two main arguments exist against it. First, the most basal *C. lorquinii* samples in the ddRAD phylogeny are blue (Figure 3). Although it is true that these individuals had admixed clusters with *C. minimus* (Figure 3) and with *C. l. carswelli* (Figure S5), they are not the geographically closest to the current *C. minimus* or *C. l. carswelli* populations, which makes it hard to imagine that they are hybrids given the long distance involved. Even more, other causes unrelated to gene flow may produce a similar result in STRUCTURE (Lawson et al., 2018). Second, this alternative hypothesis would entail a remarkably fast expansion of the blue form, resulting in nearly fixation over a vast territory (southern Iberia and northern Africa), across the Gibraltar strait. In this new area, *C. osiris* does not occur and selection against the blue from through reproductive character displacement would not act. Thus, rapid and vast distributional changes and some kind of selective advantage of the blue form over the brown one would be necessary. Taking into account the current distribution ranges (a smaller range of the brown form overlapping with *C. osiris*, and a wider distribution of the blue form on two continents), the brown-to-blue hypothesis seems less parsimonious.

The character displacement could have been triggered either by ecological niche overlap or by reproductive interference. The ecological character displacement is characterized by a selection to reduce competition (Garner et al., 2018). In butterflies, competition typically arises when two species use the same resources, namely sharing the same larval host plant (Jermy, 1985). However, the host plants of the taxa in sympatry are different: in Iberia *C. lorquinii* feeds on *Anthyllis vulneraria* and *C. osiris* on *Onobrychis* spp. (Tolman & Lewington, 2008). Furthermore, for ecological character displacement to take place, there should be a clear functional link between the displaced trait and the partitioned resource (Stuart & Losos, 2013), which is not the case for male wing colour and larval host plant.

Male colour is a sexual character in many lycaenids, which suggests reproductive character displacement as the potential mechanism involved in the colour shift. Reproductive character displacement occurs when competition for successful breeding imposes a divergent selection on the interacting species, causing a divergence of reproductive traits. In fact, male wing colour plays an important role in mate choice by female lycaenids (Bernard & Remington, 1991). At the start of the courtship females conduct a short flight to catch the interest of the patrolling males, a movement only induced if the colour of the male is correct (Bálint et al., 2012). Under situations with males of closely related species co-occurring and exhibiting the same colour, attempts or actual interspecific mating might more easily take place, resulting in a loss of fitness. Natural selection against this would lead to a colour shift in one of the species. All the characteristics of the system here studied agree with the hypothesis of reproductive character displacement, namely: (a) the displaced trait is arguably a reproductive trait; (b) character displacement is observed in populations in sympatry and not (or very rarely) in populations in allopatry; (c) sites of allopatry and sympatry do not differ appreciably in abiotic factors; (d) there was some degree of variability in the trait in the original populations (here suggested by the presence at very low frequency of the brown form in populations of *C. l. lorquinii*, allopatric with respect to *C. osiris*) upon which natural selection can act; and (e) under these circumstances, character displacement mediated by selection would be extremely fast, as it has apparently happened in *C. l. carswelli*.

The most frequent form of reproductive character displacement is reinforcement. It typically takes place when two populations meet

in secondary sympatry and there is some degree of hybrid depression that is counter-selected. There is no data about the existence of hybrids between *C. osiris* and *C. l. carswelli*, or about their fertility. Nevertheless, the fact that the whole subgenus *Cupido* seems to have diversified rapidly (1.5 Ma) according to the *COI* chronogram (Figure 2) and that we found signs of introgression between several taxa (see "Notes about introgression in the subgenus *Cupido*" in the Supporting Information), suggest the possibility that a certain degree of interspecific fertility may exist. In this case, reinforcement would be at play, the same mechanism that has been inferred to drive diversification in lycaenid butterflies of the subgenus *Agrodiaetus* (Lukhtanov et al., 2005). However, reproductive interference can also act in other ways, which we cannot discard, for example by a decrease in fitness due to time and energy lost in mate choice, unsuccessful mating attempts or in infertile mating (e.g., Friberg et al., 2013).

Reproductive character displacement in general, and reinforcement in particular, play a role in the final stages of the speciation continuum (Coyne & Orr, 2004), but it has also been proposed that they may potentially initiate speciation (Pfennig & Ryan, 2006). Since female mate choice linked to male wing colour may act as a prezygotic reproduction barrier in lycaenids, an incipient speciation event between *C. l. carswelli* and *C. l. lorquinii* could be ongoing. Even if we do not consider the taxon *carswelli* to be yet a different species, it is representative of a remarkable evolutionary phenomenon and we encourage the maintenance of its current level of protection in the Spanish autonomous communities of Murcia and Andalusia.

In conclusion, we demonstrate that *C. lorquinii* and *carswelli* are genetically closely related despite the fact that they exhibit strikingly different male phenotypes, blue and brown respectively. However, we have discovered that the brown male phenotype also occurs at very low frequency within populations of *C. lorquinii*. Consequently, we propose to treat the taxon *carswelli* as a subspecies of *C. lorquinii* (*C. l. carswelli* stat. nov.), as it constitutes a group of populations that occupy a distinct segment of the geographical range of the species, exhibit subtle genetic differences and harbour clear and nearly fixed differences in phenotype. Since *C. l. carswelli* occurs in sympatry with or very close to *C. osiris*, males of the former are likely to have experienced a colour shift due to reproductive character displacement. We argue that this colour shift could eventually trigger speciation between *C. l. carswelli* and *C. l. lorquinii*, as male colour is an important component of female mate choice. Hence, this may represent a conceptually novel speciation mechanism in butterflies, initiated by reproductive character displacement in allopatric populations that get in contact with a third taxon. Finally, as male wing colour seems to be an extremely unstable character in *Cupido* and in the Polyommatinae in general, we hypothesize that colour shifts may play a key role in the explosive diversification of this group of butterflies.

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AUTHOR CONTRIBUTIONS

J.C.H., N.A., M.M., and R.V. conceived, designed and coordinated the study. Funding was secured by R.V., and M.M. Laboratory protocols and data analyses were conducted by J.C.H. with the assistance of D.K. The manuscript was initially written by J.C.H., and R.V., along with significant contributions from the rest of the authors. All authors revised the final manuscript.

DATA AVAILABILITY STATEMENT

COI sequences were deposited in GenBank and BOLD, and can be consulted accessing the BOLD dataset dx.doi.org/10.5883/DS-CUPID, with sequence IDs listed in Table S1. Raw ddRADseq reads can be found in the Bioproject PRJNA593535. Alignments of butterfly and *Wolbachia* ddRADseq loci were uploaded to Dryad (https://doi.org/10.5061/dryad.wm37pvmhm).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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