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Erebia epiphron and *Erebia orientalis*: sibling butterfly species with contrasting histories

JOAN CARLES HINOJOSA[1,4], YERAY MONASTERIO[2], RUTH ESCOBÉS[2], VLAD DINCĂ[3] and ROGER VILA[1,*]

[1]*Institut de Biologia Evolutiva (CSIC-UPF), Passeig Marítim de la Barceloneta 37–49, 08003 Barcelona, Spain*
[2]*Asociación Española para la Protección de las Mariposas y su Medio (ZERYNTHIA), Madre de Dios 14, 26004 Logroño, Spain*
[3]*Department of Ecology and Genetics, PO Box 3000, 90014 University of Oulu, Finland*
[4]*Departament de Ciències de la Salut i de la Vida (DCEXS), Universitat Pompeu Fabra (UPF), Doctor Aiguader 88, 08003 Barcelona, Spain*

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The butterfly genus *Erebia* (Lepidoptera: Nymphalidae) is the most diverse in Europe and comprises boreo-alpine habitat specialists. Populations are typically fragmented, restricted to high altitudes in one or several mountain ranges, where habitat is relatively well preserved, but where the effects of climate change are considerable. As a result, the genus *Erebia* has become a model to study the impact of climate changes, past and present, on intraspecific genetic diversity. In this study, we inferred phylogenetic relationships among populations of the European species *Erebia epiphron* and *Erebia orientalis* using mitochondrial (*COI*) and nuclear markers (*ITS2*, *wg* and *RPS5*), and reconstructed their phylogeographical history. We confirm *E. orientalis* and *E. epiphron* as a relatively young species pair that split *c*. 1.53 (±0.65) Mya. The high genetic homogeneity of *E. orientalis*, combined with its restricted geographical range in the eastern Balkans, suggests that this taxon may be subject to inbreeding depression and displays low adaptability to potential environmental changes, which calls for close monitoring of population trends. By contrast, genetic structure was complex for *E. epiphron*, revealing an intricate phylogeographical history that included a succession of dispersal events, mixing of populations and periods of isolation in multiple refugia. Finally, we highlight southern populations that represent unique genetic lineages, which, in the case of extinction, would lead to important genetic erosion.

ADDITIONAL KEYWORDS: climate change – conservation – extinction – genetic erosion – phylogeography – speciation.

INTRODUCTION

Intraspecific genetic structure is key to elucidating the effects of past climatic changes on the distribution of organisms (e.g. Svenning & Skog, 2007; Dincă *et al.*, 2011) and, in addition, is critical for conservation prioritization and management planning (e.g. Frankham *et al.*, 2014). Alpine habitats tend to be relatively less affected by human-induced destruction or alteration compared to lowland and mid-altitude areas, but the impact of climate change on mountain biodiversity is most notable (Hoorn *et al.*, 2018). As a result, species associated with boreo-alpine habitats such as the mountain ringlet butterflies (*Erebia* Dalman, 1816) are providing models for the study of climatic shifts and their consequences (Vila *et al.*, 2005; Schmitt *et al.*, 2014).

The genus *Erebia* (Lepidoptera: Nymphalidae) comprises about 100 species distributed across the Holarctic in alpine and arctic habitats. They colonized Europe from Asian Russia about 17–23 Mya (Peña *et al.*, 2015) and experienced an extraordinary radiation during the Pleistocene glaciations (Albre *et al.*, 2008; Peña *et al.*, 2015). As a result, *Erebia* currently represents the most diverse butterfly genus in the Palearctic and harbours more species in Europe than in Asia (Tennent, 2008). Speciation in this genus has apparently been

*Corresponding author. E-mail: roger.vila@csic.es

driven by a combination of two main factors: ecological specialization, including flight time and habitat differentiation (Kuras *et al.*, 2000; Martin *et al.*, 2002; Klečková *et al.*, 2014), and allopatric distribution in both glacial and/or interglacial refugia (Vila *et al.*, 2005; Albre *et al.*, 2008), which drove each species to colonize a specific niche on one or more mountain ranges.

Their dependence on alpine habitats has resulted in a fragmented distribution across mountains in the Holarctic region, except for the northernmost latitudes, where ranges may be more extensive. Thus, *Erebia* butterflies seem to be especially vulnerable to climate change (Brierley & Kingsford, 2009; Jones *et al.*, 2009; Habel *et al.*, 2010) and genetic erosion may compromise the viability of populations and species (Bálint *et al.*, 2011; Alsos *et al.*, 2012; Rubidge *et al.*, 2012). Under the current trend of global warming (Hansen *et al.*, 2017), reductions in the ranges of mountain butterfly species due to altitudinal displacement have already been reported – for example in a southern population of *Erebia cassioides* (Hohenwarth, 1792) (Scalercio *et al.*, 2014) – and local extinctions are expected in the southern limits of species distributions (Franco *et al.*, 2006).

Given the current scenario of climate change, understanding the evolutionary histories of alpine species and identifying fractions of genetic diversity potentially under threat are of importance. We therefore focused on a widely distributed and morphologically variable alpine species with a fragmented distribution, *Erebia epiphron* (Knoch, 1783). Although this species represents a good system to assess phylogeographical patterns of alpine organisms, it has not been studied previously using genetics in a comprehensive way, but rather only for allozymes for a more restricted geographical region (Schmitt, 2006). We also included the close relative *Erebia orientalis* Elwes, 1900 with the aim of clarifying its taxonomic status and, as in *E. epiphron*, documenting its genetic structure, reconstructing its phylogeographical history and inferring which populations may harbour unique genetic lineages.

Erebia epiphron is found in the main mountain ranges of Europe, albeit sometimes restricted to small and isolated areas (Fig. 1). It is generally associated with humid habitats in mountain glades and subalpine and alpine meadows, usually above 1000 m (Tshikolovets, 2011). It is highly variable morphologically, with 11 subspecies listed by Tshikolovets (2011). Some populations are probably under threat due to global warming and it has been predicted that the species could become extinct from the Iberian Peninsula by 2080 (Settele *et al.*, 2008). We therefore aim to assess if southern populations of *E. epiphron* potentially under

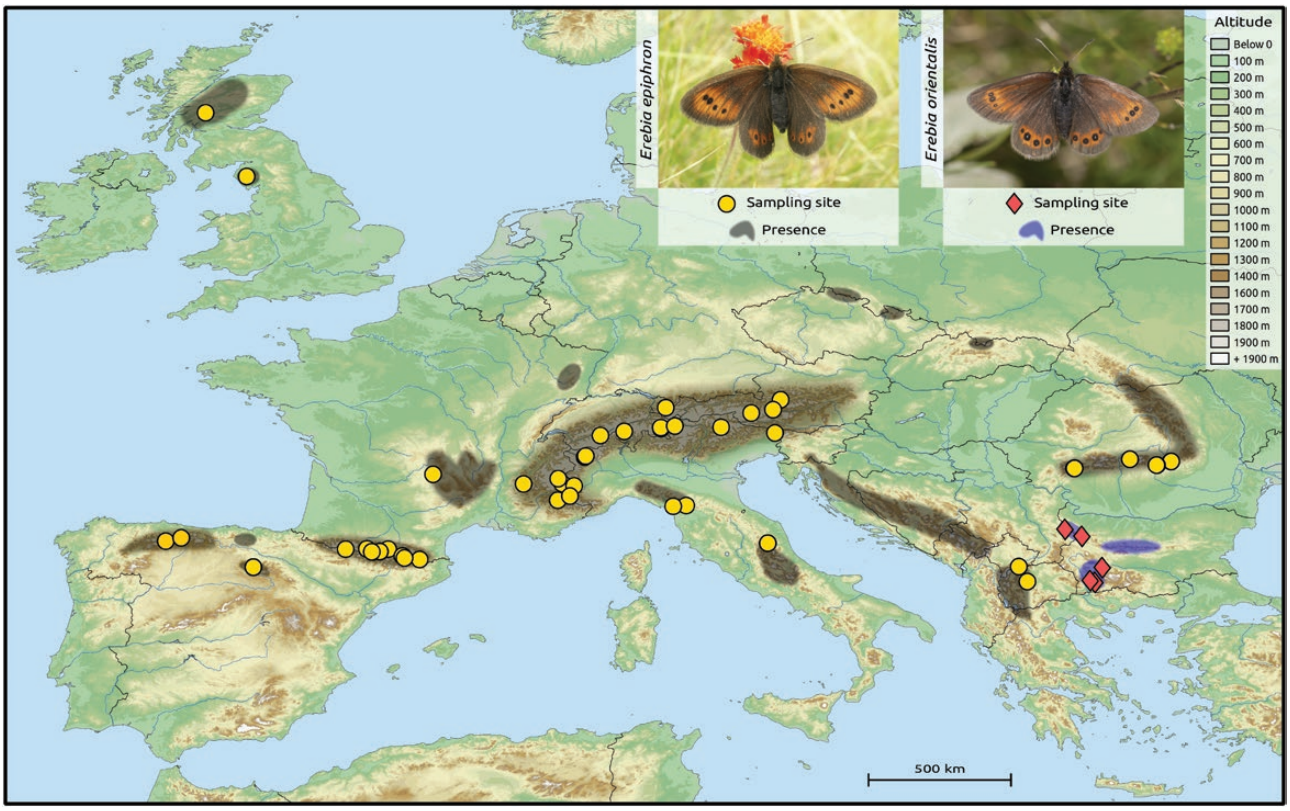

Figure 1. Approximate distribution range of *E. epiphron* and *E. orientalis* and sampling sites. Photos: Vlad Dincă.

threat from climate change have unique DNA lineages, and thus samples from the southernmost populations occurring in the three European peninsulas (Iberia, Italy and Balkans) were included. The population of Sierra de la Demanda, north-central Iberian Peninsula, is of particular conservation concern: it is the southernmost population in the peninsula, inhabits a tiny area restricted to the highest parts of these mountains, and apparently displays a low number of individuals (Monasterio & Escobés, 2015).

Erebia orientalis is endemic to mountains in western Bulgaria and eastern Serbia (Rila, Pirin and western Balkan Mountains; van Swaay *et al.*, 2010; Kudrna *et al.*, 2015) and has similar morphology and biology to *E. epiphron* (Tshikolovets, 2011). It was originally described as a subspecies of *E. epiphron* and maintained this rank for some time (Higgins, 1975), although it is now widely considered to be a different species (e.g. Varga, 2014; Peña *et al.*, 2015). Its distribution range restricted to high elevations of a few mountains in the Balkans suggests that this taxon might face similar threats due to climate change as those of the southern populations of *E. epiphron*.

Our research questions were as follows: (1) Is genetic structure compatible with the hypothesis that *E. epiphron* and *E. orientalis* are different species? (2) Were their phylogeographic histories similar? (3) Do any southern populations, presumably at risk because of climate change, display low genetic variability? (4) Do any of these southern populations represent unique genetic lineages that, in the case of extinction, would lead to important genetic erosion? By analysing nuclear (*ITS2*, *wg* and *RPS5*) and mitochondrial (*COI*) DNA markers from *E. epiphron* and *E. orientalis* we confirm the division in two species with different phylogeographical histories, despite their close kinship. *Erebia epiphron* is notably diverse genetically, and patterns suggest a complex phylogeographical history, with multiple dispersal events, mixing of populations and periods of isolation. In *E. orientalis*, the almost non-existent variability in nuclear and mitochondrial genes points to a strong bottleneck and recent contact among its populations.

MATERIAL AND METHODS

Specimens

We analysed 82 individuals for one mitochondrial gene, the barcode fragment of the cytochrome *c* oxidase subunit I (*COI*), in a dataset representative of nearly the entire distribution of *E. epiphron* and *E. orientalis* (Fig. 1). Three nuclear genes, internal transcribed spacer 2 (*ITS2*), wingless (*wg*) and 40S ribosomal protein S5 (*RPS5*), were sequenced for 29 individuals representative of the main mountain ranges and mitochondrial lineages. Information regarding collection locality and genes sequenced for each specimen studied is given in the Supporting Information (Table S1). The specimens were kept in 95% ethanol after collection and stored at –20 °C. These samples are deposited in the DNA and Tissues Collection of Institut de Biologia Evolutiva (IBE), Barcelona, Spain. Additional *COI* sequences were retrieved from GenBank (see Table S1).

DNA sequencing

DNA extraction was done following the protocol described in Vodă *et al.* (2015a). Primers LepF1 and LepR1 (5′-ATTCAACCAATCATAAAGATATTGG-3′ and 5′-TAAACTTCTGGATGTCCAAAAAATCA-3′ respectively) were used for the amplification of the *COI* gene, obtaining a 658-bp fragment. Amplification conditions were: first denaturation at 92 °C for 60 s, then 92 °C for 15 s, 48 °C for 45 s and 62 °C for 150 s for five cycles and for 30 cycles changing the annealing temperature to 52 °C, with a final extension step at 62 °C for 7 min.

ITS2 was amplified with primers ITS3La and ITS4-Euchloe (5′-GGGCATCGATGAAGAACGCAG CTAAC-3′ and 5′-TCCTCCGCTTATTGATATGCT TAA-3′, respectively) under the following conditions: first denaturation at 94 °C for 3 min, then 36 cycles of 94 °C for 35 s, 46 °C for 30 s and 72 °C for 60 s. The final extension step was done at 72 °C for 6 min. The maximum length obtained was 590 bp.

The gene *wg* was amplified with primers LepWg1 and LepWg2 (5′-GARTGYAARTGYCAYGGYATGTC TGG-3′ and 5′-ACTNCGCARCACCARTGGAATGT RCA-3′, respectively) under the following conditions: first denaturation at 95 °C for 3 min, then 40 cycles of 95 °C for 60 s, 51 °C for 60 s and 72 °C for 90 s. The final extension step was done at 72 °C for 6 min. The fragment length obtained was 403 bp.

RPS5 was amplified with primers HybrpS5degF and HybrpS5degR (5′-ATGGCNGARGARAAYTGG AAYGA-3′ and 5′-CGGTTRGAYTTRGCAACACG-3′, respectively) under the following conditions: first denaturation at 95 °C for 6 min, then 40 cycles of 95 °C for 30 s, 51 °C for 30 s and 72 °C for 90 s. The final extension step was done at 72 °C for 10 min. The fragment length obtained was 613 bp.

PCR products were purified and Sanger-sequenced by Macrogen Inc. Europe (Amsterdam, the Netherlands). All the sequences obtained in this study (Table S1) are available in GenBank (accession numbers MK074774-MK074811, MK074813-MK074840 and MK155178-MK155217) and in the dataset DS-EPIORI (DOI dx.doi.org/10.5883/DS-EPIORI) from the Barcode of Life Data Systems (http://www.boldsystems.org/).

Phylogenetic reconstruction

Nuclear sequences were concatenated in Geneious v.6.1.8 (Kearse *et al.*, 2012). *COI* and the concatenated nuclear genes were separately aligned with MAFFT v.7.304 (Katoh & Standley, 2013). The best fitting nucleotide substitution models according to jModelTest v.2.1.7 (Darriba et al., 2012) under the corrected Akaike's information criterion (AICc) were SYM+I for *RPS5*, HKY+I for *wg*, JC for *ITS2* and *GTR+I* for *COI*. *Erebia pharte* (Hübner, [1804]) was used as an outgroup for both mitochondrial (mtDNA) and nuclear (nDNA) phylogenetic analyses.

Phylogenetic reconstruction of the nuclear genes was done through a Bayesian inference obtained with Mr. Bayes v.3.2.6 (Ronquist *et al.*, 2012). Base frequencies, substitution rates and proportions of invariable sites were retrieved from jModelTest. One million generations with sampling every 1000 generations and a burn-in of 10 000 generations were selected.

COI phylogeny was reconstructed in BEAST v.1.8.0 (Drummond *et al.*, 2007) through the CIPRES Science Gateway (Miller *et al.*, 2010). Base frequencies were estimated, six gamma rate categories were selected and a randomly generated initial tree was used. Rough estimates of node ages were obtained by applying a strict clock and a normal prior distribution centred on the mean between two generally accepted substitution rates for invertebrates: 1.5% and 2.3% uncorrected pairwise distance per million years [Quek *et al.* (2004) and Brower (1994), respectively]. The standard deviation was tuned 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 the program TRACER 1.6. *Erebia pharte* (Hübner, [1804]) was used as an outgroup for both mtDNA and nDNA phylogenetic analyses.

Haplotype networks were created with TCS 1.21 (Clement *et al.*, 2000). *COI* genetic distances were calculated using MEGA v.7.0.14 (Kumar *et al.*, 2016) with the bootstrap method to estimate variance and using uncorrected *p*-distances (Collins *et al.*, 2012; Srivathsan *et al.*, 2012).

RESULTS

Nuclear DNA

The nDNA phylogeny (Fig. 2) resulted in two well-defined clades: one representing *E. epiphron* and the other *E. orientalis*, both with strong support [posterior probability (PP) = 1]. *Erebia orientalis* was always recovered as monophyletic in the trees based on single nuclear markers, while *E. epiphron* was monophyletic for *wg* and paraphyletic for *ITS2* and *RPS5* (Figs S1–S3). Although *E. epiphron* showed relatively low variability in the nuclear markers, samples from the Pyrenees and the Massif Central appeared well supported as the most basal lineage. The remaining European samples grouped with good support (PP = 0.98), but relationships within this clade remained unresolved. However, it is worth noting that the Macedonian and Romanian populations of *E. epiphron*, although geographically closest to *E. orientalis*, were not genetically closest to the latter (Fig. 2; Supporting Information, Fig. S1). Within *E. orientalis* all the *ITS2* sequences were identical, and one variable position was detected in *RPS5* and two in *wg*, but the low genetic structure did not show any geographical pattern and the separation between mountain ranges was not supported.

Mitochondrial DNA

Similarly to the nDNA phylogeny, the *COI* tree (Fig. 3) recovered *E. epiphron* and *E. orientalis* as the two main clades and the support for their monophyly was high (PP = 1 and 0.97, respectively). Their temporal separation was estimated to 1.53 (±0.65) Mya. In contrast to the nDNA phylogeny, *E. epiphron* individuals from Sierra de la Demanda were recovered (with good support) as sister to the remaining samples of this species collected across its range. The time of divergence between the Sierra de la Demanda clade and the remaining *E. epiphron* samples was estimated to *c*. 0.95 (±0.39) Mya. All specimens from the Romanian Carpathians formed a well-supported clade (PP = 1). Individuals from the Pyrenees and Massif Central also formed a moderately well-supported clade (PP = 0.92) but, unlike in the nuclear phylogeny, this *COI* clade also included specimens from the Cantabrian Mountains. Relationships among the remaining *E. epiphron* specimens were not well resolved and no clear structure was detected among samples from the Alps, Apennines and mountains of Macedonia. Specimens from England and Scotland, which are highly isolated from other *E. epiphron* populations (Fig. 1), formed a relatively well-supported clade (PP = 0.95) and even showed two sub-clades corresponding to England (PP = 0.97) and Scotland (not well supported), although the Scottish clade also included a specimen from the French Alps. As with the nuclear genes, no spatial genetic structure was found within *E. orientalis*.

The *COI* haplotype network (Fig. 4) recovered 19 haplotypes of *E. epiphron* and two haplotypes of *E. orientalis*. The minimum *p*-distance between the two species was 1.67%. Alpine samples represented eight of the 19 haplotypes obtained for *E. epiphron*. Overall, the highest *p*-distance within this species was 2.13%, while the Alps alone displayed a maximum *p*-distance of 1.52%. The Sierra de la Demanda population

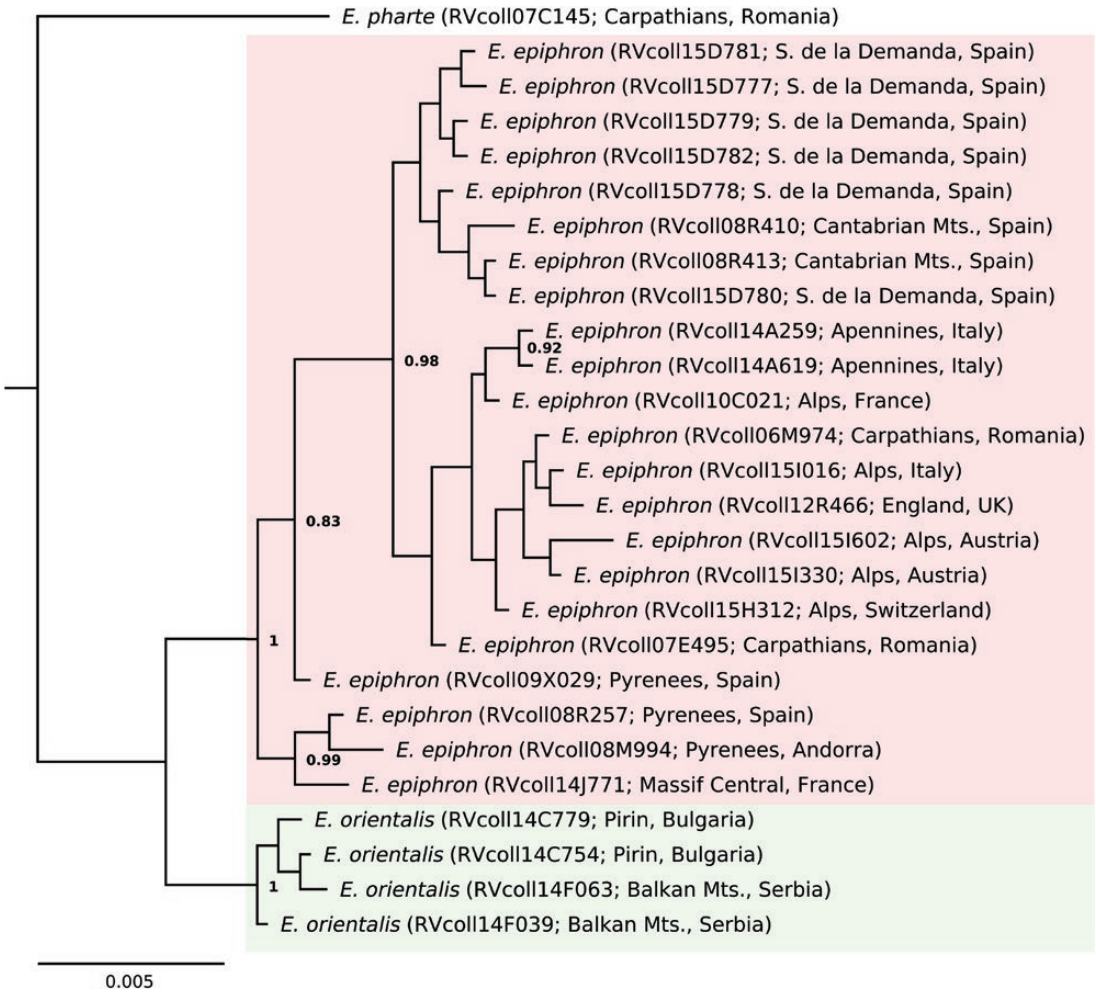

Figure 2. Nuclear DNA phylogeny based on concatenated sequences for *ITS2*, *wg* and *RPS5* obtained through Bayesian inference. Posterior probabilities > 0.7 are indicated. Scale units are presented in substitutions per site.

displayed a minimum genetic distance of 1.37% from the remaining individuals. The other mountain ranges had from one to three haplotypes that were closely related between them, with only one or two nucleotide differences. The slight exception was the Romanian Carpathians, diverging by at least four nucleotide differences from the other analysed specimens.

The two haplotypes of *E. orientalis* displayed only two nucleotide differences (0.3% divergence) and the common one was present in all sampled sites (Fig. 4). The uncommon haplotype was represented by a single sequence that was obtained from GenBank, for which we could not ascertain the quality.

DISCUSSION

Our key findings are as follows. (1) *Erebia epiphron* and *E. orientalis* split *c.* 1.53 (±0.65) Mya (Figs 2, 3) and no introgression between them has been detected despite geographical proximity (Fig. 1). (2) The genetic structure of *E. epiphron* is complex, revealing an intricate phylogeographical history that is described below in detail. (3) *Erebia orientalis* is genetically extremely homogeneous for both nuclear and mitochondrial markers, as is also the case for *E. epiphron* from Sierra de la Demanda. (4) Some of these populations isolated in the south represent unique genetic lineages that are apparently not found elsewhere.

THE RELATIONSHIP BETWEEN *E. EPIPHRON* AND *E. ORIENTALIS*

Our results support the hypothesis that *E. epiphron* and *E. orientalis* are different species. The minimum genetic distance between them is at the typical intra-/interspecific boundary (1.67% in *COI*), but they are recovered as monophyletic in the trees

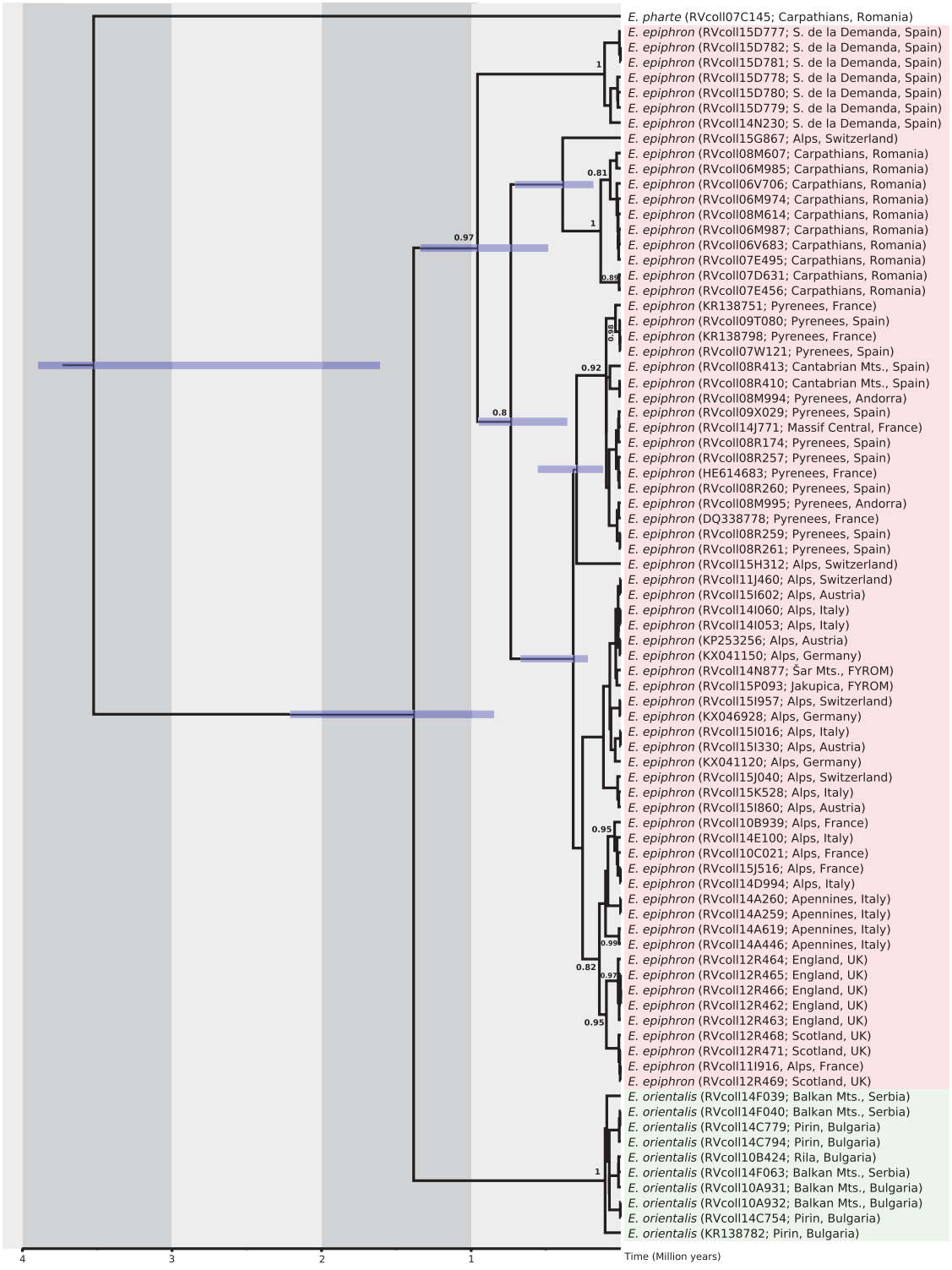

Figure 3. *COI* gene tree obtained through Bayesian inference. Posterior probabilities > 0.7 are indicated.

based on the mitochondrial *COI* (Fig. 3) and on the combined nuclear markers (Fig. 2). Moreover, the differentiation is confirmed in the trees based on each of the three nuclear genes alone, where at least one taxon is always monophyletic (Figs S1–S3). The split between *E. epiphron* and *E. orientalis* occurred *c.* 1.53 (±0.65) Mya (Fig. 3), which, despite the considerable error inherent to estimations based on a molecular

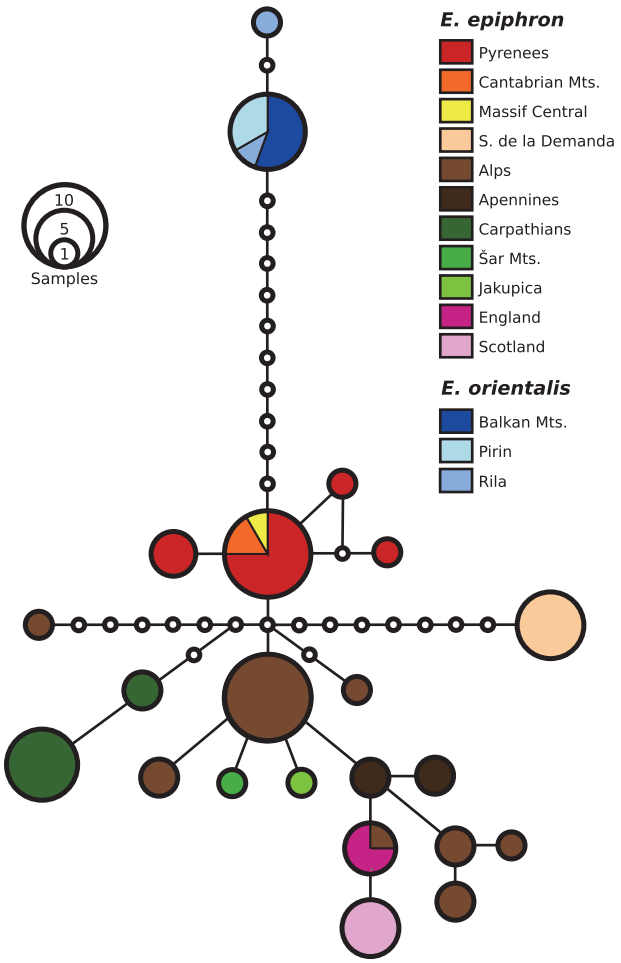

Figure 4. Maximum parsimony haplotype network based on *COI*. Every substitution is marked with a bar and the circle size is proportional to the number of samples represented. Small open circles represent unsampled haplotypes. Colours indicate the geographical origin of the specimens.

clock, is a result notably different from the *c*. 4 Mya predicted by Peña *et al.* (2015). At that time, their common ancestor probably remained isolated in Western Europe (evolving into *E. epiphron*) and the Balkan Peninsula (evolving into *E. orientalis*).

Erebia epiphron and *E. orientalis* are very similar morphologically and, in particular, they are apparently indistinguishable based on their genitalia (Higgins, 1975). Nevertheless, their genetic differentiation is clear in both nuclear and mitochondrial markers, and no gene flow was detected despite the relative proximity among eastern populations (≈150 and ≈200 km between *E. orientalis* populations and *E. epiphron* in Macedonia and Romania, respectively). It is unclear how *E. orientalis* maintained isolated from *E. epiphron* despite sharing the same habitat type and despite the repeated, even recent, geographical movements we infer in the two species. Given the geographical proximity in their ranges, it is most likely that secondary contact took place at some point. We hypothesize the existence either of a prezygotic barrier or of reproductive interference – the latter implying the lack of a prezygotic barrier and low fertility of hybrids (Vodă *et al.*, 2015b). Assuming that the two species cannot easily coexist because of biotic factors, it seems that *E. orientalis* settled in parts of the Balkan Peninsula but became surrounded by *E. epiphron*, which was able to colonize a larger area and possibly impeded expansion of *E. orientalis*. The apparent lack of fusion or introgression between these two taxa along glacial cycles represents further proof of the validity of the species.

Intraspecific relationships and phylogeographical reconstruction

Erebia epiphron showed a relatively high level of *COI* intraspecific genetic divergence (2.13% maximum *p*-distance) and the age of the crown group was inferred at *c*. 0.95 (±0.39) Mya. Thus, genetic differentiation within this species started well before the last glacial period, as Schmitt *et al.* (2006) have argued. With eight of the 19 haplotypes detected (Fig. 4) and a maximum genetic distance of 1.52%, most of the genetic diversity is concentrated in the Alps, as has also been shown for many other orophilous species (Schmitt, 2009). By contrast, nuclear genes displayed low variability and a different phylogenetic pattern with respect to the *COI* phylogeny: Pyrenean and Massif Central samples appeared to be the most basal and the other relationships were not resolved. This can be explained by the succession of glacial periods, forcing multiple bottlenecks over time followed by population mixing. However, the few substitutions detected in the nuclear markers make it difficult to use these data to reconstruct the fine-scale biogeographical events, because of potential stochasticity in the mutation and fixation processes.

The fact that the Alps, together with Iberia, harbour an important part of the genetic diversity of the species could be potentially due to two mechanisms: (1) the Alps are a contact zone for several lineages or (2) the Alps are a centre of diversification themselves. The first hypothesis would imply that the haplotypes found in the Alps are also found in other distant locations, but this is not the case. Most haplotypes in the Alps, although diverse and frequently unrelated, are private (Fig. 4). This pattern points strongly to the Alps as maintaining lineages for a long period, some of the lineages reaching these mountains independently at different times. This also implies that the Alps or areas nearby must have acted as a glacial refugium for *E. epiphron*, a hypothesis that has also been suggested

for other *Erebia* species such as *Erebia medusa* Schiffermüller, 1775 and *Erebia euryale* Esper, 1777 (Schmitt & Seitz, 2001; Haubrich & Schmitt, 2007; Albre et al., 2008; Schmitt & Haubrich, 2008).

In contrast to *E. epiphron*, genetic variability within *E. orientalis* was almost non-existent in both nuclear and mitochondrial markers throughout the species distribution. This suggests a strong bottleneck, either related to its speciation event or posteriorly, and recent contact among populations.

Based on our combined results we propose the following hypothesis for the phylogeographical history of these taxa:

1. *Erebia epiphron* and *E. orientalis* split *c.* 1.5 (±0.65) Mya, the former in the west and the latter in the east of Europe.
2. The first split within *E. epiphron* occurred *c.* 0.95 (±0.39) Mya, , more precisely between an Iberian lineage and a European lineage. The older splits – the basal lineages in the phylogenies we obtained – always involve Iberian populations: Sierra de la Demanda in *COI*, and Pyrenees + Massif Central in nuclear markers (in this case Sierra de la Demanda + Cantabrian Mts are also differentiated from the rest). Although differences exist between the types of markers and also among nuclear markers (generally involving few changes and thus with high stochasticity), it seems clear that the first split involved an Iberian lineage. The mitochondrial lineage of this Iberian lineage apparently only remains in the Sierra de la Demanda population. Later, a potential mitochondrial introgression from Europe to the Pyrenees and a recent one from the Pyrenees to the Cantabrian Mts seem to have occurred.
3. The European lineage started to differentiate *c.* 0.66 (±0.28) Mya, with a first split between an Eastern lineage – today occurring in the Carpathians and Alps – and populations in the remaining territory.
4) More recently, *c.* 0.34 (±0.21) Mya, the population that is now present in the Pyrenees and Massif Central split from the Central and European populations. Indeed, the Massif Central population was found to be almost identical genetically to the Pyrenean populations, which confirms recent gene flow between the two mountain ranges. The Pyrenees – Massif Central connection and the surprisingly limited contact with the Alps has been suggested for other boreo-alpine species (Descimon, 1995; Ronikier *et al.*, 2008) and was also found in *Erebia manto* Esper, 1777 (Schmitt *et al.*, 2014), a species with a distribution pattern similar to *E. epiphron*. These results disagree with those of Schmitt *et al.* (2006), who found a close relationship between the Pyrenean and Western Alps populations based on allozymes. They proposed a refugium that connected the Alps with the Pyrenees through the southern Massif Central during the last glaciation, but no evidence of that was found in our data.
5) Finally, after the Last Glacial Maximum individuals apparently from a Western Alps refugium – a refugium also predicted for *E. medusa* and *E. Euryale* – colonized the UK. This is suggested by the fact that Scottish individuals display a shared *COI* haplotype with a specimen from the French Alps, and the entire UK clade is closely related to other populations from this region and from the Apennines. Remarkably, the English population displays a unique haplotype, separated from the Scottish population by one substitution, so either the ancestor of these individuals had colonized from a different population or this mutation arose during the current interglacial period. Also during this period, it appears that a single population of *E. orientalis* became isolated in different mountain ranges, resulting in the current distribution.

Erebia epiphron is rather variable morphologically, even within populations. However, the subspecies currently accepted (e.g. Tolman & Lewington, 2008, Tshikolovets, 2011) are generally not genetically well supported and need revision based on additional sources of data. In addition to disentangling intra- vs. inter-population variability, the apparent existence of secondary contacts resulting in introgression events between lineages further complicates the subspecific taxonomy. There is also inconsistency of the subspecies in *E. orientalis*: it has been divided into three subspecies (Varga, 2014), *E. o. orientalis* in the Rila Mts, *E. o. infernalis* in the Pirin Mts and *E. o. macrophthalma* in the Balkan Mts, but we have not found any genetic traits supporting the validity of these subspecies.

Conservation issues

Small isolated populations in the southern range limits of cold-adapted species are likely to experiment genetic erosion, even more so under current global warming. This is evident for the Sierra de la Demanda population: all studied specimens there shared a single *COI* haplotype. It is the southernmost area where this butterfly can be found in western Europe, and it possibly inhabits one of the driest habitats known for the species, which has led to small, isolated populations where water is more plentiful (Monasterio & Escobés, 2015). Population density is quite low, with fewer than 15 sightings a day and occuping less than 20 ha on the northern slopes of the mountains (Monasterio & Escobés, 2015). These data suggest that it could be classified as Critically Endangered according to the IUCN criteria at the regional scale (IUCN, 2012).

Genetic homogeneity is also high in *E. orientalis*, with only two *COI* haplotypes detected in three mountain ranges (Fig. 4). Although populations with low genetic diversity can remain healthy and be maintained for long periods in stable environments (Habel & Schmitt 2012; Habel & Schmitt 2018), they may be more vulnerable to challenges such as climate change (Habel, 2010; Bijlsma & Loeschcke, 2012) and ecosystem alterations (Brierley & Kingsford, 2009; Jones *et al.*, 2009), as well as to new pathogens (Luquet *et al.*, 2012). At the same time, a range reduction because of a shift to higher altitudes is expected, where this possible, as observed in a southern population of *E. cassioides* (Scalercio *et al.*, 2014) –paradoxically, in that case positive effects on the population trend were documented. Within *E. epiphron* such phenomena seem to be occurring already: in Britain, Franco *et al.* (2006) detected a decline in low-altitude colonies, while Konvička *et al.* (2016) observed desynchronization of annual cohort development at low elevation in the Czech Republic.

For these reasons, and because both the *E. epiphron* population of Sierra de la Demanda and *E. orientalis* represent genetically differentiated units with restricted distributions, we advocate special protection and measures being taken to guarantee their conservation, starting with monitoring to assess their situation and population trends. Their potential extinction, more probable for the Sierra de la Demanda in the short term, would represent regrettable cases of notable genetic erosion.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Phylogenetic tree based on *ITS2* data obtained through Bayesian inference. Posterior probabilities > 0.7 are indicated. Scale units are presented in substitutions per site.

Figure S2. Phylogenetic tree based on *wg* data obtained through Bayesian inference. Posterior probabilities > 0.7 are indicated. Scale units are presented in substitutions per site.

Figure S3. Phylogenetic tree based on *RPS5* data obtained through Bayesian inference. Posterior probabilities > 0.7 are indicated. Scale units are presented in substitutions per site.

Table S1. Specimens used for the study and GenBank accession numbers for the markers sequenced. Sample IDs are indicated for those specimens deposited in the DNA and Tissues Collection of Institut de Biologia Evolutiva (IBE), Barcelona, Spain.