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

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Abstract

The butterfly genus *Erebia* (Lepidoptera: Nymphalidae) is the most diverse in Europe and comprises boreo-alpine habitat specialists. Populations are
15 typically fragmented, restricted to high altitudes in one or several mountain ranges, where habitat is relatively well preserved, but climate change effects are remarkable. As a result, the genus *Erebia* has become a model to study the impact of climate changes, past and present, onto intraspecific genetic diversity. In this study, we inferred phylogenetic relationships
20 among populations of the European species *Erebia epiphron* and *Erebia orientalis* using mitochondrial (*COI*) and nuclear markers (*ITS2*, *wg* and *RPS5*), and reconstructed their phylogeographic history. We confirm *E. orientalis* and *E. epiphron* as a relatively young species pair that split ca. 1.53 (± 0.65) million years ago. The high genetic homogeneity of *E.*
25 *orientalis*, combined with a restricted geographic range in the eastern

Balkans, suggests that this taxon may be suffering of inbreeding depression and displays low adaptability to potential environmental changes, which calls for close monitoring of population trends. Contrastingly, genetic structure was complex for *E. epiphron*, revealing an intricate phylogeographic history apparently including 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 case of extinction, would lead to important genetic erosion.

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

40 INTRODUCTION

Intraspecific genetic structure is key to elucidate effects of past climatic changes on the distribution of organisms (e.g. Svenning & Skog, 2006; Dincă *et al.*, 2011) and, in addition, is critical for conservation prioritisation and management planning (e.g. Frankham *et al.*, 2014). Alpine habitats
 45 tend to be relatively less affected by destruction or alteration compared to lowland and mid-altitude areas, nevertheless the impact of climate change on mountain biodiversity is most notable (Hoorn *et al.*, 2018). As a result, species associated to boreo-alpine habitats like the mountain ringlet butterflies (*Erebia* Dalman, 1816) are becoming an emergent model for the
 50 study of climatic shifts and their consequences (Vila *et al.*, 2005; Schmitt *et al.*, 2014).

The genus *Erebia* (Lepidoptera: Nymphalidae) comprises about a hundred species distributed across the Holarctic in alpine and arctic habitats. They colonized Europe from Asian Russia about 17-23 million years ago (Mya)
 55 (Peña *et al.*, 2015) and experienced an extraordinary radiation during the Pleistocene glaciations (Albre *et al.*, 2008; Peña *et al.*, 2015). As a result, the genus *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 is apparently driven by a combination of two
 60 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.

65 Their dependence on alpine habitats 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 due to climate change (Brierley &
Kingsford, 2009; Jones *et al.*, 2009; Habel *et al.*, 2010) and genetic erosion
70 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 of mountain butterfly species
ranges due to altitudinal displacement have already been reported –like in a
southern population of *Erebia cassioides* (Hohenwarth, 1792) (Scalercio *et*
75 *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, it is urgent to understand the
evolutionary histories of alpine species and to identify fractions of genetic
diversity potentially under threat. For that, we focused on a widely
80 distributed and morphologically variable alpine species with fragmented
distribution, *Erebia epiphron* (Knoch, 1783). Although this species
represents a good system to assess phylogeographic patterns of alpine
organisms, it has not been studied previously using genetics in a
comprehensive way, only allozymes for a more restricted geographical
85 region (Schmitt, 2006). We also included the close relative *Erebia orientalis*
Elwes, 1900 in the study with the purpose of clarifying its taxonomic status
and, as in *E. epiphron*, documenting its genetic structure, reconstructing its
phylogeographic history and inferring which populations may harbour
unique genetic lineages.

90 *Erebia epiphron* is found in the main European mountain ranges, albeit
sometimes restricted to small and isolated areas (Fig. 1). This species is
generally linked to humid habitats in mountain glades and subalpine and
alpine meadows, usually above 1000m (Tshikolovets, 2011). It is a species
highly variable morphologically, with eleven subspecies listed by
95 Tshikolovets (2011). Some of its populations are likely 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). Because of
this threat, we aim to assess if southern populations potentially under threat
by climate change have unique DNA lineages and samples from the
100 southernmost populations occurring in the three European peninsulas
(Iberia, Italy and Balkans) were included. In particular the population in
Sierra de la Demanda, in north-central Iberian Peninsula, seems to be in a
delicate situation in terms of conservation: it is the southernmost population
in the peninsula, inhabits a tiny area restricted to the highest parts of these
105 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*
110 (Tshikolovets, 2011). In fact, it was originally described as a subspecies of *E.*
epiphron and maintained this rank for a long period (Higgins, 1975),
although nowadays it is widely considered to be a different species (e.g.
Varga, 2014; Peña *et al.*, 2015). The distribution range restricted to high
elevations of 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 are: 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 case of extinction, would lead to important genetic erosion? Through the analysis of 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 phylogeographic histories, despite their close kinship. *Erebia epiphron* is notably diverse genetically and patterns suggest a complex phylogeographic 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 & METHODS

Specimens

For this study we analysed 82 individuals for one mitochondrial gene –the barcode fragment of the *cytochrome c oxidase subunit I (COI)*–, in a dataset representative for nearly all the distribution for *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 a selection of 29 individuals representative of the main mountain ranges and

140 mitochondrial lineages. Information about collecting locality and genes
sequenced for each specimen studied can be found in 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
145 retrieved from GenBank (see Table S1).

DNA sequencing

DNA extraction was done following the protocol described in Vodă *et al.*
(2015a). LepF1 and LepR1 primers (5'-ATTCAACCAATCATAAAGATATTGG-3'
and 5'-TAAACTTCTGGATGTCCAAAAAATCA-3' respectively) were employed
150 for the *COI* amplification obtaining a 658 bp fragment. 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 in 5 cycles and other 30 cycles changing the annealing temperature to
52°C with the final extension step at 62°C for 7 min.

ITS2 was amplified with ITS3La and ITS4-Euchloe primers (5'-
155 GGGCATCGATGAAGAACGCAGCTAAC-3' and 5'-
TCCTCCGCTTATTGATATGCTTAA-3', respectively) under the conditions: first
denature at 94°C for 3 min, then 94°C for 35 s, 46°C for 30 s and 72°C for 60
s in 36 cycles. The final extension step was done at 72°C for 6 min. The
maximum length obtained was 590 bp.

160 The gene *wg* was amplified with LepWg1 and LepWg2 primers (5'-
GARTGYAARTGYCAYGGYATGTCTGG-3' and 5'-
ACTNCGCARCACCARTGGAATGTRCA-3', respectively) under the following
conditions: first denature at 95°C for 3 min, then 95°C for 60 s, 51°C for 60 s

and 72°C for 90 s in 40 cycles. The final extension step was done at 72°C for
 165 6 min. The fragment length obtained was 403 bp.

RPS5 was amplified with HybrpS5degF and HybrpS5degR primers (5'-
 ATGGCNGARGARAAAYTGGAAYGA-3' and 5'-CGGTTRGAYTTTRGCAACACG-3',
 respectively) under the following conditions: first denaturation at 95°C for 6
 min, then 95°C for 30 s, 51°C for 30 s and 72°C for 90 s in 40 cycles. The
 170 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 nos XXX-XXX) and in the
 175 dataset DS-EPIORI (DOI XXXX) from the Barcode of Life Data Systems
 (<http://www.boldsystems.org/>).

Phylogenetic reconstruction

Nuclear sequences were concatenated in Geneious v6.1.8 (Kearse *et al.*,
 2012). *COI* and the concatenated nuclear genes were separately aligned
 180 with MAFFT v7.304 (Kato & Standley, 2013). The best fitting nucleotide
 substitution models according to jModelTest v2.1.7 (Darriba *et al.*, 2012)
 under 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 outgroup for both mtDNA and nDNA phylogenetic analyses.

185 Phylogenetic reconstruction of the nuclear genes was done through a
 Bayesian inference obtained with Mr. Bayes v3.2.6 (Ronquist *et al.*, 2012).
 Base frequencies, substitution rates and proportions of invariable sites were

retrieved from jModelTest. One million generations with sampling every 1,000 generations and a burn-in of 10,000 generations were selected.

190 *COI* phylogeny was reconstructed in BEAST v1.8.0 (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
195 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
200 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 outgroup for both mtDNA and nDNA phylogenetic analyses.

Haplotype networks have been created with TCS 1.21 (Clement *et al.*, 2000). *COI* genetic distances were calculated using MEGA v7.0.14 (Kumar *et al.*, 2016) with the bootstrap method to estimate variance and using
205 uncorrected p-distances (Collins *et al.*, 2012; Srivathsan *et al.*, 2012).

RESULTS

Nuclear DNA

The nuclear DNA phylogeny (Fig. 2) resulted in two well defined clades: one
210 representing *E. epiphron* and the other *E. orientalis*, both with a strong support (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* (Fig. 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 rest of European samples grouped with a good support (PP = 0.98), but relationships within this clade remained unresolved. It is however worth noting that the Macedonian and Romanian populations of *E. epiphron*, although geographically the closest to *E. orientalis*, were not genetically closest to the latter (Fig. 2 and S1). Within *E. orientalis* all the *ITS2* sequences were identical, 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 nuclear DNA 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 nuclear DNA phylogeny, *E. epiphron* individuals from Sierra de la Demanda were recovered (with good support) as sister to the rest of samples of this species collected across its range. The time of divergence between the Sierra de la Demanda clade and the rest of *E. epiphron* was estimated to ca. 0.95 (± 0.39) Mya. All specimens from the Romanian Carpathians formed a well supported clade (PP = 1). The individuals from the Pyrenees and Massif Central also formed a moderately supported clade (PP = 0.92) but, unlike in the nuclear phylogeny, this *COI* clade also included the specimens from the Cantabrian Mountains. Relationships among the rest of *E. epiphron*

specimens were not well resolved and no clear structure was detected
among samples from the Alps, Apennines, and mountains of Macedonia. The
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), but the Scottish clade also included
a specimen from the French Alps. As it happened 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 8 out 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 population of Sierra de la Demanda displayed a minimum genetic distance of 1.37% compared to the rest of 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 rest of the 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: 1) *E. epiphron* and *E. orientalis* split ca. 1.53 (± 0.65)
265 Mya (Fig. 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 phylogeographic history
described below in detail. 3) *Erebia orientalis* is genetically extremely
homogeneous for both nuclear and mitochondrial markers, as is also the
270 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
275 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 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,
280 where at least one taxon is always monophyletic (Fig. S1-S3). The split
between *E. epiphron* and *E. orientalis* occurred ca. 1.53 (± 0.65) Mya (Fig. 3),
which, despite the considerable error inherent to estimations based on a
molecular clock, is a result notably different from the ca. 4 Mya predicted by
Peña *et al.* (2015). At that moment, their common ancestor likely remained
285 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
 290 nuclear and mitochondrial markers, and no gene flow was detected despite
 relative proximity in the 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 the isolation from *E.*
epiphron despite sharing the same habitat type and of the repeated, even
 295 recent, geographical movements we infer in the two species. Given the
 geographical proximity in the ranges, it is most likely that secondary contact
 took place at some point. We hypothesise the existence of either a
 prezygotic barrier, or of reproductive interference –a phenomenon implying
 the lack of a prezygotic barrier and low fertility of hybrids (Vodă *et al.*,
 300 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 got surrounded by *E. epiphron*, which was able to colonize a
 bigger area and possibly impeded *E. orientalis* to expand. The apparent lack
 of fusion or introgression between these two taxa along glacial cycles
 305 represents another proof of the validity of the species.

Intraspecific relationships and phylogeographic 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 ca. 0.95 (± 0.39) Mya. Thus, genetic differentiation within
 310 this species started well before the last glacial period, as Schmitt *et al.*
 (2006) argued. With 8 out of the 19 haplotypes detected (Fig. 4) and a
 maximum genetic distance of 1.52%, the Alps concentrated most of the
 genetic diversity, as it has also been shown to be the case for many other
 orophilous species (Schmitt, 2009). On the other hand, nuclear genes

315 displayed low variability and a rather 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 due to the succession of glacial periods, forcing
multiple bottlenecks along time followed by mixing of the populations.
320 However, the few substitutions detected in the nuclear markers make it
hard to use these data in order to reconstruct the fine biogeographic 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:

325 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 strongly points to the
330 Alps as maintaining lineages for a long period, some of which reached these
mountains independently at different moments in history. 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*
335 Esper, 1777 (Schmitt & Seitz, 2001; Haubrich & Schmitt, 2007; Albre &
Legal, 2008; Schmitt & Haubrich, 2008).

Contrasting with *E. epiphron*, the genetic variability of *E. orientalis* was
almost inexistent in both nuclear and mitochondrial markers through all its
distribution. This suggests a strong bottleneck, either related to the
340 speciation event or posteriorly, and recent contact among populations.

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

1) *Erebia epiphron* and *E. orientalis* split ca. 1.5 (± 0.65) Mya, the former in the west and the latter in the east of Europe.

345 2) Later, ca. 0.95 (± 0.39) Mya, took place the first split within *E. epiphron*, 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: S. de la Demanda in *COI*, and Pyrenees + Massif Central in nuclear markers (in this case S. de la Demanda + Cantabrian Mts.
350 are also differentiated from the rest). Although differences exist between 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 isolate. The mitochondrial lineage of this Iberian lineage apparently only remains in the Sierra de la Demanda population. Later, a
355 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 differentiating ca. 0.66 (± 0.28) Mya, with a first split between an Eastern lineage –nowadays occurring in the
360 Carpathians and Alps– and the populations in the rest of the territory.

4) More recently, 0.34 (± 0.21) Mya, the population nowadays present in the Pyrenees and Massif Central split from the Central and European populations. Indeed, the population of Massif Central was found to be almost identical genetically to the Pyrenean populations, which confirms recent
365 gene flow between both mountain ranges. The connection Pyrenees – Massif

Central and the surprisingly limited contact with the Alps has been suggested for other boreo-alpine species (Descimon, 1995; Ronikier *et al.*, 2008) and it 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. These authors 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 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, apparently a single population of *E. orientalis* became isolated in different mountain ranges resulting in the current distribution.

Erebia epiphron is a rather variable species 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- versus inter-population variability, the apparent existence of secondary contacts resulting in introgression events between

lineages further complicates subspecific taxonomy. The inconsistency of subspecies is present in *E. orientalis* too: 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 experience genetic erosion, even more so under the current phenomenon of global warming. This is evident for the population in Sierra de la Demanda: all studied specimens 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 habitat known for the species, producing isolation of small populations in specific points where water upwellings appear (Monasterio & Escobés, 2015). The population density is quite low, with less than 15 sightings a day and occupying less than twenty hectares in the northern face of the mountains (Monasterio & Escobés, 2015). These data suggest that it could be classified as Critically Endangered according to the IUCN criteria at regional scale (IUCN, 2012).

Genetic homogeneity is also high in *E. orientalis*, with only two *COI* haplotypes detected in three mountain ranges (Fig. 4). While populations with low genetic diversity can be apparently 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 (Bijlsma & Loeschcke, 2012; Habel, 2010) 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 reduction of the range because of a shift to higher altitudes is expected, where possible, similarly to that 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 already occurring: 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 by monitoring to assess their situation and population trends. Their potential extinction, more probable for the Sierra de la Demanda in a short term, would represent regrettable cases of notable genetic erosion.

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Figures and tables

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

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.

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

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. The small black circles represent unsampled haplotypes. Colours indicate the geographic origin of the specimens.

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.

475 **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

480 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.

Sample ID	Species	Locality	Country	Population	Genbank code (COI)	Genbank code (ITS2)	Genbank code (wg)	Genbank code (RPSS)
-	<i>Erebia epiphron</i>	Former Languedoc-Roussillon	France	Pyrenees/Massif Central	KX046928	-	-	-
-	<i>Erebia epiphron</i>	Bagnères-de-Luchon, Haute-Garonne	France	Pyrenees	KX041150	-	-	-
-	<i>Erebia epiphron</i>	Vorarlberg	Austria	Alps	KX041120	-	-	-
-	<i>Erebia epiphron</i>	Ustou, Ariège	France	Pyrenees	KP253256	-	-	-
-	<i>Erebia epiphron</i>	Cauterets, Hautes-Pyrénées	France	Pyrenees	MK155196	-	-	-
-	<i>Erebia epiphron</i>	Swabia (Bavaria)	Germany	Alps	MK155184	-	-	-
-	<i>Erebia epiphron</i>	Upper Bavaria	Germany	Alps	MK155208	-	-	-
-	<i>Erebia epiphron</i>	Bavaria	Germany	Alps	MK155183	-	-	-
RVcoll06M974	<i>Erebia epiphron</i>	Săcele, Braşov	Romania	Carpathians	HQ004371	MK074778	MK074797	MK074826
RVcoll06M985	<i>Erebia epiphron</i>	Măneciu, Prahova	Romania	Carpathians	HQ004369	-	-	-
RVcoll06M987	<i>Erebia epiphron</i>	Măneciu, Prahova	Romania	Carpathians	HQ004373	-	-	-
RVcoll06V683	<i>Erebia epiphron</i>	Râu de Mori, Hunedoara	Romania	Carpathians	HQ004372	-	-	-
RVcoll06V706	<i>Erebia epiphron</i>	Uricani, Hunedoara	Romania	Carpathians	GU669667	-	-	-
RVcoll07D631	<i>Erebia epiphron</i>	Buşteni, Prahova	Romania	Carpathians	HQ004374	-	-	-
RVcoll07E456	<i>Erebia epiphron</i>	Moroeni, Dâmboviţa	Romania	Carpathians	HQ004370	-	-	-
RVcoll07E495	<i>Erebia epiphron</i>	Uricani, Hunedoara	Romania	Carpathians	HQ004375	-	MK074808	MK074837
RVcoll07W121	<i>Erebia epiphron</i>	Vielha e Mijaran, Lleida	Spain	Pyrenees	GU669854	-	-	-
RVcoll08M607	<i>Erebia epiphron</i>	Arefu, Argeş	Romania	Carpathians	HQ004377	-	-	-
RVcoll08M614	<i>Erebia epiphron</i>	Arefu, Argeş	Romania	Carpathians	HQ004376	-	-	-
RVcoll08M994	<i>Erebia epiphron</i>	El Pas de la Casa, Encamp	Andorra	Pyrenees	HM901314	-	MK074794	MK074823
RVcoll08M995	<i>Erebia epiphron</i>	El Pas de la Casa, Encamp	Andorra	Pyrenees	HM901315	-	-	-
RVcoll08R174	<i>Erebia epiphron</i>	Setcases, Girona	Spain	Pyrenees	GU669853	-	-	-
RVcoll08R257	<i>Erebia epiphron</i>	Alt Àneu, Lleida	Spain	Pyrenees	HM901357	-	MK074807	MK074836
RVcoll08R259	<i>Erebia epiphron</i>	Alt Àneu, Lleida	Spain	Pyrenees	GU669855	-	-	-
RVcoll08R260	<i>Erebia epiphron</i>	Alt Àneu, Lleida	Spain	Pyrenees	GU669848	-	-	-
RVcoll08R261	<i>Erebia epiphron</i>	Alt Àneu, Lleida	Spain	Pyrenees	GU669849	-	-	-
RVcoll08R410	<i>Erebia epiphron</i>	Lena, Asturias	Spain	Cantabrian Mountains	GU675818	-	MK074801	MK074830
RVcoll08R413	<i>Erebia epiphron</i>	Caso, Asturias	Spain	Cantabrian Mountains	GU675815	MK074776	MK074793	MK074822
RVcoll09T080	<i>Erebia epiphron</i>	Vielha e Mijaran, Lleida	Spain	Pyrenees	JF847985	-	-	-
RVcoll09X029	<i>Erebia epiphron</i>	Meranges, Girona	Spain	Pyrenees	HM901499	-	MK074792	MK074821
RVcoll10B939	<i>Erebia epiphron</i>	Uvemet-Fours, Alpes-de-Haute-Provence	France	Alps	DQ338778	-	-	-
RVcoll10C021	<i>Erebia epiphron</i>	Arvieux, Hautes-Alpes	France	Alps	KR138782	-	MK074788	MK074816
RVcoll11I916	<i>Erebia epiphron</i>	Chichilianne, Isère	France	Alps	KP870625	-	-	-
RVcoll11J460	<i>Erebia epiphron</i>	Bever, Grisons	Switzerland	Alps	KP870445	-	-	-
RVcoll12R462	<i>Erebia epiphron</i>	Cockermouth, Cumbria	United Kingdom	England	KP870916	-	-	-
RVcoll12R463	<i>Erebia epiphron</i>	Cockermouth, Cumbria	United Kingdom	England	KP870577	-	-	-
RVcoll12R464	<i>Erebia epiphron</i>	Cockermouth, Cumbria	United Kingdom	England	KP870587	-	-	-
RVcoll12R465	<i>Erebia epiphron</i>	Cockermouth, Cumbria	United Kingdom	England	KP870931	-	-	-
RVcoll12R466	<i>Erebia epiphron</i>	Cockermouth, Cumbria	United Kingdom	England	MK155216	-	MK074810	MK074839
RVcoll12R468	<i>Erebia epiphron</i>	Killin, Stirling	United Kingdom	Scotland	KP870980	-	-	-
RVcoll12R469	<i>Erebia epiphron</i>	Killin, Stirling	United Kingdom	Scotland	KP870580	-	-	-
RVcoll12R471	<i>Erebia epiphron</i>	Killin, Stirling	United Kingdom	Scotland	KP870616	-	-	-
RVcoll14A259	<i>Erebia epiphron</i>	Ussita, Macerata	Italy	Apennines	MK155192	-	MK074796	MK074825
RVcoll14A260	<i>Erebia epiphron</i>	Ussita, Macerata	Italy	Apennines	KR138751	-	-	-
RVcoll14A446	<i>Erebia epiphron</i>	Abetone, Pistoia	Italy	Apennines	MK155190	-	-	-
RVcoll14A619	<i>Erebia epiphron</i>	Massa, Lucca	Italy	Apennines	KR138798	-	MK074787	MK074815
RVcoll14D994	<i>Erebia epiphron</i>	Villar Pellice, Turin	Italy	Alps	MK155199	-	-	-
RVcoll14E100	<i>Erebia epiphron</i>	Acceglio, Cuneo	Italy	Alps	MK155180	-	-	-
RVcoll14I053	<i>Erebia epiphron</i>	Saint-Marcel, Aosta	Italy	Alps	MK155204	-	-	-
RVcoll14I060	<i>Erebia epiphron</i>	Saint-Marcel, Aosta	Italy	Alps	MK155214	-	-	-
RVcoll14J771	<i>Erebia epiphron</i>	Le Falgoux, Cantal	France	Massif Central	MK155198	-	MK074798	MK074827
RVcoll14N230	<i>Erebia epiphron</i>	Villavelayo, La Rioja	Spain	Sierra de la Demanda	HE614683	-	-	MK074819
RVcoll14N877	<i>Erebia epiphron</i>	Ljuboten	FYROM	Šar Mountains	MK155194	-	-	-
RVcoll15D777	<i>Erebia epiphron</i>	Ezcaray, La Rioja	Spain	Sierra de la Demanda	MK155181	MK074774	MK074786	MK074814
RVcoll15D778	<i>Erebia epiphron</i>	Ezcaray, La Rioja	Spain	Sierra de la Demanda	MK155185	-	MK074789	MK074817
RVcoll15D779	<i>Erebia epiphron</i>	Pazuengos, La Rioja	Spain	Sierra de la Demanda	MK155187	-	MK074790	MK074818
RVcoll15D780	<i>Erebia epiphron</i>	San Millán de Cogolla, La Rioja	Spain	Sierra de la Demanda	MK155210	MK074783	MK074805	MK074834
RVcoll15D781	<i>Erebia epiphron</i>	Pazuengos, La Rioja	Spain	Sierra de la Demanda	MK155217	-	MK074811	MK074840
RVcoll15D782	<i>Erebia epiphron</i>	Fresneda de la Sierra Tíron, Burgos	Spain	Sierra de la Demanda	MK155191	-	MK074795	MK074824
RVcoll15G867	<i>Erebia epiphron</i>	Fusio, Ticino	Switzerland	Alps	MK155195	-	-	-
RVcoll15H312	<i>Erebia epiphron</i>	Mund, Valais	Switzerland	Alps	MK155202	MK074780	MK074800	MK074829
RVcoll15I016	<i>Erebia epiphron</i>	Chiusaforte, Udine	Italy	Alps	MK155179	-	MK074785	MK074813
RVcoll15I330	<i>Erebia epiphron</i>	Kals am Großglockner, Tyrol	Austria	Alps	MK155215	MK074784	MK074809	MK074838
RVcoll15I602	<i>Erebia epiphron</i>	Ramsau am Dachstein, Styria	Austria	Alps	MK155211	-	MK074806	MK074835
RVcoll15I860	<i>Erebia epiphron</i>	Muhr, Salzburg	Austria	Alps	MK155178	-	-	-
RVcoll15I957	<i>Erebia epiphron</i>	La Punt-Chamues-ch, Grisons	Switzerland	Alps	MK155212	-	-	-
RVcoll15J040	<i>Erebia epiphron</i>	Tschier, Grisons	Switzerland	Alps	MK155213	-	-	-
RVcoll15J516	<i>Erebia epiphron</i>	Val-des-Prés, Hautes-Alpes	France	Alps	MK155197	-	-	-
RVcoll15K528	<i>Erebia epiphron</i>	Santa Cristina Gherdëina, Bolzano	Italy	Alps	MK155205	-	-	-
RVcoll15P093	<i>Erebia epiphron</i>	Studenici, Skopje	FYROM	Jakupica	MK155193	MK074777	-	-
-	<i>Erebia orientalis</i>	NA	Bulgaria	Pirin	MK155188	-	-	-
RVcoll10A931	<i>Erebia orientalis</i>	Berkovitsa, Montana	Bulgaria	Balkan Mountains	MK155186	-	-	-
RVcoll10A932	<i>Erebia orientalis</i>	Berkovitsa, Montana	Bulgaria	Balkan Mountains	MK155182	-	-	-
RVcoll10B424	<i>Erebia orientalis</i>	Yakoruda, Blagoevgrad	Bulgaria	Rila	MK155209	-	-	-
RVcoll14C754	<i>Erebia orientalis</i>	Bansko, Blagoevgrad	Bulgaria	Pirin	MK155189	MK074775	MK074791	MK074820
RVcoll14C779	<i>Erebia orientalis</i>	Bansko, Blagoevgrad	Bulgaria	Pirin	MK155207	MK074782	MK074804	MK074833
RVcoll14C794	<i>Erebia orientalis</i>	Bansko, Blagoevgrad	Bulgaria	Pirin	MK155203	-	-	-
RVcoll14F039	<i>Erebia orientalis</i>	Knjaževac, Zaječar	Serbia	Balkan Mountains	MK155206	MK074781	MK074803	MK074832
RVcoll14F040	<i>Erebia orientalis</i>	Knjaževac, Zaječar	Serbia	Balkan Mountains	MK155200	-	-	-
RVcoll14F063	<i>Erebia orientalis</i>	Knjaževac, Zaječar	Serbia	Balkan Mountains	MK155201	MK074779	MK074799	MK074828
RVcoll07C145	<i>Erebia pharte</i>	Rodna, Bistrița-Năsăud	Romania	-	HQ004435	-	MK074802	MK074831
-	<i>Erebia pronoe</i>	Bagnères-de-Luchon, Haute-Garonne	France	-	-	KC184896	-	-

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