Interaction of ecology and evolution in two *Erebia* butterflies:

A comparative study

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CHAPTER 1

General introduction

Ecosystems provide numerous services and disservices that enable the survival of humans and other living beings. Insects occupy a distinct place within ecosystems, as they influence numerous ecosystem processes. While in the past the focus was mainly on negative aspects and associations with plagues, pests, and pathogens, the ecosystem services provided by insects and their regulatory functions are now increasingly considered (Schowalter et al. 2018).

Due to their large contribution to the global fauna (Scheffers et al. 2012), insects have important provisioning, regulatory, and supporting roles in ecosystems. As a result of their high species diversity and biomass, insects are important components of numerous trophic networks, and their loss would significantly impact multiple ecosystems and further drive global species extinctions (Wilson 2016).

Especially in agricultural production, insects are of great importance. Numerous invertebrates exert important top-down control on pests such as other invertebrates, fungi, and weeds (Losey & Vaughan 2006). It is believed that approximately 80 % of all flowering plant species and 75 % of all crops rely on insect pollination (Klein et al. 2007). Furthermore, insects contribute to nutrient cycling and soil dynamics, structure, and fertility as decomposers of wood, leaves, manure, and carcasses (Losey & Vaughan 2006).

Despite their great importance for the ecosystem, there is still much that is unknown. Officially, fewer than 100 insect species are considered extinct worldwide (IUCN 2019). However, since it is estimated that 80 % of all insects are still undescribed (Storck 2018) and thus no knowledge exists about the majority of species, no statement can be made about the exact degree of endangerment of the global insect fauna. Recent studies have found a sharp decline in abundance, biomass, and area of distribution of formerly common species in several orders (Hallmann et al. 2017; Seibold et al. 2019; Wagner et al. 2019). However, trends differ largely among regions, species, and species groups and therefore cannot be generalized (Macgregor et al. 2019). Loss of insect abundance and biomass results in reduced energy flow within and between trophic levels and a decline in ecosystem services, benefits, and functions. This decline is considered a precursor to extinction (Cardoso et al. 2019). Conservation measures and monitoring intended to halt or slow this trend often focus on protecting habitats of rare and endangered species, but this has not reversed trends on a global scale or in protected areas (Filz et al. 2013).

The steady loss of habitat quality is considered one of the main drivers of the ongoing loss of biodiversity, which continues even in declared protected areas (Filz et al. 2013). Insects depend on the provision of diverse resources in small-scale areas of their habitat; however, the homogenization of landscapes has destroyed these microhabitats and with them the quality of habitats (Filz et al. 2013; Cardoso et al. 2020). This deterioration of habitat quality has contributed to the steady fragmentation of landscapes (Fischer & Lindenmayer 2007), making populations more susceptible to risks such as parasitoids or weather caprices (Melbourne & Hastings 2008). The resulting higher extinction probability may necessitate a focus on facilitating metapopulation networks to enable recolonization (Nouhys 2009). Despite these general trends, the effects on individual species cannot be extrapolated easily because their responses to these changes depend on specific characteristics such as their mobility, population structure, and ability to form metapopulations (Cardoso et al. 2020; Seibold et al. 2019). The main drivers of habitat loss include clearing of primary forests, conversion of nature to cultivated land, and changes in agricultural production (IPBES 2018).

Traditional agricultural production was characterized by historically developed, small-scale management of semi-productive land, which created a variety of open and semi-open landscapes with diverse landscape elements in which a variety of microhabitats existed (Krämer et al. 2012). Semi-natural, flower-rich grasslands became some of the most species-rich habitats in Europe (van Swaay 2002, Krämer et al. 2012). The modernization of agriculture caused an abandonment of small-

scale management practices, resulting in semi-productive areas either being abandoned and losing their open land characteristics as a result of incipient succession, being converted to residential or industrial use, or becoming part of an industrialized agricultural enterprise (Wagner et al., 2020). The latter is often characterized by large areas of monocultures, leading to a structural impoverishment of the landscape (Sánchez-Bayo & Wyckhuys 2019). This structural simplification of the landscape is exacerbated by the use of herbicides and fertilizers, which alter the composition of local flora and further degrade or destroy habitat quality.

In addition to these indirect factors, there are also direct impairments of modern agriculture that endanger insect fauna, such as the application of insecticides like neonicotinoids and fipronil. The potential hazards of these insecticides are difficult to assess due to drift, unknown mixed effects, non-lethal toxicity, and long half-lives. In addition to directly affecting insects (Wodd & Goulson 2017), these insecticides can affect other trophic levels through bioaccumulation and biomagnification (Tooker & Pearsons 2021). However, on a global scale, the massive clearing of tropical rainforests is probably causing the greatest damage to global biodiversity, although it is difficult to quantify due to a lack of inventories (Storck et al. 2018). The destruction of rainforests not only destroys habitats but also interferes with material and energy cycles, which can lead to further changes in habitats (Pielke et al. 2016).

Aside from habitat degradation caused by human activities, climate change is another major threat to global insect diversity. Insects, as ectothermic animals, are also strongly dependent on their ambient temperature. The ambient temperature determines their metabolic rate as an environmental cue, which in turn determines development and reproduction (Clarcke & Fraser 2004; van Dyck et al. 2015). In particular, extreme ambient temperatures are among the strongest evolutionary factors, which additionally have a direct impact on the population growth of a species (Waldvogel et al. 2017). With global climate warming, there is an earlier onset and longer-lasting growing seasons, from which some species may benefit. At the same time, however, decoupled, asynchronous development may also occur within communities, which may pose an increased risk of regional extinction for the species involved (van Dyck et al. 2015).

Other climate change risks include shifts in isotherms, increases in extreme weather events, decreases in predictable weather patterns, and decreases in daily temperature variability (Houghton et al. 2001). With the shift in climatic systems, large-scale changes in precipitation frequencies occur, resulting in drought stress, increased fire risk, and even flooding. Thus, the consequences of climate change extend not only to the previously mentioned phenological changes in individual ecological niches, but also to the upheavals of entire ecosystems.

Species can respond to these changes with phenological plasticity (Visser 2008; van Dyck et al. 2015). This involves shifting existing ratios within a population toward better-adapted phenotypes through directed selection. Phenological plasticity allows for fast adaptation to a changing environment but is limited by the existing gene pool. Once the potential for local adaptation is exhausted, range shifts must occur (Berg et al. 2010). For many Northern Hemisphere species, a shift of 17 km or 11 m in elevation per decade has been observed (Chen et al. 2011). However, the response of individual species to the shift in isotherms is subject to other influences such as land-use effects and precipitation conditions. Therefore, there may also be large deviations from the general trend. Even mobile orders such as butterflies follow the shift in isotherms only with a delay, due to their dependence on habitat dispersal. The discrepancy between isotherm shift and range shift results in an increased risk of extinction for many species, the so-called extinction debt. Most recorded extinction events occurred in mountainous regions where no further elevational shift was possible (Parmesan 2006).

As with responses to changes in habitat quality, responses to climatic change are difficult to predict for individual species. The effects of climate change on individual species are usually much more difficult to predict as different regions are not affected by climate change to the same extent (Devictor et al. 2012), and species-specific migration performances are often not adequately

considered in range shift modelling (Cheaib et al. 2012). A species' climatic vulnerability is highly dependent on both intrinsic factors (species, biology, ecological specialization, genetic diversity) and extrinsic factors (frequency, magnitude, and type of climatic change, competition, trophic relationships, additional stressors) (Foden et al. 2008; Dawson et al. 2011). Therefore, the responses of a species may also differ at different distribution boundaries (Kerr et al. 2015). Thus, the consideration of intrinsic and extrinsic factors at different distributional boundaries is necessary, to make a more accurate assessment of the impacts of climate change on species.

One way to study the influence of extrinsic factors is to compare populations of different regions. The comparison of different populations in the distribution range of a species allows an estimation of the theoretical ecological niche. Often, there is a discrepancy between theoretical and realized ecological niches due to constraints caused by extrinsic factors (e.g. competition, dispersal barriers), which can lead to an underestimation of the adaptive potential of a species (Peterson et al. 2011).

Phylogenetic analysis can be used to determine genetic diversity as one of the intrinsic factors and determinants of phenotypic adaptation. Likewise, phylogenetic analyses can help to identify local adaptations and can serve as a basis for decisions on conservation measures. The study of functional diversity as a link between biodiversity and ecosystem processes (Cardoso et al. 2020) is essential, especially for disturbed habitats (Ng et al. 2018) because the ecological function cannot be inferred directly based on phylogeny (Villéger et al. 2012).

The comprehensive survey of intrinsic and extrinsic factors requires large-scale monitoring methods such as Malaise, Barber, and light traps as well as transect counts. However, such an effort is often not feasible due to necessary taxonomic knowledge and economic and/or ecological constraints. Therefore, the use of metabarcoding or carefully selected indicator species of known orders such as grasshoppers or butterflies can reduce the effort to determine the consequences of the aforementioned disturbance factors and the extent and severity of biodiversity loss, abundance decline, and range shifts (Henry et al. 2019).

The choice of appropriate indicator species depends on the level of knowledge, identifiability in the habitat, and sensitive response to changes in the variable under study (McGeoch 1998). Butterflies are among the best-studied orders of insects. Records of occurrences in Europe date back to the 18th century (Habel et al. 2019). Due to these extended data record series, changes and long-term trends can be followed. Additionally, sensitive responses to gradual habitat changes (Kruess & Tscharntke 2002a), rapid generation succession and comparatively high mobility make butterflies good indicator species for habitat quality (Thomas et al. 2004, Maes & van Dyck 2005).

A significant part of the biodiversity of European butterflies is centred in the European high mountains (Dinca et al. 2021). Despite their often extreme living conditions, high mountains such as the Alps harbour numerous species, endemics, and lineages (Menchetti et al. 2021). Their prominent position as centres of diversity results in part from their altitudinal zonation and the resulting habitat diversity (Spencer & Collins 2008). This altitudinal zonation allowed numerous species to survive in these areas during past climatic oscillations and the associated alternation of optimal and pessimal phases in these ecological centres of endemism (Hewitt 2000).

Climatic oscillation, which led to the alternation between glacial and interglacial phases, occurred regularly through cyclic and non-cyclic events, especially since the Quaternary (Imbrie 1993; Hewitt 1996). During glacial periods, large parts of northern Europe and the European mountain systems were covered with ice; cold steppes spread across central Europe (Ehlers et al. 2011). Many temperate species responded by contracting their ranges and retreating to Mediterranean but also extra-Mediterranean glacial refugia (Schmitt & Varga 2012). These extra-Mediterranean refugia are mainly found at the edge of mountainous regions and act as important centres of dispersal for temperate species during interglacials. In contrast, cold-adapted species retreat into the mountains during interglacials (Schmitt 2007; Schmitt & Varga 2012).

Apart from their function as refugia, European high mountains owe much of their diversity to their location and orientation, which significantly influence the gene flow of dispersing species. Diverse hybrid zones run along the European high mountains, further contributing to the genetic diversity of these regions (Hewitt 1999). At the same time, the location of European high mountains supports disjunctions and restricted gene flow, especially in arcto-alpine and boreo-montane species, which has enabled allopatric differentiation, leading to the evolution of numerous endemics (Varga & Schmitt 2008). The most species-rich genus of European butterflies, *Erebia*, is probably the result of adaptive radiation that occurred as a combination of allopatric differentiation and ecological adaptation (Yonder et al. 2010; Pena et al. 2015). These processes have resulted in a large number of often young species, some of which are difficult to distinguish by appearance and ecology and require molecular genetic techniques for unambiguous identification (Panigaj et al. 2015).

These molecular genetic methods can be used to identify intraspecific differentiation as well. The reconstruction of intra- and interspecific phylogeny can be used to reconstruct biogeographical events, allowing conclusions to be drawn about the location and age of refugia and differentiation centres, as well as migration routes and hybridization events.

Molecular genetic markers should be chosen depending on the research question. Although they often coincide in their differentiation patterns (Zink & Barrowclough 2008), there are sometimes significant differences between individual genes or genetic markers (Chan & Levin 2005). For example, the mitochondrial sequence COI ("genetic barcode") often used for species delimitation can sometimes differ significantly from the phylogenetic patterns of nuclear genes (Toews & Brelsford 2012). These differences, termed mito-nuclear discordance, are caused by asymmetric population structures, sex-specific dispersal potentials, and different mutation rates, among other factors (Funk and Omland 2003).

The higher mutation rate of mtDNA was explained as a consequence of the oxidative environment, the absence of histones, and the lack of DNA repair (e.g. Avise 2009). However, recent studies show that the mitochondrion has structural proteins that are similar in function to histones, although the type and number of proteins differ (Bogenhangen 2012). Additionally, numerous proteins have been identified that serve for DNA repair (Stein & Sia 2017; Garcia-Lepe & Bermudez-Cruz 2019), therefore oxidative stress-induced mutations cannot be the sole cause of the higher mutation rate of mitochondrial DNA.

A further explanation for the high mutation rate of mtDNA is based on its special properties related to genetic drift. Mitochondrial DNA is haploid and uniparentally inherited, which results in an effective population size fourfold smaller than for nuclear DNA of diploid organisms with sexual reproduction (Zink & Barrowclough 2008). This smaller effective population size is associated with lower selection pressure and higher genetic drift (Lynch et al. 2016). The higher genetic drift of smaller effective population sizes may contribute to the faster accumulation of mutations, which favours faster lineage sorting, revealing younger differentiation (Funk & Omland 2003). However, mtDNA does not allow inferences about hybridization events and the recent removal of formerly existing barriers to gene flow. Therefore, phylogenetic patterns of mitochondrial markers can be particularly distorted by introgression (Rubinoff et al. 2006), which is why an analytical combination with nuclear genes is appropriate.

Another important factor to consider when analysing insect mitochondrial genes is the protobacterium *Wolbachia*. *Wolbachia* is an endoparasite that occurs worldwide in the cytoplasm of insects and can significantly affect the reproduction of its host. It can shift sex ratio in populations in favour of females by killing or feminizing male offspring to ensure its transmission into subsequent generations, thereby affecting patterns of maternally transmitted mtDNA (Jiggins 2003; Narita et al. 2006). Additionally, gene flow between infected and uninfected individuals or individuals infected with other *Wolbachia* lineages can be restricted due to cytoplasmic incompatibility, accelerating differentiation processes (Gompert et al. 2008; Smith and Fisher 2009; Sun et al. 2011). *Wolbachia* also reduces the effective population size of mitochondrial DNA, which can increase the

differentiation rate further. By removing gene flow barriers between the same *Wolbachia* lineages, it can promote interspecific introgression, which is considered a key influencing force in species adaptation and speciation (Toews and Brelsford 2012; Abbot et al. 2013).

Therefore, mtDNA-based phylogenetic studies must take into account events potentially caused by *Wolbachia*, such as gene flow barriers, introgression, asymmetric population structures, or genetic bottlenecks triggered by an initial infestation, so-called selective sweeps. Analysis of *Wolbachia* infections and different *Wolbachia* lineages can serve as additional information for biogeographic and phylogeographic reconstructions (see Lucek 2021). Consequently, a combination of diverse markers is needed to obtain a comprehensive understanding of phylogenetic structures and intra- and interspecific biodiversity.

1.1 The scope of this thesis

In this thesis, different markers and analytical techniques were combined to address questions on the biodiversity and ecology of two European butterflies of the genus *Erebia*. Representatives of this genus occur in rocky habitats, grasslands, or even open forest structures. Due to their link to open habitats, numerous representatives of this species-rich genus are threatened by habitat loss. The majority of European *Erebia* are cold-adapted species with alpine, montane, or boreal distributions. The occurrences of widespread species are often disjunct due to these habitat requirements (Sonderegger 2005), which can lead to restrictions in intraspecific gene flow. Disjunct occurrences and allopatric speciation are considered to be the main causes of species diversity within *Erebia* (Pena et al. 2015). At the same time, this circumstance offers enormous potential for previously overlooked biodiversity. The combination of different genetic markers and ecological studies can contribute to the understanding of the differentiation process of European butterflies as well as serve as a basis for conservation measures.

The first representative of the genus *Erebia* studied here is the species *Erebia pronoe*. The species is common on rocky grasslands and pastures of the European high mountains, from the montane to the alpine level (Sonderegger 2005). *Erebia pronoe* has a wide distribution range with several disjunct occurrences. Alpine butterflies and habitat specialists such as *Erebia pronoe* are often very sedentary, which may result in increased susceptibility to the effects of habitat fragmentation and degradation. At the same time, complex phylogenetic structures may emerge from diverse constraints on the intraspecific gene flow. Shifting in altitudinal ranges caused by climate change and land-use change may lead to further habitat fragmentation, thereby impairing gene flow even more. The phylogenetic study of this species provides an opportunity to analyse the consequences of historical and recent genetic isolation on intraspecific diversity. By comparing the ecological niches of different mountain regions, niche maintenance and its ecological potential can be explored.

The second representative of the genus *Erebia* is the species *Erebia aethiops.* This species is one of the exceptions within the genus *Erebia* with its adaptation to warmer, open forest habitats (van Swaay et al. 2006). *Erebia aethiops* was considered a common species of the western Palearctic, but it has already suffered major losses and is already vanishing at its northern limit in some regions such as Scotland. Despite various thermoregulatory behavioural adaptations and oligophagous character (Slamova et al. 2011; Slamova et al. 2013), it is extinct in some regions of Europe and considered endangered in other parts (Slamova et al. 2011; Kühn et al. 2018). *Erebia aethiops* responded to the decline of open forest habitats by moving to forest ecotones, where it is exposed to additional heat stress. The large-scale decline in large parts of Europe indicates insufficient compensation for habitat loss and poses the risk of a loss of intraspecific diversity and future adaptive potential.

The results of the research were published or submitted as scientific publications in peer-reviewed journals. The research focus of the publications is briefly presented in detail followed by the actual publications.

I

Erebia aethiops is a montane butterfly species that depends on open forest habitats, which were originally maintained by megaherbivores, naturally occurring fire, and human management (Samways et al. 2020). With the onset of modern forestry and the elimination of traditional management practices (such as forest pastures), the early successional stages of the forest are being lost, resulting in habitat loss for open forest species such as *Erebia aethiops*. This has caused extinctions in entire regions and the formerly common species is now considered endangered in Germany (Kühn et al.2018).

The phylogenetic analysis of *Erebia aethiops* identifies the genetic lineages and serves as a basis for conservation measures and the preservation of genetic diversity. In addition, phylogeographic analysis has been used to reconstruct its biogeography, glacial refugia and its range dynamics. These findings can be used to draw conclusions about the extra-Mediterranean glacial refugia of open forest habitats (Schmitt et al. 2009).

The identification of glacial forest refugia is mostly done by pollen analysis and radiocarbon dating of macrofossils, which are subject to various methodological limitations. Macrofossils are rare and can only be used to detect individual species, rather than existing forests, while pollen analyses are tied to the presence of sinks and only provide information on aerochore species (Comes & Kadereit 1998). Therefore, the detection of persistent, distinct genetic lineages of an open forest species can provide more reliable information on the existence of glacial forest refugia and contribute to the protection of these important habitats.

II

Mark-recapture analyses have been conducted to determine the ecological potential of *Erebia pronoe*. The realized ecological niche of a species is defined by local biotic and abiotic conditions, and by comparing two populations from different regions, a more realistic statement of the potential ecological niche can be made (Peterson et al 2011). An investigation of dispersal potential provides information on the dispersal capacity and connectivity of populations and the potential for establishing metapopulation structures. However, the determined dispersal potential of a species can be influenced by the size of the study area (Schneider 2003), so the study area was expanded to account for this.

Insects are susceptible to decoupling trends due to their dependence on environmental cues. The flight period of alpine butterflies is probably synchronized by the end of diapause and snowmelt, which are no longer as reliable, leading to difficulties in mating and potentially negative effects on population growth, density and stability (Konvicka et al. 2016; Kadlec et al. 2010). An analysis of population structure can provide information about intraspecific decoupling tendencies, while an analysis of resource use by *Erebia pronoe* is used to estimate niche flexibility and the likelihood of spatial and temporal decoupling from relevant resources (van Dyck et al. 2015).

Alpine grassland systems are subject to diverse risks, such as conflicts of use, extensification or intensification tendencies, which can lead to a loss of quality or habitat. Analysis of *Erebia pronoe* population structures can be used as an indicator of habitat quality of associated habitats (Maes and van Dyck 2005).

III

The genus *Erebia* is characterized by complex genetic structures and high phenotypic variability. Complex structures also exist at the intraspecific level, making accurate estimates of the existing diversity difficult. For example, more than eight different morphotaxa have been described for *Erebia pronoe*, which are distributed among different mountain systems, suggesting the presence of cryptic species. Large distributions with allopatric occurrences complicate gene flow and can lead to

complex phylogenetic patterns, making it difficult to assess differentiation trends and species delimitation (Dincă et al. 2019, 2021).

Studies conducted so far have focused on a subset of the whole distribution area and have been based exclusively on mitochondrial sequences. These studies indicated a differentiation between the occurrences of the Pyrenees and the occurrences of the Alps (Dincă et al. 2015; Paučulová et al. 2018), but failed to put them into context, thus not allowing an assessment of the phylogenetic structures.

In addition, possible influencing factors such as *Wolbachia* infections or introgression, which might have favoured differentiation of mitochondrial patterns (Smith et al. 2012), were not considered. Due to the lack of references and the limited data available, it was not possible to evaluate these results or estimate intraspecific diversity. To overcome these shortcomings, the study area was extended to include important occurrences from the Western Alps as a potential link to the Pyrenean region. Additionally, reference data of the sister species *Erebia melas* was incorporated. A combination of different markers (genetic, morphological and *Wolbachia* infection pattern) was used to account for the individual shortcomings of each marker and to create a more robust phylogenetic and biogeographical analysis.

1.2 References

Abbott R, Albach D, Ansell S, et al (2013) Hybridization and speciation. J Evol Biol 26:229–246. https://doi.org/10.1111/j.1420-9101.2012.02599.x

Avise JC (2009) Phylogeography: Retrospect and prospect. J Biogeogr 36:3–15. https://doi.org/10.1111/j.1365-2699.2008.02032.x

Berg MP, Toby Kiers E, Driessen G, et al (2010) Adapt or disperse: Understanding species persistence in a changing world. Glob Chang Biol 16:587–598. https://doi.org/10.1111/j.1365-2486.2009.02014.x

Bogenhagen DF (2012) Mitochondrial DNA nucleoid structure. Biochim. Biophys. Acta - Gene Regul. Mech. 1819:914–920

Cardoso P, Barton PS, Birkhofer K, et al (2020) Scientists' warning to humanity on insect extinctions. Biol Conserv 242:1-12. https://doi.org/10.1016/j.biocon.2020.108426

Chan KMA, Levin SA (2005) Leaky prezygotic isolation and porous genomes: rapid introgression of maternally inherited DNA. Evol 59:720–729. https://doi.org/10.1111/j.0014-3820.2005.tb01748.x

Cheaib A, Badeau V, Boe J, et al (2012) Climate change impacts on tree ranges: Model intercomparison facilitates understanding and quantification of uncertainty. Ecol Lett 15:533–544. https://doi.org/10.1111/j.1461-0248.2012.01764.x

Chen IC, Hill JK, Ohlemüller R, et al (2011) Rapid range shifts of species associated with high levels of climate warming. Science 333:1024–1026. https://doi.org/10.1126/science.1206432

Clarke A, Fraser KPP (2004) Why does metabolism scale with temperature? Funct Ecol 18:243–251. https://doi.org/10.1111/j.0269-8463.2004.00841.x

Comes HP, Kadereit JW (1998) The effect of Quaternary climatic changes on plant distribution and evolution. Trends Plants Sci 3:1360–1385. https://doi.org/10.1046/j.1365-294X.1998.00566.x

Dawson TP, Jackson ST, House JI, et al (2011) Beyond predictions: Biodiversity conservation in a changing climate. Science 332:53–58. https://doi.org/10.1126/science.1200303

Devictor V, van Swaay C, Brereton T, et al (2012) Differences in the climatic debts of birds and butterflies at a continental scale. Nat Clim Chang 2:121–124. https://doi.org/10.1038/nclimate1347

Dincă V, Dapporto L, Somervuo P, et al (2021) High resolution DNA barcode library for European butterflies reveals continental patterns of mitochondrial genetic diversity. Commun Biol 4:1-11. https://doi.org/10.1038/s42003-021-01834-7

Dincă V, Lee KM, Vila R, Mutanen M (2019) The conundrum of species delimitation: A genomic perspective on a mitogenetically super-variable butterfly. Proc R Soc B Biol Sci 286:1-9. https://doi.org/10.1098/rspb.2019.1311

Dincă V, Montagud S, Talavera G, et al (2015) DNA barcode reference library for Iberian butterflies enables a continental-scale preview of potential cryptic diversity. Sci Rep 5:1-12. https://doi.org/10.1038/srep12395

Ehlers J, Ehlers J, Gibbard PL, Hughes PD (2011) Quaternary glaciations- extent and chronology: A closer look, 15th edn. Elsevier

Filz KJ (2013) Global change impacts on biodiversity and their implications for species conservation. Universität Trier Abteilung für Biogeographie: 1- 210.

Fischer J, Lindenmayer DB (2007) Landscape modification and habitat fragmentation: a synthesis. Glob Ecol Biogeogr 16:265–280. https://doi.org/10.1111/j.1466-8238.2007.00287.x

Foden W, Mace GM, Vié JC, et al (2009) Species susceptibility to climate change impacts. Wildlife in a changing world–an analysis of the 2008 IUCN Red List of threatened species

Funk DJ, Omland KE (2003) Species-Level Paraphyly and Polyphyly: Frequency, Causes, and Consequences, with Insights from Animal Mitochondrial DNA. Annu Rev Ecol Evol Syst 34:397–423. https://doi.org/10.1146/annurev.ecolsys.34.011802.132421

García-Lepe UO, Bermúdez-Cruz RM (2019) Mitochondrial Genome Maintenance: Damage and Repair Pathways. In: DNA Repair- An Update. IntechOpen, London

Gompert Z, Forister ML, Fordyce JA, Nice CC (2008) Widespread mito-nuclear discordance with evidence for introgressive hybridization and selective sweeps in Lycaeides. Mol Ecol 17:5231–5244. https://doi.org/10.1111/j.1365-294X.2008.03988.x

Habel JC, Trusch R, Schmitt T, et al (2019) Long-term large-scale decline in relative abundances of butterfly and burnet moth species across south-western Germany. Sci Rep 9:1–9. https://doi.org/10.1038/s41598-019-51424-1

Hallmann CA, Sorg M, Jongejans E, et al (2017) More than 75 percent decline over 27 years in total flying insect biomass in protected areas. PLoS One 12:1-21. https://doi.org/10.1371/journal.pone.0185809

Henry E, Brammer-robbins E, Aschehoug E, et al (2019) Do substitute species help or hinder endangered species management ? Biol Conserv 232:127-130. https://doi.org/10.1016/j.biocon.2019.01.031

Hewitt G (2000) The genetic legacy of the Quaternary ice ages. Nature 405:907-913. https://doi.org/10.1038/35016000

Hewitt GM (1999) Post-glacial re-colonization of European biota. Biol J Linn Soc 68:87–112. https://doi.org/10.1111/j.1095-8312.1999.tb01160.x

Hewitt GM (1996) Some genetic consequences of ice ages. Biol J Linn Soc 58:247–276. https://doi.org/10.1111/j.1095-8312.1996.tb01434.x

Houghton J, Ding Y, Griggs D, et al (2001) Climate change 2001: The scientific basis. Cambridge University Press, Cambridge

Imbrie J, Berger A, Boyle EA, et al (1993) On the structure and origin of major glaciation cycles 2. The 100,000-year cycle. Paleoc and Paleocli 8:699–735. https://doi.org/10.1029/93PA02751

IPBES (2018) Biodiversity and Ecosystem Services for Europe and Central Asia. Bonn, Germany

IUCN (2019) The IUCN red list of threatened species. Version 2019-2.

Jiggins FM (2003) Male-killing Wolbachia and mitochondrial DNA: Selective sweeps, hybrid introgression and parasite population dynamics. Genetics 164:5–12. https://doi.org/10.1093/genetics/164.1.5

Kadlec T, Vrba P, Kepka P, et al (2010) Tracking the decline of the once-common butterfly: Delayed oviposition, demography and population genetics in the hermit Chazara briseis. Anim Conserv 13:172–183. https://doi.org/10.1111/j.1469-1795.2009.00318.x

Kerr JT, Pindar A, Galpern P, et al (2015) Climate change impacts on bumblebees converge across continents. Science 349:177–180. https://doi.org/10.1126/science.aaa7031

Klein AM, Vaissière BE, Cane JH, et al (2007) Importance of pollinators in changing landscapes for world crops. Proc R Soc B Biol Sci 274:303–313. https://doi.org/10.1098/rspb.2006.3721

Konvička M, Beneš J, Čížek O, et al (2016) Has the currently warming climate affected populations of the mountain ringlet butterfly, Erebia epiphron (Lepidoptera: Nymphalidae), in low-elevation mountains? Eur J Entomol 113:295–301. https://doi.org/10.14411/eje.2016.036

Krämer B, Poniatowski D, Fartmann T (2012) Effects of landscape and habitat quality on butterfly communities in pre-alpine calcareous grasslands. Biol Conserv 152:253–261. https://doi.org/10.1016/j.biocon.2012.03.038

Kruess A, Tscharntke T (2002) Grazing intensity and the diversity of grasshoppers, butterflies, and trap-nesting bees and wasps. Conserv Biol 16:1570–1580. https://doi.org/10.1046/j.1523-1739.2002.01334.x

Kühn E, Musche M, Harpke A, et al (2018) Tagfalter-Monitoring Deutschland: Jahresauswertung 2017. Oedippus 35:5–36

Losey JE, Vaughan M (2006) The economic value of ecological services provided by insects. Bioscience 56:311–323. https://doi.org/10.1641/0006-3568(2006)56[311:TEVOES]2.0.CO;2

Lucek K, Bouaouina S, Jospin A, et al (2021) Prevalence and relationship of endosymbiotic Wolbachia in the butterfly genus Erebia. BMC Ecol Evol 21:1–12. https://doi.org/10.1186/s12862-021-01822-9

Lynch M, Ackerman MS, Gout JF, et al (2016) Genetic drift, selection and the evolution of the mutation rate. Nat Rev Genet 17:704–714. https://doi.org/10.1038/nrg.2016.104

Maes D, van Dyck H (2005) Habitat quality and biodiversity indicator performances of a threatened butterfly versus a multispecies group for wet heathlands in Belgium. Biol Conserv 123:177–187. https://doi.org/10.1016/j.biocon.2004.11.005

McGeogh MA (1998) The selection, testing and application of terrestrial insects as bioindicators. Biol Rev 73:181–201. https://doi.org/10.1111/j.1469-185X.1997.tb00029.x

Melbourne BA, Hastings A (2008) Extinction risk depends strongly on factors contributing to stochasticity. Nature 454:100–103. https://doi.org/10.1038/nature06922

Menchetti M, Talavera G, Cini A, et al (2021) Two ways to be endemic. Alps and Apennines are

different functional refugia during climatic cycles. Mol Ecol 30:1297–1310. https://doi.org/10.1111/mec.15795

Narita S, Nomura M, Kato Y, Fukatsu T (2006) Genetic structure of sibling butterfly species affected by Wolbachia infection sweep: Evolutionary and biogeographical implications. Mol Ecol 15:1095–1108. https://doi.org/10.1111/j.1365-294X.2006.02857.x

Ng K, Barton PS, Blanchard W, et al (2018) Disentangling the effects of farmland use, habitat edges, and vegetation structure on ground beetle morphological traits. Oecologia 188:645–657. https://doi.org/10.1007/s00442-018-4180-9

Nouhuys S (2009) Metapopulation Ecology. eLS 1–9. https://doi.org/10.1002/9780470015902.a0021905

Panigaj L, Šemeláková M, Pristaš P (2015) The phylogenetic relationship of Western Carpathians Erebia spp. (Lepidoptera: Nymphalidae) based on mitochondrial cytochrome oxidase subunit I sequence analysis. Biol 70:393–399. https://doi.org/10.1515/biolog-2015-0045

Parmesan C (2006) Ecological and Evolutionary Responses to Recent Climate Change. Annu Rev Ecol Evol Syst 37:637–669. https://doi.org/10.1146/annurev.ecolsys.37.091305.110100

Paučulová L, Dzurinka M, Šemeláková M, et al (2018) Phylogeography, genetic structure and wing pattern variation of Erebia pronoe (Esper, 1780) (Lepidoptera: Nymphalidae) in Europe. Zootaxa 4441:279–297. https://doi.org/10.11646/zootaxa.4441.2.5

Peña C, Witthauer H, Klečková I, et al (2015) Adaptive radiations in butterflies: Evolutionary history of the genus Erebia (Nymphalidae: Satyrinae). Biol J Linn Soc 116:449–467. https://doi.org/10.1111/bij.12597

Peterson TA, Soberón J, Pearson RG, et al (2011) Ecological niches and geographic distributions. Princeton University Press, Oxford

Pielke RA, Mahmood R, McAlpine C (2016) Land's complex role in climate change. Phys Today 69:40–46. https://doi.org/10.1063/PT.3.3364

Rubinoff D, Cameron S, Will K (2006) A genomic perspective on the shortcomings of mitochondrial DNA for "barcoding" identification. J. Hered. 97:581–594. https://doi.org/10.1093/jhered/esl036

Samways MJ, Barton PS, Birkhofer K, et al (2020) Solutions for humanity on how to conserve insects. Biol Conserv 242:1-15. https://doi.org/10.1016/j.biocon.2020.108427

Sánchez-Bayo F, Wyckhuys KAG (2019) Worldwide decline of the entomofauna: A review of its drivers. Biol. Conserv. 232:8–27. https://doi.org/10.1016/j.biocon.2019.01.020

Scheffers B, Laurance W, Joppa L, Pimm S (2012) What we know and don't know about Earth's missing biodiversity What we know and don't know about Earth's missing biodiversity. Trends Ecol Evol 27:501–510. https://doi.org/10.1016/j.tree.2012.05.008

Schmitt T (2007) Molecular biogeography of Europe: Pleistocene cycles and postglacial trends. Front Zool 4:1–13. https://doi.org/10.1186/1742-9994-4-11

Schmitt T (2009) Biogeographical and evolutionary importance of the European high mountain systems. Front Zool 6:1–10. https://doi.org/10.1186/1742-9994-6-9

Schmitt T, Varga Z (2012) Extra-Mediterranean refugia: The rule and not the exception? Front Zool 9:1–12. https://doi.org/10.1186/1742-9994-9-22

Schneider C (2003) The influence of spatial scale on quantifying insect dispersal: An analysis of butterfly data. Ecol Entomol 28:252–256. https://doi.org/10.1046/j.1365-2311.2003.00495.x

Schowalter TD, Noriega JA, Tscharntke T (2017) Insect effects on ecosystem services -Introduction. Basic Appl Ecol 26:1-7. https://doi.org/10.1016/j.baae.2017.09.011

Seibold S, Gossner MM, Simons NK, et al (2019) Arthropod decline in grasslands and forests is associated with landscape-level drivers. Nature 574:671–674. https://doi.org/10.1038/s41586-019-1684-3

Slamova I, Klecka J, Konvicka M (2011) Diurnal Behavior and Habitat Preferences of Erebia aethiops, an Aberrant Lowland Species of a Mountain Butterfly Clade. J Insect Behav 24:230–246. https://doi.org/10.1007/s10905-010-9250-8

Slamova I, Klecka J, Konvicka M (2013) Woodland and grassland mosaic from a butterfly perspective: Habitat use by Erebia aethiops (Lepidoptera: Satyridae). Insect Conserv Divers 6:243–254. https://doi.org/10.1111/j.1752-4598.2012.00212.x

Smith MA, Bertrand C, Crosby K, et al (2012) Wolbachia and DNA barcoding insects: Patterns, potential, and problems. PLoS One 7:1-12. https://doi.org/10.1371/journal.pone.0036514

Sonderegger P (2005) Die Erebien der Schweiz (Lepidoptera: Satyrinae, Genus Erebia). Private Publication, Biel

Spencer S, Collins S (2008) Reversing the decline in butterflies and moths across Europe–the importance of particular farming practices and the implications for CAP reform. 1–18.

Stein A, Sia EA (2017) Mitochondrial DNA repair and damage tolerance. Front Biosci Landmark 22:920–943. https://doi.org/10.2741/4525

Stork NE (2018) How Many Species of Insects and Other Terrestrial Arthropods Are There on Earth ? Annu Rev Entomol 63:31–45. https://doi.org/10.1146/annurev-ento-020117-043348

Sun XJ, Xiao JH, Cook JM, et al (2011) Comparisons of host mitochondrial, nuclear and endosymbiont bacterial genes reveal cryptic fig wasp species and the effects of Wolbachia on host mtDNA evolution and diversity. BMC Evol Biol 11:1-8. https://doi.org/10.1186/1471-2148-11-86

Thomas JA, Telfer MG, Roy DB, et al (2004) Comparative Losses of British Butterflies, Birds, and Plants and the Global Extinction Crisis. Science 303:1879–1881. https://doi.org/10.1126/science.1095046

Toews DPL, Brelsford A (2012) The biogeography of mitochondrial and nuclear discordance in animals. Mol Ecol 21:3907–3930. https://doi.org/10.1111/j.1365-294X.2012.05664.x

Tooker JF, Pearsons KA (2021) Newer characters, same story: neonicotinoid insecticides disrupt food webs through direct and indirect effects. Curr Opin Insect Sci 46:50–56. https://doi.org/10.1016/j.cois.2021.02.013

van Dyck H, Bonte D, Puls R, et al (2015) The lost generation hypothesis: Could climate change drive ectotherms into a developmental trap? Oikos 124:54–61. https://doi.org/10.1111/oik.02066

van Swaay C, Warren M, Loïs G (2006) Biotope use and trends of European butterflies. J Insect Conserv 10:189–209. https://doi.org/10.1007/s10841-006-6293-4

van Swaay C (2002) The importance of calcareous grasslands for butterflies in Europe. Biol Conserv 104:315–318. https://doi.org/10.1016/S0006-3207(01)00196-3

Varga ZS, Schmitt T (2008) Types of oreal and oreotundral disjunctions in the western Palearctic. Biol J Linn Soc 93:415–430. https://doi.org/10.1111/j.1095-8312.2007.00934.x

Villéger S, Miranda JR, Hernandez DF, Mouillot D (2012) Low functional β-diversity despite high taxonomic β-diversity among tropical estuarine fish communities. PLoS One 7:1-10. https://doi.org/10.1371/journal.pone.0040679

Visser ME (2008) Keeping up with a warming world; assessing the rate of adaptation to climate change. Proc R Soc B Biol Sci 275:649–659. https://doi.org/10.1098/rspb.2007.0997

Wagner DL (2020) Insect Declines in the Anthropocene. Annu Rev Entomol 65:457–480. https://doi.org/10.1146/annurev-ento-011019-025151

Waldvogel AM, Wieser A, Schell T, et al (2017) The genomic footprint of climate adaptation in Chironomus riparius. Mol Ecol 27:1439–1456. https://doi.org/10.1111/mec.14543

Wilson EO (2016) Half-earth: our planet's fight for life. Liveright, New York

Wood TJ, Goulson D (2017) The environmental risks of neonicotinoid pesticides: a review of the evidence post 2013. Environ Sci Pollut Res 24:17285–17325. https://doi.org/10.1007/s11356-017-9240-x

Yoder JB, Clancey E, Des Roches S, et al (2010) Ecological opportunity and the origin of adaptive radiations. J Evol Biol 23:1581–1596. https://doi.org/10.1111/j.1420-9101.2010.02029.x

Zink RM, Barrowclough GF (2008) Mitochondrial DNA under siege in avian phylogeography. Mol Ecol 17:2107-2121. https://doi.org/10.1111/j.1365-294X.2008.03737.x

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Reconstruction of forest dynamics in the Western Palaearctic based on phylogeographic analysis of the ringlet butterfly *Erebia aethiops*

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Glacial refugia are centers of high biodiversity. Therefore, knowledge on their locations and reactions of associated populations and landscapes to climatic changes is crucial for conservation management. We here investigated the biogeography of a butterfly species linked to open forest habitats. Using mitochondrial and nuclear markers in combination with Bayesian simulations, we analyzed the location and age of potential glacial refugia of the species. We identified five putative refugia in Europe. Considering the ecological needs of our study species, tree density within these refugial areas, in contrast to earlier assumptions, must have exceeded the level of individually scattered trees. Our results also provide evidence that especially the refuge areas in the Carpathians were previously underestimated regarding their age: the refugia in the Southern Carpathians presented suitable conditions throughout several glacial cycles, probably since the Mindel or Riss cycles. Additionally, our analyses provided support for a forest refugium near the Tatra Mountains persisting the last glacial maximum. Our results underline the usefulness of this and probably other butterfly species as indicators of forest refugia.

Distributions of living organisms are subject to permanent change with glacial and interglacial cycles of the Pleistocene having strong effects on the ranges of animals and plants worldwide, especially in Europe[1]. During cold stages, warm-adapted species were restricted to refugia. These species in general retreated towards the equator with the onset of a glaciation; consequently, the Mediterranean peninsulas became important centers of survival and re-colonisation in the western Palearctic[2–4]. Various biogeographical studies extended the knowledge about glacial refugia and added Arctic-Alpine and Siberian faunal elements for Europe[5]. Genetic studies discovered numerous genetic lineages, often with complex structures, and thus led to the exploration of extra–Mediterranean and cryptic refugia in Europe[6].

Meanwhile, it has been shown for a larger number of temperate species representing a large array of different animal and plant groups that they have survived glacial conditions in northern, often cryptic refugia[7–9]. In a number of cases, species occupied both classical Mediterranean and extra-Mediterranean refugia[10–13]. The majority of these species are those of open land or semi-open landscapes. However, species ecologically linked to closed forest structures so far are rarely detected for these northern refugia. Nevertheless, numerous tree species have survived the last cold phase of the Pleistocene in these extra-Mediterranean refugia, according to studies using subfossil evidence of timber, pollen profiles, genetic analyses, and niche modeling[14–16]. These lines of evidence demonstrate that retreat areas for tree species were located especially at the edge of high mountain systems (i.e. Alps, Pyrenees, Carpathians). In particular, the eastern edge of the Alps, the southern slopes of the Southern Carpathians, and parts of the Carpathian Basin appear to have provided suitable conditions for the survival of

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temperate tree species[8,17,18]. Also on the Balkan Peninsula, trees were probably more widespread under glacial climatic conditions than previously assumed[15].

All this has significantly changed our understanding of the conditions in Pleistocene Europe. However, most studies have focused on the presence and density of single trees species in an area at a certain time, but not whether they also created the ecological conditions of a forest ecosystem[14,16]. To investigate this question, it is necessary to study species that are dependent on the existence of forest ecosystems. One such species that cannot survive without the existence of forest ecosystems is the Scotch Argus, *Erebia aethiops* (Esper, [1777])[19–23]. This satyrid butterfly is widespread in the western Palearctic in forested areas and can be found mostly continuously from the French Massif Central through Europe and western Siberia to the western Altai Mountains. In the north, it reaches the plains of northern central Europe only sporadically; in the south, it is missing in Iberia, and the entire south of the Balkan Peninsula. Relatively few populations are known from peninsular Italy. All over this range, the morphological differentiation is weak[23]; nevertheless, several morphology-based subspecies have been described, e.g. the southern Alps subspecies *E. aethiops rubia* Fruhstorfer, [1909]. However, many of these morphological lines were synonymized by Varga[24]. Although *E. aethiops* does not reach the Atlantic Ocean, an isolated population exists in Scotland, formerly described as subspecies *caledonia* Verity, [1911]. Further populations isolated from the main distribution area exist in the mountains of northern Turkey and in the Caucasus, which are currently treated as a separate subspecies, *E. aethiops melusina* Herrich-Schäffer, [1847].

To better understand the dynamics of forest ecosystems in Europe during the sequence of several glacial-interglacial cycles, we studied the phylogeography of *E. aethiops* across large parts of the species' range from its westernmost parts in the Massif Central in France to the eastern Carpathians and the eastern Balkan Peninsula. We supplemented our dataset with available sequences from Genbank and BOLD to account for potential northern and Asian lineages and compared this entire data set with sequence data from other closely related species of the *aethiops*-group restricted to eastern Asia. We used mitochondrial and nuclear genetic information to estimate divergence times of the main differentiation events of this butterfly depending on forests to address the following questions:

1. Where did *E. aethiops* originate from and what does this tell us about the persistence of forest ecosystems in Europe?
2. Which historical processes can explain the current distribution of *E. aethiops* and which conclusions can be drawn from this for the dynamics of forest ecosystems?
3. Where were glacial refugia of *E. aethiops* located and which new insights on the dynamics of forest ecosystems can be derived from this?

Results

Mitochondrial DNA. Concatenated COI and NDI sequences (1,212 bp) of 133 specimens from 28 populations yielded 34 haplotypes (see genetic diversity parameters in Table 1). The most common ones were H9 (24.8%), H7 (9.0%), and H1 (8.3%); all other haplotypes had frequencies less than 4%. The maximum p-distance among *E. aethiops* haplotypes was 0.0091 (Spiazzi vs. Cheile Butii; Spiazzi vs. Nanos) with an overall mean genetic distance of 0.004 (s = 0.002).

Two main groups were distinguished by the haplotype network based on both mtDNA markers; a southern Alps group, and all other populations (see Fig. 1). The Central Italian Alps group was represented by two Italian populations and haplotypes detected at Plöckenpass (on the Austrian-Italian border, and thus north of the two other populations of the Southern Alps group). This group roughly coincides with the geographic distribution of *E. aethiops rubia*. Plöckenpass exhibited a mixture of haplotypes from both main groups. While no clear structure was visible in the southern Alps group, the second main group displayed a star-like pattern, with the dominant haplotype H9 being central (found in the Alps, in populations in the Tatras, and the Romanian Carpathians), and the majority of its satellite haplotypes being restricted to the Alps. In the Tatras, another group formed a secondary star-like structure with H1 at its core. However, H1 was also found in Baile Herculane (Romania) and Trigrad (Bulgaria); the latter was dominated by two satellite haplotypes of H1. The common H7 with its two satellites was also restricted to the Tatras and the Romanian Carpathians. Only the haplotypes H5 (Cheile Butii, Southern Carpathians) and H28 (Gheorgheni, Eastern Carpathians) were exclusive for the Romanian Carpathians. The three populations sampled in Slovenia had three endemic haplotypes, that were most closely related to each other. This result partially coincided with the results of the STRUCTURE analysis of allozyme data (see below) in the divergence of the Nanos population, but was contradictory for the two populations from the Slovenian Alps (Medvodje and Sija), which, according to the allozyme data, formed a group together with the large majority of populations from the Alps (see Fig. 4). The western Balkan populations of Tresnjevik, Ropojan Valley and Valbona (all of these were not represented in the allozyme analysis) had four closely related haplotypes which were geographically restricted to this region, but were linked via H31 by just one mutational step to the most common haplotype H9.

The geographically expanded dataset with additional samples from Genbank and BOLD, based exclusively on a 429 bp COI fragment, yielded a considerably simpler haplotype network (see Fig. 2). Nonetheless, the southern Alps and the Dinaric Mountains still displayed distinct groups, whereas the western Balkans and the Carpathian region also represented the most common haplotype or direct derivates. This also applied to Germany, Scotland, and Latvia. Haplotypes from eastern Europe and Asia partly showed remarkable differentiation from the above-mentioned haplotypes. Thus, a separate lineage was located in the Pontic Mountains (northern Turkey). Despite geographic proximity and inclusion in the same morphological subspecies (i.e. *E. aethiops melusina*), samples from the western Caucasus represented a different group which genetically is most closely related to individuals from the Urals and the Altai Mountains.

	COI	NDI	Aligned haplotypes
Nucleotide diversity Pi	0.0026	0.0028	0.0039
Haplotype diversity h	0.822	0.696	0.998
Segregation sites S	21	14	35
Average number of nucleotide differences k	1.7141	1.5733	4.7344

Table 1. Genetic diversity parameters of the two separated and combined mitochondrial DNA markers of *E. aethiops*.
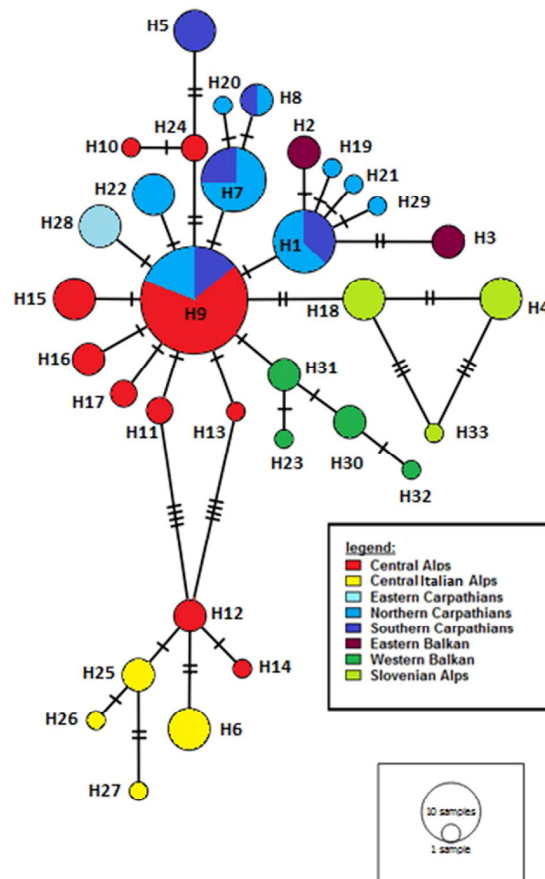

Figure 1. Minimum spanning haplotype network based on the concatenated mtDNA haplotypes (COI, NDI) of *E. aethiops*. Mutation steps are shown by the number of hatch marks.

We conducted a Bayesian analysis using BEAST to date the intraspecific splits; the splitting events of the two outgroups were estimated at 5.6 mya for *E. pronoe* and 12.1 mya for *Pararge aegeria* (see Fig. 3). The southern Alps group and the widespread group split about 560 kya. The oldest split within the latter group was estimated at 420 kya separating Slovenian specimens from those of the central Alps. Three groups branched-off between 400 and 300 kya: one group representing haplotypes found in the Tatras, the Romanian Carpathians, and Bulgaria, another group with haplotypes from the Tatras and Romanian Carpathians, and the third group with haplotypes from the Alps and Cheile Butii (southern Carpathians). The split between Slovenia and the western Balkans was estimated at 400 kya. Six of the seven haplotype groups had group posterior probabilities > 85%.

Including sequence data of the other representatives of the *aethiops* group which are restricted to eastern Asia, a RASP analysis did not deliver strong support for one of the several possible ancestral areas of *E. aethiops*. A European-Asian or a solely European origin is equally likely. However, support is given for an origin of the entire *aethiops* group in eastern Asia (see supplementary S1 and S2). Bayesian Skyline plots indicated stable female effective population sizes for the more distant past, with a decline starting at about 700 kya (Günz glaciation)
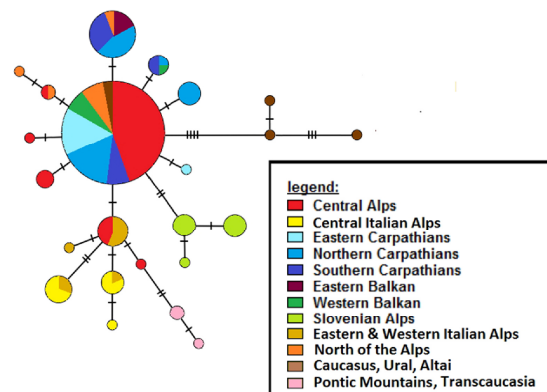
Figure 2. Minimum spanning Haplotype network of *E. aethiops* based on a COI fragment (429 bp). Mutational steps are indicated by the number of hatch marks.

resulting in a bottleneck at around 130 kya (Eem interglacial). This was followed by an ongoing phase of expansion starting in the Eem interglacial (128 kya to 115 kya B.P.)[25] (see supplementary S3).

Allozymes. We assessed the genetic diversity of allozymes over major parts of Europe; we calculated the number of alleles per locus, expected (H_e) and observed heterozygosity (H_o), the percentage of all polymorphic loci (P_{tot}) and the percentage of loci with the most common allele not exceeding 95% (P95) (see supplementary S4). The average number of alleles per locus was highest in the Apuseni Mountains, Tatras, and northern Italy. Central and western Alps populations had below-average diversities (1.62 ± 0.18 SD). Similar patterns were found for the observed heterozygosity (H_o). All loci and populations were in HWE. The 14 significant deviations from the HWE (applying to single loci in single populations) were reduced to two after Bonferroni correction (i.e. Medvodje for PGM; Fernpass for G6PDH). A test of LD revealed deviations in six populations after Bonferroni correction (see supplementary S5). Six linkage pairs were detected in Medvodje, three linkage pairs in Nanos, and Szelcepuszta. Each of the remaining three populations displayed just one case of linkage disequilibrium.

A locus-by-locus AMOVA based on a weighted average over the 20 polymorphic loci revealed the highest amount of diversity within individuals (variance component: 0.681), followed by between population variance (variance component: 0.300; F_{ST}: 0.263, $p < 0.001$), and the variance between individuals within populations (variance component: 0.159, F_{IS}: 0.19). The unweighted genetic distance[26] between all 27 populations varied from 0.015 to 0.219 with a mean of 0.062 (± 0.044 SD) (see supplementary S6). A Neighbor-Joining phenogram (NJ) based on these distances revealed four distinct groups: one group consists of the Italian populations of the central Italian Alps, a second geographically extensive group reaching from the Massif Central across the Alps to the Tatras, a third cluster consisted of the Romanian Carpathian populations; Nanos (in Slovenia) represented the fourth cluster (see supplementary S7).

The position of the Tatras group was not completely resolved. Most specimens were belonging to the extensive group, the remaining specimens to the Romanian one. Bootstrap values were weak, except for the strong support of the central Italian Alps group.

In addition to the NJ phenogram, a STRUCTURE analysis was conducted (see Fig. 4). The most likely ΔK value was five (see supplementary S8), since the value of two should be ignored[27]. K = 5 distinguished between four groups; the populations sampled in the central Italian Alps, the southern Carpathians and the eastern Balkans, Nanos, and a widespread group from the Massif Central to the Tatra populations, which is composed of two Structure groups (see supplementary S9). Geneland suggested three clusters, largely identical to the results of STRUCTURE (see supplementary S10, S11), yet, Nanos was grouped with the widespread group.

Pairwise genetic distances (derived for K = 5) were the highest for the central Italian Alps group (see supplementary S4), with the largest distance to the Nanos group (0.209 ± 0.014 SD) and the smallest distance to the Southern Carpathians—Eastern Balkans group (0.121 ± 0.008 SD). Pairwise F_{ST} values ranged from 0.009 (Sonnenstein vs. Medvodje) to 0.633 (Col des Aravis vs. Spiazzi) (see supplementary S12). A change of the main allele was observed in eight populations, where the highest number of three switches in the main allele was reached by Trigrad (Bulgaria) and Campolaro (central Italian Alps group). In addition, the central Italian Alps group is characterized by four endemic alleles, a number that was obtained in only one other group (i.e. the extensive group) (see supplementary S13).

Discussion
The center of origin of *E. aethiops* and the entire species group.
The species *E. aethiops* represents a monophyletic group together with the species *E. niphonica*, *E. neriene* and *E. alcmena*[28]. As all these species with exception of *E. aethiops* are exclusively found in eastern Asia, an Asian origin of the entire species group appears to be likely. However, the region where *E. aethiops* was evolving cannot be reconstructed unambigu-
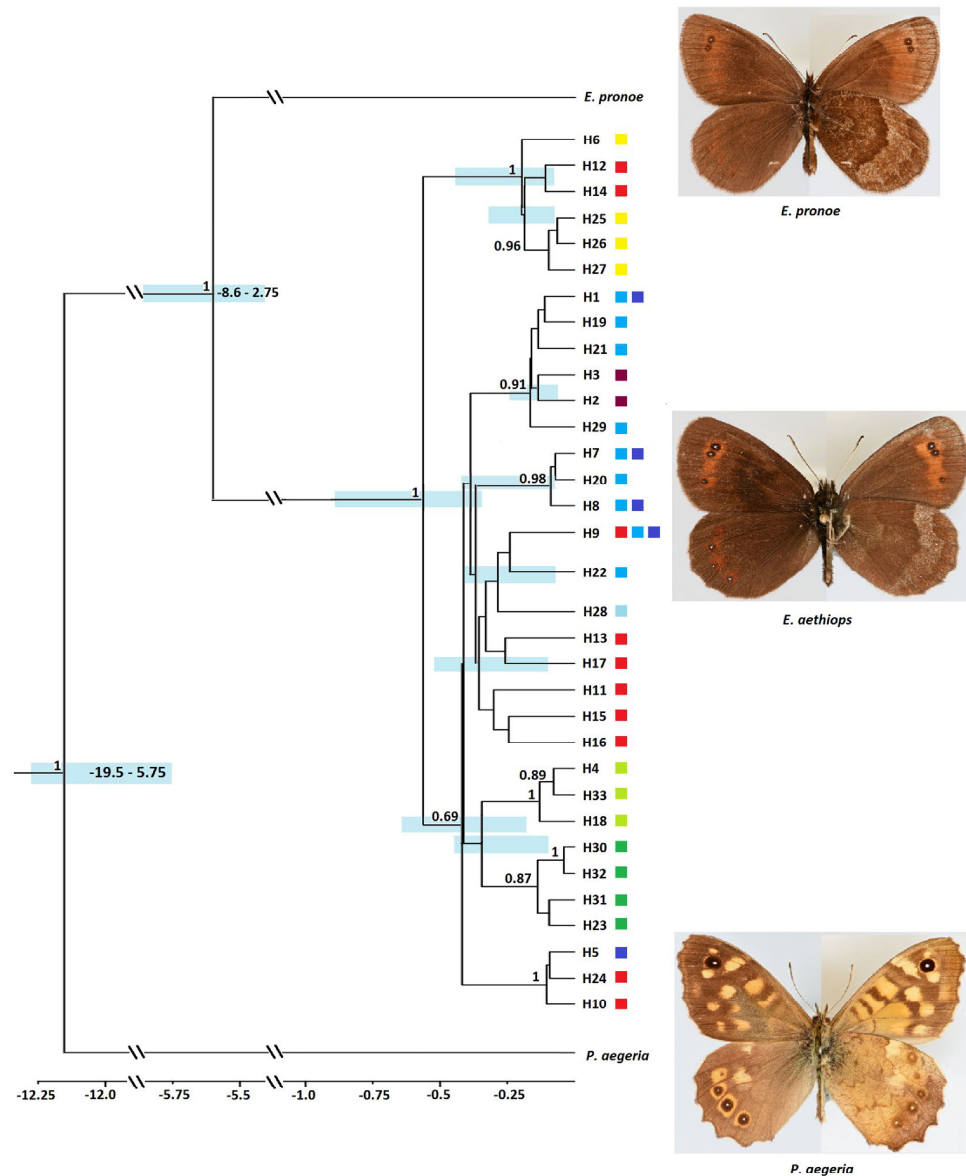
Figure 3. Bayesian phylogeny based on concatenated mtDNA haplotypes (COI, NDI) of *E. aethiops*. Number above recovered nodes: Bayesian posterior probabilities > 0.7; node bars: 95% highest posterior density in light blue bars. The geographical location of the haplotypes is given in same color scheme as in Fig. 1. Photos M. Wendt.

ously. The species' extant range stretches from western Europe to the Altai Mountains, hence allowing for several alternative areas of origin. A RASP analysis also could not point out a clear provenance (see supplementary S1). However, several aspects call for a western Palaearctic origin of the species. First, the *aethiops* group consists of five strongly supported monophyletic lineages (one per species, but two in the case of *E. niphonica*) and an unresolved backbone[28]. Apart from a limited geographic overlap between *E. neriene* and *E. aethiops* in the Altai Mountains, all lineages are distributed strictly allopatrically[28].

Consequently, an evolution in or nearby these species' current distribution ranges is much more parsimonious than a single speciation cradle in the Altai area with subsequent expansion of these lineages, but later extinction in their center of origin in the majority of cases. The fact that the ecological niches, with the exception of the more thermophilic *E. alcmena*, hardly differ suggest an allopatric origin instead of a common speciation origin. Furthermore, the ecologically rather similar species *E. aethiops* and *E. neriene* show an ecological character displacement in their limited area of sympatry in elevation levels in the Altai Mountains[29], also rejecting the
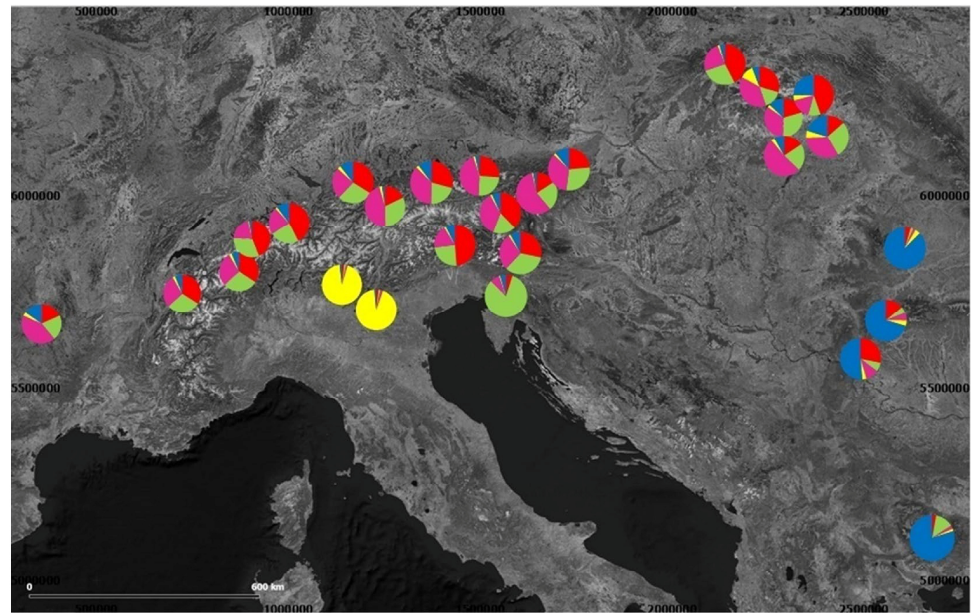
Figure 4. Cluster analysis with STRUCTURE for K = 5 based on allozyme polymorphism. The colors indicate the distinct genetic clusters to which the individuals of a population are assigned. The map was created with Qgis v.3.10.10[72] (Available online: http://qgis.osgeo.org).

hypothesis of nearby centers of origin for both species. Consequently, we suggest that the origin of *E. aethiops* is west of the Altai Mountains. Such a western Palearctic origin of *E. aethiops* was already postulated by Pena et al.[30], but based on a dataset not including its sister species analyzed by Nakatani et al.[28].

The internal genetic structures of *E. aethiops* give additional evidence for a European origin of the species because the COI sequences reported from the Pontic Mountains and Transcaucasia (both northern Turkey) on the one hand and the ones from the Urals to the Sajan Mountains on the other are branching from two opposite sides of the European network. Additionally, the sequences of the individuals from the Russian Caucasus (i.e. north of the main ridge of these mountains) are directly derived from the Ural/Sajan Mountains group, showing its maximum p-distance towards the geographically nearby populations of Transcaucasia.

Based on these data, the following evolutionary scenario and range dynamics seem the most likely: a population group expanded from Europe to Asia Minor with subsequent allopatric differentiation in the mountain ranges of northern Turkey. An additional range expansion likely occurred along a north-eastern pathway reaching as far east as the Sajan Mountains as its eastern endpoint. This expansion has given rise to a secondary one in southerly direction, reaching as far south as the Russian Caucasus. This scenario well explains why the most genetically distinct populations of this species are in such close geographic proximity: they represent the two endpoints of an pincer-like expansion rooted in Europe. As the genetic differentiation at the Eurasian level is considerably higher than in Europe alone, the time frame of these shifts must be seen prior to the beginning of the differentiation in Europe (i.e. 560 ky BP), but due to the differentiation depth, the split likely is not older than the onset of the Pleistocene.

The phylogeography of *E. aethiops* in Europe.   When looking at Europe in more detail, the oldest split within *E. aethiops*, detected at the mitochondrial and nuclear level, is between the Central Italian Alps populations and all others. The age of this split was estimated at about 560 ky BP, i.e. approximately at the beginning of the pronounced glacial-interglacial cycles with about 100,000 years per full cycle[25]. This matches other molecular clock-based estimates of divergence events of other taxa in this region[7]. Hence, it is likely that the distribution of European *E. aethiops* was covering at least parts of the Alps and its surrounding areas prior to the onset of the Günz glacial. Due to the climatic deterioration during this ice-age, which was not as severe as the following ones, but nevertheless stronger than before[25], this formerly continuous distribution area was apparently split up. Consequently, a previously functional belt of interconnected forest ecosystems at the edge of the Alps must have been disrupted by climatic changes for the first time in the Pleistocene. This apparently has led to the spatial separation into two large population groups of *E. aethiops* in Europe.

One of these two groups correspond to the lineage currently found in the Italian parts of the Alps adjoining the Po Valley. However, due to the lineage's rear edge position, it did not participate in all the following range dynamics[31]. The genetic diversity patterns at the mitochondrial and nuclear levels as well as the structure of the respective part of the mitochondrial haplotype network indicate a long-lasting persistence in the areas adjoining the Po Valley at low abundance, but largely without strong fluctuations in the total number of individuals. The

second (i.e. the widespread) group's geographic centre of origin cannot be reconstructed as clearly as in the first case, but our data suggest the eastern or south-eastern part of the Alps for several reasons. The genetic diversity at the nuclear level is still highest here today, a centre west of the Central Italian Alps group would not be compatible with the southern Alps group's rear edge status and, as known from other studies[13,32,33], the eastern and southeastern Alps probably represent one of the most important centres for glacial survival of species dwelling in forests, next to the Illyrean region, as indicated by the endemic Slovenian and Albanian haplotypes.

All further range dynamics of the widespread *E. aethiops* lineage must have taken place from this putative eastern Alpine centre of dispersal. A molecular clock analysis suggests a largely synchronous split at about 400 ky into six lineages. These lineages are also distinguished by the haplotype network, which together with the genetic distances identifies two differentiation levels within these six lineages. Thus, two comparably older lineages are found in Slovenia and Cheile Butii, with the latter located in a deep valley system in the southern Carpathians. All other haplotypes found in the Alps, northern, eastern, and southern parts of the Carpathians, western and eastern parts of the Balkan Peninsula are representatives of the younger differentiation events.

The range expansion during the mid-Mindel interstadial, which falls approximately within the time frame at about 400 ky[34], and their long-term biogeographical consequences cannot be reconstructed unambiguously. However, a range expansion over the Carpathian region almost certainly must have taken place. It must have been facilitated by a forested link between the north-eastern Alps and the Tatras and adjoining northwestern Carpathian areasduring interglacials[18]. This connection between these two regions, involving gene flow, is well documented when it comes to mountain species[35], but so far is not well-known regarding species intimately linked to forests.

The persistence of *E. aethiops* in the Carpathians since then can be explained by two partly diverging lines of evidence. The haplotype tree suggests the existence of several spatially separated glacial refugia in the southern Carpathians both during the Mindel and the Riss ice age. The haplotype network with its few slightly differentiated haplotypes partly contradicts this interpretation and requires a stronger reduction of populations in the Carpathians during these periods. The differentiated haplotype H5, restricted to the southern Carpathian location Cheile Butii, supports this hypothesis. These results are in line with previous findings that prove the survival of trees in the Carpathians during glacial periods[8,14,18], as well as the existence of extra-Mediterranean refugia of arboreal species there[6]. However, our results also indicate that the existing communities were not just habitats with single trees, but that they must have represented forest ecosystems, at least locally.

The colonisation of the western Balkan Peninsula might also go back to the Günz-Mindel interglacial, but cannot be reconstructed with certainty. Based on the haplotype tree, the expansion to this region should have taken place via Slovenia. In the subsequent glacial period, the link between the Slovenian populations and the eastern Alps should have been disrupted first, followed by the split between the western Balkans and Slovenia. However, the haplotype network and the genetic distances contradict this hypothesis and indicate independent dispersal events regarding the origin of the genetic lineages endemic to these two regions. Based on our mitochondrial data, the Slovenian populations must have become separated from those of the eastern Alps during the Mindel ice-age. This is also supported by our nuclear data. Consequently, the following range dynamics seem plausible: the forest ecosystem on the eastern and south-eastern margin of the Alps that still might have been uninterrupted during the Günz ice age must have become disconnected during the colder Mindel glacial, leading to vicariance in *E. aethiops* in this region. The high number of endemic species in Slovenia[36–38] and several phylogeographic studies[12,39,40] support a comparatively long isolation in Slovenia, also of *E. aethiops*. In this case, the Slovenian populations, similarly to the Central Italian Alps lineage, must have held a rear edge position, and the colonisation of the western Balkan Peninsula (at least as far south as Albania) must have occurred independently from the eastern Alpine region, without the Slovenian populations having any impact on them. Our data consequently suggests that forest ecosystems were present in Slovenia, just as in the Carpathian region, at least since the Mindel glacial, but probably throughout the entire Pleistocene. This goes beyond the previous knowledge of the uninterrupted existence of trees and arboreal species[8,41,42]. Furthermore, our data provide additional evidence for the importance of the western Balkan Peninsula as a Mediterranean glacial refugium for arboreal biota.

Looking at the phylogeographic structure of *E. aethiops* across the Balkan Peninsula, a frequently observed pattern is apparent, i.e. the separation between a western and an eastern Balkan lineage[43]. Whilst the northward range expansions of typical temperate species originate from their Balkan lineages during transitions from cold to warm periods, the Balkan lineages usually represent the endpoints of colonisations for cold-adapted mountain species, reaching the region from the eastern Alps and the southern Carpathians, respectively[44]. Consequently, although *E. aethiops* has to be considered a temperate forest species, it behaves like a mountain taxon in this context. Furthermore, in contrast to boreal taxa, which have been expanding into this region during ice ages, the expansion of *E. aethiops* most likely took place during warmer periods, in which forests were spreading over large parts of this region, thus facilitating the species' dispersal.

According to our genetic data, the colonisation of the before mentioned western Balkan Peninsula from the eastern Alps and the eastern Balkan Peninsula from the southern Carpathians happened within similar time horizons. However, the almost complete lack of differentiation between the eastern Balkans and the southwestern Carpathians (population Baile Herculane) supports the theory of an expansion via the Iron Gate (i.e. crossing of the Danube through the south-western Carpathians) to the Balkan Peninsula, not before the Eem interglacial. During the same time frame as this southern expansion, quick expansions out of the geographically strongly restricted Riss glacial refugia in the Carpathian region must have taken place. These expansions (that must have been associated with the complete reforestation of this region) apparently led to an area-wide distribution of *E. aethiops,* which is in line with the results of our Bayesian Skyline analysis. This hypothesis is also supported by the genetic patterns at the nuclear level, for which no detectable genetic differentiation in the southern Carpathian region exists. The mitochondrial level, however, might largely reflect the Riss glacial refugia, an apparent
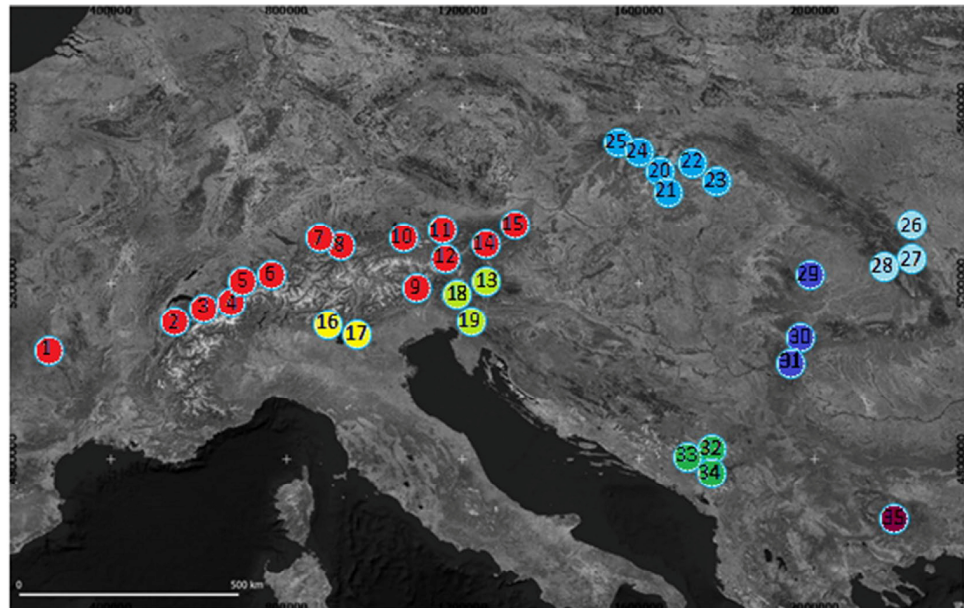
Figure 5. Geographic location of the sampled population of *E. aethiops*. Colors indicate the different genetic groups detected by genetic analyses. The map was created with Qgis v.3.10.10[72] (Available online: http://qgis.osgeo.org).

contradiction, which might be explained by different dispersal potentials of the sexes[20,22], but which needs to be investigated in more depth in future work.

The expansion within the Carpathian region during the Eem interglacial must have covered the entire arc up to the Tatras. However, both nuclear and mitochondrial data show that both the Tatras and the eastern Carpathians were populated by *E. aetiops* not only from the southern Carpathians, but also from a centre of dispersal in the eastern Alps. Such a mixture of Alpine and Carpathian elements of certain taxa in the Tatras has already been demonstrated several times[10,11]. However, the existence of numerous endemic haplotypes and allozyme alleles in the Tatras also suggests that *E. aethiops* not only colonised this region during the Eem interglacial, but was also able to survive the relatively mild Würm glacial there. A similar situation has been indicated for the eastern Carpathians, but the available data is not as robust as in the case of the Tatras. Our data thus show that not only individual trees must have been present on the southern fringe of the Tatras (and presumably also in the eastern Carpathians) during the last glacial period[8,14,17,41,42,45], but that there were local refugia in which temperate forests have survived permanently.

If one considers the species patchy distribution or lack at the Mediterranean peninsulas and the insufficient coverage of the Siberian Taiga, it becomes evident that *E. aethiops'* occurrence is also strongly influenced by its own biogeographic history. Therefore, the species cannot be considered as an indicator species of the entire western Palaearctic forest ecosystem, but within its range it can enrich the knowledge about the history and dynamics of deciduous forest structures. Thus, due to its mobility and sensitive response to environmental changes, it reacts reliably to changes in its ecosystem. Therefore, its recent distribution and genetic structure carries information not only on its own evolutionary history, but also on that of its associated ecosystem.

Material and methods

Study species. The Scotch Argus *Erebia aethiops* is a univoltine species with overwintering larvae. Eggs are laid on a wide spectrum of different grasses (Poaceae, e.g. *Molinia caerulea*, *Brachypodium pinnatum*, *Festuca* ssp., *Dactylis glomerata*, *Poa* ssp.), but also, less commonly, on sedges (Cyperaceae, e.g. *Luzula nivea*, *Carex ferruginea*, *Carex sempervirens*)[46]. The caterpillars pupate end of June, resulting in a flight season starting in July and ending at the beginning of September, with the main activity in late July and August.

Sampling design. 859 *E. aethiops* individuals representing 35 populations (8–41 specimens, mean: 30.8 per population) were sampled from 2002 to 2014 from the Massif Central in France to the Romanian Carpathians and Bulgarian mountain systems (Fig. 5, supplementary table S14). The butterflies were captured with a hand net in the field and frozen in liquid nitrogen. Specimens were subsequently stored in liquid nitrogen or in a refrigerator at − 80 °C until analyses. We added 48 sequences from BOLD and Genbank (accession numbers in Table S15 and distribution map in Fig. S16 of the supplementary) to get genetic data from parts of the range not covered by our own sampling. Since these just represented COI sequences, partly fragmentary (429 bp), only a simple

comparison was possible and not a complete analysis. The sample areas did not require permissions, except for Austria and Slovakia, whose environmental offices had issued these permits.

Genetic analyses. *DNA sequencing.* Total genomic DNA was extracted from one leg using the E.Z.N.A. Tissue DNA Kit (Omega Bio-Tek, Norcross, USA) following the manufacturer's protocol.

Variation of two mitochondrial genes was assessed for a subset of 133 individuals representing 28 populations covering all eight regions (see supplementary S14). We sequenced the barcoding region of the cytochrome c oxidase subunit I (COI, 658 bp), which provides reliable resolution at the intraspecific level as shown in several previous studies on *Erebia*[47,48], as well as the NADH dehydrogenase subunit I (NDI, 554 bp). COI was amplified with the primer pair LEP-F1 (5′-ATTCAACCAATCATAAAGATATTGG-3′) and LEP-R1 (5′-TAAACTTCT GGATGTCCAAAAAATCA-3′)[49] applying the following PCR protocol: 95 °C for 5 min, followed by 38 cycles at 95 °C for 30 s, 49 °C for 90 s, 72 °C for 60 s and terminated with a final extension step at 68 °C for 30 min. NDI was amplified using the primer pair FAW-NDI (5′-TTCAAACCGGTGTAAGCCAGG-3′) and FAW-16S (5′-TAG AATTAGAAGATCAACCAGC-3′)[50] and the following PCR protocol: 95 °C for 5 min, 33 cycles at 95 °C for 30 s, 56 °C for 90 s, 72 °C for 60 s and terminated at 68 °C for 30 min. PCR products were visualized on 1.4% agarose gels, stained with GelRed (Biotium, Fremton, USA). Amplified products were purified with a mix of FastAP and Exonuclease I (Thermo Scientific, Dreieich, Germany) and sent to Macrogen Europe (Amsterdam, Netherlands) for sequencing in both directions with the same two primers as were used for PCR.

Allozymes. 836 individuals from 27 populations (see supplementary S14) were analysed by allozyme electrophoresis. We analyzed a total of 20 allozyme loci (i.e. 6PGDH, IDH1, IDH2, GPDH, G6PDH, GAPDH, FUM, MDH1, MDH2, ME, GOT1, GOT2, PEP LGG; MPI, PK1, PK2, PK3, PGM, PGI1, PGI2) following the standard protocol by[51]. Running conditions were applied as in Schmitt and Seitz[32].

Statistical analyses. *Mitochondrial DNA.* Sequences were assembled with Geneious v. 10.2.3[52] and aligned using ClustalW[53] implemented in BioEdit v. 7.2.6.1[54]. Sequences of both genes were concatenated and checked for stop-codons with Geneious. Genbank accessions are given in Table S17 of the supplementary. The frequency of haplotypes, haplotype diversity (h), number of segregating sites (S), nucleotide diversity per gene (p_i), and the average number of nucleotide differences (k) were calculated with DNAsp v. 6[55]. The concatenated file was used to construct a Minimum Spanning haplotype network[56] with PopArt v. 1.7[57].

A Bayesian tree was reconstructed based on the mitochondrial data with BEAST v. 2.5[58]. Published data of *Pararge aegeria* (GenBank accessions in Table S17 of the supplementary) and own mtDNA sequences of *Erebia pronoe* were used as outgroups. Partitions and substitution models were estimated with Partitionfinder v. 2.1.1[59] based on the lowest Akaike Information Criterion (AIC). The HKY model with empirical base frequencies and a Gamma distribution with a category count of 4 was selected. We ran several analyses to select the best fitting tree model. The coalescent constant population model performed best and hence was chosen for the final analysis. The molecular clock was calibrated with a substitution rate of 0.0177[60]. We ran the analyses with 40 million generations sampling every 4,000 iterations. After checking the MCMC chain for convergence in Tracer v. 1.7.1[61], a burn-in of 10% was applied. Three individual runs were performed and combined using LogCombiner v. 1.8.4[62]. TreeAnnotator v. 2.5[62] was used to generate a summary tree with common ancestor heights. Figtree v. 1.4.4[61] was used for visualisation.

The same protocol was followed to obtain an input and consensus tree for a reconstruction of ancestral area analysis with RASP v. 4.2[63]. This analysis was based on the extended COI data set of *E. aethiops* and further COI sequences of *E. neriene*, *E. niphonica* and *E. alcema* obtained from Genbank and Boldsystems. The additional accession numbers are given in the RASP tree. The individual sequences were assigned occurrences coded as consecutive letters (A: Sakalin; B: Hoshu; C: zone of sympatry between *E. aethiops* and *E. neriene* in the Altai Mountains; D: Mongolia; E: China; F: Europe; G: western Altai; H: Turkey; J: Ural; I: Caucasus). Based on the results of the BioGeoBears model test, an S-DEC analysis was performed with the maxarea = 6 setting.

To model the demographic history of the populations, a Bayesian Skyline plot was generated with BEAST. Bayesian skyline analyses were run with the coalescent tree prior, in a single run with 40 million generations, log parameters were sampled every 4,000 iterations. The effective sample size and female population size was evaluated using Tracer v. 1.7.1. We used the substitution rate estimated by Papadopoulou et al.[60].

Allozymes. Alleles were labeled according to their relative mobility, starting with "1" for the slowest. Their frequency, the total number of alleles per locus (A) and further genetic diversity parameters (i.e. expected and observed heterozygosity (H_e; H_o), total number of polymorphic loci (P_{tot}) and the percentage of polymorphic alleles per locus with the most common allele not exceeding 95% (P95)) were estimated with G-Stat v. 3[64]. Tests on Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were performed with Arlequin v 3.5[65]. A sequential Bonferroni correction[66] was applied. A non-hierarchical and a hierarchical genetic variance analysis (AMOVA) were performed with Arlequin to attribute the genetic variation to the following levels: among populations, among individuals within populations, and within individuals. Additionally, pairwise F_{ST} values were estimated with Arlequin. A Neighbor Joining (NJ) phenogram[67] based on Nei's genetic distance[26] and 1,000 bootstrap replicates was generated with Phylip v. 3.67[68]. Structure v. 2.3.4[69] was used to infer the number of genetic clusters without a priori definition of populations. To ensure a better delimitation of the individual groups, which were suggested by Structure, each population was examined for a change in the main allele. We tested for genetic clusters (K) from two to 27 (i.e. the number of populations analyzed for allozymes). Ten replicates were carried out for each K, with a burn-in of 100,000 and 500,000 MCMC replicates performed thereafter. Due to the un-reliability of logarithmic probabilities, an ad hoc method was used and the

ΔK value, based on the change of the logarithmic probability of successive K values, was determined[70]. The high ΔK value for K = 2 was ignored a suggested by Hausdorf and Hennig[27]. Additionally, a GENELAND[71] analysis was conducted with ten runs, each of 250,000 generations, in an uncorrelated frequency model with the number of classes treated as unknown and variable along the MCMC chain.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. All data generated or analysed during this study are included in this published article [and its supplementary information files].

References

1. Hewitt, G. M. Some genetic consequences of ice ages. *Biol. J. Lin. Soc.* **58**, 247–276 (1996).
2. Husemann, M., Schmitt, T., Zachos, F. E., Ulrich, W. & Habel, J. C. Palearctic biogeography revisited: evidence for the existence of a North African refugium for Western Palaearctic biota. *J. Biogeogr.* **41**, 81–94. https://doi.org/10.1111/jbi.12180 (2014).
3. Hewitt, G. M. Postglacial recolonization of European biota. *Biol. J. Lin. Soc.* **68**, 87–112 (1999).
4. Hewitt, G. M. Genetic consequences of climatic oscillations in the Quaternary. *Philos. Trans. R. Soc. B Biol. Sci.* **359**, 183–195. https://doi.org/10.1098/rstb.2003.1388 (2004).
5. de Lattin, G. *Grundriß der Zoogeographie* (Gustav Fischer, Jena, 1967).
6. Schmitt, T. & Varga, Z. Extra-Mediterranean refugia: The rule and not the exception?. *Front. Zool.* **9**, 1–12. https://doi.org/10.1186/1742-9994-9-22 (2012).
7. Drees, C. *et al.* Molecular analyses and species distribution models indicate cryptic northern mountain refugia for a forest-dwelling ground beetle. *J. Biogeogr.* **43**, 2223–2236. https://doi.org/10.1111/jbi.12828 (2016).
8. Juřičková, L., Horáčková, J. & Ložek, V. Direct evidence of central European forest refugia during the last glacial period based on mollusc fossils. *Quat. Res.* **82**, 222–228. https://doi.org/10.1016/j.yqres.2014.01.015 (2014).
9. Pinceel, J., Jordaens, K., Pfenninger, M. & Backeljau, T. Rangewide phylogeography of a terrestrial slug in Europe: Evidence for Alpine refugia and rapid colonization after the Pleistocene glaciations. *Mol. Ecol.* **14**, 1133–1150. https://doi.org/10.1111/j.1365-294X.2005.02479.x (2005).
10. Gratton, P., Konopiński, M. K. & Sbordoni, V. Pleistocene evolutionary history of the Clouded Apollo (Parnassius mnemosyne): Genetic signatures of climate cycles and a "time-dependent" mitochondrial substitution rate. *Mol. Ecol.* **17**, 4248–4262. https://doi.org/10.1111/j.1365-294X.2008.03901.x (2008).
11. Hofman, S. *et al.* Phylogeography of the fire-bellied toads Bombina: Independent Pleistocene histories inferred from mitochondrial genomes. *Mol. Ecol.* **16**, 2301–2316. https://doi.org/10.1111/j.1365-294X.2007.03309.x (2007).
12. Junker, M. *et al.* Three in one-multiple faunal elements within an endangered european butterfly species. *PLoS ONE* **10**, 1–24. https://doi.org/10.1371/journal.pone.0142282 (2015).
13. Ursenbacher, S., Carlsson, M., Helfer, V., Tegelström, H. & Fumagalli, L. Phylogeography and Pleistocene refugia of the adder (*Vipera berus*) as inferred from mitochondrial DNA sequence data. *Mol. Ecol.* **15**, 3425–3437. https://doi.org/10.1111/j.1365-294X.2006.03031.x (2006).
14. Magri, D. Patterns of post-glacial spread and the extent of glacial refugia of European beech (*Fagus sylvatica*). *J. Biogeogr.* **35**, 450–463. https://doi.org/10.1111/j.1365-2699.2007.01803.x (2008).
15. Svenning, J.-C., Normand, S. & Kageyama, M. Glacial refugia of temperate trees in Europe: Insights from species distribution modelling. *J. Ecol.* **96**, 1117–1127. https://doi.org/10.1111/j.1752-4598.2012.00212.x (2008).
16. Cheddadi, R. *et al.* Imprints of glacial refugia in the modern genetic diversity of *Pinus sylvestris*. *Global Ecol. Biogeogr.* **15**, 271–282. https://doi.org/10.1111/j.1466-822x.2006.00226.x (2006).
17. Willis, K. J. & Van Andel, T. H. Trees or no trees? The environments of central and eastern Europe during the Last Glaciation. *Quatern. Sci. Rev.* **23**, 2369–2387. https://doi.org/10.1016/j.quascirev.2004.06.002 (2004).
18. Rudner, Z. E. & Sümegi, P. Recurring Taiga forest-steppe habitats in the Carpathian Basin in the Upper Weichselian. *Quatern. Int.* **76**, 177–189. https://doi.org/10.1016/S1040-6182(00)00101-4 (2001).
19. van Swaay, C., Warren, M. & Grégoire, L. Biotope use and trends of European butterflies. *J. Insect Conserv.* **10**, 189–209. https://doi.org/10.1007/s10841-006-6293-4 (2006).
20. Slamova, I., Klecka, J. & Konvicka, M. Diurnal behavior and habitat preferences of *Erebia aethiops*, an aberrant lowland species of a mountain butterfly clade. *J. Insect Behav.* **24**, 230–246. https://doi.org/10.1007/s10905-010-9250-8 (2011).
21. Burnaz, S. & Balazs, S. Contributions to the knowledge of diurnal Lepidoptera fauna of the North-Eastern part of Ţarcu Mountains (Southern Carpathians, Romania). *Buletin de Informare Entomologica* **22**, 41–52 (2011).
22. Slamova, I., Klecka, J. & Konvicka, M. Woodland and grassland mosaic from a butterfly perspective: Habitat use by *Erebia aethiops* (Lepidoptera: Satyridae). *Insect Conserv. Divers.* **6**, 243–254. https://doi.org/10.1111/j.1752-4598.2012.00212.x (2013).
23. Tshikolovets, V. V. *Butterflies of Europe & the Mediterranean area* (Tshikolovets Publications, Pardubice, 2011).
24. Varga, Z. Die Erebien der Balkanhalbinsel und Karpaten V. Übersicht der subspezifischen Gliederung und der Verbreitung der Erebia Dalman, 1816 -Arten (Lepidoptera, Nymphalidae, Saryrinae) in der Balkanhalbinsel und in den Karpaten (II. Teil). *Entomol Roman* **6**, 5–39 (2001).
25. Imbrie, J., *et al.* Milankovitch theory, the two shorter cycles can be explained radiation cycle (arising from of Geological Sciences, Brown 2 Institut d' Astronomie et de Geophysique of Earth, Atmospheric, and Planetary Earth Observatory, Columbia for Climatic and Sp. Cycle **8**, 699–735 (1993).
26. Nei, M. Genetic Distance between Populations **106**, 283–29 (1972).
27. Hausdorf, B. & Hennig, C. Species delimitation using dominant and codominant multilocus. *Markers* **59**, 491–503. https://doi.org/10.1093/sysbio/syq039 (2010).
28. Nakatani, T., Usami, S. & Itoh, T. Molecular phylogenetic analysis of the *Erebia aethiops* group (Lepidoptera, Nymphalidae). *Lepidopterol. Soc. Jpn.* **58**, 387–404 (2007).
29. Lukhtanov, V. & Lukhtanov, A. *Die Tagfalter Nordwestasiens* (Dr. Ulf Eitschberger, Marktleuthen, 1994).
30. Peña, C., Witthauer, H., Klečková, I., Fric, Z. & Wahlberg, N. Adaptive radiations in butterflies: Evolutionary history of the genus Erebia (Nymphalidae: Satyrinae). *Biol. J. Lin. Soc.* **116**(2), 449–467. https://doi.org/10.1111/bij.12597 (2015).
31. Hampe, A. & Petit, R. J. Conserving biodiversity under climate change: The rear edge matters. *Ecol. Lett.* **8**, 461–467. https://doi.org/10.1111/j.1461-0248.2005.00739.x (2005).
32. Schmitt, T., Hewitt, G. M. & Müller, P. Disjunct distributions during glacial and interglacial periods in mountain butterflies: *Erebia epiphron* as an example. *J. Evol. Biol.* **19**, 108–113. https://doi.org/10.1111/j.1420-9101.2005.00980.x (2006).

33. Schmitt, T. & Seitz, A. Intraspecific allozymatic differentiation reveals the glacial refugia and the postglacial expansions of European *Erebia medusa* (Lepidoptera: Nymphalidae). *Biol. J. Linn. Soc.* **74**, 429–458. https://doi.org/10.1006/bijl.2001.0584 (2001).

34. van Husen, D. Geological processes during the Quaternary. *Mitteilungen Der Österreichischen Geologischen Gesellschaft* **92**, 135–156 (2000).

35. Schmitt, T. Biogeographical and evolutionary importance of the European high mountain systems. *Front. Zool.* **6**, 1–10. https://doi.org/10.1186/1742-9994-6-9 (2009).

36. Albre, J., Gers, C. & Legal, L. Molecular phylogeny of the *Erebia tyndarus* (Lepidoptera, Rhopalocera, Nymphalidae, Satyrinae) species group combining CoxII and ND5 mitochondrial genes: A case study of a recent radiation. *Mol. Phylogenet. Evol.* **47**, 196–210. https://doi.org/10.1016/j.ympev.2008.01.009 (2008).

37. Fišer Pečnikar, Ž, Balant, M., Glasnović, P. & Surina, B. Seed dormancy and germination of the rare, high elevation Balkan endemic *Cerastium dinaricum* (Caryophyllaceae). *Biologia* **73**, 937–943. https://doi.org/10.2478/s11756-018-0115-5 (2018).

38. Giachino, P. M. A new species of Aphaobiella Pretner, 1949 from Grintavec Mt., Slovenia (Coleoptera: Cholevidae, Leptodirinae). *Fragm. Entomol.* **48**, 19–23. https://doi.org/10.4081/fe.2016.165 (2016).

39. Canestrelli, D., Salvi, D., Maura, M., Bologna, M. A. & Nascetti, G. One species, three Pleistocene evolutionary histories: Phylogeography of the Italian crested newt, *Triturus carnifex*. *PLoS ONE* **7**, e41754. https://doi.org/10.1371/journal.pone.0041754 (2012).

40. Previšić, A., Walton, C., KuČiniČ, M., Mitrikeski, P. T. & Kerovec, M. Pleistocene divergence of Dinaric Drusus endemics (Trichoptera, Limnephilidae) in multiple microrefugia within the Balkan Peninsula. *Mol. Ecol.* **18**, 634–647. https://doi.org/10.1111/j.1365-294X.2008.04046.x (2009).

41. Deffontaine, V. *et al.* Beyond the Mediterranean peninsulas: Evidence of central European glacial refugia for a temperate forest mammal species, the bank vole (*Clethrionomys glareolus*). *Mol. Ecol.* **14**, 1727–1739. https://doi.org/10.1111/j.1365-294X.2005.02506.x (2005).

42. Krebs, P., Pezzatti, G. B., Beffa, G., Tinner, W. & Conedera, M. Revising the sweet chestnut (*Castanea sativa* Mill.) refugia history of the last glacial period with extended pollen and macrofossil evidence. *Quat. Sci. Rev.* **206**, 111–128. https://doi.org/10.1016/j.quascirev.2019.01.002 (2019).

43. Varga, Z. Geographische Isolation und Subspeziation bei den Hochgebirgslepidopteren der Balkanhalbinsel. Acta Entomol Jugoslaviae. (1975).

44. Bhagwat, S. A. & Willis, K. J. Species persistence in northerly glacial refugia of Europe: A matter of chance or biogeographical traits?. *J. Biogeogr.* **35**, 464–482. https://doi.org/10.1111/j.1365-2699.2007.01861.x (2008).

45. Filipi, K., Marková, S., Searle, J. B. & Kotlík, P. Mitogenomic phylogenetics of the bank vole *Clethrionomys glareolus*, a model system for studying end-glacial colonization of Europe. *Mol. Phylogenet. Evol.* **82**, 245–257. https://doi.org/10.1016/j.ympev.2014.10.016 (2015).

46. Hammouti, N., Schmitt, T., Seitz, A., Kosuch, J. & Veith, M. Combining mitochondrial and nuclear evidences: A refined evolutionary history of *Erebia medusa* (Lepidoptera: Nymphalidae: Satyrinae) in Central Europe based on the COI gene. *J. Zool. Syst. Evolut. Res.* **48**, 115–125. https://doi.org/10.1111/j.1439-0469.2009.00544.x (2010).

47. Vila, M., Marí-Mena, N., Guerrero, A. & Schmitt, T. Some butterflies do not care much about topography: a single genetic lineage of *Erebia euryale* (Nymphalidae) along the northern Iberian mountains. *J. Zool. Syst. Evolut. Res.* **49**, 119–132. https://doi.org/10.1111/j.1439-0469.2010.00587.x (2011).

48. Vodă, R., Dapporto, L., Dincă, V. & Vila, R. Cryptic matters: Overlooked species generate most butterfly beta-diversity. *Ecography* **38**, 405–409. https://doi.org/10.1111/ecog.00762 (2015).

49. Hebert, P. D. N., Ratnasingham, S. & DeWaard, J. R. Barcoding animal life: Cytochrome c oxidase subunit 1 divergences among closely related species. *Proc. R. Soc. B Biol. Sci.* **270**, 1–4. https://doi.org/10.1098/rsbl.2003.0025 (2003).

50. Weller, S. J., Pashley, D. P., Martin, J. A. & Constable, J. L. Phylogeny of noctuoid moths and the utility of combining independent nuclear and mitochondrial genes. *Syst. Biol.* **43**, 194–211. https://doi.org/10.1093/sysbio/43.2.194 (1994).

51. Hebert, P. D. N., & Beaton, M. J. Methodologies for allozyme analysis using cellulose acetate electrophoresis. Zoology 32 (1993).

52. Kearse, M. *et al.* Geneious basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **28**, 1647–1649. https://doi.org/10.1093/bioinformatics/bts199 (2012).

53. Thompson, J. D., Higgins, D. G. & Gibson, T. J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673–4680. https://doi.org/10.1093/nar/22.22.4673 (1994).

54. Hall, T. A. BioEdit. *Nucleic Acids Symp. Ser.* **41**, 95–98 (1999).

55. Rozas, J. *et al.* DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Mol. Biol. Evol.* **34**, 3299–3302. https://doi.org/10.1093/molbev/msx248 (2017).

56. Bandelt, H. J., Forster, P. & Röhl, A. Median-joining networks for inferring intraspecific phylogenies. *Mol. Biol. Evol.* **16**, 37–48. https://doi.org/10.1093/oxfordjournals.molbev.a026036 (1999).

57. Leigh, J. W. & Bryant, D. POPART: Full-feature software for haplotype network construction. *Methods Ecol. Evol.* **6**, 1110–1116. https://doi.org/10.1111/2041-210X.12410 (2015).

58. Bouckaert, R. *et al.* BEAST 2.5: An advanced software platform for Bayesian evolutionary analysis. *PLoS Comput. Biol.* **15**, e1006650. https://doi.org/10.1371/journal.pcbi.1006650 (2019).

59. Lanfear, R., Frandsen, P. B., Wright, A. M., Senfeld, T. & Calcott, B. Partitionfinder 2: New methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Mol. Biol. Evol.* **34**, 772–773. https://doi.org/10.1093/molbev/msw260 (2017).

60. Papadopoulou, A., Anastasiou, I. & Vogler, A. P. Revisiting the insect mitochondrial molecular clock: The mid-aegean trench calibration. *Mol. Biol. Evol.* **27**, 1659–1672. https://doi.org/10.1093/molbev/msq051 (2010).

61. Rambaut, A., Drummond, A. J., Xie, D., Baele, G. & Suchard, M. A. Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Syst. Biol.* **67**, 901–904. https://doi.org/10.1093/sysbio/syy032 (2018).

62. Drummond, A. J., & Ho, S. Y. W. A rough guide to BEAST 1.4. Edinburgh, 1–41, http://beast-mcmc.googlecode.com/files/BEAST 14_Manual_17May2007.pdf (2007).

63. Yu, Y., Harris, A. J., Blair, C. & He, X. RASP (Reconstruct Ancestral State in Phylogenies): A tool for historical biogeography. *Mol. Phylogenet. Evol.* **87**, 46–49. https://doi.org/10.1016/j.ympev.2015.03.008 (2015).

64. Siegismund, H. R. *G-STAT, Version 3, Genetical Statistical Programs for the Analysis of Population Data.* (The Arboretum, Royal Veterinary and Agricultural University, Horsholm, Denmark, 1993).

65. Excoffier, L., Laval, G. & Schneider, S. Arlequin (version 3.0): An integrated software package for population genetics data analysis. *Evol. Bioinform.* **1**, 117693430500100. https://doi.org/10.1177/117693430500100003 (2005).

66. Rice, W. R. The sequential Bonferroni test. *Evolution* **43**, 235 (1989).

67. Saitou, N. & Nei, M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **97**, 407–425 (1987).

68. Felsenstein, J. *PHYLIP (Phylogeny Inference Package), Version 3.5.c.* (Department of Genetics, University of Washington, Seattle, Washington, 2000).

69. Pritchard, J. K., Stephens, M. & Donnelly, P. Inference of population structure using multilocus genotype data. *Genetics* **155**, 945–959 (2000).

70. Evanno, G., Regnaut, S. & Goudet, J. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Mol. Ecol.* **14**, 2611–2620. https://doi.org/10.1111/j.1365-294X.2005.02553.x (2005).
71. Guillot, G., Estoup, A., Mortier, F., & Cosson, J. F. A spatial statistical model for landscape genetics. *Genetics* **170**, 1261–1280. https://doi.org/10.1534/genetics.104.033803 (2005).
72. Team, Q. D. QGIS Geographic Information System. Open Source Geospatial Foundation Project (2020). http://qgis.osgeo.org. Accessed 05 Nov 2020.

Author contributions
M.W. and T.S. conceived and designed the study. M.W. and K.K.. performed the data acquisition. All authors aided in the interpretation of the data. M.W., M.H. and T.S. wrote the manuscript. All authors reviewed the manuscript.

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Article

Coping with Environmental Extremes: Population Ecology and Behavioural Adaptation of *Erebia pronoe*, an Alpine Butterfly Species

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Simple Summary: High alpine meadows are home to numerous endemic butterfly species. A combination of climate change and changes in agricultural practices has led to a severe decline in many species. A seemingly unaffected representative of this habitat is *Erebia pronoe*. We studied the behaviour, resource use and population structure of this species to explain its resilience and estimate its future survival potential. This species shows pronounced protandry in combination with serial eclosion. Males were significantly more active and mobile and were also caught significantly more often than females, resulting in a pronounced shift in sex ratio in the predicted population structure. The adults use a wide range of nectar plants and establish homeranges in areas of high habitat quality. Thus, *Erebia pronoe* adults use a wide array of resources combined with a slight specialisation to avoid niche overlap with closely related species. The resulting ecological flexibility seems to be an adaptation to unpredictable environmental conditions, which should be the result of a long-lasting adaptation process. Moreover, the combination of opportunism and modest specialisation should also be a good basis for coping with future changes caused by climate and land-use change.

Abstract: A mark-recapture study of the nominotypical *Erebia pronoe* in the Alps was conducted to survey its ecological demands and characteristics. Population structure analysis revealed a combination of protandry (one-week earlier eclosion of males) and serial eclosion. Significant differences between both sexes were found in population density (males: 580/ha $\pm$ 37 SE; females: 241/ha $\pm$ 66 SE), sex-ratio (2.4) and behaviour (57.7 vs. 11.9% flying). Both sexes used a wide range of nectar plants (Asteraceae, 77.3%; Dipsacaceae, 12.3%; Gentianaceae, 9.7%). The use of nectar plants shows a non-specific spectrum, which, however, completely avoids overlap with the locally co-occurring species *Erebia nivalis*. Movement patterns show the establishment of homeranges, which significantly limits the migration potential. Due to its broad ecological niche, *E. pronoe* will probably be able to react plastically to the consequences of climate change. The formation of high population densities, the unconcerned endangerment status, the unspecific resource spectrum and the sedentary character of the species make *E. pronoe* a potential indicator of the quality and general resource occurrence of alpine rupicolous grasslands.

Keywords: mark-release-recapture; movement patterns; opportunistic behaviour; partial protandry; population demography

1. Introduction

The worldwide losses in species diversity and abundance are so strong that the recent extinction of species is compared with the major extinction events in earth's history [1].

Although biodiversity loss is a global phenomenon, individual regions, habitats and species groups are particularly affected. For instance, as a result of their generally lower plasticity, specialised stenotopic species are assumed to be more vulnerable to biotic and abiotic disturbances, as they do not have the ecological flexibility to react plastically to the numerous biotic and abiotic disturbances [2]. Especially extreme habitats, with the alpine environments being one, require specific adaptations to the prevailing harsh conditions and are thus home to many specialised and narrow-ranging species [3]. For this reason, Europe's high mountains are important centres of biodiversity [4].

Habitat loss is one of the main drivers of the current biodiversity crisis [5]. With the loss of high-quality habitats, low-mobility species are affected in particular, as dispersal and/or maintenance of metapopulations is only possible to a limited extent. This is especially true for montane and alpine species, whose extinction risk is further increased by increasing isolation [6] since their habitats are inherently more susceptible to fragmentation [7] and their populations are more limited due to species–arearelationship [8]. Additional pressure is caused by the ongoing climate change, which is causing range shifts in many species in order to maintain their ecological niches [9,10]. Nevertheless, it is not possible for numerous species to keep up with climate change, thus resulting in an extinction debt [2]. Another risk of climatic change is a potential inter-and intraspecific decoupling [11–13], which can negatively affect population size [14]. Similarly, climatic conditions affect the resource supply and quality of a habitat, which in turn affects the reproductive rate [15] and thus the population size of the species found there. The population structures of a species can strongly influence behaviour [16], and low abundance can lead to negative feedback [13]. Therefore, to determine the status of a habitat or a potential indicator species, an analysis of its population structure in combination with migration patterns, resource use and behaviour must be examined.

The sensitive reaction of insects to environmental changes and their high reproductive rate qualify them as excellent indicators and study objects for changes of habitat quality [2,17]. Especially, butterflies have become a preferred study group for analysing the quality and linkage of habitats due to their ecological specialisation and mobility [6,18,19].

We have selected the butterfly species *Erebia pronoe* as a characteristic species of the endangered flower-rich montane and alpine grasslands to evaluate its ecological needs, its potential as an indicator species and its future prospects. *Erebia pronoe* is an excellent representative of this habitat type because of its overall abundance and its wide distribution in the high mountains of Europe [20] but also in numerous prime butterfly areas [21]. In this paper, the population structure and demographic development of this montane butterfly were analysed based on a mark-release-recapture study in the Austrian Alps. The region harbours the nominotypical taxon *E. pronoe pronoe*. This study provides essential information about the specific adaptations to high mountain habitats, especially regarding the potential expression of protandric structures [22,23]. Additionally, the sex-specific mobility of individuals was investigated to derive conclusions about habitat linkage and metapopulation structure in the region under investigation. We were able to analyse the effect of climatic factors on the behaviour, the sex-specific behaviour and the resourceuse of *Erebia p. pronoe*. Thus, the following two key questions are the focus of our investigation:

Does *E. p. pronoe* have specific adaptations of its population demography (e.g., modifications in protandry, prolonged eclosion phases) that can be understood as specific adaptations to its habitats, and will these allow the species to adapt to expected future climatic changes?

Does *E. p. pronoe* exhibit sufficient ecological flexibility and mobility to be able to survive the expected further changes in its alpine environment?

2. Materials and Methods

2.1. Study Species

Erebia pronoe belongs to the western Palaearctic Nymphalidae. The species can be found on wet meadows, calcareous grasslands and screes from the high montane to the

alpine zone [24]. *E. pronoe* is widespread and often common throughout the Alps. It is locally found in the Swiss and French Jura as well as in the Pyrenees, where high densities can be reached. In the Carpathians, including the High Tatras and the mountains of the Balkan Peninsula, populations of *E. pronoe* are relatively rare and small [25]. The species flies in one generation from the end of July to mid-September and hibernates as L1 larva. The larvae prefer Festucaspecies such as *F. ovina*, *F. rubra* and *F. quadriflora,* and to a lesser degree *Anthoxanthum odoratum*; breeding was successful on *Poa annua* [26].

2.2. Study Area

The study area is located in the Austrian Alps in the core of the distribution area of the nominal subspecies in the "Hohe Tauern National Park" near the "Haus der Alpinen Naturschau" (47°07′ N,12°49′ E), in the state of Salzburg. The investigated steep slope covers an area of 4.6 ha and extends at an altitude of 2250–2400m asl. The area is interspersed with gravel areas and represents a characteristic habitat of *E. pronoe*. Other suitable habitats are present within a distance of 200 m. To the north and east, it borders on the Special Protection Area "Piffkar-Fusch", where no collection was allowed. The area in the west was too steep, and the "Untere Nassfeld" in the south was too heavily grazed for working. The flat areas at the foot of the slope were grazed by 19 cows.

2.3. Mark-Release-Recapture Study

We conducted a mark-release-recapture study as the basis for our analyses of population structure and species-specific mobility parameters. Furthermore, behaviour and specific resource use were surveyed to identify specific adaptations to alpine habitats. The study was carried out from 16 July 2019 to 1 September 2019, covering most of the flight season of *E. pronoe*. The butterflies were caught with a 40 cm diameter butterfly net under suitable conditions (see [27]) from 9 am to 6 pm; to avoid day-time effects, the sampling was always started at different sections. Each individual was marked on the underside of the hind wings with a fine, waterproof pen (StabiloOHPen universal S) with an individual code consisting of letters for the day and a consecutive number. In addition, GPS coordinates, sex, wing condition (scale 1 = wing seam completely preserved; up to 4 = wing heavily damaged [28]), behaviour when being captured and current weather conditions were recorded. The same data were collected for each recapture, excluding recaptures during the same day, to avoid behavioural effects of the capture and theoretically allow complete mixing of the population [29]. We assessed the age structure of the population based on wing conditions.

The ageing per time unit was calculated sex-specifically by correlating the time intervals between capture and first recapture and the deterioration rate of wing condition. For Spearman rank correlation analyses, only days with at least five individuals per sex were considered, and their mean values were used. Furthermore, sex-specific behavioural patterns were investigated by using the X^2-test. The influence of wind and cloud cover on the behaviour was also analysed with an X^2-test. Potential sex-specific preferences of nectar plants were investigated with X^2-test and X^2-homogeneity test.

2.4. Population Demography

Based on the mark-release-recapture dataset, we conducted population modelling to assess population size as a potential indicator of habitat quality and to examine population structure for potential adaptations. The program Mark 8.2 and its module Popan [30], based on the Jolly–Seber method, was used to calculate the daily, sex-specific population size. Three parameters were calculated: φ (phi), the probability of survival; p, the probability of capture; and pent, the proportional recruitment. These parameters can be constant (.), sex-specific (g), time-factorial (t), linear (T) or quadratic (T^2) and can have additive (g + t; g + T, . . .) or interactive relationships (g × t; g × T; . . .) [31]. Furthermore, the sampling effort (i.e., the time spent in the terrain) was considered as a covariant for the probability of capture. A saturated model consisting of these parameters was verified by a goodness-of-fit

test with the program RELEASE. Based on this, various parameter combinations were calculated, and the best-supported model was determined using the corrected Akaike Information Criterion (AIC$_C$) [32] and the lowest number of parameters [33].

2.5. Wing Condition

We applied linear and non-linear mixed-effects models to illustrate the ageing of the population based on the wing condition. These models didn't converge with the female dataset. We then tested polynomial functions from the first to the 9th order to describe the ageing of the population and selected the best model based on the R^2-value. Based on the breaking points for the different stages of ageing, we used linear models to describe these periods. We used the average wing wear of at least five specimens to avoid a sample size bias.

2.6. Mobility Parameters

We analysed movement patterns in the habitat to infer migration potential as well as resource use and availability. The collected GPS data of captures and recaptures were used to reconstruct movement patterns. These were imported into QGIS 3.8.3 [34], and the direct geographic distance between capture and first recapture was determined for each individual by creating a linear distance matrix layer with the vector analyses tool in the WGS 84 (EPSG4326) Coordinate Reference System. For assessing the minimum total distance moved, we summed up the distances between all points of capture.

We performed a Shapiro–Wilk test to check for normal distribution by using the program R version 3.6.1 [35]. Since there was no normal distribution of the data (males p: 1.376×10^{-10}, females p: 0.023), the test was followed by a Mann–Whitney U test to determine differences in distances travelled by males and females and by a two-sided Spearman's rank correlation to analyse the influence of days since capturing on the distance. The travelled distances were divided into distance classes (20 m, 30 m and 50 m intervals) separately for each sex. The inverse cumulative percentage of these classes was determined, which corresponds to the probability density function, i.e., the dispersal kernel. To check for any potential artefacts caused by the chosen interval sizes, we analysed and compared three different intervals and size classes.

Based on these classes, the probabilities of dispersal flights were investigated through distance extrapolation. Two frequently applied regression analyses were used: the negative exponential function (NEF) and the inverse power function (IPF). The NEF tends to underestimate rare long-distance movements, whereas the IPF may encounter problems with "zero" movements [36]. The data were linearly transformed with a semi-ln plot for the NEF analyses or with a double-ln plot for the IPF analyses. In both equations, P stands for the proportional probability that an individual will travel at least as far as the distance D, and a for the intercept of the regression. NEF works with the dispersal constant K as the slope, whereas IPF uses the variable n as the slope, which represents the effect of distance on dispersal [22].

$$P_{NEF} = ae^{-kD} \text{ or } \ln P = \ln a - k\,D \tag{1}$$

$$\text{or } P_{IPF} = aD^{-n} \text{ or } \ln P = \ln a - n(\ln\,D) \tag{2}$$

We selected the best model and the most suitable interval size based on calculated stability indices R^2 of the calculated curves, which corresponds to the proportion of explained variance of the dependent variable by the independent variable. This allowed extrapolations of the population's proportion that should travel distances exceeding the extent of the study area. The calculations were performed separately for males and females.

3. Results

During 28 days with captures, 962 individuals (808 males; 154 females) were marked; 260 individuals (246 males; 14 females) were recaptured. This corresponds to a recapture

rate of 27.0% (30.4% males, 9.1% females) and a sexratio of five males per female. We achieved up to five recaptures for males; females were recaptured not more than once.

3.1. Demography

The best-supported model with the lowest AIC_C value and the lowest number of parameters yielded an additive effect of sex and factorial time on the survival probability phi, an additive effect of sex and survey time on the capture probability p, and an interactive effect of sex and factorial time on the proportional recruitment pent and also on the number of individuals (Table 1). This model estimated population size of 2667 males, i.e., 580/ha ($\pm$37 SE). The population size of the females was estimated at 1110 individuals, i.e., 241/ha ($\pm$66 SE). The estimated sexratio was 2.4 males per female. Estimates based on the best supported Popan model were consistent with the actual daily recapture events.

Table 1. Comparison of the best models of Popan 5.0 analyses for the estimates of the daily population size of *Erebia pronoe*: Akaike Information Criterion (AIC_C) and number of parameters used, basic variables: Probability of survival (Phi), probability of recapture (P), proportional recruitment (Pent), the total number of individuals (N), dependent variables: sex (g); factorial (t), linear (T) and quadratic (T2) dependence of time; time invested in sampling per day (hours).

Model Nr.	Model	AIC_C	Parameters
1	{Phi(g + t) p(g + hours) pent(g $\times$ t) N(g $\times$ t)}	2404.7462	34
2	{Phi(g $\times$ t) p(g $\times$ hours) pent(g + t) N(g $\times$ t)}	2408.9191	64
3	{Phi(g + t) p(g + hours) pent(g + t) N(g $\times$ t)}	2414.2151	18
4	{Phi(g + t) p(g $\times$ hours) pent(g $\times$ t) N(g $\times$ t)}	2416.0908	29
5	{Phi(g $\times$ t) p(g $\times$ hours) pent(g $\times$ t) N(g $\times$ t)}	2417.6189	30

We observed distinct protandry. Thus, the first males were observed one week before the first females; they showed a strong increase from 7 August 2019 onwards (Figure 1). The first females were marked 5 August 2019; after two weeks of parallel development to males, they exceeded the daily numbers of males on 23 August 2019. The highest estimates were obtained for males and females on 27 and 28 August 2019, respectively.
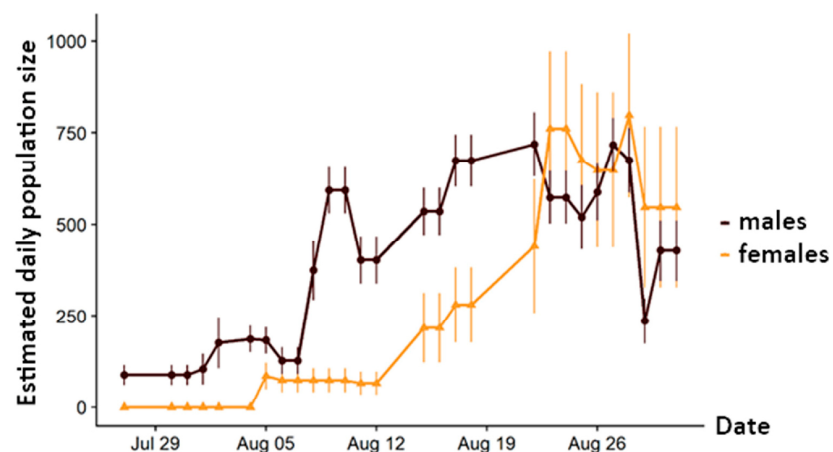
Figure 1. Estimated daily population size of both sexes of *Erebia pronoe* based on the best Popan model in MARK: Phi(g + t) p(g + hours) pent(g $\times$ t) N(g $\times$ t).

We calculated changes in the average wing condition to assess the age structure of the population (Figure 2). Males, in general, had very good wing conditions for the first 16 days of the study and did not exhibit any deterioration until 11 August 2019 ($y = -0.0238x + 1.5415$; $R^2 = 0.205$). Only from 12 August 2019 onwards, average wing

condition deteriorated continuously (y = 0.0697x + 0.1238; R^2 = 0.91) and significantly ($p < 0.001$). Due to later emergence and generally lower capture events, reliable data for females are only available for the second half of August. In this time slot, deterioration of females' wing conditions (y = 0.0617x + 0.0862; R^2 = 0.83) showed no significant difference from males ($p = 0.69$). Both sexes showed a highly significant correlation between the changes in wing condition and the time elapsed since first capture (Spearman rank correlation analysis: male rho = 0.93; female rho = 0.63; $p < 0.001$).

Figure 2. Changes in the average wing condition during the 2019 flight season of *Erebia pronoe* are categorised by sex. Males are indicated by brown circles, and females are indicated by orange triangles. Linear trends for both sexes are given and are based on the 3rd polynomial function. The 95% confidence interval of the polynomial function is given in grey.

3.2. Mobility and Movement Patterns

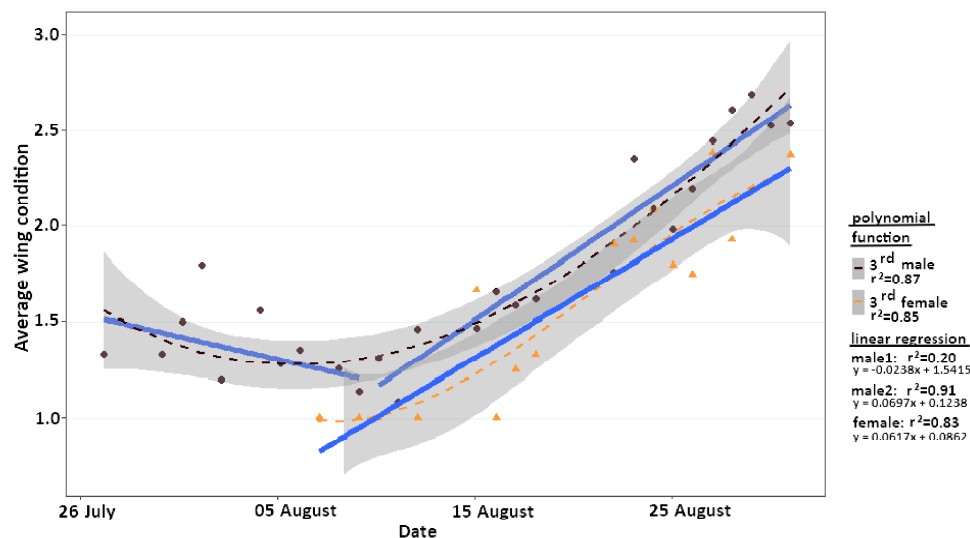*Erebia pronoe* is a species with low mobility. Males (average dispersal distance: 106 m ± 86 SE; $n = 245$) tended to move somewhat further than females (71 m ± 58 SE; $n = 16$) but not significantly so ($p = 0.15$). Both sexes moved distances below 150 m in the majority of cases, but the percentage of males exceeding this distance (24.1%) was considerably higher than for females (6.2%) (Figure 3). The longest and shortest distances detected were 423 m and 3 m for males and 219 m and 8 m for females.

We applied the NEF and IPF functions for extrapolating the potential for dispersal over longer distances. The highest stability indices (R^2) for both functions to the inverse cumulative proportion values (based on distance classes) were obtained for 30 m intervals (Table 2). For both sexes, the fit of NEF was better than the fit of IPF. Following the NEF, dispersal of 1 km or more is unlikely (Table 3). Even if the NEF is prone to underestimate rare long-distance movements, the IPF results also give a low probability for these long-distance movements (i.e., males: 3 in 1000; females: 8 in 1000), which is consistent with our observations (Figure 3).

Table 2. Stability index (R^2) of the inverse power function (IPF) and the negative exponential function (NEF) based on calculations with 20, 30 and 50 m intervals of the covered distances of *Erebia pronoe*.

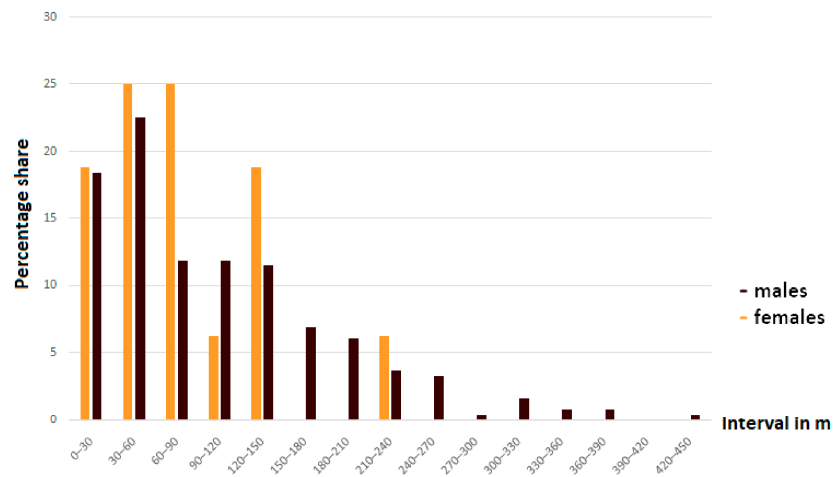|	20 m Intervals		30 m Intervals		50 m Intervals	
	IPF	NEF	IPF	NEF	IPF	NEF
Males	0.76	0.97	0.79	0.98	0.81	0.96
Females	0.79	0.94	0.83	0.93	0.80	0.93

Figure 3. Percentage distribution of the sexes of *Erebia pronoe* over 30 m distance intervals corresponding to the distances travelled when first recaptured and divided by sex.

Table 3. Percentage of *Erebia pronoe* individuals that were expected to disperse more than 1, 2, 3 or 5 km, calculated with inverse power function (IPF) and negative exponential function (NEF) based on 30 m intervals.

Distance	IPF Males	IPF Females	NEF Males	NEF Females
1 km	0.29	0.82	0.0003	3.50×10^{-5}
2 km	0.07	0.29	4.27×10^{-10}	6.50×10^{-12}
3 km	0.03	0.15	5.86×10^{-16}	1.21×10^{-18}
5 km	0.01	0.07	1.10×10^{-27}	4.15×10^{-32}

Spearman's rank correlation revealed a positive correlation of elapsed time between captures and migration distance. This effect was more pronounced in females (rho= 0.6679092, $p = 0.003252$, S = 185.97) than in males (rho = 0.1471955, $p = 0.01128$, S = 1964827).

3.3. Behavioural Differences between Sexes

Both sexes were observed with almost equal frequency in nectar uptake (Table 4). Flight activity was most frequently observed in males, whereas females were primarily encountered resting. Overall, both sexes differed highly significantly in their behaviour ($\chi^2 = 131.1$, df = 3, $p < 0.001$). The behaviour of males became more passive ($p < 0.001$) with increasing cloud cover and wind force, although this effect was only detectable for cloudiness of 70% or more and a wind force of level 3 (ES3) or more.

Table 4. Percentage of individuals of *Erebia pronoe* in four different behavioural categories, divided by sex.

	Flying	Resting	Feeding	Mating
Males	57.7	33.2	8.9	0.2
Females	11.9	75.6	11.3	1.2

3.4. Use of Nectar Plants

Members of the family Asteraceae were used as nectar sources in 77.3% of the cases. Most frequently visited were *Carlina acaulis* (37.0%), *Carduus defloratus* (18.4%) and *Leontodon hispidus* (13.2%). In addition to Asteraeceae, the family Dipsacaceae with *Scabiosa lucida*

(12.3%) and the family Gentianaceae with *Gentianella campestris* (9.7%) were important. Sex-specific preferences were not detected (χ^2 = 8.798, df = 8, p = 0.36).

4. Discussion

4.1. Population Size and Structure

Although we estimated a relatively high overall population density of 820 adult individuals per hectare, the proportion of female adults was comparatively low. This might be either caused by an actual low female population size or by biases associated with the methods. A biased sex ratio in the offspring, in emigration rates, and/or in survival rates of larvae/pupae can cause such distorted sex ratios in a population. None of these possible reasons mentioned above have so far been reported for such imbalanced sex ratios in the offspring of *Erebia*, and we have not observed such migration pattern either. The studied population displayed high levels of infection with the endoparasite *Wolbachia* (Wendt et al., unpublished), which is known to alter the sex ratio of populations in favour of the female offspring [37,38]. Higher mortality risk of the female pupae caused by the prolonged preimaginal phase is rather unlikely, considering the serial eclosion of both sexes. Therefore, we believe a bias due to methods as an explanation for the imbalanced population structure to be more likely. Studies that considered behavioural patterns found a correlation between population size and flight activity [22]. We assume that the rather low flight activity of female *E. pronoe* led to underrepresentation in marking and recapture events, which resulted in lower population estimates. Even in populations with a female bias, a higher capture probability of males can lead to a male over-representation in recapture studies [39]. It must also be considered that female behaviour and mobility are determined by numerous other factors and may not be constant. For example, factors such as larval plant availability can also lead to stronger, temporary changes in female mobility within a flight period [40]. Constant sex-specific influence may arise from landscape structures and topography. In studies on *Maculinea nausithous* and *Maculinea teleius*, strong barrier functions of topographic structures on female mobility have been demonstrated [41]. An inhibitory effect of the elevation ranges of our study area cannot be excluded and may have contributed to the low mobility of females and their underrepresentation.

4.2. Adaptation to Extreme Conditions through Protandry and Serial Eclosion

The plateau phases in the population curve and the wing condition curve indicate the constant influx of freshly eclosed specimens into the population caused by serial eclosion. This serial eclosion is more visible in the case of males and also starts earlier compared to females. The earlier onset of male eclosion is considered beneficial for male reproductive success since it allows an immediate mating of freshly eclosed females, although, in high alpine areas, protandry can be a risk, especially for isolated populations. Extreme weather events can eradicate a major part of the population, hence causing a local collapse [42]. As a result, an earlier onset of the male flight period in combination with a prolonged phase of male eclosion provides the advantages of proterandry but also mitigates the risks of local extinction caused by weather caprices. Moreover, serial eclosion reduces the intraspecific pressure of eventual mass eclosion events and the hereon emerging need for emigration. Very similar population structures have also been witnessed in other high alpine butterflies (e.g., [22]) and are assumed to be an adaptation to high alpine environments.

4.3. Resource Use

The analysis of the movement patterns of *Erebia pronoe* showed a rather sedentary behaviour and the formation of home ranges (Figure 4). These home ranges suggest a high habitat quality since the availability of resources determines the behaviour of butterflies [36,43], and apparently, the needs are fulfilled in small sub-areas of our study area. This is facilitated by the wide range of nectar plants used, which is consistent with Rapport's extended rule [44]. From early/mid-July to mid/late August, the congeneric species *Erebia nivalis* is also common in our study area [22]. The larvae of *E. nivalis* have

a biennial development but are also preferentially deposited on withered plant parts of *Festuca* species like *F. quadriflora*. Interestingly, despite the wide range of nectar plants used by *E. pronoe* and *E. nivalis*, no overlap was found. Both species use plant families that have very different flower morphology and colouration [22]. Therefore, it can be assumed that this is not a specific adaptation but a local, opportunistic use of resources. The wide range of larval food plants and nectar plants might mitigate the risk of phenological mismatch between hostplants and butterflies, a risk that has been observed several times in the context of climate change (e.g., [45]). We assume that the observed ranges of nectar plants used by *E. pronoe* rather reflect competitive mitigation than the actual foraging range of the species. The abundance of the genus *Festuca* in alpine habitats makes the competition for limited larval food plants unlikely.
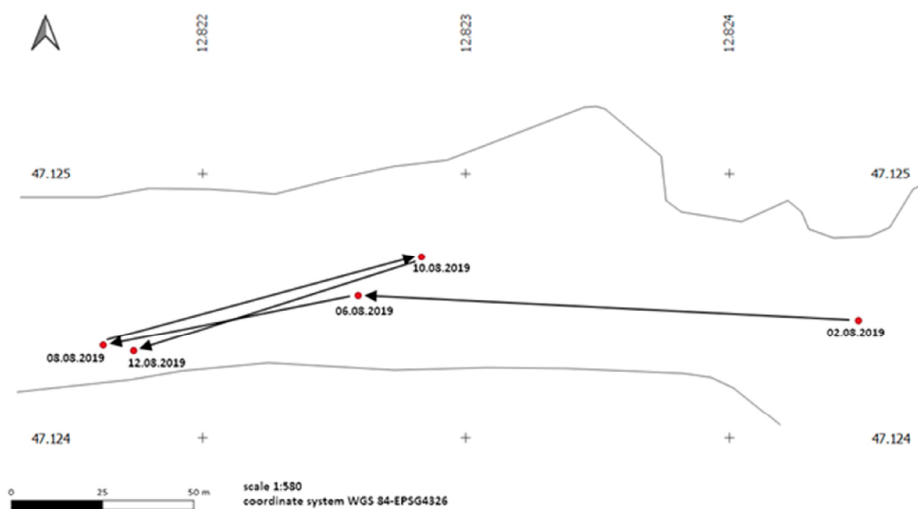

Figure 4. Example of movement patterns of a male of *Erebia pronoe* recaptured five times. The "+" symbols represent the grid of the coordinate system.

4.4. Movement Patterns

We observed low mobility and movements of short distances but with significant differences between sexes. While males were significantly more active than females, they still moved mostly short distances and travelled larger distances only to establish home ranges. As a result, the average distances travelled between capture and first recapture did not increase as a function of time. Females, on the other hand, showed significantly lower mobility than males, but the distance travelled was positively correlated with time. Both of these behavioural traits can be explained by the population structure and resource use discussed above. The wide range of nectar plants and the commonness of the larval food plants do not force females to translocate larger distances due to the abundance of all resources needed. In addition, migration always imposes a risk and reduces the time for mating and oviposition [46]. Thus, reproductive success should initially be the highest in the patch of origin and exploitation of further areas beyond only increases this success when the female has already oviposited most of her eggs. Male harassment and the chance to lay fertilised eggs in other areas might explain the later increase in observed translocation as a function of time [47]. Due to the earlier eclosion of the males, they can establish a home range and patrol it in search of freshly eclosed females. This patrolling is reflected in the increased mobility and the low but constant migration performance of the males. The establishment of home ranges can reduce the effective population size [48] and further isolate a population in a fragmented landscape. Although detrimental on a population level, the avoidance of large-scale movements might reduce the risks posed by strong

wind and bad weather in general and could be therefore beneficial on an individual level. The true migratory potential of the species may be significantly higher, as indicated by the observed maximum distances flown by the males. However, to answer this question, research in larger, potentially less resource-rich areas is required.

5. Conclusions

Erebia pronoe is a species that is well adapted to montane and alpine habitats because of the combination of moderate protandry and serial eclosion, the opportunistic use of resources as adults and its low mobility. The wide ecological niche reduces the risks of negative fitness consequences that could result from butterfly-host asynchrony. The serial eclosion of males reduces the risk of desynchronised adult emergence and the resulting risk for reproduction and stability of the population. At the same time, its relatively high abundance and stable populations make it a very good indicator species for the quality and the intactness of alpine rupicolous grasslands. It is likely that the female portion of the population is higher than predicted by our models, as previously discussed. Nonetheless, a higher population density of females would further support our statement regarding habitat quality. However, the population structure with serial eclosion and protandry is unaffected by the number of females. There might be an influence on the sedentary behaviour and migration potential during periods of resource scarcity, as may occur due to mass emergence and/or low habitat quality. However, the wide range of larval food plants and nectar plants should counteract intraspecific competition. We, therefore, assume that even a higher abundance of females would not affect the basic conclusions of our work.

Author Contributions: All authors conceived and designed the study. N.S. performed the data acquisition. M.W. and N.S. performed the analyses. All authors aided in the interpretation of the data. M.W. and T.S. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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References

1. IPBES. *The Assessment Report of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services on Pollinators, Pollination and Food Production*; Potts, S.G., Imperatriz-Fonseca, V.L., Ngo, H.T., Eds.; IPBES: Bonn, Germany, 2016; ISBN 978-92-807-3567-3.
2. Thomas, J.A. Butterfly communities under threat. *Science* **2016**, *353*, 216–218. [CrossRef]
3. Nagy, L.; Grabherr, G.; Körner, C.; Thompson, D. *Alpine Biodiversity in Europe*; Springer: Berlin, Germany, 2003.
4. Schmitt, T. Biogeographical and evolutionary importance of the European high mountain systems. *Front. Zool.* **2009**, *6*, 9. [CrossRef]
5. Sánchez-Bayo, F.; Wyckhuys, K.A.G. Worldwide decline of the entomofauna: A review of its drivers. *Biol. Conserv.* **2019**, *232*, 8–27. [CrossRef]
6. Dover, J.; Settele, J. The influences of landscape structure on butterfly distribution and movement: A review. *J. Insect Conserv.* **2009**, *13*, 3–27. [CrossRef]
7. Boggs, C.L.; Murphy, D.D. Community composition in mountain ecosystems: Climatic determinants of montane butterfly distributions. *Glob. Ecol. Biogeogr. Lett.* **1997**, *6*, 39–48. [CrossRef]
8. Dengler, J. Which function describes the species-area relationship best? A review and empirical evaluation. *J. Biogeogr.* **2009**, *36*, 728–744. [CrossRef]

9. Thomas, C.D.; Franco, A.M.A.; Hill, J.K. Range retractions and extinction in the face of climate warming. *Trends Ecol. Evol.* **2006**, *21*, 415–416. [CrossRef]

10. Chen, I.C.; Hill, J.K.; Ohlemüller, R.; Roy, D.B.; Thomas, C.D. Rapid range shifts of species associated with high levels of climate warming. *Science* **2011**, *333*, 1024–1026. [CrossRef] [PubMed]

11. Parmesan, C. Ecological and Evolutionary Responses to Recent Climate Change. *Annu. Rev. Ecol. Evol. Syst.* **2006**, *37*, 637–669. [CrossRef]

12. Parmesan, C. Influences of species, latitudes and methodologies on estimates of phenological response to global warming. *Glob. Chang. Biol.* **2007**, *13*, 1860–1872. [CrossRef]

13. Roland, J.; Keyghobadi, N.; Fownes, S. Alpine *Parnassius* Butterfly Dispersal: Effects of Landscape and Population Size. *Ecology* **2000**, *81*, 1642–1653. [CrossRef]

14. Kadlec, T.; Vrba, P.; Kepka, P.; Schmitt, T.; Konvicka, M. Tracking the decline of the once-common butterfly: Delayed oviposition, demography and population genetics in the hermit *Chazara briseis*. *Anim. Conserv.* **2010**, *13*, 172–183. [CrossRef]

15. Boggs, C.L.; Ross, C.L. The effect of adult food limitation on life history traits in *Speyeria mormonia* (Lepidoptera: *Nymphalidae*). *Ecology* **1993**, *74*, 433–441. [CrossRef]

16. Baguette, M.; Néve, G. Adult movements between populations in the specialist butterfly *Proclossiana eunomia* (Lepidoptera, *Nymphalidae*). *Ecol. Entomol.* **1994**, *19*, 1–5. [CrossRef]

17. Seibold, S.; Gossner, M.M.; Simons, N.K.; Blüthgen, N.; Müller, J.; Ambarlı, D.; Ammer, C.; Bauhus, J.; Fischer, M.; Habel, J.C.; et al. Arthropod decline in grasslands and forests is associated with landscape-level drivers. *Nature* **2019**, *574*, 671–674. [CrossRef]

18. Viljur, M.L.; Teder, T. Disperse or die: Colonisation of transient open habitats in production forests is only weakly dispersal-limited in butterflies. *Biol. Conserv.* **2018**, *218*, 32–40. [CrossRef]

19. Maes, D.; Van Dyck, H. Habitat quality and biodiversity indicator performances of a threatened butterfly versus a multispecies group for wet heathlands in Belgium. *Biol. Conserv.* **2005**, *123*, 177–187. [CrossRef]

20. Tshikolovets, V.V. *Butterflies of Europe and the Mediterranean Area*, Tshikolovets Publications, 2011.

21. van Swaay, C.A.M.; Warren, M.S. Prime Butterfly Areas of Europe: An initial selection of priority sites for conservation. *J. Insect Conserv.* **2006**, *10*, 5–11. [CrossRef]

22. Ehl, S.; Dalstein, V.; Tull, F.; Gros, P.; Schmitt, T. Specialized or opportunistic—How does the high mountain endemic butterfly *Erebia nivalis* survive in its extreme habitats? *Insect. Sci.* **2018**, *25*, 161–171. [CrossRef] [PubMed]

23. Junker, M.; Wagner, S.; Gros, P.; Schmitt, T. Changing demography and dispersal behaviour: Ecological adaptations in an alpine butterfly. *Oecologia* **2010**, *164*, 971–980. [CrossRef]

24. Huemer, P. Biodiversität von Schmetterlingen (Lepidoptera) im Gebiet des Naturparks Schlern. *Gredleriana* **2007**, *7*, 233–306.

25. Tolman, T.; Lewington, R. *Schmetterlinge Europas Und Nordwestafrikas*, 2nd ed.; Kosmos: Stuttgart, Germany, 2012.

26. Sonderegger, P. *Die Erebien der Schweiz (Lepidoptera: Satyrinae, Genus Erebia)*; Biel: Shenzhen, China, 2005.

27. Hickel, C.; Gereben-Krenn, B.-A.; Zweimüller, I.; Krenn, H.W. Wetterbedingungen für die Erfassung von Tagfaltern (Lepidoptera: Papilionoidea) in alpinen Lebensräumen in Österreich C. *Entomol. Austriaca* **2016**, *23*, 7–18.

28. Zimmermann, K.; Fric, Z.; Filipová, L.; Konvička, M. Adult demography, dispersal and behaviour of *Brenthis ino* (Lepidoptera: *Nymphalidae*): How to be a successful wetland butterfly. *Eur. J. Entomol.* **2005**, *102*, 699–706. [CrossRef]

29. Gall, L.F. The effects of capturing and marking on subsequent activity in *Boloria acrocnema* (Lepidoptera: *Nymphalidae*), with a comparison of different numerical models that estimate population size. *Biol. Conserv.* **1984**, *28*, 139–154. [CrossRef]

30. Cooch, E.G.; White, G.C. *Using Program MARK: A Gentle Introduction*. Available online: http://www.phidot.org/software/mark/docs/book/ (accessed on 10 November 2019).

31. White, G.C.; Burnham, K.P. Program mark: Survival estimation from populations of marked animals. *Bird Study* **1999**, *46*, S120–S139. [CrossRef]

32. Sugiura, N. Further analysts of the data by akaike' s information criterion and the finite corrections. *Commun. Stat.-Theory Methods* **2007**, *7*, 13–26. [CrossRef]

33. Burnham, K.; Anderson, D. *Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach*; Springer: New York, NY, USA, 2002.

34. *QGIS Development Team Quantum Geographic Information System (QGIS)*, version 3.83. Available online: https://www.qgis.org/en/site/forusers/download.html (accessed on 28 September 2019).

35. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2016.

36. Kuras, T.; Benes, J.; Fric, Z.; Konvicka, M. Dispersal patterns of endemic alpine butterflies with contrasting population structures: *Erebia epiphron* and *E. sudetica*. *Popul. Ecol.* **2003**, *45*, 115–123. [CrossRef]

37. Serbus, L.R.; Casper-Lindley, C.; Landmann, F.; Sullivan, W. The genetics and cell biology of *Wolbachia*-host interactions. *Annu. Rev. Genet.* **2008**, *42*, 683–707. [CrossRef] [PubMed]

38. Engelstädter, J.; Hurst, G.D.D. The ecology and evolution of microbes that manipulate host reproduction. *Annu. Rev. Ecol. Evol. Syst.* **2009**, *40*, 127–149. [CrossRef]

39. Nowicki, P.; Witek, M.; Skórka, P.; Settele, J.; Woyciechowski, M. Population ecology of the endangered butterflies *Maculinea teleius* and *M. nausithous* and the implications for conservation. *Popul. Ecol.* **2005**, *47*, 193–202. [CrossRef]

40. Timus, N.; Czekes, Z.; Rákosy, L.; Nowicki, P. Conservation implications of source-sink dynamics within populations of endangered *Maculinea* butterflies. *J. Insect Conserv.* **2017**, *21*, 369–378. [CrossRef]

41. Plazio, E.; Bubová, T.; Vrabec, V.; Nowicki, P. Sex-biased topography effects on butterfly dispersal. *Mov. Ecol.* **2020**, *8*, 50. [CrossRef] [PubMed]
42. Bowers, D.M.; Schmitt, J. Overcrowding leads to lethal oviposition mistakes in the Baltimore checkerspot, *Euphydryas phaeton drury* (*Nymphalidae*). *J. Lepid. Soc.* **2013**, *67*, 227–229. [CrossRef]
43. Evans, L.C.; Sibly, R.M.; Thorbek, P.; Sims, I.; Oliver, T.H.; Walters, R.J. The importance of including habitat—Specific behaviour in models of butterfly movement. *Oecologia* **2020**, *193*, 249–259. [CrossRef]
44. Stevens, G.C. The elevational gradient in altitudinal range: An extension of Rapoport's latitudinal rule to altitude. *Am. Nat.* **1992**, *140*, 893–911. [CrossRef]
45. Schweiger, O.; Settele, J.; Kudrna, O.; Klotz, S.; Kühn, I. Climate change can cause spatial mismatch of trophically interacting species. *Ecology* **2008**, *89*, 3472–3479. [CrossRef]
46. Kőrösi, Á.; Örvössy, N.; Batáry, P.; Kövér, S.; Peregovits, L. Restricted within-habitat movement and time-constrained egg laying of female *Maculinea rebeli* butterflies. *Oecologia* **2008**, *156*, 455–464. [CrossRef]
47. Mennechez, G.; Schtickzelle, N.; Baguette, M. Metapopulation dynamics of the bog fritillary butterfly: Comparison of demographic parameters and dispersal between a continuous and a highly fragmented landscape. *Landsc. Ecol.* **2003**, *18*, 279–291. [CrossRef]
48. Creel, S. Social organization and effective population size in carnivores. In *Behavioral Ecology and Conservation Biology*; Oxford University Press: Oxford, UK, 1998; pp. 246–265.

Resource over-exploitation and competition lead to endangerment of the extremely philopatric ringlet butterfly *Erebia pronoe glottis*

Abstract

A mark-recapture study of the subspecies *Erebia pronoe glottis* in the Pyrenees was conducted to survey its ecological demands and characteristics. An area intensively used by beekeepers and shepherds was studied. Population structure analysis revealed a small population of low density. Significant differences between both sexes were found in population density (males: 48/ha; females: 23/ha), sex-ratio (2.1) and behaviour (75.4 vs. 20.5% flying). Both sexes used a wide range of nectar plants (Asteraceae, 40.6%; Apiaceae 34.4%; Caprifoliaceae 18.8%). Despite the wide range of nectar plants, the available resources did not seem to allow for greater abundance. Compared to an extensively used pasture, a significant increase in flight behavior, but not in range, was observed. Movement patterns show the establishment of home ranges, which significantly limits the migration potential, which was low for both sexes (male: 101 m ± 73 SE; female 68m ±80 SE). A sedentary species such as *Erebia pronoe* does not seem to be able to avoid the pressure of resource shortage by migration. As a late-flying pollinator, *Erebia pronoe* competes seasonally for scarcer resources. These are further reduced by grazing pressure and exploited by the superior competitor *Apis mellifera*, resulting in low habitat quality and abundance.

Keywords: mark-release-recapture; resource depletion; movement patterns; population demography

Introduction

Worldwide, insect fauna is under increasing pressure, which is reflected in massive losses in diversity and abundance of numerous species (Hallmann et al. 2017; Seibold et al. 2019; Wagner 2020). One of the main causes is the loss of high-quality habitats (Sánchez-Bayo and Wyckhuys 2019). The availability of resources is one of the most significant quality parameters of habitats, along with microclimate and vegetation structures (Dover und Settele 2009, Krus & Tschartnke 2002). For a species to persist in a habitat, all significant biotic and abiotic conditions in an area must be met. In this context, a landscape can also be understood as a network of different sub-areas, with their use depending on the mobility of the respective species. If a habitat possesses only parts of the required aspects, it can also serve as a partial habitat if the mobility of a species is sufficient. Thus, in addition to size, the linkage among areas also determines the availability of resources, and subsequently survival (Melbourne and Hastings 2008).

Changes in agricultural land use have caused, among other aspects, the isolation of habitats, the destruction of their networks, and the general decrease in habitat size, which in combination reduces the overall availability of resources in an area (Storch et al. 2005; Beck et al. 2017). Furthermore, landscape restructuring exerts strong pressure on species behaviour within an area. Selection between sedentariness or increased dispersal takes place to compensate for the increased amount of matrix in a landscape. However, the (observed) dispersal potential of a species may vary depending on the size of the study area or its topography (Schneider 2003; Plazio et al. 2020a). Similarly, the size of the study area (Brown and Crone 2016) or its richness in resources (here nectar availability) (Timus

et al. 2017; Evans et al. 2020) affects the behaviour of a species in general and its movement patterns in particular. Therefore, the ecological characteristics of a species must be considered in the context of the survey conditions.

Likewise, high mobility is of great importance for numerous species to keep up with climate change and the associated shifts of range boundaries. However, even mobile taxa such as birds or butterflies cannot fully compensate for these effects, resulting in extinction debts. For example, an alpine species may follow the shift of the snowline and yet be subject to an increased risk of extinction. The earlier onset of snowmelt can result in asynchronous development of flora and fauna that depend on it, disrupting biotic interactions and removing resources, thereby increasing extinction risk (Parmesan 2006). This pressure can be met, especially by generalists, partly through a shift in their requirements (i.e. resource evasion) (Parmesan 2006).

Among insects threatened by climate change, alpine butterflies, in particular, are under great pressure due to their dependence on snow cover for the survival of their larvae (Parmesan 2006; Stuhldreher et al. 2014; Konvička et al. 2016). In addition, the majority of alpine butterflies are not able to avoid stressors through migration in the often heterogeneous high mountain landscapes because of their mostly sedentary behaviour (Junker et al. 2010; Ehl et al. 2018). This further increases the risk of biodiversity loss within the alpine insect fauna. This sensitive response of butterflies to environmental conditions and their changes as well as their high reproductive rates make them excellent indicators of habitat quality and resource availability (Maes and van Dyck 2005; Dover and Settele 2009; Viljur and Teder 2018). Therefore, the current status of local alpine butterfly populations needs to be surveyed to gain an understanding of the local ecological niche and ecological potential, and to assess the influence of various stressors. Based on these surveys, potential conservation measures can then be developed.

We have chosen the alpine butterfly species *Erebia pronoe* as a characteristic representative of alpine rupiculous grasslands (Huemer 2007). *E. pronoe* is widespread in the high mountain systems of Europe and tends to form relatively large, stable populations (Tshikolovets 2011). In a recent study performed in highly suitable habitats in high altitudes of the Eastern Alps, *E. pronoe* was found to be a low-mobility species with opportunistic use of resources to establish home ranges (Wendt et al. 2021). However, phylogeographic analyses revealed a strong differentiation within this species among and even within high mountain systems (Wendt et al. 2022). Therefore, it is unknown whether different phylogeographic lineages are ecologically similar. Furthermore, it is unknown whether populations in less suitable habitats and at lower altitudes behave similarly to the high-altitude populations analysed by Wendt et al. (Wendt et al. 2021) in highly suitable habitat of the Eastern Alps. In the here presented population ecology study, we therefore investigated the population structure of a population in the French Pyrenees by applying mark-release-recapture (MRR). This population belongs to a phylogeographic lineage strongly differentiated from the Eastern Alps (Wendt et al. 2022). Furthermore, the Pyrenean study area had three times the size of the study area assessed by Wendt et al. (Wendt et al. 2021) in the Eastern Alps; other than in the former with only moderate perturbation by pasturing, our site in the Pyrenees was strongly impacted by a larger number of apiaries and intensive high-altitude pasture by cows. Furthermore, this site was at the lower edge of the altitudinal distribution of the species and not at the upper as in the Eastern Alpine one. Studying the behaviour and mobility of both sexes and the influence of weather on these, we address the following questions:

What is the population structure and behaviour of an *E. pronoe* population at the lower altitudinal edge in the French Pyrenees?

Can behavioural differences (e.g. movement patterns, dispersal ranges, resource use) be detected compared to the Eastern Alps?

What is the influence of pastoral use and transhumance of honey bees on population structure and size?

Material & Methods

Study species

Erebia pronoe (Esper, 1780) is a representative of the western Palaearctic Nymphalidae. The species thrives on wet meadows, calcareous grasslands, and screes from the upper montane to the alpine zone (Huemer 2007). *E. pronoe* is widespread and often common throughout the Alps. It is locally found in the Swiss and French Jura as well as in the Pyrenees where high densities can be reached. In the Carpathians including the High Tatras and the mountains of the Balkan Peninsula, populations of *E. pronoe* are relatively rare and small (Tolman and Lewington 2012). The species flies in one generation from the end of July to mid-September and hibernates as L1 larva. The larvae prefer *Festuca* species such as *F. ovina*, *F. rubra,* and *F. quadriflora*, and to a lesser degree *Anthoxanthum odoratum*; breeding was successful on *Poa annua* (Sonderegger 2005).

Study area

The study area is located in the French Pyrenees in the core of the distribution area of the subspecies *Erebia pronoe glottis* near the "Parc national des Pyrénées" and close to the Station de Ski Cauterets-Le Lys (42°52' N; 0°08' W), in the arrondissement Argelès-Gazost, Department Hautes-Pyrénées. The investigated steep slope covers two study areas of 5.45 ha and 5.77 ha, respectively, and extends 1400–1650 m asl. The study areas are interspersed with gravel areas and represent a characteristic habitat of *E. pronoe*. Other suitable habitats exist within a distance of 200 m. To the east, our study areas border the protected area "Parc national des Pyrénées", where no capturing of butterflies was performed. The area in the west was too steep for performing MRR, the adjoining areas to the north and south were too heavily grazed for the occurrence of *E. pronoe*. The surrounding area was grazed by ca. 50 cows. Occasionally, a herd of horses (ca. 20) and a flock of sheep (>300) grazed in the adjacent areas. Five apiaries with 36–59 colonies each were located at a distance of 50–1,500 m from our MRR sites (Fig. 1).
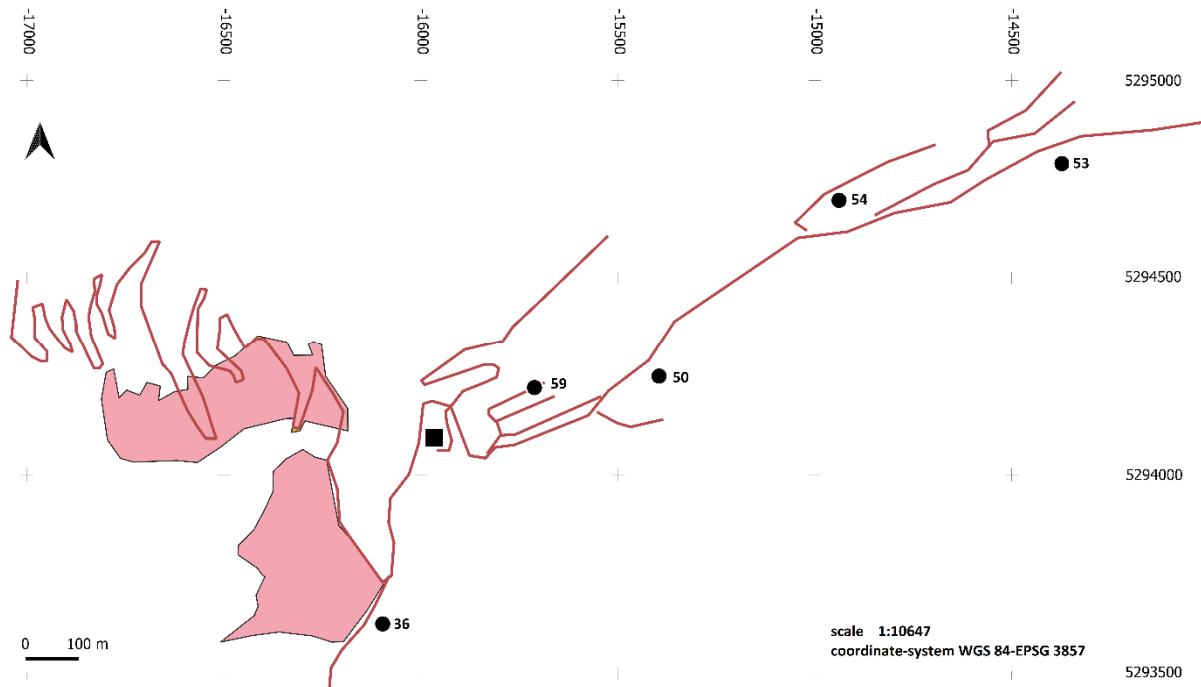
Figure 1 Sampling area in pink. Black circles mark the location of apiaries with the number of colonies given. The black square is the location of the cow shelter.

Mark-release-recapture (MRR)

We conducted a mark-release-recapture study as basis for our analyses of population structure and species-specific mobility patterns. Furthermore, behaviour and specific resource use were surveyed to identify specific adaptations to alpine habitats. The study was carried out from 20 July to 30 August 2019, covering most of the flight season of *E. pronoe*. The butterflies were caught with a butterfly net (diameter: 40 cm) from 9 am to 6 pm under suitable weather conditions (see (Hickel et al. 2016)); to avoid day-time effects, the sampling in the area was randomised and always started at different parts of the study areas. Each individual was marked on the underside of the hind wings with a fine, waterproof pen (StabiloOHPen universal S) and provided with an individual code consisting of letters for the day and a consecutive number. In addition, we recorded: GPS coordinates using the App Tourcount and a Samsung S4 mini, sex, wing condition (scale 1 = wing seam completely preserved; up to 4 = wing heavily damaged (Zimmermann et al. 2005)), behaviour when being captured and current weather conditions. The same data were collected for each recapture, excluding recaptures during the same day, to avoid behavioural effects of the capture and theoretically allow complete mixing of the population (Gall 1984). We assessed the age structure of the population based on wing conditions.

The aging per time unit was calculated sex-specifically by correlating the time intervals between capture and first recapture and the deterioration rate of wing condition. Only days with at least five individuals per sex were considered and their mean values were used for a Spearman rank correlation analyses. Potential sex-specific differences in behaviour and preferences of nectar plants were assessed by X^2 homogeneity tests. The influence of wind and cloud cover on the behaviour was analysed with X^2-tests.

Population demography

Based on the mark-release-recapture dataset, we conducted population modelling to assess population size as a potential indicator of habitat quality and to examine population structure for potential adaptations. The first five days of capture were not used for modeling as the low number of

captures interfered with the modeling process. The program Mark 8.2 and its module Popan (Cooch and White 2019), based on the Jolly-Seber method, were used to calculate the daily, sex-specific population size. Three parameters were calculated: φ (phi), the probability of survival; p, the probability of capture; and pent, the proportional recruitment. These parameters can be constant (.), sex-specific (g), time-factorial (t), linear (T) or quadratic (T^2) and can have additive (g + t; g + T, ...) or interactive relationships (g x t; g x T; ...) (White and Burnham 1999). Furthermore, the sampling effort (i.e. the time spent in the field) was considered as covariant for the probability of capture. A saturated model consisting of these parameters was verified by a goodness-of-fit test with the program RELEASE. Based on this, various parameter combinations were calculated and the best-supported model was determined using the corrected Akaike Information Criterion (AIC_C) (Sugiura 2007) and the lowest number of parameters (Burnham and Anderson 2002).

Mobility parameters

We analysed movement patterns in the habitat to infer the migration potential as well as resource use and availability. The collected GPS data of captures and recaptures were used to reconstruct the movement patterns. These were imported into QGIS 3.8.3 (QGIS Development Team 2018) and the direct geographic distance between capture and first recapture was determined for each individual by creating a linear distance matrix layer with the vector analyses tool in the WGS 84 (EPSG4326) Coordinate Reference System. For assessing the minimum total distance moved, we summed up the distances between all points of capture.

We performed a Shapiro-Wilk test to check for normal distribution by using the program R version 3.6.1 (R core Team 2017). Since there was no normal distribution of the data (males p: 1.376×10^{-10}, females p: 0.023), we used the Mann-Whitney U test to determine differences in distances travelled by males and females and the two-sided Spearman´s rank correlation to analyse the influence of days since capturing on the distances moved. In addition, an ANOVA was carried out using R to investigate whether the distances travelled differed between sexes over time. The travelled distances were divided into distance classes (20 m, 30 m, and 50 m intervals) separately for each sex. The inverse cumulative percentage of these classes was determined, which corresponds to the probability density function, i.e. the dispersal kernel. To check for any potential artefacts caused by the chosen interval sizes, we analysed and compared three different interval size classes.

Based on these classes, the probabilities of dispersal flights were investigated through distance extrapolation. Two frequently applied regression analyses were used, the negative exponential function (NEF) and the inverse power function (IPF). The NEF tends to underestimate rare long-distance movements, whereas the IPF may encounter problems with "zero" movements (Kuras et al. 2003). The data were linearly transformed with a semi-ln plot for the NEF analyses or with a double-ln plot for the IPF analyses. In both equations, "P" stands for the proportional probability that an individual will travel at least as far as the distance D, and "a" for the intercept of the regression. NEF operates with the dispersal constant K as slope, whereas IPF uses the variable n as slope, which represents the effect of distance on dispersal (Ehl et al. 2018).

$$P_{NEF}=ae^{-kD} \text{ or } \ln P = \ln a - kD$$

$$P_{IPF}=aD^{n} \text{ or } \ln P = a - n (\ln D)$$

We selected the best model and the most suitable interval size, based on calculated stability indices R^2 of the calculated curves, which corresponds to the proportion of explained variance of the dependent variable by the independent variable. This allowed extrapolations of the population´s proportion that should travel distances exceeding the extent of the study area. The calculations were performed separately for males and females.

Results

During 20 field days (20 July–30 August 2019), we marked 323 *E. pronoe* individuals (261 males; 62 females); 120 of these were recaptured (106 males; 14 females). This translates into a recapture rate of 37.2 % (males: 40.6 %; females: 22.6 %) and a sex ratio of more than four males per female. We achieved up to six recaptures for males; two females were recaptured twice.

Demography

The best-supported model with the lowest AIC$_C$ value and the lowest number of parameters yielded an additive effect of sex and linear time on the survival probability phi, an interactive effect of sex and factorial time on the capture probability p, a linear effect of time on the proportional recruitment pent and an interactive effect of sex and factorial time on the number of individuals (Tab. 1). This model estimated a population size of 535 males (± 61 SE), i.e. 48/ha, and 260 females (± 90 SE), i.e. 23/ha. The estimated sex ratio was 2.1 males per female. The modeled population structure showed no signs of protandry (see Supplementary S1).

Table 1 Comparison of the best models of Popan 5.0 analyses for the estimates of the daily population size of Erebia pronoe: Akaike Information Criterion (AIC$_C$) and number of parameters used, basic vari-ables: Probability of survival (Phi), probability of recapture (p), proportional recruitment (pent), the total number of individuals (N), dependent variables: sex (g); factorial (t), linear (T) and quadratic (T2) dependence of time; time invested in sampling per day (hours).

Model- Nr.	Model	AIC$_C$	Parameters
1	{Phi(g+T) p(g*t) pent(T) N(g*t)}	2166.3061	37
2	{Phi(g+T) p(t) pent(g+T) N(g*t)}	2169.0766	37
3	{Phi(g+T) p(g*t) pent(g+T) N(g*t)}	2171.3447	38

We calculated changes in the average wing condition to assess the age structure of the population (See Supplementary S 2). Males in general had a constant deterioration of their average wing condition (y = −0.0474x − 2067.6; R² = 0.864). Due to low numbers of captures, reliable data for females are only available for the second half of August. During this time, deterioration of females´ wing conditions was much slower than in males (y = 0.0072x − 313.93; R² = 0.022). The degree of wing deterioration was significantly correlated with the time elapsed until the first recapture in males (Spearman rank correlation analysis: rho = 0.74, p = 0.002) but not in females (rho = 0.48; p < 0.11).

Mobility and movement patterns

Mobility in the studied *E. pronoe* population was low. Thus, the average dispersal distance from capture to first recapture in males was 101 m (± 73 SE; n = 175). Females moved significantly less (68 m ± 80 SE; n = 16), (Mann-Whitney U test: W = 1950, p = 0.009). Both sexes moved distances less than 150 m in the majority of cases, but the percentage of males exceeding this distance (17.6 %) was considerably higher than for females (6.3 %) (Fig. 2). The longest and shortest distances detected were 430 m and 2 m for males, and 340 m and 8 m for females.
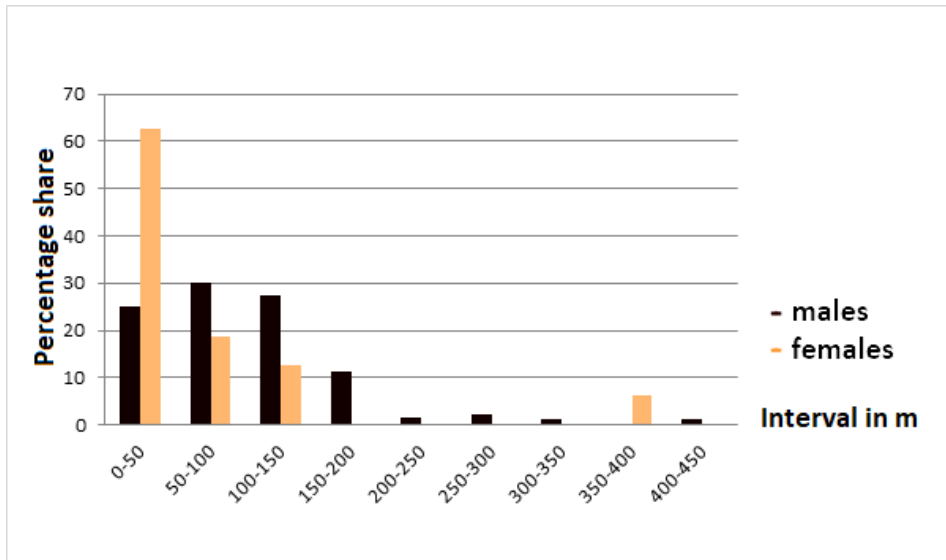
Figure 2 Dispersal of Erebia pronoe differentiated by sex. The percentage of individuals observed dispersing in one of the given 50 m distance classes is given.

Table 2 Stability index (R^2) of the inverse power function (IPF) and the negative exponential function (NEF) based on calculations with 20, 30, and 50 m intervals of the covered distances of Erebia pronoe.

	20 m intervals		30 m intervals		50 m intervals	
	IPF	NEF	IPF	NEF	IPF	NEF
males	0.85	0.98	0.87	0.98	0.90	0.98
females	0.88	0.91	0.89	0.88	0.95	0.99

We applied the NEF and IPF functions for extrapolating the potential for long-distance dispersal. The highest stability indices (R^2) for both functions to the inverse cumulative proportion values (based on distance classes) were obtained for 50 m intervals (Tab. 2). For both sexes, the fit of NEF was better than of IPF. Following NEF, dispersal of 1 km or more would be largely impossible (males: 7.07×10^{-7} %; females: 3.66×10^{-6} %). However, also the estimated values for the IPF were low (males: 0.25 %; females: 0.40 %) and the estimated proportions dispersing distances of more than 2, 3 or even 5 km were still much lower than that (Tab. 3). A Spearman rank correlation revealed a positive correlation between dispersal distance and the elapsed time between capture and first recaptures for females (rho= 0.570, p = 0.033) but not for males (rho= -0.040, p = 0.68) (Fig. 3).

Table 3 Percentage of Erebia pronoe individuals that were expected to disperse more than 1, 2, 3, or 5 km; calculated with inverse power function (IPF) and negative exponential function (NEF) based on 50 m intervals.

Distance	IPF males	IPF females	NEF males	NEF females
1 km	0.25	0.40	7.07×10^{-7}	3.66×10^{-6}
2 km	0.05	0.11	2.32×10^{-15}	5.46×10^{-14}
3 km	0.02	0.05	7.6×10^{-24}	8.15×10^{-22}
5 km	0.01	0.02	8.17×10^{-41}	1.82×10^{-37}

Behavioural differences between sexes

Both sexes were observed with almost equal frequency in resource uptake (feeding and drinking) (Tab. 4). Flight activity was most frequently observed in males, whereas females were primarily encountered resting. Overall, both sexes differed highly significantly in their behaviour ($\chi2$ = 129.6, df = 4, p < 0.001). The behaviour of males became more passive (p = 0.021) with increasing cloud cover, although this effect was only detectable for cloudiness of 60 % or more; no significant effect was observed for females (p = 0.267). There was no significant effect of wind intensity on the behaviour of both sexes (p > 0.1).

Table 4 Percentage of individuals of E. pronoe in four different behavioral categories, divided by sex.

	Flying	**Resting**	**Feeding**	**Drinking**	**Egg deposition**
males	75.4	13.3	5.3	5.8	0
females	20.5	59.0	12.8	0)	7.7

Use of nectar plants

Members of the family Asteraceae were used as nectar sources in 40.6 % of the cases. Most frequently visited were *Carlina acaulis* (25.0 %), *Carduus defloratus* (9.4 %), *Leontodon hispidus* (3.1 %), and *Cirsium eriophorum* (3.1%). In addition to Asteraeceae, the families Apiaceae with *Eryngium bourgatii* (34.4 %) and Caprifoliaceae with *Scabiosa columbaria* (18.8 %) were used. Sex-specific preferences were not observed (χ^2 = 4.354, df = 7, p = 0.74).

Discussion

Population density

With an estimated population size of about 800 individuals and a population density of about 70 individuals per hectare, the here studied population in the central Pyrenees is only about 20 % of the size and less than 10 % of the density of the eastern Alps population studied by Wendt et al. (Wendt et al. 2021). The low number of captures early in the flight period made it difficult to model the actual population structure. The lack of a plateau phase in the population structure suggests a delayed and unfinished flight period. Therefore, the actual population size is likely to exceed that modeled. Nevertheless, the observed population size and density are well below those of comparable studies. Comparing our results against other MRR studies in *Erebia* (i.e. *E. nivalis* >1.200 individuals per hectare in the eastern Alps (Ehl et al. 2018), *E. epiphron* >400 / ha, and *E. sudetica* >1200 / ha in northern Bohemia (Kuras et al. 2003) reveals a low population size and density of the here studied *E. pronoe* population. Other common alpine and arctic-alpine butterfly species also had mostly higher densities in suitable habitats, such as *Boloria pales* , *Boloria napaea* (Ehl et al. 2017), etc. However, in a comparative study of *B. pales* on grazed and ungrazed habitats in the south-eastern Carpathians, the density on the grazed sites of hence low habitat quality was about 55 individuals per hectare and thus comparable with the density obtained in our study, but was about 250 at the flower-rich and thus highly suitable ungrazed sites (Ehl et al. 2019). Consequently, the low density of *E. pronoe* in our Pyrenean population, compared within the species, among *Erebia* species, and alpine butterflies in general, most likely is the result of prevailing unsuitable conditions (see below).

If comparing high mountain butterflies with lowland species, low altitude butterflies also show a similar range of different densities than high altitude butterflies do (Junker and Schmitt 2010; Pennekamp et al. 2014). However, in suitable habitats, generalist lowland species normally have

considerably higher densities (Habel et al. 2010) than our *E. pronoe* population in the Pyrenees, and only specialist species have such low densities (Konvička et al. 2005). This further underlines the generally bad state of the here studied population of a little specialised species.
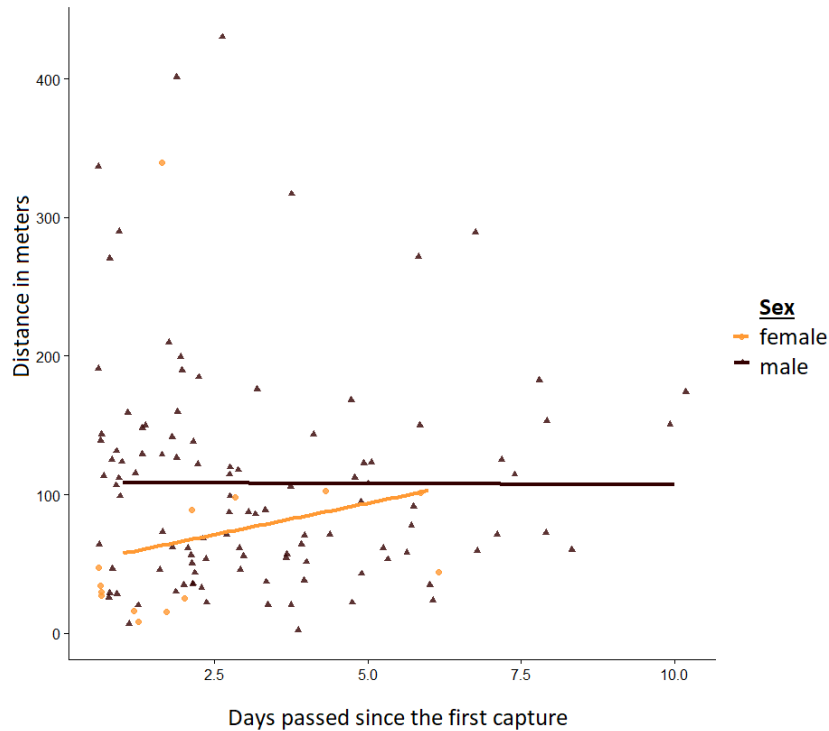

Figure 3 Correlation of migratory distance and passed time

Dispersal and behaviour

The studied population had a very sedentary behaviour and dispersal was also observed over rather short distances. However, no significant difference was obtained if compared with the eastern Alpine population (Wendt et al. 2021). This contradicts the widely accepted assumption that observed dispersal distances are positively correlated with the size of the study area (Schneider 2003). Hence, the tendency of males to establish home ranges, as already observed in the Alps and now also in the Pyrenees (Fig. 4), may lead to a spatial restriction to a sector of the study area and thus result in a limitation of the dispersal distances (Kőrösi et al. 2008). The lack of a correlation between dispersal distance and elapsed time from the first capture to the first recapture underlines the sedentary character of our male *E. pronoe*. The positive correlation in females might be explained by successive movement away from the place of origin, and hereby improves egg-laying possibilities and the spatial distribution of eggs in low-resource habitats (Evans et al. 2020; Plazio et al. 2020b) to increase fecundity. In addition, avoidance of male harassment after successful mating may also lead to an increase in female mobility as a function of time (Baguette et al. 1998). Furthermore, sex-specific adaptation to low or high energy flight styles results in a selective effect of a topographically structured habitat. Females avoid active, energy-consuming flight and prefer passive, low-energy gliding flight (Plazio et al. 2020a), leaving more energy for reproduction. This energetic aspect could be another explanation for the low flight activity and shorter range of females compared to males.
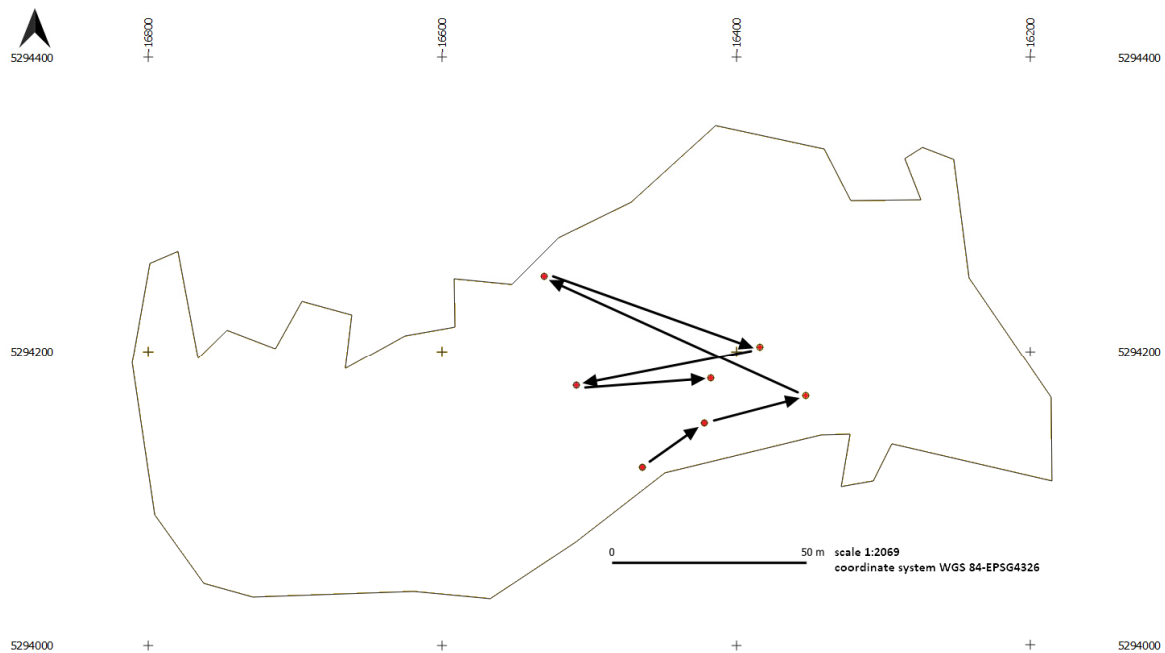
Figure 4 Example of movement patterns of a male of Erebia pronoe recaptured six times.

Males were significantly more flight active than females, as commonly observed for patrolling species (Slamova et al. 2011; Ehl et al. 2019). Overall, the studied Pyrenean population proved to be significantly more flight active than the eastern Alpine one. In the Pyrenees, we observed a behavioural shift for both sexes, with less time invested in feeding and more in flying, a trend also obtained in other species on resource-poor sites (Ehl et al. 2019; Evans et al. 2020), but without the frequently observed increase in average dispersal distance. In females, increased flight activity can be detrimental because less time remains for nectar uptake being essential for egg production and hence reproduction (Kőrösi et al. 2008; Ehl et al. 2019). Energy uptake in general is crucial for income breeders like butterflies, i.e. species in which egg maturation occurs during the adult stage and depends on resource uptake (Boggs and Murphy 1997). This also might explain the low population size in our Pyrenean population, as the number of eggs laid is among one of the most important factors determining the population size of the next generation (Leslie Hayes 1981). The higher flight activity of males on the one hand might be due to an increased effort in the search for nectar sources, but on the other hand, an indirect effect of the increased female flight activity because the activity of males changes with the appearance of females (Petit et al. 2001). Thus, the increased flight activity of females might also increase the flight activity of males. Overall, the potential for the establishment of strong metapopulation structures is considered relatively low, given the philopatry and low dispersal distances of both sexes.

Environmental stress on alpine species

Alpine species are increasingly suffering from the loss of suitable high-quality habitats. This loss is mainly driven by the change in the formerly extensive use of cultivated alpine landscapes (Huemer and Tarmann 2001; van Swaay and Warren 2006; Zöchling 2012; Jerrentrup et al. 2016). Habitat quality is a significant factor influencing behaviour (see (Ehl et al. 2019; Evans et al. 2020) and population size (Beck et al. 2017) of butterflies. The high flight activity of both sexes of the here analysed *E. pronoe* population in combination with its low population size indicates a population suffering from environmental stress. This environmental stress is most likely caused by low habitat quality, resulting from a more intensive agricultural use of our study area.

Thistle flowers represented the most important nectar sources of our Pyrenean *E. pronoe* population. This might be due to the low grazing pressure on thistles (Malo and Suárez 1995; Carpino et al. 2003), allowing that, in contrast to most other not thorny flowering plants, many of them continuously flowered in our study area characterised by high grazing intensity by cow and sheep herds. Due to the known opportunistic nectar plants use of *E. pronoe* (Wendt et al. 2021), these thorny herbs being mostly unpalatable to these ruminants were the most important sources of energy for our Pyrenean population. However, even without high grazing pressure, thistles can be the prevalent nectar resource supporting large and stable populations as in the Alps. Therefore, a lack of resources caused by grazing cannot be the exclusive reason for the low population density, so additional negative aspects of intensive grazing such as trampling damage and browsing of larval plants have also to be considered (Huemer and Tarmann 2001; Jerrentrup et al. 2016).

The intensive use of our study area by beekeepers poses another threat for the appropriate nectar supply and therefore causes a further reduction of habitat quality. Honey bees are superior nectar foragers, which fly earlier, exploit resources selectively, and also take up nectar faster than butterflies (Torné-Noguera et al. 2016; Klumpers 2017; Wignall et al. 2020), resulting in indirect competition (Henry and Rodet 2018). Honey bees have a large range of action and exert enormous competitive pressure on local insect faunas, especially near their colonies (Henry and Rodet 2018). Each of the five recorded colonies was located at a distance of less than 2 km from our study site, which is well within the average foraging distance of honey bees. Consequently, distances less than 2 km are considered high influence areas (Henry and Rodet 2018, 2020). As a result, minimum distance thresholds were not kept and upper-density limits of apiaries were exceeded in our study area, a situation for which massive impacts on the wild insect fauna have been documented (Henry and Rodet 2020). Thus, the exploitative competition exerted by honey bees leads to increased effort in nectar foraging, increased mobility, and lower population sizes of the native pollinator fauna (Henry and Rodet 2018). Furthermore, the buzzing of honey bees triggers defensive behaviour to protect against predators in caterpillars. This protective behaviour is leading to a massive decrease in the feeding rate and thus also reduces the reproductive success of a population (Tautz and Rostás 2008). Therefore, the high flight activity and low population size of *E. pronoe* in Cauterets might also be a consequence of honey bee-induced or intensified resource scarcity.

Conservation implications

Late-flying pollinators compete seasonally for the decreasing amount of available nectar (Garbuzov et al. 2020). In our study area, this scarce resource is further reduced by the high grazing pressure. The remaining nectar plants are then exploited by a superior competitor, to the point that only a comparatively small population of *E. pronoe* with low population density can survive in a supposedly natural landscape and in proximity to the Pyrenees national park.

Such small populations are at risk of being trapped in a negative feedback loop, in which low population density makes mate finding more difficult and thus favours emigration, which further weakens the population. In the future, this effect could be enhanced by an asynchronous development of both sexes. Alpine species rely on compact snow covers not only for overwintering larvae and pupae (Matter et al. 2011; Scalercio et al. 2014) but also for terminating diapause (Konvička et al. 2016). Earlier onset of snowmelt could lead to asynchronous development of both sexes, making mate finding more difficult and resulting in population decline.

The population studied here, weakened by various stressors, must be strengthened to warrant its stability. The low dispersal capacity might impede the formation of a metapopulation structure, making recolonization less likely in the event of a population collapse. Therefore, the population must be supported by increasing habitat quality. This can be done by reducing grazing pressure, which would increase resource diversity and reduce impacts such as browsing and trampling damage. In addition, indirect exclusion of competition from beekeepers needs to be mitigated,

especially in late summer, a phase with generally lower nectar availability. This should be accomplished by maintaining minimum distances and density ceilings (Henry and Rodet 2020). These measures would be beneficial for the here studied population of *E. pronoe* but also for many more alpine plant and animal species living there.

References

Baguette M, Vansteenwegen C, Convié I, Nève G (1998) Sex-biased density-dependent migration in a metapopulation of the butterfly Proclossiana eunomia. Acta Oecologica 19:17–24

Beck J, McCain CM, Axmacher JC, et al (2017) Elevational species richness gradients in a hyperdiverse insect taxon: a global meta-study on geometrid moths. Glob Ecol Biogeogr 26:412–424. https://doi.org/10.1111/geb.12548

Boggs CL, Murphy DD (1997) Community composition in mountain ecosystems: Climatic determinants of montane butterfly distributions. Glob Ecol Biogeogr Lett 6:39–48

Brown LM, Crone EE (2016) Minimum area requirements for an at-risk butterfly based on movement and demography. Conserv Biol 30:103–112. https://doi.org/10.1111/cobi.12588

Burnham K, Anderson D (2002) Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach. Springer Verlag, New York

Carpino S, Licitra G, Van Soest PJ (2003) Selection of forage species by dairy cattle on complex Sicilian pasture. Anim Feed Sci Technol 105:205–214. https://doi.org/10.1016/S0377-8401(03)00054-3

Cooch EG, White GC (2019) Using program MARK: A gentle introduction

Dover J, Settele J (2009) The influences of landscape structure on butterfly distribution and movement: A review. J Insect Conserv 13:3–27. https://doi.org/10.1007/s10841-008-9135-8

Ehl S, Böhm N, Wörner M, et al (2019) Dispersal and adaptation strategies of the high mountain butterfly Boloria pales in the Romanian Carpathians. Front Zool 16:1–16. https://doi.org/10.1186/s12983-018-0298-1

Ehl S, Dalstein V, Tull F, et al (2018) Specialized or opportunistic—how does the high mountain endemic butterfly Erebia nivalis survive in its extreme habitats? Insect Sci 25:161–171. https://doi.org/10.1111/1744-7917.12400

Ehl S, Ebertshäuser M, Gros P, Schmitt T (2017) Population demography of alpine butterflies: Boloria pales and Boloria napaea (Lepidoptera: Nymphalidae) and their specific adaptations to high mountain environments. Acta Oecologica 85:53–61. https://doi.org/10.1016/j.actao.2017.09.009

Evans LC, Sibly RM, Thorbek P, et al (2020) The importance of including habitat - specific behaviour in models of butterfly movement. Oecologia. https://doi.org/10.1007/s00442-020-04638-4

Gall LF (1984) The effects of capturing and marking on subsequent activity in Boloria acrocnema (Lepidoptera: Nymphalidae), with a comparison of different numerical models that estimate population size. Biol Conserv 28:139–154. https://doi.org/10.1016/0006-3207(84)90032-6

Garbuzov M, Balfour NJ, Shackleton K, et al (2020) Multiple methods of assessing nectar foraging conditions indicate peak foraging difficulty in late season. Insect Conserv Divers 13:532–542. https://doi.org/10.1111/icad.12420

Habel JC, Junker M, Schmitt T (2010) High dispersal ability and low genetic differentiation in the widespread butterfly species Melanargia galathea. J Insect Conserv 14:467–478. https://doi.org/10.1007/s10841-010-9275-5

Hallmann CA, Sorg M, Jongejans E, et al (2017) More than 75 percent decline over 27 years in total flying insect biomass in protected areas. PLoS One 12:. https://doi.org/10.1371/journal.pone.0185809

Henry M, Rodet G (2018) Controlling the impact of the managed honeybee on wild bees in protected areas. Sci Rep 8:1–10. https://doi.org/10.1038/s41598-018-27591-y

Henry M, Rodet G (2020) The apiary influence range: A new paradigm for managing the cohabitation of honey bees and wild bee communities. Acta Oecologica 105:103555. https://doi.org/10.1016/j.actao.2020.103555

Hickel C, Gereben-Krenn B-A, Zweimüller I, Krenn HW (2016) Wetterbedingungen für die Erfassung von Tagfaltern (Lepidoptera: Papilionoidea) in alpinen Lebensräumen in Österreich C. Entomol Austriaca 7–18

Huemer P (2007) Biodiversit{ä}t von Schmetterlingen (Lepidoptera) im Gebiet des Naturparks Schlern. Gredleriana 7:233–306

Huemer P, Tarmann GM (2001) Artenvielfalt und Bewirtschaftungsintensität: Problemanalyse am Beispiel der Schmetterlinge auf Wiesen und Weiden Südtirols. Gredleriana 1:331–418

Jerrentrup JS, Klimek S, Marchiori E, et al (2016) Impact of dairy farming on butterfly diversity in Alpine summer pastures. Agric Ecosyst Environ 232:38–45. https://doi.org/10.1016/j.agee.2016.07.023

Junker M, Schmitt T (2010) Demography, dispersal and movement pattern of Euphydryas aurinia (Lepidoptera: Nymphalidae) at the Iberian Peninsula: An alarming example in an increasingly fragmented landscape? J Insect Conserv 14:237–246. https://doi.org/10.1007/s10841-009-9250-1

Junker M, Wagner S, Gros P, Schmitt T (2010) Changing demography and dispersal behaviour: Ecological adaptations in an alpine butterfly. Oecologia 164:971–980. https://doi.org/10.1007/s00442-010-1720-3

Klumpers S (2017) Beyond random and forbidden interactions in plant-pollinator networks: how optimizing energy gain results in morphological matching among subalpine Asteraceae and their flower- visitors

Konvička M, Beneš J, Čížek O, et al (2016) Has the currently warming climate affected populations of the mountain ringlet butterfly, Erebia epiphron (Lepidoptera: Nymphalidae), in low-elevation mountains? Eur J Entomol 113:295–301. https://doi.org/10.14411/eje.2016.036

Konvička M, Čížek O, Filipová L, et al (2005) For whom the bells toll: Demography of the last population of the butterfly Euphydryas maturna in the Czech Republic

Kőrösi Á, Örvössy N, Batáry P, et al (2008) Restricted within-habitat movement and time-constrained egg laying of female Maculinea rebeli butterflies. Oecologia 156:455–464. https://doi.org/10.1007/s00442-008-0986-1

Kuras T, Benes J, Fric Z, Konvicka M (2003) Dispersal patterns of endemic alpine butterflies with contrasting population structures: Erebia epiphron and E. sudetica. Popul Ecol 45:115–123. https://doi.org/10.1007/s10144-003-0144-x

Leslie Hayes J (1981) The population ecology of a natural population of the pierid butterfly Colias alexandra. Oecologia 49:188–200. https://doi.org/10.1007/BF00349187

Maes D, van Dyck H (2005) Habitat quality and biodiversity indicator performances of a threatened butterfly versus a multispecies group for wet heathlands in Belgium. Biol Conserv 123:177–187.

https://doi.org/10.1016/j.biocon.2004.11.005

Malo JE, Suárez F (1995) Herbivorous mammals as seed dispersers in a Mediterranean dehesa. Oecologia 104:246–255. https://doi.org/10.1007/BF00328589

Matter SF, Doyle A, Illerbrun K, et al (2011) An assessment of direct and indirect effects of climate change for populations of the Rocky Mountain Apollo butterfly (Parnassius smintheus Doubleday). Insect Sci 18:385–392. https://doi.org/10.1111/j.1744-7917.2011.01407.x

Melbourne BA, Hastings A (2008) Extinction risk depends strongly on factors contributing to stochasticity. Nature 454:100–103. https://doi.org/10.1038/nature06922

Parmesan C (2006) Ecological and Evolutionary Responses to Recent Climate Change. Annu Rev Ecol Evol Syst 37:637–669. https://doi.org/10.1146/annurev.ecolsys.37.091305.110100

Pennekamp F, Garcia-Pereira P, Schmitt T (2014) Habitat requirements and dispersal ability of the Spanish Fritillary (Euphydryas desfontainii) in southern Portugal: Evidence-based conservation suggestions for an endangered taxon. J Insect Conserv 18:497–508. https://doi.org/10.1007/s10841-014-9655-3

Petit S, Moilanen A, Hanski I, Baguette M (2001) Metapopulation dynamics of the bog fritillary butterfly: movements between habitat patches. Oikos 92:491–500. https://doi.org/10.1016/S1146-609X(02)01157-8

Plazio E, Bubová T, Vrabec V, Nowicki P (2020a) Sex-biased topography effects on butterfly dispersal. Mov Ecol 8:1–13. https://doi.org/10.1186/s40462-020-00234-6

Plazio E, Margol T, Nowicki P (2020b) Intersexual differences in density-dependent dispersal and their evolutionary drivers. J Evol Biol 33:1495–1506. https://doi.org/10.1111/jeb.13688

QGIS Development Team (2018) Quantum Geographic Information System (QGIS) software version 3.83

R core Team (2017) R: A language and environment for statistical computing.

Sánchez-Bayo F, Wyckhuys KAG (2019) Worldwide decline of the entomofauna: A review of its drivers. Biol Conserv 232:8–27. https://doi.org/10.1016/j.biocon.2019.01.020

Scalercio S, Bonacci T, Mazzei A, et al (2014) Better up, worse down: Bidirectional consequences of three decades of climate change on a relict population of Erebia cassioides. J Insect Conserv 18:643–650. https://doi.org/10.1007/s10841-014-9669-x

Schneider C (2003) The influence of spatial scale on quantifying insect dispersal: An analysis of butterfly data. Ecol Entomol 28:252–256. https://doi.org/10.1046/j.1365-2311.2003.00495.x

Seibold S, Gossner MM, Simons NK, et al (2019) Arthropod decline in grasslands and forests is associated with landscape-level drivers. Nature 574:671–674. https://doi.org/10.1038/s41586-019-1684-3

Slamova I, Klecka J, Konvicka M (2011) Diurnal Behavior and Habitat Preferences of Erebia aethiops, an Aberrant Lowland Species of a Mountain Butterfly Clade. J Insect Behav 24:230–246. https://doi.org/10.1007/s10905-010-9250-8

Sonderegger P (2005) Die Erebien der Schweiz (Lepidoptera: Satyrinae, Genus Erebia). Private Publication, Biel

Storch D, Evans KL, Gaston KJ (2005) The species-area-energy relationship. Ecol Lett 8:487–492. https://doi.org/10.1111/j.1461-0248.2005.00740.x

Stuhldreher G, Hermann G, Fartmann T (2014) Cold-adapted species in a warming world - an

explorative study on the impact of high winter temperatures on a continental butterfly. Entomol Exp Appl 151:270–279. https://doi.org/10.1111/eea.12193

Sugiura N (2007) Further analysts of the data by akaike' s information criterion and the finite corrections. Commun Stat - Theory Methods 13–26. https://doi.org/https://doi.org/10.1080/03610927808827599. 73.

Tautz J, Rostás M (2008) Honeybee buzz attenuates plant damage by caterpillars. Curr Biol 18:1125–1126. https://doi.org/10.1016/j.cub.2008.10.038

Timus N, Czekes Z, Rákosy L, Nowicki P (2017) Conservation implications of source-sink dynamics within populations of endangered Maculinea butterflies. J Insect Conserv 21:369–378. https://doi.org/10.1007/s10841-016-9906-6

Tolman T, Lewington R (2012) Schmetterlinge Europas Und Nordwestafrikas, 2. Edition. Kosmos, Stuttgart

Torné-Noguera A, Rodrigo A, Osorio S, Bosch J (2016) Collateral effects of beekeeping: Impacts on pollen-nectar resources and wild bee communities. Basic Appl Ecol 17:199–209. https://doi.org/10.1016/j.baae.2015.11.004

Tshikolovets V V (2011) Butterflies of Europe and the Mediterranean area. Tshikolovets Publications.

van Swaay CAM, Warren MS (2006) Prime Butterfly Areas of Europe: An initial selection of priority sites for conservation. J Insect Conserv 10:5–11. https://doi.org/10.1007/s10841-005-7548-1

Viljur ML, Teder T (2018) Disperse or die: Colonisation of transient open habitats in production forests is only weakly dispersal-limited in butterflies. Biol Conserv 218:32–40. https://doi.org/10.1016/j.biocon.2017.12.006

Wagner DL (2020) Insect Declines in the Anthropocene. Annu Rev Entomol 65:457–480. https://doi.org/10.1146/annurev-ento-011019-025151

Wendt M, Kulanek D, Varga Z, et al (2022) Pronounced mito-nuclear discordance and various Wolbachia infections in the water ringlet Erebia pronoe have resulted in a complex phylogeographic structure. Sci Rep 12:. https://doi.org/10.1038/s41598-022-08885-8

Wendt M, Senftleben N, Gros P, Schmitt T (2021) Coping with environmental extremes: Population ecology and behavioural adaptation of erebia pronoe, an alpine butterfly species. Insects 12:. https://doi.org/10.3390/insects12100896

White GC, Burnham KP (1999) Program mark: Survival estimation from populations of marked animals. Bird Study 46:S120–S139. https://doi.org/10.1080/00063659909477239

Wignall VR, Brolly M, Uthoff C, et al (2020) Exploitative competition and displacement mediated by eusocial bees: experimental evidence in a wild pollinator community. Behav Ecol Sociobiol 74:. https://doi.org/10.1007/s00265-020-02924-y

Zimmermann K, Fric Z, Filipová L, Konvička M (2005) Adult demography, dispersal and behaviour of Brenthis ino (Lepidoptera: Nymphalidae): How to be a successful wetland butterfly. Eur J Entomol 102:699–706. https://doi.org/10.14411/eje.2005.100

Zöchling A (2012) Auswirkungen unterschiedlicher Bewirtschaftungsweisen und Nutzungsintensitäten von Almen auf die Tagfalterfauna im NP Gesäuse. Master thesis, University Wien

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Pronounced mito-nuclear discordance and various *Wolbachia* infections in the water ringlet *Erebia pronoe* have resulted in a complex phylogeographic structure

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Several morphological and mitochondrial lineages of the alpine ringlet butterfly species *Erebia pronoe* have been described, indicating a complex phylogenetic structure. However, the existing data were insufficient and allow neither a reconstruction of the biogeographic history, nor an assessment of the genetic lineages. Therefore, we analysed mitochondrial (COI, NDI) and nuclear (EF1α, RPS5) gene sequences and compared them with sequences from the sister species *Erebia melas*. Additionally, we combined this information with morphometric data of the male genitalia and the infection patterns with *Wolbachia* strains, based on a WSP analysis. We obtained a distinct phylogeographic structure within the *E. pronoe-melas* complex with eight well-distinguishable geographic groups, but also a remarkable mito-nuclear discordance. The mito-nuclear discordance in *E. melas* and *E. pronoe glottis* can be explained by different ages of *Wolbachia* infections with different *Wolbachia* strains, associated selective sweeps, and hybridisation inhibition. Additionally, we found indications for incipient speciation of *E. pronoe glottis* in the Pyrenees and a pronounced range dynamic within and among the other high mountain systems of Europe. Our results emphasize the importance of combined approaches in reconstructing biogeographic patterns and evaluating phylogeographic splits.

Butterflies are among the best-studied groups of invertebrates in Europe, but they nevertheless have great potential for harbouring previously overlooked biodiversity in the form of cryptic species[1,2]. However, unambiguous identification of cryptic species is difficult, due to similarities in morphology, behaviour, ecological niches, or individual genetic markers. Recent or rapid speciation events can promote cryptic diversity[3,4], as can be seen in the genus *Erebia*. This genus has a particularly high diversification rate and is one of the most species-rich genera of European butterflies[5]. Numerous endemics, subspecies, diverse forms, and aberrations have been described[1,2,6,7]. The high intraspecific morphological variability combined with complex genetic structures has caused difficulties in distinguishing valid species[8], and many other taxonomic and biogeographic questions have remained unanswered.

One of the unanswered questions relates to the status of the species *Erebia pronoe* (Esper, [1780]). *Erebia pronoe* is a European alpine butterfly and a character species of alpine rupicolous grasslands. There are several allopatric occurrences, due to the strong link to alpine habitats, among others in the Pyrenees, the Carpathians, and the Balkan mountain systems. Widely distributed species with allopatric distributions tend to have complex

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	COI	NDI	Combined mitochondrial DNA	EF1alpha	RPS5	Combined nuclear DNA
Nucleotid diversity Pi	0.01424	0.01332	0.01381	0.00333	0.00369	0.00346
Haplotyp diversity h	0.926	0.810	0.955	0.925	0.733	0.974
Segregation sites S	41	41	82	30	17	47
Average number of nucleotide differences k	8.90257	7.40401	16.30658	3.18257	2.13649	5.3191

Table 1. Genetic diversity parameters of the mitochondrial and nuclear DNA markers of the separated and combined sequences of *Erebia pronoe*.

phylogeographic structures, from which difficulties in species delimitation may arise[9,10]. Thus, various morphological lineages, subspecies, and hybrid forms have been described for *E. pronoe* (e.g.[11–14]). Recently, three of these described lineages have been confirmed by genetic markers[1,7]. The mitochondrial gene segments analysed in these previous studies displayed multiple entities and possible indications of overlooked cryptic species within *E. pronoe*. Their results were based on haploid genetical markers, which are prone to influences like introgression, sex-specific behavioural differences[15], and infection with parasitic bacteria such as *Wolbachia*[16,17]. Furthermore, important regions, such as the western Alps and the Balkan mountain systems, which probably served as dispersal corridors[7], were not studied. A comprehensive understanding of the phylogeographic structure of this species was therefore lacking. A dense network of sampling sites across the range of the species, comparisons between biparental nuclear and maternally inherited mitochondrial genes, screening for *Wolbachia* infections, and a comparison with closely related taxa, like the sister species *Erebia melas*[10,18], were needed to reconstruct the phylogeographic history of *E. pronoe*.

To obtain detailed information on phylogeographic patterns in this species, we sampled a dense network of sites across the entire range of *E. pronoe*: from the Pyrenees, through the entire Alpine region, and in the Carpathians, and the western and eastern Balkan mountain systems. We sequenced two nuclear and two mitochondrial gene segments and intensively screened all detected genetic lineages for infection with *Wolbachia* to address the problem of mito-nuclear discordance[19,20]. We performed a genetic comparison with the sister species *E. melas*, together with other representatives of the genus *Erebia* as outgroups, to put the phylogeographic analysis into a wider phylogenetic context. Additionally, we performed a morphometric analysis on the male genitalia to check for differences correlated with the genetically identified lineages.

We asked the following research questions:

1. Which of the morphologically described subspecies represent distinct genetic lineages?
2. Do mitochondrial and nuclear markers show similar phylogeographic patterns and how can possible differences be explained?
3. Where is the geographic origin of the species *E. pronoe* and what events led to the present distribution pattern with its phylogeographic structures?

Results

Genetic analyses. The combined sequences of COI and NDI (1181 bp) of the 124 specimens from the 27 populations had 46 haplotypes (genetic diversity parameters in Table 1). The most common haplotypes were H30 (39.2%), H17 (19.6%), H43 (19.6%), H22 (9.8%), H24 (9.8%), and H36 (9.8%). All other haplotypes had frequencies below 8%. The largest maximum p-distance between *E. pronoe* haplotypes was 0.0356 (Port de Larrau, F vs. Granchar, BG; Port de Larrau, F vs. Partnun, CH) with an overall average distance of 0.0179 (sd = 0.0127). The genetic distance between *E. pronoe* and its sister species *E. melas* ranged from p = 0.0094 (Königsstein, A; Loser, A) to 0.0376 (Port de Larrau, F); mean 0.0201 (sd = 0.0107). In both mtDNA markers, four non-silent mutations with amino acid change occurred in each Pyrenean population. Two changes in amino acid polarity occurred in each of the two mtDNA markers (see supplementary S1).

The haplotype network based on both mtDNA markers distinguishes three main groups: a Pyrenean group, a western Alps group, and a group including all other regions (see Fig. 1). The Pyrenean group, which coincides with the occurrence of the subspecies *E. pronoe glottis* Fruhstorfer, [1920], has a mean genetical distance among populations ranging from 0.0014 to 0.0034. There is no dominant haplotype in this group, but systems with satellite haplotypes are emerging, especially around H12. The western Alps group, whose occurrence coincides with the subspecies *E. pronoe psathura* Fruhstorfer, [1920], has a markedly low genetic diversity (0.0002–0.0014) and possesses a dominant haplotype, i.e. H43. The third and by far most widespread group has a complex star-shaped structure centred on the dominant haplotype H30, which occurs in the central, eastern and southern Alps. Satellite haplotypes directly derived from it with only one mutation step have been found mainly in the central and eastern Alps, but also the southern Alps (H29, H33), the High Tatras (H39), and the Romanian Carpathians (H37). Two haplotypes with quite clear differentiation are also directly derived from H30, restricted to the Valais (H17) and the central Italian Alps (H27).

The western Balkan Peninsula system (i.e. Dinaric Alps) with the central haplotype H22 is genetically more distant from the eastern Alps (0.0034) than the system from the Slovenian Alps with the central haplotype: H24 (0.0021). The eastern Balkan Peninsula also has a similar system around H35, but is less differentiated from the
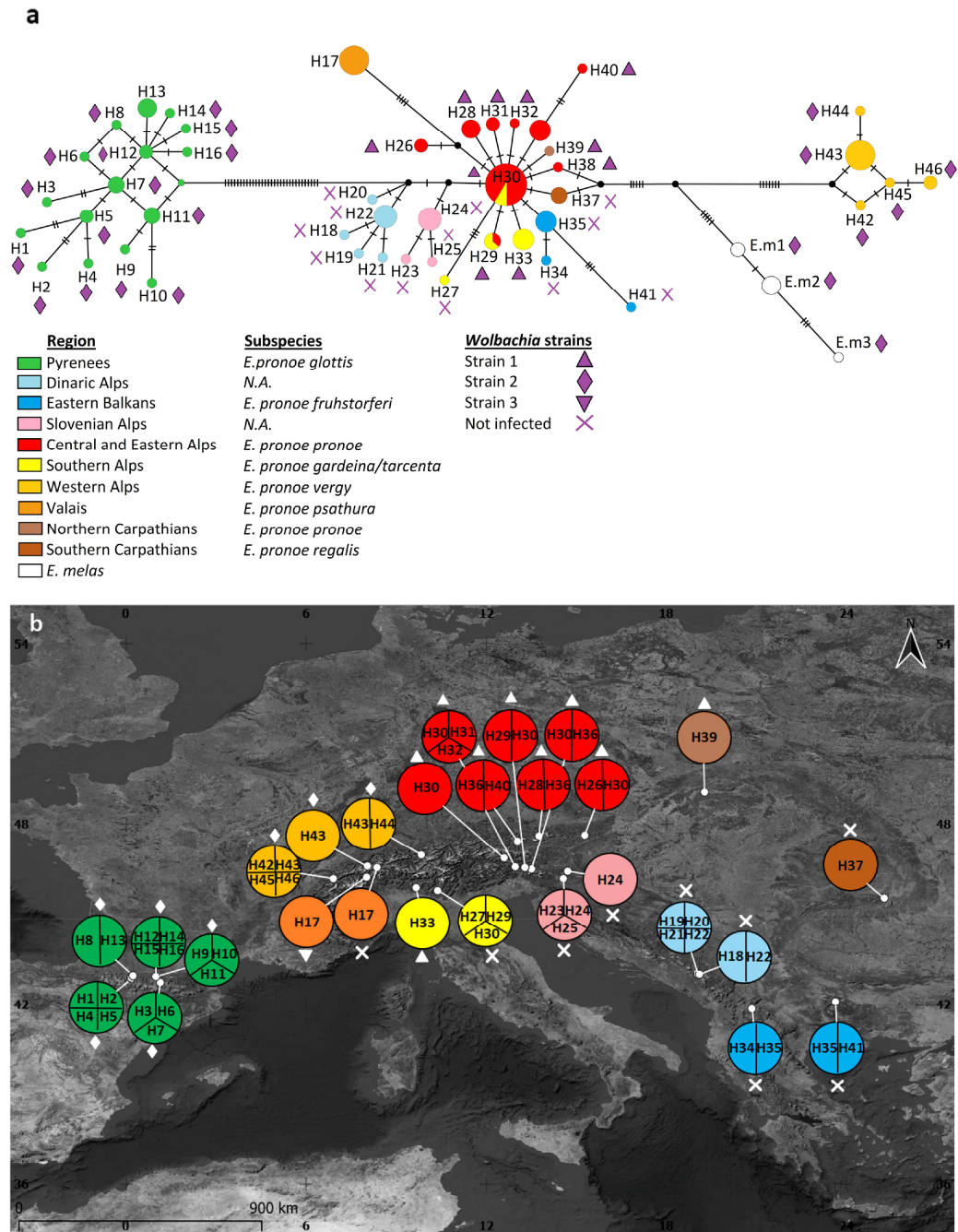
Figure 1. (**a**) TCS haplotype network based on the concatenated mtDNA haplotypes (COI, NDI) of *Erebia pronoe*. Mutational steps are shown by the number of hatch marks. The colour codes of each region and the corresponding subspecies are given in the legend (N.A. = no subspecies name available for this region). The reference haplotypes of *E. melas* are given in white. Detected *Wolbachia* infection strains are indicated by a symbol next to each haplotype. (**b**) Distribution of the identified concatenated mtDNA haplotypes (COI, NDI) among the populations of *E. pronoe*. The map was created with Qgis v.3.10.10 (Available online: http://qgis.osgeo.org).
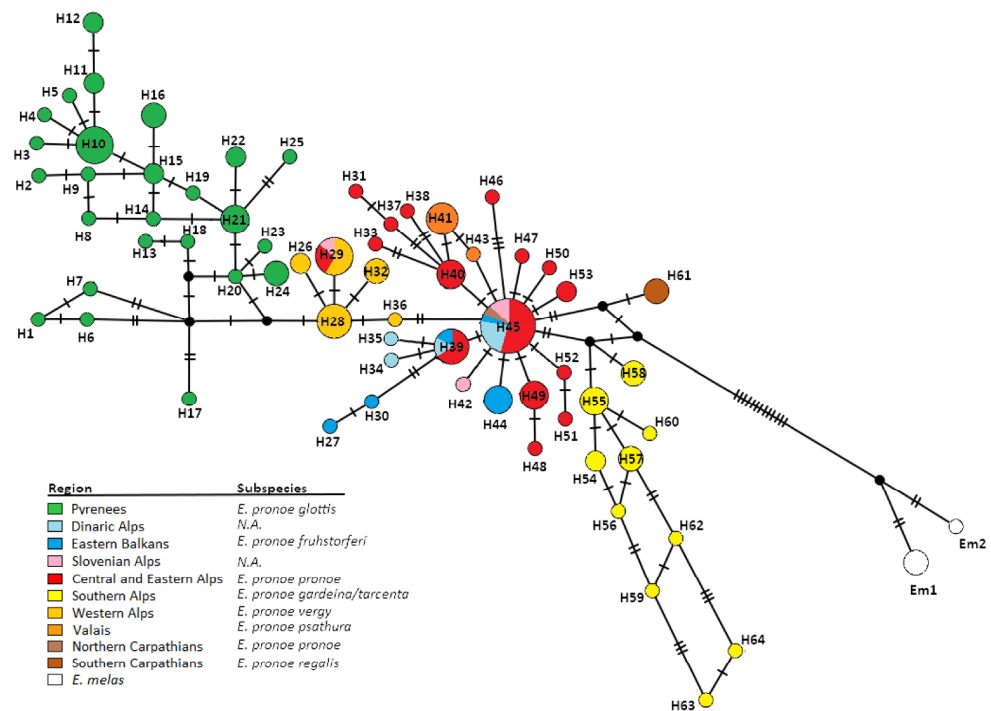
Figure 2. TCS haplotype network based on the concatenated phased nuclear DNA haplotypes (EF1α, RPS5) of *Erebia pronoe*. Mutational steps are shown by the number of hatch marks. The colour codes of each region and the corresponding subspecies are given in the legend (N.A. = no subspecies name available for this region). The reference haplotypes of *E. melas* are given in white. The geographical location of the haplotypes is given in the same colour scheme as in Fig. 1.

dominant H30 (0.0016). The haplotypes of *E. melas* have similar genetic distances to the second (westalps 0.0115) and third groups (ostalps 0.0103) and are less differentiated from them than the Pyrenean group.

The 124 combined sequences of EF1α and RPS5 (1536 bp) resulted in 68 genotypes (genetic diversity parameters in Table 1). The most frequent genotypes were G64 (21.8%), G45 (6.5%), G31 (4.8%), G23 (4.8%). All other genotypes had a frequency of less than 3% (see supplementary S2 for the distribution). The maximum p-distance between *E. pronoe* genotypes was 0.004 (Passo San Marco, I versus Pyrenees; Granchar Rila, BG; Gletsch, CH). The average genetic distance was 0.0008 (sd = 0.00056). The genetic distance between *E. melas* and *E. pronoe* ranged from 0.0074 (Grindelwald, CH; Glockner Research Station, A; Alisnica, AL; Hochkönig, A) to 0.0094 (Passo San Marco, I); with an overall mean of 0.0077 (sd = 0.00035). There was no change of amino acids in the nuclear markers of the studied populations.

The haplotype network of the phased nuclear dataset was composed of four groups, three of which had complex structures (see Fig. 2). The Pyrenean group, the western Alps group, and the widespread group had internal structures similar to the mtDNA network. However, the populations from the central Italian Alps represented a separate fourth genetic group with high genetic diversity and were not part of the widespread group as they were in the mtDNA network. However, nuclear DNA did not yield clearly separated subgroups within the widespread group for the Slovenian Alps or the western and eastern Balkan mountain systems. The Romanian Carpathians were more differentiated from the central haplotype of the widespread group based on the nuclear genes, and less so based on mtDNA markers. The haplotype H29 of the western Alps group was rarely detected in the central, eastern and Slovenian Alps. Overall, differentiation between groups was significantly lower in the nuclear genes (compared to diversity and differentiation within groups) than in the mitochondrial genes. The western Alps group clustered between the Pyrenean group and the widespread group and was closest to the Pyrenean group. The separation between the sibling species *E. pronoe* and *E. melas* was significantly more prominent (12 vs. 18 mutational steps).

All examined specimens from the Pyrenean populations were infested with *Wolbachia* strain 2. This strain was also found in all western Alps populations. All except one of the Valais specimens were without *Wolbachia* infection. However, *Wolbachia* strain 3, which was exclusively found there, differed strongly from all other *Wolbachia* sequences (average p-distance 0.1766, see Table 2). *Wolbachia* strain 1 was detected in the central, eastern, and central Italian Alps, and in the High Tatras. No *Wolbachia* infections were detected in the populations of the Romanian Carpathians, Slovenian Alps, and Balkan Peninsula. The nominotypical *E. melas* population from the south-western Carpathians (Băile Herculane, RO) was infected with *Wolbachia* strain 2.

Wolbachia strain	Wolbachia 1	Wolbachia 2
Wolbachia 1		
Wolbachia 2	0.0393	
Wolbachia 3	0.1682	0.185

Table 2. Mean genetic distance of the three genetic strains of *Wolbachia* detected in *Erebia pronoe* based on the WSP gene fragment.
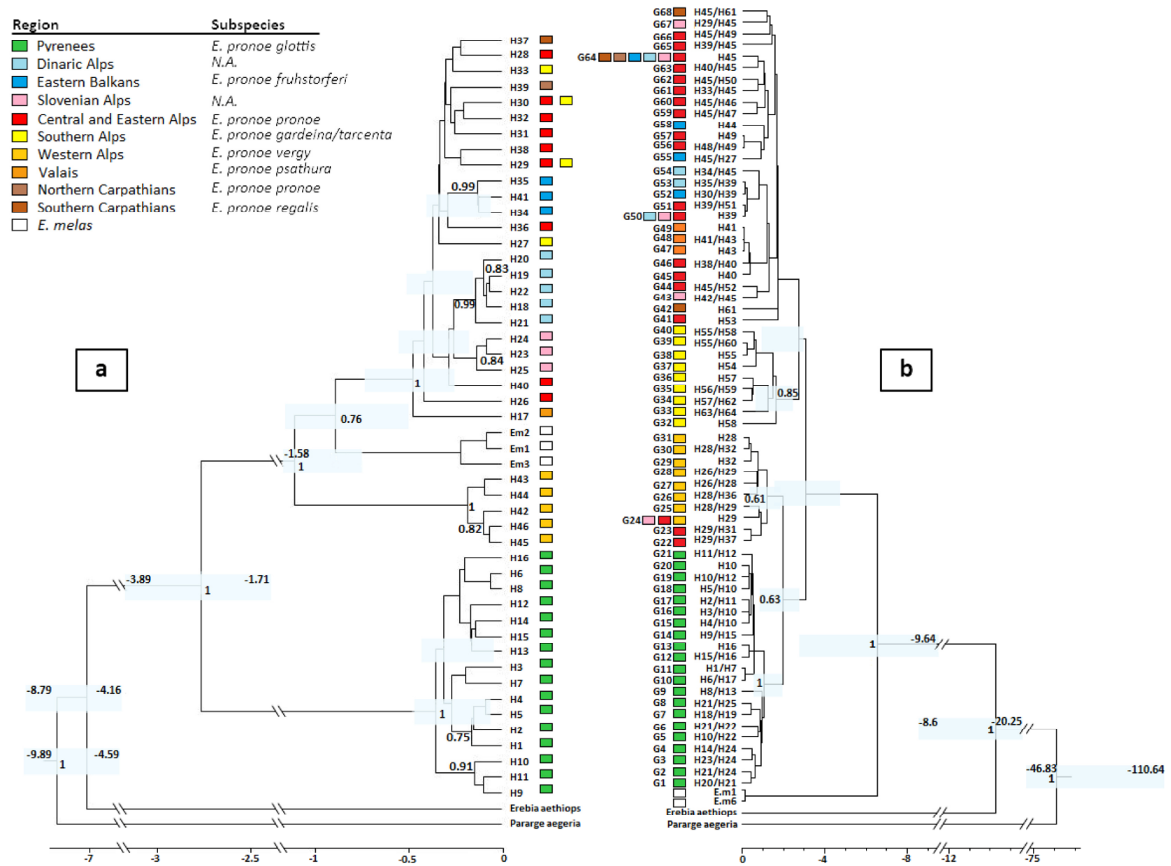

Figure 3. Bayesian phylogeny based on concatenated (**a**) mtDNA haplotypes (COI, NDI) and (**b**) nuclear haplotypes (EF1α, RPS5) of *Erebia pronoe*. Numbers next to the nodes: Bayesian posterior probabilities > 0.7; light blue node bars: 95% highest posterior density of node ages (age is explicitly given as number if bar includes a cut in the timeline). The colour codes of each region and the corresponding subspecies are given in the legend (N.A. = no subspecies name available for this region). The reference haplotypes of *E. melas* are given in white. The geographical location of the haplotypes is given in the same colours as in Fig. 1.

Model testing revealed four partitions in the mtDNA dataset: HKY + I (Gen1:1, Gen2:3), K80 + I (Gen1:2), F81 (Gen1:3), and HKY + I (Gen2:1, Gen2:2). Because K80 + I and F81 are submodels of the HKY model and cannot be selected separately, we used the HKY model instead. Model testing resulted in three partitions for the nuclear dataset: JC (Gen1:1, Gen1:3, Gen2:2), K80 + I (Gen1:2, Gen2:1), F81 (Gen2:3). The partitions suggested by the model test did not result in a successful run with stable ESS values for the nuclear data set. Therefore, several test runs were performed and the coalescent constant population size tree model with HKY substitution model gave the best results (see "Material and methods"; supplementary S3). Both trees in the Beast analysis identified the Pyrenees and western Alps as distinct groups (see Fig. 3). The Slovenian Alps, the western and the eastern Balkan mountain systems groups are separated in the mtDNA tree but are included in the widespread group in the nuclear tree. Conversely, the central Italian southern Alps are part of the widespread group in the mtDNA tree but occupy a clearly separated position in the nuclear tree. We found the strongest discordance between the two analyses and the nuclear and mitochondrial markers in the position of *E. melas*. In the mitochondrial markers,

E. melas is nested within *E. pronoe*, whereas *E. melas* is the sister to *E. pronoe* in the nuclear tree. Analysis of mismatch distributions of pairwise sequence differences revealed a bimodal distribution in the mitochondrial DNA and a geometric distribution in the nuclear dataset (see supplementary S4, S5). RASP model test reported the Dispersion-Extinction-Cladogenesis (DEC + J) as the best model for condensed trees based on AICc and LnL criteria. RASP analyses with DEC + J setting failed to separate the region of origin for either data sets, displaying every region as equally likely. In contrast, the Bayesian inference for discrete areas (BayArea) approach indicated the eastern Alpine region (region "c") as the most likely area of origin for the nuclear markers and the Carpathian region (region "e" for the mitochondrial markers (see supplementary S6–S9).

Genital morphology.   The principal component analysis showed that the first two principal components explained most of the variation (48.6% and 21.0%, see supplementary S10–S12). According to these components, the valves of *E. pronoe* and *E. melas* were distinguishable from each other, whereas an intraspecific separation within *E. pronoe* was not possible. K means clustering for k = 3 (see supplementary S13) resulted in two clusters with all *E. pronoe* lineages mixed and a third cluster with all *E. melas* specimens. Landmarks 8–10 had the highest explanatory power of the 45 landmarks (see supplementary S14).

Discussion

Erebia pronoe exhibits highly structured and strongly differentiated mitochondrial lineages, which are consistent with the distribution of previously described morphotaxa and analyses of Dincă et al.[10] These genetic lineages are also reflected to varying degrees in the nuclear markers. The observed mito-nuclear discordances can be explained by different evolutionary rates of genetic markers, the effects of *Wolbachia* infections, and introgression. These aspects are discussed in more detail in the following sections on the phylogeographic history of this species complex.

Mito-nuclear discordance and the systematic status of *Erebia melas*.   Based on genital morphology and nuclear markers, *E. melas* represents a distinct group to *E. pronoe*. The common area of origin of both species was probably located in the eastern Alps, which is supported by a RASP analysis based on the nuclear markers. However, *E. melas* acts as an ingroup of *E. pronoe* based on the mitochondrial markers, and a RASP analysis indicates a common origin for both taxa in the Carpathian region. Since most *Erebia* species in Europe have at least parts of their distribution in the Alps[21] and are adapted to Alpine environments and habitats[22,23], we consider an eastern Alpine origin of the ancestor of *E. pronoe* and *E. melas* more likely. This hypothesis subsumes the assumption that the genetic proximity on the mitochondrial level was probably caused by hybridisation and introgression events, which could have occurred as a result of several eastward advances of *E. pronoe* to the Balkan Peninsula (see below). This seems plausible, because the ability and tendency of *E. pronoe* to hybridise with other *Erebia* species have been demonstrated repeatedly[12,24,25].

The existence of *Wolbachia* strain 2 in both species, and its distribution from the Pyrenees (in *E. pronoe*) to the Balkan Peninsula (in *E. melas*) also speaks for a common origin of both species. Thus, *Wolbachia* strain 2 might represent the ancient strain present in the common ancestor of this species group, surviving today at the geographic margins (i.e. Pyrenees, western Alps, Balkan Peninsula), but which at some time was replaced in the centre of the butterfly's range (i.e. the eastern and central Alps) by strain 1. The link between co-occurrence in a common area and prevalence of one *Wolbachia* strain was also recently demonstrated in other *Erebia* species[26] and might facilitate mitochondrial introgression[27].

Intraspecific differentiation and glacial refugia of *Erebia pronoe*.   The Pyrenean region is inhabited by one of the oldest and most differentiated intraspecific lineages of *E. pronoe*. The high genetic diversity in the Pyrenees speaks for large effective population sizes throughout time, enabled by mostly altitudinal shifts in response to climatic cycles, and a lack of major genetic bottlenecks. Compared to the Pyrenean group, the genetic diversity of the western Alpine populations, also well differentiated from all other groups, is lower. This lower diversity was probably the result of repeated cold stage retreat to a geographically more restricted refugium at the foot of the south-western Alps, a well-known refugial area for numerous species[28].

We cannot say conclusively whether the populations in the Pyrenean region or in the western Alps differentiated first, due to the contradictory genetical markers. The higher evolutionary rate of the mitochondrial markers, the allopatric distribution, and the hybridisation with diverse *Erebia* species may have led to a greater differentiation of the Pyrenees and/or a loss of the genetic link between the western Alps and the Pyrenees. Since a link between the western Alps and the Pyrenees is still well reflected in the nuclear data set and by the shared *Wolbachia* strain 2, we consider the most likely scenario to be an early Pleistocene or even Pliocene expansion from the western Alps to the Pyrenees, with subsequent isolation and differentiation. Thus, the Pyrenees-western Alps populations might first have separated as one group from an eastern Alps group s.l., as suggested by nuclear information, and not in two independent events, as suggested by mitochondrial genes.

Simultaneously to the split between western Alps and Pyrenees, a separation of the eastern Alpine group s.l. into a southern Alpine subgroup and an eastern Alpine subgroup should have occurred. The southern Alpine subgroup displays a high genetic diversity in their nuclear markers, but a significantly lower diversity in the mtDNA. This might be explained by the existence of a cold-stage refugial area in the southern Alps or their margin, supporting the constant survival of large populations, but also a reshaping of the mtDNA patterns through introgression from the eastern Alpine subgroup during secondary contact when both subgroups expanded into formerly glaciated east-central Alpine areas. The isolated occurrence of *Wolbachia* strain 1 and mitochondrial haplotypes H29 and H30 (shared with the eastern Alps subgroup) in the southern Alps further support the hypothesis of gene flow from the eastern Alpine region into the southern Alpine populations and vice versa.

The eastern Alpine subgroup probably survived glacial periods in a large, cohesive refugium at the eastern edge of the Alps, as has been demonstrated for numerous other species[28]. This area is also seen as a potential centre of origin of the entire taxon. From there, a recent (most likely postglacial) dispersal must have taken place, which should be responsible at least partly for the star-like pattern of this group in both mitochondrial and nuclear haplotype networks. However, further dispersal events out of the eastern Alps during previous inter-glacials and maybe even going back to the Pliocene have to be postulated to explain the entire range dynamics in *E. pronoe*.

Apparently, multiple advances out of the eastern Alps into the Balkan mountain systems have taken place from several independent glacial refugia in the region, as indicated by the different mtDNA lineages in Slovenia, western Balkan mountains, and eastern Balkan mountains. A separation between the eastern and western Balkans, and hence also separate glacial refugia in both areas, was frequently observed for mountain taxa[28,31]. This pattern may have resulted from a succession of independent dispersal events from the eastern Alps throughout the younger Pleistocene, with subsequent regional extinction events and/or independent dispersal events across the Carpathians, as has been demonstrated for numerous other species[29].

A similar pattern of two independent colonisation events also applies to the Carpathians. Thus, the highly isolated populations in the south-eastern Carpathians must go back to an older expansion out of the eastern Alps. This probably took place during one of the last interglacial phases. The route most likely followed the Carpathian arc, but only a few populations survived at their south-eastern edge. This underlines the phylogeographic independence of this part of the Romanian Carpathians, which is also supported by studies on numerous other mountain species[30–32]. On the other hand, the Tatra mountains, as the northernmost part of the Carpathians, were colonised very recently, most likely postglacially, out of the eastern Alpine area. The strong and rather recent link between these two areas is also supported by phylogeographic studies on many taxa[30,33,34].

Because of the slower evolutionary rate of nuclear DNA and the resulting incomplete lineage sorting, nuclear markers can contribute little to the reconstruction of these presumably recent events. In line with that, the Valais lineage also has little nuclear differentiation but is clearly distinguished from the western and eastern Alpine lineages by the exclusive mtDNA haplotype H17 and *Wolbachia* strain 3. The presence of a single, highly differentiated mtDNA haplotype and an exclusive *Wolbachia* strain indicates a selective sweep. This lineage most likely represents a chronological relict of an interglacial expansion of the eastern Alpine subgroup to the western-central Alps surviving since then in this area, finding glacial refugia in nearby unglaciated areas and becoming infested by a *Wolbachia* strain not present in any other *E. pronoe* lineage, hence accelerating its differentiation.

Another selective sweep was probably the cause of the mito-nuclear unconformity in the southern Alps lineage. The occurrence of the mtDNA haplotypes H29 and H30 and the *Wolbachia* strain 1 indicate mitochondrial hybridisation between the eastern and southern Alpine lineages during an expansive interglacial phase. As a result, *Wolbachia* infection probably occurred, which might have impoverished the mitochondrial diversity of the southern Alps lineage.

Consequences for subspecific differentiation in *Erebia pronoe*.

In general, the support given by our data for the so-far described subspecies decreases from west to east. *Erebia pronoe glottis* Fruhstorfer, 1920, distributed in the Pyrenees, represents the best-supported subspecies. Fixed mitochondrial amino acid changes emphasize the distinctness of this taxon, which might be well advanced in the process of speciation; we cannot even exclude the possibility that it has already reached full species rank. The genetic separation of the western Alps from the Valais, geographically separated along the main Alpine ridge, justifies the recognition of the taxa *E. pronoe vergy* (Ochsenheimer, 1807) and *E. pronoe psathura* Fruhstorfer, 1920, respectively, and is supported by both marker sets as well as by the existence of two different *Wolbachia* strains. The eastern Alpine subgroup resembles the nominotypical *E. pronoe pronoe*. The existence of at least one lineage in the southern Alpine area is supported by both marker sets. A finer separation based on the mitochondrial markers is not possible, because of recent introgression events affecting east Alpine haplotypes, as also indicated by the existence of *Wolbachia* strain 1. This population group could be assigned to the taxon *E. pronoe gardeina* Schawerda, 1924, or to *E. pronoe tarcenta* Fruhstorfer, 1920, considering their ranges. Nevertheless, a final decision requires further regional studies. *Erebia pronoe fruhstorferi* Warren, 1933 was accepted to be widely distributed in the Balkan mountain systems. However, our data suggest independent lineages in the western and eastern Balkan mountain systems of which only the eastern populations can be assigned to this taxon. The lineage of the Slovenian Alps is primarily based on mitochondrial markers and morphological characteristics[7]. The existence of an independent lineage for the highly isolated populations in the southern Carpathians, justifies the subspecies status of *E. pronoe regalis* Hormuzachi, 1937. Both marker sets display a differentiation, which was more pronounced in the nuclear than in the mitochondrial DNA.

Material and methods

Study species. The water ringlet butterfly *Erebia pronoe* (Esper 1780) is a typical species of gravel-interspersed rough grasslands and also wet meadows of the high montane to alpine zone[35]. *Erebia pronoe* is widespread from the Cordillera Cantabrica through the Pyrenees and into the Alps. However, the species occurs only as scattered and isolated populations in the Carpathians and the Balkan mountain systems. *Erebia pronoe* is a univoltine species with a flight period from late July to mid-September and overwinters as a L1 larva. The larvae feed on *Festuca ovina* and *F. quadriflora*[22].

Sampling design. 124 specimens representing 27 populations (1–8 specimens, mean: 4.6 specimens, see Fig. 1b; supplementary S15) were collected from the Pyrenees to the Tatra Mountains and the Balkan Peninsula

during the summers 2000–2014. Butterflies were captured with a hand net and frozen in liquid nitrogen in the field. The specimens were stored in a freezer at − 80 °C until analysis.

Genetic analysis. *DNA sequencing..* Genomic DNA was extracted from one leg using the EZNA Tissue DNA Kit (Omega Bio-Tek, Norcross, USA). The manufacturer's protocol was followed.

Variation in two mitochondrial genes and two nuclear genes was examined for the entire dataset. We sequenced the barcoding region encoding a fragment of cytochrome c oxidase subunit I (COI, 658 bp) and NADH dehydrogenase subunit I (NDI, 554 bp). COI was amplified with the primer pair LEP-F1 (5′-ATTCAA CCAATCATAAAGATATTGG-3′) and LEP-R1 (5′-TAAACTTCTGGATGTCCAAAAAATCA-3′)[36] using the following PCR protocol: 95 °C for 5 min, followed by 38 cycles at 95 °C for 30 s, 49 °C for 90 s, 72 °C for 60 s, and terminated with a final extension step at 68 °C for 30 min. NDI was amplified using the primer pair FAW-NDI (5′-TTCAAACCGGTGTAAGCCAGG-3′) and FAW-16S (5′-TAGAATTAGAAGATCAACCAGC-3′)[37] and the following PCR protocol: 95 °C for 5 min, 33 cycles at 95 °C for 30 s, 56 °C for 90 s, 72 °C for 60 s, and terminated at 68 °C for 30 min. Furthermore, the eukaryotic translation elongation factor 1 alpha (EF1α, 957 bp) and ribosomal protein S5 (RPS5, 610 bp) were sequenced. The nuclear markers isocitrate dehydrogenase (IDH, 709 bp), Nedd2-like caspase (NC, 610 bp), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 663 bp), and wingless (WG, 400 bp) did not provide sufficient variability among the Pyrenees, the western, southern, and eastern Alps. Therefore, only a test series of each region was examined and no further analysis of these markers was performed. For PCR protocols and primers, see[38,39].

The *Wolbachia* surface protein-coding gene (WSP, 549 bp) was amplified with the primer pair wsp81F (5′-TGG TCC AAT AAG TGA TGAAGA AAC-3′) and wsp 691R (5′-AAA AAT TAA ACG CTA CTC CA-3′)[40] using the following PCR protocol: 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s, 54 °C for 90 s, 72 °C for 60 s, and terminated with a final extension step at 68 °C for 30 min.

The PCR products were loaded onto a 1.4% agarose gel and stained with GelRed (Biotium, Fremton, USA) to check for successful amplification. Primers and dNTPs were deactivated with a mixture of FastAP and exonuclease I (Thermo Scientific, Dreieich, Germany). The amplified products were shipped to Macrogen Europe (Amsterdam, The Netherlands). Both sense and antisense strands were sequenced.

Data analyses. Our mitochondrial and nuclear sequences were assembled using GENEIOUS v. 10.2.3 and aligned using CLUSTALW implemented in BIOEDIT v. 7.2.6.1. The sequences of each marker set were concatenated and checked for stop codons using GENEIOUS. The GenBank accession numbers are given in the data availability statement and in more detail with the corresponding haplotypes/genotypes in supplementary S16. The nuclear dataset was phased in DNASP with default settings. Haplotype frequency, haplotype diversity (h), number of segregating sites (S), nucleotide diversity per gene (pi), and average number of nucleotide differences (k) were calculated using DNASP v. 6. The combined dataset was used to construct a TCS haplotype network with default settings using POPART v. 1.7. A mismatch analysis was performed separately for both marker sets in R using the packages 'adegenet' and 'pegas'.

A Bayesian tree was reconstructed based on the mitochondrial dataset and based on the nuclear dataset using BEAST v. 2.5. Published data for *Pararge aegeria* and *Erebia aethiops* were used as outgroups. The partition and substitution models were estimated using PARTITIONFINDER v. 2.1.1 based on the lowest Akaike Information Criterion (AIC). For the mtDNA dataset, the HKY model with empirical base frequencies and a gamma distribution with a category number of 4 were selected. We performed several analyses testing every option offered by Beast to select the best-fitting tree model. Based on the logarithmic likelihood and the explained sum of square (ESS) values, the coalescent constant population model performed best and was therefore selected for the final analysis. We used a relaxed clock log normal setting for the molecular clock and a clock rate of 0.0177[41].

For the nuclear dataset, using the settings proposed by Partitionfinder led to the early termination of the analysis. We obtained stable analyses and parameter values with ESS above 200, by avoiding partitions and using the HKY model with empirical base frequencies and a gamma distribution with a category number of 4. Based on the logarithmic likelihood and the explained sum of square (ESS) values, the coalescent exponential population model performed best and was therefore selected for the final analysis. We used a relaxed clock log normal setting for the molecular clock and a clock rate of 0.00177[41].

We performed the analyses with 40 million generations, collecting every 4000 iterations. After checking the MCMC chain for convergence in TRACER v. 1.7.1, a burn-in of 10% was applied. Three individual runs were performed and combined with LOGCOMBINER v. 1.8.4. TREEANNOTATOR v. 2.5 was used to generate a summary tree with common ancestral probability. FIGTREE v. 1.4.4 was used for the visualization.

The same protocol was followed to obtain an input and consensus tree for both datasets for an ancestral occurrence reconstruction analysis using RASP v. 4.2. This analysis was performed for both marker sets of *E. pronoe* and the outgroups of *E. melas*, *E. aethiops*, and *P. aegeria*. The individual sequences were assigned to occurrences coded as consecutive letters (A: Pyrenees; B: western Alps; C: eastern Alps; D: Balkan mountain systems; E: Carpathians). Based on the results of the BioGeoBears model test, an S-DEC analysis was performed with the maxarea = 5 setting, as well as a Bayesian Binary MCMC with 50,000 cycles and K81 instead of JC.

Preparation of genitalia. The genital capsule was removed with dissecting needles and macerated in a 10% potassium hydroxide solution for at least twelve hours. The genital apparatus was cleaned and the valvae and aedeagus were separated from the tegumen. The specimens were then fixed in euparal or Canada balsam on a microscope slide. Images were captured using a camera (Leica CFV450) mounted on a binocular microscope. To combine images of different depths of focus, we used a focus stacking technique (Helicon Focus). A combination of 6 landmarks and 39 sliding semi-landmarks[42] was used with the help of TPS software[43–45]. A general

Procrustes analysis was performed to obtain relative warps. The calculation of K Means clustering, hierarchical clustering based on Euclidian distance, and the generation of graphs were performed using R version 3.3.2[46], the implemented package R stats, and the R package networkD3 0.4[47].

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. All data generated or analysed during this study are included in this published article [and its supplementary information files]. GenBank accession number MZ190632-MZ190677 (*E.p* CO1); MZ345012-MZ345057 (*E.p* ND1); MZ190563-MZ190631 (*E.p* RPS5); MZ190749-MZ190750 (*E.m* RPS5); MZ190678-MZ190746 (*E.p* Ef1alpha); MZ190747-MZ190748 (*E.m* Ef1alpha), MZ358189-MZ358191 (WSP); MZ358183-MZ358185 (*E.m* CO1); MZ358186-MZ358189 (*E.m.* ND1).

References

1. Dincă, V. *et al.* DNA barcode reference library for Iberian butterflies enables a continental-scale preview of potential cryptic diversity. *Sci. Rep.* **5**, 20 (2015).
2. Vodă, R., Dapporto, L., Dincă, V. & Vila, R. Cryptic matters: Overlooked species generate most butterfly beta-diversity. *Ecography (Cop.)* **38**, 405–409 (2015).
3. Reidenbach, K. R. *et al.* Patterns of genomic differentiation between ecologically differentiated M and S forms of anopheles gambiae in West and Central Africa. *Genome Biol. Evol.* **4**, 1202–1212 (2012).
4. Gustafsson, A. L. S. *et al.* Genetics of cryptic speciation within an arctic Mustard, *Draba nivalis. PLoS One* **9**, 25 (2014).
5. Wiemers, M., Chazot, N., Wheat, C. W., Schweiger, O. & Wahlberg, N. A complete time-calibrated multi-gene phylogeny of the European butterflies. *Zookeys* **2020**, 97–124 (2020).
6. Schmitt, T., Louy, D., Zimmermann, E. & Habel, J. C. Species radiation in the Alps: Multiple range shifts caused diversification in Ringlet butterflies in the European high mountains. *Org. Divers. Evol.* **16**, 791–808 (2016).
7. Paučulová, L., Dzurinka, M., Šemeláková, M., Csanády, A. & Panigaj, Ľ. Phylogeography, genetic structure and wing pattern variation of *Erebia pronoe* (Esper, 1780) (Lepidoptera: Nymphalidae) in Europe. *Zootaxa* **4441**, 279–297 (2018).
8. Gratton, P. *et al.* Testing Classical Species Properties with Contemporary Data: How 'bad Species' in the Brassy Ringlets (*Erebia tyndarus* complex, Lepidoptera) Turned Good. *Syst. Biol.* **65**, 292–303 (2016).
9. Dincă, V., Lee, K. M., Vila, R. & Mutanen, M. The conundrum of species delimitation: A genomic perspective on a mitogenetically super-variable butterfly. *Proc. R. Soc. B Biol. Sci.* **286**, 25 (2019).
10. Dincă, V. *et al.* High resolution DNA barcode library for European butterflies reveals continental patterns of mitochondrial genetic diversity. *Commun. Biol.* **4**, 14 (2021).
11. Mortera, H. Una nueva subespecie de *Erebia pronoe* (Esper[1780]) (Lepidoptera: Nymphalidae, Satyrinae) de los Picos de Europa (Asturias, España). *Boletín SEA* **28**, 63–65 (2001).
12. Michel, F., Meglécz, E., Martin, J. F. & Descimon, H. *Erebia serotina* Descimon & de Lesse 1953 (Lepidoptera: Nymphalidae), a recurrent hybrid between two distantly related species. *Ann. Soc. Entomol. Fr.* **49**, 100–116 (2013).
13. Fruhstorfer, H. Altes und Neues über Erebien. *Arch. Nat.* **84**, 83–108 (1920).
14. Warren, B. C. S. Notes on Erebiid species. *Entomol. Rec.* **45**, 40–41 (1933).
15. Toews, D. P. L. & Brelsford, A. The biogeography of mitochondrial and nuclear discordance in animals. *Mol. Ecol.* **21**, 3907–3930 (2012).
16. Jiggins, F. M. Male-killing *Wolbachia* and mitochondrial DNA: Selective sweeps, hybrid introgression and parasite population dynamics. *Genetics* **164**, 5–12 (2003).
17. Smith, M. A. *et al. Wolbachia* and DNA barcoding insects: Patterns, potential, and problems. *PLoS One* **7**, 25 (2012).
18. Peña, C., Witthauer, H., Klečková, I., Fric, Z. & Wahlberg, N. Adaptive radiations in butterflies: Evolutionary history of the genus *Erebia* (Nymphalidae: Satyrinae). *Biol. J. Linn. Soc.* **116**, 449–467 (2015).
19. Gompert, Z., Forister, M. L., Fordyce, J. A. & Nice, C. C. Widespread mito-nuclear discordance with evidence for introgressive hybridization and selective sweeps in *Lycaeides. Mol. Ecol.* **17**, 5231–5244 (2008).
20. Narita, S., Nomura, M., Kato, Y. & Fukatsu, T. Genetic structure of sibling butterfly species affected by *Wolbachia* infection sweep: Evolutionary and biogeographical implications. *Mol. Ecol.* **15**, 1095–1108 (2006).
21. Kudrna, O., Pennerstorfer, J. & Lux, K. *Distribution Atlas of European Butterflies and Skippers* (Wissenschaftlicher Verlag-Peks, 2015).
22. Sonderegger, P. *Die Erebien der Schweiz (Lepidoptera: Satyrinae, Genus Erebia)* (Private Publication, 2005).
23. Kudrna, O. *et al.* Distribution Atlas of Butterflies in EuropeGesellschaft für Schmetterlingsschutz e.V, Halle. (2011).
24. Popescu-Gorj, A. A rare hybrid within the genus Erebia (Lep. Satyridae). *Trav. Muséum d'His-toire Nat. Grigore Antipa* **14**, 267–271 (1974).
25. Lalanne Cassou, B., Lalanne Cassou, C., Hinojosa, J. C., Vila, R. & Mérit, X. Étude d'un nouvel hybride d'Erebia pyrénéens *Erebia pronoe* (Esper, 1780) femelle x *Erebia meolans* (de Prunner, 1798) mâle. *Lépidoptères Rev. Lépidoptéristes Fr.* **27**, 85–95 (2018).
26. Lucek, K. Prevalence and relationship of endosymbiotic *Wolbachia* in the butterfly genus *Erebia.* (2021).
27. Sahoo, R. K. *et al.* Evolution of Hypolimnas butterflies (Nymphalidae): Out-of-Africa origin and Wolbachia-mediated introgression. *Mol. Phylogenet. Evol.* **123**, 50–58 (2018).
28. Schmitt, T. Biogeographical and evolutionary importance of the European high mountain systems. *Front. Zool.* **6**, 1–10 (2009).
29. Varga, Z. Geographische isolation und subspeziation bei den Hochgebirgs-Lepidopteren der Balkanhalbinsel. *Acta Entomol. Jugoslaviae* **11**, 5–39 (1975).
30. Zhang, L. B., Comes, H. P. & Kadereit, J. W. Phylogeny and quaternary history of the European montane/alpine endemic *Soldanella* (Primulaceae) based on ITS and AFLP variation. *Am. J. Bot.* **88**, 2331–2345 (2001).
31. Pauls, S. U., Lumbsch, H. T. & Haase, P. Phylogeography of the montane caddisfly *Drusus discolor*: Evidence for multiple refugia and periglacial survival. *Mol. Ecol.* **15**, 2153–2169 (2006).
32. Ujvárosi, L. *et al.* Divergence and speciation in the Carpathians area: Patterns of morphological and genetic diversity of the crane fly *Pedicia occulta* (Diptera:Pediciidae). *J. North Am. Benthol. Soc.* **29**, 1075–1088 (2010).
33. Schmitt, T., Habel, J. C., Rödder, D. & Louy, D. Effects of recent and past climatic shifts on the genetic structure of the high mountain Yellow-spotted ringlet butterfly *Erebia manto* (Lepidoptera, Satyrinae): A conservation problem. *Glob. Chang. Biol.* **20**, 2045–2061 (2014).
34. Ronikier, M. & Zalewska-Gałosz, J. Independent evolutionary history between the Balkan ranges and more northerly mountains in *Campanula alpina* s.l. (Campanulaceae): Genetic divergence and morphological segregation of taxa. *Taxon* **63**, 116–131 (2014).
35. Huemer, P. Biodiversit{ä}t von Schmetterlingen (Lepidoptera) im Gebiet des Naturparks Schlern. *Gredleriana* **7**, 233–306 (2007).

36. Hebert, P. D. N., Ratnasingham, S. & DeWaard, J. R. Barcoding animal life: Cytochrome c oxidase subunit 1 divergences among closely related species. *Proc. R. Soc. B Biol. Sci.* **270**, 1–4 (2003).
37. Weller, S. J., Pashley, D. P., Martin, J. A. & Constable, J. L. Phylogeny of noctuoid moths and the utility of combining independent nuclear and mitochondrial genes. *Syst. Biol.* **43**, 194–211 (1994).
38. Wahlberg, N. & Wheat, C. W. Genomic outposts serve the phylogenomic pioneers: Designing novel nuclear markers for genomic DNA extractions of lepidoptera. *Syst. Biol.* **57**, 231–242 (2008).
39. Wahlberg, N., Peña, C., Ahola, M., Wheat, C. W. & Rota, J. PCR primers for 30 novel gene regions in the nuclear genomes of lepidoptera. *Zookeys* **2016**, 129–141 (2016).
40. Braig, H. R., Zhou, W., Dobson, S. L. & O'Neill, S. L. Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. *J. Bacteriol.* **180**, 2373–2378 (1998).
41. Papadopoulou, A., Anastasiou, I. & Vogler, A. P. Revisiting the insect mitochondrial molecular clock: The mid-aegean trench calibration. *Mol. Biol. Evol.* **27**, 1659–1672 (2010).
42. Bookstein, F. L. Landmark methods for forms without landmarks: Localizing group differences in outline shape. *Proc. Work. Math. Methods Biomed. Image Anal.* **1**, 279–289 (1996).
43. Rohlf, F. J. *tpsDig2. Ecology and Evolution and Ecology* (State University of New York at Stony Brook, 2017).
44. Rohlf, F. J. *tpsRelw. Ecology and Evolution and Ecology* (State University of New York at Stony Brook, 2017).
45. Rohlf, F. J. *tpsUtil. Ecology and Evolution and Ecology* (State University of New York at Stony Brook, 2019).
46. R core Team. *R: A Language and Environment for Statistical Computing.* (2017).
47. Allaire, J. J., Gandrud, C., Russell, K. & Yetman, C. networkD3: D3 JavaScript Network Graphs from R. *R Packag.* version 0.4 (2017).

Author contributions

M.W. and T.S. conceived and designed the study. T.S. sampled most of the material; partly assisted by L.R. and Z.V.; L.R. sampled the material in Romania. M.W. and D.K. performed the data acquisition. D.K. and M.W. performed the morphometric analysis. All other analyses were performed by M.W. All authors aided in the interpretation of the data. M.W., Z.V. and T.S. wrote the manuscript. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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CHAPTER 6
General Discussion

Europe and the Northern Hemisphere in general are subject to quasi-periodic glacial-interglacial alternations, which have occurred with 100 kyr cycles since the mid-Pleistocene Transition (Clark et al. 2006). Each separate glacial cycle, the amplitude of which increased in magnitude with the mid-Pleistocene Transition, has had a profound impact on climatic and ecological conditions in Europe. As climatic and ecological conditions change, the ranges of species change, which in turn significantly influences their differentiation. According to their refugia and later centres of dispersal, the fauna of Europe is roughly divided into Mediterranean, continental, and alpine / arcto-alpine species. During a glacial period, Mediterranean species retreat from northern and central Europe and concentrate on at least one of the Mediterranean peninsulas, though extra-Mediterranean refugia have also been recorded for the Würm glacial period. Continental species retreat from northern and central Europe similarly but maintain their distributional centres mostly in extra-Mediterranean glacial refugia. Another distribution type with extra-Mediterranean glacial refugia are boreo-montane species, which are linked to boreal coniferous forests and the mountain forest zone. Arcto-alpine species spread during glacial periods over the cold steppes. With the end of a glacial period, they retreat to their refugia in the northern tundra belt and/or in the high mountains of Europe (Schmitt 2009).

Collapsing distributional boundaries are often associated with local extinctions rather than migration (Bennet et al. 1991), causing very dynamic regions such as northern Europe to exhibit genetic impoverishment compared to more stable regions such as the Mediterranean peninsulas due to the constant alternation of genetic bottlenecks and founder effects (Hewitt 2004). If persistence in refugia is not possible during pessimal periods, an affected species becomes extinct. However, if there is no renewed gene flow after the onset of differentiation between two or more sub-regions that have become disjunct, the differentiation of the sub-regions will continue (Willis and Niklas 2004). Yet, the range dynamics associated with glacial cycles can also prevent the onset of differentiation processes or overwrite differentiations that have already begun, resulting in evolutionary stasis.

Although poikilothermic species are typically more affected by climatic changes than homeothermic species (Richards 1973), the genus *Erebia* survived the Quaternary with its diverse glacial cycles as a very species-rich genus. Unlike other genera, *Erebia* even increased in species richness during climatically much more extreme phases of the Neogene (Peña et al. 2015). The genus' high diversity can be attributed to its climatic niche specialization, which includes very narrow climatic niches and pronounced niche maintenance (Klečková et al. 2023). In addition, general niche preservation and niche overlap among sister taxa have supported allopatric speciation of the genus, allowing differentiation to proceed even during glacial maxima. The differentiation processes induced by glacial-interglacial cycles are found at the interspecific level and are detectable at the intraspecific level of *Erebia* (e.g., (Vila et al. 2005; Albre et al. 2008).

The studied species *Erebia aethiops* and *Erebia pronoe* also show various differentiations, which probably have their origin in the Mindel glacial, which exceeded the preceding Günz glacial in its intensity (Clark et al. 2006). Although the intensity of the glacial period determined the general degree of glaciation and permafrost spreading in Europe, regions were affected differently depending on latitude and altitude (Hewitt 2004). Accordingly, the distributional ranges of *Erebia aethiops* and *Erebia pronoe* were also affected to different degrees due to their respective ecological niches, which partly explains the differences in phylogenetic and biogeographic patterns of both species.

The phylogenetic patterns of *Erebia aethiops* are comparably shallow despite its presumed origin at the end of the Pliocene (Klečková et al. 2023). Only the isolated Asian occurrences suggest greater differentiation and longer-lasting isolation. This could not be investigated in more detail due to the limited data set but is considered likely. The low differentiation along the main Alpine ridge indicates

intense gene flow and thus lower population isolation. *Erebia aethiops*, with a distribution from the colline to montane altitudes, is exposed to warmer climatic conditions than typical alpine species like *Erebia pronoe*. Due to this adaptation to warmer habitats, flight activity is less constrained by lower temperatures in the morning hours. Higher temperatures may be more likely to limit the flight activity of males, which cool off in the shade of shrubs and trees when threatened with overheating (Slamova et al. 2011). By exploiting a variety of microhabitats and sex-specific behavioural adaptations, *Erebia aethiops* can maintain high flight activity throughout the flight season. In this context, open forests not only provide the necessary microhabitat structures but also promote butterfly orientation and dispersal along forest edges (Junker and Schmitt 2010). Thus, the ecological niche of *Erebia aethiops* results in higher mobility and dispersal potential (Slamova et al. 2013), which supports the formation of stable metapopulations and gene flow between occurrences (Baguette et al. 2000). The better connectivity of occurrences supports evolutionary stasis and complicates differentiation processes, resulting in less differentiated phylogenetic structures compared to *Erebia pronoe*.

In contrast, *Erebia pronoe*, with its link to humid and stony alpine meadows, is constrained in gene flow under both glacial and interglacial conditions. During glacial periods, the distribution was disjunct due to the required proximity to high mountain systems, which mitigated drought stress (Schmitt 2009; Schmitt and Varga 2012). With the transition to an interglacial or interstadial period, a shift in altitude occurred, also leading to disjunct distributions in different high mountains. Disjunct distributions and restricted gene flow can also occur within mountains, as shown by the differentiations within the Pyrenees, the Western Alps, and the Balkans (Schmitt 2009).

The extreme climatic conditions of high mountains require thermoregulatory adaptations of *Erebia pronoe*, which led to the formation of narrow climatic niches with a high degree of niche conservation. Niche conservation in narrow climatic niches has caused dispersal or isolation during glacial-interglacial cycles along high mountain altitudes, which may have contributed to the genus's high diversity in European high mountains (Schmitt et al. 2016; Klečková et al. 2023). Larger dispersal efforts are not required to follow vertical shifts in altitude (Schmitt 2009) and are also subject to a trade-off between mobility and fecundity (Tigreros and Davidowitz 2019). Further, a filtering effect results from the topographic structures of the high mountains, directing the flight behaviour of females toward more passive forms of flight. Thus, acting as a selection pressure against more active flight and risky migration toward safe dispersal and reproduction (Plazio et al. 2020a, b).

Selection toward philopatric behaviour and against greater dispersal performance explains the observed low dispersal potential of *Erebia pronoe*, which is also found in other *Erebia* species (Polic et al. 2014; Ehl et al. 2016). The dispersal behaviour of butterflies is considered plastic, allowing them to respond to environmental stimuli like resource availability (Hovestadt and Nieminen 2009). This includes the formation of home ranges, which are considered to represent a response to the sufficient availability of resources on sub-plots. The formation of home ranges is accompanied by a restriction of dispersal behaviour, which has been demonstrated for other mountain species (e.g., (Polic and Grill 2013; Ehl et al. 2018), and seems plausible for the population of *Erebia pronoe* in the Hohe Tauern.

In contrast, there are influencing factors that are expected to increase the dispersal performance. These include the size of the study area, population density, and lack of resources (Schneider 2003; Timus et al. 2017; Evans et al. 2020). Accordingly, a significant increase in dispersal performance was expected in the Pyrenean population of *Erebia pronoe*. It would be necessary to survey another reference plot to determine whether the population was incapable of adjusting the dispersal performance because it had reached its limits or whether a response did not occur because the stimulus of the influencing factors was insufficient.

As mentioned earlier, larger dispersal efforts are not required of alpine butterflies to shift their altitudinal range (Schmitt 2009). However, the formation of metapopulation networks may be

impeded by high philopatry (Baguette et al. 2000). The formation of metapopulations enables a species to survive in regions with sub-optimal habitats (Nouhuys 2009). In addition, metapopulations also contribute to regional gene flow and biodiversity conservation (Vanden Broeck et al. 2017; Junker et al. 2021). The extent to which *Erebia pronoe* is capable of forming metapopulation networks remains questionable. Especially since the occurrence of phylogenetic structures within individual high-altitude systems also suggests regional constraints on gene flow.

Despite some significant differences in the two species´ phylogenies, *Erebia aethiops* showed the most differentiation at its range limits. Species are usually exposed to less favourable weather conditions at their range margins (Melbourne and Hastings 2008), which can result in greater fluctuations in abundance (Thomas et al. 1994). According to the drift-barrier hypothesis, as population size decreases, so does the selection pressure acting on populations. The decrease in selection pressure results in a state of effective evolutionary neutrality, which promotes gene drift (Lynch et al. 2016). The drift-barrier hypothesis thus provides an explanation for the stronger differentiations at the range margins of both studied *Erebia* species.

Despite the weaker differentiation of mtDNA between populations of *Erebia aethiops*, the changes of major alleles of allozymes and the presence of endemic alleles and major allele changes in rear-edge populations like Spiazzi, Campolaro, and Trigrad also suggest local adaptations. The high infection rate with *Wolbachia* in the Alps (Lucek et al. 2021) also suggests an influence of this endoparasite on gene flow and mtDNA phylogenies. Infestations of different *Wolbachia* strains at the distributional limits of Asian occurrences (Ilinsky and Kosterin 2017) may have contributed to the greater differentiation of these regions, just as in *Erebia pronoe*.

In these isolated populations at the distribution boundaries of a species, lower selection pressure and increased genetic drift may result in the accumulation of mutations in alleles. The asexual inheritance and smaller effective population size of the haploid genome of mtDNA provide lower selection pressure, which favours the accumulation of mutation (Lynch 1996). The mitochondrial genome encodes only a fraction of the proteins necessary for its function, while the majority is encoded in mitochondrially targeted genes (N-mtDNA) in the nucleus (Sloan et al. 2017). Processes like cellular respiration, mitochondrial translation, the formation of the OXPHOS system, or the formation of ribosomes rely on the interaction of mtDNA and N-mtDNA. The accumulation of mutations in mtDNA can have a negative impact on the interaction of mtDNA and N-mtDNA, resulting in incompatibilities (Sloan et al. 2017). These incompatibilities can arise within a population or occur when populations come into contact with each other. If mito-nuclear incompatibilities occur between populations, they can prevent hybridization upon secondary contact without the formation of a "fitness valley" (see Bateson-Dobzhansky-Muller incompatibilities, (Orr 1996) and thus support advancing speciation or differentiation. Because of nuclear alleles, the heterogametic sex carries an increased risk of mito-nuclear incompatibilities (see Haldane's rule, (Toews and Brelsford 2012), which can lead to asymmetric introgression.

If mito-nuclear incompatibilities occur within a population, males are particularly affected, since mutations that are not deleterious to females can accumulate in the mtDNA without selection against them (see "the mother's curse"; (Gemmell et al. 2004). If the potential of N-mtDNA to adapt to the mtDNA change is exceeded, introgression to maintain mtDNA and N-mtDNA compatibility can occur (Sloan et al. 2017). In general, this favours the introgression of mtDNA (Currat et al. 2008). Introgression of mtDNA can also occur interspecifically and is aided in establishing distributional boundaries by population abundance differences (see Hubbs effect, (Hubbs 1955).

The Pyrenees' strong differentiations and *Erebia pronoe's* pronounced mito-nuclear discordance are most likely the results of a combination of the previously mentioned processes. In turn, the fixed nature of amino acid changes in each individual studied in the region suggests positive selection and local adaptation (Toews and Brelsford 2012; Abbott et al. 2013). The uniform amino acid pattern that occur in all known species hybridizing with *Erebia pronoe* in the Pyrenees, as well as evidence of

backcrossing that has occurred (Lalanne Cassou et al. 2018), suggest interspecific introgression as a major cause of the strong differentiation of this region.

Reproductive isolation is considered a central feature of many species' concepts, yet there is multiple evidence for the successful mating of distantly related *Erebia* species. Butterflies in general have high mating success, yet there is also some evidence of female reproductive failure (female mating failure, or "FMF", (Rhainds 2010, 2019). Diverse ecological traits such as flight activity, population density, sex ratio, choosiness, and mating synchrony influence female mating success. While male mating failure results only in individual reductions in reproduction, lifetime female mating failure also potentially negatively affects overall population abundance. In order to avoid mating failure and preserve the high energy and time investment in reproduction, diverse evolutionary adaptations were developed to ensure female mating success (Rhainds 2010, 2019). Females may seek males or locations attractive to males in the face of lower population densities and mate encounter probabilities. Another phenomenon is the lowered selectivity in mate choice in the absence of fertilization or low numbers of males (Rhainds 2010).

The lack or failure of reproductive isolation may be a result of mating failure avoidance. In Michel et al. (2013), a protandry-induced overlap of *Erebia epiphron* females and *Erebia pronoe* males, caused by protandry, has been implicated as a cause for the regular occurrence of the hybrid *Erebia serotina*. As likely as this explanation for the occurrence of *Erebia serotina* is, it does not explain the cases of interspecific hybridization of *Erebia pronoe* females with the earlier-flying *Erebia meolans* males. Therefore, attention should be paid to the intraspecific sex ratio. Asymmetry of the intraspecific sex ratio increases the risk of FMF, which leads to an adaptation in the female mating behaviour and a subsequent increasing likelihood of interspecific hybridization.

As mentioned above, intraspecific asymmetry may be a natural consequence of portrandry. Another reason for asymmetry in intraspecific sex ratio may be infection with *Wolbachia*. Especially during the first infection, the sex ratio of a population can be massively disrupted by effects such as male killing (Werren et al. 2008). Because of the high rate of *Wolbachia* infection in the Pyrenees and its ability to infect across species, population structure-influencing events in the Pyrenees were or are likely. With the low prospect of mating success, the probability of behavioural adaptation in *Erebia* females is increased and may promote cross-species gene flow.

Given the widespread occurrence of *Wolbachia* and the frequency of protandry in butterflies, asymmetries in sex ratio and subsequent promoted interspecific hybridization should be more common. The high proportion of barcode sharing and fertile hybridization within the European butterflies suggest this. An estimated 16 % of European butterflies engage in interspecific hybridization, of which about half produce fertile offspring (Descimon and Mallet 2010). Furthermore, 15 % of European butterfly species share genetic barcodes, which cannot be attributed solely to methodological errors (Dincă et al. 2021). Instead, diverse cases of barcode sharing within the genus *Erebia*, in combination with low genetic distances between the closest related species, indicate further interspecific gene flow, supporting the hypothesis presented here.

These cases of barcode sharing and complex phylogenies cannot be resolved with the analysis of mitochondrial markers alone. Only by a combination of different markers facilitates the construction and interpretation of these phylogenies. At the same time, when interpreting the discovered patterns, factors influencing gene flow, such as migratory potential, population structure, or *Wolbachia* infections of a species, must be taken into consideration (see Toews and Brelsford 2012). Only by combining different genetic markers and considering ecological aspects can a possible assessment of phylogenetic structures be made. Nevertheless, phylogenetic categorization remains difficult, especially for allopatric distributions such as the Pyrenean occurrences of *Erebia pronoe* and the Asian occurrences of *E. aethiops*. Differentiation and speciation are ongoing processes that pass through numerous stages and can therefore complicate categorizations (Descimon and Mallet 2010).

Regardless of the category used, sub-areas in an allopatric distribution are significant centres of differentiation that contribute significantly to biodiversity (Mutanen et al. 2012), as has also been shown in the species presented here. However, the inferred interest for potential conservation action must consider the specific conditions of the occurrence. Smaller occurrences are potentially more vulnerable to intrinsic and extrinsic stressors (Melbourne and Hastings 2008) and could be stabilized by lower levels of fragmentation and isolation. Here, the population strength of the regions to be connected must also be considered, as gene flow occurs towards the smaller (Hubbs 1955) and expanding populations (Currat et al. 2008). If hybridization and subsequent introgression occur after secondary contact has occurred, previously not combined alleles might be combined, which carries the risk of incompatibilities and hybrid breakdown (Orr 1996). Thus, bringing previously isolated occurrences into contact may be associated with risks to occurrence stability and diversity, which depend, among other things, on the duration of isolation or the degree of differentiation.

Another potential risk to be considered is the introduction of *Wolbachia* into an uninfected population. In *Erebia pronoe*, secondary contact between the Eastern Alps and the Southern Alps resulted in the introduction of *Wolbachia* and the loss of mitochondrial diversity in the Southern Alps. A similar scenario would be likely for populations in the Valais and Balkan mountains. The populations in both regions are uninfected or barely infected with *Wolbachia* and would thus be susceptible to infection as a result of hybridization with the highly infected populations of the eastern Alps. A subsequent selective sweep might then also lead to a loss of mitochondrial differentiation in these regions. In contrast, the high infection rate with a competing *Wolbachia* strain could protect populations in the Western Alps from a selective sweep and subsequent loss of mtDNA differentiation. The extent to which a population can be protected from *Wolbachia* infection in the long term is difficult to assess due to the horizontal transmission of *Wolbachia* demonstrated for other *Erebia* species as well as differences in immune response depending on the *Wolbachia* strain (Siozios et al. 2008; Zug and Hammerstein 2015; Lucek et al. 2021).

Similarly, little is known about the prevalence of *Wolbachia* in European occurrences of *E. aethiops*. Apart from one Swiss region, previous studies have only been done for Asian populations (Ilinsky and Kosterin 2017; Lucek et al. 2021). In *E. aethiops*, a potential loss of intraspecific diversity would be expected in case of increased gene flow between the Eastern Alps and the Southern Alps or the Balkan mountains occurrences. The Plöckenpass population is grouped as a central and eastern Alps population based on allozymes but clearly separates from the central and eastern Alps and groups with the southern Alpine populations based on mtDNA. This suggests unilateral introgression and might indicate the presence of different *Wolbachia* strains in *E. aethiops* populations in Europe. Isolation in different glacial refugia supports infection with different *Wolbachia* strains, as demonstrated for *Erebia pronoe* and other *Erebia* species (Lucek et al. 2021). Potential infection with different *Wolbachia* strains would restrict gene flow between putatively contiguous occurrences and contribute to the fragmentation of *Erebia aethiops* occurrences. Therefore, studies on range fragmentation must take genetic aspects into account, too. This may require balancing the protection of biodiversity and the protection of species.

Regardless of the focus of potential conservation measures, a primary focus must be on maintaining or enhancing habitat quality (see Thomas et al. 2011; Krämer et al. 2012; Wagner 2020). Most *Erebia* species require structurally rich habitats to utilize microclimate in microhabitats for thermoregulation and reduction of climatic stress (see Stuhldreher et al. 2014; Kleckova and Klecka 2016; Konvička et al. 2016). Species are often under climatic stress, especially at their distributional boundaries (Hiyama et al. 2012). However, these regions are also important centres of diversity and speciation (Klečková et al. 2023), as in the case of *Erebia aethiops* and *Erebia pronoe*. These speciation processes require an ecological opportunity and freely available resources, which require high habitat quality (Yoder et al. 2010; Peña et al. 2015). Accordingly, with the large-scale decline in habitat quality in many landscapes in Europe, important speciation centres are being threatened, as also shown by the example of *Erebia pronoe* in Cauterets.

With the transition from summer to autumn, a natural scarcity of nectar resources occurs (Couvillon et al. 2015; Garbuzov et al. 2020). The wide range of nectar plants used in the Hohe Tauern and Cauterets allows *Erebia pronoe* to use the scarcer resources in a flexible and opportunistic manner. Thus, a competitive situation with overlapping flight periods with other species such as *Erebia nivalis* can be largely avoided, and a stable population with a high abundance, such as at the site in the Hohe Tauern, can be established. Still, the low abundance at the Cauterets site indicates that opportunistic use was not sufficient to facilitate a larger population. Several stressors interacted with one another and increased the competitive pressure in the region. The high grazing pressure had reduced the resource supply in the area. At the same time, a resource competitor that is superior in many aspects was introduced in large numbers. The resource depletion caused by grazing has less impact on the introduced honeybee, since *Apis mellifera* can respond to resource scarcity by adjusting dispersal performance (Steffan-Dewenter and Kuhn 2003), which *Erebia pronoe* apparently is unable to do. Honey bees are also superior to butterflies and other naturally occurring pollinators in other aspects of nectar uptake (Torné-Noguera et al. 2016; Klumpers 2017; Wignall et al. 2020). The resulting exploitative competition of honey bees correlates with their colony numbers and proximity to natural pollinator networks (Henry and Rodet 2018). The Cauterets population was subjected to competition in both density and distance that resulted in a decline in nectar uptake of over 50 % among natural pollinators even under more favourable conditions (Henry and Rodet 2020). The resulting additional depletion of resources can lead to further degradation of habitat quality and affect natural pollinator networks (Henry and Rodet 2018; Ropars et al. 2019, 2020; Wignall et al. 2020), as in the case of the *Erebia pronoe* population at Cauterets.

The loss or decline of indicator species such as *Erebia pronoe* or *Erebia aethiops* is not only problematic for intraspecific diversity but suggests broader problems for the resident community. As was evident at the Cauterets site, the decline in abundance of *Erebia pronoe* indicates a fundamental disturbance of the habitat due to overuse. With the loss of key species, co-extinction can occur in disturbed communities, leading to further declines in biodiversity and ecosystem services. Therefore, comprehensive monitoring is needed to determine the recent status of the study species and its associated habitat, allowing us to examine the effects of cumulative stressors on habitat quality, which may only become apparent upon closer examination. Since resources for conservation are often limited, prioritization is necessary. Comprehensive phylogenetic and biogeographic studies are needed to allow prioritization of lineages and regions of particular conservation value. These studies must be based on a combination of different markers to compensate for the methodological shortcomings of individual markers. The resulting complex systems may require balancing between different aspects of biodiversity, as the examples presented here have shown.

Summary and outlook

In this work, the necessity of considering ecological aspects for the interpretation of phylogenetic patterns was demonstrated. The use of single genetic marker systems has often proven to be insufficient for the reconstruction of reliable phylogenies and could have led to misinterpretations of phylogenetic and biogeographic structures, especially in the case of *Erebia pronoe*. Currently, the application of next-generation sequencing techniques is often not possible for biogeographic and phylogenetic studies due to time and cost constraints, which is why the combination of diverse genetic markers still has its justification and can also serve as a basis for subsequent NGS analyses. In this work, we demonstrated how the information of the different markers can be combined and how possible contradictions between markers can be resolved. In particular, the importance of incorporating information about *Wolbachia* infections and the need to identify the diverse *Wolbachia* strains were highlighted. In addition, we considered the influence of *Wolbachia* on abundance and population structure and how these aspects, together with other ecological factors such as dispersal potential, may contribute to interspecific hybridization. On this basis, a hypothesis for the frequent hybridization of *Erebia pronoe* with other *Erebia* species was developed.

By comparing two realized ecological niches of *Erebia pronoe* in the field, a more accurate idea of the potential niche was obtained. The identified population structure and resource use allow the species to respond to biotic and abiotic stressors by providing flexibility and hedging against the potential stochasticities of its habitat. Simultaneously, the limits of this flexibility at Cauterets site were demonstrated by the cumulative stressors of anthropogenic use. The negative impact of exploitative competition has been demonstrated for natural pollinators such as wild bees (e.g., (Henry and Rodet 2018; Ropars et al. 2020), but there have been few studies on the potential exploitation competition between honey bees and butterflies. Existing studies have surveyed butterflies only incidentally, resulting in a small amount of data with no statistical power (Osteen et al. 2018; Ropars et al. 2019; Wignall et al. 2020). Furthermore, these studies were conducted at a time of high nectar supply, allowing for spatial or temporal niche partitioning that may mask the competition effect (Balfour et al. 2015). As agricultural systems continue to become more impoverished, there is an increasing shift of apiaries to natural and semi-natural landscapes, which could further increase pressure on natural pollinators. This subtle form of habitat degradation needs more attention to protect insect biodiversity. To ensure better protection of natural pollinators, new limits for apiaries need to be determined. The density and distance limits proposed by Henry and Rodet (2020) were established with respect to wild bees under conditions of high resource availability. Future research needs to identify thresholds for less potent pollinators and under less optimal resource availability to derive recommendations for other habitats, seasons and pollinators.

References

Abbott R, Albach D, Ansell S, et al (2013) Hybridization and speciation. J Evol Biol 26:229–246. https://doi.org/10.1111/j.1420-9101.2012.02599.x

Albre J, Gers C, Legal L (2008) Molecular phylogeny of the Erebia tyndarus (Lepidoptera, Rhopalocera, Nymphalidae, Satyrinae) species group combining CoxII and ND5 mitochondrial genes: A case study of a recent radiation. Mol Phylogenet Evol 47:196–210. https://doi.org/10.1016/j.ympev.2008.01.009

Baguette M, Petit S, Queva F (2000) Population spatial structure and migration of three the same habitat butterfly species within network : consequences for conservation. J Appl Ecol 37:100–108.https://doi.org/10.1046/j.1365-2664.2000.00478.x

Balfour NJ, Gandy S, Ratnieks FLW (2015) Exploitative competition alters bee foraging and flower choice. Behav Ecol Sociobiol 69:1731–1738. https://doi.org/10.1007/s00265-015-1985-y

Bennett KD, Tzedakis PC, Willis KJ (1991) Quaternary refugia of north European trees. J Biogeogr 18:103-115. https://doi.org/10.2307/2845248

Clark PU, Archer D, Pollard D, et al (2006) The middle Pleistocene transition: characteristics, mechanisms, and implications for long-term changes in atmospheric pCO2. Quat Sci Rev 25:3150–3184. https://doi.org/10.1016/j.quascirev.2006.07.008

Couvillon MJ, Riddell Pearce FC, Accleton C, et al (2015) Honey bee foraging distance depends on month and forage type. Apidologie 46:61–70. https://doi.org/10.1007/s13592-014-0302-5

Currat M, Ruedi M, Petit RJ, Excoffier L (2008) The hidden side of invasions: Massive introgression by local genes. Evolution 62:1908–1920. https://doi.org/10.1111/j.1558-5646.2008.00413.x

Descimon H, Mallet J (2010) Bad Species. Ecology 219–249

Dincă V, Dapporto L, Somervuo P, et al (2021) High resolution DNA barcode library for European butterflies reveals continental patterns of mitochondrial genetic diversity. Commun Biol 4:1-11. https://doi.org/10.1038/s42003-021-01834-7

Ehl S, Dalstein V, Tull F, et al (2016) Specialized or opportunistic—how does the high mountain

endemic butterfly Erebia nivalis survive in its extreme habitats? Insect Sci 25:161–171. https://doi.org/10.1111/1744-7917.12400

Ehl S, Hostert K, Korsch J, et al (2018) Sexual dimorphism in the alpine butterflies Boloria pales and Boloria napaea: differences in movement and foraging behavior (Lepidoptera: Nymphalidae). Insect Sci 25:1089–1101. https://doi.org/10.1111/1744-7917.12494

Evans LC, Sibly RM, Thorbek P, et al (2020) The importance of including habitat - specific behaviour in models of butterfly movement. Oecologia. https://doi.org/10.1007/s00442-020-04638-4

Garbuzov M, Balfour NJ, Shackleton K, et al (2020) Multiple methods of assessing nectar foraging conditions indicate peak foraging difficulty in late season. Insect Conserv Divers 13:532–542. https://doi.org/10.1111/icad.12420

Gemmell NJ, Metcalf VJ, Allendorf FW (2004) Mother's curse: The effect of mtDNA on individual fitness and population viability. Trends Ecol Evol 19:238–244. https://doi.org/10.1016/j.tree.2004.02.002

Henry M, Rodet G (2018) Controlling the impact of the managed honeybee on wild bees in protected areas. Sci Rep 8:1–10. https://doi.org/10.1038/s41598-018-27591-y

Henry M, Rodet G (2020) The apiary influence range: A new paradigm for managing the cohabitation of honey bees and wild bee communities. Acta Oecologica 105:103555. https://doi.org/10.1016/j.actao.2020.103555

Hewitt GM (2004) Genetic consequences of climatic oscillations in the Quaternary. Philos Trans R Soc B Biol Sci 359:183–195. https://doi.org/10.1098/rstb.2003.1388

Hiyama A, Taira W, Otaki JM (2012) Color-pattern evolution in response to environmental stress in butterflies. Front Genet 3:1–6. https://doi.org/10.3389/fgene.2012.00015

Hovestadt T, Nieminen MJ (2009) Costs and benefits of dispersal in butterflies. In: Settele J, Shreeve T, Konvička M, Dyck H Van (eds) Ecology of butterflies in Europe. Cambridge University Press, pp 97–106

Hubbs CL (1955) Hybridization between Fish Species in Nature. Syst Zool 4:1-20. https://doi.org/10.2307/2411933

Ilinsky Y, Kosterin OE (2017) Molecular diversity of Wolbachia in Lepidoptera: Prevalent allelic content and high recombination of MLST genes. Mol Phylogenet Evol 109:164–179. https://doi.org/10.1016/j.ympev.2016.12.034

Junker M, Konvicka M, Zimmermann K, Schmitt T (2021) Gene-flow within a butterfly metapopulation: the marsh fritillary Euphydryas aurinia in western Bohemia (Czech Republic). J Insect Conserv 25:585–596. https://doi.org/10.1007/s10841-021-00325-8

Junker M, Schmitt T (2010) Demography, dispersal and movement pattern of Euphydryas aurinia (Lepidoptera: Nymphalidae) at the Iberian Peninsula: An alarming example in an increasingly fragmented landscape? J Insect Conserv 14:237–246. https://doi.org/10.1007/s10841-009-9250-1

Kleckova I, Klecka J (2016) Facing the heat: Thermoregulation and behaviour of lowland species of a cold-dwelling butterfly genus, Erebia. PLoS One 11:1–16. https://doi.org/10.1371/journal.pone.0150393

Klečková I, Klečka J, Faltýnek FZ, et al (2023) Climatic niche conservatism and ecological diversification in the Holarctic cold-dwelling butterfly genus Erebia. Insect Syst Divers 7:1-23. https://doi.org/10.1093/isd/ixad002

Klumpers S (2017) Beyond random and forbidden interactions in plant-pollinator networks: how optimizing energy gain results in morphological matching among subalpine Asteraceae and their flower- visitors. Doctoral dissertation, Leiden University

Konvička M, Beneš J, Čížek O, et al (2016) Has the currently warming climate affected populations of the mountain ringlet butterfly, Erebia epiphron (Lepidoptera: Nymphalidae), in low-elevation mountains? Eur J Entomol 113:295–301. https://doi.org/10.14411/eje.2016.036

Krämer B, Poniatowski D, Fartmann T (2012) Effects of landscape and habitat quality on butterfly communities in pre-alpine calcareous grasslands. Biol Conserv 152:253–261. https://doi.org/10.1016/j.biocon.2012.03.038

Lalanne Cassou B, Lalanne Cassou C, Hinojosa JC, et al (2018) Étude d'un nouvel hybride d'Erebia pyrénéens Erebia pronoe (Esper, 1780) femelle x Erebia meolans (de Prunner, 1798) mâle. Lépidoptères - Rev des Lépidoptéristes Fr 27:85–95

Lucek K, Bouaouina S, Jospin A, et al (2021) Prevalence and relationship of endosymbiotic Wolbachia in the butterfly genus Erebia. BMC Ecol Evol 21:1–12. https://doi.org/10.1186/s12862-021-01822-9

Lynch M (1996) Mutation accumulation in transfer RNAs: Molecular evidence for Muller's ratchet in mitochondrial genomes. Mol Biol Evol 13:209–220. https://doi.org/10.1093/oxfordjournals.molbev.a025557

Lynch M, Ackerman MS, Gout JF, et al (2016) Genetic drift, selection and the evolution of the mutation rate. Nat Rev Genet 17:704–714. https://doi.org/10.1038/nrg.2016.104

Melbourne BA, Hastings A (2008) Extinction risk depends strongly on factors contributing to stochasticity. Nature 454:100–103. https://doi.org/10.1038/nature06922

Michel F, Meglécz E, Martin JF, Descimon H (2013) Erebia serotina Descimon & de Lesse 1953 (Lepidoptera: Nymphalidae), a recurrent hybrid between two distantly related species. Ann la Soc Entomol Fr 49:100–116. https://doi.org/10.1080/00379271.2013.774741

Mutanen M, Hausmann A, Hebert PDN, et al (2012) Allopatry as a Gordian Knot for Taxonomists: Patterns of DNA Barcode Divergence in Arctic-Alpine Lepidoptera. PLoS One 7:1-9. https://doi.org/10.1371/journal.pone.0047214

Nouhuys S (2009) Metapopulation Ecology. eLS 1–9. https://doi.org/10.1002/9780470015902.a0021905

Orr HA (1996) Dobzhansky, Bateson, and the genetics of speciation. Genetics 144:1331–1335. https://doi.org/10.1093/genetics/144.4.1331

Osteen JM, Necarsulmer AM, Brown JJF, Nguyen NH (2018) and Native Pollinators on Late-Blooming Desert Scrub. CEC:1–7. https://doi.org/10.21973/N39S9F

Peña C, Witthauer H, Klečková I, et al (2015) Adaptive radiations in butterflies: Evolutionary history of the genus Erebia (Nymphalidae: Satyrinae). Biol J Linn Soc 116:449–467. https://doi.org/10.1111/bij.12597

Plazio E, Bubová T, Vrabec V, Nowicki P (2020a) Sex-biased topography effects on butterfly dispersal. Mov Ecol 8:1–14. https://doi.org/10.1186/s40462-020-00234-6

Plazio E, Margol T, Nowicki P (2020b) Intersexual differences in density-dependent dispersal and their evolutionary drivers. J Evol Biol 33:1495–1506. https://doi.org/10.1111/jeb.13688

Polic D, Fiedler K, Nell C, Grill A (2014) Mobility of ringlet butterflies in high-elevation alpine grassland: effects of habitat barriers, resources and age. J Insect Conserv 18:1153–1161.

https://doi.org/10.1007/s10841-014-9726-5

Polic D, Grill A (2013) Mobility patterns of alpine Erebia populations: Does a large road act as a dispersal barrier? 5th Symp Res Prot Areas, 10 to 12 June 2013, Mittersill 5:591–596

Rhainds M (2010) Female mating failures in insects. Entomol Exp Appl 136:211–226. https://doi.org/10.1111/j.1570-7458.2010.01032.x

Rhainds M (2019) Ecology of female mating failure/lifelong virginity: a review of causal mechanisms in insects and arachnids. Entomol Exp Appl 167:73–84. https://doi.org/10.1111/eea.12759

Richards S (1973) Temperature regulation. Wykeham Publications, London, Great Britain

Ropars L, Affre L, Schurr, et al (2020) Land cover composition, local plant community composition and honeybee colony density affect wild bee species assemblages in a Mediterranean biodiversity hot-spot. Acta Oecologica 104:1–35. https://doi.org/10.1016/j.actao.2020.103546

Ropars L, Dajoz I, Fontaine C, et al (2019) Wild pollinator activities negatively related to honey bee colony densities in urban context. PLoS One 14:1–16. https://doi.org/10.1101/667725

Schmitt T (2009) Biogeographical and evolutionary importance of the European high mountain systems. Front Zool 6:1–10. https://doi.org/10.1186/1742-9994-6-9

Schmitt T, Louy D, Zimmermann E, Habel JC (2016) Species radiation in the Alps: multiple range shifts caused diversification in Ringlet butterflies in the European high mountains. Org Divers Evol 16:791–808. https://doi.org/10.1007/s13127-016-0282-6

Schmitt T, Varga Z (2012) Extra-Mediterranean refugia: The rule and not the exception? Front Zool 9:1–12. https://doi.org/10.1186/1742-9994-9-22

Schneider C (2003) The influence of spatial scale on quantifying insect dispersal: An analysis of butterfly data. Ecol Entomol 28:252–256. https://doi.org/10.1046/j.1365-2311.2003.00495.x

Siozios S, Sapountzis P, Ioannidis P, Bourtzis K (2008) Wolbachia symbiosis and insect immune response. Insect Sci 15:89–100. https://doi.org/10.1111/j.1744-7917.2008.00189.x

Slamova I, Klecka J, Konvicka M (2011) Diurnal Behavior and Habitat Preferences of Erebia aethiops, an Aberrant Lowland Species of a Mountain Butterfly Clade. J Insect Behav 24:230–246. https://doi.org/10.1007/s10905-010-9250-8

Slamova I, Klecka J, Konvicka M (2013) Woodland and grassland mosaic from a butterfly perspective: Habitat use by Erebia aethiops (Lepidoptera: Satyridae). Insect Conserv Divers 6:243–254. https://doi.org/10.1111/j.1752-4598.2012.00212.x

Sloan DB, Havird JC, Sharbrough J (2017) The on-again, off-again relationship between mitochondrial genomes and species boundaries. Mol Ecol 26:2212–2236. https://doi.org/10.1111/mec.13959

Steffan-Dewenter I, Kuhn A (2003) Honeybee foraging in differentially structured landscapes. Proc R Soc B Biol Sci 270:569–575. https://doi.org/10.1098/rspb.2002.2292

Stuhldreher G, Hermann G, Fartmann T (2014) Cold-adapted species in a warming world - an explorative study on the impact of high winter temperatures on a continental butterfly. Entomol Exp Appl 151:270–279. https://doi.org/10.1111/eea.12193

Thomas CD, Hill JK, Anderson BJ, et al (2011) A framework for assessing threats and benefits to species responding to climate change. Methods Ecol Evol 2:125–142. https://doi.org/10.1111/j.2041-210X.2010.00065.x

Thomas JA, Moss D, Pollard E (1994) Increased fluctuations of butterfly populations towards the northern edges of species' ranges. Ecography 17:215–220. https://doi.org/10.1111/j.1600-

0587.1994.tb00096.x

Tigreros N, Davidowitz G (2019) Flight-fecundity tradeoffs in wing-monomorphic insects. Adv In Insect Phys 56:1–41. https://doi.org/10.1016/bs.aiip.2019.02.001

Timus N, Czekes Z, Rákosy L, Nowicki P (2017) Conservation implications of source-sink dynamics within populations of endangered Maculinea butterflies. J Insect Conserv 21:369–378. https://doi.org/10.1007/s10841-016-9906-6

Toews DPL, Brelsford A (2012) The biogeography of mitochondrial and nuclear discordance in animals. Mol Ecol 21:3907–3930. https://doi.org/10.1111/j.1365-294X.2012.05664.x

Torné-Noguera A, Rodrigo A, Osorio S, Bosch J (2016) Collateral effects of beekeeping: Impacts on pollen-nectar resources and wild bee communities. Basic Appl Ecol 17:199–209. https://doi.org/10.1016/j.baae.2015.11.004

Vanden Broeck A, Maes D, Kelager A, et al (2017) Gene flow and effective population sizes of the butterfly Maculinea alcon in a highly fragmented, anthropogenic landscape. Biol Conserv 209:89–97. https://doi.org/10.1016/j.biocon.2017.02.001

Vila M, Vidal-Romaní JR, Björklund M (2005) The importance of time scale and multiple refugia: Incipient speciation and admixture of lineages in the butterfly Erebia triaria (Nymphalidae). Mol Phylogenet Evol 36:249–260. https://doi.org/10.1016/j.ympev.2005.02.019

Wagner DL (2020) Insect Declines in the Anthropocene. Annu Rev Entomol 65:457–480. https://doi.org/10.1146/annurev-ento-011019-025151

Werren JH, Baldo L, Clark ME (2008) Wolbachia: Master manipulators of invertebrate biology. Nat Rev Microbiol 6:741–751. https://doi.org/10.1038/nrmicro1969

Wignall VR, Brolly M, Uthoff C, et al (2020) Exploitative competition and displacement mediated by eusocial bees: experimental evidence in a wild pollinator community. Behav Ecol Sociobiol 74:1-15. https://doi.org/10.1007/s00265-020-02924-y

Willis KJ, Niklas KJ (2004) The role of Quaternary environmental change in plant macroevoultion: the exception or the rule?. Proc R Soc B BiolSci 359: 159-172. https://doi.org/10.1098/rstb.2003.1387

Yoder JB, Clancey E, Des Roches S, et al (2010) Ecological opportunity and the origin of adaptive radiations. J Evol Biol 23:1581–1596. https://doi.org/10.1111/j.1420-9101.2010.02029.x

Zug R, Hammerstein P (2015) Wolbachia and the insect immune system: What reactive oxygen species can tell us about the mechanisms of Wolbachia-host interactions. Front Microbiol 6:1–16. https://doi.org/10.3389/fmicb.2015.01201

SUPPLEMENTARY MATERIAL DIRECTORY

Additional results are presented in the supplementary material in the attached CD-ROM. A PDF version of the whole thesis is also contained. The numbering of the single chapters is conform to the chapter-numbers in the thesis.

PDF file dissertation Martin Wendt.

CHAPTER S2 Reconstruction of forest dynamics in the Western Palaearctic based on phylogeographic analysis of the ringlet butterfly *Erebia aethiops*

Results
- Figure of the Graphical output from Dispersal-Extinction-Cladogensis (DEC) analysis.
- Table of the Model test by RASP v. 4.2.
- Figure of the Bayesian Skyline Plot of *E. aethiops* based on mtDNA data.
- Table of the allozyme diversity patterns of *E. aethiops*.
- Table of the linkage pairs of alleles by populations of *E. aethiops.*
- Table of the mean genetic distance of the four main geo. groups of *E. aethiops* based on the allozyme data.
- Table of the Neighbor Joining phenogram based on the genetic distance (Nei 1972) of 27 populations of *E. aethiops.*
- Table of the Structure K-values. Estimates of cluster number (K) from STRUCTURE analyses using allozyme polymorphisms for (a) all species and populations analysed, calculated for K= 1–27.
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CHAPTER S4 Resource over-exploitation and competition lead to endangerment of the extremely philopatric ringlet butterfly *Erebia pronoe glottis*

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- Figure of the Estimated daily population size of both sexes of *Erebia pronoe* based on the best Popanmodel in MARK: Phi(g+T) p(g*t) pent(T) N(g*t).
- Changes in the average wing condition during the 2019 flight season of *Erebia pronoe* are categorised by sex.

CHAPTER S5 Pronounced mito-nuclear discordance and various *Wolbachia* infections in the water ringlet *Erebia pronoe* have resulted in a complex phylogeographic structure

Results
- Table of the non-silent mutation in the mtDNA marker of E. pronoe in the Pyrenees populations with
- details of the base pairs.
- Figure of the distribution of the identified concatenated nuclear DNA haplotypes (Ef1α, RPS5) haplotypes among the populations of *E. pronoe*.
- Table of the tested Beast tree models with respective posterior, likelihood prior values and the number of ESS values below 200 for the nuclear data set.
- Figure of the mismatch distribution analysis of the mtDNA markers of *E. pronoe* generated in R using the packages "adegenet" and "pegas".
- Figure of the mismatch distribution analysis of the nuclear DNA markers of E. pronoe generated in R using the packages "adegenet" and "pegas".
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- Table of the results of the RASP Model Test for the mtDNA dataset.
- Figure of the graphical output from Bayesian analysis (exported from RASP).
- Table of the results of the RASP Model Test for the nuclear DNA dataset.
- Figure of the K means Clustering (K = 3) (49 landmarks) of penis valves of *E. pronoe* and *E. melas* as reference species.
- Figure of the cluster Dendrogram (49 landmarks) of penis valves of *E. pronoe* and *E. melas* as reference species.
- Table of the principal component analysis with singular values and percentage explained by the relative warps.
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- Table of the relative contribution of each landmark of the valves of *E. pronoe*.
- Table of the geographical location of the analysed populations of *E. pronoe.*
- Table of the GenBank accession numbers and corresponding haplotypes and genotypes.

ACKNOWLEDGEMENT

CURRICULUM VITAE

VI

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I hereby certify that I have not used any aids other than those indicated for the preparation of this dissertation, and that the results of other participants as well as passages and quotations taken verbatim and in terms of content from other works are identified as such. The thesis has not been submitted in the same or similar form to any other examination office or has been accepted by them as part of an examination.

Martin Wendt
Berlin,18.03.2023
_____

Interaction of ecology and evolution in two *Erebia* butterflies: A comparative study

SUPPLEMENTARY MATERIAL

Zur Erlangung des
Doktorgrades der Naturwissenschaften (Dr. rer. nat.)

der

Naturwissenschaftlichen Fakultät I – Biowissenschaften –
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Halle-Wittenberg,

vorgelegt

von Herr Martin Wendt

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Legend
a) Region Code

Letter	Region
A	Sakhalin
B	Honshu
C	Sayan Mountains
D	Mongolia
E	China
F	Europe
G	Western Altai Mountains
H	Küre Mountains, Pontic Mountains, Transcaucasia
J	Ural Mountains
I	Caucasia

b) Color Code

LEGEND

*	C	CFI	DF	FIJ
A	CD	CFIJ	E	FJ
ACE	CDF	CFJ	F	G
ADE	CE	CG	FGI	H
AE	CF	CGIJ	FGIJ	I
AEF	CFG	CGJ	FGJ	J
B	CFGI	D	FH	
BE	CFGIJ	DE	FI	

c) Backbone Nodes in Detail

NODE 73		NODE 76		NODE 117		NODE 120		NODE 121	
E	0.5090	CFIJ	0.2916	FH	0.8211	CFIJ	0.1305	AEF	0.0572
AE	0.3388	FGIJ	0.2887	F	0.1789	FGIJ	0.1292	*	0.9428
ACE	0.0772	CFGI	0.1306			F	0.1276		
ADE	0.0749	CGIJ	0.0734			FIJ	0.0871		
*	0.0001	FIJ	0.0731			FI	0.0856		
		CFI	0.0718			CFI	0.0718		
		FGI	0.0708			FGI	0.0704		
						CFGI	0.0641		
						CFGIJ	0.0567		
						*	0.1770		

S1: Graphical output from Dispersal-Extinction-Cladogensis (DEC) analysis (exported from RASP). Graphical results of ancestral distributions at each node of the *aethiops* group obtained by DEC analysis. Pie charts at each node show probabilities of alternative ancestral ranges with the posterior probability for the node next to it. a) Region codes; b) Color key to possible ancestral ranges at different nodes; black with an asterisk represents other ancestral ranges; c) backbone nodes in detail. Tip labels have the area codes and the accessions number or rather the haplotype number.

S2: Model test by RASP v. 4.2 with log of the Likelihood, number of parameters and corrected Akaike Information Criterion.

Model	LnL	parameters	AICc
DEC	-110.8	2	225.9
DEC+J	-106.2	3	218.8
DIVALIKE	-103.9	2	212
DIVALIKE+J	-101.6	3	209.5
BAYAREALIKE	-108.1	2	220.5
BAYAREALIKE+J	-93.14	3	192.7
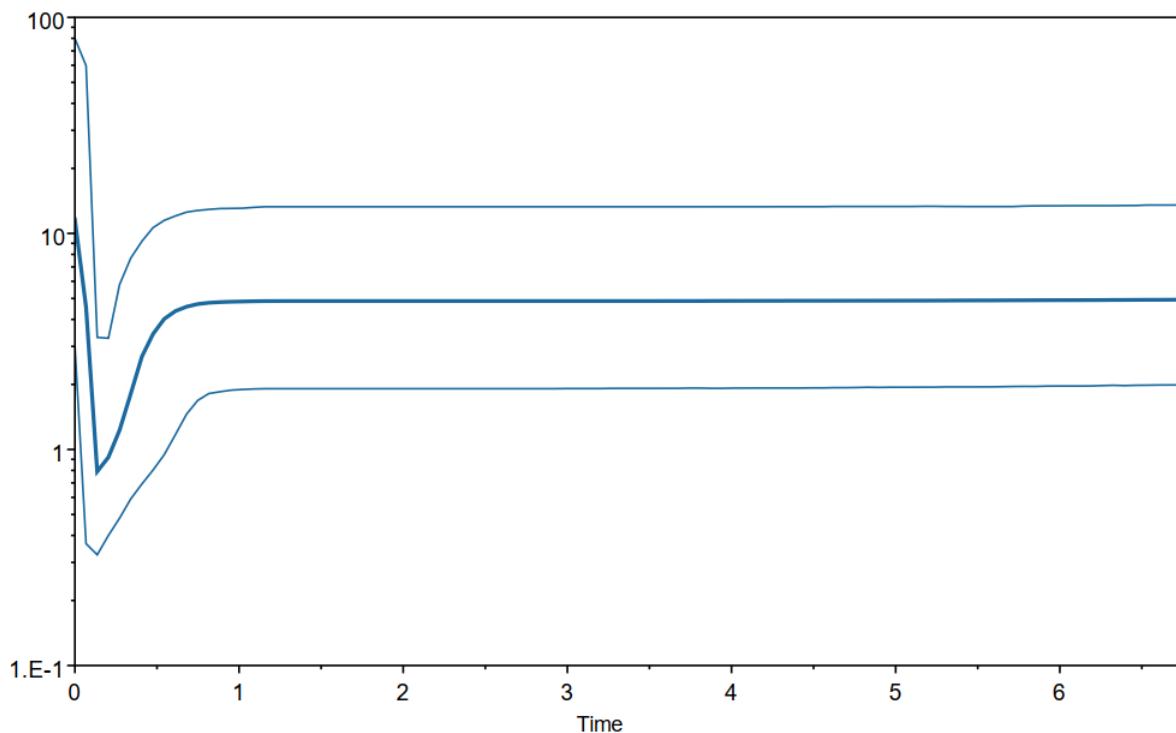
S3: Bayesian Skyline Plot of *E. aethiops* based on mtDNA data.

S4: Allozyme diversity patterns of *E. aethiops*. The following values are given: mean number of alleles A, percentage of the expected and observed heterozygosity He and Ho, percentage of all polymorphic loci Ptot and of loci with the most common allele not exceeding 95 % P95. Values based on an insufficient number of individuals are given in parenthesis and are excluded from the calculation of means.

Country	Location	He	Ho	A	Ptot	P95	N
F	Artout	10,1	10,4	(1,39)	(33,3)	(33,3)	8,0
F	Col des Aravis	4,6	4,8	(1,28)	(22,2)	16,7	14,0
CH-VS	Ausserberg	6,0	5,4	1,44	38,9	16,7	42,0
CH-BE	Grindelwald	6,8	5,3	(1,33)	(22,2)	16,7	18,8
CH-UR	Klausenpass	6,2	6,5	1,50	38,9	16,7	36,0
A-NT	Fernpass	9,3	7,2	1,67	61,1	33,3	38,9
A-NT	Holzleithner Sattel	6,4	5,6	1,83	72,2	33,3	39,5
I	Plöckenpass	5,8	4,9	1,44	33,3	11,1	37,7
A-SB	Sonnenstein	8,3	7,8	1,94	66,7	22,2	39,9
A-ST	Loser	8,4	9,0	(1,40)	(44,4)	(44,4)	8,0
A-SB	Seetal	5,3	5,3	1,44	27,8	11,1	36,9
SLO	Medvodje	6,2	5,3	1,72	50,0	27,8	37,5
A-ST	Kaisertal	7,7	6,6	1,61	55,6	33,3	39,3
A-ST	Seebergsattel	8,1	7,1	1,83	61,1	27,8	37,6
I	Spiazzi	4,8	4,6	1,56	33,3	22,2	26,0
I	Campolaro	8,2	7,6	2,00	55,6	27,8	38,0
SK	Tokariny	6,8	7,8	1,67	50,0	11,1	36,9
SK	Pusté pole	11,2	9,7	2,06	66,7	38,9	36,0
H	Szelcepuszta	7,8	8,2	(1,39)	27,8	27,8	21,1
H	Haragistya	10,9	12	1,72	61,1	38,9	27,8
SK	Kojšovska holá	6,9	6,6	1,72	50,0	22,2	37,7
H	Gyertyánkút	10,0	9,4	(1,39)	(33,3)	27,8	14,9
RO	Baile Herculane	7,0	7,1	(1,33)	(22,2)	(22,2)	7,9
RO	Cheile Buţii	10,5	10,0	1,89	61,1	33,3	37,9
RO	Cheile Runcului	11,2	11,7	1,78	55,6	27,8	39,8
SLO	Nanos	9,3	9,4	1,78	50,0	27,8	40,7
BG	Trigrad	14,1	14,0	1,61	50,0	27,8	35,7
	MEAN	8,1	7,8	1,62	46,1	26,0	
	SD	2,3	2,5	0,22	15,1	8,8	
F	*C. oedippus*	25,3	20,1	2,88	87,5	62,5	95,5

S5 Linkage pairs of alleles by populations of *E. aethiops.*

Population/ Linkagepairs	Medvodje	Nanos	Szelcepuszta	Sonnenstein	Kaisertal	Grindelwald
1	GOT1-GOT2	6PGDH-IDH1	6PGDH-GOT2	6PGDH-PEP	6PGDH-GOT1	IDH2-PGM
2	GOT1-ME	6PDGH-IDH2	6PGDH-PGM			
3	GOT1-PGI	IDH1-IDH2	GOT2-PGM			
4	GOT2-ME					
5	GOT2-PGI					
6	ME-PGI					

S6: Mean genetic distance of the four main geo. groups of *E. aethiops* based on the allozyme data.

	Central Alps	Eastern Balkan	Southern Alps	Southern Carpathians
Eastern Balkan	0,079 ±0,012			
Southern Alps	0,153 ±0,018	0,169 ±0,008		
Southern Carpathians	0,059 ±0,018	0,046 ±0,004	0,121 ±0,008	
Western Balkan	0,056 ±0,016	0,059 ±0,0	0,209 ±0,014	0,108 ±0,010
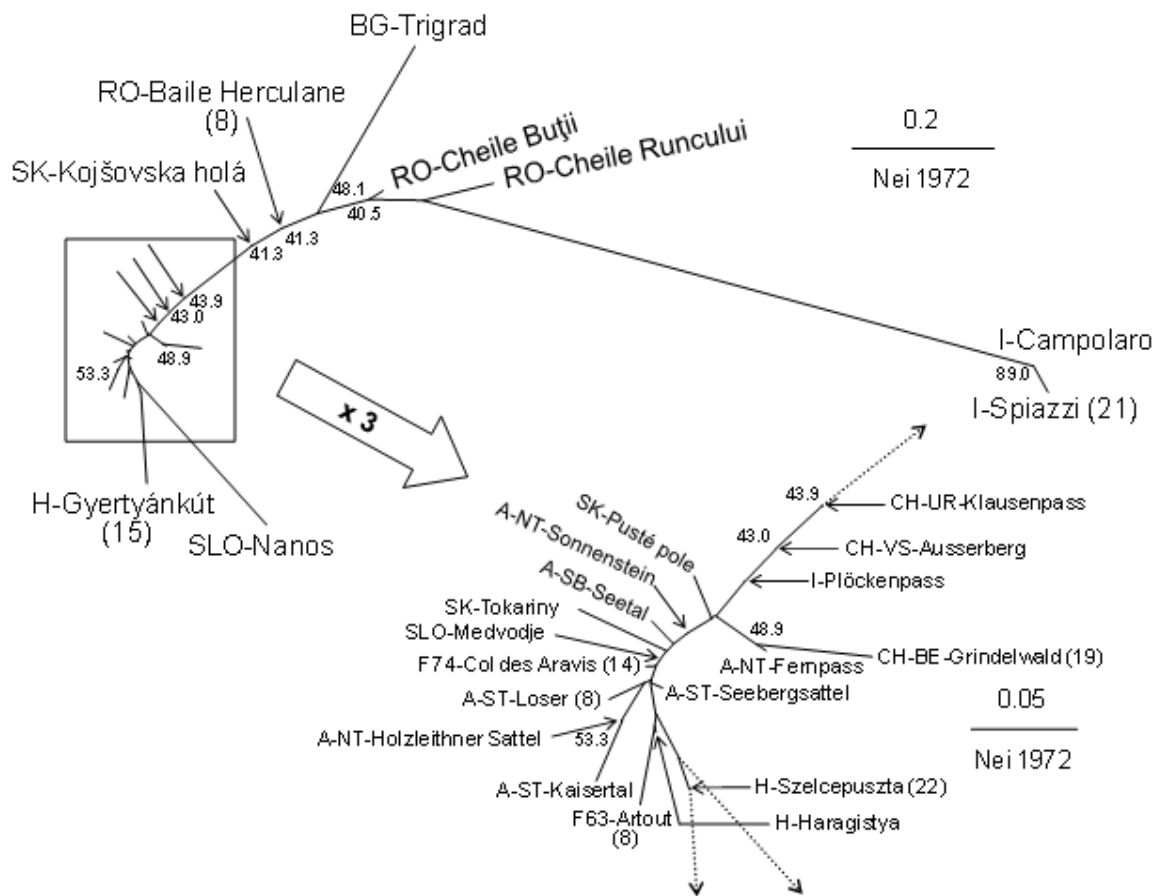
S7: Neighbor Joining phenogram based on the genetic distance (Nei 1972) of 27 populations of *E. aethiops*. Bootstrap values > 40 % are given at the branches. The box was magnified three times.

S8: Structure K-values. Estimates of cluster number (K) from STRUCTURE analyses using allozyme polymorphisms for (a) all species and populations analysed, calculated for K= 1–27. Ln(Pr) is the mean log-likelihood probability calculated by the program STRUCTURE. SD is the standard deviation calculated from ten independent runs. The ad hoc statistic ΔK is not applicable for K= 1 and the highest K value, and not proper for K= 2 (Hausdorf and Hennig 2010).

K	LN(PR)	±SD	ΔK
1	-6712.8500	0.0527	NA
2	-6079.6000	2.7673	180.412529
3	-5945.6000	23.6026	8.210523
4	-5617.8100	28.2878	8.214135
5	-5522.3800	4.3645	25.423579
6	-5537.9100	22.3992	0.609397
7	-5539.7900	12.1479	2.474496
8	-5511.6100	14.1166	0.570250
9	-5491.4800	28.7081	2.599966
10	-5545.9900	36.9710	1.188769
11	-5644.4500	36.9469	0.900481
12	-5709.6400	27.1023	1.718303
13	-5821.4000	124.6410	0.816264
14	-6034.9000	218.3150	0.886792
15	-6054.8000	202.7125	0.849134
16	-6246.8300	214.2018	0.991728
17	-6226.4300	192.6670	0.634255
18	-6328.2300	189.4610	0.236566
19	-6474.8500	261.2760	NA
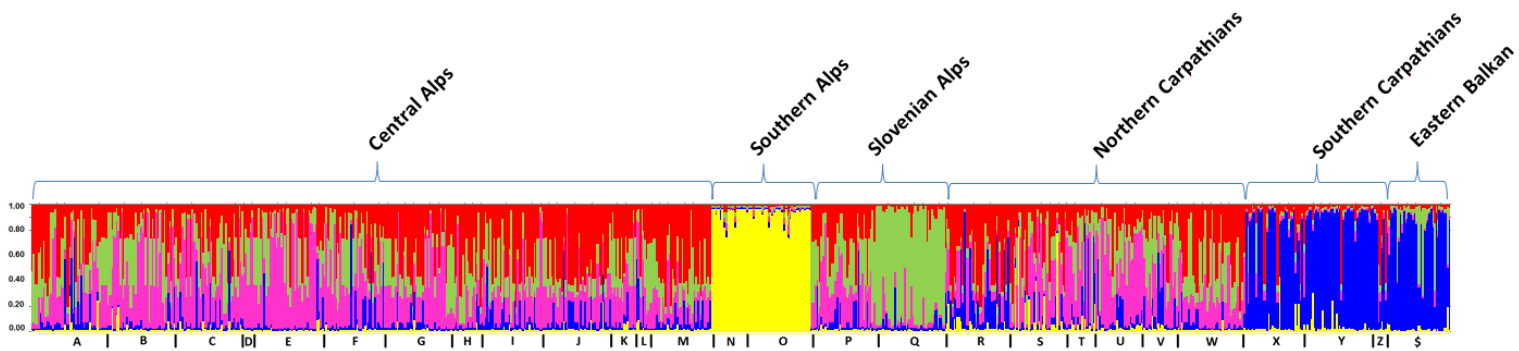
S9: Bayesian structure analysis of the *E. aethiops* populations using STRUCTURE software (Pritchard et al., 2000). The analysis was performed for five given groups (K = 5).

A:Fernpass,B:Holzleitner Sattel, C:Sonnenstein, D:Loser, E:Kaisertal, F:Seebergsattel, G:Seetal, H:Grindelwald, I:Klausenpass, J:Ausserberg, K:Col des Aravis, L:Artout, M:Ploeckenpass,N:Spiazzi, O:Campolaro, P:Medvodje, Q:Nanos, R: Kojšovska holá, S: Pusté pole, T: Gyertyánkút, U: Haragistya, V: Szelcepuszta, W: Tokariny, X: Cheile Buţii, Y: Cheile Runcului, Z: Baile Herculane, $:Trigrad
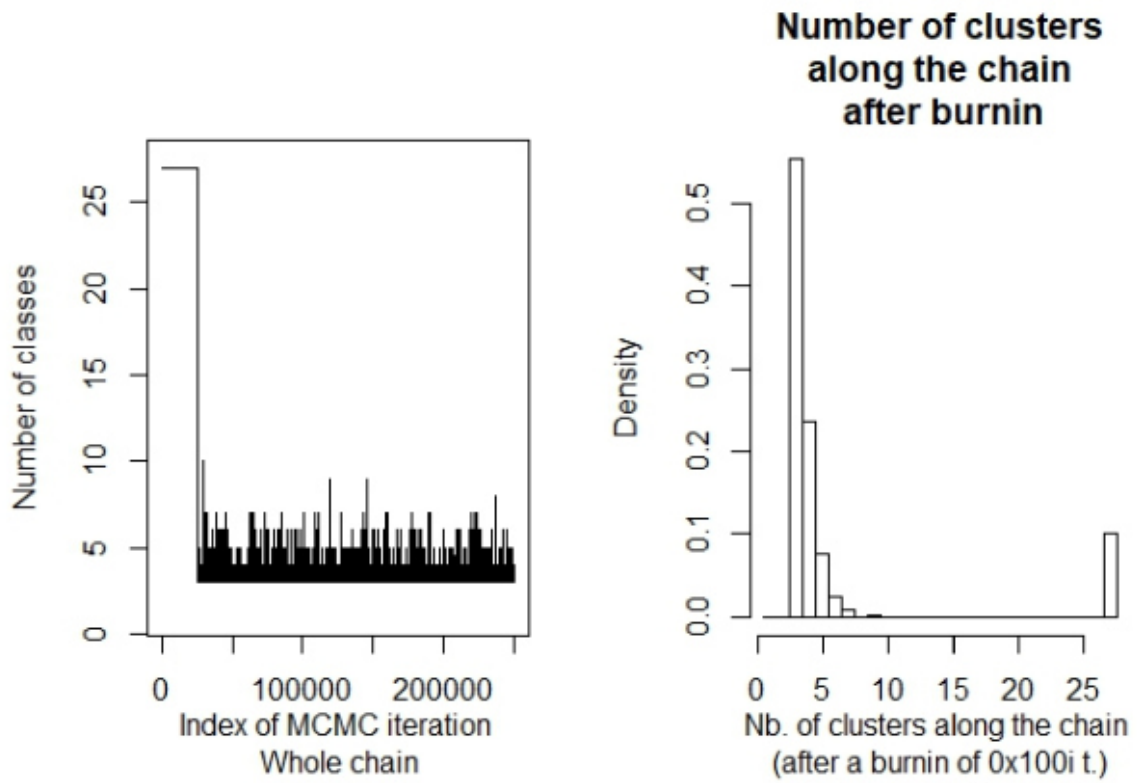
S10: Number of populations along the MCMC run by Geneland.
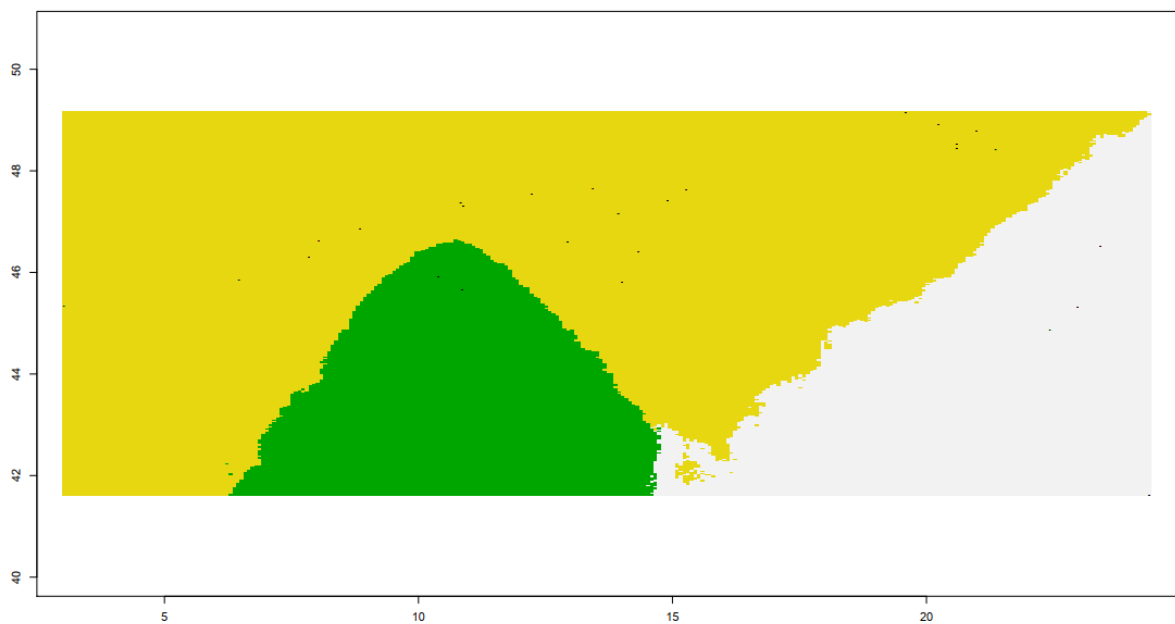
S11: Map of population membership by Geneland based on the allozyme data of *E. aethiops*

S12: Pairwise F_{ST} values of the four main geo. groups of *E. aethiops* based on the allozyme data

	Central Alps	Eastern Balkan	Central Italian Alps	Southern Carpathians
Eastern Balkan	0,284 ± 0,043			
Southern Alps	0,567 ± 0,046	0,498 ± 0,013		
Southern Carpathians	0,276 ± 0,080	0,164 ± 0,020	0,467 ± 0,034	
Western Balkan	0,243 ± 0,053	0,224 ±0,0	0,623 ± 0,004	0,416 ± 0,017

S13: Private allele and changes of the main allele by populations of *E. aethiops*

Location	Switch in the main allele	Endemic alleles
Fernpass		
HolzleithnerSattel		
Sonnenstein		PGM-1
Loser		
Kaisertal		
Seebergsattel		IDH2-1
Seetal		
Grindelwald		ME-1
Klausenpass		
Ausserberg	PGM-3(4)	
Col des Aravis		
Artout		GOT-6
Plöckenpass		
Medvodje		
Haragistya		
Szelcepuszta		
Gyertyánkút		
Tokariny		
Pusté pole		GOT-4, MDH1-1, MDH1-3
Spiazzi	PEP-3(2), PGM-3(4)	G6PDH-1, GOT-5

Campolaro	GOT2-1(3), PEP-3(2), PGM-3(4)	FUM-1, GOT-2
Kojšovskaholá	PGM-3(4)	
CheileBuţii	PGM-3(4)	
CheileRuncului	IDH1-5(2), PGM-3(4)	IDH1-3, MDH1-4
BaileHerculane	PGM-3(4)	
Nanos		
Trigrad	IDH1-5(2), PGI-3(5), PGM-3(4)	IDH2-5

S14: Geographical sample location of all *E. aethiops* samples including geographic coordinates and altitude.

No.		Location	Hight [m NN]	Coordinates
1.		Artout	1250	45°20'16''N 03°01'25''E
2.		Col des Aravis	1700	45°51'54''N 06°27'22''E
3.		Val-d´Ílliez	890	46°07'03''N 07°01'19''E
4.		Ausserberg	1200	46°19'10''N 07°51'33''E
5.		Grindelwald	1800	46°38'43''N 08°01'44''E
6.		Klausenpass	1800	46°52'21''N 08°52'18''E
7.		Fernpass	1250	47°21'53''N 10°50'01''E
8.		HolzleithnerSattel	1100	47°18'24''N 10°53'13''E
9.		Plöckenpass	1450	46°36'10''N 12°56'13''E
10.		Sonnenstein	1200	47°32'58''N 12°15'55''E
11.		Loser	1550	47°39'29''N 13°26'46''E
12.		Seetal	1300	47°09'11''N 13°56'16''E
13.		Medvodje	1550	46°25'25''N 14°19'02''E
14.		Kaisertal	1050	47°25'08''N 14°54'31''E
15.		Seebergsattel	1300	47°37'44''N 15°16'44''E
16.		Spiazzi	900	45°39'26''N 10°51'18''E
17.		Campolaro	1550	45°54'37''N 10°23'04''E
18.		Sija	500	46°11'41''N 13°50'01''E
19.		Nanos	800	45°48'22''N 14°00'51''E
20.		Szelcepuszta	500	48°31'35''N 20°35'02''E

21.		Haragistya	550	48°26'30''N 20°32'18''E
22.		Kojšovskaholá	1100	48°46'35''N 20°59'11''E
23.		Gyertyánkút	700	48°26'30''N 21°21'52''E
24.		Pusté pole	950	48°53'10''N 20°14'21''E
25.		Tokariny	800	49°09'45''N 19°37'49''E
26.		Pascani	460	47°06'23''N 26°24'38''E
27.		Covacipeter	1360	46°40'40''N 25°43'13''E
28.		Gheorgheni	980	46°34'45''N 25°33'46''E
29.		CheileRuncului	600	46°30'48''N 23°26'18''E
30.		CheileBuţii	950	45°18'00''N 22°58'29''E
31.		BaileHerculane	350	44°51'57''N 22°25'02''E
32.		Tresnjevik	1580	42°44'19''N 19°41'01''E
33.		Ropojantal	1400	42°29'13''N 19°48'27''E
34.		Valbona	1350	42°30'01''N 19°56'50''E
35.		Trigrad	1350	41°36'32''N 24°23'45''E

S15: Bold and Genebank accession numbers of COI sequences fragment (429bp) of *E. aethiops with colour code, E. neriene, E. niphonica und E. alcmena.*

Accession number	Species/Color code of *E. aethiops*	Database	Location
MK186285.1	red	Genbank	Graubunden Piz Beverin Switzerland
MK186282.1	orange	Genbank	Bern Neuveville Switzerland
MK186281.1	yellow	Genbank	Locarno Switzerland
KT782504.1	orange	Genbank	Riga Latvia
HQ563606.1	orange	Genbank	Bavaria Schmidmühlen Germany
HQ004360.1	light blue	Genbank	Suceava pas Pascanu Romania
HQ004359.1	light blue	Genbank	Suceava ValeaBistritei Romania
HQ004358.1	light blue	Genbank	Brasov Racos Romania
HQ004357.1	light blue	Genbank	Suceava Sunatori Romania
HQ004355.1	light blue	Genbank	Brasov cabana Babarunca Romania
HM391826.1	red	Genbank	Bavaria Wimbachschloss Germany
FJ663461.1	brown	Genbank	East Kazakhstan
KX040921.1	red	Genbank	Bavaria Oytal Germany
AB324832.1	brown	Genbank	Middle Urals Serga river Russia
AB324834.1	brown	Genbank	Alan Range Sayan Russia
AB324833.1	brown	Genbank	Caucasus Teberda Russia
KR138772	pink	Genbank	Achalkalaki Georgia
KR138741	pink	Genbank	Ersizlerdere Turkey
MN138605	red	Genbank	Bavaria Oberstdorf Germany
BIBSA1199-15	gold	Bold	Vipiteno Sant Antonio Italy
BIBSA125-15	gold	Bold	Liguria San Martino Italy
BIBSA201-15	gold	Bold	Alpedella Gianna Italy
BIBSA934-15	gold	Bold	Rifugio Monte Mongioie Italy
EULEP2778-15	orange	Bold	Isle of Arran Scotland
EULEP593-15	red	Bold	Isere France
EULEP617-15	orange	Bold	Thüringen Altremda Germany
EULEP980-15	red	Bold	Ginzlingen Austria
EZHBA304-07	brown	Bold	Iskitim Russia
EZHBA306-07	brown	Bold	Iskitim Russia
GBLAB766-13	orange	Bold	Bavaria Fischerhäuser Isarauen Germany
GWORR684-10	red	Bold	Bavaria Jachenau Germany
LEATC177-13	gold	Bold	Margreid Italy
LEPAA411-16	orange	Bold	Waldenburg Switzerland
LEPAA839-16	red	Bold	Uznach Switzerland
LOWAB239-09	pink	Bold	Ispir Turkey
OXB258-15	orange	Bold	Kirkby Stephen Smardale Great Britain
OXB259-15	orange	Bold	Kirkby Stephen Smardale Great Britain
OXB260-15	orange	Bold	Kirkby Stephen Smardale Great Britain
OXB603-15	yellow	Bold	Madonna di Campiglio Italy
OXB724-15	gold	Bold	Col de Visentin Italy
PHLAI521-13	red	Bold	Greit Austria
WMB1643-13	red	Bold	Isere France
WMB1867-13	red	Bold	Col du Noyer France

WMB3680-14	red	Bold	Piste Sky Laye France
WMB5027-14	gold	Bold	Monte Calvaria France
WMB837-13	gold	Bold	Mompantero Vecchio Italy
AB324849	*E. niphonica*	Genbank	Sakhalin Reg. Russia
LC340527	*E. neriene niphonica*	Genbank	Akaishi Mts. Japan
AB306501	*E. niphonica*	Genbank	Ishikawa Japan
AB306500	*E. niphonica*	Genbank	Nagano Japan
EZHBA341-07	*E. neriene*	Bold	Todzha interm Russia
AB324842	*E. neriene*	Genbank	Hangay Mts. Mongolia
AB324841	*E. neriene*	Genbank	Terelj Mongolia
AB324838	*E. neriene*	Genbank	Khabarovsk Russia
AB324837	*E. neriene*	Genbank	Khabarovsk Russia
KM111616	*E. alcmena*	Genbank	
AB324835	*E. alcmena*	Genbank	Shaanxi China
LC340541	*E. alcmena*	Genbank	Datong-Shan China
AB324836	*E. alcmena*	Genbank	Qinghai China
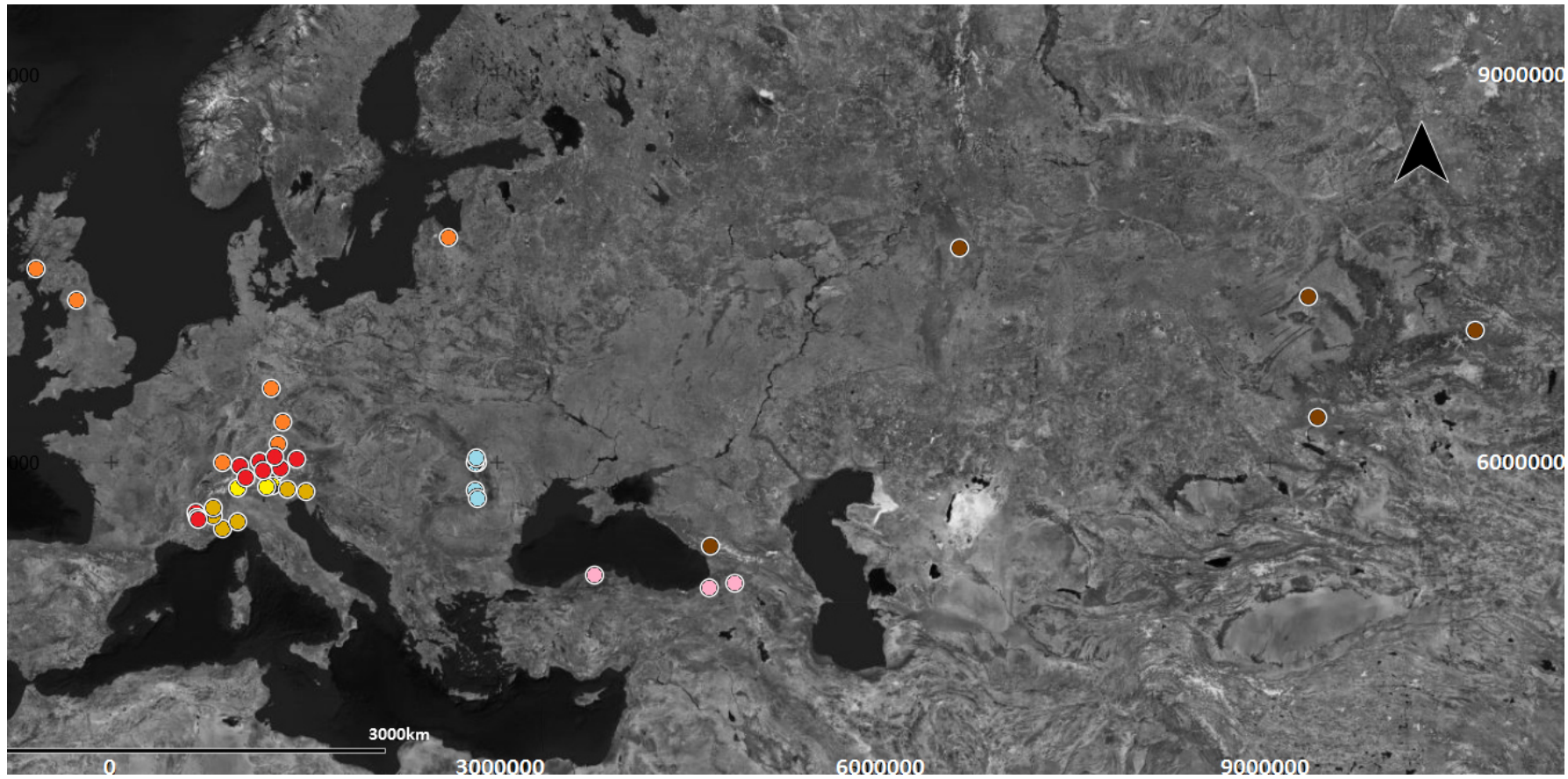
S16: Geographic location of the 48 COI sequence samples from BOLD and Genbank of *E. aethiops*. The geographical location of the haplotypes is given in same color scheme as in The map was created with Qgis v.3.10.10[72] (Available online: http://qgis.osgeo.org).

S17: Nucleotide sequences and GenBank accession numbers of *E. aethiops*; *E. pronoe* and *P. aegeria.*

Nucleotide sequence CO1	GenBank accession number	Nucleotide sequence ND1	GenBank accession number
BankIt2309627 H1	MT017932	BankIt2309627 H1	MT017966
BankIt2309627 H10	MT017933	BankIt2309627 H10	MT017967
BankIt2309627 H11	MT017934	BankIt2309627 H11	MT017968
BankIt2309627 H12	MT017935	BankIt2309627 H12	MT017969
BankIt2309627 H13	MT017936	BankIt2309627 H13	MT017970
BankIt2309627 H14	MT017937	BankIt2309627 H14	MT017971
BankIt2309627 H15	MT017938	BankIt2309627 H15	MT017972
BankIt2309627 H16	MT017939	BankIt2309627 H16	MT017973
BankIt2309627 H17	MT017940	BankIt2309627 H17	MT017974
BankIt2309627 H18	MT017941	BankIt2309627 H18	MT017975
BankIt2309627 H19	MT017942	BankIt2309627 H19	MT017976
BankIt2309627 H2	MT017943	BankIt2309627 H2	MT017977
BankIt2309627 H20	MT017944	BankIt2309627 H20	MT017978
BankIt2309627 H21	MT017945	BankIt2309627 H21	MT017979
BankIt2309627 H22	MT017946	BankIt2309627 H22	MT017980
BankIt2309627 H23	MT017947	BankIt2309627 H23	MT017981
BankIt2309627 H24	MT017948	BankIt2309627 H24	MT017982
BankIt2309627 H25	MT017949	BankIt2309627 H25	MT017983
BankIt2309627 H26	MT017950	BankIt2309627 H26	MT017984
BankIt2309627 H27	MT017951	BankIt2309627 H27	MT017985
BankIt2309627 H28	MT017952	BankIt2309627 H28	MT017986
BankIt2309627 H29	MT017953	BankIt2309627 H29	MT017987
BankIt2309627 H3	MT017954	BankIt2309627 H3	MT017988
BankIt2309627 H30	MT017955	BankIt2309627 H30	MT017989
BankIt2309627 H31	MT017956	BankIt2309627 H31	MT017990
BankIt2309627 H32	MT017957	BankIt2309627 H32	MT017991
BankIt2309627 H33	MT017958	BankIt2309627 H33	MT017992
BankIt2309627 H4	MT017959	BankIt2309627 H4	MT017993
BankIt2309627 H5	MT017960	BankIt2309627 H5	MT017994
BankIt2309627 H6	MT017961	BankIt2309627 H6	MT017995
BankIt2309627 H7	MT017962	BankIt2309627 H7	MT017996
BankIt2309627 H8	MT017963	BankIt2309627 H8	MT017997
BankIt2309627 H9	MT017964	BankIt2309627 H9	MT017998
BankIt2309627 EP_GF1	MT017965	BankIt2309627 EP_GF1	MT017999
Pararge aegeria	MH089839.1	*Pararge aegeria*	KM592968.1

CHAPTER S4 Resource over-exploitation and competition lead to endangerment of the extremely philopatric ringlet butterfly *Erebia pronoe glottis*

S 2 Estimated daily population size of both sexes of *Erebia pronoe* based on the best Popan model in MARK: Phi(g+T) p(g*t) pent(T) N(g*t).

S 3 Changes in the average wing condition during the 2019 flight season of *Erebia pronoe* are categorised by sex.
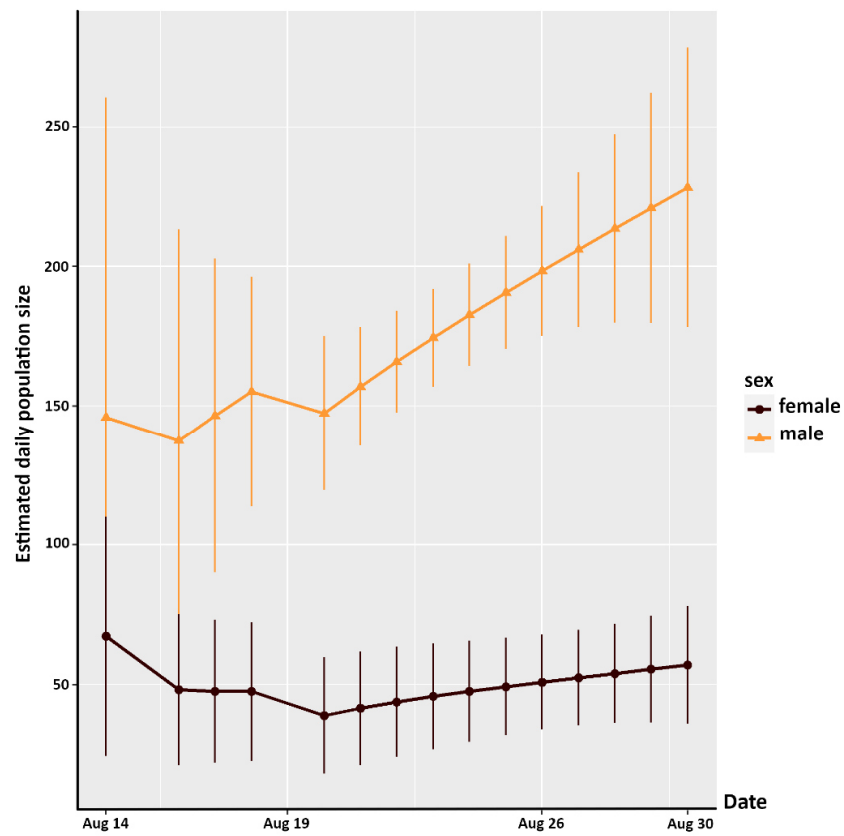
CHAPTER S5 Pronounced mito-nuclear discordance and various *Wolbachia* infections in the water ringlet *Erebia pronoe* have resulted in a complex phylogeographic structure

S1 Non-silent mutation in the mtDNA marker of *E. pronoe* in the Pyrenees populations with details of the base pairs.

	CO1	ND1
	27 AA.: Asparagin(ASN)->Threonin(THR)	6 AA.:Threonin(THR)->Methionin(Met) (polar->unpolar)
	29 AA.: Serin(SER)->Glycin(Gly) (polar-unpolar)	13 AA.: Glycin(Gly)-> Valin(Val)
	30 AA.: Phenylalanin(Phe)->Serin(Ser) (unpolar ->polar)	68 AA.: Methionin(Met)-> Valin(Val)
	67 AA.: Valin(Val)->Isoleucin(Ile)	122 AA.: Methionin(Met)->Threonin(THR) (unpolar->polar)
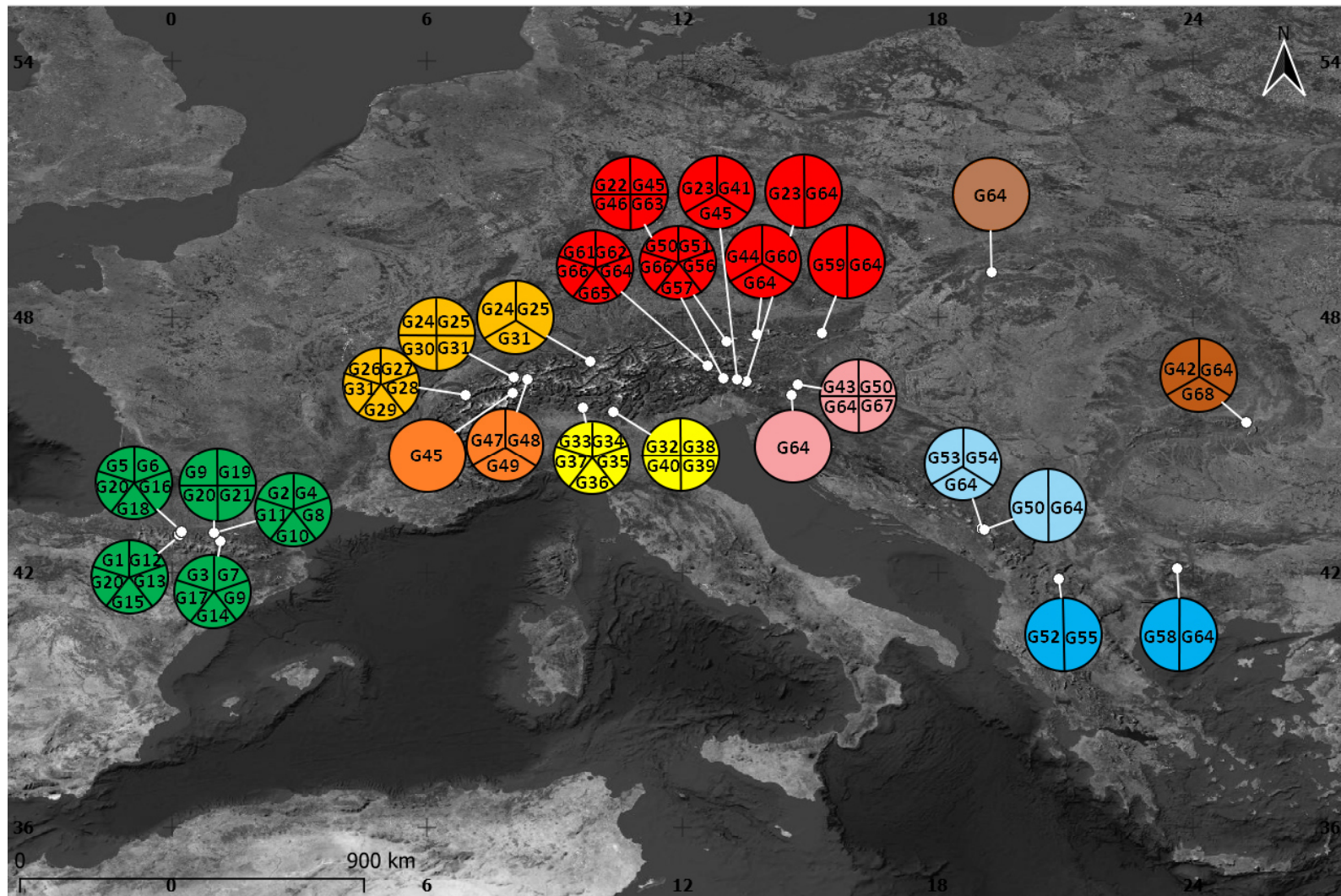
S 2 Distribution of the identified concatenated nuclear DNA haplotypes (Ef1α, RPS5) haplotypes among the populations of *E. pronoe*. The map was created with Qgis v.3.10.10 (Available online: http://qgis.osgeo.org).

S 3 Tested Beast tree models with respective posterior, likelihood prior values and the number of ESS values below 200 for the nuclear data set of *E. pronoe*.

	Posterior	Likelihood	Prior	ESS < 200
Yule model (with partition)	45672.036	-2511.205	48183.241	28
Calibrated Yule model (with partition)	-	-	-	-
Birth Death model (with partition)	-1191.461	-2910.297	1718.836	10
Coalescent constant population model (with partition)	88973.685	-2917.832	9189.517	31
Coalescent exponential population model (with partition)	-49485.186	-2480.265	-51965.451	24
Yule model	-3386.803	– 3277.856	-108.948	5
Calibrated Yule model	-	-	-	-
Birth Death model	-3342.947	– 3296.598	-46.35	5
Coalescent constant population model	-3528.819	– 3293.915	-234.904	-
Coalescent exponential population model	-3520.63	-3293.249	-227.381	-
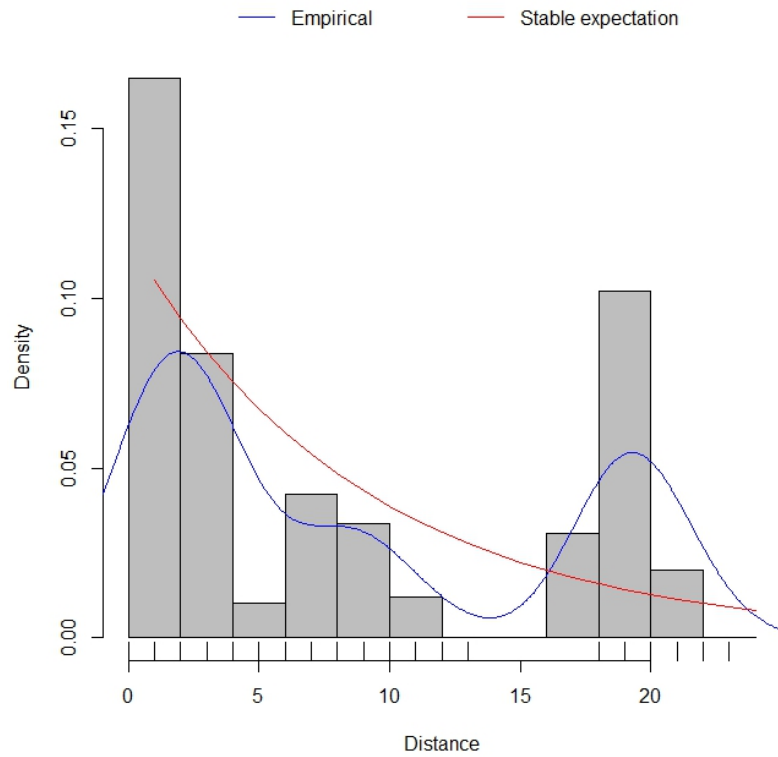
S4 Mismatch distribution analysis of the mtDNA markers of *E. pronoe* generated in R using the packages "adegenet" and "pegas".

S5 Mismatch distribution analysis of the nuclear DNA markers of *E. pronoe* generated in R using the packages "adegenet" and "pegas".

Result of combined:

Region Code	Region
A	Pyrenees
B	Western Alps
C	Eastern Alps
D	Balkans
E	Carpathians

a) Region codes

b) color keys

*
A
AC
B
BC
BE
C
CD
D
E
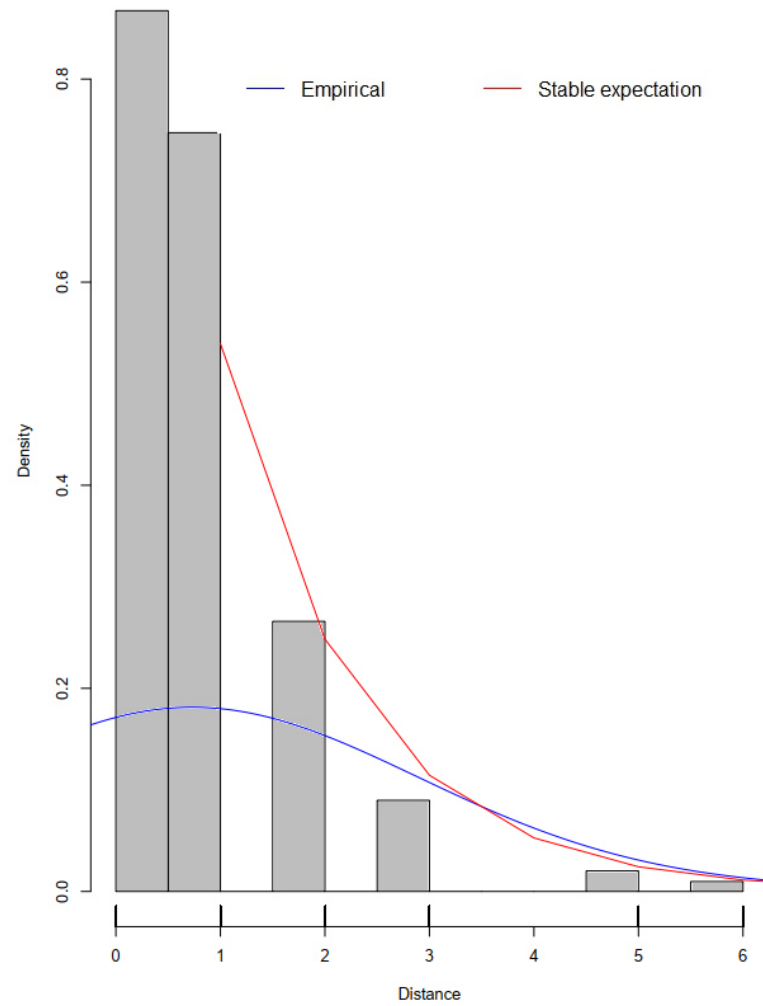
S6 Graphical output from Bayesian analysis (exported from RASP). Graphical results of ancestral distributions at each node of the *E. pronoe* group obtained by Bayesian Binary MCMC analysis based on the mtDNA dataset. Pie charts at each node show probabilities of alternative ancestral ranges with the posterior probability for the node next to it. a) Region codes; b) Color key to possible ancestral ranges at different nodes; black with an asterisk represents other ancestral ranges. Tip labels contain the area codes and correspond to the GenBank accessions codes of S15.

S7 Results of the RASP Model Test for the mtDNA dataset.

	LnL	numparams	d	e	j	AICc	AICc_wt
DEC	-43.77	2	0.1	0.14	0	91.81	4.80E-06
DEC+J	-31.85	3	1.00E-12	2.90E-08	0.016	70.24	0.23
DIVALIKE	-40.2	2	0.11	4.80E-09	0	84.67	0.0002
DIVALIKE+J	-30.65	3	1.00E-12	1.00E-12	0.017	67.82	0.77
BAYAREALIKE	-51.1	2	0.15	0.85	0	106.5	3.10E-09
BAYAREALIKE+J	-36.73	3	1.00E-07	0.23	0.022	80	0.0017

Result of combined:

(D) EP ALI2
73
4 (C) EP PLOE1
75
74 (C) EP GF3
7 5 (C) EP GF5
(C) EP LO1
76
5 (C) EP SE3
81
(C) EP HO3
78
98 (C) EP HO5
79
(D) EP POSHA2
80
85 (D) EP GRR3
(E) EPKOE1
82
4 (C) EP MED3
83
84 (C) EP GF4
(C) EP GF2
(C) EP HO4
86
20 (C) EP HO2
93 88
60 (D) EP POSHA1
89
20 (D) EP ALI3
15
(D) EP ALI1
(B) EP GLET1
94
(B) EP GLET2
98
100 (B) EP GLET3
96 11
(C) EP PLOE3
73
(C) EP PLOE2
99
95 55 (C) EP LO4
5 (C) EP MED4
(E) EPKOE3
(C) EP PPK3
(C) EP PGD5
100
40 (C) EP PGD2
102
27 (C) EP PGD1
13
33 (C) EP PSM4
(C) EP PSM5
106
38 (C) EP PSM3
104
55 (C) EP PSM1
105
35 (C) EP PSM2
(C) EP PGD3
(A) EP ETAR5
(A) EP LDS3
2 (A) EP PDL2
(A) EP LDS4
(A) EP CAU3
50 (A) EP CAU2
(A) EP CAU1
100
(A) EP ETAR2
7 (A) EP PDL4
27 (A) EP PDL5
41
(A) EP CT3
99 (A) EP CT5
(A) EP PDL1
10 (A) EP CT4
90 (A) EP ETAR3
12 (A) EP CT2
12 (A) EP LDS5
12 5 (A) EP LDS1
48 (A) EP ETAR1
10 (A) EP CT1
63 (A) EP CAU5
(B) EP GRIN1
4 (B) EP GRIN5
4 (A) EP VAIL1
(A) EP VAIL2
(A) EP VAIL3
(A) EP VAIL5
61
(B) EP GRIN3
8 (B) EP GRIN4
(C) EP OI1
16
23 (C) EP PLOE5
(E) ROBH 1
(E) ROBH 6
100
(E) EaetRo1
(C) PA1
100

a) Region codes

Region Code	Region
A	Pyrenees
B	Western Alps
C	Eastern Alps
D	Balkans
E	Carpathians

b) color keys

-	
A	
AB	
AC	
B	
BC	
C	
CD	
CE	
D	
E	

77   42   0

S8 Graphical output from Bayesian analysis (exported from RASP). Graphical results of ancestral distributions at each node of the *E. pronoe* group obtained by Bayesian Binary MCMC analysis based on the nuclear DNA dataset. Pie charts at each node show probabilities of alternative ancestral ranges with the posterior probability for the node next to it. a) Region codes; b) Color key to possible ancestral ranges at different nodes; black with an asterisk represents other ancestral ranges. Tip labels contain the area codes and correspond to the GenBank accessions codes of S15.

S9 Results of the RASP Model Test for the nuclear DNA dataset.

	LnL	numparams	d	e	j	AICc	AICc_wt
DEC	-82.3	2	0.027	0.037	0	168.8	3.60E-10
DEC+J	-60.22	3	1.00E-12	1.00E-12	0.03	126.8	0.47
DIVALIKE	-84.49	2	0.039	0.05	0	173.1	4.00E-11
DIVALIKE+J	-60.74	3	1.00E-12	1.00E-12	0.031	127.8	0.28
BAYAREALIKE	-113.5	2	0.045	0.11	0	231.1	1.10E-23
BAYAREALIKE+J	-60.84	3	1.00E-07	1.00E-07	0.031	128	0.25
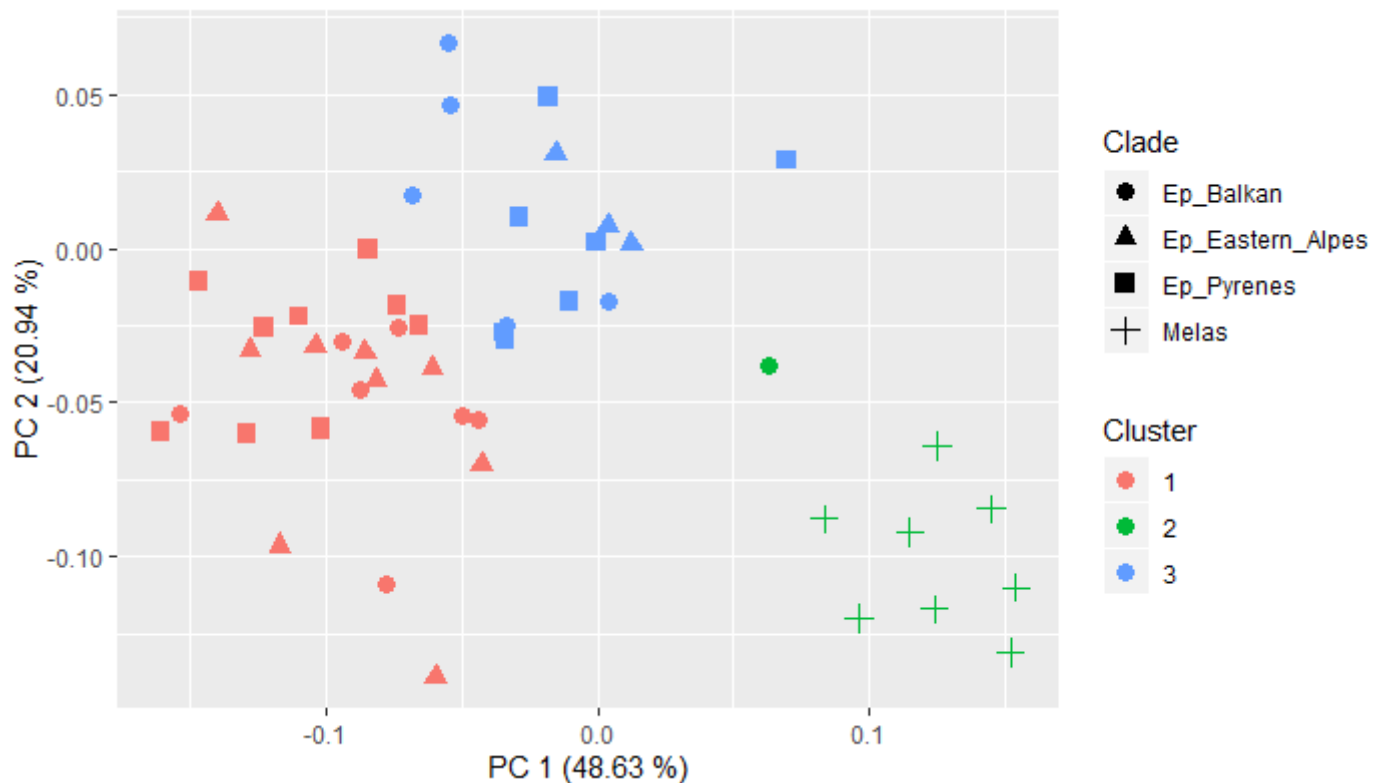
S10 K means Clustering (K = 3) (49 landmarks) of penis valves of *E. pronoe* and *E.melas* as reference species. First principal component (PC1) is on the x-axis, second principal component(PC2) on the y-axis.
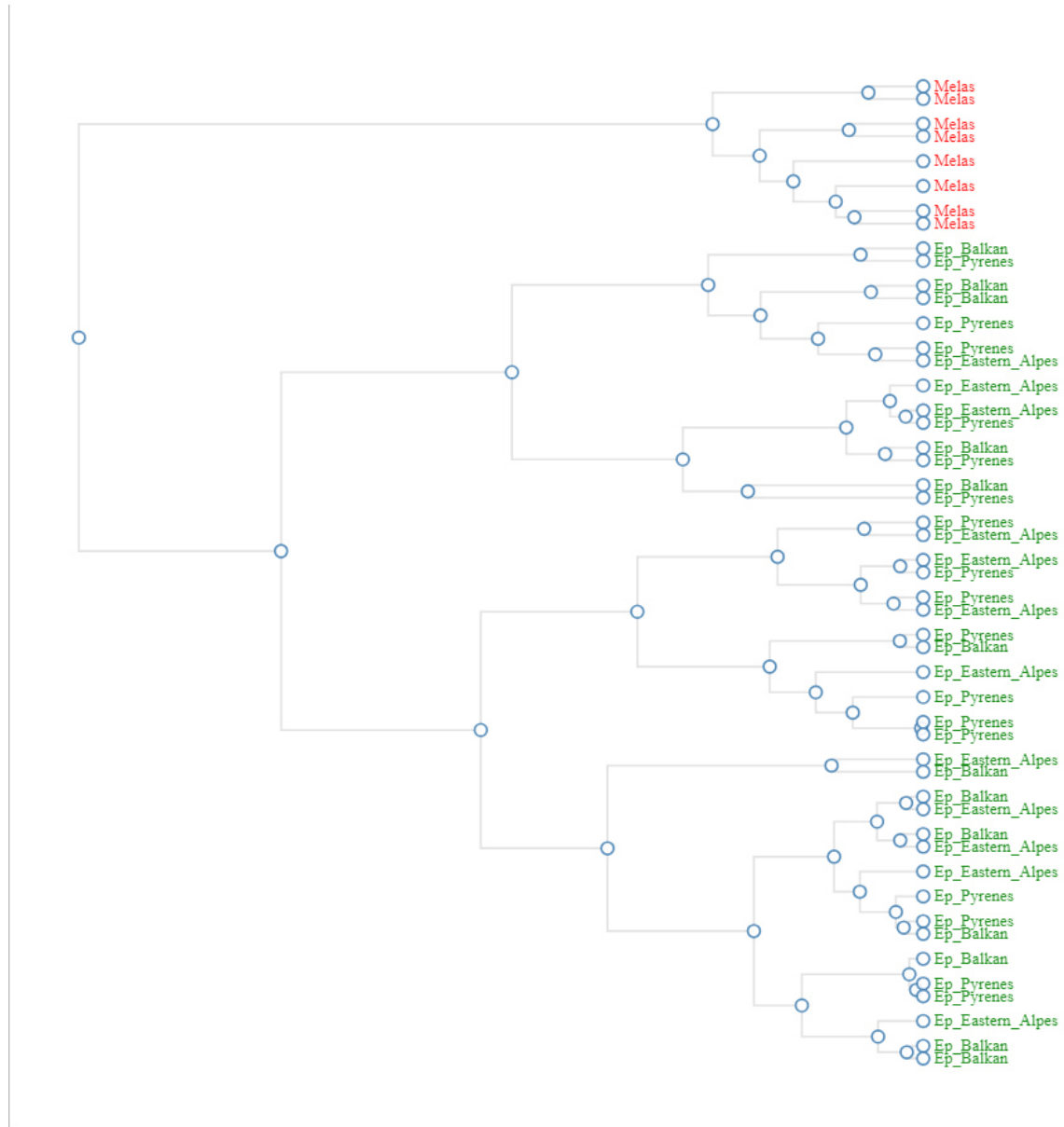
S11 Cluster Dendrogram (49 landmarks) of penis valves of *E. pronoe* and *E.melas* as reference species. Based on the euclydian distance.

S12 Principal component analysis with singular values and percentage explained by the relative warps.

PCs	SV	%	Cum %	PCs	SV	%	Cum %
1	0,64939	48,63%	48,63%	30	0,02264	0,06%	99,67%
2	0,42609	20,94%	69,57%	31	0,02092	0,05%	99,72%
3	0,28317	9,25%	78,82%	32	0,01928	0,04%	99,76%
4	0,19879	4,56%	83,37%	33	0,01779	0,04%	99,80%
5	0,15164	2,65%	86,03%	34	0,01663	0,03%	99,83%
6	0,14500	2,42%	88,45%	35	0,01544	0,03%	99,86%
7	0,13359	2,06%	90,51%	36	0,01489	0,03%	99,88%
8	0,12546	1,82%	92,32%	37	0,01264	0,02%	99,90%
9	0,10298	1,22%	93,55%	38	0,01231	0,02%	99,92%
10	0,08978	0,93%	94,48%	39	0,01186	0,02%	99,94%
11	0,08103	0,76%	95,23%	40	0,01122	0,01%	99,95%
12	0,07887	0,72%	95,95%	41	0,01013	0,01%	99,96%
13	0,07189	0,60%	96,55%	42	0,00904	0,01%	99,97%
14	0,06158	0,44%	96,98%	43	0,00820	0,01%	99,98%
15	0,05759	0,38%	97,37%	44	0,00727	0,01%	99,98%
16	0,05291	0,32%	97,69%	45	0,00598	0,00%	99,99%
17	0,04953	0,28%	97,97%	46	0,00578	0,00%	99,99%
18	0,04539	0,24%	98,21%	47	0,00535	0,00%	100,00%
19	0,04348	0,22%	98,43%	48	0,00443	0,00%	100,00%
20	0,04162	0,20%	98,63%	49	0,00359	0,00%	100,00%
21	0,03880	0,17%	98,80%				
22	0,03793	0,17%	98,97%				
23	0,03374	0,13%	99,10%				
24	0,03098	0,11%	99,21%				
25	0,03065	0,11%	99,32%				
26	0,02650	0,08%	99,40%				
27	0,02566	0,08%	99,47%				
28	0,02503	0,07%	99,55%				
29	0,02345	0,06%	99,61%				
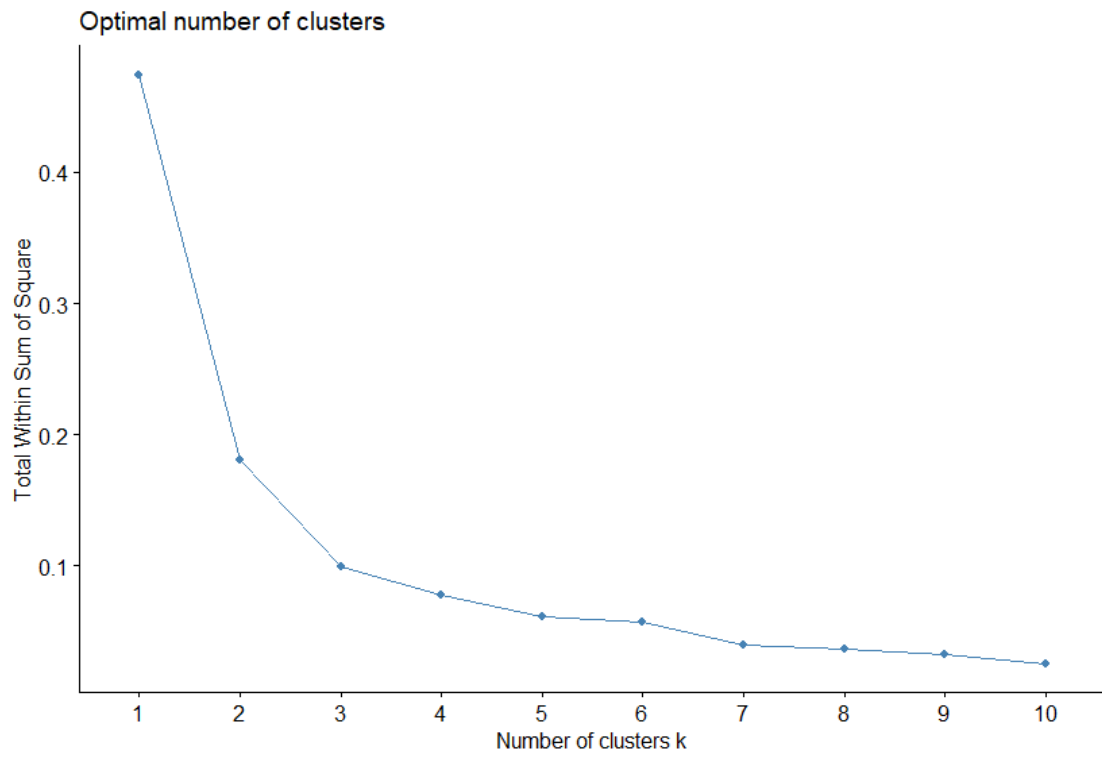
S13 Optimal number of cluster K for the morphology of penis valves of *E. pronoe* and *E.melas* as reference species.

S14 Relative contribution of each landmark of the valves of *E. pronoe*.

Landmark Nr.	SS	Landmark Nr.	SS	Landmark Nr.	SS
1	0,00087	17	0,02099	33	0,00022
2	0,00146	18	0,01329	34	0,00010
3	0,00067	19	0,00836	35	0,00007
4	0,00259	20	0,00246	36	0,00006
5	0,01421	21	0,00067	37	0,00005
6	0,03325	22	0,00067	38	0,00005
7	0,06644	23	0,00061	39	0,00004
8	0,13907	24	0,00051	40	0,00007
9	0,15045	25	0,00024	41	0,00014
10	0,12320	26	0,00051	42	0,00014
11	0,10489	27	0,00092	43	0,00011
12	0,08705	28	0,00055	44	0,00052
13	0,07672	29	0,00022	45	0,00139
14	0,06073	30	0,00019		
15	0,04918	31	0,00020		
16	0,03562	32	0,00023		

S 15 Geographical location of the analysed populations of *E. pronoe*

Population	Kürzel	East	North	
Alisnica	Ali	019.03630648	43.01950597	04.08.2014
Babky	Bab	019.25575314	49.06373272	01.08.2007
Cauterets	Cau	000.14444951	42.88263512	10.08.2013
Col de Tourmalet	CT	000.18655137	42.88357926	04.08.2013
Etang de Areau	Etar	001.13755862	42.73844451	26.07.2013
GlocknerForschungsstation	GF	012.59021205	46.87349964	14.08.2012
Gletsch	Glet	008.34917261	46.53948009	09.08.2005
Grindelwald	Grin	008.34917261	46.53948009	06.08.2003
GrancharRila	GRR	023.62406505	42.10001195	07.08.2011
Hochkönig	HO	013.03669011	47.44197926	13.08.2006
Königsstein	KÖ	025.25521250	45.54982944	13.08.2019
Lac de Sayen	LDS	000.22713762	42.94865481	09.08.2013
Loser	LO	013.73884252	47.60825146	05.08.2007
Medvodje	Med	014.71519500	46.41662963	18.07.2007
Oisternig	OI	013.49895954	46.49479040	05.08.2006
Partnun	Pat	009.83871852	46.96376659	07.08.2003
Port de Laurrau	PDL	000.99509131	42.92420538	12.08.2013
Peca	Peca	014.57227828	46.18152147	06.08.2006
PassoGroce Domini	PGD	010.37757524	45.77453490	12.08.2007
Plöckenpass	Ploe	012.95933898	46.58354109	04.08.2006
PoscenskiKraj	Popkra	019.08818954	42.99469424	02.08.2014
Popova Shapka	Posha	020.83895555	41.83845630	07-08.08.2011
PassoPromollo	PPR	013.27387304	46.54113982	17.08.2005
Passo San Marco	PSM	009.65906214	45.87224175	13.08.2007
Seebergsattel	SE	015.27724122	47.63096436	16.07.2007
Simplonpass	SIM	008.01663321	46.21699937	08.08.2005
Vail d´Illiez	Vail	006.89124460	46.16815996	08.08.2005

S16 GenBank accession numbers and corresponding haplotypes and genotypes.

mtDNA	CO1	ND1	Haplotypes	nDNA	RPS5	EF1alpha	Genotypes
EP_Ali1	MZ190632	MZ345012	H20	EPALI1	MZ190563	MZ190678	G54
EP_Ali2	MZ190633	MZ345013	H21	EPALI2	MZ190564	MZ190679	G64
EP_Ali3	MZ190634	MZ345014	H19	EPALI3	MZ190565	MZ190680	G53
EP_Ali4	MZ190635	MZ345015	H22	EPCAU1	MZ190566	MZ190681	G21
EP_Bab1	MZ190636	MZ345016	H39	EPCAU2	MZ190567	MZ190682	G20
EP_CAU1	MZ190637	MZ345017	H16	EPCAU3	MZ190568	MZ190683	G19
EP_CAU2	MZ190638	MZ345018	H12	EPCAU5	MZ190569	MZ190684	G9
EP_CAU4	MZ190639	MZ345019	H14	EPCT1	MZ190570	MZ190685	G8
EP_CAU5	MZ190640	MZ345020	H15	EPCT2	MZ190571	MZ190686	G4
EP_CT1	MZ190641	MZ345021	H10	EPCT3	MZ190572	MZ190687	G11
EP_CT2	MZ190642	MZ345022	H11	EPCT4	MZ190573	MZ190688	G2
EP_CT4	MZ190643	MZ345023	H9	EPCT5	MZ190574	MZ190689	G10
EP_Etar1	MZ190644	MZ345024	H6	EPETAR1	MZ190575	MZ190690	G7
EP_Etar2	MZ190645	MZ345025	H3	EPETAR2	MZ190576	MZ190691	G14
EP_Etar3	MZ190646	MZ345026	H7	EPETAR3	MZ190577	MZ190692	G3
EP_KOE1	MZ190647	MZ345027	H37	EPETAR5	MZ190578	MZ190693	G17
EP_POPKRA3	MZ190648	MZ345028	H18	EPGF2	MZ190579	MZ190694	G65
EP_GF1	MZ190649	MZ345029	H30	EPGF3	MZ190580	MZ190695	G62
EP_Glet1	MZ190650	MZ345030	H17	EPGF4	MZ190581	MZ190696	G66

EP_Grin1	MZ190651	MZ345031	H43	EPGF5	MZ190582	MZ190697	G61
EP_GRR1	MZ190652	MZ345032	H35	EPGLET1	MZ190583	MZ190698	G49
EP_GRR2	MZ190653	MZ345033	H41	EPGLET2	MZ190584	MZ190699	G48
EP_HO1	MZ190654	MZ345034	H36	EPGLET3	MZ190585	MZ190700	G47
EP_HO3	MZ190655	MZ345035	H40	EPGRIN1	MZ190586	MZ190701	G31
EP_LDS1	MZ190656	MZ345036	H13	EPGRIN3	MZ190587	MZ190702	G25
EP_LDS3	MZ190657	MZ345037	H8	EPGRIN4	MZ190588	MZ190703	G24
EP_LO1	MZ190658	MZ345038	H28	EPGRIN5	MZ190589	MZ190704	G30
EP_LO4	MZ190659	MZ345039	H38	EPGRR3	MZ190590	MZ190705	H58
EP_Med1	MZ190660	MZ345040	H24	EPHO2	MZ190591	MZ190706	G51
EP_Pat2	MZ190661	MZ345041	H44	EPHO3	MZ190592	MZ190707	G57
EP_PDL1	MZ190662	MZ345042	H4	EPHO4	MZ190593	MZ190708	G50
EP_PDL2	MZ190663	MZ345043	H2	EPHO5	MZ190594	MZ190709	G56
EP_PDL3	MZ190664	MZ345044	H5	EPLDS1	MZ190595	MZ190710	G6
EP_PDL4	MZ190665	MZ345045	H1	EPLDS3	MZ190596	MZ190711	G16
EP_PECA1	MZ190666	MZ345046	H23	EPLDS4	MZ190597	MZ190712	G18
EP_PECA2	MZ190667	MZ345047	H25	EPLDS5	MZ190598	MZ190713	G5
EP_PGD1	MZ190668	MZ345048	H29	EPLO1	MZ190599	MZ190714	G60
EP_PGD2	MZ190669	MZ345049	H27	EPLO4	MZ190600	MZ190715	G44
EP_PLOE3	MZ190670	MZ345050	H31	EPMED3	MZ190601	MZ190716	G67
EP_PLOE5	MZ190671	MZ345051	H32	EPMED4	MZ190602	MZ190717	G43
EP_POSHA2	MZ190672	MZ345052	H34	EPOI1	MZ190603	MZ190718	G23
EP_PSM1	MZ190673	MZ345053	H33	EPPDL1	MZ190604	MZ190719	G1

EP_SE2	MZ190674	MZ345054	H26	EPPDL2	MZ190605	MZ190720	G15
EP_Vail1	MZ190675	MZ345055	H42	EPPDL4	MZ190606	MZ190721	G13
EP_Vail2	MZ190676	MZ345056	H46	EPPDL5	MZ190607	MZ190722	G12
EP_Vail5	MZ190677	MZ345057	H45	EPPGD1	MZ190608	MZ190723	G40
				EPPGD2	MZ190609	MZ190724	G39
WSP1	MZ358189			EPPGD3	MZ190610	MZ190725	G32
WSP2	MZ358190			EPPGD5	MZ190611	MZ190726	G38
WSP3	MZ358191			EPPLOE1	MZ190612	MZ190727	G63
				EPPLOE2	MZ190613	MZ190728	G45
				EPPLOE3	MZ190614	MZ190729	G46
				EPPLOE5	MZ190615	MZ190730	G22
				EPPOSHA1	MZ190616	MZ190731	G52
				EPPOSHA2	MZ190617	MZ190732	G55
				EPPPR3	MZ190618	MZ190733	G41
				EPPSM1	MZ190619	MZ190734	G36
				EPPSM2	MZ190620	MZ190735	G33
				EPPSM3	MZ190621	MZ190736	G35
				EPPSM4	MZ190622	MZ190737	G37
				EPPSM5	MZ190623	MZ190738	G34
				EPSE3	MZ190624	MZ190739	G59
				EPVAIL1	MZ190625	MZ190740	G29
				EPVAIL2	MZ190626	MZ190741	G28
				EPVAIL3	MZ190627	MZ190742	G27

				EPVAIL5	MZ190628	MZ190743	G26
				EPKOE1	MZ190629	MZ190744	G68
				EPKOE3	MZ190630	MZ190745	G42
				Erebia melas1	MZ190749	MZ190747	G69
				Erebia melas6	MZ190750	MZ190748	G70
				Erebia aethiops	MZ190631	MZ190746	