

Genetic assessment and climate modelling of the Iberian specialist butterfly *Euchloe bazae* (Lepidoptera: Pieridae)

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Abstract

1. Although both positive (expansion of thermophilous species) and negative effects (retraction of cold-adapted species) have been attributed to global warming, range shifts may be constrained by the ecological traits of species.
2. This can be especially true for highly specialised organisms like the Spanish Greenish Black-tip (*Euchloe bazae*), an Iberian endemic specialist of semiarid steppes with a debated taxonomic and conservation status.
3. Here, we first seek to clarify the taxonomic status of this butterfly and its populations by using multilocus phylogenetic inference. Then, we update its distribution range and employ ecological niche modelling, combined with other sources of data, to re-evaluate its conservation status.
4. Our results confirm *E. bazae* as a well-differentiated species with one of the most restricted distribution ranges among the European butterflies.
5. We demonstrate that its two disjunct populations, regarded as subspecies, are genetically differentiated and should be treated as independent management units.
6. Climate models under two future emission scenarios suggest an increase of the area climatically suitable. However, the suitability of the areas currently occupied is estimated to decrease, meaning that rapid range shifts could be required for the survival of the species.
7. Given the presumably low dispersal capabilities of *E. bazae*, its current restricted distribution, high degree of population fragmentation (divided in two distant populations), low intrapopulation genetic variability, decline in extension and abundance, low densities, high yearly fluctuations, and numerous threats to its habitat, we consider that their populations are extremely fragile and we propose to consider it 'Endangered' [EN B1ab(i,ii,iii,iv); B2ab(i,ii,iii,iv)].

Resum (Catalan)

1. Tot i que s'han atribuït tant efectes positius (expansió d'espècies termòfiles) com negatius (contracció d'espècies adaptades al fred) a l'escalfament global, canvis en les distribucions poden estar condicionats pels trets ecològics de les espècies.

Paula Escuer and Joan C. Hinojosa contributed equally to this work.

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2. Això pot ser especialment cert per a organismes altament especialitzats com la grogueta del desert (*Euchloe bazae*), un endemisme ibèric especialista d'estepes semiàrides amb un estatus taxonòmic i de conservació debatut.
3. En aquest estudi, primer cerquem esclarir l'estatus taxonòmic d'aquesta papallona i de les seves poblacions mitjançant la inferència filogenètica multilocus. També actualitzem la seva distribució i fem servir la modelització de nínxols ecològics combinada amb altres dades per reavaluar el seu estatus de conservació.
4. Els nostres resultats confirmen que *E. bazae* és una espècie ben diferenciada i amb una de les distribucions més restringides entre les papallones europees.
5. Demostrem que els seus dos nuclis de població, cadascun considerat una sub-espècie, són diferents genèticament i s'han de tractar com a unitats de gestió independents.
6. Els models climàtics elaborats amb dos escenaris d'emissions futurs suggereixen un augment de l'àrea climàticament adient. No obstant això, s'estima que la idoneïtat de l'àrea actualment ocupada disminuirà, el que podria requerir canvis ràpids en la seva distribució per garantir-ne la supervivència.
7. Tenint en compte que *E. bazae* disposaria d'una capacitat de dispersió baixa, la distribució actual reduïda, l'alt grau de fragmentació (dividida en dues poblacions llunyanes), la baixa variabilitat genètica intrapoblacional, la disminució de l'extensió i l'abundància, les baixes densitats, les elevades fluctuacions anuals i les nombroses amenaces al seu hàbitat, considerem que les poblacions d'aquesta papallona són extremament fràgils i proposem considerar-la "En perill" [EN B1ab(i,ii,iii,iv); B2ab(i,ii,iii,iv)].

KEYWORDS

climate change, conservation, ecological niche modelling, phylogenetics, xerothermophilous

INTRODUCTION

Several recent publications have drawn attention to the increasing evidence of an ongoing global insect decline (see a review by Wagner et al., 2021), and terms such as 'insect apocalypse' and 'insect collapse' are becoming increasingly popular. The decrease in insect abundance affects almost all orders, and butterflies (Papilionoidea superfamily) are not an exception (Warren et al., 2021). In Europe, probably the best-studied region regarding long-term population trends, significant decreases have been reported: the combined index of abundance has declined by 30% for grassland butterflies since 1990 (van Swaay et al., 2020). In this context, the latest Red List of European Butterflies (van Swaay et al., 2010) classifies 37 out of 435 assessed species (9%) as threatened (3 critically endangered, 12 endangered, and 22 vulnerable) and 44 species (10%) as near threatened. However, this is considered a conservative estimate (van Swaay et al., 2011) and further studies are required in order to evaluate in more detail the status of these species.

The reasons that explain the decline of butterflies are numerous, but they are largely linked to their ecology. Butterflies frequently display a metapopulation pattern (Thomas & Hanski, 1997), that is, a

network of interconnected populations that inhabit patches where their ecological requirements are fulfilled – generally open areas with the presence of larval host plants and sources of nectar for the adults. Thus, habitat alteration linked to human activity has the potential to erode the metapopulation network by reducing the availability and/or suitability of these patches. This can trigger a chain reaction characterised by local extinctions, increasing isolation between populations (i.e. increasing fragmentation) and the reduction of genetic diversity (Berwaerts et al., 1997; Hill et al., 2006).

The main factors responsible for habitat alteration are the combination of the abandonment of the land (reducing the availability of open areas) and the implementation of an intensive agricultural model in which agrochemicals (pesticides, herbicides and fungicides) are omnipresent (Herrando et al., 2016; Settele et al., 2009; Warren et al., 2021). Additionally, in the last decades, climate change arose as one of the main pressures exerted over butterfly populations and their habitats. Butterfly distributions seem to be highly sensitive to climatic changes (e.g. Mattila et al., 2011; Scalerio et al., 2014). Although it could be argued that global warming has both positive (expansion of thermophilous species) and negative (retraction of cold-adapted species) effects on them, this view represents a simplification of a more complex reality. Indeed, the response of populations to global

warming is conditioned by several other factors intrinsic to the biology of each species (Mattila et al., 2011; Melero et al., 2016; Stefanescu et al., 2011) and the degree of population fragmentation (Bellard et al., 2012; Leadley et al., 2010), especially for species with limited ranges (Devictor et al., 2008; Parmesan, 2006). Hence, the fate of highly specialised thermophilous species with small ranges remains unclear in relation to the effect of global warming.

An example of a thermophilous species with a restricted distribution is the Iberian endemic *Euchloe bazae* Fabiano, 1993. It is a specialist of semiarid steppes and, prior this study, it has been recorded in only twelve 10 km × 10 km Universal Transverse Mercator (UTM) squares (Munguira et al., 2017). Two subspecies have been described, corresponding to two groups of allopatric populations: one is located in the Ebro Valley, northern Iberia (*E. bazae iberiae* Back, Olivares and Leestmans, 2005), and the other in Hoya de Baza, southern Iberia (*E. bazae bazae* Fabiano, 1993). Since 2019, *E. bazae* is classified as 'endangered' by the Spanish Catalogue of Threatened Species (officially as 'Catálogo Español de Especies Amenazadas'). It is the third species of butterfly to achieve this top degree of protection in Spain but, contrastingly, its status is settled as 'least concern' (van Swaay et al., 2015) in the IUCN Red List and as 'vulnerable' in the European Red List of butterflies (van Swaay et al., 2010).

The study of *E. bazae* presents additional challenges since its taxonomic status (of the species as a whole and of its subspecies) and the phylogenetic relationships with relatives require clarification. In fact, this butterfly was originally described as a subspecies of *Euchloe charlonia* (Donzel, 1842), a taxon from North Africa and the Middle East, and this status has been maintained by several authors (e.g. García-Barros et al., 2004; Lafranchis, 2004; Tolman & Lewington, 2008). Only recently, it has increasingly been recognised as a distinct species (e.g. García-Barros et al., 2013; Tshikolovets, 2011; Wiemers et al., 2018). Its taxonomic status and its placement within the *charlonia* group (subgenus *Elphinstonia*) – which, according to Back et al. (2006), includes *E. bazae*, *E. charlonia*, *E. lucilla* Butler, 1886, *E. penia* (Freyer, 1852) and *E. transcaspica* (Staudinger 1892) – is supported by mitochondrial DNA barcodes (Back et al., 2006; Dincă et al., 2015, 2021), but no nuclear DNA evidence has been published.

In this study, (1) we assess the taxonomic status of the taxon *bazae* and its putative subspecies, and (2) we re-evaluate its conservation status, paying special attention to the impact of climate change. To address the first point, we sequenced mitochondrial (*COI*, *ND1*) and nuclear (*wg*, *ITS2*) DNA markers and studied the evolutionary history of the western Palearctic species of the *charlonia* group sensu Back et al. (2006). For the second goal, we provide an updated distribution range at high resolution (1 km × 1 km UTM grid), which we use in climate-based distribution modelling to estimate the past and present distribution of the species, as well as to forecast the impact of climate change on its distribution in the near future. We integrated these predictions with current range and density data, observed trends, genetic evidence, and a review of habitat threats, to re-evaluate the conservation status of *E. bazae* using the IUCN criteria.

MATERIALS AND METHODS

Presence/absence records

Presence records for *E. bazae* were obtained in field surveys conducted by the authors from 2008 to 2021. GPS coordinates were recorded at high resolution and then translated into a UTM grid of a 1 × 1 km resolution. Absence coordinates (no record for this species) were obtained from localities where long-term weekly or by-weekly monitoring has been performed for at least 2 years under the programmes BMS España (<https://butterfly-monitoring.net/es/spain-bms>) and the Catalan BMS (www.catalanbms.org).

Sampling, DNA extraction and sequencing

A total of 49 samples were used for DNA analyses (Table S1; Figure 1a). We obtained 42 cytochrome c oxidase subunit I sequences (*COI*; fragments *COIa* or barcode and *COIb*), 41 NADH dehydrogenase 1 (*ND1*), 33 internal transcribed spacer 2 (*ITS2*) and 34 wingless (*wg*) sequences; six additional *COIa* sequences were retrieved from GenBank (Table S1). These sequences included all the species of the *charlonia* species group except for the central Asian taxon *E. lucilla*, a species closely related to *E. transcaspica* (Back et al., 2006). Individuals of *E. tomyris*, *E. crameri* and *E. belemia* were used as outgroups.

Total genomic DNA was extracted using Chelex 100 resin, 100–200 mesh, sodium form (Biorad), under the following protocol: one leg was removed and introduced into 100 µl of Chelex 10% to which 5 µl of Proteinase K (20 mg/ml) were added. The samples were incubated overnight at 55°C in a shaker and were subsequently incubated at 100°C for 15 min. Primers and PCR protocols used for the amplification of *COIa*, *COIb*, *ND1*, *wg* and *ITS2* are provided in Tables S2 and S3. Universal tails were included in all primers. PCR products were purified and Sanger sequenced by MacroGen Inc. Europe (Amsterdam, the Netherlands). All sequences have been deposited in GenBank (Table S1).

Genetic analyses

Sequences were visualised, edited and aligned with Geneious Prime 2019.0.3 (<https://www.geneious.com>). Uncorrected p-distances were calculated in Geneious Prime for the *COI* barcode region and *ITS2*. Haplotype diversity (*Hd*) and nucleotide diversity (π) were estimated for the barcode region using the functions *hap.div* and *nuc.div* implemented in pegas v1.0-1 (Paradis, 2010) R package; the sample RVcoll06K685 was excluded from these analyses due to the presence of missing data.

Two phylogenies were constructed in BEAST v2.5.0 (Bouckaert et al., 2014), one based on the nuclear markers and another using mitochondrial markers. Distinct partitions were assigned for each gene, using the best model according to jModelTest (Darriba et al., 2012). Four rate categories (if gamma was included) were used

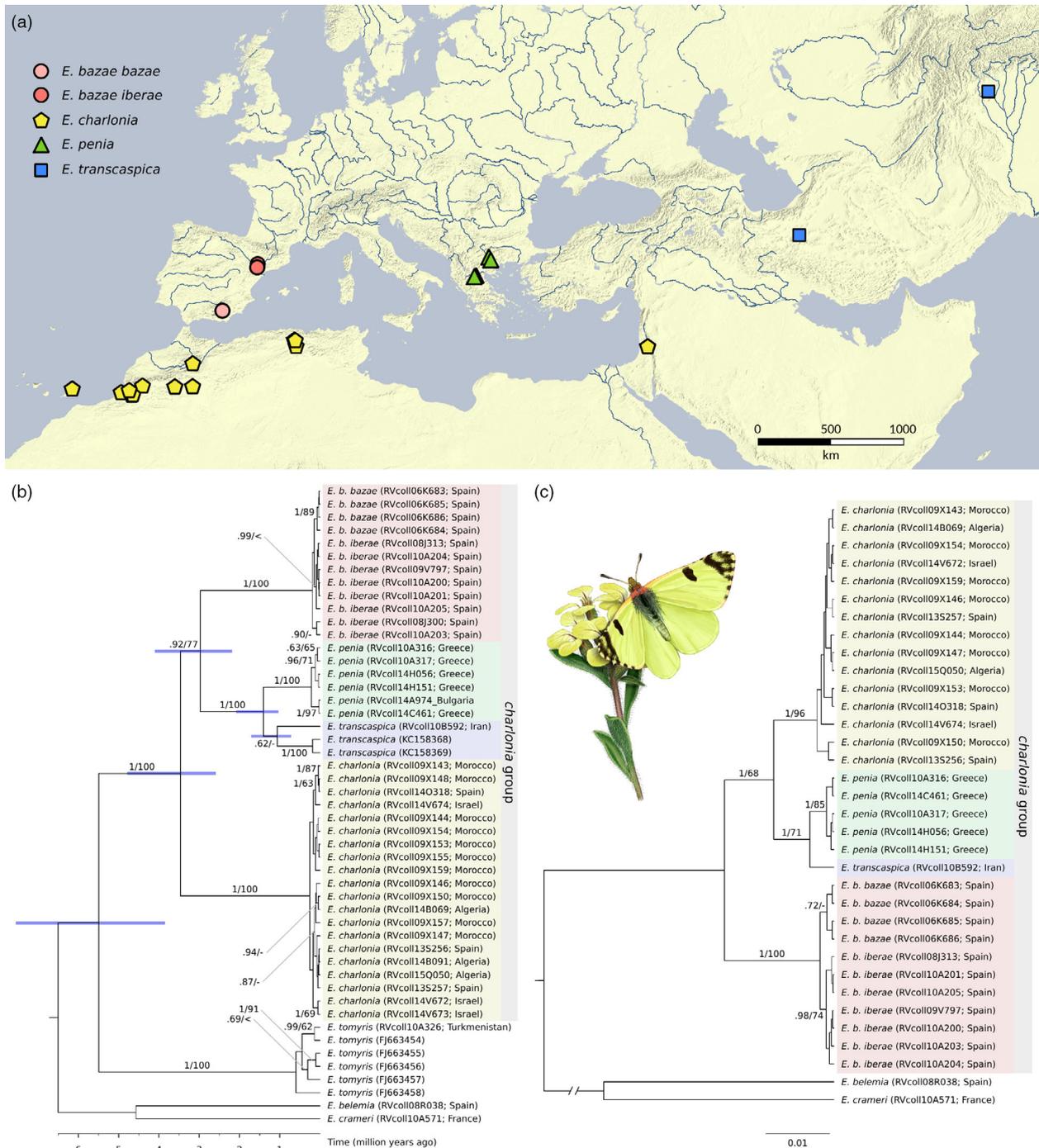


FIGURE 1 (a) Sampling sites of taxa of the *charlonia* group included in this study: *Euchloe bazae*, *E. charlonia*, *E. penia* and *E. transcaspica*. (b, c) Phylogenies obtained through Bayesian inference. Posterior probabilities (left, values >0.6) and bootstrap supports (right, values >60) of the nodes are indicated. (b) Mitochondrial DNA phylogeny (COI + COIb + ND1); the x axis indicates time in millions of years and the blue bars show the 95% HPD range for the posterior distribution of node ages. (c) Nuclear DNA phylogeny (ITS2 + wg); scale units are presented in substitutions per site

and base frequencies were estimated. In the mitochondrial phylogeny, the COI fragment was used to obtain rough estimates of node ages. These estimates were obtained by applying a strict clock and a normal prior distribution centred on the mean between two commonly used substitution rates for invertebrates: 1.5% and 2.3% uncorrected pairwise distance per million years (Quek et al., 2004 and

Brower, 1994; respectively). The standard deviation was tuned so that the 95% confidence interval of the posterior density coincided with the 1.5% and 2.3% rates. Parameters were retrieved using two independent runs of 20 million generations each and convergence was checked with TRACER 1.7.1 (Rambaut et al., 2018). A 10% burn-in was applied and results from both runs were merged.

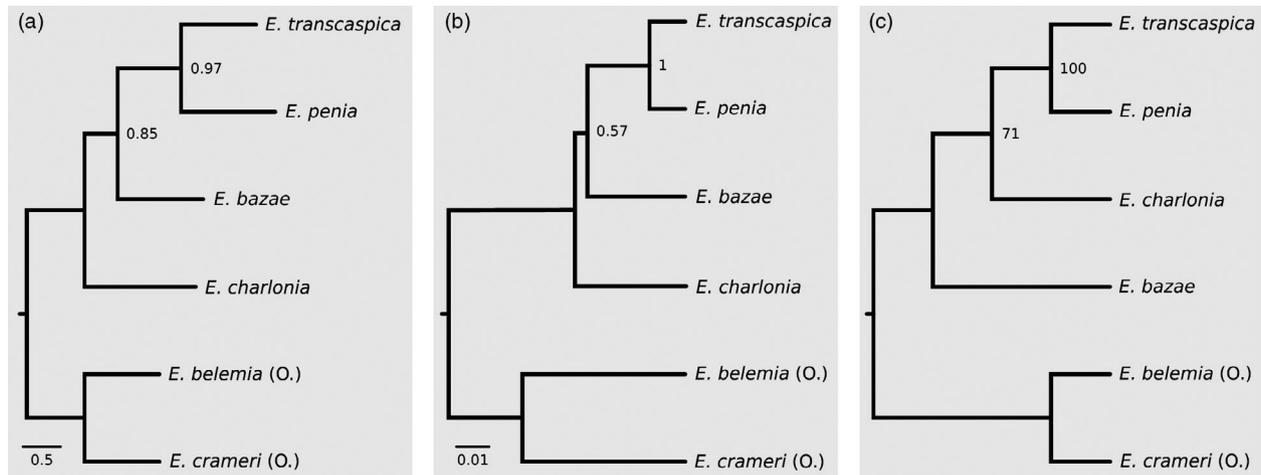


FIGURE 2 Species trees estimated using mitochondrial and nuclear genes. Species tree obtained with (a) ASTRAL (branch lengths are expressed in coalescent units), (b) *BEAST (branch lengths in substitutions per site) and (c) SVDquartets

Species trees were estimated using both nuclear and mitochondrial loci with three methodologies. First, we ran *Beast (Heled & Drummond, 2009) using the best model indicated by jModelTest for each gene. Four rate categories (if gamma was included) were selected and base frequencies were estimated. Parameters were estimated using two independent runs of 30 million generations each and convergence was checked with TRACER 1.7.1. A burn-in of 10% was applied. Second, we used SVDQuartets (Chifman & Kubatko, 2015) implemented in PAUP* v4.0a169 (Swofford, 2003) selecting 1000 bootstrap replicates and maintaining the other parameters as default. Third, we obtained a species tree in ASTRAL v5.7.5 (Zhang et al., 2018) using the default settings. In this case, the gene trees were retrieved by maximum likelihood (ML) inference in CIPRES (Miller et al., 2010) and by using RAxML-HPC2 v8.2.12 (Stamatakis, 2014).

Ecological niche modelling

Raster layers for 19 standard climatic variables at 30 arc-sec (1 km approximately) resolution were downloaded from WorldClim v1.4 (<http://www.worldclim.org/>) for current times and future scenarios, and at 2.5 arcmin (4.1 km approximately) for past scenarios. All layers were cropped for the extension of the Iberian Peninsula. Principal component analyses were run to select the least multicollinear variables. We selected the biologically most meaningful variables for *E. bazae* that were also correlated less than 80% to the rest of the variables: BIO3 (isothermality), BIO7 (temperature annual range), BIO8 (mean temperature of the wettest quarter), BIO15 (precipitation seasonality), BIO16 (precipitation of the wettest month), and BIO18 (precipitation of the warmest quarter).

To project the potential distribution using current, future and past climate data, we employed an ensemble forecasting approach implemented in the package biomod2 v3.3-7.1 (Thuiller et al., 2009). We loaded 121 presence points and a total of 111 true absences and the current climatic layers of selected variables and proceeded setting

the parameters for the ensemble forecast of species distribution models. We set the number of replicates to 10 and included projections from three regression methods [generalised linear model (GLM), generalised additive model (GAM), and multiple adaptive regression splines (MARS)], and one machine-learning method [random forest (RF)]. Then we proceeded to evaluate the accuracy of the ensemble models; for that, three different parameters were calculated, namely area under the ROC curve (AUC), the true skill statistic (TSS) and the kappa statistic.

We first projected the ensemble models for their current distributions. Subsequently, we loaded future and past climate layers to predict the distributions. For that, we used the Intergovernmental Panel on Climate Change (IPCC) future projections data, using the same climatic variables as in the present models. We used statistical downscaling (delta method) layers based on the Community Climate System Model 4 (CCSM4) created by the Community Earth System Model (CESM) for the Last Glacial Maximum (LGM) and for the years 2050 and 2070, in which two varied Representative Concentrations Pathways (RCP) were selected. For future predictions, the chosen models were: A1 (CMIP5, RCP4.5), a low-emission scenario with a human-induced global warming of 1.8 °C, and A2 (CMIP5, RCP8.5), a high-emission scenario with a warming of 3.7 °C – see a more detailed explanation of the scenarios in IPCC (2014). Finally, we calculated the response curves of the biologically most meaningful climatic variables to visualise which ones and how they influence the species distribution.

RESULTS

Species estimated occurrence

Based on 13 years of field surveys (2008–2021), *E. bazae* was recorded in a total of 58 1 × 1 km UTM squares, with two main areas of occurrence. One area are 43 1 × 1 km² in Hoya de Baza (southern Iberia) and

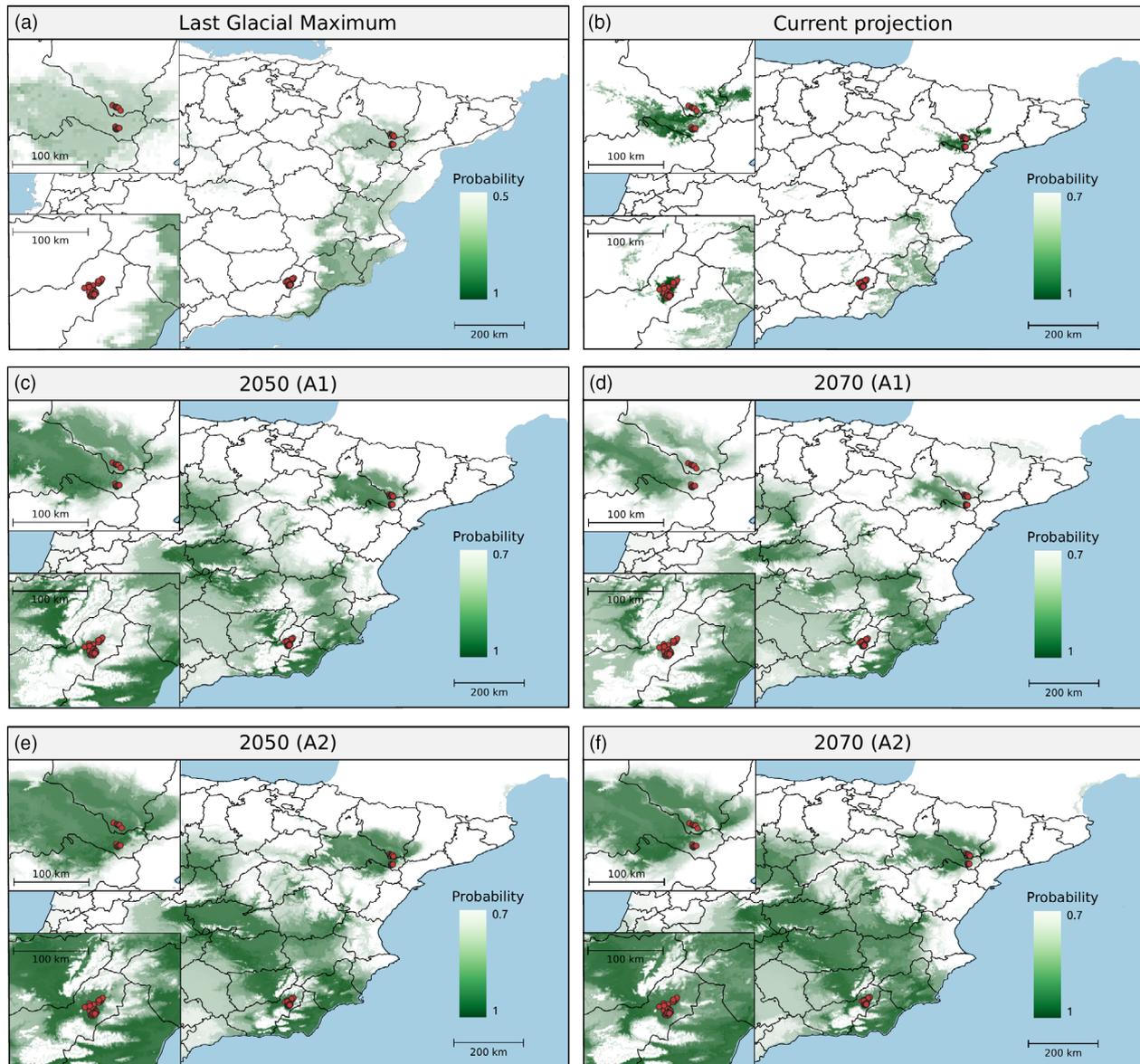


FIGURE 3 Ecological niche models for *Euchloe bazae* for (a) the Last Glacial Maximum (LGM; ~21,000 years before present), (b) present, (c, e) 2050 and (d, f) 2070. Future projections consider scenarios of (c, d) low emissions (A1) and (e, f) high emissions (A2). Red dots represent *E. bazae* records

corresponds to *E. b. bazae*. The other corresponds to *E. b. iberae*, which inhabits the Ebro Valley (northern Iberia) in 15×1 km UTM squares, and is divided in two subpopulations separated by ~20 km: Barranco de Valcuerna (north of the Ebro River, 7×1 km²) and south-eastern Caspe (south of the Ebro River, 8×1 km²). The resulting area of occupancy (AOO) = 58 km² (*E. b. bazae* = 43 km² and *E. b. iberae* = 15 km²). The extent of occurrence (EOO) = 385 km² (*E. b. bazae* = 210 km² and *E. b. iberae* = 175 km²).

Genetic distances, phylogenetic relationships, and divergence times

Based on the COI barcode region (Table S4), *E. bazae* and *E. penia* displayed the highest interspecific divergence (minimum p-distance

= 5.84%, mean p-distance = 6.26%) while the lowest differentiation was found between *E. penia* and *E. transcaspica* (minimum = 2.55%, mean = 2.95%). In *E. bazae*, the haplotype diversity (H_d) and nucleotide diversity (π) were 0.600 and 0.0012, respectively. The maximum intraspecific p-distance for the species was 0.46%, the mean intraspecific p-distance was 0.14%, the mean p-distance within *E. b. bazae* was 0.09%, and the mean p-distance within *E. b. iberae* was 0.04%. The minimum p-distance between the two subspecies was 0.15% and the mean p-distance was 0.23%. Overall, *E. b. iberae* and *E. b. bazae* were differentiated by three fixed substitutions: one in COI, one in ND1 and another in wg. Based on ITS2 (Table S5), the highest interspecific divergence was found between *E. bazae* and *E. charlonia* (minimum p-distance = 3.57%, mean p-distance = 3.82%), and again *E. penia* and *E. transcaspica* displayed the lowest differentiation (minimum = 0.50%, mean = 0.57%).

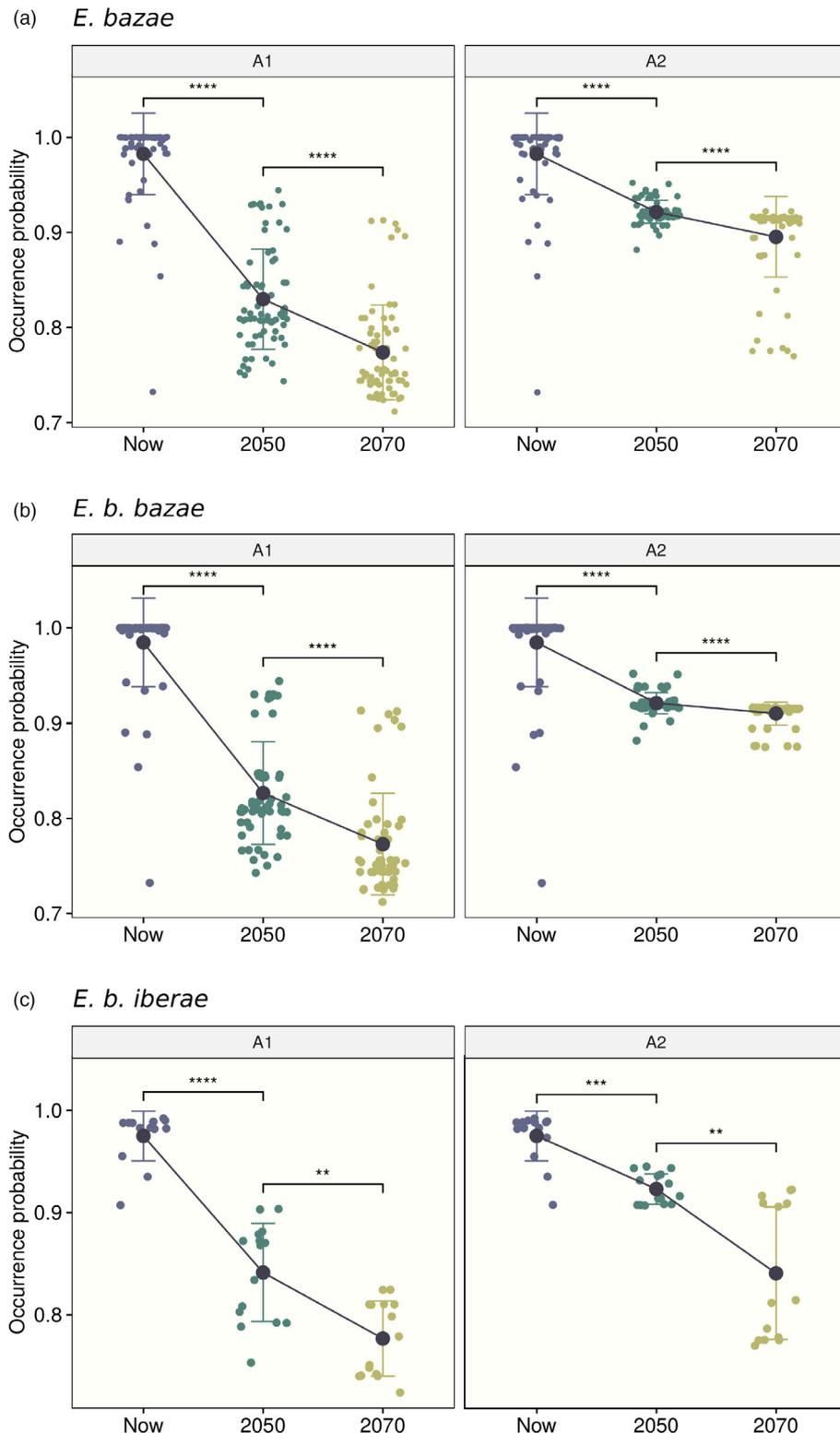


FIGURE 4 Evolution of the estimated suitability of the areas currently inhabited by *Euchloe bazae* from the present to 2070, under scenarios of low emissions (A1) and high emissions (A2). Results are presented considering (a) the overall sites currently inhabited by the species and the sites currently inhabited only by the subspecies (b) *E. b. bazae* and (c) *E. b. iberae*. Means and standard deviations are shown. Asterisks represent statistical significance (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$)

The mitochondrial DNA (mtDNA) phylogeny (Figure 1b; Figure S1a) recovered the *charltonia* group as a well-supported monophyletic group. *Euchloe bazae*, *E. penia* and *E. charltonia* were each monophyletic, with very

good support, but the monophyly of *E. transcaspica* was poorly supported. *Euchloe charltonia* was recovered as the most basal species within the group, while *E. bazae* was sister [albeit moderately supported: posterior

probability (PP) = 0.92; bootstrap support (BS) = 77%] to the well-supported clade formed by *E. penia* and *E. transcaspica*. According to our estimations, the *charlonia* group split from the *tomyris* group ca. 5.5 (3.7–7.5) million years ago (mya). The *charlonia* group started diversifying ca. 3.5 (2.5–4.7) mya, during the Pliocene, when *E. charlonia* split from the rest. *Euchloe bazae* split ca. 3.0 (2.1–4.0) mya and *E. penia* and *E. transcaspica* clades split ca. 1.4 (0.9–2.0) mya. Regarding *E. bazae*, the subspecies *E. b. bazae* was recovered as monophyletic (PP = 1; BS = 89%) while the monophyly of *E. b. iberae* was unresolved.

Similarly to mtDNA, the nuclear DNA (nDNA) phylogeny (Figure 1c; Figure S1b) recovered *E. bazae*, *E. penia* and *E. charlonia* as monophyletic with good supports. The monophyly of *E. transcaspica* could not be assessed since only one individual was available. The two subspecies of *E. bazae* formed two clades, although these were poorly differentiated and only *E. b. iberae* was relatively well supported (PP = 0.89; BS = 74%).

Euchloe transcaspica and *E. penia* formed a clade in both the mtDNA phylogeny and the nDNA phylogeny. However, the two phylogenies did not agree in the positions of *E. bazae* and *E. charlonia*: while the mtDNA phylogeny recovered *E. charlonia* as the most basal species of the group and *E. bazae* was sister to *E. transcaspica* + *E. penia*, in the nDNA phylogeny *E. bazae* was the most basal and *E. charlonia* was sister to *E. transcaspica* + *E. penia*.

The different methods used for species tree inference (Figure 2) were also discordant regarding the basal position in the group. *Euchloe bazae* was recovered as sister to *E. transcaspica* + *E. penia* and hence *E. charlonia* was the most basal taxon in the ASTRAL (local posterior probability = 0.85) and *BEAST (PP = 0.57) species trees. In contrast, SVDquartets placed *E. bazae* in the basal position and *E. charlonia* as sister to *E. transcaspica* + *E. penia* (BS = 71%).

Climate modelling trends

From the selected variables, those that mostly affected the probability of occurrence of *E. bazae* were (Figure S2; Table S6): the precipitation of the warmest quarter (BIO18), the temperature annual range (BIO7; the difference between the maximum temperature in the warmest month and the minimum temperature of the coldest month) and the precipitation of the wettest month (BIO16). The presence of the species is predicted when the annual range of temperatures is higher than 30°C, the annual precipitations drop below 200 mm, and the precipitation of the warmest quarter is below 50 mm. These results highlight the preference of *E. bazae* for very dry continental climates.

The LGM projection (Figure 3a) estimated that the area climatically suitable for the species may be wider than the one at present, particularly in the eastern part of the Iberian Peninsula. The area of Hoya de Baza where *E. b. bazae* occurs was estimated as unsuitable in the LGM and thus was likely colonised postglacially, probably from more coastal regions. In contrast, the populations in the Ebro Valley could have persisted since the LGM. Interestingly, a tight corridor could have connected northern and southern populations through the valleys of the Túrria and Jiloca rivers.

The model projection to present conditions (Figure S3a) shows two areas with very high (>0.9) occurrence probabilities. These areas correspond to the approximate distribution of the two extant populations of *E. bazae*: the population from the Ebro Valley in northern Iberia (*E. b. iberae*) and the population from Hoya de Baza in southern Iberia (*E. b. bazae*). The estimated climatically suitable area in the Ebro Valley is considerably larger than the area delimited by empirical observations. When considering areas with occurrence probabilities >0.7, the potential distribution further increases in the Ebro Valley and it also expands along the Mediterranean coast in the south (Figure 3b). Assuming that the species does not in fact occur in a wider area, this may indicate that values between 0.7 and 0.9 (or even higher in the Ebro Valley) do not offer proper climatic conditions for the species or, if they are, other factors limit its occurrence – for example, the absence of larval host plant or the difficulty to colonise such suitable localities, possibly due to habitat fragmentation. The model projections to future scenarios predict an increase of the climatically suitable area compared to present. In Scenario A1 (low emissions), a potential range expansion by 2050 (Figure 3c; Figure S3b) is followed by a slight reduction by 2070 (Figure 3d; Figure S3c). Overall, the Scenario A2 (high emissions) predicts higher increases of the climatically suitable area (Figure 3e,f; Figure S3d-e).

In both A1 and A2 scenarios, predictions show that the suitability of the currently occupied areas will decrease significantly for the two subspecies (Figure 4). The mean of the probability of occurrence in the areas with presence records is 0.983 for the present but decreases to 0.830 in A1 and 0.921 in A2 for 2050, and to 0.774 in A1 and 0.895 in A2 for 2070. This is an overall decline in the occurrence probability of 21.2% (A1) and 9.0% (A2) by 2070 compared to the present. The decline is especially high in localities where *E. b. iberae* is currently present. The current mean of the probability of occurrence where this subspecies has been recently recorded is 0.975, but it descends to 0.841 in A1 and 0.923 in A2 for 2050 and to 0.777 in A1 and 0.841 in A2 for 2070. This represents a decline of 20.3% (A1) and 13.7% (A2) by 2070. For *E. b. bazae*, the current mean of the probability of occurrence where it is present is 0.985, but it drops to 0.827 in A1 and 0.921 in A2 for 2050 and to 0.773 in A1 and 0.910 in A2 for 2070. In this subspecies, the estimated overall declines until 2070 are much lower in A2 (7.6%) but reach 21.5% in A1.

DISCUSSION

Evolutionary history of the *charlonia* species group

The mtDNA and nDNA phylogenies (Figure 1b,c) were discordant regarding the most basal taxon of the *charlonia* species group, a position that is alternated between *E. charlonia* – according to mtDNA and confirming the results of Back et al. (2006) – and *E. bazae* – according to nDNA. Coalescent-based methods also did not agree in the position of these two species, although two out of three placed *E. charlonia* at the base (Figure 2). In fact, the 95% HPD ranges for the

posterior distribution of node ages widely overlapped (Figure 1b) and it seems probable that both species emerged about the same time. Thus, during the Pliocene, the *charlonia* group was divided into three lineages: (1) *E. charlonia*, currently present from North Africa to the Middle East, (2) the Iberian taxon *E. bazae*, and (3) *E. penia* + *E. transcaspica* – *E. lucilla* would also be included in this lineage according to Back et al. (2006) –, a group distributed from the Balkan Peninsula to Central Asia. The emergence of these three groups could be the result of the fragmentation of populations as a consequence of the Zanclean flood, although our oldest estimation for the diversification of the *charlonia* species group is 4.7 mya and the Mediterranean was refilled about 5.3 mya (Roveri et al., 2014). The desiccation of the Mediterranean Sea could have favoured the expansion of the group by connecting continents and creating new areas with arid climates (Rouchy & Caruso, 2006). This expansion through the Mediterranean Basin and Europe would have ended with the Zanclean flood and during the Pliocene, an epoch when evidence pinpoints that Europe was unsuitable for these species: it included periods when most of Europe and the Mediterranean were more humid than today (Fauquette et al., 1999; Haywood et al., 2000; Jost et al., 2009) and dry steppe habitats appeared to be scarce in Central Europe (e.g. Popescu et al., 2010).

Our results fully confirm that *E. bazae* is a valid species, with an estimated age of ca. 3 million years. The distance values obtained from comparisons between *E. bazae* and the rest of the species are typically interspecific in both *COIa* and *ITS2*. For *COIa* the minimum interspecific distance is 4.79% with respect to *E. charlonia*, while the median of the minimum interspecific distances with the nearest neighbour is 2.1% for European Papilionoidea (Dincă et al., 2021). The minimum distance for the *ITS2* marker is 3.17% with respect to *E. transcaspica* and similar for the rest (compare with values obtained for other taxa, for example, Hinojosa et al., 2021; Wiemers et al., 2010). The validity of the subspecies *E. b. bazae* (southern population) and *E. b. iberae* (northern population), as suggested by Back et al. (2006), also has a genetic basis: they are genetically distinct, with fixed mutations in both the mtDNA and the nDNA. Thus, no trace of ongoing or recent gene flow between subspecies is observed. Based on the low genetic distance between the two subspecies, the two populations were possibly in contact during one or several of the most recent Pleistocene ice ages. Despite the distance that currently separates them (~450 km), the ecological niche modelling (ENM) results showed that in the LGM they could have been connected through the valleys of the Túrria and Jiloca rivers, although most of the estimated area of distribution was close to the Mediterranean coast, where mean annual temperatures were warmer and reached up to 20°C (Braconnot et al., 2007).

The response of *E. bazae* to climate change

The response curves of the selected climatic variables (Figure S2) pinpoint that *E. bazae* is linked to dry (low precipitation in the wettest month and warmest quarter) continental (high-temperature annual

ranges) climate. Given this situation, estimating the impact of global warming on the distribution of this species is not straightforward and indeed the ENM results suggested a relatively complex situation (Figure 3). For 2050, model predictions based on both Scenarios A1 (low emissions) and, especially, A2 (high emissions) showed a potential increase of the climatically suitable area. In contrast, model predictions for the year 2070 presented a range reduction with respect to that estimated for 2050, in general for Scenario A1 and in the Ebro Valley area for A2. In fact, this pattern characterised by an initial increase of the suitable range followed by its decrease in longer term is not exclusive for *E. bazae* and was estimated for 25%–33% of the European butterflies (Settele et al., 2008).

It is worth noting that a significant decrease in the suitability of the area that the species currently occupies is estimated in both scenarios and for both subspecies already in 2050 (Figure 4). Considering that the mean of the occurrence probability in areas currently occupied by the species is well above 0.9, but it will drop below this value in the next decades – except for *E. b. bazae* in Model A2 –, this may force populations to shift their distribution in a very short period of time. In changing landscapes, butterfly distributions are dependent on the capacity of the species to colonise new areas (Thomas et al., 1998), which relies on factors related to their biology and ecology such as specialisation (which includes larval host specialisation and habitat specificity), voltinism, and the duration of flight period (Sekar, 2012). *Euchloe bazae* presents high larval host plant and habitat specialisation, univoltinism and remarkably short flight time and, hence, it is expected to have a poor capacity to face distribution shifts. Despite this, the two subspecies could have distinct reactions to changing environments as they use distinct larval host plants: *E. b. bazae* feeds exclusively on *Eruca vesicaria* (L.) Cav. and *E. b. iberae* depends mainly on *Vella aspera* Pers. (Munguira et al., 2015). *Eruca vesicaria* is an opportunistic annual plant distributed in all the Iberian Peninsula, capable of colonising disturbed areas such as fields and roadsides. This could be a crucial factor to enable the spread of *E. b. bazae*. Contrastingly, *V. aspera* is endemic to north-eastern Iberia with a restricted distribution range (Recasens et al., 1988). Although it can be locally abundant (Goñi Martínez & Sanz Trullén, 2019), it is not an annual plant and requires several years to develop. Furthermore, it has been suggested that the populations of this plant were heavily fragmented recently (Perez-Collazos et al., 2008) and that they could decline due to changes in land management (Puente, 2013). These factors could lead to a spatial mismatch between *E. b. iberae* and its host plant, as suggested for other butterflies (e.g. Schweiger et al., 2008).

Re-evaluation of the conservation status of *E. bazae*

Munguira et al. (2015, 2017) elaborated a recovery plan detailing the factors that threatened the species, and in 2019 *E. bazae* was listed as ‘endangered’ in the Spanish Catalogue of Threatened Species mostly based on the information provided by this plan – see consultation CC 24/2017 by which ‘EN’ status is requested and the order

TEC/596/2019 by which this status is approved. Here, we list the following concerns derived from the results we obtained plus additional ones gathered by Munguira et al. (2015):

(1) *Euchloe bazae* has an extremely restricted distribution and it is present in only three locations worldwide. The species AOO is 58 km² and the EOO is 385 km², which are among the lowest for European endemic butterflies.

(2) The distribution is highly fragmented. The two subpopulations of *E. b. iberae* are separated by ~20 km and the single population of *E. b. bazae* is ~450 km far from them. No current gene flow has been detected between the two subspecies, and their isolation dates back to, at least, the Last Glacial Maximum, when they could have been in contact according to climate distribution models. The two subspecies are genetically differentiated in both nuclear and mitochondrial markers. They are also morphologically and ecologically distinct and hence are confirmed as two evolutionarily significant units (ESUs) and management units (MUs).

(3) An additional subpopulation existed in La Granja d'Escarp – the only one inhabiting Catalonia and located more than 20 km away from the current closest population –, but it is now regarded as extinct (Munguira et al., 2017; Vila et al., 2018). As a potential cause for extinction, a wildfire that occurred on 2003 can be mentioned, as it affected the region where the species and the host plant were recorded. Nevertheless, stochastic extinction processes are not uncommon in small isolated populations of butterflies and extinction sites cannot be easily recolonised because of a deficient metapopulation structure.

(4) A decrease in abundance of the population of *E. b. bazae* has been reported (Olivares & Jiménez, 2008).

(5) The density of adult individuals is very low. According to Munguira et al. (2017), densities were 400 adults/km² in *E. b. bazae* and 1,700 adults/km² in *E. b. iberae*. They found these densities to be lower than other endangered Iberian species with very restricted range, such as the endemics from Sierra Nevada (southern Iberia) *Polyommatus golgus* (Hübner, 1813) (3,500–5,500 adults/km², classified as VU) and *Agriadetes zullichi* Hemming, 1933 (1,200–13,300 adults/km², classified as EN).

(6) High yearly fluctuations in the abundance of adults have been reported (Munguira et al., 2015; Olivares & Jiménez, 2008).

(7) *Euchloe bazae* displays a low genetic diversity. For the COI barcode region, and although the haplotype diversity (*Hd*) is not especially low (0.600), other parameters indicate low diversity, namely: nucleotide diversity (π) = 0.0012, mean p-distance = 0.14% and maximum p-distance = 0.46%. Genetic distance values are considerably smaller than the average for the European butterflies –average intra-specific distance = 0.41%; average maximum intraspecific distance = 1.19% (Dincă et al., 2021). Genetic variability for the rest of markers is not higher, but it is harder to place into a broader context because of lack of data for other species. These results suggest a low potential for adaptation due to low genetic variability for natural selection to work on, as well as a substantial risk of inbreeding depression (Gaggiotti & Hanski, 2004).

(8) Climate change may force the populations of *E. bazae* to disperse since a significant decrease in the suitability of their current locations is predicted. Even though new locations with higher climatic

suitability are predicted, rapid range shifts could be difficult for an ecological specialist.

(9) Their habitat is experiencing several alterations (Munguira et al., 2015; Puente, 2013), including the introduction of more intensive agriculture practises, overgrazing, abandonment of the land, *Pinus halepensis* Mill. plantations, urban development, and wildfires.

For these reasons, we believe that *E. bazae* fit into the threshold of an endangered species for the B criteria (IUCN, 2012) because of its low AOO and EOO, (a) the high species distribution fragmentation and the low number of locations, (b.i) the observed decline in the extent of occurrence, (b.ii) the observed decline in the area of occupancy, (b.iii) the projected loss of habitat quality and (b.iv) the observed decline in number of locations or subpopulations. Thus, we propose to change the IUCN category for *E. bazae* from 'least concern' in the IUCN Red List and 'vulnerable' in the European Red List to 'endangered' [EN B1ab(i,ii,iii,iv); B2ab(i,ii,iii,iv)].

The results derived from this study not only are important to protect *E. bazae*, but more generally highlight that, even in thermophilous species, climate change is a risk if their degree of specialisation hinder a rapid response to the changing environment. In such cases, conservation and monitoring efforts cannot be dismissed.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS' CONTRIBUTION

Paula Escuer, Joan C. Hinojosa and Roger Vila conceived, designed and coordinated the study. Funding was secured by Roger Vila. Fieldwork to obtain samples and presence data was done by Joan C. Hinojosa, Helena Romo, Miguel L. Munguira, Javier Olivares, Vlad Dincă, Gerard Talavera and Roger Vila. Laboratory protocols were conducted by Joan C. Hinojosa, Carla Minguet-Parramona and Vlad Dincă. Genetic data were analysed by Joan C. Hinojosa and ENM analyses were performed by Paula Escuer and Gerard Talavera. The manuscript was initially written by Paula Escuer, Joan C. Hinojosa and Roger Vila, along with significant contributions from the rest of the authors.

DATA AVAILABILITY STATEMENT

DNA sequences are openly available in GenBank at <https://www.ncbi.nlm.nih.gov/genbank/>, reference numbers are indicated in Supplementary

Table S1. Ecological Niche Modelling input and output are available in figshare at <http://doi.org/10.6084/m9.figshare.19410947>.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Appendix S1 Supporting Information

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