Received: 18 November 2020 | Revised: 3 May 2021 | Accepted: 6 May 2021

DOI: 10.1111/mec.15981

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

The Pleistocene species pump past its prime: Evidence from European butterfly sister species

Sam Ebdon[1] | Dominik R. Laetsch[1] | Leonardo Dapporto[2] | Alexander Hayward[3] | Michael G. Ritchie[4] | Vlad Dincă[5] | Roger Vila[6] | Konrad Lohse[1]

[1]Institute of Evolutionary Biology, University of Edinburgh, Edinburgh, UK

[2]ZEN Laboratory, Dipartimento di Biologia, Università di Firenze, Firenze, Italy

[3]Centre for Ecology and Conservation, University of Exeter, Cornwall, UK

[4]Centre for Biological Diversity, School of Biology, University of St Andrews, Fife, UK

[5]Ecology and Genetics Research Unit, University of Oulu, Oulu, Finland

[6]Institut de Biologia Evolutiva (CSIC - Universitat Pompeu Fabra), Barcelona, Spain

Correspondence
Sam Ebdon, Institute of Evolutionary Biology, University of Edinburgh, Edinburgh, EH9 3FL, UK.
Email: sam.ebdon@ed.ac.uk

Funding information
Biotechnology and Biological Sciences Research Council; Natural Environment Research Council; H2020 European Research Council

Abstract

The Pleistocene glacial cycles had a profound impact on the ranges and genetic make-up of organisms. While it is clear that the contact zones that have been described for many sister taxa are secondary and have formed in the current interglacial, it is unclear when the taxa involved began to diverge. Previous estimates based on small numbers of loci are unreliable given the stochasticity of genetic drift and the contrasting effects of incomplete lineage sorting and gene flow on gene divergence. Here, we use genome-wide transcriptome data to estimate divergence for 18 sister species pairs of European butterflies showing either sympatric or contact zone distributions. We find that in most cases, species divergence predates the mid-Pleistocene transition or even the entire Pleistocene period. We also show that although post-divergence gene flow is restricted to contact zone pairs, they are not systematically younger than sympatric pairs. This suggests that contact zones are not limited to the initial stages of the speciation process, but can involve notably old taxa. Finally, we show that mitochondrial divergence and nuclear divergence are only weakly correlated and mitochondrial divergence is higher for contact zone pairs.

1 | INTRODUCTION

Divergence in allopatry provides a simple null model of speciation (Mayr, 1947). Following geographic isolation and given enough time, reproductive isolation is inevitable as incompatibilities will eventually become fixed as a result of genetic drift and/or selection (Bateson, 1909; Dobzhansky, 1937; Muller, 1942). Taxa that evolved partial reproductive isolation in allopatry may come into secondary contact as a result of range shifts and—depending on their degree of reproductive isolation and niche overlap—either form a contact zone or invade each other's range (Barton, 1985; Pigot, 2013). If allopatric divergence dominates speciation, then local alpha diversity for a given clade cannot accrue until secondary sympatry is achieved (Weir & Price, 2011). Thus, the forces that facilitate or hamper secondary sympatry and the timescale over which this occurs have profound consequences both for speciation and for the spatial distribution of species diversity. While modern ranges only provide a snapshot of the dynamic history of range shifts, understanding the

extent to which current range overlap between closely related species can be explained by their speciation history and *vice versa* has been at the core of speciation research (Coyne & Orr, 2004).

The glacial cycles of the Pleistocene had a profound effect on current diversity of temperate ecosystems (Hewitt, 1996, 2001; Hofreiter, 2009). Populations of temperate taxa in Europe were isolated in ice-free refugia around the Mediterranean basin (Iberia, Italy, the Balkans and the larger Mediterranean islands) as glaciers encroached. The observation that the geographic ranges of many young taxa are restricted to individual glacial refugia in southern Europe (Dennis et al., 1991; Hewitt, 1996, 1999, 2011; Schmitt, 2007) suggests that this repeated separation into and expansion out of glacial refugia has played a major role in their origin. The availability of allozyme and mitochondrial (*mt*) data in the 80s and 90s has spurred an abundance of case studies on intra- and interspecific diversity of European taxa including detailed investigations of hybrid zones in taxa ranging from fire-bellied toads (Kruuk et al., 1999), the house mouse (Boursot et al., 1996), grasshoppers (Barton, 1980 and Butlin & Hewitt, 1985), to plants (Bacilieri et al., 1996) and marine mussels (Skibinski & Beardmore, 1979). The pervading evidence from these studies is that genetic diversity within and in, many cases, divergence between species is structured by refugia (Dapporto et al., 2019; Hewitt, 1996; Schmitt, 2007).

1.1 | When was divergence between sister species initiated?

While it is clear that the hybrid zones we observe today are secondary contacts that formed after the last glacial maximum and may have formed many times over throughout the Pleistocene, it is far from clear when divergence between the sister taxa involved was initiated. One possibility is that the Pleistocene glacial cycles initiated species divergence directly by separating populations into allopatric refugia (i.e. a 'species pump' *sensu* Haffer, 1969). Another possibility is that the initial divergence between sister species predates the Pleistocene, and so, any build-up of reproductive isolation during the Pleistocene (e.g. via the fixation of intrinsic incompatibilities and/or reinforcement) occurred in populations that were already partially diverged. If the Pleistocene species pump hypothesis is correct, we would expect sister species divergence times to be concentrated during or at the beginning of the mid-Pleistocene transition 0.8–1.2 million years ago (MYA), which marks the onset of continent-wide glacial cycling (Bishop et al., 2011). The idea that Pleistocene divergence acted as a species pump was first proposed in the context of American faunas (Avise et al., 1998; Bernatchez & Wilson, 1998; Haffer, 1969), but has dominated phylogeographic studies on European sister taxa (e.g. Habel et al., 2008; Hewitt, 1996, 2000; Schmitt, 2007; Schoville et al., 2012). In contrast, other studies including some of the early work on European contact zones (Barton & Hewitt, 1985; Butlin & Hewitt, 1985) conclude that the taxa involved in such secondary contacts may substantially predate the Pleistocene (Abbott et al., 2000; Hewitt, 1996; Klicka & Zink, 1997; Spooner & Ritchie, 2006). Similarly, Pleistocene climate forcing is insufficient in explaining divergence in an Amazonian butterfly suture zone (Dasmahapatra et al., 2010). Thus, it remains unclear to what extent divergence between sister taxa was initiated by 'Pleistocene species pump' dynamics or has an older, deeper origin?

A corollary for the hypothesis of allopatric speciation in different refugia is that range overlap is secondary. Since species can more easily invade each others' ranges once sufficient premating barriers and ecological differentiation have developed, we would expect species pairs with overlapping ranges to be older overall than those without range overlap, all else being equal (Coyne & Orr, 2004). Support for this prediction comes from comparative studies showing that the proportion of range overlap (degree of sympatry (Chesser & Zink, 1994)) is positively (albeit weakly) correlated with genetic divergence (Barraclough & Vogler, 2000; Pigot & Tobias, 2013). However, a recent study in *Chorthippus* grasshoppers shows that subspecies that hybridize across contact zones can be older than currently sympatric species (Nolen et al., 2020).

1.2 | Mitonuclear discordance

Age estimates for recently diverged taxa have largely relied on single-locus phylogenies and ignored incomplete lineage sorting. Hewitt (2011) summarizes age estimates for European hybrid-zones taxa including mammals, insects, amphibians and reptiles, which range from hundreds of thousands to several million years ago. However, given that these estimates are based on different markers and calibrations, the extent to which glacial cycles have initiated speciation events remains unknown. Estimates based on mitochondrial (*mt*) data are particularly unreliable for at least three reasons. First, the mutation rate of mtDNA is highly erratic (Galtier et al., 2009). Second, given the stochasticity of coalescence, the ancestry of a single locus (however well resolved) is a very poor measure of species divergence. In the absence of gene flow, divergence at a single locus may substantially predate the onset of species divergence, while it may be much more recent in the presence of gene flow (Knowles & Carstens, 2007; Wang & Hey, 2010). Mitonuclear discordance in both directions has been found in a large number of animal systems (Toews & Brelsford, 2012) including several closely related species of European butterflies (Dincă et al., 2019; Hinojosa et al., 2019; Wiemers et al., 2010). Finally, mtDNA does not evolve neutrally since transmission of mitochondria is completely linked to maternal inheritance of endosymbionts such as *Wolbachia* and *Spiroplasma* and, in organisms with Z/W sex determination, of the W chromosome. Thus, *mt* diversity and divergence may be driven largely by selective sweeps (including introgression sweeps) rather than neutral gene flow and genetic drift (Galtier et al., 2009; Hurst & Jiggins, 2005; Jiggins, 2003; Martin et al., 2020).

1.3 | European butterflies as a model group

Testing whether climate-induced Pleistocene range shifts have triggered speciation or patterned older splits between species requires

replication both at the level of genetic loci and at the level of speciation events. Although we can now generate WGS data for any species, there are surprisingly few reliable estimates for the onset of divergence between European sister species and such estimates are lacking even for well-studied contact zone taxa (but see Nolen et al., 2020; Nürnberger et al., 2016).

Lepidoptera are arguably the best-studied arthropod family: European butterflies provide a unique opportunity to investigate divergence and speciation processes comparatively (Dapporto et al., 2019). Near-complete information on geographic ranges and key life history traits (e.g. voltinism and host plant range) is available (Kudrna, 2019; Tolman & Lewington, 2013). Additionally, the taxonomy of all 496 European species is well resolved (Wiemers et al., 2018) and a complete, multilocus phylogeny of all European taxa exists (Dapporto et al., 2019). This, combined with extensive DNA barcode reference libraries (Dapporto et al., 2019; Dincă et al., 2021), facilitates the identification of species (especially in the case of cryptic taxa) and provides extensive sampling of sister species pairs, many of which abut at narrow contact zones (Dennis et al., 1991; Platania et al., 2020; Vodă et al., 2015) (Figure 1). Secondary contact zones have been described in detail for several European taxa, including *Spialia orbifer* and *S. sertorius* (Lorkovic, 1973), the Italian *Pontia* hybrid zone (Porter et al., 1997) and the contacts between *Iphiclides podalirus* and *I. feisthamelii* and between *Melanargia galathea* and *M. lachesis* along the Pyrenees (Habel et al., 2017; Wohlfahrt, 1996, Gaunet et al., 2019).

Here, we use European butterflies as a model system to investigate to what extent the divergence times between sister species in this group are concentrated in the Pleistocene, as predicted by the Pleistocene species pump hypothesis, and test how well recent sister species fit a null model of divergence in allopatry. Although European butterflies have been studied intensively, with few exceptions (see Talla et al., 2017), robust estimates of divergence required for any systematic comparison of speciation are lacking. We generate RNA-seq data for 18 sister species pairs and ask the following specific questions:

(i) Has speciation been initiated during the Pleistocene, as envisaged by the species pump hypothesis, or did the glacial cycles pattern pre-existing, older subdivisions?
(ii) Are sister species pairs that form contact zones younger than pairs that overlap in range?
(iii) Is there evidence for gene flow between contact zone species?
(iv) How strongly correlated are mitochondrial and nuclear divergence and do contact zone pairs show increased mitonuclear discordance?

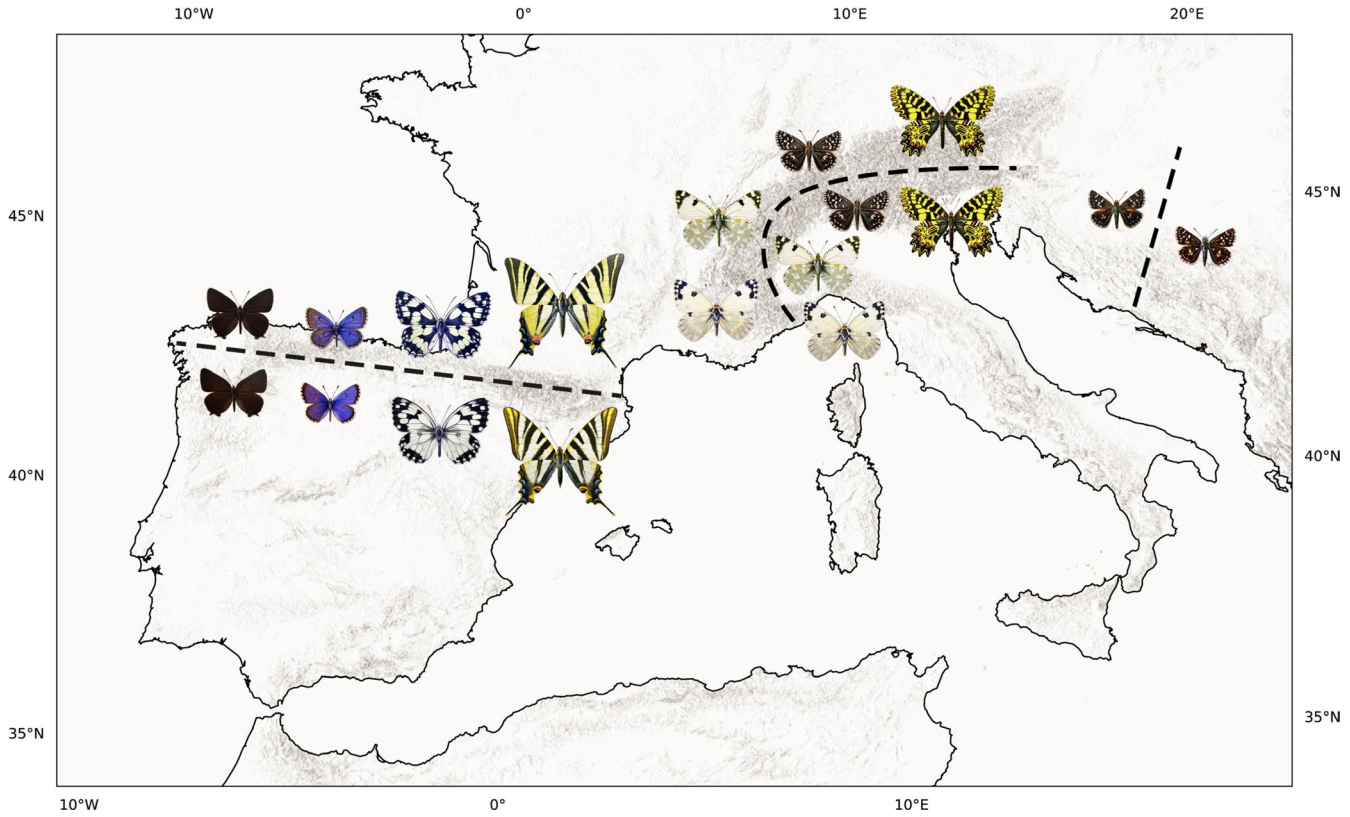

FIGURE 1 Nine of the 18 sister species pairs of butterfly in which we quantified genome-wide divergence meet at contact zones in southern Europe. In the left group, from left to right across northern Iberia are *Satyrium*, *Pseudophilotes*, *Melanargia* and *Iphiclides*. In the centre group, from bottom to top across the Alps are *Pontia*, *Euchloe*, *Pyrgus*, and *Zerynthia*. Finally, on the right across the Balkans is the genus *Spialia*

2 | METHODS

2.1 | Sampling and molecular work

We identified true sister species pairs in the European butterfly phylogeny (Wiemers et al., 2018; Dapporto et al., 2019). Species pairs involving island and mountain endemics were excluded, as these cannot achieve secondary sympatry. We also did not consider species pairs that are unlikely to have originated in Europe, for example sister pairs involving North American taxa. Following these criteria, we sampled 18 sister species pairs (Table 1). Our sampling includes 7.3% of European butterfly species (Wiemers et al., 2018) and almost all 'good' butterfly sister species pairs in Europe (Descimon & Mallet, 2009).

Field sampling was conducted over multiple seasons (2016–2019) at several locations across southern and central Europe (Portugal, Spain, France, Hungary, and Romania) targeting known glacial refugia (and avoiding localities close to known contact zones) whenever possible. Samples were hand-netted in the field, flash-frozen in a liquid nitrogen dry shipper (Voyageur 12) and stored at −70°C shortly after capture (wings were retained for identification). Specimen identifications were confirmed for 14 species that are difficult to identify based on morphology (morphological characters are subtle and/or internal) but for which COI barcodes are diagnostic of morphoID using LepF/R primers (Hajibabaei et al., 2006) and existing reference databases (Dincă et al., 2021). We were unable to obtain fresh material for *Erebia euryale* and *E. ligea*, and *Fabriciana adippe* and *F. niobe* (two remaining sister pairs meeting our sampling criteria).

RNA extractions were prepared by dividing individuals bilaterally (including all parts of the body: head, thorax and abdomen) and using one side. RNA was extracted following a hybrid protocol: samples were homogenized in TRIzol, and RNA was eluted using the Purelink RNA Purification Kit protocol after a DNA digestion step. Extracted RNA was submitted to Edinburgh Genomics to generate automated TruSeq-stranded mRNA-seq libraries. Libraries were sequenced on an Illumina NovaSeq platform using 100-bp paired-end reads after poly-A selection. For each species, where possible, we generated RNA-seq data for two samples, one male and one female from different localities. Transcriptome data for 66 samples (across 38 species) were generated and analysed previously by Mackintosh et al., (2019). Of these, 26 samples from 13 species are included in the present analysis (Table S1).

2.2 | Generating transcriptome assemblies

Reads were processed following the pipeline developed by Mackintosh et al., (2019). Reads were trimmed and checked for quality using FastQC v0.11.8 (Andrews et al., 2010) both before and after trimming with FastP v0.20.0 (Chen et al., 2018) using MultiQC v1.7 (Ewels et al., 2016) to visualize the results. Trimmed reads were assembled into *de novo* transcriptomes using Trinity v2.8.5 (Grabherr et al., 2011), pooling data sets by species.

Transcriptome completeness was assessed using BUSCO v5 (Simão et al., 2015) on full transcriptome assemblies using the *lepidopteraodb10* database, with scores ranging from 84 to 96% (Table S2). Transcripts were processed with Transdecoder v5.5 (Haas et al., 2016) and retained based on BLAST (Camacho et al., 2009) and HMMER (Finn et al., 2011) homology search results. Read pairs from each sample were mapped against the respective species transcriptome, composed of the longest isoform of each complete protein-coding transcript, using BWA MEM (Li, 2013). Coverage at mapped sites was determined using GATK CallableLoci v3.5 (McKenna et al., 2010). Sites with at least 10-fold coverage and a minimum MAPQ of 1 in each sample were considered suitable for variant calling. Callable loci were intersected between individuals using BEDTools v2.28 (Quinlan & Hall, 2010), and variants were called using FreeBayes v1.3.1 (Garrison & Marth, 2012) and filtered for unbalanced SNPs and missing genotypes (RPL ≥ 1 && RPR ≥ 1 && SAF ≥ 1 && SAR ≥ 1 && N_MISSING = 0) using BCFtools filter v0.1.19 (Li, 2011).

To generate comparable data sets across all samples, Orthofinder v2.3.3 (Emms & Kelly, 2015) was used to cluster proteins into orthogroups. Orthogroups were labelled single-copy orthologues (SCOs) if one protein of each taxon was present. Genus single-copy orthologues (GSCOs) were diagnosed based on the presence of single-copy proteins within the focal pair. Protein sequences from each orthogroup were used to align corresponding DNA sequences using Translatorx v12.0 (Abascal et al., 2010).

Data were generated for 36 species (18 sister pairs) from five families. For 17 pairs, data were generated from 665 SCOs from high-quality transcriptomes (BUSCO scores >90%). For the pair of *Zerynthia* species (one of which, *Zerynthia polyxena*, was sampled as a larva), GSCOs (5000 orthologues) were used to avoid restricting the SCOs for other pairs. With the exception of the *Zerynthia* pair, all analyses are based on SCO to enforce consistent comparisons across pairs. While the SCO data set is much smaller than the pair GSCO data sets and likely enriched for conserved and highly expressed genes, this has very little impact on estimates of divergence and diversity at fourfold degenerate (4D) sites, as these are highly correlated (Figure S1 and Mackintosh et al., 2019).

2.3 | Estimating gene and population divergence

For each species pair, we calculated the average pairwise gene divergence d_{xy} at fourfold degenerate (4D) sites using sequence alignments for one or two diploid samples from each species. This calculation is implemented in the script orthodiver.py (www.github.com/samebdon/orthodiver).

Species split times were estimated using two different approaches. First, we used a simple rescaling of mean genetic divergence and diversity to obtain a lower bound of the species divergence time. Assuming a simple null model of divergence without gene flow, neutrality and an infinite site mutation model, net mean divergence $d_a = d_{xy} - \pi$ (Nei & Li, 1979) is directly proportional to species divergence time $T = \frac{d_a}{2\mu}$. Here, μ is the *de novo* mutation rate per generation (per base).

TABLE 1 Estimates of mean gene divergence (d_{xy}), net gene divergence (d_a) and differentiation (F_{st}) at 4D sites and lower bounds for species divergence times for 18 sister species pairs of European butterfly

Sister 1			Sister 2									
Species	π	Gen y^{-1}	Species	π	Gen y^{-1}	d_{xy}	d_a	Split time (MYA)	F_{st}	Degree of sympatry	Contact zone	Known to hybridize
Brenthis daphne	0.0046	1	*B. ino*†	0.0094	1	0.0246	0.0176	3.04 (1.60, 6.89)	0.716	0.74	No	No
Colias alfacariensis†	0.0243	2–3	*C. hyale*‡	0.0211	2–3	0.0387	0.0159	0.92 (0.48, 2.05)	0.412	0.70	No	No
Euchloe ausonia	0.0250	2	*E. crameri*†	0.0352	2	0.0715	0.0416	3.58 (1.89, 7.99)	0.582	0.00	Yes	No
Gonepteryx cleopatra	0.0104	1	*G. rhamni*	0.0156	1	0.0448	0.0316	5.48 (2.89, 12.2)	0.705	0.97	No	Yes
Iphiclides feisthamelii‡	0.0079	1–3	*I. podalirius*‡	0.0052	1–3	0.0275	0.0204	1.20 (0.63, 2.68)	0.747	0.00	Yes	Yes
Lasiommata megera†	0.0385	2–3	*L. petropolitana*‡	0.0065	1	0.0543	0.0316	2.75 (1.45, 6.13)	0.586	0.43	No	No
Leptidea reali†	0.0077	1–2	*L. sinapis*‡	0.0093	1–3	0.0153	0.0068	0.47 (0.25, 1.04)	0.444	1.00	No	No
Melanargia galathea†,‡	0.0152	1	*M. lachesis*†	0.0145	1	0.0389	0.0227	4.14 (2.18, 9.24)	0.597	0.20	Yes	Yes
Pieris mannii†	0.0100	3	*P. rapae*‡	0.0198	3–4	0.0678	0.0525	2.61 (1.37, 5.81)	0.779	1.00	No	No
Polyommatus eros‡	0.0104	1	*P. icarus*‡	0.0174	1–3	0.0529	0.0382	3.37 (1.78, 7.51)	0.724	1.00	No	Yes
Pontia daplidice†,‡	0.0063	3	*P. edusa*	0.0159	3	0.0516	0.0401	2.33 (1.22, 5.19)	0.777	0.00	Yes	Yes
Pseudophilotes baton	0.0080	1–2	*P. panoptes*	0.0131	1	0.0276	0.0171	1.97(1.03, 4.39)	0.619	0.00	Yes	No
Pyrgus malvae‡	0.0164	1–2	*P. malvoides*†	0.0176	1–2	0.0362	0.0192	1.66 (0.87, 3.70)	0.531	0.04	Yes	Yes
Satyrium esculi†	0.0076	1	*S. ilicis*	0.0036	1	0.0432	0.0378	6.49 (3.42, 14.5)	0.869	0.06	Yes	No
Satyrus actaea†	0.0261	1	*S. ferula*†	0.0074	1	0.0663	0.0493	8.53 (4.50, 19.0)	0.745	0.26	No	No
Spialia orbifer†,‡	0.0331	2	*S. sertorius*	0.0418	2	0.0671	0.0292	2.55 (1.35, 5.69)	0.438	0.12	Yes	No
Thymelicus acteon‡	0.0154	2	*T. sylvestris*‡	0.0208	1	0.0848	0.0671	7.66 (4.04, 17.1)	0.790	0.99	No	No
Zerynthia cassandra	0.0033	1	*Z. polyxena*	0.0032	1	0.0312	0.0279	4.82 (2.54, 10.7)	0.895	0.00	Yes	No

Gen y^{-1} is the number of generations per year. Samples from species marked with a † were barcoded to confirm correct identification. Species where *Wolbachia* presence was confirmed by Duplouy and Hornett (2018) and Hinojosa et al., (2019) are marked with a ‡.

We assumed $\mu = 2.9 * 10^{-9}$, an estimate of the spontaneous mutation rate obtained from parent–offspring trios of South American *Heliconius melpomene* butterflies (Keightley, Pinharanda, et al., 2014). Since both violations of the mutation model (back-mutations) and the demographic model (gene flow) reduce d_a, this time estimate is a lower bound of the true species divergence time. We converted estimates of species divergence time (T) into years (τ) using the mean generation time of each pair (Table 1). Information on generation times was compiled from Collins Butterfly Guide (Tolman & Lewington, 2013) (Table 1). For species in which generation times vary with latitude, we assumed the minimum generation time of the southern part of the range. This is a reasonable long-term average, given that European glacial refugia are located around the Mediterranean.

Second, we estimated species split times using a multilocus coalescent approach. We considered the distribution of pairwise differences in blocks of a fixed length of 4D sites to fit an isolation-with-migration (IM) model and a nested history of strict divergence to each species pair. In the absence of recombination within blocks, the distribution of pairwise differences has been derived analytically (Lohse et al., 2011; Wilkinson-Herbots, 2012). We obtained maximum-likelihood parameter estimates under both models and used likelihood-ratio tests for model comparisons in *Mathematica* v11.3 (File S1). The block length for each pair was chosen to give an average of three pairwise differences between sister species per block.

However, given the high rate of recombination (relative to mutation) in butterflies (Martin et al., 2016, 2019) and the substantial span of 4D blocks, we expect multilocus inference (assuming no within-block recombination) to be biased. In particular, recombination is known to lead to (upwardly) biased estimates of divergence time (Wall, 2003).

Given this and other limitations (see Discussion), we will focus on the more simple and robust estimates of species divergence based on d_a throughout.

As an additional test for gene flow, we compared the observed distributions of pairwise differences with analytic expectations under a model of strict divergence without gene flow given the estimates of T and ancestral N_e obtained from d_a and mean π (which cannot be affected by recombination).

Thus, in the absence of gene flow, we would expect the empirical distribution of pairwise differences to be narrower than the analytic expectation (Wall, 2003) due to recombination. In contrast, wider-than-expected distributions are indicative of post-divergence gene flow. We re-sampled (without replacement) 10,000 data sets of equal size as the observed data from the expected distribution of each species and tested whether the likelihood of the observed distribution of pairwise differences falls within the distribution of likelihoods expected under a null model of strict divergence.

2.4 | Estimating range size and overlap

Geographic ranges were quantified as follows: we obtained occurrence data over Europe for all focal species with a resolution of 60′ latitude and 30′ longitude by critically revising the data from the Distribution Atlas of European Butterflies and Skippers (Kudrna et al., 2011) and by adding data from Roger Vila's collection stored at Institut de Biologia Evolutiva (Barcelona) (Figures S2–S4). To calculate range overlap, we applied the biodecrypt function (Platania et al., 2020) of the recluster R package (Dapporto et al., 2013). This function computes alpha hull with a given concavity (α) and evaluates the area of overlap among pairs of species. We used $\alpha = 2$ and $\alpha = 3$ for species with discontinuous and continuous distributions in Europe, respectively. We quantified the range overlap of each species pair and calculated the degree of sympatry as:

$$\text{Sympatry} = \frac{\text{Overlap}_{A,B}}{\min(\text{Area}_{A,B})}, \quad (1)$$

that is the fraction of the smaller range involved in the overlap. In the following, we consider sister pairs with a degree of sympatry ≤0.2 contact zone pairs and those with a degree of sympatry >0.2 sympatric. Based on this, we classified nine pairs as contact zone taxa. However, since there are only two species pairs with intermediate levels of sympatry (>0.2 and <0.7), our comparisons of contact zone and sympatric pairs are robust to a wide range of thresholds.

2.5 | Mitochondrial diversity and divergence

Sequence alignments for the COI barcode locus were obtained from the BOLD database (Ratnasingham & Hebert, 2007) for all 18 sister species pairs. These sequences, together with associated information, are publicly available in the data set DS-EUGENMAP (dx.doi.org/10.5883/DS-EUGENMAP) on BOLD at www.boldsystems.org and were originally produced by Dincă et al. (2021). For each species, we included all available sequence records from Europe (ranging from 21 in *E. crameri* to 429 in *L. sinapis* (Table S1)). Mean pairwise diversity (π) within species and divergence (d_{xy}) across all sites were computed using DnaSP (Librado & Rozas, 2009).

We obtained the average gene divergence time for each pair from the multilocus-calibrated phylogeny of European butterflies of Wiemers et al., (2020) as half of patristic distances calculated with distTips function of the adephylo R package (Jombart & Dray, 2010). The correlation between our estimates of species divergences and these node ages was explored with standardized major axis (SMA) regression, using the 'sma' function of the 'smatr' R package. SMA estimates slope and intercept and tests whether slope differs from one.

3 | RESULTS

3.1 | Most European butterfly sister species predate the Pleistocene

Mean gene divergence (d_{xy}) at 4D sites between sister species ranged from 0.015 to 0.085, with a mean of 0.047 (Table 1, Figure 2) across

the 18 pairs. We contrasted sympatric and allopatric samples in one genus where it was possible to confirm that sampling location has negligible impact on estimates of divergence (*M. galathea* (Romania): *M. lachesis* (Spain) d_x = 0.039, Spain: Spain = 0.038). Divergence between samples from the same localities typically varied by 0.0005.

Species divergence times obtained from d_a at fourfold degenerate sites (4D) ranged from 0.47 (0.2, 1.0) (*Leptidea*) to 8.5 (4.5, 19) (*Satyrus*) MYA, with a mean of 3.8 MYA (Figure 2). The distribution of divergence estimates does not suggest there is synchronicity in divergence independent of absolute values. Even though these point estimates are lower bounds of species divergence (see Discussion), they not only substantially predate the mid-Pleistocene transition (15 out of 18 pairs) but also, in the majority of cases (11 out of 18 pairs), are older than the entire Quaternary period ≈2.6 MY (Table 1). Of the seven taxa with Pleistocene τ estimates, three fall in the early Pleistocene: *Pseudophilotes* (1.97 (1.0, 4.4) MYA), *Pontia* (2.33 (1.2, 5.2) MYA) and *Spialia* (2.55 (1.3, 5.7) MYA). However, when accounting for the (known) uncertainty in the mutation rate estimate we used as a molecular clock (Keightley, Pinharanda, et al., 2014), we can rule out a divergence time more recent than the mid-Pleistocene transition or the Quaternary period for 13 and four sister species pairs, respectively (Figure 2).

We find that estimates of sister species divergence based on the distribution of pairwise differences are highly correlated (Pearson's correlation coefficient = 0.79) with the estimates based on mean d_a (Figure S5). The IM model fit significantly better than a strict divergence model for two species pairs (*Pontia* and *Colias*), and, as expected, T estimates under the IM model for these two species pairs are older (3.30 vs 0.919 and 5.08 vs 2.33 MYA, respectively) than estimates based on d_a (Table S3). However, we find that sampling blocks of a fixed length resulted in a consistent downward bias in d_{xy} of on average 10%. This is likely a result of selecting for long and likely conserved transcripts. In the light of this, and given that blocks based on 4D sites violate other key assumptions of multilocus inference, in particular, no recombination within loci and known phase, we caution against over-interpreting these model-based estimates (see Discussion) and focus on the simpler and more robust estimates of sister species divergence based on d_a throughout.

3.2 | Sister pairs that form contact zones are not significantly younger than sympatric pairs

There are two reasons to expect species pairs that form contact zones to be younger than sympatric pairs: first, if speciation under a null model of divergence in allopatry is initiated by periods of vicariance, the formation of a contact zone (parapatry) represents an earlier stage in the transition to complete reproductive isolation and substantial range overlap (sympatry) (Coyne & Orr, 2004). Second, any gene flow across contact zones would reduce d_a and hence our estimate of species divergence. While the nine pairs that form contact zones (degree of sympatry ≤0.2) have a lower net divergence (d_a = 0.0287, SD = 0.00930) than the nine sympatric pairs (degree of sympatry >0.2, d_a = 0.0347, SD = 0.0195; Table 1), this difference is not significant (t = −0.82999, df = 11.478, p = 0.210). Additionally, we find no relationship between the degree of sympatry and d_a (t = 0.723, df = 16, p = 0.480). Similarly, we may expect pairs that are still able to form hybrids (i.e. for which F1s have been observed in the wild) to be younger than those that do not. However, contrary to this expectation, we again find no significant difference in d_a between pairs which do and do not hybridize (d_a 0.0293 and 0.0329, respectively, t = −0.582, df = 15.861, p = 0.284).

3.3 | Evidence for recent gene flow in some contact zone pairs

The empirical distribution of pairwise differences deviates significantly from the expectation under a strict divergence model in a majority of species pairs (12 out of 18) (Figure 3 & S6). Of these, eight pairs have narrower distributions than expected, compatible with recombination within blocks, and four pairs have wider distributions than expected, compatible with post-divergence gene flow (*Pseudophilotes*, *Pontia*, *Iphiclides* and *Zerynthia*). While the eight pairs with narrower distributions are equally split between contact and sympatric pairs, all four taxa with wider distributions are contact zone pairs (Figure 3). However, given the limited number of pairs overall, this difference between contact zones and sympatric pairs is not significant (Fisher's exact test, p = 0.0901).

3.4 | Pervasive mitonuclear discordance in contact zone species pairs

Our estimates of species divergence are based on average net divergence (d_a) across many hundreds of genes and are robust to how orthologues are filtered (Figure S1). Given that previous studies on European butterflies have been largely based on mitochondrial (*mt*) phylogenies, an obvious question is to what extent *mt* divergence is correlated with mean nuclear divergence. We find that both d_a and d_{xy} at COI are positively but only weakly correlated with mean nuclear divergence (Figure 4). The correlation is weaker for d_a than for d_{xy} (R^2 = 0.27 and 0.31, respectively), which is unsurprisingly given that mitochondrial diversity (and hence d_a) is both inherently random and may be affected by selective sweeps. Comparing the relation between *mt* and nuclear d_a between contact zone and sympatric pairs, we find a shallower slope for contact zone pairs (0.29 compared to 0.99; Figure 4). This difference, although not significant (p = 0.09), appears to be driven by the reduced *mt* diversity in contact zone compared with sympatric pairs (mean π = 0.0030, SD = 0.0014 and π = 0.0047, SD = 0.0031, respectively; t = 1.5763, df = 11.324, p = 0.0712). This suggests that *mt* diversity may be more strongly affected by selective sweeps in contact zone species than in sympatric pairs. We find no corresponding signal for any difference in nuclear

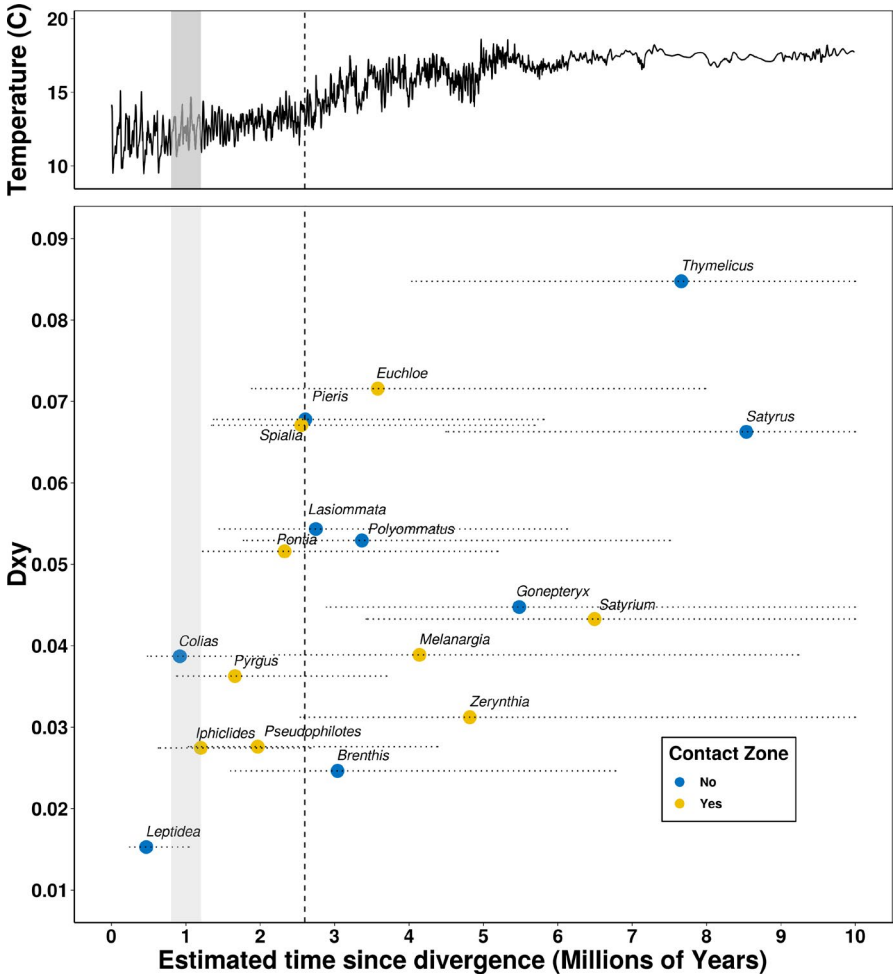

FIGURE 2 Species divergence time estimates (τ) plotted against mean genetic divergence (d_{xy}) for 18 European butterfly sister species pairs. Pairs which abut at contact zones (degree of sympatry ≤ 0.2) are shown in yellow, sympatric pairs with substantial range overlap (> 0.2) in blue. The vertical dashed line represents the beginning of the Pleistocene (2.6 MYA), and the vertical grey bar indicates the mid-Pleistocene transition (0.8–1.2 MYA). The horizontal dotted lines represent the 95% confidence intervals of τ estimates given the uncertainty in the mutation rate used for calibration ($1.3–5.9 \times 10^{-9}$ (Keightley, Pinharanda, et al., 2014)). The temperature data (5-point running means of global surface temperature) are taken from Hansen et al., (2013)

diversity between contact zones and sympatric pairs (t = −0.0139, df = 31.539, p = 0.506) and, in general, no correlation between nuclear and *mt* diversity (Figure S7 and Mackintosh et al., (2019)).

Our estimates for the lower bound of sister species divergence differ substantially from the ages of the corresponding nodes in the Wiemers et al., (2020) phylogeny for individual pairs (Figure S8). This is unsurprising given that the latter are largely informed by mtDNA data (Figure S9). However, perhaps surprisingly (given the difference in calibration, data and inference approach), our estimates are not consistently older or younger than the node ages of Wiemers et al., (2020) (t_{paired} = −1.105, df = 17, p = 0.285). A standardized major axis regression shows a significant relationship (R^2 = 0.3657, p = 0.00780), a slope (1.377) not different from one (r = 0.3786, p = 0.121) and an intercept (−0.5750) not different from zero (Figure S8).

3.5 | Genetic diversity does not correlate with relative range size

Genetic diversity at 4D sites within all 36 species ranged from 0.32% to 4.2% with a mean of 1.5%. Given the *H. melpomene* mutation rate of $\mu = 2.9 \times 10^{-9}$ (Keightley, Pinharanda, et al., 2014), these correspond to effective population sizes ranging from 280,000 to 3,600,000 with a mean of 1,300,000 (assuming $\theta = 4N_e mu$). Mackintosh et al. (2019) tested whether neutral genetic diversity across European butterflies correlates with geographic range and found no significant relation across 38 taxa. Our sampling of species pairs allows for a simpler, alternative test of the potential relationship between diversity and range size using sister-clade comparisons, which is less sensitive to potential phylogenetic correlates and uncertainty in current range estimates. If diversity is a function of range size, we expect the species in a pair with the larger range to have higher genetic diversity than the species with the smaller range. We indeed find a difference in the expected direction, 0.0167 (SD = 0.0114) vs 0.0139 (SD = 0.00865), although the effect of relative range size is not significant (t_{paired} = 1.127, df = 17, p = 0.138; Figure S10).

4 | DISCUSSION

We quantify and compare genome-wide divergence across 18 sister species pairs of European butterfly. Simple estimates for the onset of species divergence based on net gene divergence (d_a) and a direct mutation rate estimate for butterflies suggest that the majority of pairs have diverged before the onset of the major Pleistocene glacial

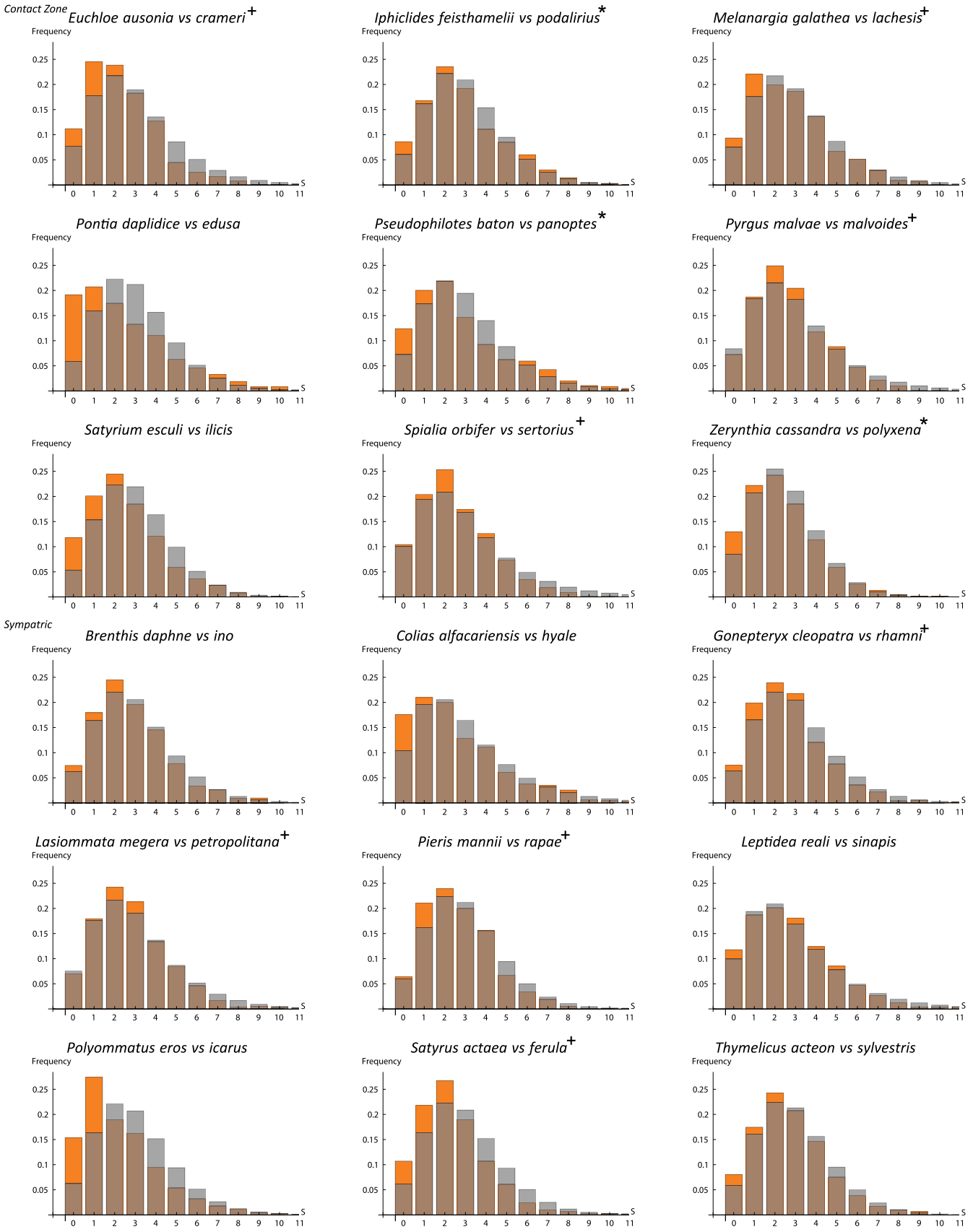

FIGURE 3 The distribution of pairwise differences, *S* in blocks of a fixed length of 4D sites in contact (upper box) and sympatric (lower box) pairs. The observed distribution in single-copy orthologues is shown in orange, and the expectation under a history of strict divergence (estimated from π and d_a), in grey. Pairs that show wider-than-expected distributions are marked with an asterisk (*), and species that show narrower-than-expected distributions are marked with a plus (+)

cycles. Our results support the notion that the modern contact zones are secondary between species that began to diverge much earlier, in the Pliocene or early Pleistocene. Thus, even though the current ranges of many taxon pairs reflect glacial refugia, their initial divergence during the Pliocene or early Pleistocene is unlikely to have been triggered by repeated cycles of range connectivity and vicariance into refugia, as envisaged by the species pump hypothesis and phylogeographic studies based on *mt* and allozyme data (e.g. Habel et al., 2008; Lai & Pullin, 2004; Schmitt, 2007; Todisco et al., 2010; Zinetti et al., 2013) because substantial glaciation across continents did not develop (Bishop et al., 2011) until the 'mid-Pleistocene transition' from 0.8 to 1.2 MYA and the shift from 41,000- to 100,000-year glacial cycles. Given the antiquity of most sister species, it is perhaps unsurprising that we do not find any relationship between current range overlap and the time since divergence. Specifically, species pairs that form contact zones are not significantly younger than pairs that broadly overlap in range. However, we do find that strong signals of post-divergence gene flow are restricted to contact zone pairs. It is likely that the absence of sympatric pairs with significant gene flow reflects a simple survivorship bias: any incipient species pairs with significant gene flow might have already collapsed. Likewise, we are more likely to observe old contact zones pairs that have survived repeated glacial cycles.

Our finding that *mt* divergence between sister species is only weakly correlated with mean nuclear divergence and the possibility that net *mt* divergence may be greater for contact zone than sympatric species pairs as a result of reduced genetic diversity (note that the differences in d_a and mean π between contact zone and sympatric pairs are marginally non-significant, $p = 0.09$ and 0.07, respectively) could suggest that contact zone species may be subject to more frequent selective sweeps linked to mitochondria. Such sweeps may be acting on *mt* variation directly or indirectly through maternally inherited genomes or chromosomes (e.g. *Wolbachia* (Jiggins, 2003) and the W chromosome) and have been documented in a number of Lepidopteran systems (Gaunet et al., 2019; Graham & Wilson, 2012; Kodandaramaiah et al., 2013; Martin et al., 2020; Ritter et al., 2013) (see Table 1 for species in this study with confirmed *Wolbachia* presence). Our results raise the intriguing possibility that such sweeps could play a role in the build-up of reproductive isolation (Giordano et al., 1997; Rokas, 2000; Shoemaker et al., 1999).

4.1 | Sources of dating uncertainty and bias

Since we have assumed the simplest possible demographic null model of species divergence without gene flow using d_a, our estimates of divergence between sister species based on d_a should be interpreted as lower bounds. Any gene flow between sister species would reduce d_a and species divergence estimates both by decreasing d_{xy} and by potentially increasing π (in the recipient species).

Calibrating absolute split times involves assumptions about both the generation time and the mutation rate. We have assumed that the mutation rate is the same (per generation) across all species pairs, irrespective of their generation time, and applied a direct laboratory estimate of the per generation mutation rate from the tropical butterfly *H. melpomene*. While there is good evidence for a generation time effect on mutation rates in invertebrates (Thomas et al., 2010), our assumption of a simple linear relationship between generation time and sequence divergence may be overly simplistic. In particular, if temperate European species, which have longer average generation times than *H. melpomene*, have a higher per generation mutation rate, we would have overestimated the age of sister species. In contrast, given that generation time varies between populations, species and likely through time, our use of the average minimum generation time (within each pair) as a proxy for the long-term generation time is conservative: assuming longer average generation times would yield even older estimates species divergence. Likewise, while our assumption that 4D sites evolve under strict neutrality may be unrealistic, it is conservative with respect to our inference of old sister species divergence. For example, assuming that a fraction of 0.22 of 4D sites is under strong selective constraint due to codon usage bias (Lawrie et al., 2013) would result in underestimation of T by 22%. Given these uncertainties in calibration, our absolute time estimates should be interpreted with caution until direct mutation rate estimates for temperate butterflies are available. However, in the absence of information on mutation rate heterogeneity across Lepidoptera, our main conclusion that divergence between most sister species of European butterflies predates the Pleistocene would still hold if mutation rates were higher by a factor of two. Given that the direct estimate of the *de novo* mutation rate in *H. melpomene* is similar to spontaneous mutation rate estimates for other insects (Keightley et al., 2014), this seems extremely unlikely. While our split time estimates may be surprising in the light of previous phylogeographic studies on European butterflies based on *mt* diversity (e.g. Habel et al., 2008; Lai & Pullin, 2004; Schmitt, 2007; Todisco et al., 2010; Zinetti et al., 2013), our divergence estimate for *Leptidea reali* and *L. sinapis*, the youngest and only pair for which divergence has been estimated based on genome-wide data before, is lower than previous estimates (Talla et al., 2017).

4.2 | Glacial cycling and the Messinian salinity crisis

Taking our estimates of species splits at face value, the divergence of eleven species pairs predates the onset of Pleistocene glacial cycling >2.6 MYA (Gibbard & Head, 2009). This is not compatible with the idea that, overall, recent speciation events in European butterflies were initiated by the range shifts into and out of glacial refugia during the Pleistocene. However, our age estimates do of course not rule out that Pleistocene range shifts and vicariance may have played an important role in completing speciation processes, for example through reinforcement and/or the evolution of intrinsic incompatibilities.

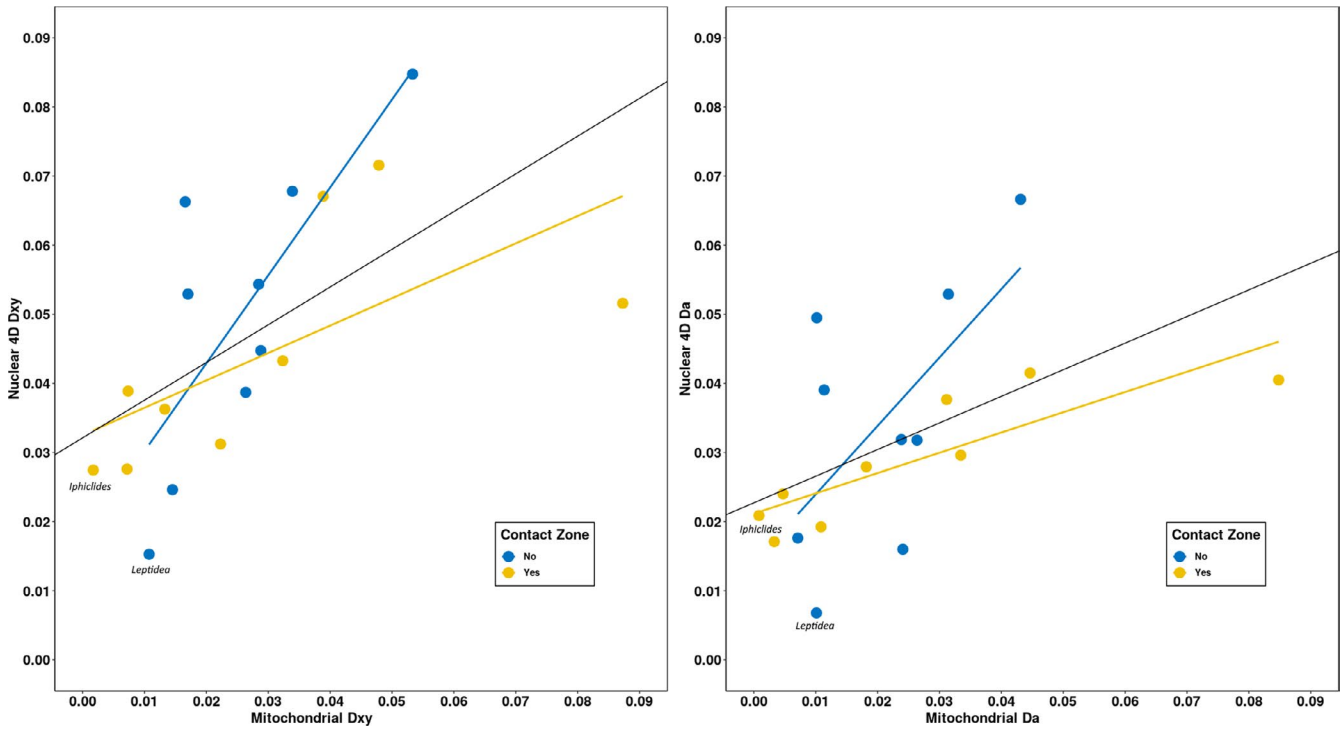

FIGURE 4 Mitochondrial d_{xy} (left) and d_a (right) are weakly correlated with mean nuclear divergence (R^2 = 0.3565 and 0.2732, respectively). The coloured lines show the interactions for pairs that form contact zones and sympatric pairs. The two highlighted pairs (*Iphiclides* and *Leptidea*) have known *Wolbachia*-associated sweeps and low $mt\pi$ (and so high mtd_a)

A major geographic event that could have initiated species divergence in Europe before the onset of Pleistocene glacial cycling is the Messinian salinity crisis (MSC) ≈ 6 MYA during which the Mediterranean greatly reduced in size (Krijgsman et al., 1999). As a consequence, Europe and Africa were connected across the strait of Gibraltar until the Zanclean flood when the Atlantic reconnected to the re-expanding Mediterranean sea. It is plausible that this created a strong dispersal barrier for many species that previously had continuous distributions around the Mediterranean basin and may have initiated divergence into the east and west European/Mediterranean sister taxa. While the MSC has been considered a plausible trigger of species divergence in amphibians (Nürnberger et al., 2016) and reptiles (Kaliontzopoulou et al., 2011) and butterflies in the *Melitaea* radiation (Leveneu et al., 2009), this remains of course speculation.

4.3 | Do European butterfly species fall within the grey zone of speciation?

Roux et al., (2016) conducted a comparative analysis of divergence and gene flow across 61 pairs of sister taxa and found that pairs with net synonymous divergence of >2% rarely show evidence for ongoing gene flow. In contrast, taxa with d_a between 0.5% and 2% may show some evidence for ongoing gene flow and ambiguous species status, suggesting that speciation may be incomplete. While our five youngest pairs (*Brenthis*, *Colias*, *Leptidea*, *Pseudophilotes* and *Pyrgus*) fall in this 'grey zone of speciation', we only find evidence for gene flow in one (*Pseudophilotes*). In contrast, we find a clear gene flow signal in three more diverged pairs: *Iphiclides*, d_a = 2.09%; *Zerynthia*, d_a = 2.79%; and *Pontia*, d_a = 4.05%. However, as we have focused sampling on 'good species' *sensu* Mallet (Descimon & Mallet, 2009) we are missing the recent (intraspecific) end of the continuum of divergence described by Roux et al., (2016). It will be interesting to test to what extent intraspecific divergence times between different refugial populations of butterflies are concentrated in the mid-Pleistocene, a pattern that has been found for other herbivorous insect and their parasitoids (Bunnefeld et al., 2018), and quantify the overlap between inter- and intraspecific divergence times. Nevertheless, our contrasting finding of both gene flow signals in old contact zone pairs (e.g. *Pontia*) and no evidence for gene flow (and complete sympatry) in the youngest pair (*Leptidea*) suggests that the 'grey zone of speciation' may be very wide indeed for European butterflies.

4.4 | Outlook

Given the challenges of demographic inference from transcriptome data (in particular the high relative recombination rate in butterflies), we have deliberately resisted the temptation to overinterpret models of demographic history. Our goal was instead to establish robust and comparable lower bounds for the age of butterfly sister species in Europe. Being based on mean divergence at 4D sites, these lower

bounds for species ages make minimal assumptions and are unaffected by recombination. Likewise, we have decided to focus on a simple and conservative diagnostic for introgression.

Resolving these speciation processes in greater detail will require examination of whole-genome data from larger samples under realistic models of speciation history. Fitting explicit models of speciation to whole-genome sequence data, ideally including both selection and gene flow will undoubtedly refine estimates for the onset of divergence between these species pairs and overcome many of the biases inherent in basing such inferences on transcriptome data. Perhaps even more importantly, it would allow us to quantify the likely endpoints (if present) of speciation processes. While it is straightforward to determine lower bounds for the onset of divergence under simple null models that assume no gene flow, as we have done here, estimating upper bounds of species divergence in the presence of gene flow is a much harder inference problem. As pointed out by Barton and Hewitt (1985), the initial time of divergence may be unknowable given that post-divergence gene flow eventually erases all information about this parameter. Although current and historic levels of gene flow between European butterfly sister species remain to be determined, our results already suggest that their speciation histories are older and potentially slower than had been assumed by previous phylogeographic studies based on mt data. It will be fascinating to understand the evolutionary forces that drive both this general pattern and its exceptions, in particular, the selection responsible for the origin of very young but complete (in terms of reproductive isolation) cryptic species such as *Leptidea* (Talla et al., 2019) and the recently discovered *Spialia rosae* (Hernández-Roldán et al., 2016).

ACKNOWLEDGEMENTS

This work was supported by an ERC starting grant (ModelGenomLand). SE is supported by an EastBio studentship from the British Biological Sciences Research Council (BBSRC). KL is supported by a fellowship from the Natural Environment Research Council (NERC, NE/L011522/1). VD is supported by the Academy of Finland (Academy Research Fellow, decision no. 328895). RV acknowledges support from project PID2019-107078GB-I00/AEI/10.13039/501100011033. LD acknowledges support from project 'Ricerca e conservazione sugli Impollinatori dell' Arcipelago Toscano e divulgazione sui Lepidotteri del parco'. AH is supported by a Biotechnology and Biological Sciences Research Council (BBSRC) David Phillips Fellowship (BB/N020146/1). We thank Alex Mackintosh and Simon Martin for helpful comments on an earlier draft, Richard Lewington for permission to reproduce his butterfly illustrations, Andrés de la Filia for help in the molecular laboratory and Edinburgh Genomics for sequencing.

AUTHOR CONTRIBUTIONS

SE, DRL, RV and KL conceived the study. SE performed molecular labwork and analysed the data. DRL, LD and KL provided tools for analyses. AH, VD and RV contributed samples. KL, DRL and MGR supervised the project. SE, KL and LD wrote the paper with input from all coauthors.

ETHICAL APPROVAL

Field sampling of butterflies was conducted in compliance with the School of Biological Sciences Ethics Committee at the University of Edinburgh and the ERC ethics review procedure. Permissions for field sampling were obtained from the Generalitat de Catalunya (SF/639), the Gobierno de Aragon (INAGA/500201/24/2018/0614 to Karl Wotton) and the Gobierno del Principado de Asturias (014252). The samples for *Z. cassandra* from Elba were collected after permission from the Italian Ministero dell' Ambiente e della Tutela del Territorio e del Mare (Prot. 0012493/ PNM 24/06/2015).

DATA AVAILABILITY STATEMENT

Read data are available from the ENA at PRJEB43082. Sequence alignments for the COI barcode locus were obtained from the dataset DS-EUGENMAP (dx.doi.org/10.5883/DS-EUGENMAP) on BOLD at www.boldsystems.org and were originally produced by Dincă et al., (2021). The script used for calculating diversity and divergence is available at https://github.com/samebdon/orthodiver/blob/master/orthodiver.py.

ORCID

Sam Ebdon https://orcid.org/0000-0002-9321-4686
Dominik R. Laetsch https://orcid.org/0000-0001-7887-0186
Leonardo Dapporto https://orcid.org/0000-0001-7129-4526
Alexander Hayward https://orcid.org/0000-0001-7413-718X
Michael G. Ritchie https://orcid.org/0000-0001-7913-8675
Vlad Dincă https://orcid.org/0000-0003-1791-2148
Roger Vila https://orcid.org/0000-0002-2447-4388
Konrad Lohse https://orcid.org/0000-0001-9918-058X

REFERENCES

Abascal, F., Zardoya, R., & Telford, M. J. (2010). TranslatorX: multiple alignment of nucleotide sequences guided by amino acid translations. *Nucleic Acids Research*, *38*, W7–W13. ISSN 0305-1048. https://doi.org/10.1093/nar/gkq291

Abbott, R. J., Smith, L. C., Milne, R. I., Crawford, R. M., Wolff, K., & Balfour, J. (2000). Molecular analysis of plant migration and refugia in the arctic. *Science*, *289*(5483), 1343–1346.

Wiemers, M., Balletto, E., Dincă, V., Fric, Z. F., Lamas, G., Lukhtanov, V., Munguira, M. L., van Swaay, C., Vila, R., Vliegenthart, A., Wahlberg, N., & Verovnik, R. (2018). An updated checklist of the European butterflies (Lepidoptera, Papilionoidea). *ZooKeys*, *811*, 9-45. ISSN 1313-2989

Andrews, S. (2010). *Fastqc: a quality control tool for high throughput sequence data*.

Avise, J. C., Walker, D., & Johns, G. C. (1998). Speciation durations and Pleistocene effects on vertebrate phylogeography. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *265*(1407), 1707–1712.

Bacilieri, R., Ducousso, A., Petit, R. J., & Kremer, A. (1996). Mating system and asymmetric hybridization in a mixed stand of European oaks. *Evolution*, *50*(2), 900–908. https://doi.org/10.1111/j.1558-5646.1996.tb03898.x

Barraclough, T. G., & Vogler, A. P. (2000). Detecting the geographical pattern of speciation from species-level phylogenies. *The American Naturalist*, *155*(4), 419–434. https://doi.org/10.1086/303332

Barton, N. (1980). The fitness of hybrids between two chromosomal races of the grasshopper Podisma pedestris. *Heredity*, *45*(1), 47–59. https://doi.org/10.1038/hdy.1980.49

Barton, N. H., & Hewitt, G. M. (1985). Analysis of hybrid zones. *Annual Review of Ecology and Systematics*, *16*(1), 113–148. https://doi.org/10.1146/annurev.es.16.110185.000553

Bateson, W. (1909). *Heredity and variation in modern lights*. Cambridge University Press.

Bernatchez, L., & Wilson, C. C. (1998). Comparative phylogeography of nearctic and palearctic fishes. *Molecular Ecology*, *7*(4), 431–452. https://doi.org/10.1046/j.1365-294x.1998.00319.x

Bishop, M. P., Björnsson, H., Haeberli, W., Oerlemans, J., Shroder, J. F., & Tranter, M. (2011). *Encyclopedia of snow, ice and glaciers*. Springer Science & Business Media.

Boursot, P., Din, W., Anand, R., Darviche, D., Dod, B., Von Deimling, F., Talwar, G., & Bonhomme, F. (1996). Origin and radiation of the house mouse: mitochondrial DNA phylogeny. *Journal of Evolutionary Biology*, *9*(4), 391–415. https://doi.org/10.1046/j.1420-9101.1996.9040391.x

Bunnefeld, L., Hearn, J., Stone, G. N., & Lohse, K. (2018). Whole-genome data reveal the complex history of a diverse ecological community. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(28), E6507–E6515. ISSN 1091-6490. https://doi.org/10.1073/pnas.1800334115

Butlin, R., & Hewitt, G. (1985). A hybrid zone between Chorthippus parallelus parallelus and Chorthippus parallelus erythropus (Orthoptera: Acrididae): behavioural characters. *Biological Journal of the Linnean Society*, *26*(3), 287–299. https://doi.org/10.1111/j.1095-8312.1985.tb01637.x

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. L. (2009). Blast+: architecture and applications. *BMC Bioinformatics*, *10*(1), 421. https://doi.org/10.1186/1471-2105-10-421

Chen, S., Zhou, Y., Chen, Y., & Gu, J. (2018). fastp: an ultra-fast all-in-one fastq preprocessor. *Bioinformatics*, *34*(17), i884–i890. https://doi.org/10.1093/bioinformatics/bty560

Chesser, R. T., & Zink, R. M. (1994). Modes of speciation in birds: a test of Lynch's method. *Evolution*, *48*(2), 490–497. https://doi.org/10.1111/j.1558-5646.1994.tb01326.x

Coyne, J. A., & Orr, H. A. (2004). *Speciation*. Sunderland, MA.

Dapporto, L., Cini, A., Vodă, R., Dincă, V., Wiemers, M., Menchetti, M., Magini, G., Talavera, G., Shreeve, T., Bonelli, S., Casacci, L. P., Balletto, E., Scalercio, S., & Vila, R. (2019). Integrating three comprehensive data sets shows that mitochondrial DNA variation is linked to species traits and paleogeographic events in European butterflies. *Molecular Ecology Resources*, *19*(6), 1623–1636. https://doi.org/10.1111/1755-0998.13059

Dapporto, L., Ramazzotti, M., Fattorini, S., Talavera, G., Vila, R., & Dennis, R. L. (2013). recluster: an unbiased clustering procedure for beta-diversity turnover. *Ecography*, *36*(10), 1070–1075. https://doi.org/10.1111/j.1600-0587.2013.00444.x

Dasmahapatra, K. K., Lamas, G., Simpson, F., & Mallet, J. (2010). The anatomy of a 'suture zone' in amazonian butterflies: a coalescent-based test for vicariant geographic divergence and speciation. *Molecular Ecology*, *19*(19), 4283–4301.

Dennis, R., Williams, W., & Shreeve, T. (1991). A multivariate approach to the determination of faunal structures among european buttery species (Lep-idoptera: Rhopalocera). *Zoological Journal of the Linnean Society*, *101*(1), 1–49.

Descimon, H., & Mallet, J. (2009). Bad species. Ecology of butterflies. *Europe*, *500*(C), 219.

Dincă, V., Dapporto, L., Somervuo, P., Vodă, R., Cuvelier, S., Gascoigne-Pees, M., Huemer, P., Mutanen, M., Hebert, P. D., & Vila, R. (2021). High resolution dna barcode library for european butterflies reveals continental patterns of mitochondrial genetic diversity. *Communications Biology*, *4*(1), 1–11.

Dincă, V., Lee, K. M., Vila, R., & Mutanen, M. (2019). The conundrum of species delimitation: a genomic perspective on a mitogenetically super-variable buttery. *Proceedings of the Royal Society B*, *286*(1911), 20191311.

Dobzhansky, T. (1937). *Genetics and the Origin of Species*. 11. Columbia University Press.

Duplouy, A., & Hornett, E. (2018). Uncovering the hidden players in lepidoptera biology: the heritable microbial endosymbionts. *PEERJ 6*, e4629.

Emms, D. M., & Kelly, S. (2015). Orthofinder: solving fundamental biases in whole genome comparisons dramatically improves orthogroup inference accuracy. *Genome Biology*, *16*(1), 157. https://doi.org/10.1186/s13059-015-0721-2

Ewels, P., Magnusson, M., Lundin, S., & Käller, M. (2016). Multiqc: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*, *32*(19), 3047–3048. https://doi.org/10.1093/bioinformatics/btw354

Finn, R. D., Clements, J., & Eddy, S. R. (2011). Hmmer web server: interactive sequence similarity searching. *Nucleic Acids Research*, *39*(suppl 2), W29–W37. https://doi.org/10.1093/nar/gkr367

Galtier, N., Nabholz, B., Glémin, S., & Hurst, G. (2009). Mitochondrial DNA as a marker of molecular diversity: a reappraisal. *Molecular Ecology*, *18*(22), 4541–4550. https://doi.org/10.1111/j.1365-294X.2009.04380.x

Garrison, E., & Marth, G. (2012). *Haplotype-based variant detection from short read sequencing*. arXiv preprint arXiv:1207.3907

Gaunet, A., Dinca, V. E., Dapporto, L., Montagud, S., Voda, R., Schär, S., Badiane, A., Font, E., & Vila, R. (2019). Two consecutive Wolbachia-mediated mitochondrial introgressions obscure taxonomy in palearctic swallowtail butterflies (Lepidoptera, Papilionidae). *Zoologica Scripta*, *48*, 507–519. https://doi.org/10.1111/zsc.12355

Gibbard, P., & Head, M. J. (2009). The definition of the Quaternary system/era and the Pleistocene series/epoch. *Quaternaire*, *20*(2), 125–133. https://doi.org/10.4000/quaternaire.5086

Giordano, R., Jackson, J. J., & Robertson, H. M. (1997). The role of Wolbachia bacteria in reproductive incompatibilities and hybrid zones of Diabrotica beetles and Gryllus crickets. *Proceedings of the National Academy of Sciences*, *94*(21), 11439–11444. https://doi.org/10.1073/pnas.94.21.11439

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B. W., Nusbaum, C., Lindblad-Toh, K., … Regev, A. (2011). Full-length transcriptome assembly from rna-seq data without a reference genome. *Nature Biotechnology*, *29*(7), 644. https://doi.org/10.1038/nbt.1883

Graham, R. I., & Wilson, K. (2012). Male-killing Wolbachia and mitochondrial selective sweep in a migratory African insect. *BMC Evolutionary Biology*, *12*(1), 204. https://doi.org/10.1186/1471-2148-12-204

Haas, B., Papanicolaou, A. et al (2016). *Transdecoder (find coding regions within transcripts)*.

Habel, J. C., Meyer, M., El Mousadik, A., & Schmitt, T. (2008). Africa goes europe: The complete phylogeography of the marbled white buttery species complex Melanargia galathea/M. lachesis (Lepidoptera: Satyridae). *Organisms Diversity & Evolution*, *8*(2), 121–129.

Habel, J. C., Vila, R., Vodă, R., Husemann, M., Schmitt, T., & Dapporto, L. (2017). Differentiation in the marbled white buttery species complex driven by multiple evolutionary forces. *Journal of Biogeography*, *44*(2), 433–445.

Haffer, J. (1969). Speciation in amazonian forest birds. *Science*, *165*(3889), 131–137.

Hajibabaei, M., Janzen, D. H., Burns, J. M., Hallwachs, W., & Hebert, P. D. (2006). DNA barcodes distinguish species of tropical Lepidoptera.

Proceedings of the National Academy of Sciences, *103*(4), 968–971. https://doi.org/10.1073/pnas.0510466103

Hansen, J., Sato, M., Russell, G., & Kharecha, P. (2013). Climate sensitivity, sea level and atmospheric carbon dioxide. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences*, *371*(2001), 20120294. https://doi.org/10.1098/rsta.2012.0294

Hernández-Roldán, J. L., Dapporto, L., Dincă, V., Vicente, J. C., Hornett, E. A., Šíchová, J., Lukhtanov, V. A., Talavera, G., & Vila, R. (2016). Integrative analyses unveil speciation linked to host plant shift in Spialia butterflies. *Molecular Ecology*, *25*(17), 4267–4284.

Hewitt, G. M. (1996). Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society*, *58*(3), 247–276. https://doi.org/10.1006/bijl.1996.0035

Hewitt, G. M. (1999). Post-glacial re-colonization of European biota. *Biological Journal of the Linnean Society*, *68*(1–2), 87–112. https://doi.org/10.1111/j.1095-8312.1999.tb01160.x

Hewitt, G. (2000). The genetic legacy of the Quaternary ice ages. *Nature*, *405*(6789), 907–913.

Hewitt, G. M. (2001). Speciation, hybrid zones and phylogeography—or seeing genes in space and time. *Molecular Ecology*, *10*(3), 537–549. https://doi.org/10.1046/j.1365-294x.2001.01202.x

Hewitt, G. M. (2011). Quaternary phylogeography: the roots of hybrid zones. *Genetica*, *139*(5), 617–638. https://doi.org/10.1007/s10709-011-9547-3

Hinojosa, J. C., Koubínová, D., Szenteczki, M. A., Pitteloud, C., Dincă, V., Alvarez, N., & Vila, R. (2019). A mirage of cryptic species: Genomics uncover striking mitonuclear discordance in the buttery Thymelicus sylvestris. *Molecular Ecology*, *28*(17), 3857–3868.

Hofreiter, M., & Stewart, J. (2009). Ecological change, range uctuations and population dynamics during the Pleistocene. *Current Biology*, *19*(14), R584–R594, ISSN 0960–9822. https://doi.org/10.1016/J.CUB.2009.06.030

Hurst, G. D., & Jiggins, F. M. (2005). Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. *Proceedings of the Royal Society B: Biological Sciences*, *272*(1572), 1525–1534.

Jiggins, F. M. (2003). Male-killing Wolbachia and mitochondrial DNA: Selective sweeps, hybrid introgression and parasite population dynamics. *Genetics*, *164*(1), 5–12. https://doi.org/10.1093/genetics/164.1.5

Jombart, T., & Dray, S. (2010). adephylo: exploratory analyses for the phylogenetic comparative method. *Bioinformatics*, *26*, 1907–1909. https://doi.org/10.1093/bioinformatics/btq292

Kaliontzopoulou, A., Pinho, C., Harris, D. J., & Carretero, M. A. (2011). When cryptic diversity blurs the picture: a cautionary tale from Iberian and north African Podarcis wall lizards. *Biological Journal of the Linnean Society*, *103*(4), 779–800. https://doi.org/10.1111/j.1095-8312.2011.01703.x

Keightley, P. D., Ness, R. W., Halligan, D. L., & Haddrill, P. R. (2014). Estimation of the spontaneous mutation rate per nucleotide site in a Drosophila melanogaster full-sib family. *Genetics*, *196*(1), 313–320, ISSN 0016–6731. https://doi.org/10.1534/genetics.113.158758

Keightley, P. D., Pinharanda, A., Ness, R. W., Simpson, F., Dasmahapatra, K. K., Mallet, J., Davey, J. W., & Jiggins, C. D. (2014). Estimation of the spontaneous mutation rate in heliconius melpomene. *Molecular Biology and Evolution*, *32*(1), 239–243. https://doi.org/10.1093/molbev/msu302

Klicka, J., & Zink, R. M. (1997). The importance of recent ice ages in speciation: a failed paradigm. *Science*, *277*(5332), 1666–1669. ISSN 0036-8075. https://doi.org/10.1126/science.277.5332.1666

Knowles, L. L., & Carstens, B. C. (2007). Delimiting species without monophyletic gene trees. *Systematic Biology*, *56*(6), 887–895. https://doi.org/10.1080/10635150701701091

Kodandaramaiah, U., Simonsen, T. J., Bromilow, S., Wahlberg, N., & Sperling, F. (2013). Deceptive single-locus taxonomy and phylogeography: Wolbachia-associated divergence in mitochondrial DNA is not reflected in morphology and nuclear markers in a buttery species. *Ecology and Evolution*, *3*(16), 5167–5176.

Krijgsman, W., Hilgen, F., Raffi, I., Sierro, F., & Wilson, D. (1999). Chronology, causes and progression of the Messinian salinity crisis. *Nature*, *400*(6745), 652–655.

Kruuk, L. E., Gilchrist, J. S., & Barton, N. H. (1999). Hybrid dysfunction in fire-bellied toads (Bombina). *Evolution*, *53*(5), 1611–1616.

Kudrna, O. (2019). *Distribution of butterflies and skippers in Europe: (Lepidoptera: Rhopalocera, Grypocera): 24 years mapping European butterflies (1995-2019): final report*. Spolecnost pro Ochranu Motylu (SOM).

Kudrna, O., Harpke, A., Lux, K., Pennerstorfer, J., Schweiger, O., Settele, J., & Wiemers, M. (2011). *Distribution atlas of butterflies in Europe*. Gesellschaft für Schmetterlingsschutz.

Lai, B. C. G., & Pullin, A. S. (2004). Phylogeography, genetic diversity and conservation of the large copper buttery Lycaena dispar in Europe. *Journal of Insect Conservation*, *8*(1), 27–36.

Lawrie, D. S., Messer, P. W., Hershberg, R., & Petrov, D. A. (2013). Strong purifying selection at synonymous sites in d. melanogaster. *PLOS Genetics*, *9*(5), 1–18. https://doi.org/10.1371/journal.pgen.1003527

Leveneu, J., Chichvarkhin, A., & Wahlberg, N. (2009). Varying rates of diversification in the genus melitaea (lepidoptera: Nymphalidae) during the past 20 million years. *Biological Journal of the Linnean Society*, *97*(2), 346–361. https://doi.org/10.1111/j.1095-8312.2009.01208.x

Li, H. (2011). A statistical framework for snp calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. *Bioinformatics*, *27*(21), 2987–2993. https://doi.org/10.1093/bioinformatics/btr509

Li, H. (2013). *Aligning sequence reads, clone sequences and assembly contigs with bwa-mem*. arXiv preprint arXiv:1303.3997.

Librado, P., & Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, *25*(11), 1451–1452. https://doi.org/10.1093/bioinformatics/btp187

Lohse, K., Harrison, R. J., & Barton, N. H. (2011). A general method for calculating likelihoods under the coalescent process. *Genetics*, *189*(3), 977–987. ISSN 0016–6731. https://doi.org/10.1534/genetics.111.129569

Lorkovic, Z. (1973). 150 jahre bis zur entdeckung der präimaginalstadien von Spialia orbifer hbn. (Lep., Hesperiidae). *Acta Entomologica Yugoslavica*, *9*, 67–70.

Mackintosh, A., Laetsch, D. R., Hayward, A., Charlesworth, B., Waterfall, M., Vila, R., & Lohse, K. (2019). The determinants of genetic diversity in butterflies. *Nature Communications*, *10*(1), 3466. ISSN 2041-1723. https://doi.org/10.1038/s41467-019-11308-4

Martin, S. H., Davey, J. W., Salazar, C., & Jiggins, C. D. (2019). Recombination rate variation shapes barriers to introgression across buttery genomes. *PLoS Biology*, *17*(2), e2006288.

Martin, S. H., Möst, M., Palmer, W. J., Salazar, C., McMillan, W. O., Jiggins, F. M., & Jiggins, C. D. (2016). Natural selection and genetic diversity in the buttery Heliconius melpomene. *Genetics*, *203*(1), 525–541.

Martin, S. H., Singh, K. S., Gordon, I. J., Omufwoko, K. S., Collins, S., Warren, I. A., Munby, H., Brattström, O., Traut, W., Martins, D. J., Smith, D. A. S., Jiggins, C. D., Bass, C., & ffrench-Constant, R. H. (2020). Whole-chromosome hitchhiking driven by a male-killing endosymbiont. *PLoS Biology*, *18*(2), e3000610. https://doi.org/10.1371/journal.pbio.3000610

Mayr, E. (1947). Ecological factors in speciation. *Evolution*, *1*(4), 263–288. https://doi.org/10.1111/j.1558-5646.1947.tb02723.x

McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella, K., Altshuler, D., Gabriel, S., Daly, M., DePristo, M. A. (2010). The genome analysis toolkit: a mapreduce framework for analyzing next generation dna sequencing data. *Genome Research*, *20*(9), 1297–1303. https://doi.org/10.1101/gr.107524.110

Muller, H. (1942). Isolating mechanisms, evolution, and temperature. *Biological Symposia*, *6*, 71–125.

Nei, M., & Li, W. H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences*, *76*(10), 5269–5273. ISSN 0027–8424. https://doi.org/10.1073/pnas.76.10.5269

Nolen, Z. J., Yildirim, B., Irisarri, I., Liu, S., Groot Crego, C., Amby, D. B., Mayer, F., Gilbert, M. T. P., & Pereira, R. J. (2020). Historical isolation facilitates species radiation by sexual selection: Insights from chorthippus grasshoppers. *Molecular Ecology*, *29*(24), 4985–5002.

Nürnberger, B., Lohse, K., Fijarczyk, A., Szymura, J. M., & Blaxter, M. L. (2016). Para-allopatry in hybridizing fire-bellied toads (Bombina bombina and B. variegata): Inference from transcriptome-wide coalescence analyses. *Evolution*, *70*(8), 1803–1818.

Pigot, A. L., & Tobias, J. A. (2013). Species interactions constrain geographic range expansion over evolutionary time. *Ecology Letters*, *16*(3), 330–338. https://doi.org/10.1111/ele.12043

Platania, L., Vodă, R., Dincă, V., Talavera, G., Vila, R., & Dapporto, L. (2020). Integrative analyses on western palearctic Lasiommata reveal a mosaic of nascent buttery species. *Journal of Zoological Systematics and Evolutionary Research*, *58*, 809–822.

Porter, A. H., Wenger, R., Geiger, H., Scholl, A., & Shapiro, A. M. (1997). The Pontia daplidice-ed usa hybrid zone in northwestern Italy. *Evolution*, *51*(5), 1561–1573. https://doi.org/10.2307/2411208

Quinlan, A. R., & Hall, I. M. (2010). Bedtools: a flexible suite of utilities for comparing genomic features. *Bioinformatics*, *26*(6), 841–842.

Ratnasingham, S., & Hebert, P. D. (2007). Bold: The barcode of life data system (http://www.barcodinglife.org). *Molecular Ecology Notes*, *7*(3), 355-364.

Ritter, S., Michalski, S. G., Settele, J., Wiemers, M., Fric, Z. F., Sielezniew, M., Šašic, M., Rozier, Y., & Durka, W. (2013). Wolbachia infections mimic cryptic speciation in two parasitic buttery species, Phengaris teleius and P. nausithous (Lepidoptera: Lycaenidae). *PLoS One*, *8*(11), e78107.

Rokas, I. (2000). Wolbachia as a speciation agent. *Trends in Ecology & Evolution*, *15*(2), 44. https://doi.org/10.1016/S0169-5347(99)01783-8

Roux, C., Fraisse, C., Romiguier, J., Anciaux, Y., Galtier, N., & Bierne, N. (2016). Shedding light on the grey zone of speciation along a continuum of genomic divergence. *PLoS Biology*, *14*(12), e2000234. https://doi.org/10.1371/journal.pbio.2000234

Schmitt, T. (2007). Molecular biogeography of europe: Pleistocene cycles and postglacial trends. *Frontiers in Zoology*, *4*(1), 11. https://doi.org/10.1186/1742-9994-4-11

Schoville, S. D., Roderick, G. K., & Kavanaugh, D. H. (2012). Testing the Pleistocene species pump' in alpine habitats: lineage diversification of flightless ground beetles (Coleoptera: Carabidae: Nebria) in relation to altitudinal zonation. *Biological Journal of the Linnean Society*, *107*(1), 95–111.

Shoemaker, D. D., Katju, V., & Jaenike, J. (1999). Wolbachia and the evolution of reproductive isolation between Drosophila recens and Drosophila subquinaria. *Evolution*, *53*(4), 1157–1164.

Simão, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V., & Zdobnov, E. M. (2015). Busco: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics*, *31*(19), 3210–3212. https://doi.org/10.1093/bioinformatics/btv351

Skibinski, D., & Beardmore, J. (1979). A genetic study of intergradation between Mytilus edulis and Mytilus galloprovincialis. *Experientia*, *35*(11), 1442–1444. https://doi.org/10.1007/BF01962773

Spooner, L., & Ritchie, M. (2006). An unusual phylogeography in the bushcricket Ephippiger ephippiger from southern France. *Heredity*, *97*(6), 398–408. https://doi.org/10.1038/sj.hdy.6800884

Talla, V., Johansson, A., Dincă, V., Vila, R., Friberg, M., Wiklund, C., & Backström, N. (2019). Lack of gene ow: narrow and dispersed differentiation islands in a triplet of Leptidea buttery species. *Molecular Ecology*, *28*(16), 3756–3770.

Talla, V., Suh, A., Kalsoom, F., Dincă, V., Vila, R., Friberg, M., Wiklund, C., & Backström, N. (2017). Rapid increase in genome size as a consequence of transposable element hyperactivity in wood-white (Leptidea) butterflies. *Genome Biology and Evolution*, *9*(10), 2491–2505.

Thomas, J. A., Welch, J. J., Lanfear, R., & Bromham, L. (2010). A generation time effect on the rate of molecular evolution in invertebrates. *Molecular Biology and Evolution*, *27*(5), 1173–1180. ISSN 0737-4038. https://doi.org/10.1093/molbev/msq009

Todisco, V., Gratton, P., Cesaroni, D., & Sbordoni, V. (2010). Phylogeography of Parnassius apollo: hints on taxonomy and conservation of a vulnerable glacial buttery invader. *Biological Journal of the Linnean Society*, *101*(1), 169–183.

Toews, D. P., & Brelsford, A. (2012). The biogeography of mitochondrial and nuclear discordance in animals. *Molecular Ecology*, *21*(16), 3907–3930. https://doi.org/10.1111/j.1365-294X.2012.05664.x

Tolman, T., & Lewington, R. (2013). *Collins British buttery guide*. Collins.

Vodă, R., Dapporto, L., Dincă, V., & Vila, R. (2015). Why do cryptic species tend not to co-occur? A case study on two cryptic pairs of butterflies. *PLoS One*, *10*(2), e0117802.

Wall, J. D. (2003). Estimating ancestral population sizes and divergence times. *Genetics*, *163*(1), 395–404. ISSN 0016–6731. https://doi.org/10.1093/genetics/163.1.395

Wang, Y., & Hey, J. (2010). Estimating divergence parameters with small samples from a large number of loci. *Genetics*, *184*(2), 363–379. https://doi.org/10.1534/genetics.109.110528

Weir, J. T., & Price, T. D. (2011). Limits to speciation inferred from times to secondary sympatry and ages of hybridizing species along a latitudinal gradient. *The American Naturalist*, *177*(4), 462–469. https://doi.org/10.1086/658910

Wiemers, M., Chazot, N., Wheat, C. W., Schweiger, O., & Wahlberg, N. (2020). A complete time-calibrated multi-gene phylogeny of the European butterflies. *ZooKeys*, *938*, 97.

Wiemers, M., Stradomsky, B. V., & Vodolazhsky, D. I. (2010). A molecular phylogeny of Polyommatus s. str. and Plebicula based on mitochondrial COI and nuclear ITS2 sequences (Lepidoptera: Lycaenidae). *European Journal of Entomology*, *107*(3), 325.

Wilkinson-Herbots, H. M. (2012). The distribution of the coalescence time and the number of pairwise nucleotide differences in a model of population divergence or speciation with an initial period of gene ow. *Theoretical Population Biology*, *82*(2), 92–108.

Wohlfahrt, T. (1996). Vergleichende untersuchun gen über die größe und form der augenecken am analende der hinterügel von Iphiclides podalirius podalirius (linnaeus, 1758) und I. podalirius feisthamelii (duponchel, 1832). *Spixiana*, *19*, 281-288.

Zinetti, F., Dapporto, L., Vovlas, A., Chelazzi, G., Bonelli, S., Balletto, E., & Ciofi, C. (2013). When the rule becomes the exception. no evidence of gene ow between two Zerynthia cryptic butterflies suggests the emergence of a new model group. *PLoS One*, *8*(6), e65746.

SUPPORTING INFORMATION

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How to cite this article: Ebdon, S., Laetsch, D. R., Dapporto, L., Hayward, A., Ritchie, M. G., Dincă, V., Vila, R., & Lohse, K. (2021). The Pleistocene species pump past its prime: Evidence from European butterfly sister species. *Molecular Ecology*, 00, 1–15. https://doi.org/10.1111/mec.15981