Does divergent selection predict the evolution of mate preference and reproductive isolation in the tropical butterfly genus Melinaea (Nymphalidae: Ithomiini)?

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Abstract

1. Many studies have shown that speciation can be facilitated when a trait under divergent selection also causes assortative mating. In Müllerian mimetic butterflies, a change in wing colour pattern can cause reproductive isolation. However, colour pattern divergence does not always lead to reproductive isolation. Understanding how divergent selection affects speciation requires identifying the mechanisms that promote mate preference and/or choosiness.

2. This study addresses whether shifts in wing colour pattern drives mate preference and reproductive isolation in the tropical butterfly genus *Melinaea* (Nymphalidae: Ithomiini), and focuses on five taxa that form a speciation continuum, from subspecies to fully recognized species.
3. Using genetic markers, wing colour pattern quantification, male pheromone characterization and behavioural assays of mating preference, we characterize the extent of genetic and phenotypic differentiation between taxa and compare it to the level of reproductive isolation.
4. We show strong premating isolation between the closely related species *M. satevis* and *M. marsaeus*, in addition to genetic and phenotypic (colour pattern and pheromones) differentiation. By contrast, *M. menophilus* and *M. marsaeus* consist of pairs of subspecies that differ for colour pattern but that cannot be differentiated genetically. Pheromonal differentiation of subspecies was significant only for *M. marsaeus*, although most individuals were indistinguishable. *Melinaea menophilus* and *M. marsaeus* also differ in the strength of assortative mating, suggesting that mate preference has evolved only in *M. marsaeus*, consistent with selection against maladaptive offspring, as subspecific "hybrids" of *M. marsaeus* have intermediate, non-mimetic colour patterns, unlike those of *M. menophilus* which display either parental phenotypes.
5. We conclude that a shift in colour pattern per se is not sufficient for reproductive isolation, but rather, the evolution of assortative mating may be caused by selection against maladaptive intermediate phenotypes. This study suggests that mate preference and assortative mating evolve when adaptive, and that even in the early stages of divergence, reproductive isolation can be nearly complete due to mating preferences.

Keywords: assortative mating, colour pattern, hybrids, mimicry, magic traits, reinforcement, sexual selection, speciation

Introduction

A key aspect of evolutionary biology is determining the factors that promote population diversification and the processes that initiate progress towards speciation. Divergence in both mating preference and cues are expected to reduce mating between populations and increase reproductive isolation (Boughman 2001), and many studies have highlighted the importance of traits that are under divergent ecological selection that also contribute to assortative mating (e.g. Jiggins *et al.* 2001; Servedio *et al.* 2011; Maan & Seehausen 2012; Jiang, Bolnick & Kirkpatrick 2013). Because the trait subject to divergent selection can directly lead to assortative mating, gene flow is reduced, and these "magic traits" can be the first step in speciation (Servedio *et al.* 2011). However, assortative mating requires the evolution of both divergent cues and preferences, and divergence in one of these alone will not automatically lead to reproductive isolation (Maan & Seehausen 2012).

To understand how divergent selection affects reproductive isolation and hence speciation, we need to identify the mechanisms that generate mating assortment. What is currently needed are detailed studies of closely related taxa that span the speciation continuum, such as populations or species pairs, that are under divergent ecological selection and that vary strongly in their degree of reproductive isolation. A comparative approach based on natural replicates also offers a powerful means with which to study the conditions conducive for speciation. Mimetic organisms, whereby multiple co-occurring unpalatable species converge on the same warning signal and effectively share the cost of educating predators, are especially well suited for studies on speciation, as species

often consist of multiple subspecies diverging for adaptive traits such as wing colour pattern, which can then cause reproductive isolation through sexual and natural selection against phenotypic intermediates (Jiggins *et al.* 2001; Naisbit, Jiggins & Mallet 2001; Merrill *et al.* 2012; Arias *et al.* 2016).

The tribe Ithomiini (ca. 390 species) represents the largest radiation of mimetic butterflies in the Neotropics, where they numerically dominate forest butterfly communities, and have been instrumental in the discovery and description of Müllerian and Batesian mimicry in the 19th century (Bates 1862; Müller 1897). Indeed, the tribe is thought to drive mimicry in many Lepidoptera (Brown & Benson 1974; Beccaloni 1997). However, due to the difficulty in breeding and maintaining ithomiines in captivity, no study has, until now, investigated mate choice and mating behaviour in this tribe. Here we present the first experimental test of reproductive isolation in the tribe Ithomiini, using the genus *Melinaea*.

The genus *Melinaea* consists of at least 14 species and over 70 subspecies (Lamas 2004; but see also McClure & Elias 2017; McClure *et al.* 2018) distributed across much of the Neotropics and is oligophagous on the plant subfamily Solandreae (Solanaceae; Willmott & Freitas 2006). A recent assessment of diversification rates in the tribe revealed that a clade of eight species in the genus experienced an extremely rapid and recent radiation (Chazot *et al.* 2017) in agreement with previous studies using mitochondrial and nuclear genes, and rapidly evolving microsatellite markers, that show little genetic differentiation among taxa of this clade (Whinnett *et al.* 2005; Elias *et al.* 2007; Dasmahapatra *et al.* 2010; McClure & Elias 2017). The *Melinaea* of north-eastern Peru (San Martín and Loreto departments) are of particular interest for speciation studies, as multiple species, many consisting of different subspecies, are present and overlap in distribution. Different subspecies are characterised by different wing colour patterns which are associated with

distinct mimetic communities, including with the polymorphic *Heliconius numata*, whose different morphs are co-mimics to different *Melinaea* taxa (Brown & Benson 1974; Beccaloni 1997). As a result, distribution is often parapatric, with a different dominant taxon in each locality, and a transition or contact zone where different taxa co-occur. Colour patterns are used in mate recognition in a range of mimetic organisms (Jiggins *et al.* 2001; Jiggins *et al.* 2006; Merrill *et al.* 2012), and this may also be the case in the genus *Melinaea*, although this has never before been investigated in Ithomiini. However, Jiggins *et al.* (2006) have demonstrated a phylogenetic pattern of speciation that is correlated with changes in wing colour pattern in the genus *Ithomia* (Ithomiini), which strongly suggests that this may be the case. In addition to colour pattern, pheromones may also play an important role in mate recognition and reproductive isolation. Indeed, Ithomiini male butterflies collect pyrrolizidine alkaloids (PA) which are thought to provide toxicity and pheromone precursors (see Schulz *et al.* 2004 and references therein). Furthermore, as in other ithomiines, male butterflies have hairpencils on their posterior wings that are modified androconial scales used to diffuse these compounds (see e.g. Edgar, Culvenor & Pliske 1975). Premating isolation is expected to be especially strong since females appear to mate only once (i.e. are monandrous; McClure & Elias 2017). Indeed, mistakes or mating with subpar males likely impose a high cost to females, and they are therefore expected to be choosy.

This paper focuses on five *Melinaea* taxa thought to form a speciation continuum, from subspecies to fully recognized species (Lamas 2004): *M. menophilus* ssp. nov. 1 and *M. men. hicetas*, *M. marsaeus phasiana* and *M. mar. rileyi*, and finally *M. satevis cydon*. Previous studies have shown that these taxa utilize the same hostplant, *Juanulloa parasitica* (McClure & Elias 2016; McClure & Elias 2017). As such, McClure & Elias (2016) suggested that diversification in these taxa was likely driven by shifts in colour pattern linked to co-occurring Müllerian mimics

and the resulting predation pressure rather than hostplant shifts. Using artificial models of the polymorphic and Müllerian co-mimic *Heliconius numata*, Chouteau et al. (2016) and Arias et al. (2016) have shown that migrants and intermediate phenotypes respectively, possess locally unrecognized warning signals and suffer greater predator attack frequencies. As the Müllerian co-mimics *Heliconius numata* and *Melinaea* are undistinguishable to predators (Llaurens, Joron & Théry 2014), the results of these studies can be extrapolated to the genus *Melinaea*.

The main purpose of this study is to uncover the factors that drive reproductive isolation (and therefore, speciation) between different mimetic taxa, and what, if anything, promotes the evolution of mating preference and/or choosiness. Although most studies have focused on mating cues (Servedio *et al.* 2011; Maan & Seehausen 2012), determining the evolutionary consequences of divergent selection on reproductive isolation requires studies of the variation that exist in both mating cues and preferences in diverging taxa. The types of isolation that exist between partially isolated taxa in nature are of great interest, as they can provide insight as to what mechanisms are important in the early stages and which processes are then important in driving reproductive isolation and speciation. Using genetic markers (microsatellites), wing colour pattern quantification and vision models for butterflies and their avian predators, male pheromone characterization and behavioural assays of mating preference, we characterize the extent of genetic and phenotypic differentiation for five *Melinaea* taxa, and compare it to the level of mate preference (as a measure of premating isolation). We then discuss the factors that best explain differential progress towards speciation in light of our results.

Material and Methods

Butterfly sampling. Butterflies were collected in north-eastern Peru from 2011 to 2016. Collection localities consisted of premontane forest habitats near Tarapoto (Rio Shilcayo basin: 6°27’30’’S, 76°21’00’’W), Shapaja (6°36’56’’S, 76°09’61’’W) and Chazuta (6°57’05’’S, 76°13’75’’W), and lowland forest on Pongo-Baranquita road (6°17’53’’S, 76°14’38”W) and Shucushyacu (5°57’20’’S, 75°53’06’’W). Various sites a few kilometres apart were sampled within each locality. The number of individuals of each taxon used to measure genetic differentiation, pheromone characterization and colour pattern quantification are found in Table 1.

Individuals that were phenotypically intermediate between *M. mar. phasiana* and *M. mar. rileyi* were considered to be putative hybrids. To test if the occurrence of putative hybrids deviated from expectations if mating were random, a Pearson’s χ^2 test was done on the observed frequencies obtained from the data and by calculating expected frequencies based on Hardy-Weinberg equilibrium (Table S1). This was done both for the entire distribution (i.e. all localities were pooled) and for the contact zone, where hybridization may be more common.

Rearing conditions. Gravid wild caught females were kept in 2x2x2 m outdoor insectaries under ambient conditions in Tarapoto, San Martín, where all rearing was carried out (see McClure & Elias 2016 for further information). Butterflies were provided with nourishment in the form of sugar water solution and bee pollen. All species in this study use *J. parasitica* as a host plant (McClure & Elias 2016; McClure & Elias 2017), and as such, potted *J. parasitica* plants were used for oviposition, and larvae collected in the cages were reared individually in transparent plastic containers in the shade behind a nearby building under ambient conditions. Larvae were checked daily for food replacement and cleaning, and leaves were offered ad libitum.

159 Newly emerged butterflies were kept segregated by sex in outdoor insectaries until use,
160 with sugar water solution and bee pollen for nourishment, and pyrrolizidine alkaloid sources in the
161 form of withered *Heliotropium* sp. (Boraginaceae) and Eupatorieae (Asteraceae).
162
163 **No-choice mating experiments.** To test for reproductive isolation, no-choice experiments were
164 used as they examine whether mating can occur, when no alternatives are present (a situation more
165 likely to reflect what happens in nature). Strict preference in a choice situation does not preclude
166 the possibility of accepting a mate when no alternative is present. Trials were carried out with four
167 males and four females, unrelated, and of either the same or different taxa. Trials lasted for four
168 days or until a mating event occurred and 12 replicates were done for each combination. For trials
169 between different taxa, half of the replicates were done using each reciprocal cross so as to control
170 for potentially different mating probabilities. For trials between the closely related species *M.*
171 *satevis cydon* and *M. marsaeus*, half of the replicates were done using each of the *M. marsaeus*
172 subspecies. However, the results of these reciprocal crosses were not found to be statistically
173 different (*M. menophilus*: χ^2=1.2, df=1, p=0.273; *M. marsaeus*: no mating was observed; *M.*
174 *marsaeus* x *M. satevis cydon*: no mating was observed; see Table 2), and were therefore pooled.
175 *Melinaea marsaeus* was used with *M. satevis cydon* to test assortative mating between closely
176 related species as McClure & Elias (2017) have shown, using microsatellite markers, that *M.*
177 *menophilus* clusters separately from *M. marsaeus* and *M. satevis cydon*. The latter two species are
178 therefore more closely related, and provide a relevant comparison after reproductive isolation is
179 complete.
180 McClure & Elias (2017) reported that copula lasted anywhere between a little over an hour
181 up to 24 h. As such, cages were checked hourly between 6 AM and 6 PM (hours during which

there is daylight) every day for mating events. To further ensure that no mating events took place unnoticed, the presence of a spermatophore was ascertained by palping the females' abdomen at the end of the experiment. Males, regardless of whether they were mated, and females that did not mate, were occasionally re-used, but only after 7-10 days had elapsed to prevent habituation and no more than once. Females that mated were not re-used.

Mating probabilities P_{ij} between *i*-type females and *j*-type males relative to the probability of mating within types were estimated using likelihood in order to test between hypotheses (McMillan, Jiggins & Mallet 1997; Naisbit, Jiggins & Mallet 2001). The probability of mating occurring can be calculated by maximizing the $\log_e$-likelihood expression:

$$L(P_{ij}) = m_{ij} \log_e(P_{ij}) + (N_{ij} - m_{ij}) \log_e(1 - P_{ij})$$

N and m are the total number of trials and the number of trials where mating occurred, respectively. Fitting models with different numbers of parameters (i.e., same versus different mating probabilities for different types of crosses) enabled to test for differences in the mating probability across trials using a likelihood ratio test with $G=2\Delta\log_e L$, which asymptotically follows a $\chi 2$-distribution (Edwards 1972). As such, we were able to test whether individuals of different taxa mate less frequently than those of the same taxon, or if all crosses are either equal or all significantly different from one another.

An index of premating isolation similar to what was used by Coyne & Orr (1989) was also calculated using the expression:

$$1 - \frac{frequency of heterospecific mating}{frequency of conspecific mating}$$

This index ranges from -∞ (complete disassortative mating) through 0 (no mating isolation) to 1 (complete mating isolation).

Genetic differentiation. Samples used in this study were preserved in either ethanol or in salt-saturated 20% dimethylsulphoxide (DMSO) with ethylenediaminetetraacetic acid (EDTA). Individuals were genotyped at 12 microsatellite markers developed for *Melinaea*, using primers and PCR conditions from McClure et al. (2014). The extent of genetic differentiation and admixture, and the number of possible genetic clusters (or distinct groups), was assessed in three ways. First, STRUCTURE version 2.3.4 (Pritchard, Stevens & Donnelly 2000) was used on the data, run with 500 000 updates of the Markov chain after an initial 'burn-in' of 50 000 updates for one to five genetic clusters (K=1-5), with five replicates at each value of K. The method described by Evanno et al. (2005), based on the second-order rate of change of the log likelihood and implemented in STRUCTURE HARVESTER (Earl & vonHoldt 2012), was used to determine the number of clusters that best describe the data. A factorial correspondence analysis was also used on the data using GENETIX (Belkhir *et al.* 1996). Finally, Fst values for each pair of taxa were calculated using GENEPOP version 4.2 (Raymond & Rousset 1995).

Differentiation of colour pattern. Differentiation in colour pattern was quantified using Colour Pattern Modelling (CPM) described by Le Poul et al. (2014). Photographs, taken under standardized conditions (see Le Poul *et al.* 2014 for details), of the dorsal and ventral sides of forewings and hindwings of each specimen were used. The CPM automatically detects and eliminates the background in the pictures and each pixel of the butterfly wing image is automatically attributed a colour, which is then categorized into one of three major colours present in the wing patterns (that is, black, orange or yellow). Hind and forewings are aligned separately using a rigid transformation, and similarity (accounting both for wing shape and pattern) with a wing model that averages all wing images is maximized recursively. After alignment, the position

of each pixel in the wing image is considered homologous among all individuals, enabling a pixel-by-pixel analysis of pattern variation.

In order to link divergence in colour pattern to reproductive isolation (specifically, prezygotic isolation driven by mate choice, and postzygotic isolation driven by predation), the value of each pixel was re-calculated by incorporating models of animal vision (see Arias *et al.* 2016 for details) and by using the precise colour spectrum of each colour (see Llaurens, Joron & Théry 2014). Models of animal vision, based on the sensitivities of photoreceptors present in their eyes, allow inferences about the colour contrasts and the conspicuousness of the different colours that can be perceived by different animals. As such, two avian vision systems that vary in their sensitivity to ultra-violets (i.e. wavelengths below 400nm) and one butterfly vision system were performed. All vision systems consist of four photoreceptors. Specifically, the quantum catches, which is the relative amount of light captured by each photoreceptor when observing a given colour (Iriel & Lagorio 2010), was applied using the method described in Vorobyev & Osorio (1998) and assuming a Weber fraction of 0.05 for all vision systems. A light environment corresponding to both small and large gaps in a forest canopy (computed as in Llaurens, Joron & Théry 2014) was used. As the photoreceptor sensitivities of the birds composing the predator community in this study system are not known, the calculations were based on the two main vision systems found in birds. The blue tit (*Parus caeruleus*) was used for vision with ultra-violet sensitive pigments (UVS), with cone proportion and sensitivity as described by Hart et al. (2000), and the shearwater (*Puffinus pacificus*) was used for vision with violet sensitive pigments (VS), as described by Hart (2004). To model butterfly vision, the photoreceptors sensitivity was computed using the visual sensitivity peaks reported for *Danaus plexippus* (the monarch, the closest relative of *Melinaea* for which sensitivity peaks are known) in Stalleicken, Labhart & Mouritsen (2006) and Blackiston,

Briscoe & Weiss (2011), applying Stavenga (2010)'s equations. Since Blackiston, Briscoe & Weiss (2011) reported the existence of a dark orange filter in the long wavelength receptor, which effectively gives rise to a fourth type of receptor, we also modelled this filtered photoreceptor by digitizing the spectrum reported in McCulloch, Osorio & Briscoe (2016) for *Heliconius erato* (the closest butterfly species – also a Nymphalidae – for which the spectrum has been characterized, and whose long wavelength photoreceptor peaks at the same value as *D. plexippus*) using the software Graph Grabber 2.0 (www.quintessa.org/). The vision model for *D. plexippus* is available in ESM. The precise colour spectrum of each colour detected by CPM (black, orange and yellow) were those taken on *Melinaea* by Llaurens, Joron & Théry (2014). Quantum catches for each photoreceptor in birds and butterflies were estimated using AVICOL (Gomez 2006), under large light gap and small light gap light conditions. The phenotypic variation (variation among all pixels common between all wings) after accounting for animal vision was summarized using a principle component analysis (PCA). Differences between groups were tested using ADONIS (PERMANOVA) in the R package Vegan (Oksanen *et al.* 2016), followed by a pairwise comparison (i.e. post hoc test) with Bonferroni correction (pairwiseAdonis: Martinez Arbizu 2017). Finally, differentiation between taxa was measured as Euclidean distances between centroids in PCA space.

Chemical analysis. Preliminary tests of female wing extracts failed to reveal any compounds, so all further tests focused on males. The hairpencils (i.e. androconial scales) of 6-10 males per taxa were dissected and extracted individually in 100 µl of ultrapure dichloromethane (Sigma-Aldrich®) shortly after capture. Samples were kept at -20°C until analysis in Montreal, Canada, by gas chromatography/mass spectrometry (GC/MS) with an Agilent 7890A-5975C. Using pulsed

splitless injection, 2 µL of each extract was injected on an Agilent HP-5MS column (30 m x 250 µm x 0.25 µm) with the inlet maintained at 250°C. Helium was used as a carrier gas and flow rate was of 1.5411 mL/min. Temperature gradient was programmed from 50°C to 300°C at a rate of 8°C/min for a total run time of 39min. Kovats' retention indices (RIs) were computed using n-alkanes from C8 to C20 that were eluted under the same conditions as the samples (external standards). Compounds were identified by comparison of mass spectra and gas chromatographic retention indices to those in the literature and the NIST library. A data matrix of all the compounds for each individual was aligned using GCAligner 1.0 (Dellicour & Lecocq 2013). Relative concentrations were determined by peak area analysis and differences between groups were visualized using nonmetric multidimensional scaling (NMDS) ordination based on Bray-Curtis similarity matrix, using the function metaMDS in the R package Vegan (Oksanen *et al.* 2016). Differences between groups were tested using ADONIS (PERMANOVA) in the R package Vegan, followed by a pairwise comparison (i.e. Post hoc test) with Bonferroni correction (pairwiseAdonis: Martinez Arbizu 2017). If differences within species (i.e. between subspecies) were found to be significant, this was followed with a non-parametric Mann-Whitney U test so as to test for differences in the amount of each compound. Finally, differentiation between taxa was measured as Euclidean distances between centroids.

Results

Distribution. Partial geographical isolation is observed between the subspecies of both *M. marsaeus* and *M. menophilus*, with uneven abundance at the different localities. Distribution and relative frequency of the five different taxa in each region is shown in Fig. S1. The general pattern of distribution for these subspecies pairs consists in one subspecies being present in premontane

297 forest (*M. men.* ssp. nov. 1 and *M. mar. phasiana*) and the other in lowland forest (*M. men. hicetas*
298 and *M. mar. rileyi*). The different subspecies are considered parapatric, and both species have a
299 transition or contact zone in the lowlands near Pongo, a known suture and hybrid zone (Whinnett
300 *et al.* 2005; Dasmahapatra *et al.* 2010). Finally, the third species, *M. satevis cydon*, is a lowland
301 species.

302 Overall, the number of potential *M. marsaeus* hybrids (based on intermediate phenotypes)
303 is much lower than expected under random mating (4.4% observed *vs.* 45.8% expected; see Table
304 S1). This is also true in the contact zone where a strong hybrid deficit is observed (12.5% observed
305 *vs.* 42.97% expected; see Table S1). Of 34 phenotypically "pure" females that were collected in
306 the field and used to produce broods, two produced offspring of intermediate "hybrid" phenotypes
307 (i.e. 5.9% of females). This is putatively the result of mating between *M. mar. phasiana* and *M.*
308 *mar. rileyi*.

309 No putative *M. menophilus* hybrids were observed. This is consistent with McClure & Elias
310 (2017) who reported that progeny of crosses between *M. men. hicetas* and *M. men.* ssp. nov. 1
311 possess either of the parental phenotypes.

312

313 **No-choice mating experiments.** Mating events were much more prevalent within taxa for both
314 the closely related species *M. satevis cydon* and *M. marsaeus* ($p<0.01$), and within the *M.*
315 *marsaeus* subspecies ($p<0.01$). However, this was not true for *M. menophilus* ($p>0.05$). Table 2
316 shows mating probabilities both within and between subspecies, and between closely related
317 species. Both the closely related species *M. marsaeus* and *M. satevis cydon* showed strong
318 premating isolation, as did the *M. marsaeus* subspecies (index of premating isolation=1). By

contrast, the subspecies of *M. menophilus* showed no assortative mating (index of premating isolation=0).

Genetic differentiation. Both STRUCTURE (Fig. S2) and the factorial correspondence analyses (GENETIX; Fig. 1) detected low levels of structuring (K=3; Delta K peak=7.5), with the three groups corresponding to the three species (*M. menophilus*, *M. marsaeus* and *M. satevis cydon*). Subspecies clustered together and presented high levels of admixture, as also evidenced by low Fst values (Fst within *M. menophilus* = 0.01 and *M. marsaeus* < 0.01; Table 3). The species *M. marsaeus* and *M. satevis cydon* were also found to be closely related (Fst = 0.02-0.04; Table 3).

Differentiation of colour pattern. Fig. S3 shows the average wing colour patterns, calculated by the CPM, for each *Melinaea* taxon (Fig. S3a) and the heatmaps (Fig. S3b) generated to visualize how each of the three colours (black, orange and yellow) vary (from blue to red) across the wings.

Because results were identical for animal visions under both light conditions (large and small light gaps), only models based on small light gaps, which likely replicate conditions in primary forest where natural populations of *Melinaea* occur, are shown. Similarly, results for both avian vision (VS and UVS vision) were the same, and as such, only results for UVS vision (i.e. the blue tit) are discussed here, although results for VS vision are shown in Fig. S4.

Differentiation in colour pattern was significant for all taxa and putative hybrids, both under butterfly (PERMANOVA ADONIS: $F = 79.39$; $df = 5$; $p = 0.001$; Fig. 2a) and avian (PERMANOVA ADONIS: $F = 73.30$; $df = 5$; $p = 0.001$; Fig. 2b) vision. A pairwise post hoc test with Bonferroni correction shows all groups as being significantly different from each other ($p = 0.001$). However, differentiation of the two subspecies of *M. menophilus*, which differ for a single

yellow band, appears greater under the butterfly vision model than under the avian vision model. Euclidean distances between centroids of pairs of taxa are presented in Table 3.

Chemical analysis. A total of six compounds (Table 4) were identified, four of which were common to all taxa, albeit at different ratios (Fig. 3), and two were unique to *M. menophilus*. A comparison of the different chemical extracts was found to be significantly different (PERMANOVA ADONIS: F=27.60; df=4; p=0.001) and the NMDS ordinal plot shows the three species as being completely separate, but the subspecies as clustering together (Fig. 4). A pairwise post hoc test with Bonferroni correction confirmed that the closely related species *M. marsaeus* and *M. satevis cydon* are significantly different from each other (p = 0.015). The subspecies of *M. menophilus* (p = 1.0) were not found to be significantly different, but the subspecies of *M. marsaeus* were (p = 0.02). This difference appears to be the result of a difference in the ratio between the ΔC21 acid (U = 7; p = 0.002) and the C21 acid (U = 4; p < 0.01; Fig. 3). However, most individuals of both subspecies, in addition to the potential hybrid, possess the same intermediate ratio of the two compounds (Figs 3 & 4). Euclidean distances between centroids of pairs of taxa are presented in Table 3 and show increasing levels of differentiation with increasing reproductive isolation.

Discussion

Synchrony between assortative mating and divergent selection can trigger rapid speciation. Indeed, when mate choice is based on an ecologically important trait, divergence in that trait can facilitate reproductive isolation and speciation, even with gene flow (Servedio *et al.* 2011; Kopp *et al.* 2018 and references therein). Mimicry is a good example of a trait under strong ecological divergent

selection that can also be used as a mating cue, and this has been shown for many different organisms, including fish (Hypoplectrus coral reef fishes: Puebla *et al.* 2007), frogs (Dendrobates: Reynolds & Fitzpatrick 2007) and butterflies (Heliconius: Jiggins *et al.* 2001; Merrill *et al.* 2012). Because the evolution of mate choice is thought to be an important process generating and maintaining biological diversity, determining which traits and corresponding selective pressures initiate differentiation is important, but understanding the causes of speciation also requires studies associated with diverging preference and/or increased choosiness (Maan & Seehausen 2012). In the poison frog *Ranitomeya imitator*, Twomey et al. (2016) found that although colour pattern diverges repeatedly, genome-wide divergence occurs only when there is mate preference, resulting in assortative mating. Similarly, in the mimetic *Heliconius* butterflies, Chouteau et al. (2017) showed that *H. numata* is a panmictic population despite the presence of polymorphism as a result of disassortative mating, an unusual feature in Müllerian mimetic organisms.

To understand how divergent selection affects speciation, we need to know how it affects the evolution of reproductive isolation. Servedio & Boughman (2017) suggested that the ideal empirical evidence to evaluate how the evolution of choosiness affects speciation would result from testing whether evolutionary changes in choosiness are associated with changes in assortative mating among species and reduction in gene flow, preferably by comparing early to late stages of speciation. The genus *Melinaea* is therefore especially pertinent in furthering our understanding of the evolution of reproductive isolation and speciation as the genus has undergone a rapid and recent diversification, and consists of pairs of taxa that differ in their degree of differentiation and assortative mating, with some in the very early stages of speciation.

Our results show strong premating isolation between the closely related species *M. satevis cydon* and *M. marsaeus*, in addition to genetic and phenotypic differentiation, both for the colour

388 pattern and pheromones. This is consistent with McClure & Elias (2017) who observed that mating
389 between sympatric species were extremely rare, including between these two closely related
390 species, and that these crosses never produced any eggs. Reproductive isolation may not be as
391 strong between allopatric species, however, and McClure et al. (2018) reported having successfully
392 crossed the allopatric species *M. satevis cydon* and *M. tarapotensis* (formerly *M. satevis*
393 *tarapotensis*: see McClure *et al.* 2018). These crosses successfully produced viable hybrid
394 offspring, and although most of the gametes of these hybrids had an unbalanced genome and a
395 degenerative appearance, some hybrids produced a small proportion (4%) of viable offspring in
396 backcrosses (McClure *et al.* 2018). In regards to the sympatric species *M. satevis cydon* and *M.*
397 *marsaeus*, strong pre- and post-mating isolation may prevent the costly production of hybrids with
398 possible genetic incompatibilities.

399 Premating isolation was also observed between the subspecies of *M. marsaeus*, but not
400 those of *M. menophilus*, despite the absence of genetic differentiation between subspecies of both
401 these species. McClure and Elias (2017) observed mating pairs of *M. menophilus*, and reported
402 that these crosses were fertile and resulted in viable progeny. Chemical differentiation was not
403 significantly different between the subspecies of *M. menophilus*, but was significantly different
404 between the subspecies of *M. marsaeus*. This difference appears to be driven by a difference in the
405 ratio between the ΔC21 acid and the C21 acid. However, whether this difference can be perceived
406 by the butterflies and whether it is biologically significant remains unknown. Furthermore, this
407 difference was not present in all individuals, with many individuals of both subspecies and the
408 potential hybrid possessing the same ratio. This suggests that even if this difference is biologically
409 significant, it is not the sole trait used for mate recognition. As such, colour pattern is likely the

410 first trait to diversify and be used in mate recognition. Chemical differentiation may only occur
411 subsequently, reinforcing mate recognition and premating isolation.

412 Differentiation of colour pattern was significantly different between subspecies of both
413 species, but this differentiation was found to be more pronounced between the subspecies of *M.*
414 *marsaeus*. This was especially true when differentiation was modelled on bird vision, thought to
415 be the main predators. A study by Llaurens et al. (2014) that compared the colour pattern of
416 *Heliconius numata* with that of their Müllerian co-mimics *Melinaea* found that the colour contrast
417 of yellow against a black background was greater for butterflies than for birds. The authors
418 suggested that this variation in colour, likely undetectable to birds, might be used by butterflies to
419 distinguish between mating partners without losing the benefits of mimicry. As such, migrants
420 between populations of *M. marsaeus* are likely to suffer higher levels of predator attacks because
421 they are strongly non-mimetic outside their habitat (Chouteau, Arias & Joron 2016), which can
422 directly reduce gene flow between populations by lowering the rate of heterospecific encounters.
423 Differences in the distribution of the two species may also be due to differences in the strength of
424 disruptive selection in the form of predation. In *M. menophilus*, where both phenotypes differ in
425 the presence or absence of a single yellow band, the overlap in distribution is wide and both
426 phenotypes occur to some extent throughout their range. In *M. marsaeus*, where both phenotypes
427 differ more considerably, area of contact is narrow and each phenotype is almost exclusively
428 present at either end of the distribution.

429 Furthermore, putative hybrids between *M. mar. phasiana* and *M. mar. rileyi* possess
430 intermediate non-mimetic colour patterns and likely suffer intense frequency-dependent predation
431 similar to what is observed in the perfect co-mimic *Heliconius numata* (Arias *et al.* 2016), which
432 can further decrease gene flow and drive the spread of alleles for enhanced mate preference and/or

choosiness in a reinforcement-like process. In *Heliconius* butterflies, Merrill et al. (2012) suggested that selection against hybrids was as strong as selection against migrants (in this case, a non-mimetic control species). Progeny of crosses between *M. men. hicetas* and *M. men.* ssp. nov. 1 do not produce phenotypic intermediates, but rather possess either of the parental phenotypes, with the *hicetas* phenotype appearing to be at least partly dominant (McClure & Elias 2017). Although currently untested, differences in colour pattern within *M. menophilus* may be the result of a single locus with dominance, and this genetic architecture may differ from other *Melinaea* species, including *M. marsaeus*. Nevertheless, as there are no intermediate phenotypes produced in *M. menophilus*, selective pressure against mating between taxa is likely reduced and rampant gene flow can be expected, thereby inhibiting the fixation of preference or increased choosiness alleles. Yukilevich (2012) demonstrated that, in *Drosophila*, asymmetries in the strength of premating isolation between species pairs matches the cost of producing hybrids. As such, at least in *M. marsaeus*, mating preference may have directly evolved in response to selection against maladaptive offspring of intermediate phenotypes.

In this study we show that the absence of ecological adaptations other than colour pattern (see McClure & Elias 2016) does not preclude the evolution of mating isolation. In fact, through the maintenance of a spatial mosaic of mimetic colour patterns, predation on Müllerian mimics constrains geographical distribution and allows for different species or subspecies, even those with similar ecological niches, to exist in different regions (Aubier, Joron & Sherratt 2017). This study also suggests that mate preference and assortative mating evolve adaptively in response to divergent selection, and that even in the early stages of speciation, reproductive isolation can be nearly complete due to mating preferences, as seen in *M. marsaeus*. But perhaps surprisingly, we also show that changes in traits used for mate recognition, such as colour pattern, does not

456 invariably lead to reproductive isolation, as demonstrated by the equal hetero- and conspecific
457 mating probabilities observed in *M. menophilus*. Nevertheless, populations of *M. menophilus*
458 remain partly segregated by colour pattern, likely as a result of selection against immigrants.
459 Mallet & Barton (1989) showed selection against immigrants across a hybrid zone to be of 52%
460 where two races of *H. erato* meet, sufficient to maintain a cline in colour pattern, despite random
461 mating. But because *M. menophilus* does not produce any phenotypic hybrids, it is presently
462 difficult to evaluate the true occurrence of heterospecific mating in the field.

463 In conclusion, we find that premating isolation in *Melinaea* arises early and quickly, with
464 apparently no intermediate levels of premating isolation, despite a continuum of genetic and
465 phenotypic differentiation. Our results suggest that colour patterns adapted to different mimicry
466 rings may be used in mate recognition. However, reproductive isolation, as a result of mate
467 preference and/or increased choosiness, and variable progress towards speciation is consistent with
468 selection against maladaptive hybrids rather than a change in colour pattern per se. Uncovering the
469 evolutionary cause of assortative mating requires the comparative analyses of the strength of
470 assortative mating across different taxa subject to different selective pressures or genetic
471 architectures (Jiang, Bolnick & Kirkpatrick 2013). The exceptional conditions present in the region
472 of Tarapoto, north-eastern Peru, where multiple species form concordant contact or hybrid zones
473 between taxa of lowland and premontane forests (Dasmahapatra *et al.* 2010) offer an optimal
474 natural setting to investigate the evolution of assortative mating across a large range of taxa.

475

476 **Authors' contributions.** MMc and ME designed and coordinated the study. MMc collected the
477 samples and field data, performed the experiments, carried out the molecular lab work, analysed
478 the molecular, chemical and experimental data and drafted the manuscript. LM and AF analysed

the chemical extracts, and BF identified the chemical compounds. ME performed butterfly vision modelling and CH, MMo, YLP and ME analysed the colour pattern of the wings. ME obtained the funding and helped draft the manuscript. All authors gave final approval for publication.

Data accessibility. Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.008b59c (McClure et al. 2019)

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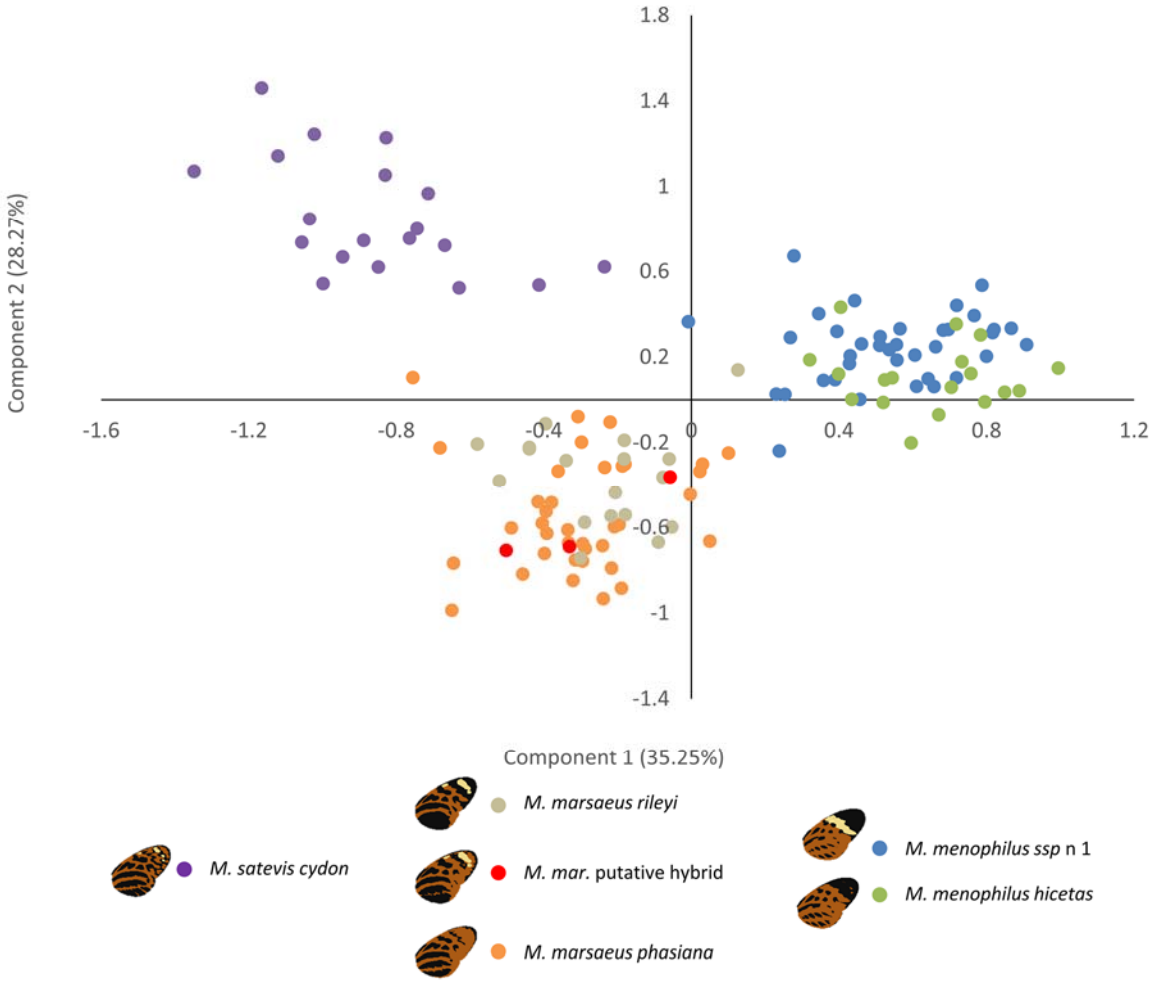
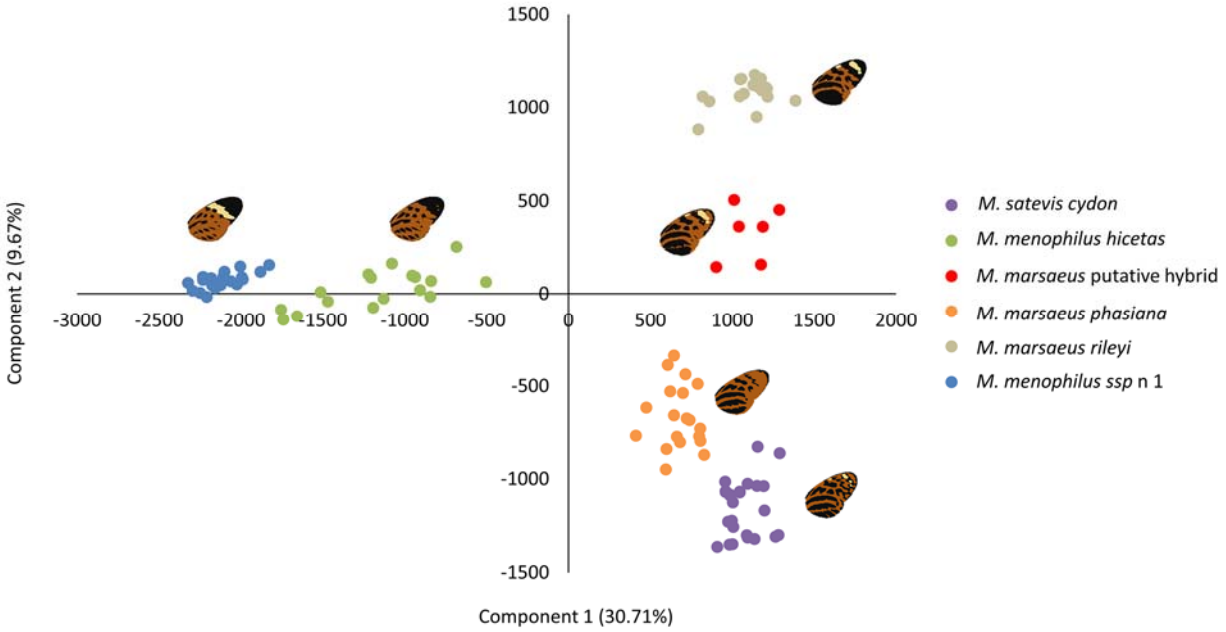


Figure 1: Factorial correspondence analysis for five *Melinaea* taxa and putative hybrids between subspecies of *M. marsaeus* on 12 microsatellite loci computed using the program GENETIX

661 **a**



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663 **b**

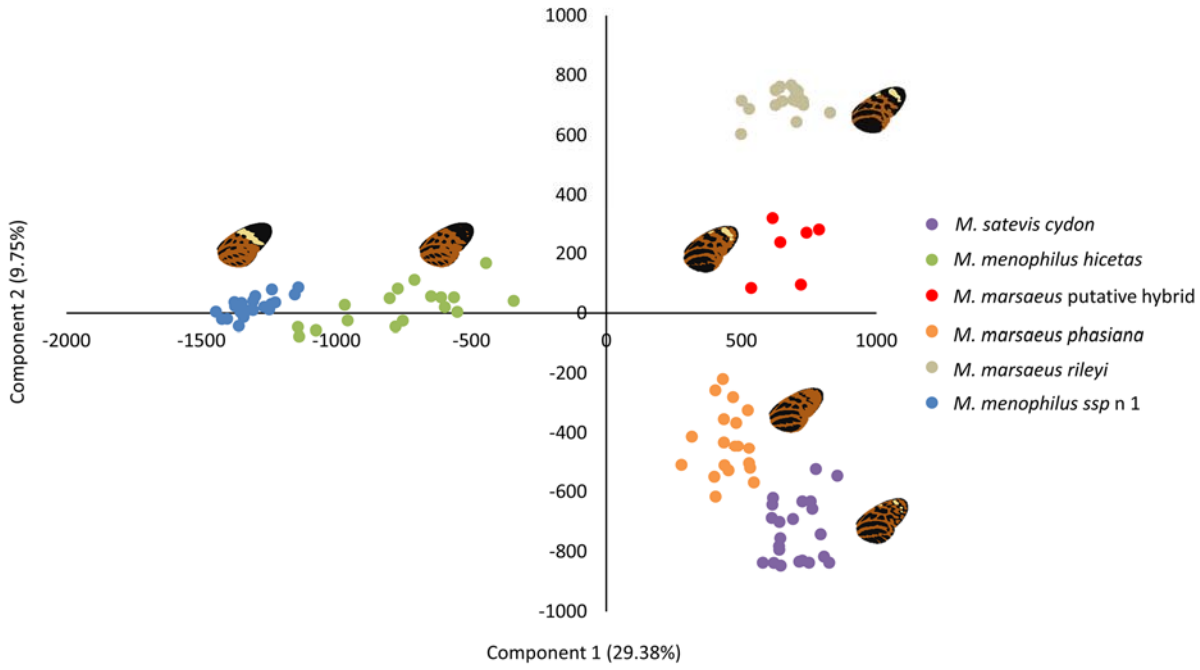

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Figure 2: Principal component analysis showing the variation in the colour pattern of five *Melinaea* taxa and putative hybrids between subspecies of *M. marsaeus* as quantified by Colour Pattern Modelling and modelled on **a)** butterfly vision and **b)** UVS bird vision

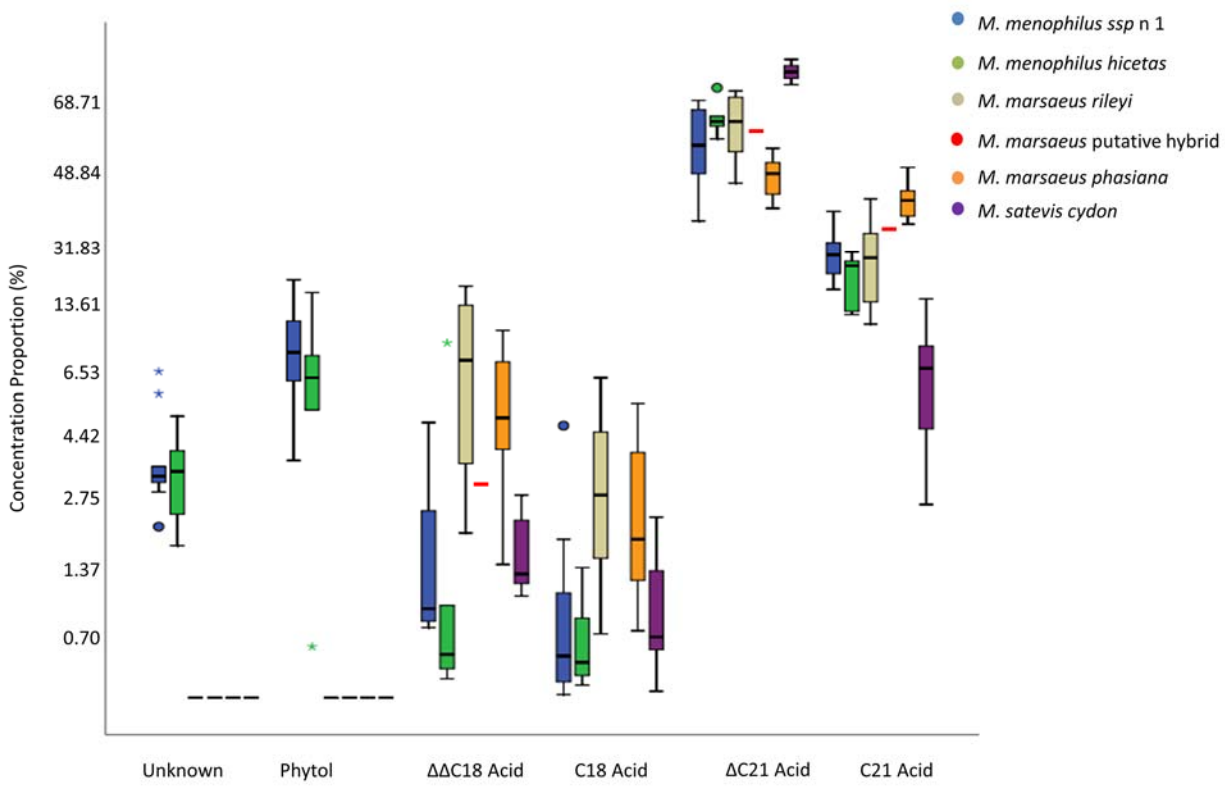
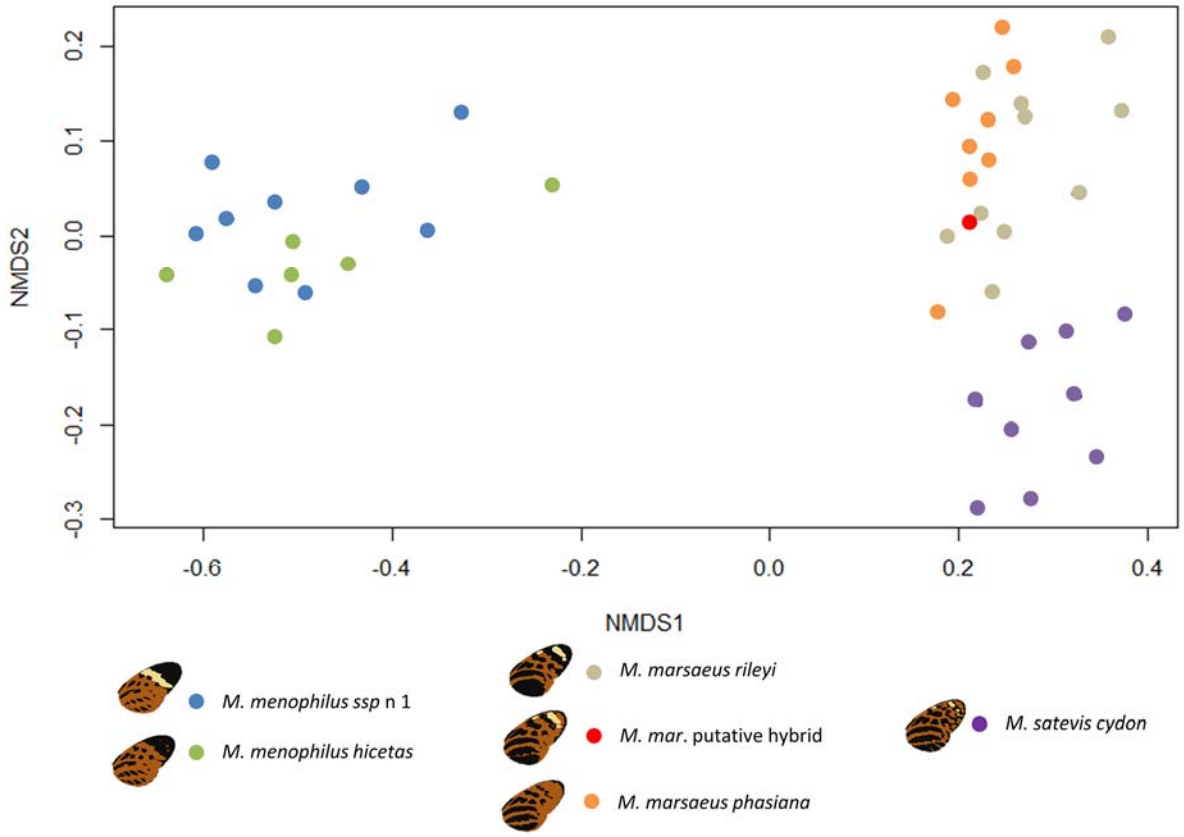


Figure 3: Proportion of the different compounds present in the chemical profile of five *Melinaea* taxa and one putative hybrid between subspecies of *M. marsaeus* obtained by GC-MS



673

674 **Figure 4:** NMDS ordination plot based on Bray-Curtis distances calculated on the chemical profile
675 obtained by GC-MS of five *Melinaea* taxa and one putative hybrid between subspecies of *M.*
676 *marsaeus*

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678

679 **Table 1.** The number of individuals of each *Melinaea* taxon used to measure genetic
680 differentiation, pheromone characterization and colour pattern quantification

	Genetic differentiation	**Pheromone characterization**	**Colour pattern quantification**
M. menophilus ssp. nov. 1	37	9	20
M. menophilus hicetas	18	6	17
M. marsaeus rileyi	18	10	20
M. marsaeus putative hybrid	3	1	6
M. marsaeus phasiana	37	8	19
M. satevis cydon	19	9	22

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Table 2. Observed mating probabilities within and between different *Melinaea* taxa and the resulting best fitting model for each (i.e. whether different taxa mate less frequently than those of the same taxon, or if all crosses are either equal or all significantly different from one another). Different lower-case letters indicate significant differences of $p<0.05$ for each cross, based on the best fitting model obtained.

No-choice mating experiments	Number of trials	Number of mating	Mating probability	Best fitting model
M. menophilus ssp. nov. 1 x *M. menophilus* ssp. nov. 1	12	9	0.75^{a}	$P_{ii}=P_{jj}=P_{ij}=P_{ji}$
M. menophilus ssp. nov. 1 x *M. menophilus hicetas*	12	8	0.67^{a}	
M. menophilus hicetas x M. menophilus hicetas	12	7	0.58^{a}	
M. marsaeus rileyi x M. marsaeus rileyi	12	8	0.67^{a}	$P_{ii}=P_{jj}\neq P_{ij}=P_{ji}$
M. marsaeus rileyi x M. marsaeus phasiana	12	0	0^{b}	
M. marsaeus phasiana x M. marsaeus phasiana	12	6	0.5^{a}	
M. marsaeus x M. marsaeus	24	14	0.58^{a}	$P_{ii}\neq P_{jj}\neq P_{ij}=P_{ji}$
M. marsaeus x M. satevis cydon	12	0	0^{b}	
M. satevis cydon x M. satevis cydon	12	11	0.92^{c}	

690 **Table 3.** Measures of genetic differentiation (Fst), colour pattern differentiation as perceived by butterflies and birds (Euclidean
691 distances between group centroids), pheromone differentiation and the index of premating isolation (where 0=no mating isolation,
692 1=complete mating isolation) for different pairs of *Melinaea* taxa. For clarity and ease of comparison, a relative value ranging from 0 to
693 1, calculated as the absolute Euclidean distance value divided by the maximum value observed in the dataset, is included in brackets for
694 colour pattern and pheromones.

Pairs of taxa	**Fst**	**Colour pattern distances (butterflies)**	**Colour pattern distances (birds)**	**Pheromone distances**	**Index of premating isolation**
M. menophilus* ssp. nov. 1 & *M. men. hicetas	0.013	1.47×10^3 (0.63)	0.85×10^3 (0.57)	10.65 (0.20)	0
M. marsaeus phasiana* & *M. mar. rileyi	0.006	2.19×10^3 (0.94)	1.42×10^3 (0.94)	22.46 (0.42)	1
M. marsaeus phasiana* & *M. mar. putative hybrid		1.38×10^3 (0.59)	0.91×10^3 (0.60)		
***M. marsaeus rileyi* & *M. mar.* putative hybrid**		1.19×10^3 (0.51)	0.79×10^3 (0.52)		
M. satevis cydon & M. mar. phasiana	0.04	1.83×10^3 (0.78)	1.20×10^3 (0.80)	53.24 (1.00)	1
M. satevis cydon* & *M. mar. rileyi	0.02	2.33×10^3 (1.00)	1.51×10^3 (1.00)	32.04 (0.60)	1
***M. satevis cydon* & *M. mar.* putative hybrid**		1.85×10^3 (0.79)	1.20×10^3 (0.80)		

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Table 4. Compounds identified in extracts of male hair pencils (i.e. androconial scales) of different *Melinaea* taxa (* indicates identification through NIST)

Retention index	Compound identification	*Melinaea* taxa
1202.68	Unknown	*M. menophilus*
2114.63	Phytol*	*M. menophilus*
2438.06	ΔΔC18 acid	all taxa
2454.92	Fatty acid ester	all taxa
2638.32	ΔC21 acid	all taxa
2661.19	C21 acid	all taxa

Supplementary Information & Figures

Table S1. Expected frequency of *M. marsaeus* hybrids based on Hardy-Weinberg equilibrium. Shown are the results for the Pearson's χ^2 test comparing the expected and the observed frequencies of putative hybrids for the entire distribution (i.e. all localities were pooled) and for the contact/hybrid zone specifically

	Exp(*M. mar. phasiana*)	Exp(*M. mar.* hybrid)	Exp(*M. mar. rileyi*)	χ2 (df=1)	*p*
Contact zone	2.34	10.31	11.34	12.07	$p<0.001$
Total distribution	23.09	83.83	76.09	149.74	$p<0.001$

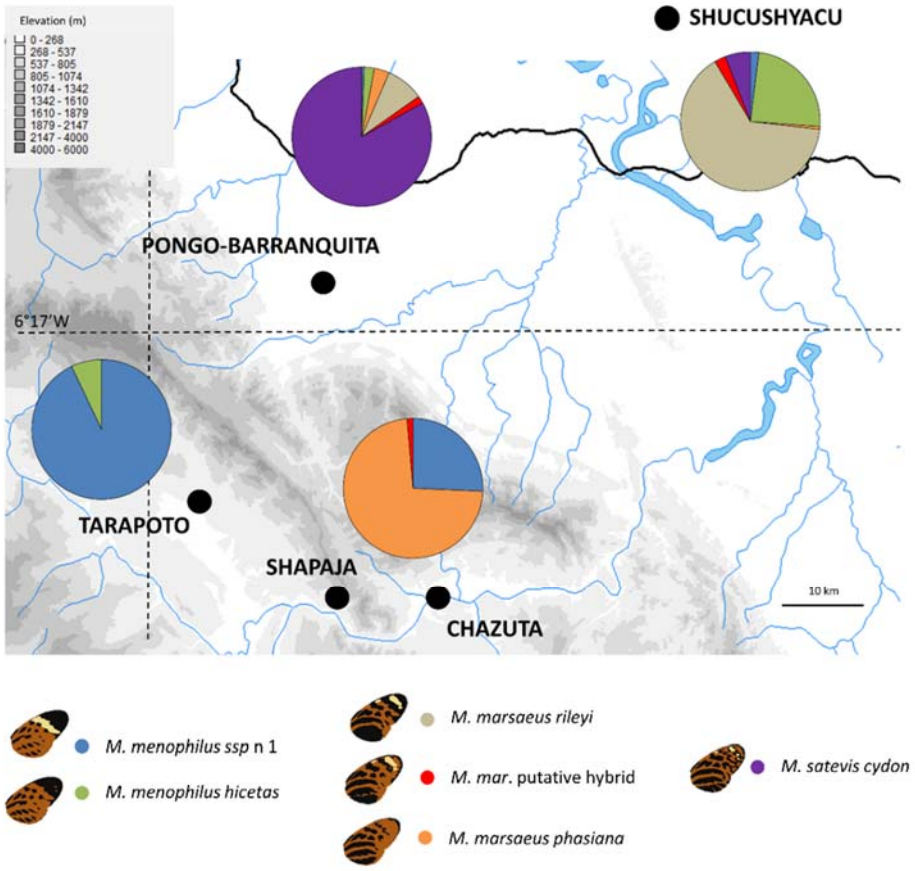
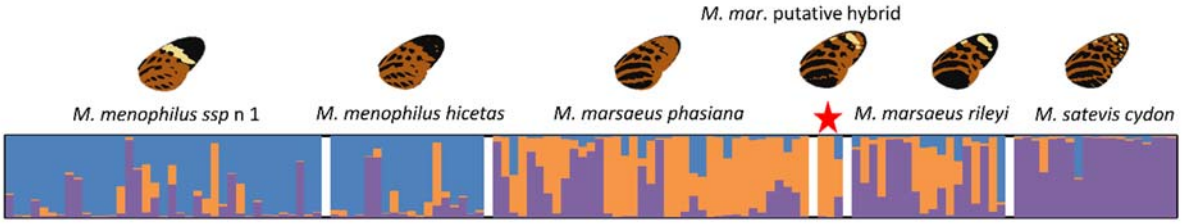


Figure S1: Distribution of five different *Melinaea* taxa and putative hybrids between subspecies of *M. marsaeus* in north-eastern Peru



712

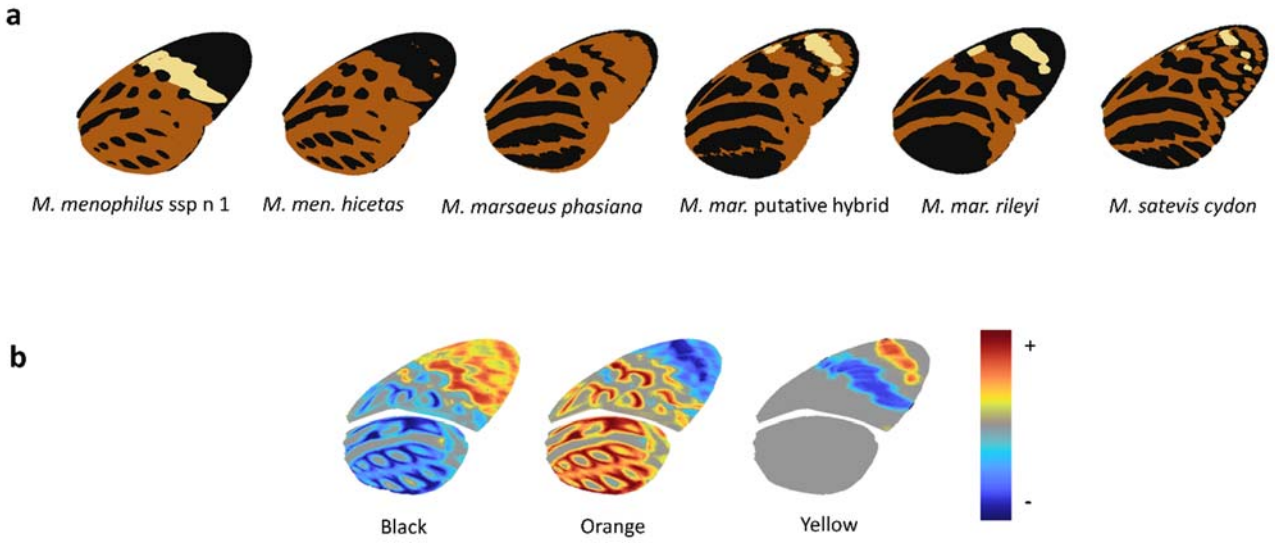
713 **Figure S2:** STRUCTURE plot based on 12 polymorphic microsatellite loci for 5 different

714 *Melinaea* taxa and putative hybrids between subspecies of *M. marsaeus* (indicated with a red star).

715 Bar colours represent posterior possibilities of assignment to inferred genotypic group

716

717



718

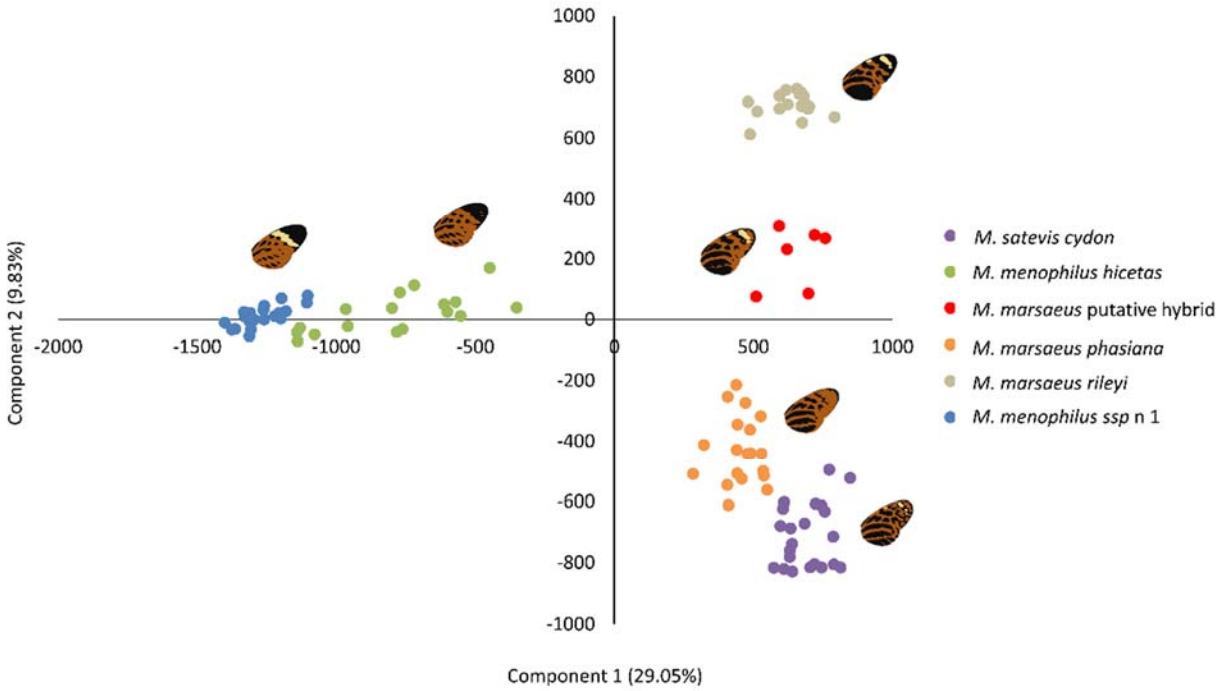
719 **Figure S3: a)** Average wing colour patterns for the five *Melinaea* taxa and putative hybrids

720 between subspecies of *M. marsaeus* and **b)** the heatmaps generated to visualize the degree of

721 variation (from blue to red) across taxa for each of the three colours (black, orange and yellow)

722 across the wing

723



724

725 **Figure S4:** Principal component analysis showing the variation in the colour pattern of five
726 *Melinaea* taxa and putative hybrids between subspecies of *M. marsaeus* as quantified by Colour
727 Pattern Modelling and modelled on VS bird vision

728

729

730

731 **Supplementary Files Available Online**

732 **ESM File.** Vision modelling of four effective photoreceptors of the monarch butterfly, *Danaus*
733 *plexippus*, based on sensitivity peaks reported by Stalleicke et al. (2006) and Blackiston et al.
734 (2011), and of a dark orange filter reported by Blackiston et al. (2011) and extrapolated from the
735 spectrum presented for *Heliconius erato* in McCulloch et al. (2016). Relative proportions of
736 photoreceptors are 1:1:3:3.

737