



**HAL**  
open science

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

Melanie McClure, Louisa Mahrouche, Céline Houssin, Monica Monllor, Yann Le Poul, Brigitte B. Frerot, Alexandra Furtos, Marianne Elias

### ► To cite this version:

Melanie McClure, Louisa Mahrouche, Céline Houssin, Monica Monllor, Yann Le Poul, et al.. Does divergent selection predict the evolution of mate preference and reproductive isolation in the tropical butterfly genus *Melinaea* (Nymphalidae: Ithomiini)?. *Journal of Animal Ecology*, 2019, 88 (6), pp.940-952. 10.1111/1365-2656.12975 . mnhn-02165459

**HAL Id: mnhn-02165459**

**<https://hal-mnhn.archives-ouvertes.fr/mnhn-02165459>**

Submitted on 28 Jun 2019

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

3

4 McClure, M.\*<sup>1</sup>, Mahrouche, L.<sup>2</sup>, Houssin, C.<sup>1</sup>, Monllor, M.<sup>1</sup>, Le Poul, Y.<sup>3</sup>, Frérot, B.<sup>4</sup>, Furtos, A.<sup>2</sup>,  
5 Elias, M.<sup>1</sup>

6

7 <sup>1</sup>Institut Systématique Évolution Biodiversité (ISYEB), Centre National de la Recherche  
8 Scientifique, MNHN, Sorbonne Université, EPHE, Paris, France

9 <sup>2</sup>Centre régional de spectrométrie de masse, Département de chimie, Université de Montréal,  
10 Montréal, Canada

11 <sup>3</sup>Faculty of Biology, LMU Munich, Planegg-Martinsried, Germany

12 <sup>4</sup>Institut d'Écologie et des sciences de l'Environnement, IEES - INRA UMR 1392, Versailles  
13 cedex, France

14 \*Corresponding author. E-mail: [mel\\_mcclure@hotmail.com](mailto:mel_mcclure@hotmail.com)

15

16 **Abstract**

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

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

45

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

48

## 49 **Introduction**

50 A key aspect of evolutionary biology is determining the factors that promote population  
51 diversification and the processes that initiate progress towards speciation. Divergence in both  
52 mating preference and cues are expected to reduce mating between populations and increase  
53 reproductive isolation (Boughman 2001), and many studies have highlighted the importance of  
54 traits that are under divergent ecological selection that also contribute to assortative mating (e.g.  
55 Jiggins *et al.* 2001; Servedio *et al.* 2011; Maan & Seehausen 2012; Jiang, Bolnick & Kirkpatrick  
56 2013). Because the trait subject to divergent selection can directly lead to assortative mating, gene  
57 flow is reduced, and these "magic traits" can be the first step in speciation (Servedio *et al.* 2011).  
58 However, assortative mating requires the evolution of both divergent cues and preferences, and  
59 divergence in one of these alone will not automatically lead to reproductive isolation (Maan &  
60 Seehausen 2012).

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

69 often consist of multiple subspecies diverging for adaptive traits such as wing colour pattern, which  
70 can then cause reproductive isolation through sexual and natural selection against phenotypic  
71 intermediates (Jiggins *et al.* 2001; Naisbit, Jiggins & Mallet 2001; Merrill *et al.* 2012; Arias *et al.*  
72 2016).

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

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

92 distinct mimetic communities, including with the polymorphic *Heliconius numata*, whose different  
93 morphs are co-mimics to different *Melinaea* taxa (Brown & Benson 1974; Beccaloni 1997). As a  
94 result, distribution is often parapatric, with a different dominant taxon in each locality, and a  
95 transition or contact zone where different taxa co-occur. Colour patterns are used in mate  
96 recognition in a range of mimetic organisms (Jiggins *et al.* 2001; Jiggins *et al.* 2006; Merrill *et al.*  
97 2012), and this may also be the case in the genus *Melinaea*, although this has never before been  
98 investigated in Ithomiini. However, Jiggins *et al.* (2006) have demonstrated a phylogenetic pattern  
99 of speciation that is correlated with changes in wing colour pattern in the genus *Ithomia*  
100 (Ithomiini), which strongly suggests that this may be the case. In addition to colour pattern,  
101 pheromones may also play an important role in mate recognition and reproductive isolation.  
102 Indeed, Ithomiini male butterflies collect pyrrolizidine alkaloids (PA) which are thought to provide  
103 toxicity and pheromone precursors (see Schulz *et al.* 2004 and references therein). Furthermore,  
104 as in other ithomiines, male butterflies have hairpencils on their posterior wings that are modified  
105 androconial scales used to diffuse these compounds (see e.g. Edgar, Culvenor & Pliske 1975).  
106 Premating isolation is expected to be especially strong since females appear to mate only once (i.e.  
107 are monandrous; McClure & Elias 2017). Indeed, mistakes or mating with subpar males likely  
108 impose a high cost to females, and they are therefore expected to be choosy.

109         This paper focuses on five *Melinaea* taxa thought to form a speciation continuum, from  
110 subspecies to fully recognized species (Lamas 2004): *M. menophilus* ssp. nov. 1 and *M. men.*  
111 *hicetas*, *M. marsaeus phasiana* and *M. mar. rileyi*, and finally *M. satevis cydon*. Previous studies  
112 have shown that these taxa utilize the same hostplant, *Juanulloa parasitica* (McClure & Elias  
113 2016; McClure & Elias 2017). As such, McClure & Elias (2016) suggested that diversification in  
114 these taxa was likely driven by shifts in colour pattern linked to co-occurring Müllerian mimics

115 and the resulting predation pressure rather than hostplant shifts. Using artificial models of the  
116 polymorphic and Müllerian co-mimic *Heliconius numata*, Chouteau et al. (2016) and Arias et al.  
117 (2016) have shown that migrants and intermediate phenotypes respectively, possess locally  
118 unrecognized warning signals and suffer greater predator attack frequencies. As the Müllerian co-  
119 mimics *Heliconius numata* and *Melinaea* are undistinguishable to predators (Llaurens, Joron &  
120 Théry 2014), the results of these studies can be extrapolated to the genus *Melinaea*.

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

135

## 136 **Material and Methods**

137 **Butterfly sampling.** Butterflies were collected in north-eastern Peru from 2011 to 2016.  
138 Collection localities consisted of premontane forest habitats near Tarapoto (Rio Shilcayo basin:  
139 6°27'30''S, 76°21'00''W), Shapaja (6°36'56''S, 76°09'61''W) and Chazuta (6°57'05''S,  
140 76°13'75''W), and lowland forest on Pongo-Baranquita road (6°17'53''S, 76°14'38''W) and  
141 Shucushyacu (5°57'20''S, 75°53'06''W). Various sites a few kilometres apart were sampled  
142 within each locality. The number of individuals of each taxon used to measure genetic  
143 differentiation, pheromone characterization and colour pattern quantification are found in Table 1.

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

150

151 **Rearing conditions.** Gravid wild caught females were kept in 2x2x2 m outdoor insectaries under  
152 ambient conditions in Tarapoto, San Martín, where all rearing was carried out (see McClure &  
153 Elias 2016 for further information). Butterflies were provided with nourishment in the form of  
154 sugar water solution and bee pollen. All species in this study use *J. parasitica* as a host plant  
155 (McClure & Elias 2016; McClure & Elias 2017), and as such, potted *J. parasitica* plants were used  
156 for oviposition, and larvae collected in the cages were reared individually in transparent plastic  
157 containers in the shade behind a nearby building under ambient conditions. Larvae were checked  
158 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*:  $\chi^2=1.2$ ,  $df=1$ ,  $p=0.273$ ; *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

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

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

$$191 L(P_{ij}) = m_{ij} \log_e(P_{ij}) + (N_{ij} - m_{ij}) \log_e(1 - P_{ij})$$

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

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

$$201 1 - \frac{\text{frequency of heterospecific mating}}{\text{frequency of conspecific mating}}$$

202 This index ranges from  $-\infty$  (complete disassortative mating) through 0 (no mating isolation) to 1  
203 (complete mating isolation).

204

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

218  
219 **Differentiation of colour pattern.** Differentiation in colour pattern was quantified using Colour  
220 Pattern Modelling (CPM) described by Le Poul et al. (2014). Photographs, taken under  
221 standardized conditions (see Le Poul *et al.* 2014 for details), of the dorsal and ventral sides of  
222 forewings and hindwings of each specimen were used. The CPM automatically detects and  
223 eliminates the background in the pictures and each pixel of the butterfly wing image is  
224 automatically attributed a colour, which is then categorized into one of three major colours present  
225 in the wing patterns (that is, black, orange or yellow). Hind and forewings are aligned separately  
226 using a rigid transformation, and similarity (accounting both for wing shape and pattern) with a  
227 wing model that averages all wing images is maximized recursively. After alignment, the position

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

230 In order to link divergence in colour pattern to reproductive isolation (specifically,  
231 prezygotic isolation driven by mate choice, and postzygotic isolation driven by predation), the  
232 value of each pixel was re-calculated by incorporating models of animal vision (see Arias *et al.*  
233 2016 for details) and by using the precise colour spectrum of each colour (see Llaurens, Joron &  
234 Théry 2014). Models of animal vision, based on the sensitivities of photoreceptors present in their  
235 eyes, allow inferences about the colour contrasts and the conspicuousness of the different colours  
236 that can be perceived by different animals. As such, two avian vision systems that vary in their  
237 sensitivity to ultra-violets (i.e. wavelengths below 400nm) and one butterfly vision system were  
238 performed. All vision systems consist of four photoreceptors. Specifically, the quantum catches,  
239 which is the relative amount of light captured by each photoreceptor when observing a given colour  
240 (Irié & Lagorio 2010), was applied using the method described in Vorobyev & Osorio (1998) and  
241 assuming a Weber fraction of 0.05 for all vision systems. A light environment corresponding to  
242 both small and large gaps in a forest canopy (computed as in Llaurens, Joron & Théry 2014) was  
243 used. As the photoreceptor sensitivities of the birds composing the predator community in this  
244 study system are not known, the calculations were based on the two main vision systems found in  
245 birds. The blue tit (*Parus caeruleus*) was used for vision with ultra-violet sensitive pigments  
246 (UVS), with cone proportion and sensitivity as described by Hart *et al.* (2000), and the shearwater  
247 (*Puffinus pacificus*) was used for vision with violet sensitive pigments (VS), as described by Hart  
248 (2004). To model butterfly vision, the photoreceptors sensitivity was computed using the visual  
249 sensitivity peaks reported for *Danaus plexippus* (the monarch, the closest relative of *Melinaea* for  
250 which sensitivity peaks are known) in Stalleicken, Labhart & Mouritsen (2006) and Blackiston,

251 Briscoe & Weiss (2011), applying Stavenga (2010)'s equations. Since Blackiston, Briscoe &  
252 Weiss (2011) reported the existence of a dark orange filter in the long wavelength receptor, which  
253 effectively gives rise to a fourth type of receptor, we also modelled this filtered photoreceptor by  
254 digitizing the spectrum reported in McCulloch, Osorio & Briscoe (2016) for *Heliconius erato* (the  
255 closest butterfly species – also a Nymphalidae – for which the spectrum has been characterized,  
256 and whose long wavelength photoreceptor peaks at the same value as *D. plexippus*) using the  
257 software Graph Grabber 2.0 ([www.quintessa.org/](http://www.quintessa.org/)). The vision model for *D. plexippus* is available  
258 in ESM. The precise colour spectrum of each colour detected by CPM (black, orange and yellow)  
259 were those taken on *Melinaea* by Llaurens, Joron & Théry (2014). Quantum catches for each  
260 photoreceptor in birds and butterflies were estimated using AVICOL (Gomez 2006), under large  
261 light gap and small light gap light conditions. The phenotypic variation (variation among all pixels  
262 common between all wings) after accounting for animal vision was summarized using a principle  
263 component analysis (PCA). Differences between groups were tested using ADONIS  
264 (PERMANOVA) in the R package Vegan (Oksanen *et al.* 2016), followed by a pairwise  
265 comparison (i.e. post hoc test) with Bonferroni correction (pairwiseAdonis: Martinez Arbizu  
266 2017). Finally, differentiation between taxa was measured as Euclidean distances between  
267 centroids in PCA space.

268

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

274 splitless injection, 2  $\mu$ L of each extract was injected on an Agilent HP-5MS column (30 m x 250  
275  $\mu$ m x 0.25  $\mu$ m) with the inlet maintained at 250°C. Helium was used as a carrier gas and flow rate  
276 was of 1.5411 mL/min. Temperature gradient was programmed from 50°C to 300°C at a rate of  
277 8°C/min for a total run time of 39min. Kovats' retention indices (RIs) were computed using n-  
278 alkanes from C8 to C20 that were eluted under the same conditions as the samples (external  
279 standards). Compounds were identified by comparison of mass spectra and gas chromatographic  
280 retention indices to those in the literature and the NIST library. A data matrix of all the compounds  
281 for each individual was aligned using GCAAligner 1.0 (Dellicour & Lecocq 2013). Relative  
282 concentrations were determined by peak area analysis and differences between groups were  
283 visualized using nonmetric multidimensional scaling (NMDS) ordination based on Bray-Curtis  
284 similarity matrix, using the function metaMDS in the R package Vegan (Oksanen *et al.* 2016).  
285 Differences between groups were tested using ADONIS (PERMANOVA) in the R package Vegan,  
286 followed by a pairwise comparison (i.e. Post hoc test) with Bonferroni correction (pairwiseAdonis:  
287 Martinez Arbizu 2017). If differences within species (i.e. between subspecies) were found to be  
288 significant, this was followed with a non-parametric Mann-Whitney U test so as to test for  
289 differences in the amount of each compound. Finally, differentiation between taxa was measured  
290 as Euclidean distances between centroids.

291

## 292 **Results**

293 **Distribution.** Partial geographical isolation is observed between the subspecies of both *M.*  
294 *marsaeus* and *M. menophilus*, with uneven abundance at the different localities. Distribution and  
295 relative frequency of the five different taxa in each region is shown in Fig. S1. The general pattern  
296 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

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

321

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

328

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

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

337 Differentiation in colour pattern was significant for all taxa and putative hybrids, both  
338 under butterfly (PERMANOVA ADONIS:  $F = 79.39$ ;  $df = 5$ ;  $p = 0.001$ ; Fig. 2a) and avian  
339 (PERMANOVA ADONIS:  $F = 73.30$ ;  $df = 5$ ;  $p = 0.001$ ; Fig. 2b) vision. A pairwise post hoc test  
340 with Bonferroni correction shows all groups as being significantly different from each other ( $p =$   
341 0.001). However, differentiation of the two subspecies of *M. menophilus*, which differ for a single



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

344  
345 **Chemical analysis.** A total of six compounds (Table 4) were identified, four of which were  
346 common to all taxa, albeit at different ratios (Fig. 3), and two were unique to *M. menophilus*. A  
347 comparison of the different chemical extracts was found to be significantly different  
348 (PERMANOVA ADONIS: F=27.60; df=4;  $p=0.001$ ) and the NMDS ordinal plot shows the three  
349 species as being completely separate, but the subspecies as clustering together (Fig. 4). A pairwise  
350 post hoc test with Bonferroni correction confirmed that the closely related species *M. marsaeus*  
351 and *M. satevis cydon* are significantly different from each other ( $p = 0.015$ ). The subspecies of *M.*  
352 *menophilus* ( $p = 1.0$ ) were not found to be significantly different, but the subspecies of *M.*  
353 *marsaeus* were ( $p = 0.02$ ). This difference appears to be the result of a difference in the ratio  
354 between the  $\Delta$ C21 acid ( $U = 7$ ;  $p = 0.002$ ) and the C21 acid ( $U = 4$ ;  $p < 0.01$ ; Fig. 3). However,  
355 most individuals of both subspecies, in addition to the potential hybrid, possess the same  
356 intermediate ratio of the two compounds (Figs 3 & 4). Euclidean distances between centroids of  
357 pairs of taxa are presented in Table 3 and show increasing levels of differentiation with increasing  
358 reproductive isolation.

359

## 360 **Discussion**

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

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

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

386         Our results show strong premating isolation between the closely related species *M. satevis*  
387 *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  $\Delta$ C21 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

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

447         In this study we show that the absence of ecological adaptations other than colour pattern  
448 (see McClure & Elias 2016) does not preclude the evolution of mating isolation. In fact, through  
449 the maintenance of a spatial mosaic of mimetic colour patterns, predation on Müllerian mimics  
450 constrains geographical distribution and allows for different species or subspecies, even those with  
451 similar ecological niches, to exist in different regions (Aubier, Joron & Sherratt 2017). This study  
452 also suggests that mate preference and assortative mating evolve adaptively in response to  
453 divergent selection, and that even in the early stages of speciation, reproductive isolation can be  
454 nearly complete due to mating preferences, as seen in *M. marsaeus*. But perhaps surprisingly, we  
455 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

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

482

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

485

486 **Funding.** This research was funded by a CNRS ATIP grant and ANR grant (SPECREP) awarded  
487 to ME and by the Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT)  
488 as a PDF award to MMc.

489

490 **Acknowledgements.** We thank the Peruvian authorities and Dr Gerardo Lamas (Museo de  
491 Historia Natural, Universidad Mayor de San Marcos) for research permits (236-2012-AG-DGFFS-  
492 DGEFFS, 201-2013-MINA-GRI-DGFFS/DGEFFS and 002-2015-SERFOR-DGGSPFFS). We  
493 also thank Mario Tuanama, Ronald Mori-Pezo and Javier Bacigalupo for their precious help in the  
494 field. Molecular work was carried out at the Service de Systématique Moléculaire du Muséum  
495 National d'Histoire Naturelle (CNRS-UMR 2700). We thank Violaine Llaurens, Doris Gomez and  
496 Monica Arias for providing spectra for *Melinaea* taxa, ambient light files and quantum catches for  
497 birds to perform the vision modelling. We thank Doris Gomez and Adriana Briscoe for advice on  
498 butterfly vision modelling.

499 **References**

- 500 Arias, M., le Poul, Y., Chouteau, M., Boisseau, R., Rosser, N., Théry, M. & Llaurens, V. (2016).  
501 Crossing fitness valleys: empirical estimation of a fitness landscape associated with  
502 polymorphic mimicry. *Proceedings of the Royal Society of London, Series B: Biological*  
503 *Sciences*, **283**, DOI: 10.1098/rspb.2016.0391.
- 504 Aubier, T., Joron, M. & Sherratt, T.N. (2017). Mimicry among unequally defended prey should be  
505 mutualistic when predators sample optimally. *American Naturalist*, **189**, 267-282.
- 506 Bates, H. (1862). Contributions to an insect fauna of the Amazon valley: Lepidoptera: Heliconidae.  
507 *Transactions of the Linnean Society of London*, **25**, 495-566.
- 508 Beccaloni, G. (1997). Ecology, natural history and behaviour of ithomiine butterflies and their  
509 mimics in Ecuador (Lepidoptera: Nymphalidae: Ithomiinae). *Tropical Lepidoptera*, **8**, 103-  
510 124.
- 511 Belkhir, K., Borsa, P., Chikhi, L., Raufaste, N. & Bonhomme, F. (1996). GENETIX 4.0.4, Logiciel  
512 sous Windows TM pour la génétique des populations. *Laboratoire Génome, Populations,*  
513 *Interactions, CNRS UMR 5000, Université de Montpellier II, Montpellier, France,*  
514 (<http://www.univ-montp2.fr/~genetix/genetix/constr.htm#download>).
- 515 Blackiston, D., Briscoe, A. & Weiss, M. (2011). Color vision and learning in the monarch butterfly,  
516 *Danaus plexippus* (Nymphalidae). *The Journal of Experimental Biology*, **214**, 509-520.
- 517 Boughman, J. (2001). Divergent sexual selection enhances reproductive isolation in sticklebacks.  
518 *Nature*, **411**, 944-948.
- 519 Brown, K.J. & Benson, W. (1974). Adaptive polymorphism associated with multiple Müllerian  
520 mimicry in *Heliconius numata* (Lepid. Nymph.). *Biotropica*, **6**, 205-228.



521 Chazot, N., Willmott, K., Lamas, G., Freitas, A., Piron-Prunier, F., Arias, C., Mallet, J., De-Silva,  
522 D. & Elias, M. (2017). Renewed diversification following Miocene landscape turnover in  
523 a Neotropical butterfly radiation. *BioRxiv* <https://doi.org/10.1101/148189>, Reviewed &  
524 recommended by PCI Evolutionary Biology  
525 (<http://dx.doi.org/10.24072/pci.evolbiol.100032>).

526 Chouteau, M., Arias, M. & Joron, M. (2016). Warning signals are under positive frequency-  
527 dependent selection in nature. *Proceedings of the National Academy of Sciences of the*  
528 *United States of America*, **113**, 2164-2169.

529 Chouteau, M., Llaurens, V., Piron-Prunier, F. & Joron, M. (2017). Polymorphism at a mimicry  
530 supergene maintained by opposing frequency-dependent selection pressures. *Proceedings*  
531 *of the National Academy of Sciences of the United States of America*, **114**, 8325-8329.

532 Coyne, J. & Orr, H. (1989). Patterns of speciation in *Drosophila*. *Evolution*, **43**, 362-381.

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

536 Dellicour, S. & Lecocq, T. (2013). GCALIGNER 1.0: an alignment program to compute a multiple  
537 sample comparison data matrix from large eco-chemical datasets obtained by GC. *Journal*  
538 *of Separation Science*, **36**, 3206-3209.

539 Earl, D. & vonHoldt, B. (2012). STRUCTURE HARVESTER: a website and program for  
540 visualizing STRUCTURE output and implementing the Evanno method. *Conservation*  
541 *Genetics Resources*, **4**, 359-361.

542 Edgar, J., Culvenor, C. & Pliske, T. (1975). Isolation of a lactone, structurally related to the  
543 esterifying acids of pyrrolizidine alkaloids, from the costal fringes of male ithomiinae.  
544 *Journal of Chemical Ecology*, **2**, 263-270.

545 Edwards, A. (1972). Likelihood. Cambridge, Cambridge University Press.

546 Elias, M., Hill, R.I., Willmott, K.R., Dasmahapatra, K.K., Brower, A.V.Z., Mallet, J. & Jiggins,  
547 C.D. (2007). Limited performance of DNA barcoding in a diverse community of tropical  
548 butterflies. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **274**,  
549 2881-2889.

550 Evanno, G., Regnaut, S. & Goudet, J. (2005). Detecting the number of clusters of individuals using  
551 the software STRUCTURE: a simulation study. *Molecular Ecology*, **14**, 2611-2620.

552 Hart, N. (2004). Microspectrophotometry of visual pigments and oil droplets in a marine bird, the  
553 wedge-tailed shearwater *Puffinus pacificus*: topographic variations in photoreceptor  
554 spectral characteristics. *Journal of Experimental Biology*, **207**, 1229-1240.

555 Hart, N., Partridge, J., Cuthill, I. & Bennett, A. (2000). Visual pigments, oil droplets, ocular media  
556 and cone photoreceptor distribution in two species of passerine bird: the blue tit (*Parus*  
557 *caeruleus* L.) and the blackbird (*Turdus merula* L.). *Journal of Comparative Physiology*,  
558 *A*, **186**, 375-387.

559 Iriel, A. & Lagorio, M. (2010). Implications of reflectance and fluorescence of *Rhododendron*  
560 *indicum* flowers in biosignaling. *Photochemical and Photobiological Sciences*, **9**, 342-348.

561 Jiang, Y., Bolnick, D. & Kirkpatrick, M. (2013). Assortative mating in animals. *American*  
562 *Naturalist*, **181**, E125-E138.

563 Jiggins, C., Mallarino, R., Willmott, K. & Bermingham, E. (2006). The phylogenetic pattern of  
564 speciation and wing pattern change in Neotropical *Ithomia* butterflies (Lepidoptera:  
565 Nymphalidae). *Evolution*, **60**, 1454-1466.

566 Jiggins, C., Naisbit, R., Coe, R. & Mallet, J. (2001). Reproductive isolation caused by colour  
567 pattern mimicry. *Nature*, **411**, 302-305.

568 Kopp, M., Servedio, M., Mendelson, T., Safran, R., Rodríguez, M., Hauber, M., Scordato, E.,  
569 Symes, L., Balakrishnan, C., Zonana, D. & Sander Van Doorn, G. (2018). Mechanisms of  
570 assortative mating in speciation with gene flow: connecting theory and empirical research.  
571 *American Naturalist*, **191**, 1-20.

572 Lamas, G. (2004). Atlas of Neotropical Lepidoptera. Checklist: Part 4A. Hesperioidea-  
573 Papilionoidea. Gainesville, Scientific Publishers.

574 Le Poul, Y., Whibley, A., Chouteau, M., Prunier, F., Llaurens, V. & Joron, M. (2014). Evolution  
575 of dominance mechanisms at a butterfly mimicry supergene. *Nature communications*, **5**,  
576 DOI: 10.1038/ncomms6644.

577 Llaurens, V., Joron, M. & Théry, M. (2014). Cryptic differences in colour among Müllerian  
578 mimics: how can the visual capacities of predators and prey shape the evolution of wing  
579 colours? *Evolutionary Biology*, **27**, 531-540.

580 Maan, M. & Seehausen, O. (2012). Magic cues versus magic preferences in speciation.  
581 *Evolutionary Ecology Research*, **14**, 779-785.

582 Mallet, J. & Barton, N. (1989). Strong natural selection in a warning-color hybrid zone. *Evolution*,  
583 **43**, 421-431.

584 Martinez Arbizu, P. (2017). pairwiseAdonis: Pairwise multilevel comparison using adonis. *R*  
585 *package version 0.0.1*.

586 McClure, M., Chouteau, M., Bernard, A. & Elias, M. (2014). The development and  
587 characterization of polymorphic microsatellite loci for the genus *Melinaea* (Nymphalidae,  
588 Ithomiini). *Conservation Genetics Resources*, **6**(4), 891-893.

589 McClure, M., Dutrillaux, B., Dutrillaux, A.-M., Lukhtanov, V. & Elias, M. (2018). Heterozygosity  
590 and chain multivalents during meiosis illustrate ongoing evolution as a result of multiple  
591 holokinetic chromosome fusions in the genus *Melinaea* (Lepidoptera, Nymphalidae).  
592 *Cytogenetic and Genome Research*, **153**, 213-222.

593 McClure, M., Mahrouche, L., Houssin, C., Monllor, M., Le Poul, Y., Frérot, B., Furtos, A. & Elias,  
594 M. (2019). Data from: Does divergent selection predict the evolution of mate preference  
595 and reproductive isolation in the tropical butterfly genus *Melinaea* (Nymphalidae:  
596 Ithomiini)? Dryad Digital Repository. <https://doi.org/10.5061/dryad.008b59c>.

597 McClure, M. & Elias, M. (2016). Unravelling the role of host plant expansion in the diversification  
598 of a Neotropical butterfly genus. *BMC Evolutionary Biology*, **16**, 128-134.

599 McClure, M. & Elias, M. (2017). Ecology, life history, and genetic differentiation in Neotropical  
600 *Melinaea* (Nymphalidae: Ithomiini) butterflies from north-eastern Peru. *Zoological*  
601 *Journal of the Linnean Society*, **179**, 110-124.

602 McCulloch, K., Osorio, D. & Briscoe, A. (2016). Sexual dimorphism in the compound eye of  
603 *Heliconius erato*: a nymphalid butterfly with at least five spectral classes of photoreceptor.  
604 *Journal of Experimental Biology*, **219**, 2377-2387.

605 McMillan, W., Jiggins, C. & Mallet, J. (1997). What initiates speciation in passion-vine  
606 butterflies? *Proceedings of the National Academy of Sciences of the United States of*  
607 *America*, **94**, 8628-8633.

608 Merrill, R., Wallbank, R., Bull, V., Salazar, P., Mallet, J., Stevens, M. & Jiggins, C. (2012).  
609 Disruptive ecological selection on a mating cue. *Proceedings of the Royal Society of*  
610 *London B*, **279**, 4907-4913.

611 Müller, F. (1897). *Ituna* and *Thyridia*; a remarkable case of mimicry in butterflies. *Transactions*  
612 *of the Entomological Society of London*.

613 Naisbit, R.E., Jiggins, C.D. & Mallet, J. (2001). Disruptive sexual selection against hybrids  
614 contributes to speciation between *Heliconius cydno* and *H. melpomene*. *Proceedings of the*  
615 *Royal Society of London. Series B: Biological Sciences*, **268**, 1849-1854.

616 Oksanen, J., Blanchet, F., Kindt, R., Legendre, R., McGlinn, D., Minchin, P., O'Hara, R., Simpson,  
617 G., Solymos, P., Stevens, M., Szoecs, E. & Wagner, H. (2016). Vegan: Community  
618 ecology package. *R package version 2.4-1*.

619 Pritchard, J., Stevens, M. & Donnelly, P. (2000). Inference of population structure using multilocus  
620 genotype data. *Genetics*, **155**, 945-959.

621 Puebla, O., Bermingham, E., Guichard, F. & Whiteman, E. (2007). Colour pattern as a single trait  
622 driving speciation in *Hypoplectrus* coral reef fishes? *Proceedings of the Royal Society of*  
623 *London, Series B: Biological Sciences*, **274**, 1265-1271.

624 Raymond, M. & Rousset, F. (1995). An exact test for population differentiation. *Evolution*, **49**,  
625 1280-1283.

626 Reynolds, R. & Fitzpatrick, B. (2007). Assortative mating in poison-dart frogs based on an  
627 ecologically important trait. *Evolution*, **61**, 2253-2259.

628 Schulz, S., Beccaloni, G., Brown, K., Boppré, M., Freitas, A., Ockenfels, P. & Trigo, J. (2004).  
629 Semiochemicals derived from pyrrolizidine alkaloids in male ithomiine butterflies

630 (Lepidoptera: Nymphalidae: Ithomiinae). *Biochemical Systematics and Ecology*, **32**, 699-  
631 713.

632 Servedio, M. & Boughman, J.W. (2017). The role of sexual selection in local adaptation and  
633 speciation. *Annual Review of Ecology, Evolution and Systematics*, **48**, 85-109.

634 Servedio, M., Sander Van Doorn, G., Kopp, M., Frame, A. & Nosil, P. (2011). Magic traits in  
635 speciation: 'magic' but not rare? *Trends in Ecology & Evolution*, **26**, 389-397.

636 Stalleicken, J., Labhart, T. & Mouritsen, H. (2006). Physiological characterization of the  
637 compound eye in monarch butterflies with focus on the dorsal rim area. *Journal of*  
638 *Comparative Physiology, A*, **192**, 321-331.

639 Stavenga, D. (2010). On visual pigment templates and the spectral shape of invertebrate  
640 rhodopsins and metarhodopsins. *Journal of Comparative Physiology, A*, **196**, 869-878.

641 Twomey, E., Vestergaard, J., Venegas, P. & Summers, K. (2016). Mimetic divergence and the  
642 speciation continuum in the the mimic poison frog *Ranitomeya imitator*. *American*  
643 *Naturalist*, **187**, 205-224.

644 Vorobyev, M., Osorio, D., Bennett, A., Marshall, N. & Cuthill, I. (1998). Tetrachromacy, oil  
645 droplets and bird plumage colours. *Journal of Comparative Physiology, A*, **183**, 621-633.

646 Whinnett, A., Zimmermann, M., Willmott, K.R., Herrera, N., Mallarino, R., Simpson, F., Joron,  
647 M., Lamas, G. & Mallet, J. (2005). Strikingly variable divergence times inferred across an  
648 Amazonian butterfly 'suture zone'. *Proceedings of the Royal Society of London. Series B:*  
649 *Biological Sciences*, **272**,(2525-2533).

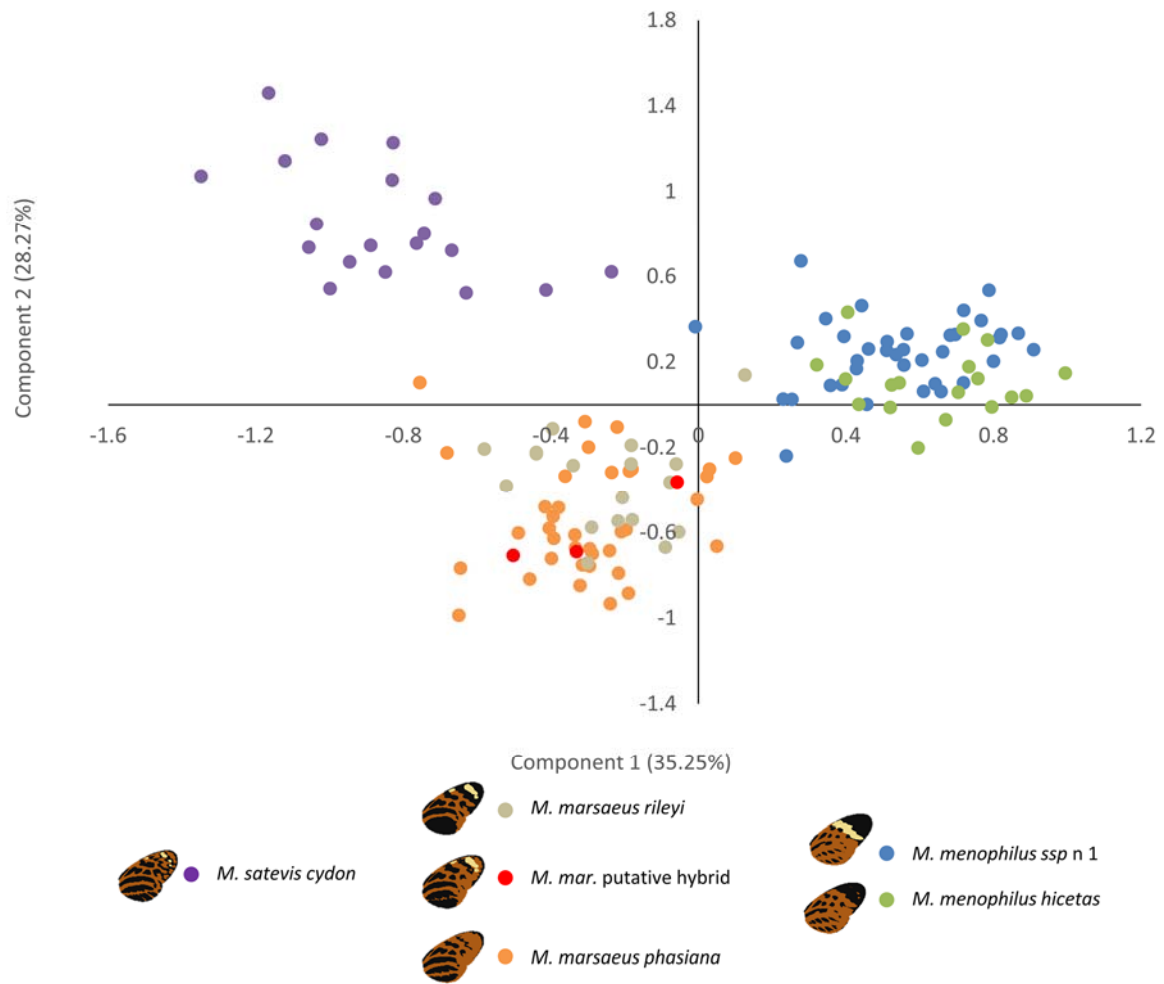
650 Willmott, K.R. & Freitas, A. (2006). Higher-level phylogeny of the Ithomiinae (Lepidoptera:  
651 Nymphalidae): classification, patterns of larval hostplant colonization and diversification.  
652 *Cladistics*, **22**, 297-368.

653 Yukilevich, R. (2012). Asymmetrical patterns of speciation uniquely support reinforcement in  
654 *Drosophila*. *Evolution*, **66**, 1430-1446.

655

656

657 **List of Figures**



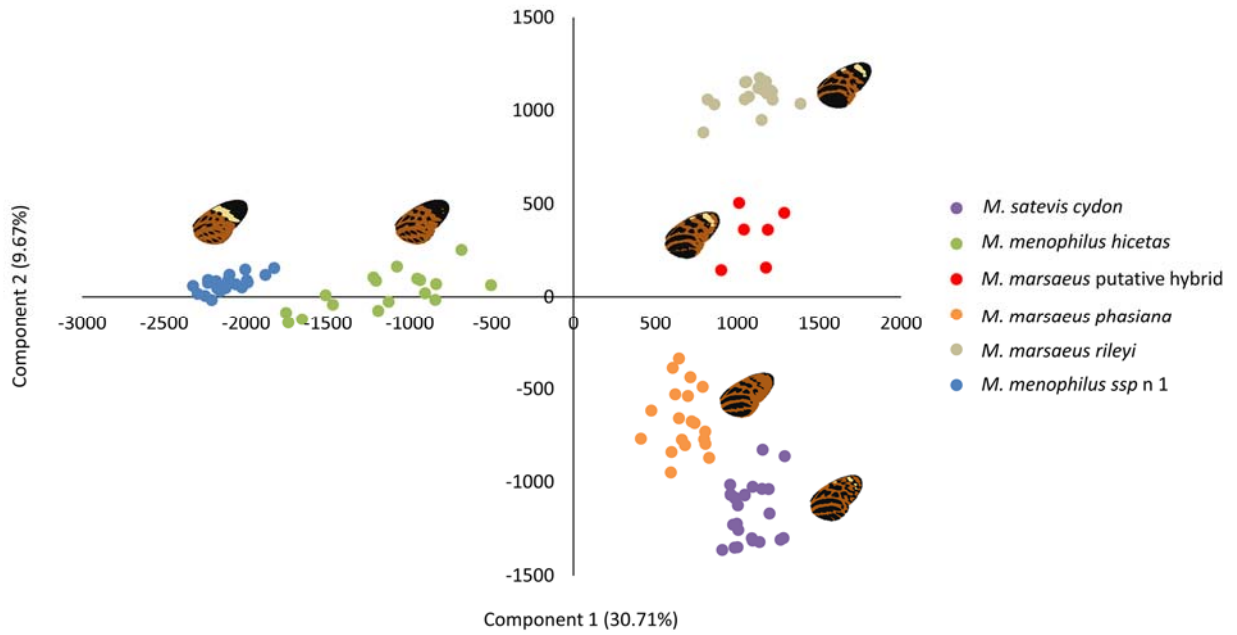
658

659 **Figure 1:** Factorial correspondence analysis for five *Melinaea* taxa and putative hybrids between

660 subspecies of *M. marsaeus* on 12 microsatellite loci computed using the program GENETIX

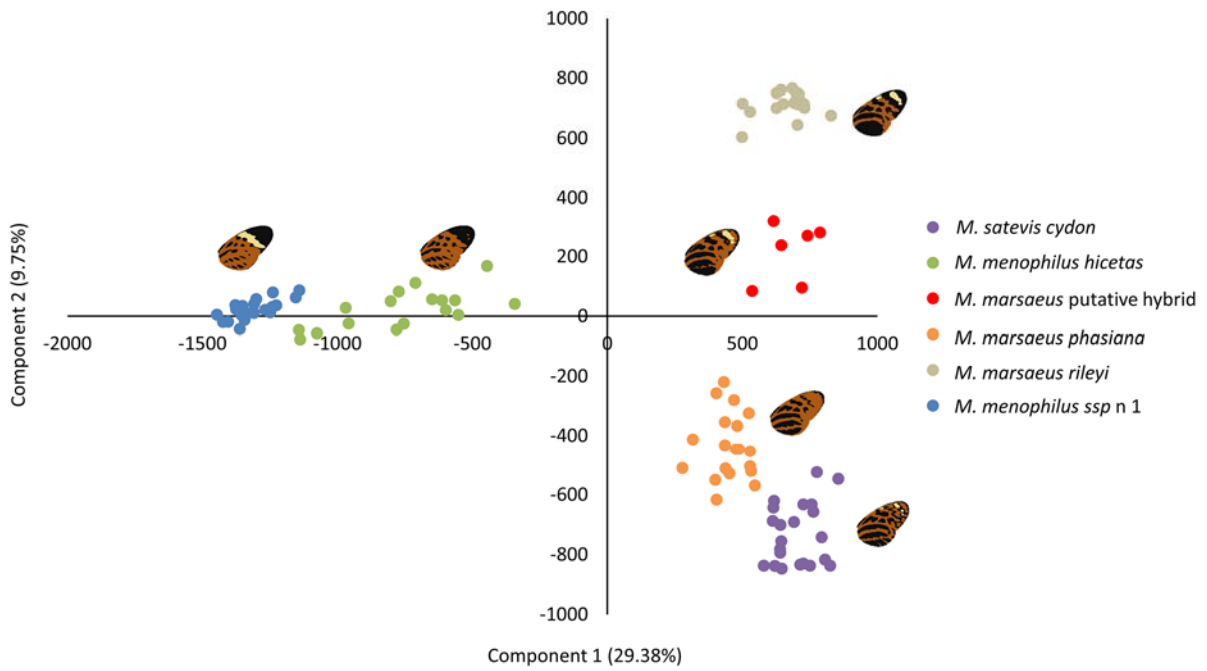


661 **a**



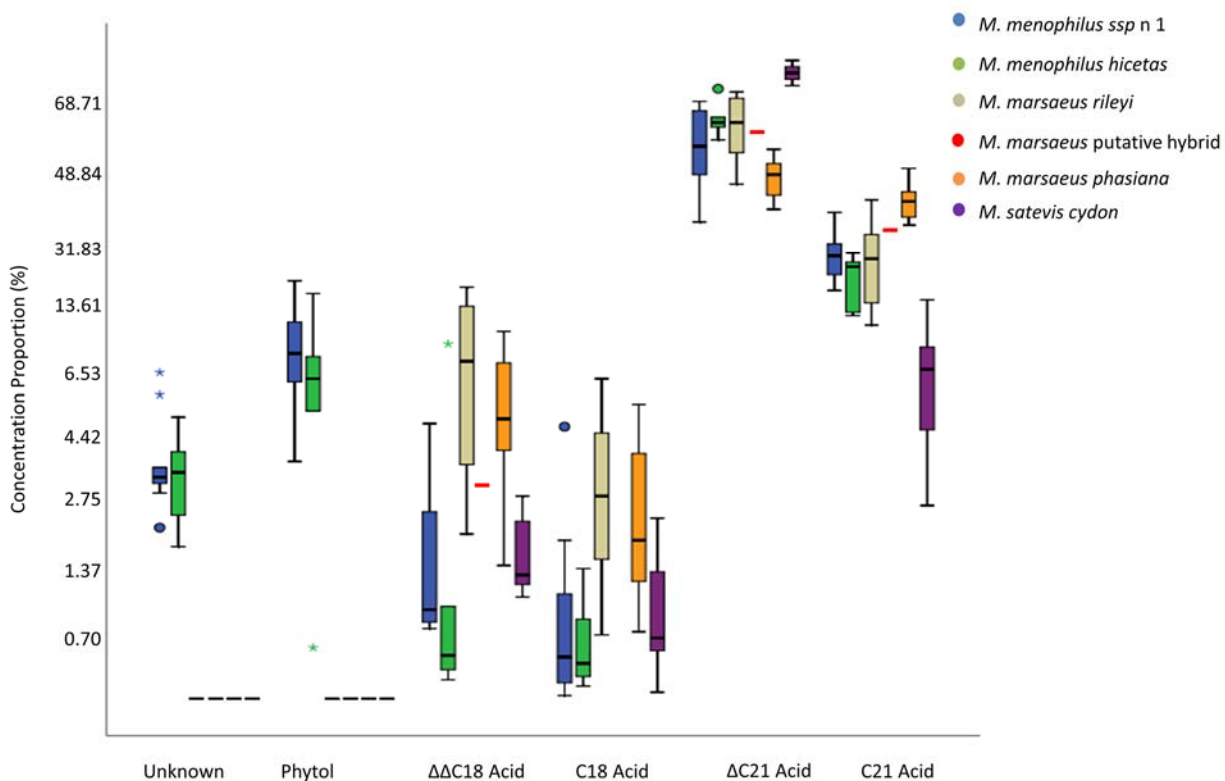
662

663 **b**

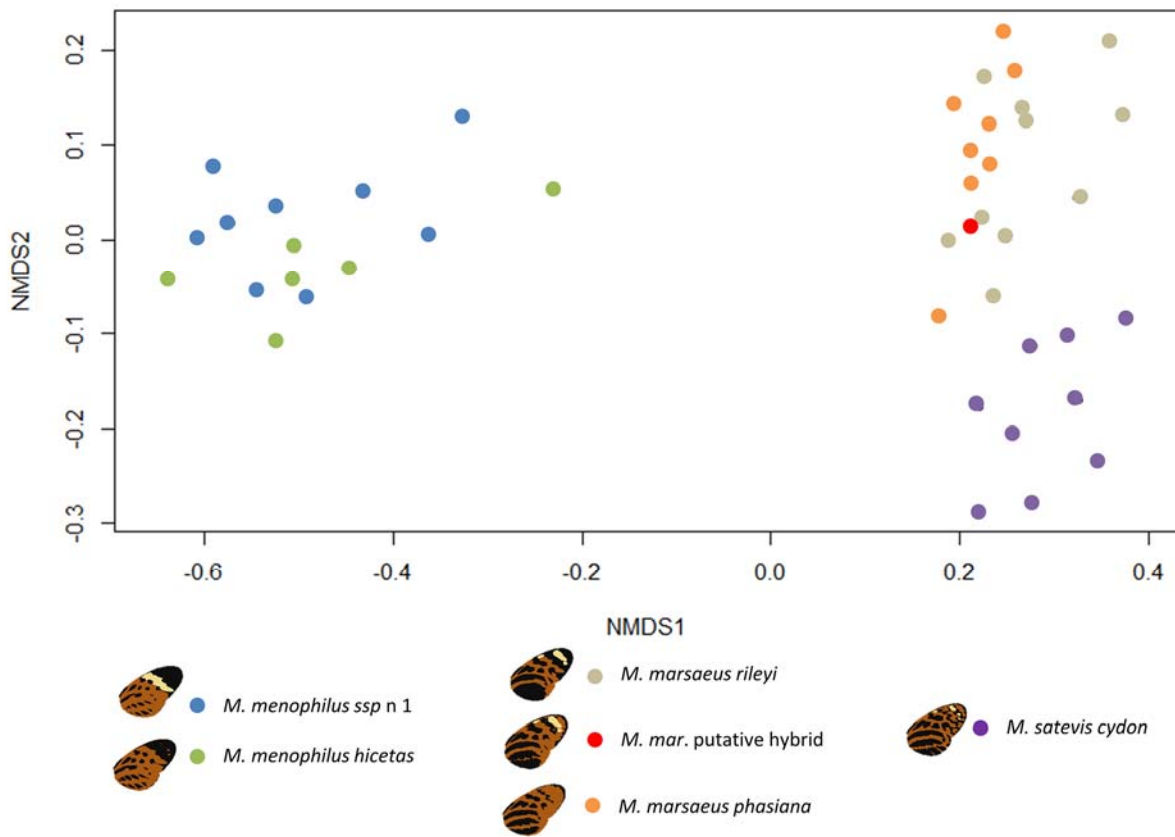


664

665 **Figure 2:** Principal component analysis showing the variation in the colour pattern of five  
 666 *Melinaea* taxa and putative hybrids between subspecies of *M. marsaeus* as quantified by Colour  
 667 Pattern Modelling and modelled on a) butterfly vision and b) UVS bird vision  
 668



669  
 670 **Figure 3:** Proportion of the different compounds present in the chemical profile of five *Melinaea*  
 671 taxa and one putative hybrid between subspecies of *M. marsaeus* obtained by GC-MS  
 672



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*

677

678

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

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

681  
 682  
 683

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

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

688

689

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.

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

695

696

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

699

<b>Retention index</b>	<b>Compound identification</b>	<b><i>Melinaea</i> taxa</b>
1202.68	Unknown	<i>M. menophilus</i>
2114.63	Phytol*	<i>M. menophilus</i>
2438.06	$\Delta\Delta$ C18 acid	all taxa
2454.92	Fatty acid ester	all taxa
2638.32	$\Delta$ C21 acid	all taxa
2661.19	C21 acid	all taxa

700

701

702 **Supplementary Information & Figures**

703 **Table S1.** Expected frequency of *M. marsaeus* hybrids based on Hardy-Weinberg equilibrium.

704 Shown are the results for the Pearson’s  $\chi^2$  test comparing the expected and the observed

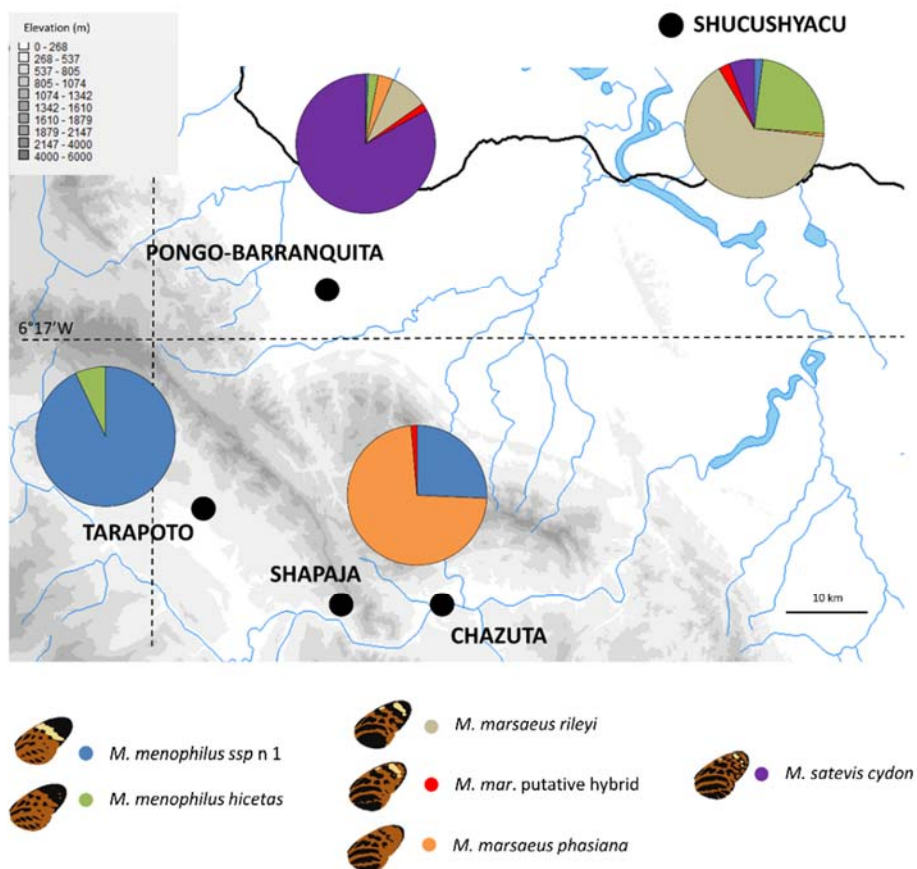
705 frequencies of putative hybrids for the entire distribution (i.e. all localities were pooled) and for

706 the contact/hybrid zone specifically

	Exp( <i>M. mar. phasiana</i> )	Exp( <i>M. mar. hybrid</i> )	Exp( <i>M. mar. rileyi</i> )	$\chi^2$ (df=1)	<i>p</i>
Contact zone	2.34	10.31	11.34	12.07	<i>p</i> <0.001
Total distribution	23.09	83.83	76.09	149.74	<i>p</i> <0.001

707

708

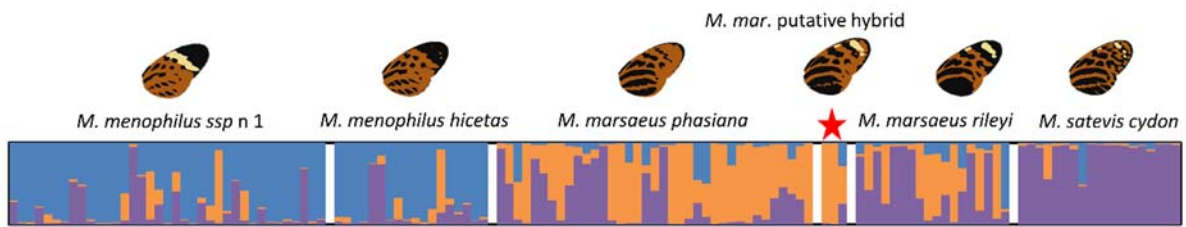


709

710 **Figure S1:** Distribution of five different *Melinaea* taxa and putative hybrids between subspecies

711 of *M. marsaeus* in north-eastern Peru





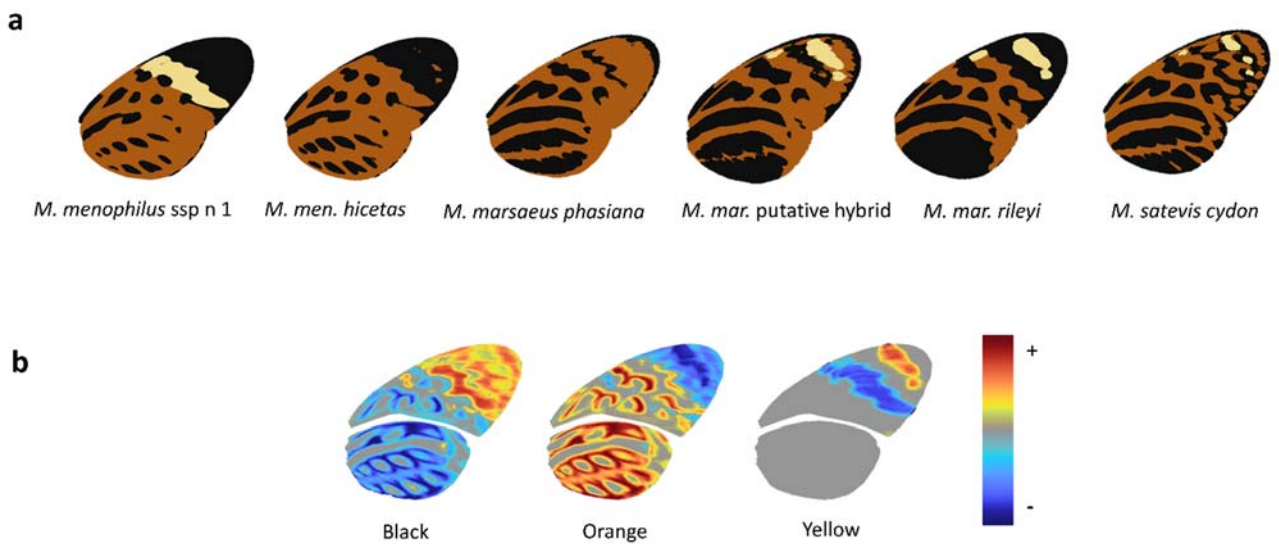
712

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

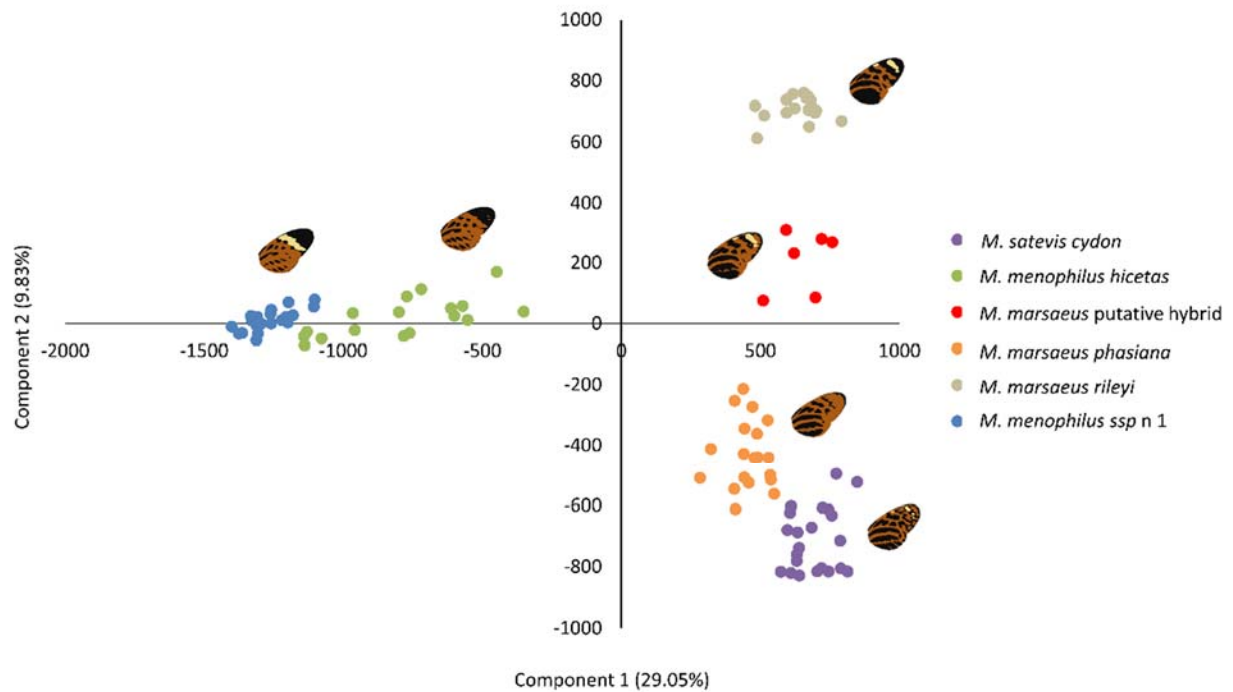
719 **Figure S3: a)** Average wing colour patterns for the five *Melinaea* taxa and putative hybrids

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