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Selection on male sex pheromone composition contributes to butterfly reproductive isolation

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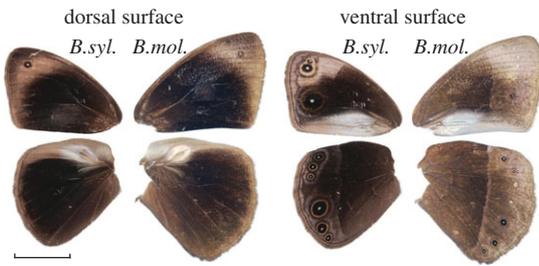
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Selection can facilitate diversification by inducing character displacement in mate choice traits that reduce the probability of maladaptive mating between lineages. Although reproductive character displacement (RCD) has been demonstrated in two-taxa case studies, the frequency of this process in nature is still debated. Moreover, studies have focused primarily on visual and acoustic traits, despite the fact that chemical communication is probably the most common means of species recognition. Here, we showed in a large, mostly sympatric, butterfly genus, a strong pattern of recurrent RCD for predicted male sex pheromone composition, but not for visual mate choice traits. Our results suggest that RCD is not anecdotal, and that selection for divergence in male sex pheromone composition contributed to reproductive isolation within the *Bicyclus* genus. We propose that selection may target olfactory mate choice traits as a more common sensory modality to ensure reproductive isolation among diverging lineages than previously envisaged.

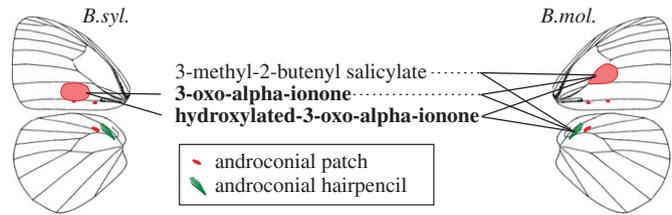
1. Introduction

Species diversification is typically a by-product of drift or ecological adaptation of populations evolving in allopatry. Sometimes, when formerly separated lineages come into secondary contact, selection can increase divergence in their mate choice traits to reduce the risk of maladaptive mating. This process is termed reproductive character displacement (RCD) and includes reinforcement, a special case where gene flow is ongoing between lineages [1,2]. Once controversial, RCD has received theoretical support [3]. Empirical evidence for RCD has also accumulated mostly from studies comparing a pair of species [4–10], whereas the frequency of RCD in nature is best addressed using large assemblages of recently diverged species [11–16]. Moreover, these studies have focused primarily on visual and acoustic mate choice traits, despite the fact that chemical communication is ubiquitous and probably the most common means of species recognition across living taxa worldwide [17,18]. Compared with visual, acoustic or electric signals, chemical signals such as sex pheromones are unique in that their perception requires specific combinations of chemical receptors [19–21]. Therefore, chemical signals may ensure a high level of privacy in communication between interacting organisms, and their evolution may be less dependent on selective forces other than mate choice. Thus, while these studies demonstrate that RCD has occurred, the assessment of how frequently RCD occurs in nature [22] should thus focus on large assemblages of closely related species and on mate choice traits most commonly used in species recognition—i.e. chemical mate choice traits [17,18]. In this study, we tested for the presence of RCD in a chemical mate choice trait in contributing to the evolution of recurrent reproductive isolation under natural selection.

allopatric species: *B. sylvicolus* / *B. mollitia*

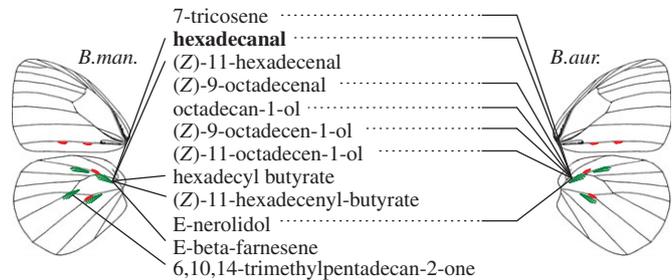


androconial diagrams and localization of pMSP components on wings



two pMSP components shared out of three

sympatric species: *B. mandanes* / *B. auricruda*



one pMSP component shared out of 12

Figure 1. Morphological (left) and chemical (right) diversity of males of an allopatric (upper panels) and a sympatric (lower panels) pair of sister species. Scale bar, 1 cm. Names of shared pMSP compounds are in bold.

The more than 80 species of the sub-Saharan butterfly genus *Bicyclus* (Lepidoptera, Nymphalidae) are strikingly similar morphologically. This speciose group diversified during the Miocene, and pairs of sister species differentiated between 0.5 and 5 million years ago [23]. Up to 20 species can be captured in a day in a single forest patch, where males pursue, and court females from various *Bicyclus* species and rare heterospecific matings have been observed in captivity and in nature. This suggests that interspecific interactions are frequent and it raises the question of how reproductive isolation occurs between these sympatric and morphologically similar species (figure 1). Interestingly, the key taxonomic trait used to distinguish *Bicyclus* species is the number and position of androconia [24], the wing structures thought to be involved in the production and emission of male sex pheromone. Furthermore, it has been demonstrated that male sex pheromone (MSP) plays a key role in mate choice in the model species *Bicyclus anynana* [25–27]. After landing behind a female, *B. anynana* males flicker their wings and erect their androconial hair, probably favouring the dissemination of MSP at short range [25]. Therefore, we postulated that selection on differentiation in the sex pheromone composition has produced recurrent RCD in this butterfly genus. Building on knowledge from the model species *B. anynana* [25–27], we first developed a methodology to select from complex chemical profiles the components most likely to form the male sex pheromone for over 30 *Bicyclus* species. Accounting for the potential effect of the environment, we then assessed the presence of a recurrent pattern of character displacement in the predicted sex pheromone composition across the genus. We next compared the rate of evolution of the predicted sex pheromones with that of other, morphological, *Bicyclus* mate choice traits (the wing eye-spots [28,29]). Finally, we examined the extent to which the evolution of composition of the predicted sex pheromone and of androconia are coupled.

2. Material and methods

(a) Sampling

We sampled three males and two females of 32 *Bicyclus* species in four African countries (electronic supplementary material,

figure S1) using fruit-baited traps. The different androconia and the remaining part of the wings of freshly killed individuals were stored in separate glass vials filled with *n*-heptane as a solvent for chemical compounds extraction and further gas-chromatography and mass-spectrometry analyses (GC-MS; electronic supplementary material, note S1).

(b) Selection of the predicted male sex pheromone dataset

We developed a standardized routine to select the compounds most likely composing the MSP among the more than 40 compounds identified per species. We based this method on data collected for *B. anynana*, for which the behaviourally active chemicals forming the MSP have been identified using gas chromatography-electroantennographic detection (GC-EAD) and behavioural assays [25–27]. We called the selected compounds the ‘predicted male sex pheromone’ (pMSP) components. We first removed any peak smaller than 10 ng, to homogenize the detectability of compounds between samples and to permit the identification of all selected peaks. Despite the ability of GC-EAD to detect the presence of olfactory receptors for chemicals present in minute amounts, the *B. anynana* MSP was shown to be formed by the most abundant, male-specific and repeatable compounds [25]. We thus selected the compounds on average more than five times more abundant in males than in females and present in at least two out of three sampled males. This first selection still resulted in more compounds than known from data obtained using GC-EAD and behavioural assays in *B. anynana*, in which the MSP components are the top third of the most abundant compounds [25–27]. Moreover, MSP components that experience directional sexual selection are usually more abundant than other compounds (see [30] and examples in Lepidoptera [31,32], Hymenoptera [33], lizards [34] or elephants [35]). Thus, from the first selection, we kept the top third most abundant compounds for each *Bicyclus* species, forming the dataset of pMSP components used in all subsequent analyses (electronic supplementary material, table S1). Of note, we did not include the Malawian laboratory stock of *B. anynana* from which the selection procedure was designed but rather a field-caught Ugandan *B. anynana* population (electronic supplementary material, note S1).

(c) Scoring of morphological traits

We coded the presence and absence of 20 androconial structures grouped according to their position in relation to the wing veins and dorsal or ventral wing surface (electronic supplementary material, note S1). We coded presence or absence of the 17 eyespots and two wing bands (electronic supplementary material, note S1) separated in two groups according to their presence on the ventral or dorsal wing surfaces because they evolve under different selection pressures: the ventral eyespots probably evolve under natural selection, whereas the dorsal characters evolve under sexual selection [28,29].

(d) Test of reproductive character displacement

First, we coded each trait (pMSP, androconia, dorsal forewing eyespots as well as ventral fore- and hindwing eyespots) as a series of binary characters (present or absent). We compared the pMSP composition between species taken pair by pair. Because most *Bicyclus* species do not share any pMSP component (see Results), in 85% of the species pairs, the commonly used Jaccard distance (proportion of pMSP differences) has a value of 1. Yet, pairs of *Bicyclus* species differ widely in their number of pMSP differences. Therefore, we partitioned the pMSP composition into two complementary variables: the number of pMSP differences and the number of shared pMSP components, per pair of species. There was no correlation between the numbers of pMSP components shared and different between species, supporting our partitioning approach (Mantel test with 9999 permutations, $n = 496$ pairs of species, $\rho = -0.11$, $p = 0.15$). To allow meaningful comparison across traits, we also scored the other, morphological, traits similarly (shared versus different) although these matrices showed significant correlations for these morphological traits (androconia $\rho = -0.52$, $p < 0.01$, ventral eyespots $\rho = -0.35$, $p < 0.01$ and dorsal eyespots $\rho = -0.64$, $p < 0.01$). Yet, using the Jaccard distance for these morphological traits did not change our results (not shown). Second, pMSPs were also compared between species based on their amounts, and using the Euclidian distance on log-transformed amounts of pMSP components.

We tested whether the variability of pMSP composition between pairs of species could be explained by the role of this trait in reproductive isolation. We expected larger differences in pMSP composition or amounts between sympatric than between allopatric pairs of species [12]. Because androconia (via pMSP) and dorsal eyespots are also linked to mate choice, we also expected a RCD pattern for these traits. We defined sympatry and allopatry solely based on our field data such that sympatric pairs of species had been caught simultaneously in the same fruit-baited trap. Extensive work in museums has, however, confirmed that our assignation of sympatry and allopatry was relevant (O. Brattström 2014, personal observation). We performed multiple regressions on the pMSP matrices using linear models in the ecodist R package [36–38] to test whether sympatry or allopatry explained the variation in pMSP composition between pairs of species. This is a non-parametric method that creates a null distribution of the estimated parameters by permuting the data and does not rely on the assumption of independence of each observation (each pair of species). We included the following control factors in the model: phylogenetic distance between species within the pairs (phylogenetic tree based on COI, COII and EF1 α genes; electronic supplementary material, note S2); and forest versus savannah habitats, as humid environments such as rainforests may affect olfactory communication [39]. We did not include an interaction term between the phylogenetic distance and sympatry, because this statistical method has not been validated for models with an interaction term between variables (P. Legendre 2012, personal communication).

(e) Estimation of the rates of evolution of the chemical and morphological traits

We mapped each series of binary characters (pMSP components, androconia, dorsal forewing eyespots and ventral fore- and hindwing eyespots) on the phylogenetic tree (electronic supplementary material, note S2). We measured the rate of evolution as a turnover of character presence and absence across the *Bicyclus* phylogeny. For this, we fitted a symmetrical (equal rates of gain and loss) continuous time Markov model to each trait using a Bayesian framework in the program BAYESTRAITS multistate [40]. Although an asymmetrical model better fitted the androconia and ventral eyespot traits than a symmetrical model, both yielded similar results for comparing the rate of evolution between all traits (electronic supplementary material, figure S5).

3. Results

(a) High specificity of predicted sex pheromone composition

The 32 sampled *Bicyclus* species displayed on average 42 ± 17 chemical volatile compounds (mean \pm s.d.). Chemical profiles were found to be repeatable between conspecifics when three males and two females were analysed using gas chromatography-mass spectrometry (GC–MS) analyses (electronic supplementary material, notes S1 and S4). For the 32 *Bicyclus* species, we selected a total of 75 compounds that probably formed the composition of their respective MSP (3.7 ± 2.1 pMSP components per species, mean \pm s.d.; figure 2 and electronic supplementary material, table S1 and figure S2a). These pMSP components showed a wide diversity of chemical structures, and for some of them have been previously identified as sex pheromone components in moths [41]. Of the *Bicyclus* pMSP components, 51% were fatty acid derivatives, 20% terpenes and terpenoids, 2% aromatics, 6% carotene derivatives and the remaining 21% miscellaneous or of unknown origin (figure 2 and electronic supplementary material, table S1). Although the behavioural activity of the male odours has yet to be demonstrated in bioassays, the low within-species variability of chemical profiles (Spearman rank correlation R (mean \pm s.d.) = 0.81 ± 0.15 and 0.74 ± 0.18 in males and females, respectively; electronic supplementary material, figure S7) contrasted with the high between-species variability of male chemical profiles ($R = 0.18 \pm 0.16$; Wilcoxon rank test $p < 2.2 \times 10^{-16}$, electronic supplementary material, figure S7; 71% of pMSP components were species-specific, and each pMSP component was shared by 1.6 species on average of 32; figure 2 and electronic supplementary material, table S1 and figure S2b) and is consistent with a role of the pMSP in species recognition. This pattern of high species-specificity remained true when we considered the additional *Bicyclus* lineages in which the 75 pMSP components were found as trace compounds, but had not been selected as pMSP components in these latter species (grey cells in figure 2b); each pMSP component or its trace was shared by 4.5 species on average of 32. Moreover, we observed that most observed changes in pMSP composition arose recently in the evolution of the *Bicyclus* genus (figure 2 and electronic supplementary material, note S2) and some pairs of sympatric species can display a remarkably large number of pMSP differences (figure 1).

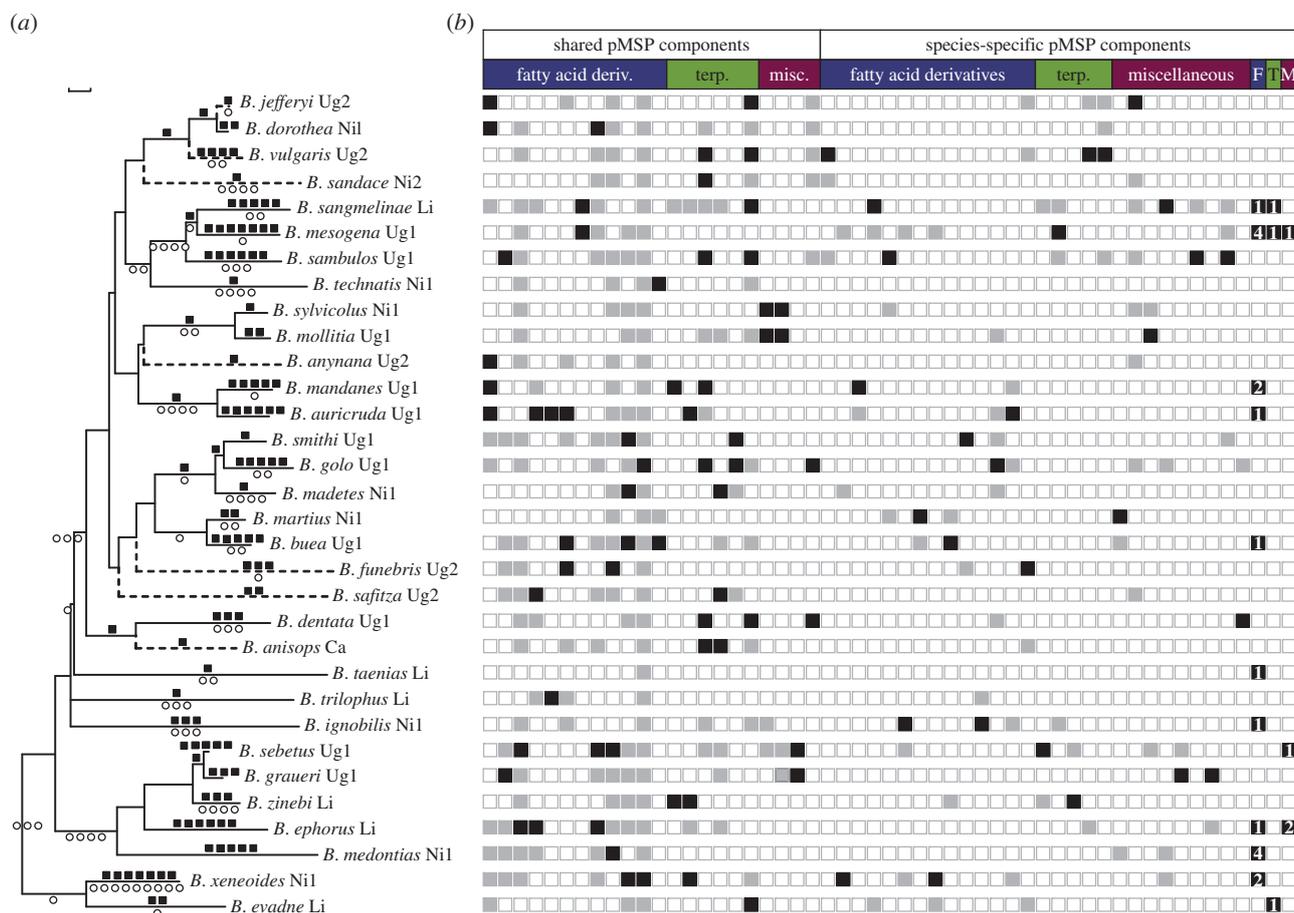


Figure 2. Wide diversity and evolution of the pMSP composition and of androconia across the phylogeny of *Bicyclus*. (a) Bayesian consensus tree of the sampled species based on mitochondrial genes COI and COII, and nuclear gene EF1 α . Filled squares and open circles represent changes in presence and absence for pMSP components and androconia, respectively (electronic supplementary material, note S2). Plain and dotted tree branches represent forest and savannah habitats respectively. The scale bar above the tree represents 1% of genetic divergence. Codes following species names correspond to sampling locations: Li for Liberia (Sapo); Ni for Nigeria (1, Afi mountains and 2, Yankari); Ca for Cameroon (Fossong Ellelem) and Ug for Uganda (1, Kibale and 2, Mburo; see the electronic supplementary material, figure S1). (b) The left part of the table shows the pMSP components (black cells) shared by two or more *Bicyclus* species and the right part of the table shows the pMSP components specific to one *Bicyclus* species. Grey cells represent pMSP components found in additional *Bicyclus* species as trace compounds but not selected as pMSP components in these latter species. Black cells in the three last columns represent numbers of additional strictly specific pMSP components. The pMSP components are classified in three chemical types: fatty acid derivatives, terpenes and terpenoids, and miscellaneous (see the identification of individual compounds in the electronic supplementary material, table S1).

(b) Reproductive isolation by olfactory communication

These results suggest that pMSP composition could be involved in premating reproductive isolation between *Bicyclus* species. To test this hypothesis, we next compared pMSP composition between sympatric and allopatric pairs of species while controlling for phylogenetic distance within pairs. The number of pMSP differences was greater between species in sympatric pairs, compared with allopatric pairs (permutation test; 9999 permutations; $n = 496$ pairs of species; effect size = 0.66; $p = 0.009$; figure 3a). By contrast, the number of shared pMSP components was solely determined by phylogenetic distance (permutation test; 9999 permutations; $n = 496$ pairs of species; effect size = -2.49; $p < 0.001$; electronic supplementary material, figure S8a and note S6). Therefore, RCD appears to develop by the recurrent accumulation of new, distinct, chemical components and not by a reduction in the number of shared pMSP components. Several factors contribute to the robustness of this result. First, we used a conservative test, defining as sympatric only those pairs of species caught simultaneously in the same fruit-baited trap. Because we expected sympatric pairs to be chemically more different than allopatric pairs, mistakes

in the assignation of sympatry or allopatry would homogenize the differences in pMSP composition expected between the sympatric and the allopatric groups of species and as such decrease the observed RCD pattern. Second, the number of pMSP differences tended to decrease with phylogenetic distance within pairs of sympatric species (figure 3a). Although we could not test the significance of this effect (P. Legendre 2012, personal communication), it suggests that the pMSP divergence is owing to interactions between closely related species in sympatry, which is expected for RCD, because less closely related species are less likely to hybridize [42]. In this regard, considering only the most recently diverged pairs of species tripled the effect size of sympatry (model using only species pairs separated by less than 0.15 of phylogenetic distance in figure 3a; $n = 38$ pairs, $R^2 = 0.31$; effect size = 1.77 and $p = 0.02$ for 'sympatry'; effect size = 2.03 and $p = 0.07$ for 'habitat'; effect size = 5.48 and $p = 0.59$ for 'phylogeny', electronic supplementary material, table S2). Third, the significantly higher number of pMSP component differences between sympatric compared with allopatric species pairs was robust to the deletion of three pairs of allopatric species which could be considered

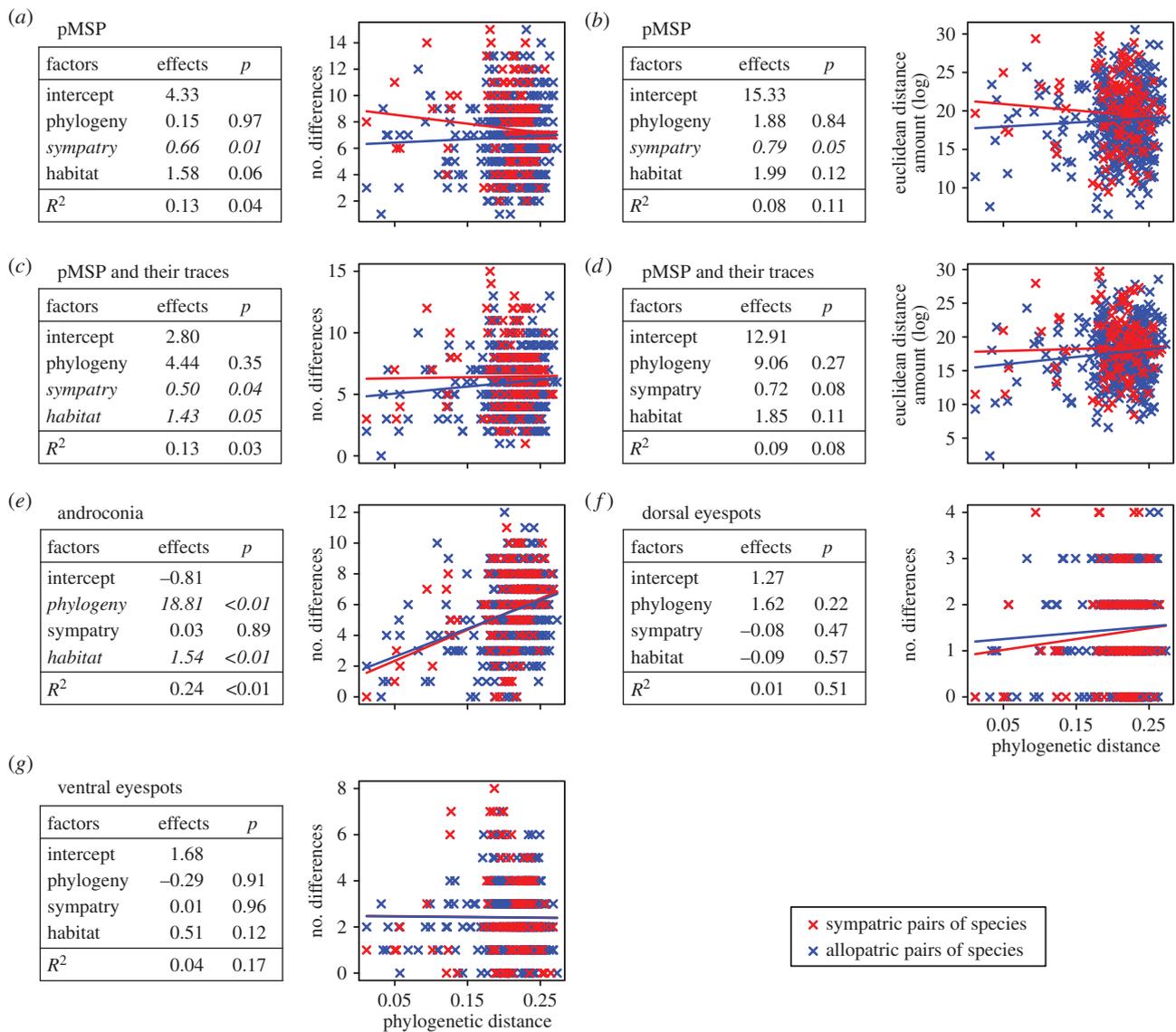


Figure 3. Reproductive character displacement for pMSP composition but not for other, morphological, adaptive traits. The following traits were tested for RCD: (a–d) pMSP composition, (e) androconia, (f) dorsal eyespots and (g) ventral eyespots. Panels (a,c) represent the number of differences in presence and absence of pMSP components; panels (b,d) represent the Euclidean distances on amount of pMSP component; panels (a,b) involve our selection of 75 pMSP components only, whereas panels (c,d) also include the pMSP components when found as traces in additional *Bicyclus* species ('grey cells' in figure 2b). On the left of each panel, the statistical output of the following model is given in a table for each trait: *phenotypic difference between species* ~ *intercept* + *phylogeny* + *sympatry* + *habitat*. Significant effects are italicized. On the right of each panel, graphs show the phenotypic differences between species against their phylogenetic distance, using the following model: *phenotypic difference between species* ~ *intercept* + *phylogeny* + *sympatry* + *habitat* + *phylogeny: sympatry*. These graphs thus include the interaction term for illustration sake, and the effect of the habitat is averaged. Of note, the results for differences in pMSP composition (a) are robust to the removal of the three bottom left allopatric species pairs (electronic supplementary material, figure S9).

as outliers in our dataset (electronic supplementary material, note S5 and figure S9). Fourth, the RCD pattern was also found when we tested for the variation in amount, and not simply in the presence or absence, of the pMSP components across the *Bicyclus* phylogeny: sympatric pairs of species displayed significantly higher differences in amount in pMSP components than allopatric pairs of species (figure 3b; $p = 0.05$). Fifth, the RCD pattern remained when we included the pMSP components found as traces in the additional *Bicyclus* lineages, either considering their presence or absence (figure 3c; $p = 0.04$) or as a trend when considering their variation in amount (figure 3d; $p = 0.08$).

As controls, we tested for the presence of RCD in other adaptive, morphological, traits: dorsal forewing eyespots

(important in mate choice [28]), ventral wing eyespots (important in predator avoidance [28]) and androconia (putatively important for pheromone production). For these traits, the numbers of shared characters and of differences are comparable for sympatric and allopatric species pairs, and both depend on phylogenetic distance (figure 3e–g and electronic supplementary material, figure S8 and note S6). In contrast to the olfactory trait, neither of these morphological traits evolved character displacement between sympatric closely related lineages. The evolution of androconia, in particular, seems not to be linked to the signal they release, as specific structures are not associated with specific pMSP components (electronic supplementary material, note S7).

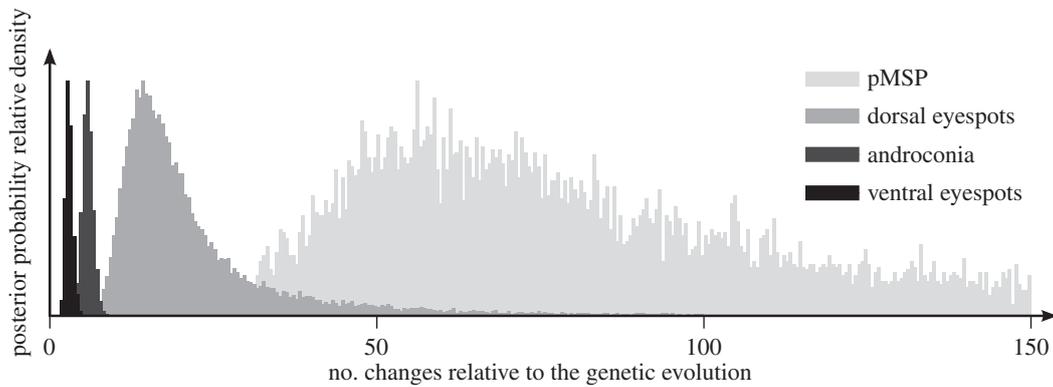


Figure 4. Higher rate of character change for pMSP composition compared with morphological traits. The distribution of evolutionary rates for, from left to right, ventral eyespots, androconia, dorsal eyespots and pMSP based on Bayesian estimation. The most probable rates of both gains and losses (number of change relative to genetic evolution) are 2.9, 5.7, 14.4 and 59.7, respectively. Similar results were found when gains and losses were estimated as two independent parameters (electronic supplementary material, figure S5).

(c) Limited habitat-driven evolution of predicted male sex pheromone composition

In addition to RCD, ecological character displacement can also drive divergence of mate choice traits when natural selection occurs to reduce ecological niche competition [42,43]. We considered two ecologically significant factors that could affect pMSP evolution: habitat type (forest versus savannah) and host plant specificity [44]. Habitat type had, indeed, a marginally significant effect on pMSP differentiation (effect size = 1.58 and $p = 0.06$; figure 3a and electronic supplementary material, figures S9 and S10), but was not responsible for the pattern of pMSP character displacement observed between closely related sympatric species pairs. The effect of habitat type was due to a higher number of pMSP components in forest than in savannah species (generalized estimated equation; $n = 32$ species; effect size = 4.52; $p < 0.01$; electronic supplementary material, note S8), which suggests that a higher diversity of olfactory signals may compensate for the potential impairment of olfactory communication in humid environments like rainforests [39]. The number of species (higher in forests) could not be separated from the effect of habitat owing to the reduced number of sampled localities ($n = 6$; electronic supplementary material, note S8). Of note, forest species also displayed a larger number of androconia, but not of eyespots, compared with savannah species (as shown indirectly by the electronic supplementary material, figure S10).

Ecological character displacement could also result from adaptation to different host plants at the larval stage to avoid competition, which could induce modification of MSP composition and reproductive isolation at the adult stage [45]. Although host plant specificity remains unknown for most *Bicyclus* species, 56% of pMSP components are fatty acid derivatives which can be produced *de novo* by insects [46,47]. The remaining 44% of pMSP components may be, but are not necessarily, derived from host plant use (e.g. [48] and figure 2). Moreover, these potentially host-plant-derived components did not show higher species specificity than fatty-acid-derived pMSP components (shared respectively by 1.65 ± 1.43 and 1.48 ± 0.93 species; mean \pm s.d.). In the laboratory, rearing *B. anynana* larvae on grasses closely related to its assumed natural host plant (*Oplismenus* sp.) or on maize (*Zea mays*), the usual laboratory host plant for this butterfly,

did not affect the chemical profiles of adults [25]. These results suggest that host plant adaptation may have only a limited effect on pMSP composition or differentiation across the *Bicyclus* genus.

(d) Differential fusion or species sorting do not explain the pattern of predicted male sex pheromone evolution

Two other alternative processes to RCD (species sorting and differential fusion [12,49]) might explain the greater divergence of pMSP composition in sympatric compared with allopatric species pairs. These processes occur if, after the differentiation of pMSP composition between species evolved in allopatry, secondary contact results in the merging of insufficiently differentiated lineages (differential fusion) or the local extinction of one (species sorting), whereas those lineages that have evolved sufficiently strong reproductive isolation are able to coexist. We found, however, that allopatric pairs of *Bicyclus* species were not younger than sympatric ones (electronic supplementary material, note S9) which would be expected if a subset of the sympatric species (the youngest) had merged or went extinct in sympatry during secondary contact owing to weak reproductive isolation [42]. Thus, our data suggest that neither differential fusion nor species sorting explain the recurrent pattern of pMSP diversification across the genus.

(e) Rapid evolution of sex pheromone composition

In the few documented case studies, species interactions caused the evolution of RCD over very short evolutionary timescales in nature, i.e. in a small number of generations [5] or a few thousand years [7]. Thus, we expect that any mating traits which produce reproductive isolation by RCD should evolve rapidly. Although the actual rates of change must be treated with caution (electronic supplementary material, note S3), the results of the comparison suggest that pMSP have diverged over four times more rapidly than dorsal eyespots, another mate choice trait in *B. anynana* [29], which, in turn, have evolved more than twice as fast as androconia (figure 4). This relatively high turnover of pMSP

composition across species is in agreement with the involvement of this trait in premating reproductive isolation [5,7,50].

4. Discussion

Our results correlate the local co-occurrence of closely related *Bicyclus* species to the larger differences in composition, and in amount, of their major male volatile compounds. This suggests a link between the establishment, or increase, of reproductive isolation between *Bicyclus* species and the recurrent differentiation of their major male volatile compounds.

Our results are based on a selection method targeting the volatile wing compounds most likely acting as male secondary sexual traits. We based our selection method on criteria established in the field of sexual selection and widely supported across taxa and across phenotypic traits: we picked male-specific, repeatable and most abundant compounds as these are most likely under directional selection for largest possible amounts by females. These characteristics are indeed the ones of the male sex pheromone identified using electrophysiological and behavioural experiments in the model species *B. anynana* [25–27]. The use of a selection method also has the advantage to avoid circular reasoning, as we show that the compounds chosen on one basis (male sex specificity, repeatability and amount in males) show a biological pattern of species specificity and RCD. However, our selection of pMSP components probably contains some chemicals not playing the role of sex pheromone, and we probably missed some sex pheromone components in our pMSP selection. We may also have included as pMSP, compounds that have other function in reproduction, such as anti-aphrodisiacs. Yet, the behaviour of *B. anynana* does not suggest the existence of anti-aphrodisiacs (e.g. no pupal mating like in *Heliconius* [51]). Anti-aphrodisiacs are transferred between male and female abdomens, which were not analysed here. Moreover, traits involved in male–male competition for accessing females, such as anti-aphrodisiacs, are unlikely to be under selection for increased differentiation in sympatry compared with allopatry. Altogether, our criteria maximized the probability of selecting compounds acting as male secondary sexual traits under sexual selection by females, and the inclusion of compounds with other functions in our pMSP selection, such as anti-aphrodisiacs, would have blurred the RCD pattern. Future behavioural studies using synthetic sex pheromone perfumes as in Nieberding *et al.* [26] are needed to confirm the role of the selected compounds in sexual communication and reproductive isolation in field-caught populations of *Bicyclus* sharing some pMSP components.

Importantly, the presence of a recurrent RCD pattern is stronger for the chemical trait when we use their pattern of presence and absence (figure 3a), rather than their variation in amount across species (figure 3b). In addition, the RCD pattern held true for the chemical trait when we included the pMSP components found scattered as trace compounds in other *Bicyclus* lineages ('grey cells' in figure 2b). This was expected as, notwithstanding our selection method, the majority of male major compounds are simply absent in related species. The traces of pMSP components found in other *Bicyclus* species are either present: (i) at low amount, in that case we expect the female to reject the male, as shown in *B. anynana* in which mate choice is based on

maximal amounts of MSP [26,27]; or (ii) in a female, in that case, the chemical cue is not going to be a problem as females rarely court other females in *B. anynana*. Thus, in *Bicyclus* butterflies, the presence or absence of pMSP components probably allows species recognition. Our results do not exclude that, as is found in moths [17,18], variations in ratios between compounds or the presence of minor chemical compounds may participate to reproductive isolation in this genus, together with the presence of abundant pMSP components.

Our results show that incipient *Bicyclus* species initially share some compounds (electronic supplementary material, figure S4a) and achieve reproductive isolation by accumulating more rapidly pMSP differences in sympatry than in allopatry (figure 3a). With time, differences in pMSP components continue to accumulate between species and shared pMSP components are lost, because pMSP turnover is high (figure 4), which is why most *Bicyclus* species currently do not share any pMSP component anymore. Importantly, it does not imply that all pMSP differences appeared to achieve reproductive isolation in sympatry, as allopatric pairs of species also display a substantial number of pMSP differences (figure 3a).

Experimental evolution of rapid reproductive isolation by RCD of sex pheromones in a pair of *Drosophila* species [5] supports the hypothesis that the evolution of chemical communication may contribute to reproductive isolation by RCD. Direct experimental evidence that links sex pheromone composition and reproductive isolation was repeatedly found in Lepidoptera in which the presence of a heterospecific sex pheromone compound typical of a closely related sympatric species, decreases attraction to traps baited with female pheromones [52]. In *B. anynana*, the absence of the MSP decreases the mating success of males [25]. This suggests that differences in MSP composition may have an effect on species recognition in addition to mate choice, because males of other *Bicyclus* species lacking the MSP typical of *B. anynana* males would have a low mating success when courting *B. anynana* females [53].

Natural selection is traditionally flagged for RCD as a response to the production of maladaptive hybrids (historical explanation [2]). Yet, the avoidance of maladaptive mating by females can be considered as promoted by sexual selection for good genes [54]. Moreover, divergent sexual selection produces rapid diversification of secondary sexual traits [55] such as observed for pMSP composition. It is notoriously difficult to separate natural and sexual selection in driving the evolution of secondary sexual traits and many authors see the two processes as a continuum from species recognition to mate choice [53,56]. Here, we cannot exclude natural or sexual selection in the evolution of pMSP composition as the two processes can produce the same RCD pattern.

Together, our data provide large-scale evidence of recurrent RCD in an olfactory mate choice trait. We propose that chemical communication displays unique properties compared with visual, acoustic or electric [50] modes of communication, which may facilitate its role in the evolution of premating reproductive isolation. First, we found similar pMSP components in distantly related *Bicyclus* lineages and most pMSP components in one *Bicyclus* species could be found scattered as trace compounds in a few other *Bicyclus* lineages (in 14% of the species on average, noted by grey cells in figure 2b). This large phenotypic standing variation, as found in other species [41,57], suggests that each *Bicyclus*

species has access to most of the enzymes involved in the biosynthesis of the pMSP components. Thus, the wide diversity and the rapid evolution (figures 2 and 4) of sex pheromone composition in *Bicyclus* may be explained by the lack of intrinsic biosynthetic constraints, as has been found in *Drosophila* and some moths [58–60]. Second, sex pheromone components are qualitative, discrete, signals that require specific one-to-one corresponding chemical receptors to allow communication to take place [19–21], which gives rise to a unique ‘lock-and-key’ privacy in communication between courting individuals. Interestingly, the stimulation of the sex pheromone-specific olfactory receptor is sufficient to trigger complete courtship behaviour in the silk moth *Bombyx mori* [20] and *Ostrinia* Lepidoptera [21], and the replacement of a sex pheromone receptor by that of another species is sufficient to induce male courtship towards hetero-specific females [20]. Thus, we expect that the turnover of sex pheromone receptors expressed in specific olfactory neurons can elicit the same adaptive courtship behaviour in response to newly produced sex pheromone components without additional modification of hard-wired circuits that link the olfactory neurons to higher neuronal centres. This provides a mechanism by which such qualitative chemical signals can undergo rapid evolution. We propose that, relative to other modes, chemical communication is: (i) an *open* niche as the diversity of potential new chemical compounds is seemingly unlimited; (ii) *private* provided the ‘lock-and-key’ nature of olfactory receptors and sex pheromone components, and as such evolves under a limited number of selective forces (i.e. illicit receivers such as predators or parasitoids); and (iii) *labile* given that a turnover in sex pheromone

signals and receptors can take place without disrupting the subsequent adaptive behavioural courtship steps. As such, this ubiquitous mode of communication may act as a more general driver of reproductive isolation by RCD than previously envisaged.

Data accessibility. The new DNA sequences are accessible on GenBank, accession nos. KC786271–KC786277. The raw GC–MS data, the different trait matrices and the different tree files are accessible on Dryad public repository (doi:10.5061/dryad.768sd).

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