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What shapes the continuum of reproductive isolation? Lessons from *Heliconius* butterflies

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The process by which species evolve can be illuminated by investigating barriers that limit gene flow between taxa. Recent radiations, such as *Heliconius* butterflies, offer the opportunity to compare isolation between pairs of taxa at different stages of ecological, geographical, and phylogenetic divergence. Here, we report a comparative analysis of existing and novel data in order to quantify the strength and direction of isolating barriers within a well-studied clade of *Heliconius*. Our results highlight that increased divergence is associated with the accumulation of stronger and more numerous barriers to gene flow. Wing pattern is both under natural selection for Müllerian mimicry and involved in mate choice, and therefore underlies several isolating barriers. However, pairs which share a similar wing pattern also display strong reproductive isolation mediated by traits other than wing pattern. This suggests that, while wing pattern is a key factor for early stages of divergence, it may become facultative at later stages of divergence. Additional factors including habitat partitioning, hybrid sterility, and chemically mediated mate choice are associated with complete speciation. Therefore, although most previous work has emphasized the role of wing pattern, our comparative results highlight that speciation is a multi-dimensional process, whose completion is stabilized by many factors.

1. Introduction

Studies of speciation have long contrasted allopatric and sympatric speciation, speciation through sexual versus natural selection, and ecological versus non-ecological speciation. However, these contrasts do not always reflect the diversity of processes involved in divergence and the challenge is to reach an integrated understanding of speciation [1–3]. Species divergence involves multiple different traits and processes that can lead to reproductive isolation [4]. These include adaptation to local environmental conditions, pre-mating isolation, and post-mating effects that reduce the fitness of hybrids. To untangle the evolutionary processes at play, it is useful to quantify the relative importance of the factors reducing gene flow between diverging populations [5].

Speciation is a continuous process and we can typically only observe the results of divergence at a specific stage, not the process in its entirety. For instance, incompatibilities between extant species may not reveal the ecological and evolutionary forces initially causing divergence [6]. Conversely, ecotypes or subspecies at early divergence may shed light on factors favouring early divergence, but speciation is not a necessary outcome [3,7] and the challenge of speciation with gene flow might not be its initiation but its progression and
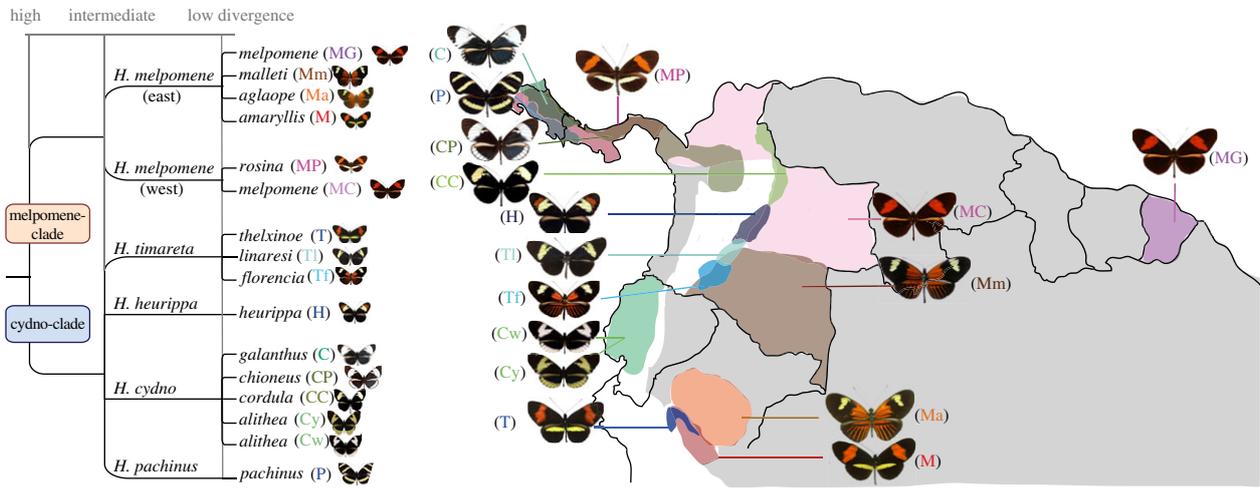
Figure 1. Geographical range and relationships of the taxa included in this study. Grey areas represent areas harbouring other subspecies of *H. cydno*, *H. timareta*, and *H. melpomene* which we did not include in our analyses. *H. m. melpomene* and *H. m. malleti* have a wide range through South America but were represented only in the country where they were studied. Phylogeny is adapted from [13,14]. Range localization is adapted from [15].

completion [8]. In that context, a useful way to study speciation as a continuous process is to compare multiple pairs of incipient or closely related species which vary in their extent of divergence, possibly depicting stages along the so-called speciation continuum. While keeping in mind that those pairs of taxa may or may not become pairs of species, and that there may be more than one trajectory of divergence, studying those pairs within the speciation continuum framework is informative of the mechanisms underlying divergence and explaining the different levels attained [7–11].

With a large diversity of recently diverged species and sub-species, the radiation of *Heliconius* butterflies is an excellent system for studying speciation with gene flow [12]. Within *Heliconius*, two sister-clades, melpomene-clade and cydno-clade, each contain a large number of local representatives across the Neotropics (figure 1). They provide replicate pairs of taxa distributed along a continuum of divergence, notably spanning the 'grey zone of speciation' [11], providing an opportunity to assess the factors shaping reproductive isolation along the speciation process. *Heliconius melpomene* is considered a single taxonomic species but comprises two lineages with significant genetic differentiation between western and eastern populations on either side of the Andes [13,14]. The cydno-clade includes four lineages, described as taxonomic species, *Heliconius cydno*, *Heliconius pachinus*, *Heliconius timareta*, and *Heliconius heurippa*. Across their range, representatives of the cydno-clade are typically broadly sympatric with *H. melpomene* and hybridize at low frequency [16–18], offering an opportunity to study both pre- and post-mating factors of reproductive isolation, even between clades that diverged about 2 Ma [13].

Research on speciation in *Heliconius* butterflies has put emphasis on behavioural pre-mating isolation, found to be strong in most pairs of taxa [19–22]. However, other factors affecting differentiation such as microhabitat partitioning [23], hybrid fertility [24,25], hybrid survival in the wild [26], and hybrid mating success [27] have also received some attention. Here, to provide an extensive comparison across the whole clade, we conducted a joint re-analysis of those published data with new data and quantified the contribution to reproductive isolation of each isolating component.

Most studies focus on pairs of species diverging in wing colour pattern. Wing pattern has been termed a 'magic trait' causing speciation, because disruptive selection and assortative mating operate directly on the same trait, wing pattern, thereby coupling two key forms of reproductive isolation [19,26,28–30]. First, *Heliconius* wing patterns are warning signals under strong natural selection for Müllerian mimicry [31,32]. Individuals not fitting one of the warning patterns recognized by predators suffer a higher risk of predation and there is evidence for selection against immigrant and hybrid wing patterns [26,31,32]. Second, wing patterns are also involved in mate recognition in *Heliconius*, and males typically preferentially court females displaying their own colour pattern [19,21,27,33]. The loci controlling colour pattern appear to be tightly linked to mate preference loci, which may help maintain the association between signal and preference [20,34]. Consequently, wing pattern divergence causes reproductive isolation both through hybrid unfitness and assortative mating, and in *Heliconius*, speciation is indeed frequently associated with a colour pattern shift [28,35,36].

Cases of mimicry between closely related species were unknown in *Heliconius* until the discovery of a new cryptic subspecies of *H. timareta* in sympatry with its co-mimic *H. melpomene* [18,37–39]. Less is known about the mechanisms responsible for reproductive isolation between these species pairs with similar wing patterns, but this will be important in understanding the role of mimicry shifts in reproductive isolation. Indeed, wing-pattern similarity may be predicted to increase the frequency of heterospecific mating, as well as increase the survival of hybrid adults, and so may weaken both pre-mating and post-mating isolation.

In this study, we investigated the mechanisms involved in the build-up of reproductive isolation, by means of a comparative analysis on this subclade of *Heliconius* butterflies. We combine new data with data collected from the existing literature. The numerous studies of *Heliconius* taxon-pairs at various levels of divergence allow us to evaluate the relative importance of different barriers to gene flow and their emergence along a continuum of divergence. We have applied a unified framework for the quantification of isolating barriers that facilitates these comparisons [5]. By contrasting co-mimetic versus non-mimetic pairs of species, we also specifically address the importance of wing pattern as a 'magic trait' for reproductive isolation in *Heliconius*.

Table 1. Strength of reproductive isolation associated with each barrier to gene flow. RI ranges from 0 (non-significant barrier) to 1 (full isolation). For each pair of species, the two lines correspond to the two possible directions of heterospecific mating with the female/mother given first. Barriers that could not be estimated are not shown and a dash indicated barriers that could not be estimated but are likely non-significant. The grey scale describes the continuum of divergence with the 'high' category corresponding to pairs of taxa involving a representative of the melpomene-clade and the cydno-clade, and 'intermediate' and 'low' including pairs of taxa belonging to the same clade, respectively with (*) and without ('n.s') significant genetic divergence [14,33,40].

divergence	F_{ST} RAD [14]	F_{ST} AFLP [40]	F_{ST} AFLP [33]	#1 / #2	♀ X ♂	spatial	mating	F_1 egg	F_1 larva	F_1 adult	F_1 mating with #1	F_1 mating with #2	F_1 fertility
high divergence	0.34	0.23 *		H. c. chioneus (CP) / H. m. rosina (MP)	CPxMP	0.74	1	0		0.35	0.20	0.52	0.32
					MPxCP	0.74	1	0		0.35			0.15
	0.35	0.25 *		H. c. cordula (CC) / H. m. melpomene (MC)	CCxMC		0.82	0	0				0.29
					MCxCC		0.88	0	0				0.18
	0.42	0.35 *		H. heurippa (H) / H. m. melpomene (MC)	HxMC	0.91	0.93	0	0		0.44	0.29	0.27
					MCxH	0.91	0.90	0	0		0.75	0.20	0
	0.44	0.29 *		H. c. chioneus (CP) / H. m. melpomene (MG)	CPxMG	0.78		0					0.48
					MGxCP		1						0.34
	0.36			H. t. thelxinoe (T) / H. m. amaryllis (M)	TxM	0.63	0.86	0	0	—	0.48	0	0.33
					MxT	0.63	0.85	0	0	—	0.87	0	0.16
		0.21 *		H. t. florencia (Tf) / H. m. malleti (Mm)	TfxMm	0.48	0.90	0		—			0.33
					MmxTf	0.48	0.96	0		—	0.52	1	0.19
intermediate	0.38	0.35 *		H. heurippa (H) / H. c. cordula (CC)	CCxH		0.56	0	0				0
					HxCC		0.98	0	0				0
		0.07 *	0.17 *	H. c. galanthus (C) / H. pachinus (P)	CxP		0.83	—	—		0	0.94	—
					PxC		1	—	—		0	0.94	—
	0.37	0.30 *		H. m. rosina (MP) / H. m. melpomene (MG)	MGxMP		1	0	—				0
					MPxMG		0.48		—				0.32
low			0.001 (n.s.)	H. c. alithea white/yellow (Cw/Cy)	CyxCw		0.26	—	—	0.18	—	0.26	—
					CwxCy		0.07	—	—	0.18	—	0.26	—
		0.02 (n.s.)		H. t. florencia (Tf) / H. t. linaresi (Tl)	TfxTl		0.02	0	—				0
					TfxTl		0.48	0	—				0
	0.16			H. m. amaryllis (M) / H. m. aglaope (Ma)	MaxM		0.40	—	—				—
					MxMa		0	—	—				—

2. Material and methods

(a) Species studied and the continuum of divergence

The pair of taxa examined display variable levels of genetic divergence which we here sort into three broad categories. Firstly, phylogenies support a split between the cydno-clade and the melpomene-clade about 1.5–2 Ma [13], so pairs of taxa involving a representative of the melpomene-clade and a representative of the cydno-clade are replicates describing the evolution of divergence (with gene flow) since the original split between those two sister-clades. They were called 'pairs at high divergence'. All of them are sympatric pairs except for the comparison Panama/French Guiana. Secondly, each clade comprises pairs of taxa with significant genetic divergence (table 1; electronic supplementary material, table S2) and consistent genetic clustering [14] and were considered at 'intermediate divergence'. Within the cydno-clade, those pairs correspond to taxonomic species replacing each other in parapatry such as *H. cydno galanthus*/*H. pachinus* [20,41], or *H. cydno cordula*/*H. heurippa* [25,42]. Within the melpomene-clade, pairs at intermediate divergence correspond to allopatric taxonomic subspecies of *H. melpomene* that belong to the eastern and western lineage [19]. Thirdly, other within-clade pairs of taxa do not exhibit significant genome-wide differentiation and were considered at 'low divergence' [14,33,40]. Those correspond to sympatric white/yellow morphs of *H. cydno alithea* [20,33] and to parapatric races of *H. timareta* [43,44] or *H. melpomene* [21].

New data are provided for the co-mimics *H. t. thelxinoe*/*H. m. amaryllis*, *H. t. florencia*/*H. m. malleti* and three non-mimetic pairs *H. heurippa*/*H. c. cordula*/*H. m. melpomene* in the electronic supplementary material. For the other pairs of taxa, data were taken from the literature and re-analysed in the unified framework. References used for each barrier are given in the electronic supplementary material, table S1.

(b) General framework: quantifying the strength of reproductive isolation

We quantified the strength of reproductive isolation for each isolating barrier following [5,45]. Briefly, the index RI offers a linear quantification of reproductive isolation associated with the presence of a given barrier relative to expectations in the absence of all barriers. It allows a direct link to gene flow: RI = 1 when isolation prevents gene flow, whereas RI = 0 if the probability that gene flow does not differ from expectations without this barrier [5]. Confidence interval for the index can be drawn from confidence interval on the data (electronic supplementary material, table S3).

The strength of RI provided by each pre-mating/post-mating barrier is estimated with the expression:

$$RI_{barrier1} = 1 - 2 \times \frac{H_1}{H_1 + C_1},$$

where H_1 is the frequency of heterospecific mating/the fitness of hybrids and C_1 the frequency of conspecific mating/the fitness of pure individuals.

RI was calculated separately for both directions of crosses $(A \times B/B \times A)$. We summarize hereafter how each barrier was investigated. Detailed methods are given in the electronic supplementary material.

(c) Local co-occurrence

Although taxa may overlap in range at a broad geographical scale, encounter rates between conspecifics or heterospecifics still differ. For four pairs of species collected in locations equally distributed along a transition zone between microhabitats (electronic supplementary material, figure S1), we use collection data as a proxy for natural encounter rates, and draw an estimate of the expected number of heterospecific versus conspecific matings which we use to calculate RI associated with probabilities of co-occurrence, $RI_{co-occurrence}$.

(d) Behavioural pre-mating isolating barriers

Heliconius males usually patrol the habitat, approach females, and perform courtship characterized by intense wing flapping over the female. Females can accept or reject mating [46]. Most studies have investigated male attraction by visual cues (on models), male preference towards live females, and mating. Those three facets of mate choice were analysed separately to dissect their respective contribution to sexual isolation. Achieved mating, which reflects the multiple aspects of mate choice by both sexes leading to a mating event, was used for the whole comparison between barriers.

(i) Visual cues

In all studies, male preference for different visual cues has been estimated by presenting a group of males with a model made with dead female wings dissected and by recording courtship towards each model.

(ii) Male choice

In all studies, individually marked males were monitored for courtship during a short time-interval when presented with a heterospecific and a conspecific freshly emerged, virgin female (live-female experiment).

(iii) Achieved mating

To investigate mating achievement, most studies have simulated a natural situation, either with a no-choice experiment in which a virgin female (conspecific or heterospecific) is presented to males for 48 h, or with a tetrad experiment, where four individuals, one male and one female of each species, were kept until the first mating occurred.

(e) Post-mating isolating barriers

(i) F_1 hatch rate, hybrid sterility

Most studies quantified egg hatching rate in heterospecific crosses of first generation (F_1) and second generation (backcrosses), which allows inferring F_1 male and female fertility. Mated females were kept in individual cages with various fresh shoots of several *Passiflora* species. Eggs were collected on a regular schedule, stored individually in small plastic cups and checked daily for hatching.

(ii) Hybrid survival

Hybrid survival was recorded only for four pairs. In all cases, larvae were raised in individual plastic containers for the first
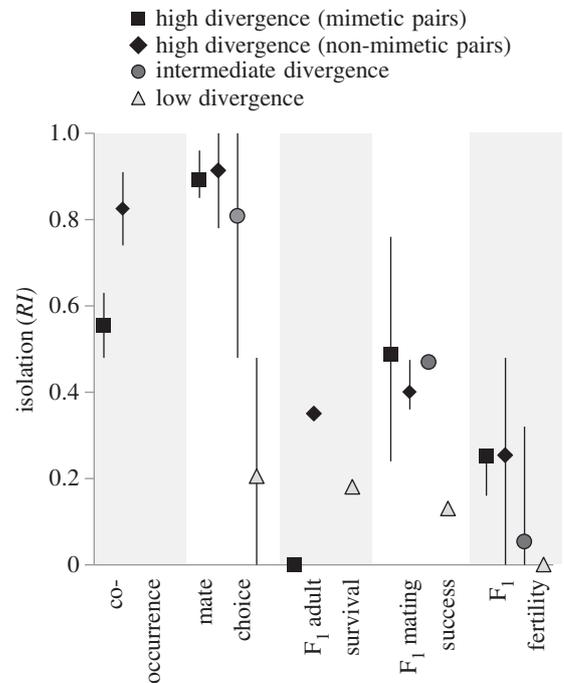

Figure 2. Mean strength of reproductive isolation for each relevant isolating barrier. RI associated with each barrier averaged by stage of divergence. The bars range from minimal to maximal values. See detailed values of RI in table 1.

instars. Then, they were gathered by family group in a larger box and fed *ad libitum* on young shoots of *Passiflora* sp. Survival rate was calculated for each family as the proportion of larvae growing until imago.

(iii) Hybrid adult fitness

Survival was estimated experimentally in Panama for *H. m. rosina*, *H. c. chioneus*, and their F_1 hybrids, from attack rates on artificial models made with plasticine and paper wings exposed during 3 days in the wild [26]. Survival was also estimated by mark-release-resight in Ecuador on the yellow and white morphs of *H. c. alithea* and let us infer predation against F_1 hybrids which are white [31].

Hybrid ability to mate has been investigated with no-choice experiments, live-female experiments, or using wing models (electronic supplementary material, table S1).

3. Results

(a) Co-occurrence

For four highly divergent species pairs that overlap on a large portion of their range, local co-occurrence was finely quantified (electronic supplementary material, figure S1) to estimate the probability of encounters (table 1 and figure 2). We found that relative differences in species frequencies contribute significantly to RI in both mimetic and non-mimetic pairs ($RI_{co-occurrence} = 0.48-0.91$).

This heterogeneous microspatial distribution corresponds to microhabitat transition, suggesting microhabitat partitioning between taxa. For instance, *H. c. chioneus* and *H. m. rosina* feed on different pollen sources and *H. c. chioneus* occupies tall forest habitats where its co-mimic *Heliconius sapho* is abundant, whereas *H. m. rosina* is frequent in edge habitats typical of its co-mimic *Heliconius erato* [23]. Similarly, with increasing altitude, *H. t. thelxinoe*, *H. t. florencia*, or *H. heurippa*
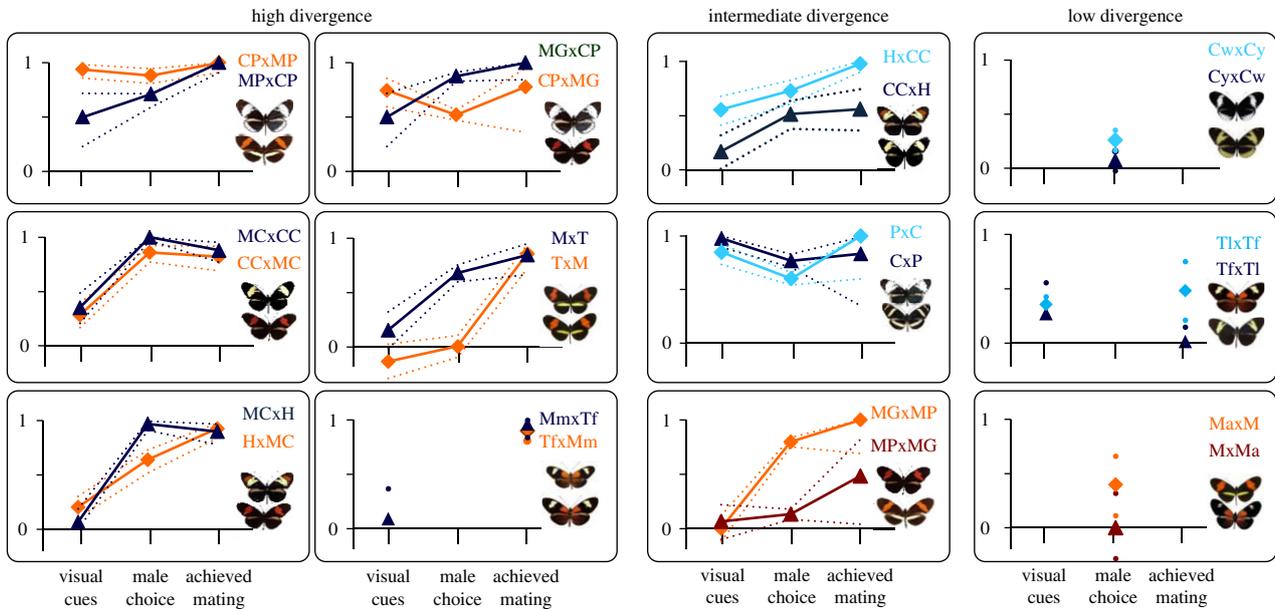
Figure 3. 'RI' associated with each behavioural pre-mating barrier to gene flow. For each pair of species, the two colours correspond to the two possible directions of heterospecific mating (female given first). Dotted lines are the confidence intervals.

progressively replace the local *H. melpomene* representative, and are also associated with closed forested habitats.

(b) Behavioural pre-mating isolating barriers
(i) Visual cues
At high divergence, isolation due to male preference based on models (visual cues only) is strong for pairs with different colour patterns (figure 3). It is generally higher in the direction involving *H. melpomene* males ($RI_{colour} = 0.75$–0.94, except for *H. c. cordula*/*H. m. melpomene* at $RI_{colour} = 0.28$) than in the other direction involving cydno-clade males ($RI_{colour} = 0.35$–0.5). Colour preference is lower for *H. heurippa*/ *H. m. melpomene* than between other pairs diverging in colour pattern ($RI_{colour} = 0.07/0.2$), likely due to the intermediate pattern of *H. heurippa*, which includes the red band of *H. m. melpomene*. In the co-mimetic pairs, males do not discriminate between models, as expected given the high visual similarity of the two species.

At intermediate divergence, colour preference remains an isolating factor, although its strength varies depending on the pair considered. RI_{colour} reaches $0.85/0.98$ for *H. c. galanthus*/ *H. pachinus* but only $0.17/0.56$ for *H. heurippa*/*H. c. cordula*. It is zero for the allopatric *H. m. rosina*/*H. m. melpomene*, probably because of the red forewing band shared by the two subspecies.

At low divergence, between *H. t. florencia* and *H. t. linaresi*, some preference is observed ($RI_{colour} = 0.27/0.35$).

(ii) Male choice
At high divergence, male preference for conspecific over heterospecific living females is stronger than observed with models (figure 3), suggesting that a wider range of proximal cues are available, such as chemical signals or behavioural cues, and influence male courtship decision leading to a higher RI ($RI_{malechoice} = 0.64$–1).

The use of proximal versus long-range visual cues by males seems to depend on the direction of the heterospecific interaction: *H. melpomene* males indeed respond to wing models with a very strong choice based on colour cues, and

appear to show little discrimination when presented with females with a similar pattern (*timareta*). By contrast, *H. cydno* or *H. heurippa* males show some discrimination against *H. melpomene* models, but it is weaker than for *H. melpomene* males [19,34], and choice is generally enhanced by real-females cues. Moreover, in the mimetic pair, *H. t. thelxinoe* males strongly prefer conspecific over heterospecific females using close-range chemical cues [22].

At intermediate and at low divergence, a limited amount of RI due to male courtship behaviour is sometimes observed ($RI_{malechoice} = 0.5$–0.78 and 0–0.4, respectively), although the strength of isolation is generally weaker and more asymmetric than at high divergence.

(iii) Achieved mating
At high divergence, the total index of sexual isolation is high for all pairs and in both directions of crosses ($RI_{mating} = 0.78$–1). RI estimated using achieved mating is higher than when estimated based on model or live-female experiments (figure 3), suggesting that female response and contact interactions (beyond male courtship) also contribute to pre-mating isolation, especially for the mimetic pairs.

At intermediate divergence, isolation is generally high, though asymmetric (table 1), such as for *H. c. cordula*/ *H. heurippa* ($RI_{mating} = 0.56/0.98$) or between allopatric populations of *H. melpomene* ($RI_{mating} = 0.65/1$). RI estimated on total mating is again higher than RI estimated on experiments with models, suggesting that close-range cues and male–female interactions may also be relevant at intermediate divergence.

By contrast, at low divergence between the parapatric races *H. t. florencia*/*H. t. linaresi*, RI is much lower. It is observed only in one direction (TnxTf, $RI_{mating} = 0.48$) and largely explained by colour pattern preference.

(c) Post-mating isolating barriers
(i) F₁ egg and larval survival
At high divergence, F_1 hybrids show no significant reduction in hatch rate leading to a null contribution to RI (table 1).

Oviposition preferences for different *Passiflora* hosts generally constitute an axis of differentiation between the melpomene-clade and the cydno-clade, *H. melpomene* being generally more specialized than its local cydno-clade counterpart [18,37,47] with some exception in Colombia where *H. melpomene* has a diverse range of oviposition plants [48].

Hybrid larval survival has only been tested in three pairs at high divergence but shows no significant reduction in survival, leading to a null contribution to RI (table 1). This suggests neither hybrid viability breakdown related to genetic incompatibilities nor incapacity to metabolize the host plant are acting in these pairs. For *H. c. cordula*/*H. m. melpomene* and *H. heurippa*/*H. m. melpomene* hybrids (electronic supplementary material, table S7), this result corresponds to expectations because the hybrids were fed on a common host plant (*Passiflora oesterdii*). However, this may be surprising for the *H. t. thelxinoe*/*H. m. amaryllis* hybrids, which were fed on the maternal host plant (electronic supplementary material, table S4).

Testing survival in experimental conditions with unlimited access to food, fewer parasites, and no competition might have underestimated the importance of efficient host-plant use in hybrid growth. We can note, for instance, that, in semi-natural conditions, early-stage *H. melpomene* larvae from central America had a higher survival rate on *Passiflora menispermifolia* than on other *Passiflora* species [47], while in insectaries, similar growth rates have been achieved for various species of *Passiflora* [49]. In Peru, several preliminary attempts of feeding *H. m. amaryllis* larvae and backcrosses towards *H. m. amaryllis* with *Passiflora edulis* or *Passiflora granadilla* (well accepted by *H. t. thelxinoe*) led to higher mortality rate.

(ii) F$_1$ adult survival

Adult mortality due to predation was estimated only for the hybrids between *H. c. chioneus*/*H. m. rosina*. Its contribution to isolation was significant with RI = 0.35.

In the co-mimetic pairs, F$_1$ hybrids are visually similar to the parents and predation is not expected to participate in RI.

In other cases, F$_1$ hybrids may also be similar to one parent (*H. c. galanthus*/*H. pachinus* hybrids being like *H. c. galanthus* [20], *H. heurippa*/*H. m. melpomene* hybrids being similar to *H. m. melpomene* [25]). For *H. c. alithea*, heterozygotes at the K locus of *H. c. alithea* are white [33], which introduces asymmetry in isolation and an estimated RI$_{adult\ survival}$ of 0.36 or 0, respectively, in areas dominated by yellow or white, thus mean RI$_{adult\ survival}$ = 0.18.

(iii) F$_1$ mating success

At high divergence, in non-mimetic as well as co-mimetic pairs, mate discrimination against F$_1$ hybrids appears as an additional isolating barrier (figure 2), although its strength is highly variable and asymmetric, depending on the parental partner tested (RI$_{F1success}$ = 0–0.87, (table 1) electronic supplementary material, tables S5–6, S8–9).

At intermediate divergence, for *H. c. galanthus*/*H. pachinus* F$_1$ hybrids, whose phenotype is similar to the *H. c. galanthus* parent, mating discrimination is also exerted by *H. pachinus* males, resulting in asymmetric isolation (RI$_{F1success}$ = 0/0.94).

(iv) Fertility of F$_1$ adults

At high divergence, the estimated isolating strength of hybrid sterility is intermediate compared with other factors (figure 2) and asymmetric (table 1) (RI$_{fertility}$ = 0.27–0.48 in one direction, RI$_{fertility}$ = 0–0.34 in the other direction).

F$_1$ males are fully fertile except for the allopatric pair *H. c. chioneus*/*H. m. melpomene* which show a slight reduction in fertility [24].

Female F$_1$ fertility is more complex. All studies involving crosses between a *H. cydno*/*heurippa*/*timareta* mother and a *melpomene* father found complete sterility of female F$_1$ (electronic supplementary material, table S4) [24,25]. In the other direction of crosses, i.e. a *melpomene* mother and a *cydno*/*timareta*/*heurippa* father, F$_1$ fertility is highly variable. At the extremes, all *H. m. melpomene* × *H. heurippa* females tested were fully fertile [25], whereas *H. m. melpomene* (French Guiana) × *H. c. chioneus* (Panama) females were all sterile [24]. For most other pairs, partial fertility was reported [24,43] (electronic supplementary material, table S10) with an intriguing non-uniform pattern. For instance, in *H. m. amaryllis* × *H. t. thelxinoe* hybrids, some hybrid females had a lower fertility than pure females, while others were completely sterile and others completely fertile (electronic supplementary material, table S4).

At intermediate or low divergence, no significant reduction in fertility was found except for the allopatric pair *H. m. rosina* (Panama)/*H. m. melpomene* (French Guiana) with lower fertility for F$_1$ female (and possibly males) hybrids [50], resulting in RI$_{fertility}$ = 0.43 in one direction.

4. Discussion

Quantifying reproductive isolation throughout a speciose clade of *Heliconius* butterflies shows that different levels of genetic divergence correspond to marked quantitative and qualitative differences in RI. Higher divergence is associated with both the accumulation of additional barriers and the strengthening of a common set of barriers, although some axes of differentiation are quite labile depending on the ecological context.

The diversity of taxa at different levels of divergence and strengths of reproductive isolation has been characterized as a 'speciation continuum'. This does not necessarily imply that these actually represent sequential stages in speciation, nor that any particular example is on an inevitable path towards complete speciation. For example, different stages might be at equilibrium between divergence and gene flow or correspond to qualitatively different pathways to differentiation. Nevertheless, the 'speciation continuum' is useful and perhaps analogous to the manner in which those studying the evolution of complex structures, such as the eye or the flagellum, infer past evolutionary trajectories from the comparative study of apparently intermediate structures in extant animals. Such examples provide support for the plausibility of a particular route towards a complex structure, or in the present case a route towards complete speciation, but do not prove that any particular evolutionary route has been taken in nature. Our analysis therefore allows assessment of the roles that different factors might take in shaping divergence, while accepting that the current array of divergence states does not necessarily represent successive stages along a unique path to speciation.

(a) Is reproductive isolation driven by a single trait or multi-dimensional factors?

Isolation in the face of gene flow requires that certain factors counter the effects of recombination between alleles that

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characterize diverging taxa [8,51–53]. This might include strong disruptive selection on a single (large-effect) trait [54], an association between ecological divergence and reproductive isolation (via a 'magic' trait, for instance [29]), or the coupling of several isolating barriers [51]. Diverging *Heliconius* taxa showing a shift in colour pattern meet all those criteria, making colour pattern divergence a major initiator and driver of reproductive isolation in this group [28,35].

Given that colour-pattern differentiation underlies the main isolating barriers (predation, mate choice, and habitat partitioning) and that all those barriers operate at low, intermediate, and high divergence, one may wonder whether increased isolation results from the 'stronger selection' scenario [54], under which barriers associated with colour pattern differences are strengthened along the continuum of divergence. This is the case, for instance, in *Pundamilia* cichlid fish, in which increased isolation is associated with increased divergence on one main axis of differentiation: male colouration in relation to habitat transparency [55]. The alternative hypothesis would be that increased isolation is the product of 'multifarious selection' [54], with the addition of independent traits and more isolating barriers at higher divergence [56,57]. For instance, between colour-pattern races of poison frog, isolation is much higher for a pair which also exhibit size differences associated with habitat specialization [58].

Those predictions can be tested by comparing the strength of the barriers potentially associated with colour pattern divergence along the *Heliconius* continuum. The lower stages of divergence reported in *Heliconius* correspond to wing-pattern races, for which selection causes genetic differentiation only around wing-patterning loci [39] and maintain weak isolation. At this stage, selection on different mimicry associations maintains spatial segregation through predation against migrants [31,32], and is likely to cause post-mating isolation through predation against non-mimetic hybrids. The third barrier, male preference based on colour, is already acting at low-divergence, but its contribution is variable and asymmetric. What is the fate of those barriers at higher divergence? Isolation due to *predation* against hybrids has not been quantified in many pairs of taxa. It does appear stronger for the *H. c. chioneus × H. m. rosina* hybrids (high divergence), than for *H. c. alithea* F₁ (low divergence) for instance. It is worth noting that predation itself is of the same magnitude in both cases, reducing the survival of any deviant form by about 30%. Reproductive isolation due to predation is thus lower in *C. alithea* heterozygotes because they are similar to one parent (white), while *H. c. chioneus × H. m. rosina* hybrids differ from both parents and suffer from predation in all habitats. Therefore, isolation against hybrids depends on dominance and segregation of colour patterns in hybrids, with the hybrid being generally more different at a higher level of divergence (except for the mimetic pairs). *Habitat partitioning* gets stronger at high divergence. Just like for pairs of taxa at low divergence, fine-scale partitioning between taxa at high divergence may follow the distribution of their co-mimics, as observed, for instance, between *H. c. chioneus* and *H. m. rosina* across the transition from closed forest to edge habitat [23]. However, habitat specialization for closed forests is also exhibited by other members of the cydno-clade such as *H. timareta* (co-mimic with *H. melpomene*) or *H. heurippa* (no co-mimic), suggesting that microspatial partitioning at high divergence is not only conditioned by mimicry, but also by other ecological preferences which remain unknown but may involve abiotic conditions,

adaptation to altitude, or host plants. The component of *mate choice* clearly attributable to visual cues, deduced from experiments with models, is generally strengthened at high and intermediate divergence, though not consistently between species. In addition, assortative mating is likely to involve a chemical component for most pairs of taxa at high divergence. Again, as hybrids tend to be quite different from parental species at higher divergence, sexual selection against hybrids is also stronger at high divergence. Overall, increased isolation does involve a strengthening of isolating barriers directly linked to colour pattern differences, but higher reproductive isolation also rests largely on the addition of other isolating dimensions.

To assess the relative importance of colour pattern shift at later stages of speciation, it is also useful to consider species pairs that do not exhibit colour pattern divergence, such as the co-mimics *H. timareta*/*H. melpomene*. Genomic evidence suggests that these species were initially divergent in colour pattern and became co-mimics after secondary introgression of wing-pattern alleles from *H. melpomene* into *H. timareta* [59]. Under this scenario, if colour pattern divergence plays an important role in the isolation of species at higher divergence, reproductive isolation is expected to be weakened secondarily by mimicry and gene flow. Such collapse of differentiation has sometimes been observed, notably between pairs of taxa that rely on one main axis of differentiation, habitat related, for instance [60]. Compared with *H. c. chioneus*/*H. m. rosina*, the co-mimics *H. t. thelxinoe*/*H. m. amaryllis* indeed display an approximately 2% reduction in total estimated reproductive isolation and a slightly lowered genomic divergence [61]. Both in the Colombian and Peruvian mimetic pairs, natural hybrids are also marginally more frequent (1–3%) [18,37]. This reduction in reproductive isolation between co-mimics follows the prediction but shows that lifting the wing-pattern barrier has a rather limited effect on species differentiation because reproductive isolation relies on multiple other isolating mechanisms (habitat specialization, assortative mating based on chemical communication [22], partial hybrid sterility, and likely host-plant divergence). This implies that reproductive isolation between pairs at a high level of divergence is strong enough to allow the secondary loss of certain barriers to gene flow, in this case via the introgression of wing-pattern alleles, without compromising genome-wide differentiation. Consistent with this idea, but at yet deeper levels of divergence within the genus *Heliconius*, the co-mimics *H. erato* and *H. melpomene*, are visually attracted to each-other yet never hybridize, owing to strong differences in other courtship signals and natural history [62]. Generally, our analysis supports the hypothesis that multiple diverging dimensions add cumulatively to reproductive isolation and favour the completion of speciation in the face of gene flow [54].

(b) How do isolating mechanisms evolve?

The continuum of reproductive isolation spanned in this study also corresponds to a continuum of time since divergence, raising the questions of how the multiple barriers accumulate through time, which result from selection, which are a by-product of isolation through drift, and what is the relative importance of ecological and non-ecological processes.

Pre-mating sexual isolation stands out as one of the strongest barriers at all levels of divergence and gets stronger along the continuum of divergence. This observation is consistent with the rapid evolution of pre-mating isolation generally

reported for speciation with gene flow [2], in fish [7,63], *Drosophila* [45], or plants [56]. As with darter fish [64], the rapid evolution of strong assortative mating in *Heliconius* appears to be associated with sexual selection, notably for chemosensory traits [65] which, as indicators of mate quality, are common targets of sexual selection [66].

An increase in pre-zygotic isolation between hybridizing populations may also reflect reinforcement, under selection against interspecific mating [67]. In *Drosophila*, for instance, the fast evolution of mate choice has been linked to reinforcement processes, with pre-mating isolation being stronger for pairs with geographical overlap [45] and pairs with higher hybridization costs [68]. Here, higher stages of divergence are characterized by a decrease in hybrid fitness, such that stronger pre-mating isolation may reflect stronger selection against hybridization. In addition, the higher geographical overlap seen in pairs at high divergence also provides more opportunities for selection against hybridization to operate. Evidence for reinforcement comes from higher pre-mating isolation observed in the sympatric *H. c. chioneus*/*H. m. rosina* than in the allopatric *H. c. chioneus*/*H. m. melpomene* as well as an increased mate choice between *H. c. galanthus* and *H. pachinus* in populations close to the contact zone [41].

Under a hypothesis of reinforcement, pre-mating isolation comes as a response to hybrid unfitness, so it may seem paradoxical to observe rather weak or moderate post-mating barriers. It could be that their current contributions do not reflect their past importance or that the accumulation of several weak barriers is sufficient to select for assortative mating. Our analysis may also underestimate the strength of extrinsic post-mating barriers, which are experimentally more difficult to assess. Notably, little is known about the ecology of hybrids, and poor hybrid performance may represent a significant barrier when parental species occur in markedly different microhabitats (e.g. altitude for *H. timareta*/*H. melpomene*).

Habitat specialization associated with fine-scale spatial segregation and host-plant divergence is observed for all pairs at high divergence but for none at low divergence. Interestingly, parapatric species at intermediate divergence do not show clear habitat or host-plant differences either, suggesting that habitat specialization might be one of the key barriers allowing geographical overlap and leading to high divergence. Such a transition from parapatric, ecologically similar morphs to overlapping microhabitat-specialized taxa is also reported along the stickleback speciation continuum [7] and perhaps constitutes a tipping point in the evolution of isolation [10].

The last post-mating barrier widely observed at high divergence but generally absent at lower levels of divergence is hybrid female sterility (with the exception of allopatric races of *H. melpomene* [50]). This result is quite general in the literature: when speciation occurs with gene flow, post-mating incompatibilities tend to accumulate more slowly than ecological and pre-mating isolation [45,63,69], and follow Haldane's rule by first affecting the heterogametic sex [70]. Generally, the strongest isolation was found between allopatric pairs

coming from distant areas (Panama versus French Guiana), whereas in sympatry, F_1 female sterility can be variable, from fully sterile to fully fertile, suggesting that sterility is variably affected by local gene flow. *Heliconius* female sterility is typically caused by interactions between the Z chromosome and autosomal loci [24,25,50]. Among sympatric pairs of taxa at high divergence such as *H. timareta*/*H. melpomene* or *H. cydno*/*H. melpomene*, Z chromosomes are very divergent, while autosomes show a strong signal of admixture [61]. Admixture might prevent the accumulation of incompatibilities on autosomes (or may allow its purge following secondary contact), therefore limiting the evolution of female sterility. Such a hypothesis would question the stability of this intrinsic barrier, traditionally assumed to be irreversible.

5. Conclusion

We have quantified most of the known components of reproductive isolation across a recent adaptive radiation. Contrasting pairs of hybridizing taxa showing different levels of divergence suggest that speciation involves the strengthening of some isolating barriers but, importantly, seems to require the accumulation of additional barriers. Indeed, the synergistic action of wing pattern shifts and other isolating mechanisms appears to be important for reproductive isolation in *Heliconius*, especially at early stages of divergence. Nevertheless, the case of co-mimetic hybridizing species reveals that certain isolating barriers, and especially wing pattern differences, may in fact be quite labile or partially reversible. This shows that a seemingly key factor in the early stages of differentiation may have its role taken over by other barriers at later stages of divergence. A key promoter of the stability and completion of species divergence thus appears to be the multi-dimensionality of reproductive isolation.

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