ASSORTATIVE MATING PREFERENCES AMONG HYBRIDS OFFERS A ROUTE TO HYBRID SPECIATION

Maria C. Melo,1,2 Camilo Salazar,3,4 Chris D. Jiggins,5,6 and Mauricio Linares1,7

1Instituto de Genética, Universidad de los Andes, Carrera 1E No 18a–10, P.O.Box 4976, Santafé de Bogotá D.C., Colombia.
   2E-mail: mar-melo@uniandes.edu.co
3Smithsonian Tropical Research Institute. Apartado 0843-03092, Panamá, República de Panamá
   4E-mail: salazarc@si.edu
5Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ. United Kingdom
   6E-mail: c.jiggins@zoo.cam.ac.uk
6E-mail: mlinares@uniandes.edu.co

Received October 8, 2008
Accepted December 26, 2008

Homoploid speciation generates species without a change in chromosome number via introgressive hybridization and has been considered rare in animals. Heliconius butterflies exhibit bright aposematic color patterns that also act as cues in assortative mating. Heliconius heurippa has a color pattern that can be recreated by introgression of the H. melpomene red band into an H. cydno genetic background. Wild H. heurippa males show assortative mating based on color pattern and we here investigate the origin of this preference by studying first-generation backcross hybrids between H. melpomene and H. cydno that resemble H. heurippa. These hybrids show assortative mating preferences, showing a strong preference for their own color pattern over that of either parental species. This is consistent with a genetic basis to wing pattern preference and implies, first, that assortative mating preferences would facilitate the initial establishment of a homozygous hybrid color pattern by increasing the likelihood that early generation hybrids mate among themselves. Second, once established such a lineage would inherit assortative mating preferences that would lead to partial reproductive isolation from parental lineages.

KEY WORDS: Homoploid speciation, introgression, mating preference, pleiotropy, transgressive segregation.

Homoploid hybrid speciation is the establishment of a reproducively isolated lineage through hybridization without a change in chromosome number (Coyne and Orr 2004). It is therefore distinct from the numerous examples of hybrid speciation through allopolyploidization in plants (Ramsey and Schemske 2002). Although skepticism about the importance of this process in animal speciation remains, several cases of homoploid hybrid speciation have recently been reported (Mallet 2007; Mavérez and Linares 2008).

The best-studied case of homoploid hybrid speciation is in Helianthus sunflowers. Large blocks of the genome of Helianthus anomalus are derived in similar proportions from each of its putative parental species (Ungerer et al. 1998). In this case, genes involved in epistatic hybrid incompatibilities contribute to isolation of the hybrid lineage from its parental taxa (Buerkle et al. 2000). Moreover, the generation of novel ecological traits through hybridization has also facilitated establishment of the hybrid lineage (Rieseberg et al. 2003). In contrast to the situation in Helianthus, hybrid species might also form through introgression of just a few loci from one species into another. If such introgression were to generate a novel adaptive trait(s) with pleiotropic effects on reproductive isolation it could lead to rapid speciation (Jiggins et al. 2008). We have recently termed this model “hybrid trait speciation” (Jiggins et al. 2008). This process might play an
important role in recent radiations in which genetic incompatibilities between hybridizing species are absent. In effect, “hybrid trait specification” is an extension of the “magic trait” model of speciation, in which divergence in a single trait under ecological selection also causes reproductive isolation as a side effect, facilitating speciation (Gavrilets 2004). Examples include beak morphology in Darwin finches, which is correlated with vocalizations, shifts in color patterns and female preferences in sticklebacks, and disruptive selection in predatory mimicry associated with assortative mating in hamlet fish (Boughman 2001; Podos 2001; Puebla et al. 2007).

Heliconius butterflies are known for their aposomatic color pattern diversity and usually form Müllerian mimicry rings both with other Heliconius species and more distantly related taxa (Jiggins et al. 2001). The genetic basis of these color patterns can be studied through artificial hybridization (Naisbit et al. 2003), and such experiments show that few loci of major effect and some modifiers that improve the mimetic resemblance are involved in controlling color pattern diversity (Naisbit et al. 2003). Natural hybridization and gene flow can occur at various levels of divergence, from races through species (Mallet et al. 2007), suggesting that hybridization could have a role in shaping diversity in this genus.

Helianthus cydno and H. melpomene are good examples of naturally hybridizing species (Mallet et al. 2007). Although sympatric across much of their range, they show distinct ecology and color pattern phenotype (Estrada and Jiggins 2002). Heliconius melpomene usually has a red and yellow color pattern and mimics H. erato, whereas H. cydno has white and yellow patterns and mimics species in the sara-sapho clade. This shift in color pattern due to mimicry also acts as a cue in mating preference between these species (Jiggins et al. 2001). Interestingly, three species belonging to the H. cydno species complex, H. timareta, H. pachinus, and H. heurippa have all been proposed as hybrid species (Linares 1989; Mallet 1999; Gilbert 2003). In particular, the color pattern of H. heurippa was reconstructed by laboratory crosses between the adjacent geographical races of H. cydno and H. melpomene (Mavárez et al. 2006). Heliconius cydno cordula and H. melpomene melpomene have yellow and red bands, respectively, and are found in sympathy near to the current range of H. heurippa (Mavárez et al. 2006). Previous work has shown that H. heurippa uses its color pattern as a cue in assortative mating leading to reproductive isolation (Mavárez et al. 2006). However, nothing is known of the origin of this mate preference. It has been speculated that the distinctive mate preferences of H. heurippa might have arisen subsequent to the establishment of the novel color pattern, in populations already fixed for the H. heurippa pattern (Mavárez et al. 2006). Alternatively, combining mate preference alleles inherited from the two parental species might have led to the establishment of the novel pattern of assortative mating more directly through the process of hybridization. Here we investigate the latter possibility by testing the mating preference of first-generation backcross hybrids with a color pattern similar to that of H. heurippa.

Methods

Experimental Populations and Reconstruction of Heliconius Heurippa

Heliconius melpomene melpomene, H. heurippa, and H. c. cordula were collected in Virgen de Chirajara (Colombia, 4°12'48"N, 73°47'70"W), Buenavista (Colombia, 4°10'30"N, 73°40'41"W) and San Cristobal (Venezuela, 7°47'56"N, 72°11'56"W), respectively. These sites are located in the eastern Colombian Andes. Experimental populations for each species were established from field mated females in 2 × 3 × 2 m insectaries in La Vega, Colombia following methods described previously (Jiggins et al. 2001). Here we study first-generation backcross hybrids that phenotypically resemble H. heurippa. First, a female H. c. cordula was mated with an H. m. melpomene male. F₁ males were then backcrossed to H. c. cordula females and the phenotypic class of backcross offspring most resembling H. heurippa was used for mate choice experiments. The selected hybrids had Bb N°N NN Brbr color pattern genotypes (following the Mavárez et al. 2006 loci nomenclature), being heterozygous for the red forewing band (B), homozygous for the yellow forewing band (N), and heterozygous for the brown pincer-like mark (Br, see Mavárez et al. 2006). Note that these are not the “reconstructed” H. heurippa phenotypes reported previously that are genetically homozygous for all major color pattern alleles, and they differ somewhat from H. heurippa in possessing the heterozygous brown pincer mark on the underside of the hindwing (see fig. 2 in Mavárez et al. 2006). Nonetheless, we are specifically interested in these individuals for two reasons. First, the most direct route to generation of a homozygous H. heurippa pattern is through intercrossing these phenotypes, so we are interested in the probability that these phenotypes would mate in the wild (Mavárez et al. 2006). Although the relative probability of establishment of this particular pattern would require knowledge of mating preferences among all hybrid phenotypes, nonetheless the establishment of any hybrid pattern would be facilitated if hybrids mate assortatively. Most importantly, however, these are the first generation of hybrids to show the combined red and yellow forewing band characteristic of H. heurippa, so we are interested in the establishment of assortative mating preferences among individuals that possess this phenotype.

Color Pattern Models

In butterflies males generally search for females using visual cues (Rutowski 1991; Vane-Wright and Boppré 1993). In particular,
Heliconius males are attracted by bright colors at distances of 20 m or more. Thus, males appear adapted to use bright color patterns as a long-range attraction signal to detect mates (Jiggins et al. 2001, 2004). At close quarters, males also show preferences based in part on color pattern, in general preferring their own color pattern. In addition, pheromonal and/or tactile signals are also important at a close range (Jiggins et al. 2001; Jiggins et al. 2004).

The preference of Heliconius heurippa for the hybrid color pattern

Five males of H. c. cordula were exposed to female wing pattern models in 2 × 2 × 2 m insectaries. In each trial, one model was a control, having the same pattern as the males being tested. The other corresponded to the experimental color pattern, in this case the hybrid wings described above. Models were made from dissected natural wings and hung by transparent nylon lines in the center of spherical area of 60 cm (Jiggins et al. 2001). Entering to the sphere was considered an approach, whereas a sustained fluttering directed toward the model was a courtship (Jiggins et al. 2001). Approaches and courtships were recorded in 25 replicates of 5 min each. Males were replaced in each replica. This experiment was repeated for H. heurippa and H. m. melpomene males.

Reconstructed Heliconius heurippa mating preference

The backcross hybrid males obtained here were then tested for their preference using the same experimental design except that individuals were tested individually (i.e., each reconstructed male was tested with its own pattern and with a different pattern at the same time). Hybrid males were separately tested for their preference for H. heurippa, H. c. cordula, and H. m. melpomene patterns, relative to a control (see results for details).

Statistical analysis

For both experiments the corresponding probabilities of approach and courtship were calculated using a likelihood. The model used here was developed and maximized following Jiggins et al. (2001) and Mavárez et al. (2006). For all experiments, a probability value of one was assigned to the control color pattern model with the same pattern as the males being tested, with the objective being to estimate relative probabilities of experimental approach and courtship. A likelihood-ratio test (LRT) with \( G = 2 \Delta \log L \), which asymptotically follows a \( \chi^2 \) distribution (Edwards 1972), was used to determine whether probabilities of approach/courtship directed toward the experimental model differed from probabilities of approach/courtship directed toward the control pattern model.

Results

Heliconius heurippa males did not distinguish between their own color pattern and that of the backcross hybrid model, that is the laboratory created BbN\( ^N \)N\( ^N \) Brbr hybrid with the pattern similar to H. heurippa (hereafter the backcross model, Fig. 1). Neither approach nor courtship probabilities differed between control and experimental patterns \( (G_1 = 1.363, P > 0.05 \) and \( G_1 = 0.981, P > 0.05 \), respectively). In contrast both H. melpomene and H. cydno males were significantly less likely to approach the backcross model as compared to their own pattern \( (G_1 = 67.736, P < 0.05 \) and \( G_1 = 32.825, P < 0.05 \), respectively). This reflects the pattern seen when these two species were evaluated for their courtship behavior toward the wild H. heurippa color pattern (Mavárez et al. 2006). Assortative mate preferences for courtship behavior were even stronger than that for approach (Fig 1; \( G_1 = 60.64, P < 0.05 \) and \( G_1 = 51.741, P < 0.05 \) respectively).

Sixteen reconstructed H. heurippa individuals were obtained from seven F\( _1 \) males backcrossed to H. cydno females. A total of 881 approaches and 132 courtships were recorded when these individuals were tested. Unsurprisingly, these individuals do not distinguish H. heurippa and backcross models \( (G_1 = 0.397, P > 0.05 \) and \( G_1 = 0.581, P > 0.05 \), respectively). Subsequently, H. heurippa models were used as controls in experiments with these hybrids that involve parental species models (approach and courtship data are shown in Table 1). In both experiments, the backcross hybrid males were significantly less likely to approach either parental pattern as compared to that of H. heurippa \( (G_1 = 90.302, P < 0.05 \) and \( G_1 = 7.586, P < 0.05 \) for approach and courtship of H. cydno, respectively and \( G_1 = 65.267, P < 0.05 \) and \( G_1 = 14.699, P < 0.05 \) for approach and courtship of H. melpomene, respectively). In addition, there was significant heterogeneity between individuals in their response to the parental patterns, \( (G_{15} = 139.6, P < 0.05 \) and \( G_{15} = 95.0, P < 0.05 \) for experiments with H. cydno and H. melpomene, respectively). Nonetheless, all individuals showed more approaches to the H. heurippa pattern so the direction of preference was consistent between individuals. In contrast, there was no heterogeneity among this same set of individuals when tested against H. heurippa vs. backcross hybrid models \( (G_{15} = 5.4, \text{NS}) \).

Discussion

We have shown that the first-generation backcross hybrids between H. cydno and H. melpomene that phenotypically resemble H. heurippa (BbN\( ^N \)N\( ^N \) Brbr color pattern genotypes) show similar mate preferences to wild H. heurippa. In addition, the parental species, H. cydno and H. melpomene, discriminate against the color pattern of these first-generation backcross hybrids in a manner similar to their response to wild H. heurippa. This suggests that hybrid offspring of interspecific crosses could become established as populations partially reproductively isolated from their parental species very rapidly, and therefore offers insights into the
route by which *H. heurippa* could have become established as a hybrid species.

Although we show strong mating preferences among hybrid individuals, our experiments do not resolve exactly how these came about. It seems most likely that preferences have a genetic basis, but we cannot rule out a learned response given our current data. Nonetheless, previous experiments have shown no evidence that *H. melpomene* males learn their color pattern preferences from contact with other individuals (Jiggins et al. 2004). It remains possible that individuals might be able to somehow determine their own color pattern and thereby learn an assortative mating preference, although this is perhaps unlikely. Previous experiments in which males were allowed to emerge in the dark and prevented from visual inspection of their own patterns had no effect on mating behavior (Crane 1955). In addition, *H. cydno* and *H. pachinus* are predominantly white and yellow, respectively. Their F₁ hybrids are phenotypically similar to the *H. cydno* parents, but show intermediate mate preferences as expected for a genetically determined, rather than a learned response (Kronforst et al. 2006). The genetic mapping of preference would otherwise be consistent with individuals learning to prefer their own patterns, which would make “preference” a direct pleiotropic effect of color pattern phenotype (Kronforst et al. 2006). Similarly, F₁ hybrids between *H. melpomene rosina* (red forewing band) and *H. cydno chioneus* (white forewing band) from Panama prefer mating with themselves more frequently than with either species, despite being phenotypically most similar to *H. melpomene* (Naisbit et al. 2001). Hence, the most likely explanation for the hybrid mate preferences observed here is that they have a genetic basis, such that genes for preferring yellow from *H. cydno*, and those for preferring red from *H. melpomene* combine to produce the hybrid mate preference for a yellow and red pattern. This could be considered a form of transgressive segregation, whereby hybrid trait values lie outside the range of the two parents (Mallet 2007). Hence, a new aposematic signal coupled with a novel mating preference could have been rapidly generated by hybridization.
Further crosses will be required to determine the genetic basis of preference that leads to this pattern.

We have previously proposed a scenario for the origin of *H. heurippa* whereby *H. cydno* and *H. melpomene* initially overlapped and hybridized near to the current range of *H. heurippa*. A period of reduced intensity of stabilizing selection on color pattern could have led to an increase in the number of hybrids, as observed currently in a natural population in San Cristobal, Venezuela (Mavarez et al. 2006). As selection was established again, whichever novel hybrid form was most abundant and had a successful aposematic signal would have become favored by positive frequency-dependent selection and been driven to fixation.

We previously speculated that the proto-*H. heurippa* would have gained assortative mating preferences, and hence reproductive isolation from parental species, subsequent to its establishment as a geographic population with a distinct color pattern phenotype. However, the data presented here suggest a much more direct route whereby assortative mating would arise as an instantaneous result of the hybrid origin of the new color pattern. Furthermore, the data also imply that in the early stages of hybridization, sexual selection would have favored the establishment of proto-*H. heurippa* hybrids as they would have mated most readily with other such hybrids. Nonetheless, determining the relative probability of establishment of the *H. heurippa* pattern, as opposed to alternative hybrid phenotypes, would require knowledge of the mating preferences for all hybrid genotypes, combined with an estimation of the frequency with which they are generated in natural populations. Similar modeling of other hybrid speciation scenarios suggests that this is likely to be a fruitful approach if further mate preference data can be obtained (Buerkle et al. 2000; Gavrilets et al. 2007).

Several examples of animal homoploid hybrid species have been proposed but in most cases little is known about how they attained reproductive isolation from their parental species (Mallet 2007; Mavarez and Linares 2008). In *Rhagoletis* sp., *Lycaeides* sp., and *Cottus gobio* group, novel ecological adaptations have been identified that might have contributed to reproductive isolation in sympathy/parapathy (Mallet 2007; Mavarez and Linares 2008). However, not all homoploid hybrid species have shown ecological differentiation from parents, suggesting other mechanisms such as sexual selection could be involved in reproductive isolation. Studies on *Lycaeides* sp., *Rhagoletis* sp., and cichlid fish have shown that hybrid species exhibit assortative mating with their parents (Mavarez and Linares 2008). In the case of *Xiphophorus clemensiae*, a parental female mating preference for long-tailed males enhances the opportunity for hybridization, but it is not clear how the intermediate tail length hybrid would be reproductively isolated from long- and short-tailed parents. Assortative mating was also recently observed in a homoploid hybrid *Pogonomymex* ant species and one of its parental species, but nothing is known of how these mate preferences arose (Schwander et al. 2008). Our experiments therefore extend previous results by showing how hybridization could have had a direct role in generating assortative mating in an incipient hybrid species.

**ACKNOWLEDGMENTS**

We would like to thank Banco de la República grant 2297, Los Andes University science faculty, and Fondo Colombiano de Investigaciones Científicas y Proyectos Especiales Francisco José de Caldas COLCIENTIAS grant 120440520138 for funding to CS and ML. CJ was funded by the Royal Society, BBSRC and the Leverhulme Trust.

**LITERATURE CITED**


---

**Table 1. Results of experiments on individual backcross males involving pure species models.**

<table>
<thead>
<tr>
<th>Male ID</th>
<th><em>H. cydno model</em></th>
<th><em>H. melpomene model</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cydno control</td>
<td>melpomene control</td>
</tr>
<tr>
<td>1</td>
<td>5 0 14 3</td>
<td>4 0 10 1</td>
</tr>
<tr>
<td>9</td>
<td>3 0 3 0</td>
<td>2 1 6 1</td>
</tr>
<tr>
<td>8</td>
<td>2 1 9 2</td>
<td>6 0 9 0</td>
</tr>
<tr>
<td>10</td>
<td>8 0 13 1</td>
<td>1 0 7 0</td>
</tr>
<tr>
<td>21</td>
<td>2 0 12 1</td>
<td>2 0 14 0</td>
</tr>
<tr>
<td>3</td>
<td>2 0 17 0</td>
<td>6 0 11 1</td>
</tr>
<tr>
<td>4</td>
<td>2 0 12 0</td>
<td>3 0 11 1</td>
</tr>
<tr>
<td>5</td>
<td>4 1 8 7</td>
<td>4 0 11 0</td>
</tr>
<tr>
<td>16</td>
<td>3 2 5 3</td>
<td>2 0 5 5</td>
</tr>
<tr>
<td>104</td>
<td>5 0 28 2</td>
<td>6 0 20 1</td>
</tr>
<tr>
<td>103</td>
<td>2 0 15 0</td>
<td>2 0 14 0</td>
</tr>
<tr>
<td>105</td>
<td>4 10 10 0</td>
<td>0 0 12 0</td>
</tr>
<tr>
<td>10–1</td>
<td>2 0 23 0</td>
<td>0 0 10 0</td>
</tr>
<tr>
<td>10–2</td>
<td>16 2 27 6</td>
<td>13 0 27 4</td>
</tr>
<tr>
<td>10–3</td>
<td>5 1 36 1</td>
<td>12 0 15 0</td>
</tr>
<tr>
<td>10–4</td>
<td>7 0 12 1</td>
<td>5 0 18 1</td>
</tr>
<tr>
<td>Total</td>
<td>72 17 234 37</td>
<td>70 1 200 15</td>
</tr>
<tr>
<td>Mean</td>
<td>4.50 1.06 14.6 2.31</td>
<td>4.38 0.063 12.50 0.938</td>
</tr>
<tr>
<td>SD</td>
<td>3.60 2.49 9.68 2.94</td>
<td>3.67 0.250 5.59 1.48</td>
</tr>
</tbody>
</table>


**Associate Editor: W. O. McMillan**