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Polymorphic Butterfly Reveals the Missing Link in Ecological Speciation

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Ecological speciation occurs when ecologically based, divergent selection causes the evolution of reproductive isolation. There are many empirical examples of this process; however, there exists a poorly characterized stage during which the traits that distinguish species ecologically and reproductively segregate in a single population. By using a combination of genetic mapping, mate-choice experiments, field observations, and population genetics, we studied a butterfly population with a mimetic wing color polymorphism and found that the butterflies exhibited partial, color-based, assortative mate preference. These traits represent the divergent, ecologically based signal and preference components of sexual isolation that usually distinguish incipient populations. Because mate choice in Heliconius butterflies provides a clear example of a trait involved in ecological speciation (12), Heliconius butterflies are chemically defended and warningly colored. Their evolutionary history has been marked by widespread divergence of color patterns among closely related species and geographic subpopulations (13). This divergence is combined with convergence among distantly related species as a result of natural selection for Müllerian mimicry (13), which is mimicry among mutually protected species. Furthermore, closely related species and geographic subpopulations that differ in mimetic wing patterns generally exhibit color pattern–based assortative mate preference, whereby males preferentially approach and court females that share their color pattern (14, 15). Hence, selection for mimicry may generate premating reproductive isolation and precipitate speciation. For example, H. cydno galanthus and H. pachinus in Costa Rica are closely related, interfertile species that have different wing color patterns as a result of divergent natural selection to match different mimicry models, H. sapho and H. Hewitsoni (Fig. 1A). DNA sequence data support the relative order of diversification, with the models being five times more divergent at mitochondrial DNA (mtDNA) than their mimics (16). The shift in mimicry between H. cydno and H. pachinus involves both color and pattern (Fig. 1B) and is accompanied by assortative mating (Fig. S1), which is mediated by male preference for white versus yellow wing color (14, 17). Because hybrids have rarely been collected (18, 19), it appears that this assortative mate preference prevents substantial hybridization where the two species meet (Fig. 1A).

In western Ecuador, H. cydno althea is locally polymorphic for the same white/yellow shift that generates premating isolation between H. cydno galanthus and H. pachinus in Costa Rica. As in Costa Rica, the color shift in H. cydno althea appears to be caused by selection for mimicry, with the yellow morph matching H. eleuchia and the white morph matching H. sapho (Fig. 1A). Again, DNA sequence data support the relative order of diversification (16). Furthermore, field observations have demonstrated that althea morph frequencies track those of H. sapho and H. eleuchia over time and space (20), and transplant experiments have demonstrated...
strated that the fitness of *alithea* morphs is determined by the local abundance of the models (20).

*H. cydno alithea* is polymorphic for both color (white versus yellow) and pattern (presence versus absence of melanin patches, Fig. 1A). The genetic basis of this color and pattern variation was determined by crosses (16), which revealed that color is controlled by a single Mendelian locus with a dominant white allele and a recessive yellow allele. Pattern is controlled by a second, unlinked Mendelian locus, with the presence of melanin dominant to the absence of melanin.

*H. cydno galanthus* and *H. pachinus* from Costa Rica differ at five major color patterning loci, two of which have virtually identical phenotypes to those seen segregating in *alithea*. The *K* locus, which is tightly linked to the gene *wingless* (14), controls white versus yellow, whereas the unlinked *Ac* locus (21) controls the presence versus absence of melanin in the same region of the forewing. Backcrosses between yellow *alithea* and white *galanthus* showed tight linkage between color and *wingless* [logarithm of the odds (lod) score = 11.0], just as in crosses between *galanthus* and *pachinus* (lod score = 18.2).

Assortative mate preference between *H. cydno galanthus* and *H. pachinus* is mediated by the *K* locus in that males recognize conspecific females based on color (14, 17). Furthermore, male preference for white versus yellow is physically linked to the *K* locus (14). To determine whether there is color-based mate preference in *H. cydno alithea*, we tested 36 wild-caught and 139 captive-reared males for their preference for white or yellow *alithea* females (16). In total, we observed 1644 courtships by 115 males (Fig. 2). White and yellow males had divergent courtship preferences (likelihood-ratio test, $G_{11} = 35.18$, $P = 2.32 	imes 10^{-4}$), with yellow males exhibiting a pronounced preference for yellow females (Wald’s test, $Z = 6.143$, $P < 10^{-9}$). White males had no ...

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**Fig. 1.** Parallel divergence and Müllerian mimicry in *Heliconius* butterflies. (A) In Costa Rica, co-mimetic species pairs are restricted to opposite drainages. In western Ecuador, *H. eleuchia*, *H. sapho*, and polymorphic *H. cydno alithea* (red arrows point to alternate *Ac* phenotypes) co-occur. (B) Spectral reflectance measurements of light (white or yellow) and dark (black or iridescent blue) wing patches show concordance between co-mimics and parallel divergence in Costa Rica and Ecuador (averaged across 10-nm intervals; error bars are standard deviation).

**Fig. 2.** Distribution of *H. cydno alithea* mate preference indices for yellow [yellow bars, maximum likelihood estimate = 0.36 (0.423 to 0.299)] and white [white bars, maximum likelihood estimate = 0.54 (0.604 to 0.475)] males. The preference index ($x$ axis) is the proportion of courtship and attempted mating events that were directed toward white females; a preference index of 1 indicates complete preference for white, whereas 0 indicates complete preference for yellow. All males with two or more courtship events are shown.
significant preference \((Z = 1.142, P = 0.254)\). Male preference was also affected by pattern differences: Yellow females with the melanic \(Ac\) patch were more attractive than yellow females without it \((G_{12} = 27.24, P = 7.14 \times 10^{-7}\)). Male \(Ac\) phenotype and status (wild versus captive) had no effect on preference. Field observations were also consistent with our experimental results, showing no color bias in white males' mating behavior \((G_1 = 0.97, P = 0.33)\) but significant bias in yellow males' behavior toward yellow females \((G_1 = 10.98, P = 9 \times 10^{-5}\)).

The difference in mate preference between the \(H.\ cydno alithea\) morphs may be explained if the color forms actually represent partially isolated subspecies with overlapping distributions, much like sympatric host races in phytophagous insects. Thus, we genotyped white and yellow \(alithea\) specimens at over 800 amplified fragment length polymorphisms (AFLPs), sequenced 1600 base pairs of mtDNA, and generated comparative data for \(H.\ cydno galanthus\), \(H.\ pachinus\), and three outgroup species: \(H.\ melpomene\), \(H.\ atthis\), and \(H.\ ismenius\). Phylogenetic and population genetic analyses showed that closely related \(alithea\), \(galanthus\), and \(pachinus\) were differentiated (Fig. 3), but there was no genetic differentiation between white and yellow \(alithea\): analysis of molecular variance (AMOVA)-based \(F_{ST} = 0.001 (P = 0.35)\) for AFLPs and \(F_{ST} = 0.057 (P = 0.10)\) for mtDNA. Furthermore, clustering of the AFLP data with the program STRUCTURE (22) failed to detect any color-associated subdivision in \(alithea\) (Fig. 3B), and constraining the morphs to form separate clusters resulted in a very poor fit to the data \([\text{difference in ln(likelihood)} = -319]\). We also tested this hypothesis by examining patterns of linkage disequilibrium (LD) between the unlinked color-patternning loci \(K\) and \(Ac\) (16). Strong assortative mating between the morphs should generate LD among unlinked markers, yet we found no association between \(K\) and \(Ac\) in 68 wild-caught butterflies \((P = 1.00)\). These results, combined with frequent observations of mixed mating in greenhouse cultures, indicate that the \(alithea\) morphs are not reproductively isolated and hence appear to represent a single polymorphic interbreeding population.

Another mechanism that could explain mimicry-based mate preference in \(alithea\) is genetic linkage between color and preference. Male color preference co-segregates with alleles at the color locus \(K\) in crosses between \(H.\ cydno galanthus\) and \(H.\ pachinus\), and first-generation hybrids have intermediate preference (14). Our \(alithea\) data support this hypothesis, because yellow males \((K^yK^y\), where \(y\) indicates yellow allele\) exhibited yellow preference, but white males, the majority \((\approx 83\%)\) of which were likely to be heterozygous at \(K\) \((K^yK^w\), where \(W\) indicates white allele\), did not. We hypothesize that this genetic association involves a mechanism that prevents recombination, such as a single locus controlling both traits or an inversion, because otherwise, interbreeding between the morphs would dissociate them over time. Single-locus control of signal and preference is unlikely, generally (23), but may be facilitated in this case because omomochrome pigments color nymphalid butterfly wings (24) and serve as lateral filtering pigments in insect eyes (25). Lastly, the association between color and preference in \(alithea\) is not likely to be due to self-referent phenotype matching, whereby males recognize their own color and preferentially court females that display the same color, because this would result in a white-biased preference among white males, similar to the yellow bias observed in yellow males.

\(H.\ cydno alithea\) may represent a very early stage in the evolution of reproductive isolation, when the same traits that distinguish sister species, in this case mimicry and color-based mate preference, segregate in a single population (supporting online material). To examine the relationship between \(alithea\) and other examples of ecological speciation, we reviewed the literature and scored 20 relevant biological systems for criteria that define the progression of speciation (table S1). A total of 10 criteria were summarized as the following five categorical variables (table S2): number of divergent phenotypic traits, genetic basis of these traits, presence versus absence of postzygotic reproductive isolation, degree of spatial segregation, and strength of genetic dif-

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**Fig. 3. Population genetics of \(H.\ cydno alithea\), \(H.\ cydno galanthus\), and \(H.\ pachinus\).** (A) Bootstrap neighbor-joining tree of polymorphic AFLP markers (bootstrap values > 50% are shown). (B) STRUCTURE clustering (no-admixture and admixture) on AFLP data correctly identified the three populations at \(K = 3\), but clustering at \(K = 4\) resulted in a reduced likelihood and did not subdivide \(alithea\) by color. (C) Bootstrap neighbor-joining tree of mtDNA sequences (bootstrap values > 50% are shown). In each panel, individuals are designated by colored symbols (triangles for the Costa Rican taxa \(H.\ cydno galanthus\) and \(H.\ pachinus\), circles for \(H.\ cydno alithea\); white or yellow indicates wing color).
fication. Multiple correspondence analysis of these variables revealed that H. cydno alithea differed from other examples of ecological speciation in that divergence is based on a single-locus trait and is not accompanied by postzygotic isolation or background genetic differentiation (fig. S2). Research on other examples of ecological speciation has revealed populations that may also be in an early stage of divergence (26–28), suggesting that continued examination of these and other systems will reveal a continuum in the trajectory of ecological speciation, as is evident in Heliconius butterflies.

References and Notes

A Type I–Secreted, Sulfated Peptide Triggers XA21-Mediated Innate Immunity

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The rice Xa21 gene confers immunity to most strains of the bacterium Xanthomonas oryzae pv. oryzae (Xoo). Liquid chromatography–tandem mass spectrometry analysis of biologically active fractions from Xoo supernatants led to the identification of a 194-amino acid protein designated Ax21 (activator of XA21-mediated immunity). A sulfated, 17-amino acid synthetic peptide (axY22) derived from the N-terminal region of Ax21 is sufficient for activity, whereas peptides lacking tyrosine sulfation are biologically inactive. Using communoprecipitation, we found that XA21 is required for axY22 binding and recognition. axY22 is 100% conserved in all analyzed Xanthomonas species, confirming that XA21 is a pattern-associated molecular pattern and that XA21 is a pattern recognition receptor.

In 1995 we showed that the rice Xa21 resistance gene, which encodes a protein with predicted leucine-rich repeat (LRR), transmembrane, extracellular domain, conferred immunity to diverse strains of the Gram-negative bacterium Xanthomonas oryzae pv. oryzae (Xoo). Liquid chromatography–tandem mass spectrometry analysis of biologically active fractions from Xoo supernatants led to the identification of a 194-amino acid protein designated Ax21 (activator of XA21-mediated immunity). A sulfated, 17-amino acid synthetic peptide (axY22) derived from the N-terminal region of Ax21 is sufficient for activity, whereas peptides lacking tyrosine sulfation are biologically inactive. Using communoprecipitation, we found that XA21 is required for axY22 binding and recognition. axY22 is 100% conserved in all analyzed Xanthomonas species, confirming that XA21 is a pattern-associated molecular pattern and that XA21 is a pattern recognition receptor.