Supporting Online Material

Materials and methods

Spectral reflectance

Color measurements were made with a USB2000 spectrometer (Ocean Optics, Dunedin, FL) fitted with a 400 µm UV/VIS optical fiber (Ocean Optics, Dunedin, FL) with a Spectralon white standard (Labsphere, North Sutton, NH). A custom made adaptor ensured that measurements were taken at a 45° angle relative to the subject surface. Measurements were taken at homologous areas in the forewing colored patch and proximal dark area. Measurements were all taken in the same orientation with the probe angled posteriorly, viewing the wing from behind. Three wild-caught individuals of each species/phenotype were measured.

Genetics of color and pattern

Genetics of color patterning in alithea – Previous work with *H. cydno galanthus* and *H. pachinus* has shown that these two species differ at multiple, unlinked Mendelian color patterning loci (S*1*-*S3*), two of which have phenotypes similar to the variation segregating in *H. cydno alithea*. The *K* locus controls white vs. yellow on the forewing with the white allele dominant to yellow (S*3*). The *Ac* locus controls the presence vs. absence of melanin in the proximal region of the forewing and hindwing, with presence of melanin dominant to absence (S*2*). Similar segregation occurs in crosses between color pattern races of *H. melpomene* and in interspecific crosses between *H. cydno* and *H. melpomene* (S*1, S4, S5*).

To examine the genetic basis of color pattern variation in *H. cydno alithea*, we isolated field-caught individuals with alternative phenotypes into divergent population cages: one for yellow individuals without the *Ac* melanin patch ("triangle" phenotype) and one for white individuals with the *Ac* melanin patch ("bar" phenotype). Offspring from the former cage were always yellow triangle, while the latter hosted a large population of all four color-pattern combinations. This suggested that *Heliconius cydno alithea* would follow the basic mode of inheritance for color-pattern traits with white being dominant to yellow and bar being dominant to triangle.

We confirmed these observations by rearing 15 experimental broods between *alithea* males and females of various color and pattern phenotypes and comparing observed and expected offspring phenotype ratios with *G*-tests. As predicted, offspring of white parents (inferred *K* heterozygotes) fit the expected 3:1 ratio (52 white to 21 yellow offspring, $G_1 = 0.54$, $P = 0.46$). Test crosses of a white (heterozygote) against a yellow homozygote fit the expected 1:1 ratio (16:17 white: yellow $G_1 = 0.03$, $P = 0.86$), while 100% of the offspring of yellow parents were yellow (21 offspring). These combined data are consistent with a hypothesis of a single locus, with two alleles and white dominant to yellow ($G_2 = 0.57$, $P = 0.75$). For the *Ac* locus, we

observed that presence of the melanic patch was dominant to absence. Three *Ac* phenotypes were observed in laboratory crosses, triangle, bar, and intermediate (denoting intermediate melanization). Offspring of bar parents showed both bars and triangles while offspring of bar and triangle parents produced all three phenotypes, either bar and intermediate or bar and triangle (23 offspring from 5 broods). Triangle phenotypes, when crossed together, always produced triangle offspring (113 offspring from 10 broods). Combined, these data are consistent with a hypothesis of a single locus with 2 or 3 alleles and presence of melanin (bar and intermediate) dominant to absence (G_2 = 3.36, P = 0.187).

Inferring K homology across systems – Previous genetic mapping with an F2 intercross between *H. cydno galanthus* and *H. pachinus* (*S3*) showed that the *K* locus is tightly linked to the gene *wingless* (LOD = 18.2). To verify the homology of *K* between Costa Rica and Ecuador, we generated 3 broods segregating white vs. yellow by backcrossing F1 males (*galanthus*/yellow *alithea*) to yellow *alithea* females. We genotyped 41 of these BC offspring and found no recombination between *wingless* and *K* (LOD = 11.0). LOD scores were calculated with Joinmap (*S6*).

Measuring LD in nature – Pronounced assortative mating by color in *H. cydno alithea* should result in baseline genetic differentiation and generate linkage-disequilibrium among unlinked markers. As an additional test for genetic differentiation between white and yellow *alithea* morphs, we examined the extent of linkage-disequilibrium between the unlinked color patterning loci *K* and *Ac* in 68 wild-caught individuals. All individuals were collected in May, October and November, 2008. The alternate *Ac* phenotypes were at equal frequency in white and yellow butterflies (Fisher's exact test, P = 1.00): out of 28 white butterflies, 7 had the dominant *Ac* patch (bar phenotype), 21 did not (triangle phenotype) while for 40 yellow butterflies, 11 had the dominant *Ac* patch (bar phenotype), 29 did not (triangle phenotype).

Measuring mate preference

Experimental butterflies – Wild-caught *H. cydno alithea* butterflies were collected from several polymorphic sampling locations in Pichincha Province, Ecuador, during October and November, 2008. Captive males were collected from two polymorphic populations at the *Heliconius* Butterfly Works farm in Mindo, Ecuador. All males were collected as adults so they had access to females prior to the experiment.

Courtship experiments - Experimental males were numbered on the wing with a marker and placed in a 1.75 m^3 cage as a group where they were presented with one virgin white female and one virgin yellow female at the same time. Immediately after courting or attempting to mate a female, the males were caught and their identity was recorded. Butterflies were fed sugar water and given access to flowers for nectar and pollen.

Statistical analysis – Male butterfly color preference was modeled with generalized linear mixed models with binomial response and logit link function (*S7, S8*). Fixed effects (treatments)

included male color-pattern, the color-pattern of contrasting females and male status (wild versus captive). Random effects included subject (male number) and unique trial (a unique combination of males and females in the experimental cage on a given trial day). The overall "full" model, allowing for interactions, had 19 fixed effect parameters (Akaike Information Criterion [AIC] = 836.03) and did not indicate overdispersion (maximum estimated dispersion parameter, from quasibinomial fit, was between 1.09 and 1.18). Since the study included over 580 preference scores from over 60 unique trials, we retained the binomial error family. Due to a lack of overdispersion and relatively high sample size we tested for random and fixed effects using likelihood ratio tests (*S7*). First, we used likelihood ratio tests to test for random effects. Both unique trial (G_1 = 28.87, P = 7.74 x 10^{-8}) and male number (G_1 = 5.71, P = 0.017) were significant, meriting their retention in all other models and overall use of GLMMs. Male status (G_{10} = 9.33, P = 0.501) and male & female color-pattern interaction (G_9 = 9.24, P = 0.416) were not significant. Male color pattern (G_{12} = 35.36, P = 4.11 x 10^{-4}) and female color pattern (G_{12} = 27.31, P = 6.97 x 10^{-3}) were both significant. The effect of male color pattern was due to male color (G_{11} = 35.18, P = 2.32 ×10^{-4}), not male pattern (G_{11} = 9.85, P = 0.544). The effect of female pattern on male choice was largely due to yellow females (G_{12} = 27.24, P = 7.14 ×10^{-3}) not white females (G_{12} = 11.03, P = 0.526). The effects of slopes within a model were assessed with Z tests (*S7*). Inspection of AIC support these findings: the lowest AIC was for the sub-model including male color and female pattern differences among yellow females (AIC = 819.16, 5 parameters). All analyses were carried out with the lme4 package in R (*S9*). Alternative model tests and approximate confidence intervals, which were based on approximate degrees of freedom [Wald's tests, see ref (*S7*)] did not change these conclusions. Generalized linear mixed effects models is an active area of research, so all analyses were double checked by an independent statistician modeling average number of courtships using mixed models in SAS.

Courtship observations in nature – We reviewed records of chasing and mating behavior from field observations of polymorphic *H. cydno alithea* populations made in 1992 -1998 (*S10, S11*). Over the course of these field seasons, 12 events were noted in which one *alithea* butterfly chased another, which is the first step of courtship. An additional six events showed courtship related behavior including circle-chasing, male hovering over a non-receptive female and copulation. In each case we classified each butterfly as the 'instigator' or 'recipient' of the behavior. Consistent with our experimental results, we found that the white morph instigated behaviors with the two colors equally (7 white, 6 yellow) while the yellow morph showed a bias toward yellow (0 white, 5 yellow). Because the white morph frequency averaged 0.67 across the locations in which these field observations occurred (*10, 11*), the chasing behavior of the white morph was not different than that expected by chance (G_1 = 0.97, P = 0.33) but that of the yellow morph was significantly biased in favor of yellow (G_1 = 10.98, P = 9×10^{-4}).

Mate preference data for H. cydno galanthus and H. pachinus – For comparison to the *alithea* preference data, we present previously published (*S3, S12*) mate preference data for *H. cydno galanthus* and *H. pachinus* from Costa Rica in Fig S1. The conditions for these experiments were similar to those for the *alithea* experiments except males were presented with *H. cydno galanthus* and *H. pachinus* females in an enclosure at the *Heliconius* rearing facility at the

University of Texas. Males for these experiments were collected from greenhouse cultures (two separate *H. pachinus* populations and three *H. cydno galanthus* populations) that had recently been established with individuals collected in Costa Rica. The tested individuals were a mixture of naïve males, who had no prior access to females, and experienced males, who were collected as adults from population cages.

Genetic data and analyses of genetic differentiation

mtDNA data and analysis – We sequenced a region of mitochondrial DNA from 32 *H. cydno alithea* (15 white, 17 yellow), 16 *H. cydno galanthus*, 16 *H. pachinus*, and 2 individuals from each of three outgroup taxa, *H. melpomene cythera*, *H. atthis*, and *H. ismenius,* using published methods and primers (*S13*). The sequenced region spans the COI, tRNA-Leu, and COII genes. The aligned dataset consisted of 1615 sites, of which 167 were polymorphic. 22 sites were polymorphic in *alithea*. We generated a bootstrap (1000 pseudo-replicates) neighbor-joining tree from these data (Fig 3C) using the p-distance method in MEGA4 (*S14*), with missing data excluded in pairwise comparisons. We also estimated genetic differentiation between white and yellow *alithea* using the AMOVA approach implemented in Arelquin (*S15*). The probabilities of the F_{ST} estimates were estimated by permuting haplotypes between populations 1000 times. We ran two separate AMOVA analyses. First, we estimated F_{ST} between all white and yellow *alithea* samples (collected from 5 locations) and found no significant differentiation (F_{ST} = 0.057, P = 0.10). Second, we estimated F_{ST} between the 9 white and 7 yellow samples from a single collecting location. This analysis revealed complete absence of differentiation between co-occurring individuals of the two morphs (F_{ST} = -0.066, P = 0.496). Finally, we used these sequences, and comparative data from GenBank, to estimate pairwise divergence between putative mimicry models (*hewitsoni* vs. *sapho* in Costa Rica, *sapho* vs. *eleuchia* in Ecuador) and mimics (*cydno galanthus* vs. *pachinus* in Costa Rica, white vs. yellow *cydno alithea* in Ecuador) in MEGA4. The mimicry models in Costa Rica, *H. sapho* and *H. hewitsoni*, belong to the pupal-mating *Heliconius* clade but are quite distantly-related with average mitochondrial DNA divergence of 7.11% (0.66% SE). Average divergence between *H. cydno galanthus* and *H. pachinus*, on the other hand, is only 1.26% (0.24% SE). Similarly, *H. eleuchia* and *H. sapho* are distantly-related members of the same pupal-mating clade (average mtDNA divergence = 7.77% [0.68% SE]) while the *alithea* morphs are not differentiated (average mtDNA divergence = 0.38% [0.11% SE]).

AFLP data and analysis – We genotyped the same 70 individuals included in the mtDNA analysis with 8 AFLP primer pairs using a Plant Mapping Kit (Applied Biosystems, Foster City, CA); EcoRI-ACT/MseI-CAT, EcoRI-ACT/MseI-CAG, EcoRI-ACT/MseI-CTG, EcoRI-ACT/MseI-CTT, EcoRI-ACA/MseI-CAT, EcoRI-ACA/MseI-CAG, EcoRI-ACA/MseI-CTG, and EcoRI-ACA/MseI-CTT. We sized and scored AFLP fragments using Genemapper software (Applied Biosystems, Foster City, CA). The entire dataset consisted of 1268 polymorphisms, of which 813 were polymorphic in *alithea*. We generated a bootstrap (1000 pseudo-replicates) neighbor-joining tree from these data (Fig 3A) using PAUP (*S16*). This tree clearly distinguished *H. cydno galanthus*, *H. pachinus*,

and *H. cydno alithea* but revealed no color-associated clustering within *alithea*. We explicitly tested for genetic differentiation between *alithea* color morphs using STRUCTURE-based clustering (*S17*) and AMOVA-based estimates of F_{ST}. Admixture and no-admixture STRUCTURE clustering of the AFLP data from *H. cydno galanthus*, *H. pachinus*, and *H. cydno alithea* correctly identified the three groups at K=3 (Fig 3B). Clustering at K=4 resulted in a reduced likelihood (Δ lnL = -195 with the no-admixture model, Δ lnL = -27 with the admixture model) and did not subdivide *alithea* by color (Fig 3B) indicating no genetic differentiation between the *alithea* morphs. We also performed admixture and no-admixture clustering with only the *alithea* samples and polymorphisms, at K=2, with and without constraining the morphs to form separate clusters (USEPOPINFO setting). Unconstrained admixture clustering of *alithea* at K=2 split all individuals at similar proportions between the two clusters (average assignment for each individual = 0.825 to cluster 1, 0.175 to cluster 2) and showed no difference between white and yellow. Forcing the *alithea* morphs to form separate clusters with constrained admixture clustering produced a very poor fit to the data (Δ lnL = -319). The same comparison with no-admixture clustering produced similar results. AMOVA-based F_{ST} values, estimated with Arlequin, revealed highly significant differentiation among *H. cydno galanthus*, *H. pachinus*, and *H. cydno alithea* (F_{ST} = 0.166, $P < 10^{-5}$) but no differentiation between the *alithea* color morphs (F_{ST} = 0.001, P = 0.343).

Comparing *H. cydno alithea* to other examples of ecological speciation

Systems and scoring - We identified 19 additional biological systems that represent probable examples of ecological speciation. These include three other *Heliconius* comparisons (*H. cydno galanthus*/*H. pachinus*, *H. erato*/*H himera*, and *H. melpomene*/*H. cydno*), classic examples of ecological speciation (e.g. *Timema* walking sticks, benthic/limnetic threespine stickleback, *Pundamilia* cichlids, *Mimulus* monkeyflowers, *Rhagoletis* host races) and others (Table S1). We reviewed the literature and scored all systems, plus *H. cydno alithea*, for 10 criteria which provide evidence for ecological speciation or define the amount of divergence between the species/populations, i.e. define their stage in the speciation process (Table S1). All systems met two basic criteria consistent with ecological speciation; 1) evidence for ecologically-based divergent selection, and 2) assortative mating/mate preference.

Notes about scoring – For each system, we counted the number of phenotypic traits that have been shown to differ between species/populations. We list these traits under the system-specific references below. This count does not include divergent preferences that result in assortative mating (e.g. pheromone preference in *Ostrinia nubilalis*) but does include divergent traits that may serve as mate cues (e.g. pheromone blend in *Ostrinia nubilalis*). These trait counts are conservative because we are necessarily limited to only those traits that have been investigated in each system. We also counted certain composite traits as one trait (e.g. color pattern, feeding morphology, body form and behavior in various systems). The genetic basis of traits is unknown in a number of systems but we included this category because it provides an indication of the minimum amount of genomic differentiation that accompanies ecological

divergence. *H. cydno alithea* is polymorphic for both color and *Ac* pattern and each trait is controlled by a separate locus. This system was scored as single-locus genetic basis because the *Ac* pattern phenotypes are at identical frequencies in the two color morphs (see analysis above and in main text) and only color has been shown to influence mimicry (*S11*). Other systems that differed in a single trait (*Oophaga pumilio* and *H. cydno*/*pachinus*) were scored as polygenic because color pattern variation is suspected to be polygenic in *Oophaga pumilio* (see below) and known to be polygenic between *H. cydno* and *H. pachinus* (see below). Systems in which differentiation involves two or more traits, and no data exist regarding genetic basis of one or more traits, were scored as presumably polygenic. In some cases this assumption was supported by limited or circumstantial data or comments from previous papers. Given the uncertainty regarding scoring the 'genetic basis of traits' category, we also performed MCA (see below) with this category removed, which produced similar results.

For postzygotic isolation, we scored presence/absence of both intrinsic and extrinsic isolation. For extrinsic postzygotic isolation, we scored some systems as 'presumably yes' if hybrids are known to be intermediate and the natural history of the system suggests that intermediate phenotypes are likely to have reduced fitness. For instance, hybrids between *O. pumilio* color morphs display intermediate aposematic patterns and thus may not be recognized by predators as being protected. Thus, we scored this system as 'presumably yes' for extrinsic postzygotic isolation. For 'spatial segregation', we scored systems as 'broad' if their ranges include large regions with no overlap, 'fine' if they are broadly sympatric but differ in microhabitat (e.g. benthic/limnetic stickleback, *H. cydno* and *H. melpomene*) and 'none' if there is very little or no spatial segregation. We scored strength of genetic differentiation, based on mitochondrial (chloroplast in plants) and nuclear markers. For these two criteria, we scored systems as 'weak' if F_{ST} values were statistically significant but less than 0.2, 'strong' if F_{ST} values were statistically significant and greater than 0.2 or gene trees at presumably neutral loci showed complete lineage sorting, and 'none' if estimates of genetic differentiation were not statistically significant. For systems in which multiple nuclear loci were analyzed, F_{ST} values and statistical significance refer to summaries across all markers, not just a subset of data.

There are multiple examples in which a given system displayed variation in the criterion being scored. In these cases, we scored them according to the predominate pattern. For instance, color morphs of *O. pumilio* generally do not overlap but there are a few polymorphic populations. Given the rarity of polymorphism in this system, we scored it as "broad" spatial segregation. When in doubt, our default scoring for all 10 criteria made the system in question as similar to our scoring for *H. cydno alithea* as possible so these are generally conservative estimates.

Category summary and MCA analysis - We summarized the 10 criteria into five categorical variables (Table S2). For this, we binned 'number of divergent traits' into categories of one, few (2 or 3), or many (4 or more). Information regarding intrinsic and extrinsic postzygotic isolation was combined into one category, as was information for mitochondrial and nuclear genetic differentiation. For the 'strength of genetic differentiation' variable, we categorized systems as 'no' if there was no significant differentiation in either category, 'weak' if both were weak,

'strong' if both were strong, and 'mix' if there was a combination of strong differentiation in one category and no, weak, or unknown in the other. R (*S9*) was used to perform multiple correspondence analysis (MCA) on these five variables. MCA was used because it is most appropriate for the categorical nature of the data (*S18*). The analysis indicated that the first two MCA axes (principal inertias) explained 86.6% of the variation in the dataset. The first axis explained the majority of variation (79.3%) with three variables most important: 1) genetic basis of divergent traits, 2) presence or absence of postzygotic isolation, and 3) degree of genetic differentiation. The results demonstrated that *H. cydno alithea* is well differentiated from other systems along axis 1. Principal components analysis (PCA) and correspondence analysis with a doubling transformation (*S18*) produced similar results.

Supporting Text

The trajectory of ecological speciation in *H. cydno alithea*

Our research has demonstrated partial color-based assortative mate preference in polymorphic *H. cydno alithea*. The novelty of this result is that we have discovered the basic building blocks of ecological speciation segregating in an interbreeding population. Thus, this system may provide insight into the process by which a single population transitions from random mating to reproductive isolation. Currently, the *alithea* color morphs are not strongly isolated. Our courtship experiments demonstrate a shift in preference with yellow males preferring to court yellow females and white males generally showing no preference. This does not appear to result in pronounced assortative mating in nature because we find no evidence of genetic differentiation between the morphs or LD between the unlinked color patterning loci. The partial mate preference observed in *alithea* is probably insufficient, in itself, to cause speciation. However, there are multiple scenarios under which the raw material present in the *alithea* system could drive the color morphs toward greater reproductive isolation and eventual speciation.

1. Preference shift in the white morph – if genetic variation for preference changes such that white preference is dominant to yellow preference, butterflies heterozygous for color and preference (currently white with no preference) would prefer white. Over generations, this could reduce the frequency of color heterozygotes in the population and lead to a homozygous yellow form with yellow preference and a largely homozygous white form with white preference.

2. Increased influence of *Ac* on mimicry – There is no predator-imposed selection against individuals that are heterozygous at the color locus because they are white and thus mimetic. If the unlinked *Ac* locus also contributed to mimicry, then there would be an opportunity for selection against intermediate, non-mimetic phenotypes and increased reproductive isolation. We do not currently know what influence the *alithea Ac* pattern phenotype has on mimicry. It may contribute to mimicry with the melanic bar phenotype producing a better match to *H. sapho* and the non-melanic triangle phenotype producing a

better match to *H. eleuchia* (*S10*). However, the fact that the *Ac* alleles are at equal frequency in white and yellow morphs suggests that either a) *Ac* does not influence mimicry strongly, or b) the *Ac* polymorphism is recent and has not had time to sort out between the morphs. If *Ac* does contribute to mimicry, now or in the future, then predator-imposed natural selection against mismatched phenotypes could result in two subgroups, white/bar and yellow/triangle, and a level of extrinsic postzygotic isolation separating them. This, in turn, would also provide fuel for the evolution of even greater premating isolation via reinforcement.

3. Change in distribution of mimicry models – The geographic distribution of the *alithea* morphs is ultimately determined by the distributions of the models, *H. sapho* and *H. eleuchia* (*S10*). If model ranges shift over time, perhaps as a consequence of changes in the distributions of their host plants *Passiflora macrophylla* and *P. tina* (*S10, S11*), this could result in large geographic regions dominated by a single model. This in turn would generate local selection for only one *alithea* morph and cause migrants that do not match the local color to have reduced fitness (resembling the parapatric distributions of *cydno*/*sapho* and *pachinus*/*hewitsoni* in Costa Rica). Again, this would generate an additional level of reproductive isolation and push the system toward speciation.

The possibility also exists that this speciation scenario is playing in reverse, with the *H. cydno alithea* color polymorphism and color/preference association actually representing the mixed remnants of formerly separate subspecies that have now fused. Our results refute this hypothesis however. This despeciation scenario predicts remnant evidence of genetic differentiation, spatial segregation, and color/pattern association but we find none of these in *alithea*. There are some *alithea* subpopulations that are only yellow, but these occur in both the north and south of its range (*S10*).

Regardless of whether the *alithea* morphs ever speciate themselves, this system gives us a window into the process by which the building blocks of ecological speciation are generated. Divergent natural selection to utilize novel ecological niches (in this case mimicry niche space) generates initial reproductive isolation on which additional layers can build. Speciation is generally a cumulative process, with multiple levels of reproductive isolation all contributing to generate a barrier to gene flow. Our experimental results and the speciation scenarios outlined above provide a route by which species and subspecies diversity in *Heliconius* may have originated.

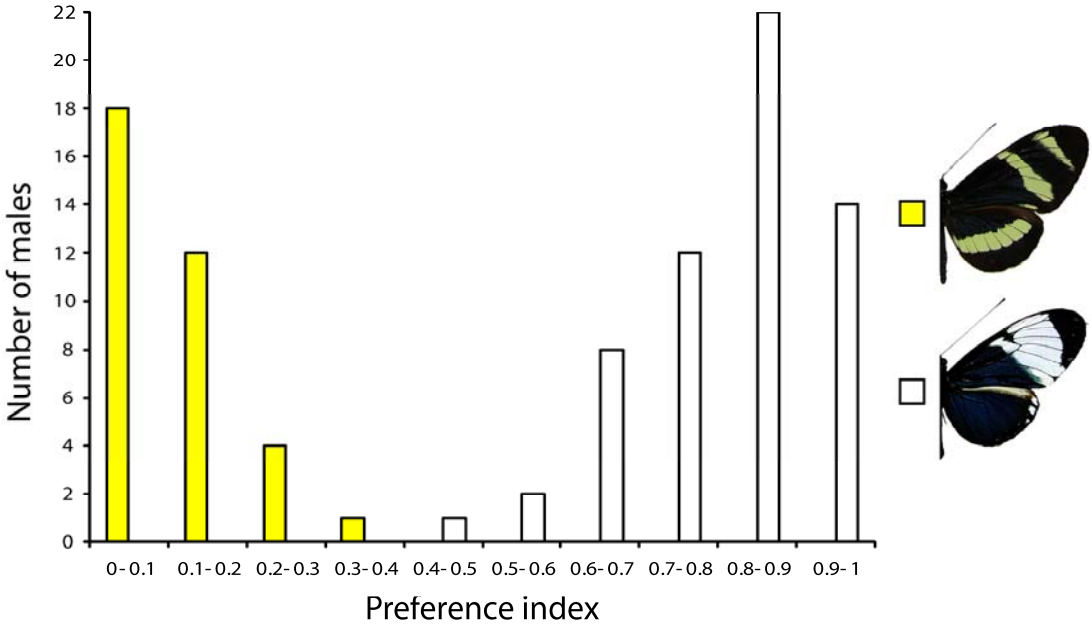

Fig S1. Distribution of mate preference indices for *H. pachinus* (yellow bars) and *H. cydno galanthus* (white bars) males. The preference index (x-axis) is the proportion of courtship and attempted mating events that were directed toward *H. cydno galanthus* females; a preference index of 1 indicates complete preference for *galanthus* whereas 0 indicates complete preference for *pachinus* (data combined from refs *S3* & *S12*).

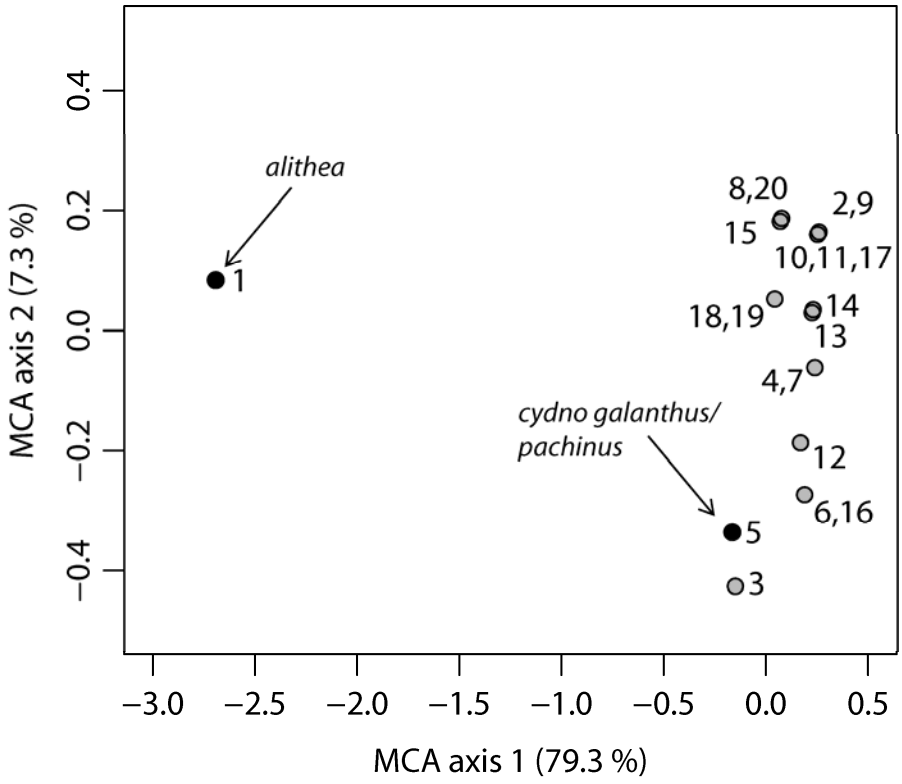

Fig S2. Multiple correspondence analysis (MCA) of biological systems representing examples of ecological speciation. Twenty systems, including *H. cydno alithea*, were scored for five categorical variables that characterize their stage in the speciation process (Table S2). MCA based on these variables revealed that *H. cydno alithea* is highly differentiated along the first axis, primarily due to the fact that the divergent trait is controlled by a single locus and has no accompanying postzygotic isolation or genetic differentiation.

Table S1. Comparison of systems representing examples of ecological speciation.

System	Ecological selection	Number of divergent traits	Genetic basis of traits	Postzygotic Isolation		Mate preference	Assortative mating	Spatial segregation[2]	Genetic differentiation[3]	
				Intrinsic	Extrinsic				mtDNA	nuclear
Heliconius cydno alithea	yes	1	single locus	no	no	yes	no	none	no	no
Timema cristinae	yes	4	polygenic?[1]	no	yes?	yes	yes	fine	weak	weak
Oophaga pumilio	yes	1	polygenic?	no	yes?	yes	yes	broad	strong	strong
Gasterosteus aculeatus (benthic/limnetic)	yes	7	polygenic	no	yes	yes	yes	fine	strong	strong
H. cydno & *H. pachinus*	yes	1	polygenic	no	yes?	yes	yes	broad	strong	weak
H. erato & *H. himera*	yes	3	polygenic	no	yes	yes?	yes	broad	strong	strong
H. cydno & *H. melpomene*	yes	5	polygenic	yes	yes	yes	yes	fine	strong	strong
Ostrinia nubilalis	yes	2	polygenic?	no	yes	yes	yes	none	weak	weak
Pundamilia cichlids	yes	6	polygenic	no	yes?	yes	yes	fine	no?	weak
Rhagoletis pomonella	yes	2	polygenic	no	yes	no	yes	fine	no?	weak
Littorina saxatilis	yes	3	polygenic?	yes	yes?	yes	yes	fine	weak?	weak
Mimulus lewisii & *M. cardinalis*	yes	4	polygenic	yes	yes	n/a	yes	broad	no?	strong
Zeiraphera diniana (Larch budmoth)	yes	4	polygenic?	no	yes?	yes	yes	fine	no	strong
Acyrthosiphon pisum (Pea aphid)	yes	2	polygenic	no?	yes	no?	yes	fine	?	strong
Amphilophus citrinellus & *A. zaliosus*	yes	5	polygenic?	no	yes?	yes	yes	none	weak	weak
Gambusia hubbsi	yes	2	polygenic?	no	yes?	yes	yes?	broad	strong	strong

Anthoxanthum odoratum	yes	3	polygenic?	no?	yes	n/a	yes	fine	weak	weak
Nesospiza buntings	yes	5	polygenic?	no?	yes?	yes	yes	none	no	weak & strong
Howea palms	yes	4	polygenic?	no?	yes?	n/a	yes	none	?	strong
Hypoplectrus hamlet fish	yes	2	polygenic?	no	yes	yes	yes	none	weak	weak

[1] '?' means presumably based on indirect evidence.
[2] 'broad' refers to large regions without overlap, 'fine' refers to separation by microhabitat.
[3] 'weak' refers to statistically significant but $F_{ST} < 0.2$, 'strong' refers to statistically significant and $F_{ST} > 0.2$, or complete lineage sorting in presumably neutral gene trees.

Systems scored in Table S1

***Heliconius cydno alithea*:**
Number of divergent traits: color, this paper and (*S11*)
Genetic basis of traits: single locus, this paper and (*S10*)
Intrinsic postzygotic isolation: this paper and (*S10*)
Extrinsic postzygotic isolation: this paper and (*S11*)
Spatial segregation: this paper and (*S10, S11*)
Strength of mtDNA differentiation: this paper
Strength of nuclear differentiation: this paper
Ecological selection: this paper and (*S11*)
Mate preference: this paper
Assortative mating: this paper

***Timema cristinae*:**
Number of divergent traits: color pattern, body form, behavior, and host (*S19-S21*)
Genetic basis of traits: presumably polygenic (*S20*)
Intrinsic postzygotic isolation: (*S22*)
Extrinsic postzygotic isolation: presumably yes (*S22, S23*)
Spatial segregation: (*S20, S21*)
Strength of mtDNA differentiation: (*S21, S23, S24*)
Strength of nuclear differentiation: (*S21, S25*)
Ecological selection: (*S19, S20, S22, S23, S26, S27*)
Mate preference: (*S21-S23*)
Assortative mating: (*S21-S23*)

***Oophaga pumilio* (Stawberry poison dart frog):**
Number of divergent traits: color pattern (*S28, S29*)
Genetic basis of traits: presumably polygenic (*S30*)
Intrinsic postzygotic isolation: (*S30*)
Extrinsic postzygotic isolation: unknown – presumably yes (*S30*)
Spatial segregation: (*S28, S29, S31, S32*)
Strength of mtDNA differentiation: (*S32, S33*)
Strength of nuclear differentiation: (*S32, S34*)
Ecological selection: (*S35-S38*)
Mate preference: (*S37-S39*)
Assortative mating: (*S31*)

Benthic/limnetic Three-spine Stickleback (*Gasterosteus aculeatus*):
Number of divergent traits: body form (size and shape), nuptial color, lake microhabitat, trophic morphology, diet, courtship behavior, and body armor (*S40-S45*)
Genetic basis of traits: (*S45-S51*)
Intrinsic postzygotic isolation: (*S40, S41,S43*)

Extrinsic postzygotic isolation: (*S52*)
Spatial segregation: (*S40, S41*)
Strength of mtDNA differentiation: (*S53*)
Strength of nuclear differentiation: (*S40, S41*)
Ecological selection: (*S54*)
Mate preference: (*S44*)
Assortative mating: (*S55-S57*)

***Heliconius cydno* & *H. pachinus*:**
Number of divergent traits: wing pattern (*S1-S3*)
Genetic basis of traits: (*S1-S3*)
Intrinsic postzygotic isolation: (*S1-S3, S58*)
Extrinsic postzygotic isolation: presumably yes (*S12*)
Spatial segregation: (*S12, S58*)
Strength of mtDNA differentiation: (*S58*)
Strength of nuclear differentiation: (*S58*)
Ecological selection: this paper & (*S58*)
Mate preference: (*S3, S12*)
Assortative mating: (*S12*)

***Heliconius erato* & *H. himera*:**
Number of divergent traits: wing pattern, habitat, morphology (genitalia & wing shape) (*S59, S60*)
Genetic basis of traits: (*S60-S63*)
Intrinsic postzygotic isolation: (S*62*)
Extrinsic postzygotic isolation: (*S59, S62*)
Spatial segregation: (*S59, S60*)
Strength of mtDNA differentiation: (*S64, S65*)
Strength of nuclear differentiation: (*S64, S66*)
Ecological selection: (*S59, S60, S62*)
Mate preference: presumably yes (*S62, S67*)
Assortative mating: (*S62, S67*)

Heliconius cydno* & *H. melpomene:
Number of divergent traits: wing pattern, microhabitat, host plants, flight kinematics, morphology (body size, genitalia, etc.): (*S1, S4, S68-S71*)
Genetic basis of traits: (*S4*)
Intrinsic postzygotic isolation: (*S72, S73*)
Extrinsic postzygotic isolation: (*S74*)
Spatial segregation: (*S68*)
Strength of mtDNA differentiation: (*S13, S58, S75, S76*)
Strength of nuclear differentiation: (*S13, S58, S66, S76-S78*)
Ecological selection: (*S66, S79*)

Mate preference: (*S74, S79*)
Assortative mating: (*S74, S79*)

European Corn-borer (*Ostrinia nubilalis*):
Number of divergent ecological traits: Pheromones, host plants (*S80-S82*)
Genetic basis of traits: Presumably polygenic (*S80, S83*)
Intrinsic postzygotic isolation: (*S83*)
Extrinsic postzygotic isolation: (*S84*)
Spatial segregation: (*S82, S85*)
Strength of mtDNA differentiation: F_{ST} = 0.046, *P* = 0.037, our analysis of data in (*S86*)
Strength of nuclear differentiation: (*S85, S87, S88*)
Ecological selection: (*S82, S86*)
Mate preference: (*S80*)
Assortative mating: (*S82, S88*)

***Pundamilia* cichlids:**
Number of divergent traits: water depth, male coloration, spectral sensitivity of LWS opsin, diet, body size, male display rate (*S89-S93*)
Genetic basis of traits: (*S94-S97*)
Intrinsic postzygotic isolation: (*S98*)
Extrinsic postzygotic isolation: (*S96, S97*)
Spatial segregation: (*S91, S95*)
Strength of mtDNA differentiation: presumably no or weak (*S99, S100*)
Strength of nuclear differentiation: (*S95*)
Ecological selection: (*S90, S91, S95*)
Mate preference: (*S91, S93-S96, S98*)
Assortative mating: (*S91*)

Apple Maggot Fly (*Rhagoletis pomonella*):
Number of divergent ecological traits: host preference and timing of eclosion (*S101, S102*)
Genetic basis of traits: (*S103*)
Intrinsic postzygotic isolation: (*S104*)
Extrinsic postzygotic isolation: (*S105*)
Spatial segregation: (*S102*)
Strength of mtDNA differentiation: presumably no (*S106*)
Strength of nuclear differentiation: (*S107*)
Ecological selection: (*S101, S102*)
Mate preference: (*S102*)
Assortative mating: (*S102*)

Rough Periwinkle (*Littorina saxatilis*):
Number of divergent ecological traits: Habitat, brood size, embryo size (*S108*)
Genetic basis of traits: Unknown, presumably polygenic (*S109, S110*)

Intrinsic postzygotic isolation: (*S108*)
Extrinsic postzygotic isolation: Presumably yes (*S111*)
Spatial segregation: (*S108, S110*)
Strength of mtDNA differentiation: (J. Grahame, Pers. comm.)
Strength of nuclear differentiation: (*S109, S110, S112*)
Ecological selection: (*S108, S111*)
Mate preference: (*S113*)
Assortative mating: (*S112*)

***Mimulus lewisii* & *M. cardinalis*:**
Number of divergent traits: elevation, vegetative features (leaf shape, leaf serration, stem height), floral characteristics (flower color, petal shape, corolla width, nectar guides, amount of nectar), flowering time (*S114-S119*)
Genetic basis of traits: (*S115-S117*)
Intrinsic postzygotic isolation: (*S118*)
Extrinsic postzygotic isolation: (*S118, S119*)
Spatial segregation: (*S114, S118*)
Strength of mtDNA differentiation: chloroplast, presumably no or weak (*S120*)
Strength of nuclear differentiation: (*S121*)
Ecological selection: (*S114, S116, S119*)
Mate preference: NA
Assortative mating: (*S116, S118, S119*)

Larch Budmoth (*Zeiraphera diniana*)
Number of divergent ecological traits: size, pheromones, host preference, phenology (*S122-S126*)
Genetic basis of traits: Presumably polygenic (*S124*)
Intrinsic postzygotic isolation: (*S124*)
Extrinsic postzygotic isolation: (*S123, S125*)
Spatial segregation: (*S125*)
Strength of mtDNA differentiation: None (Dres and Mallet, unpublished data)
Strength of nuclear differentiation: (*S123-S125*)
Ecological selection: (*S125*)
Mate preference: (*S125*)
Assortative mating: (*S125*)

Pea Aphid (*Acyrthosiphon pisum*):
Number of divergent ecological traits: host plant, wing morphology (*S127-S130*)
Genetic basis of traits: (*S127, S131*)
Intrinsic postzygotic isolation: presumably none (*S131*)
Extrinsic postzygotic isolation: (*S129, S130*)
Spatial segregation: (*S127, S129, S130*)
Strength of mtDNA differentiation: Unknown

Strength of nuclear differentiation: (*S127, S130*)
Ecological selection: (*S127, S131*)
Mate preference: Presumably no (*S131*)
Assortative mating: (*S127, S129, S130*)

Apoyo lake Cichlids (*Amphilophus citrinellus* and *A. zeliosus*):
Number of divergent traits: body shape, trophic morphology (pharyngeal jaws), diet, escape behavior, and courtship behavior (*S132-S135*)
Genetic basis of traits: unknown – presumably polygenic
Intrinsic postzygotic isolation: (*S136*)
Extrinsic postzygotic isolation: unknown – presumably yes (*S135*)
Spatial segregation: (*S135*)
Strength of mtDNA differentiation: (*S135*)
Strength of nuclear differentiation: (*S135*)
Ecological selection: (*S135*)
Mate preference: (*S136*)
Assortative mating: (*S136*)

***Gambusia hubbsi*:**
Number of divergent traits: body shape (multiple components including size of caudal peduncle & head size) & swimming performance (*S137, S138*)
Genetic basis of traits: presumably polygenic (*S137, S139*)
Intrinsic postzygotic isolation: (*S137, S138*)
Extrinsic postzygotic isolation: presumably yes (*S138*)
Spatial segregation: (*S138*)
Strength of mtDNA differentiation: (*S138*)
Strength of nuclear differentiation: (*S138*)
Ecological selection: (*S138*)
Mate preference: (*S138*)
Assortative mating: presumably yes (*S138*)

***Anthoxanthum odoratum*:**
Number of divergent traits: flowering time, height, yield (*S140*)
Genetic basis of traits: presumably polygenic
Intrinsic postzygotic isolation: presumably no
Extrinsic postzygotic isolation: (*S140, S141*)
Spatial segregation: (*S140, S141*)
Strength of mtDNA differentiation: chloroplast (*S141*)
Strength of nuclear differentiation: (*S141*)
Ecological selection: (*S140, S141*)
Mate preference: NA
Assortative mating: (*S140, S141*)

***Nesospiza* buntings:**
Number of divergent traits: bill depth, wing length, diet, song, plumage color (*S142*)
Genetic basis of traits: presumably polygenic
Intrinsic postzygotic isolation: presumably no (*S142*)
Extrinsic postzygotic isolation: presumably yes (*S142*)
Spatial segregation: (*S142*)
Strength of mtDNA differentiation: (*S142*)
Strength of nuclear differentiation: (*S142*)
Ecological selection: (*S142*)
Mate preference: (*S142*)
Assortative mating: (*S142*)

***Howea* palms:**
Number of divergent traits: inflorescence spike number, leaf shape, flowering time, soil pH (*S143*)
Genetic basis of traits: presumably polygenic
Intrinsic postzygotic isolation: unknown
Extrinsic postzygotic isolation: presumably yes (*S143*)
Spatial segregation: (*S143*)
Strength of mtDNA differentiation: unknown
Strength of nuclear differentiation: (*S143*)
Ecological selection: (*S143*)
Mate preference: NA
Assortative mating: (*S143*)

***Hypoplectrus* hamlet fish:**
Number of divergent traits: color pattern, association time with mimicry model (*S144*)
Genetic basis of traits: presumably polygenic
Intrinsic postzygotic isolation: presumably no (*S144, S145*)
Extrinsic postzygotic isolation: presumably yes (*S144*)
Spatial segregation: (*S144*)
Strength of mtDNA differentiation: (*S145*)
Strength of nuclear differentiation: (*S144*)
Ecological selection: (*S144*)
Mate preference: (*S144*)
Assortative mating: (*S144*)

Table S2. Scoring of categorical variables for MCA analysis.

System name	System #	Number of divergent traits[1]	Genetic basis of traits?	Postzygotic Isolation (intrinsic and/or extrinsic	Spatial segregation?	Strength of genetic differentiation
Heliconius cydno alithea	1	one	single locus	no	none	no
Timema cristinae	2	many	polygenic	yes	fine	weak
Oophaga pumilio	3	one	polygenic	yes	broad	strong
Gasterosteus aculeatus (benthic/limnetic)	4	many	polygenic	yes	fine	strong
H. cydno & *H. pachinus*	5	one	polygenic	yes	broad	mix[2]
H. erato & *H. himera*	6	few	polygenic	yes	broad	strong
H. cydno & *H. melpomene*	7	many	polygenic	yes	fine	strong
Ostrinia nubilalis	8	few	polygenic	yes	none	weak
Pundamilia cichlids	9	many	polygenic	yes	fine	weak
Rhagoletis pomonella	10	few	polygenic	yes	fine	weak
Littorina saxatilis	11	few	polygenic	yes	fine	weak
Mimulus lewisii & *M. cardinalis*	12	many	polygenic	yes	broad	mix
Zeiraphera diniana (Larch budmoth)	13	many	polygenic	yes	fine	mix
Acyrthosiphon pisum (Pea aphid)	14	few	polygenic	yes	fine	mix
Amphilophus citrinellus & *A. zaliosus*	15	many	polygenic	yes	none	weak
Gambusia hubbsi	16	few	polygenic	yes	broad	strong
Anthoxanthum odoratum	17	few	polygenic	yes	fine	weak
Nesospiza buntings	18	many	polygenic	yes	none	mix
Howea palms	19	many	polygenic	yes	none	mix
Hypoplectrus hamlet fish	20	few	polygenic	yes	none	weak

[1] few = 2 or 3, many = 4 or more. See Table S1.
[2] mix = combination of strong differentiation in one category with no, weak, or unknown in another. See Table S1.

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