Evolution, 60(6), 2006, pp. 1254–1268

MULTILOCUS ANALYSES OF ADMIXTURE AND INTROGRESSION AMONG HYBRIDIZING *HELICONIUS* BUTTERFLIES

MARCUS R. KRONFORST,[1] LAURA G. YOUNG, LAUREN M. BLUME, AND LAWRENCE E. GILBERT
Section of Integrative Biology, University of Texas, Austin, Texas 78712

Abstract.—Introgressive hybridization is an important evolutionary process and new analytical methods provide substantial power to detect and quantify it. In this study we use variation in the frequency of 657 AFLP fragments and DNA sequence variation from 15 genes to measure the extent of admixture and the direction of interspecific gene flow among three *Heliconius* butterfly species that diverged recently as a result of natural selection for Müllerian mimicry, and which continue to hybridize. Bayesian clustering based on AFLP genotypes correctly delineated the three species and identified four *H. cydno*, three *H. pachinus*, and three *H. melpomene* individuals that were of mixed ancestry. Gene genealogies revealed substantial shared DNA sequence variation among all three species and coalescent simulations based on the Isolation with Migration (IM) model pointed to interspecific gene flow as its cause. The IM simulations further indicated that interspecific gene flow was significantly asymmetrical, with greater gene flow from *H. pachinus* into *H. cydno* ($2Nm = 4.326$) than the reverse ($2Nm = 0.502$), and unidirectional gene flow from *H. cydno* and *H. pachinus* into *H. melpomene* ($2Nm = 0.294$ and 0.252, respectively). These asymmetries are in the directions expected based on the genetics of wing patterning and the probability that hybrids of various phenotypes will survive and reproduce in different mimetic environments. This empirical demonstration of extensive interspecific gene flow is in contrast to a previous study which found little evidence of gene flow between another pair of hybridizing *Heliconius* species, *H. himera* and *H. erato*, and it highlights the critical role of natural selection in maintaining species diversity. Furthermore, these results lend support to the hypotheses that phenotypic diversification in the genus *Heliconius* has been fueled by introgressive hybridization and that reinforcement has driven the evolution of assortative mate preferences.

Key words.—Admixture, gene flow, *Heliconius*, hybridization, introgression, isolation with migration, Lepidoptera.

Received January 3, 2006. Accepted April 7, 2006.

Hybridization can influence the process of speciation in a variety of ways (Barton 2001). In some instances hybridization can promote diversification. If hybrid fitness is reduced, natural selection may favor enhanced prezygotic isolation and drive speciation to completion (Butlin 1987). In addition, hybridization can bring together new combinations of genes which might be favorable in certain environments, or allow the exploitation of a previously unavailable niche which, in the long term, can lead to the formation of new species (Burke and Arnold 2001). On the other hand, hybrids can act as corridors for gene exchange between incipient species which may inhibit further divergence by breaking up favorable genetic correlations and transporting, between species, alleles responsible for divergent characters (Ortíz-Barrientos et al. 2002). However, gene flow does not necessarily impede divergence or cause incipient species to fuse. When natural selection drives and maintains ecological divergence between species, gene flow can have a variable impact throughout the genome. Regions of the genome that are under divergent selection can accrue and maintain significant differentiation while gene flow homogenizes the rest of the genome (Emelianov et al. 2004; Turner et al. 2005). Although there are a number of instances in which natural selection for ecological divergence has driven speciation in the absence of geographic barriers (Bush 1994; Feder et al. 1994; Schluter 1998), it is unclear to what extent gene flow persists throughout the process.

Robust quantification of gene flow among young species has traditionally been challenging given the potential for shared ancestral variation. Today however, high-throughput DNA genotyping and sequencing, combined with a variety of new analytical methods, offer substantial power to estimate the extent of introgression among hybridizing taxa (Mallet 2005). Methods like the clustering algorithm implemented in Pritchard et al.'s (2000) STRUCTURE software use multilocus genotype data to infer discrete genetic clusters and estimate admixture among them. Others, like the coalescent simulation method of Hey and Nielsen (2004), use multilocus DNA sequence data from predefined populations to estimate parameters such as effective population sizes, time of population divergence, and between population migration rates. Although relatively new, these approaches have proven effective in estimating admixture and introgression in a wide variety of organisms (Mallet 2005). Here we apply both approaches to measure the extent of contemporary admixture and historical gene flow among three hybridizing butterfly species.

Neotropical *Heliconius* butterflies are unpalatable, aposematic, and have undergone a recent adaptive radiation in wing color patterns as a result of natural selection for Müllerian mimicry. Historically, this radiation was believed to be the result of allopatric divergence while species and races were isolated to Pleistocene refugia (Brown et al. 1974; Sheppard et al. 1985; Turner and Mallet 1996). This explanation is now in doubt because divergence times among races and species are considerably older than the last glacial advance (Brower 1994a, 1996), comimetic taxa have experienced very different demographic and evolutionary histories (Brower 1996; Flanagan et al. 2004), and mtDNA haplotypes are shared among races; a pattern that is inconsistent with long-term reductions in population sizes (Brower 1994a, 1996). Rather, diversification within the genus appears to have been fueled largely

[1] Present address: Department of Ecology and Evolutionary Biology, Rice University, Houston, Texas 77005; E-mail: mkronforst@mail.utexas.edu.

© 2006 The Society for the Study of Evolution. All rights reserved.

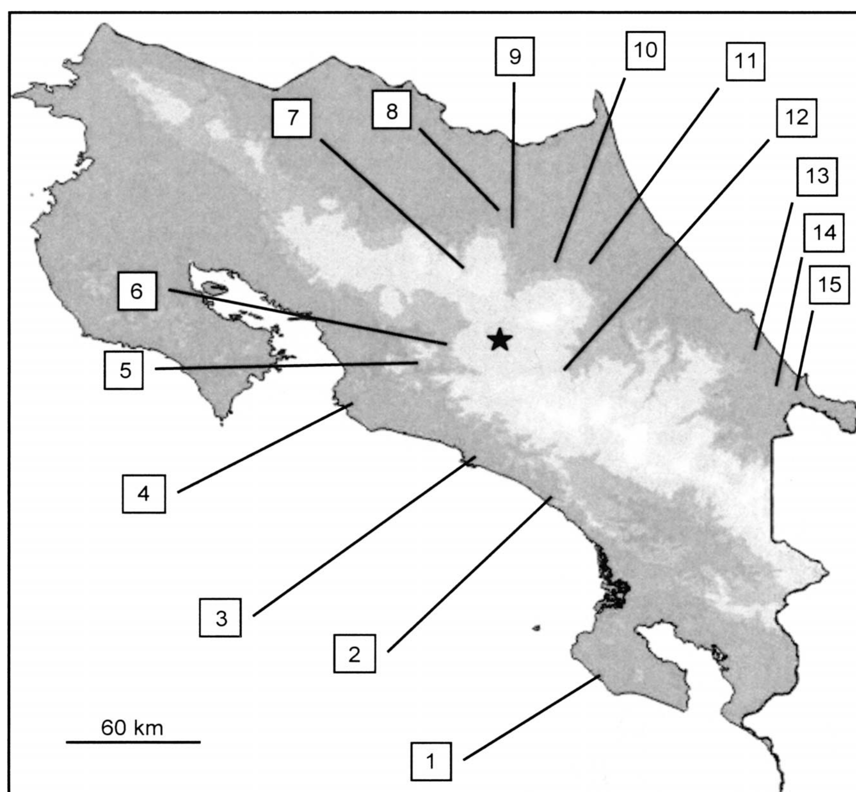

FIG. 1. Map of Costa Rica with sampling locations. Locations are as follows: 1, Sirena Biological Station, Corcovado National Park; 2, Dominical; 3, Manuel Antonio National Park; 4, Carara Biological Reserve; 5, Santiago de Puriscal; 6, Finca Hamadryas; 7, Cariblanco; 8, La Selva Biological Station; 9, Horquetas; 10, Guapiles; 11, Guacimo; 12, Tapantí National Park; 13, Bananito; 14, Hitoy Cerere Biological Reserve; 15, Vesta. Costa Rica's capital, San José (indicated by a star), is located within the Meseta Central, which is a corridor between the coastal drainages. Shading indicates topographic relief.

by ecological differentiation, divergence in wing patterns, and the evolution of assortative mating preferences (McMillan et al. 1997; Mallet et al. 1998; Jiggins et al. 2001). As a consequence of recent diversification, reproductive isolation is incomplete among a number of species; approximately 25% of *Heliconius* species are known to hybridize in nature (Mallet et al. 1998). Thus, phenotypic, behavioral, and ecological divergence has occurred in the presence of potential gene flow.

In Costa Rica, *H. melpomene*, *H. cydno*, and *H. pachinus* come into contact and occasionally hybridize. Sister species *H. melpomene* and *H. cydno*, which diverged within the last 1.5 million years (Beltrán et al. 2002), are sympatric throughout Central and northern South America where each has radiated into a variety of racial forms to match different mimicry models. Although *H. melpomene* and *H. cydno* differ in mimicry, host-plant use (Smiley 1978), and habitat specialization (Estrada and Jiggins 2002), they continue to hybridize at low frequency. F_1 and backcross hybrids are easily produced in captivity and at least 60 have been collected from their overlapping range in the last century (Mallet et al. 2003). Three of these field-caught hybrids were found in Costa Rica. Consistent with Haldane's Rule, F_1 hybrid males are fertile while females are sterile (Naisbit et al. 2002).

Heliconius pachinus, a lineage within the *H. cydno* clade (Brower 1994b, 1996; Brower and Egan 1997; Beltrán et al. 2002), is similar to *H. cydno* in every respect aside from wing pattern. In Costa Rica, the local race of *H. cydno*, *H. c. galanthus*, is restricted to the Caribbean drainage, *H. pachinus* is restricted to the Pacific drainage, and *H. melpomene rosina* is distributed throughout the parapatric distributions of each. *Heliconius cydno galanthus* and *H. pachinus* hybridize freely in captivity and approximately 10 hybrids have been collected in the field (L. Gilbert, pers. obs.). Despite their parapatric distributions, there is no definable hybrid zone between *H. cydno* and *H. pachinus* because of extensive habitat degradation in Costa Rica's Meseta Central. This plateau is the only potential point of contact in Costa Rica due to a series of mountain ranges that run the length of the country (Fig. 1). Most *cydno*/*pachinus* hybrids have been found on the Caribbean drainage, immediately adjacent to the Meseta Central (L. Gilbert, pers. obs.). Hybrids between these two are completely fertile. Although *H. pachinus* and *H. melpomene rosina* hybridize in captivity and are sympatric throughout the Pacific drainage of Costa Rica and northern Panama, there are no known field-caught hybrids between them. Laboratory-reared hybrids exhibit the same female-limited sterility that characterizes crosses between *H. cydno* and *H. melpomene* (Gilbert 2003).

Heliconius melpomene, *H. cydno*, and *H. pachinus* each have 21 chromosomes and the color pattern differences among them are largely controlled by only five or six loci (Gilbert 2003; Naisbit et al. 2003). Hybridization is limited but persistent, thus the large portion of the genome not re-

sponsible for color pattern or ecological differences may be experiencing substantial gene flow. On the other hand, hybridization does not necessarily result in introgression. Hybrids possess recombinant, nonmimetic color patterns and therefore are subject to intense predation. Manipulative and transplant experiments have documented elevated predation rates on novel patterns (Benson 1972; Mallet and Barton 1989; Kapan 2001) and selection against hybrids in one *H. erato* racial hybrid zone was estimated to be $s \approx 0.5$ (Mallet and Barton 1989; Mallet 1993). Furthermore, recombinant wing patterns are not recognized by parental species as potential mates and thus hybrids are subject to disruptive sexual selection (Naisbit et al. 2001). In the case of hybrids between *H. melpomene* and either *H. cydno* or *H. pachinus*, females are sterile and both sexes may be subject to disruptive ecological selection as well. Although the production of fertile hybrids makes interspecific gene flow possible, hybrids may be ineffective at transferring genes across the species boundary. In fact, the only study to address genetic differentiation and introgression between hybridizing *Heliconius* species found very minimal interspecific gene flow. Jiggins et al. (1997) surveyed 30 allozyme loci and mitochondrial DNA haplotypes across a hybrid zone between *H. erato* and *H. himera*. Despite 5–10% hybrids in mixed populations, the species showed no sign of homogenization near the hybrid zone and very little evidence of gene flow across it.

In this study we use hundreds of amplified fragment length polymorphisms (AFLPs) and comparative DNA sequence data for 15 genes to measure the extent and direction of interspecific gene flow among *H. melpomene*, *H. cydno*, and *H. pachinus* in Costa Rica. Specifically, we address three questions. First, is there evidence for contemporary admixture among *H. cydno*, *H. pachinus*, and *H. melpomene* in Costa Rica? Second, what are the historical rates of introgression among the three species? And third, is gene flow between species symmetrical and if not, are asymmetries consistent with data on the genetics of color patterning, hybrid fitness, and mate preference?

Materials and Methods

Specimens

We collected 56 *H. cydno*, 44 *H. pachinus*, and 27 *H. melpomene* specimens from various locations throughout Costa Rica (Appendix; Fig. 1). None of the sampled butterflies exhibited phenotypic evidence of recent introgression except one *H. cydno* male from location 8. This individual had a *H. cydno* wing pattern but showed partial expression of the *H. pachinus* proximal ''shutter'' on the fore- and hindwings, indicating heterozygosity at the wing patterning locus *Ps* (Nijhout et al. 1990; Gilbert 2003). All specimens were collected in the field as adults between June and August 2000 or 2002. Tissue was preserved in 95% ethanol and total genomic DNA was extracted using a DNeasy Tissue Kit (Qiagen, Valencia, CA) or a standard phenol/chloroform extraction protocol.

Generating AFLPs

We typed each of the 127 individuals with amplified fragment length polymorphisms (AFLPs). The AFLP technique produces a large number of polymorphic markers that are distributed throughout the genome and therefore is a useful tool for population genetic analyses (Mueller and Wolfenbarger 1999). The basic procedure involves digesting genomic DNA with two restriction enzymes, annealing adaptors of known sequence to the ends of these restriction fragments, and then narrowing the overall number of fragments by performing two increasingly selective rounds of polymerase chain reaction (PCR) amplification (Vos et al. 1995). We generated markers using the PE Applied Biosystems AFLP plant mapping kit (PE Applied Biosystems, Foster City, CA) and separated fragments with an ABI Prism 3100 genetic analyzer (PE Applied Biosystems). Four selective primer combinations were used to generate fragments; EcoRI-ACT/MseI-CAT, EcoRI-ACT/MseI-CTG, EcoRI-ACA/MseI-CAT, and EcoRI-ACA/MseI-CTG.

AFLP Data Analyses

We sized and scored AFLP fragments between 50 and 500 bp using ABI GENEMAPPER software version 3.7 (PE Applied Biosystems). Fragments with a peak height below 100 reflectance units were scored as an absence. To identify instances of admixture we employed the Bayesian/Markov chain Monte Carlo method implemented in STRUCTURE (Pritchard et al. 2000; Falush et al. 2003). With this method individuals can be assigned to populations under the assumption of no admixture, in which case the probability of each individual originating from each inferred population is estimated. Alternatively, an admixture model allows individuals to be of mixed ancestry and estimates the proportion of each individual's genotype that can be traced to each of the inferred populations. Although there is uncertainty associated with estimating allele frequencies from dominant markers like AFLPs, a recent advance in the models employed by STRUCTURE accounts for this uncertainty in the clustering process by allowing the user to define a null allele at each locus (vers. 2.2). Based on the partial genotypic data supplied to the model, the identity of the null allele and the allele frequencies from the inferred populations of origin, STRUCTURE 2.2 estimates the probability of each possible diploid genotype at each locus and uses these to iteratively update genotype parameters of the model.

We applied the AFLP data to the STRUCTURE models in two ways. To assess the power of the method to discriminate the three species we first used the no-admixture model and performed naive clustering, providing only genetic data and no prior information regarding the identity of individuals. Given our a priori expectation of three genetic clusters, and the fact that our questions relate only to species-level differentiation and gene flow, all analyses assumed three populations. To detect gene flow we repeated this same analysis using the admixture model. In each case a burn-in of 10,000 iterations was followed by 10^6 iterations of data collection. We assessed support for possible instances of mixed ancestry in two ways. First, as part of the admixture clustering, we computed the 95% posterior probability interval around each individual admixture proportion using the ANCESTDIST option. Individuals for whom the probability interval of the genome proportion derived from the population of origin did

not include one are likely to have experienced introgression. Second, we performed an additional round of admixture clustering, this time indicating the population of origin for each individual and setting the prior probability of each individual having pure ancestry from its assigned population at 0.95 (USEPOPINFO option, MIGRPRIOR = 0.05). Using this model we estimated the probability that each individual was a member of each of the other populations (was misclassified) or had an ancestor from each of the other populations within the last three generations (GENSBACK = 3). This run had a burn-in of 10,000 iterations followed by 90,000 iterations of data collection.

Locus Selection and DNA Sequencing

We sequenced multiple haplotypes for one mitochondrial and 14 nuclear loci from *H. cydno*, *H. pachinus*, *H. melpomene*, and the closely related outgroup, *H. hecale* (Table 1). Eight of the 15 selected loci (*apterous*, *cubitus interruptus*, *Distal-less*, *engrailed*, *invected*, *patched*, *scalloped*, and *wingless*) potentially play a role in wing color patterning (Carroll et al. 1994; Keys et al. 1999; Brunetti et al. 2001; Reed and Gilbert 2004; Reed and Serfas 2004) and three (*cinnabar*, *scarlet*, and *white*) are members of the ommochrome biosynthesis pathway which is responsible for the formation and depositing of colors on the developing wings of Nymphalid butterflies (Reed and Nagy 2005). The four remaining loci are standard regions used for phylogenetics (*Elongation factor* 1 − α and mtDNA: *Cytochrome Oxidase I* and *II*) or intron-containing regions of nuclear genes that have been characterized in *Heliconius* (*Mannose phosphate isomerase* and *Triose phosphate isomerase*). All 14 nuclear loci have been placed on a *H. cydno* genetic map (M. Kronforst and L. Gilbert, unpubl. data).

We developed polymerase chain reaction (PCR) primers for *ap, Dll, en, inv, ptc, sd, st, w,* and *wg* by comparing amino acid and nucleotide sequences among a variety of insects including *Drosophila melanogaster*, *Precis coenia*, and *Bombyx mori* (Kronforst 2005). Primers for *cinnabar* and a *scarlet* sequence from *H. melpomene* were provided by R. Reed (Duke University). Primers for *Tpi, Mpi,* and *ci* were developed by A. Tobler in the lab of W. O. McMillan (Beltrán et al. 2002), *Ef1*α primers are from Cho et al. (1995), *CO* primers are from Simon et al. (1994), and the reverse *wg* primer is from Brower and DeSalle (1998). All sequenced regions aside from *Ef1*α and *CO* contained at least one intron. Primers and details of the sequenced regions have been published previously or are available from the authors.

To survey haplotype diversity, we selected five to seven *H. cydno*, *H. pachinus*, *H. melpomene*, and *H. hecale* individuals for DNA sequencing. Based on their AFLP genotypes, one *H. cydno* and one *H. melpomene* specimen selected for DNA sequencing had experienced recent introgression. PCR products were amplified in 10 μl reaction volumes using a touch-down thermal cycling profile which consisted of an initial denaturing step at 94°C for 2 min followed by 94°C for 30 sec, 65°C for 30 sec, 72°C for 1 min for 15 cycles with the annealing temperature reduced 1°C/cycle, then 25 cycles at an annealing temperature of 50°C. For *CO,* the same profile was used without the step-down (40 cycles with an annealing temperature of 50°C).

For loci that exhibited within-species length variation (*ap, ci, Dll, en, inv, Mpi, ptc, sd, st, Tpi, w, wg*) PCR products were pooled by species and cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA.). Approximately four to 10 clones were then sequenced from each species pool (occasionally fewer for *H. hecale*). Although this approach allowed us to rapidly survey species-level genetic variation at each locus, it does suffer from two potential complications. First, sequencing cloned PCR products can reveal single-base errors and in vitro recombination that occur during PCR amplification (Kobayashi et al. 1999; Beltrán et al. 2002), both of which could not be detected as artifacts by our haplotype screening method. We sought to minimize single-base substitution errors by amplifying PCR products with a 9:1 mixture of *Taq* polymerase and the proofreading polymerase *Pfx* (Invitrogen). However, undetected single-base errors are unlikely to influence phylogenetic analyses or coalescent simulations because they occur randomly and thus would appear as singleton polymorphisms (Beltrán et al. 2002). In addition, in vitro recombination is quite rare (Beltrán et al. 2002) and although it may have influenced our gene tree estimates, any potential impact on our analyses of introgression was eliminated because we only studied a single region of each gene that exhibited no evidence of recombination (see below).

A second potential analytical complication to result from our haplotype screening method was that when identical or nearly identical haplotypes were sequenced from the same species pool, it was impossible to know whether these represented two PCR copies of the same haplotype or multiple occurrences of the same haplotype in the population. To partially overcome this issue, we directly sequenced individual PCR products for a subset of loci (*ap, Dll, inv, Mpi, ptc, st, Tpi, wg*) and used the cloned sequences to identify the haplotypes carried by some or all individuals. For *Dll,* we directly sequenced haplotypes from 12 *H. cydno*, 13 *H. pachinus*, 12 *H. melpomene*, and 11 *H. hecale* individuals. For loci that did not exhibit length variation (*cn, Ef1*α, *CO*), PCR products were sequenced directly. For *cn, Ef1*α*, Mpi, Tpi, and CO* only one *H. hecale* individual was sequenced and for *CO,* we sequenced 50 *H. cydno* and 41 *H. pachinus* samples. All PCR products were sequenced in both directions using Big Dye version 3 (PE Applied Biosystems) and analyzed with an ABI Prism 3100 automated sequencer (PE Applied Biosystems). Sequences have been deposited in GenBank under accession numbers AY744577–AY744672, AY745254–AY745278, AY745315–AY745335, AY745356–AY745490, and DQ448305–DQ448516.

The datasets for *Mpi, Tpi,* and *CO* were supplemented with sequences available in GenBank (accession numbers *Mpi*: AF413731, AF413734, AF413739–AF413744, AF516220, AY332417–AY332422, AY332461–AY332464, *Tpi*: AF413778, AF413782–AF413790, AY329804, AY329805, AY329839–AY329843, *CO*: U08482, U08483, U08500, U08518, U08520, U08523, U08524, U08544, AF413672–AF413674, AF413679, AF413683, AF413707). All of these sequences have previously been reported by Brower (1994a, b), Beltrán et al. (2002), and Flanagan et al. (2004). Because Brower (1994a,b) and Beltrán et al. (2002) analyzed larger

TABLE 1. Details of the sequenced loci.

Locus	Species	No. haplotypes	No. polymorphic sites (S)	Population mutation rate (π/bp)	Minimum number of recombination events (R_M)	Length of fully aligned dataset (bp)
apterous (*ap*)	*cydno*	7	17	0.03942	1	533
	pachinus	8	10	0.03450	0	
	melpomene	8	0	0.00000	—	
	hecale	2	0	0.00000	—	
cubitus interruptus (*ci*)	*cydno*	8	9	0.01320	0	345
	pachinus	8	18	0.02270	1	
	melpomene	8	24	0.03506	2	
	hecale	8	17	0.02395	0	
cinnabar (*cn*)	*cydno*	5	4	0.00466	1	515
	pachinus	5	5	0.00505	0	
	melpomene	5	2	0.00233	1	
	hecale	1	—	—	—	
Cytochrome oxidase (*CO*)	*cydno*	54	18	0.00374	—	589
	pachinus	43	13	0.00227	—	
	melpomene	9	1	0.00085	—	
	hecale	3	2	0.00226	—	
Distal-less (*Dll*)	*cydno*	22	32	0.01885	6	608
	pachinus	24	35	0.02139	4	
	melpomene	21	47	0.03533	7	
	hecale	13	18	0.01337	3	
Elongation factor 1α (*Ef1*α)	*cydno*	5	7	0.00259	1	1240
	pachinus	5	2	0.00065	0	
	melpomene	5	5	0.00162	0	
	hecale	2	0	0.00000	—	
engrailed (*en*)	*cydno*	4	21	0.03681	0	392
	pachinus	5	23	0.03323	0	
	melpomene	5	12	0.01847	0	
	hecale	7	13	0.01641	0	
invected (*inv*)	*cydno*	9	43	0.03180	4	482
	pachinus	8	27	0.01734	0	
	melpomene	9	29	0.01962	1	
	hecale	9	23	0.03322	0	
Mannose phosphate isomerase (*Mpi*)	*cydno*	8	53	0.05766	2	454
	pachinus	12	12	0.01311	0	
	melpomene	10	43	0.04161	0	
	hecale	4	13	0.01928	0	
patched (*ptc*)	*cydno*	6	22	0.01265	3	687
	pachinus	6	5	0.00243	0	
	melpomene	6	8	0.00487	0	
	hecale	6	2	0.00098	0	
scalloped (*sd*)	*cydno*	8	38	0.03418	4	549
	pachinus	8	21	0.01745	0	
	melpomene	8	16	0.01593	1	
	hecale	8	22	0.02218	0	
scarlet (*st*)	*cydno*	10	19	0.01631	0	504
	pachinus	8	16	0.00887	0	
	melpomene	7	30	0.02829	0	
	hecale	10	30	0.02345	1	
Triose phosphate isomerase (*Tpi*)	*cydno*	11	25	0.01561	1	614
	pachinus	11	22	0.01455	0	
	melpomene	10	15	0.00732	1	
	hecale	3	7	0.00763	0	
white (*w*)	*cydno*	4	28	0.04213	0	428
	pachinus	4	40	0.06215	0	
	melpomene	4	39	0.05228	0	
	hecale	3	51	0.09671	0	
wingless (*wg*)	*cydno*	11	19	0.00493	3	1403
	pachinus	8	15	0.00569	0	
	melpomene	5	13	0.00402	0	
	hecale	5	9	0.00316	1	

portions of *CO* then that which was sequenced here, all analyses of mtDNA data were performed after trimming the dataset to the same 589 bp region. An additional *H. hecale Ef1α* haplotype (AY090168) was also included.

DNA Sequence Analyses

Chromatograms were edited and contigs aligned using the program BioEdit (Hall 1999) and sequences were aligned using Clustal X 1.8 (Higgins and Sharp 1988) and by eye. For each locus we calculated the number of polymorphic sites, nucleotide diversity per base pair (Nei 1987), and the minimum number of recombination events (Hudson and Kaplan 1985) for each species using DnaSP 3.5 (Rozas and Rozas 1999).

We developed gene genealogies for each locus using distance and parsimony methods of phylogenetic reconstruction as well as Bayesian inference. Distance-based trees were estimated using the neighbor-joining method implemented in MEGA 2.1 (Kumar et al. 2001) based on uncorrected pairwise proportional differences with gaps excluded. The strength of support for each node was assessed by bootstrapping (1000 replicates). Maximum-parsimony (MP) trees were also constructed with MEGA 2.1, using the close-neighbor-interchange heuristic search option, excluding gaps, weighting all sites equally, and starting with a random tree. All MP trees for each locus were used to construct a strict consensus tree. Finally, we used MrBayes 3.0 (Huelsenbeck and Ronquist 2001) to develop gene trees and estimate posterior probabilities for each node with parameters estimated based on the default GTR + I + Γ model. Four Metropolis-Coupled Markov chains were run for 80,000 generations following 20,000 burn-in generations, sampling every ten generations, starting from a random tree. The branching patterns of the three trees (NJ, MP, and Bayesian) were compared for consistency.

Finally, to estimate historical rates of introgression among species we applied the combined DNA sequence data to the Isolation with Migration model implemented in the program IM (Nielsen and Wakeley 2001, Hey and Nielsen 2004, Won and Hey 2005). This Markov chain Monte Carlo method uses DNA sequences from a pair of populations to infer six model parameters; population mutation rates for both extant populations as well as the ancestor, time since divergence, and per gene migration rates in both directions. Since IM can only accommodate two populations at a time, we performed all three pairwise comparisons among *H. cydno*, *H. pachinus*, and *H. melpomene*. Furthermore, IM cannot accommodate gaps or missing data in DNA alignments or regions that exhibit evidence of recombination (Hey and Nielsen 2004). Therefore, for each comparison we removed all gaps from each alignment and tested the data for evidence of recombination using the four-gamete test (Hudson 1985) in DnaSP 3.5. Loci with evidence of recombination were divided into nonrecombining sections using the algorithm of Hudson and Kaplan (1985), and a single section was included in the IM analysis. To preserve as much genealogical information as possible, we choose the section of each gene with the most sequence variation. Following Won and Hey (2005), variation in the mitochondrial region *CO* that was consistent with recombination was removed. For each comparison, we ran IM with 10 Metropolis-coupled chains for 20 million steps of data collection following a 300,000 step burn-in.

We converted the maximum-likelihood estimate and 90% highest posterior density (HPD) interval for each parameter to biologically relevant units of effective population size (N), population migration rate ($2Nm$), or time in years since divergence using an average (geometric mean) per gene mutation rate which we estimated by comparison with sequences from *H. hecale*. Assuming a rate of mitochondrial evolution of 1.1–1.2% per lineage per million years (Brower 1994a), *H. hecale* is estimated to have split from the common ancestor of the three ingroup taxa approximately two million years ago. Given four million years of evolution between each species and *H. hecale*, our estimate of the average mutation rate per locus per year was 2.121×10^{-6} for the *cydno*/*pachinus* comparison, 2.438×10^{-6} for the *cydno*/*melpomene* comparison, and 2.535×10^{-6} for the *pachinus*/*melpomene* comparison. Conversion of population mutation rates to effective population sizes also requires a generation time. Following Kronforst and Fleming (2001), we used an estimate of 45 days per generation.

RESULTS

AFLP Clustering

We identified and scored a total of 664 presumptive AFLP loci, seven of which were monomorphic. Naive, no-admixture

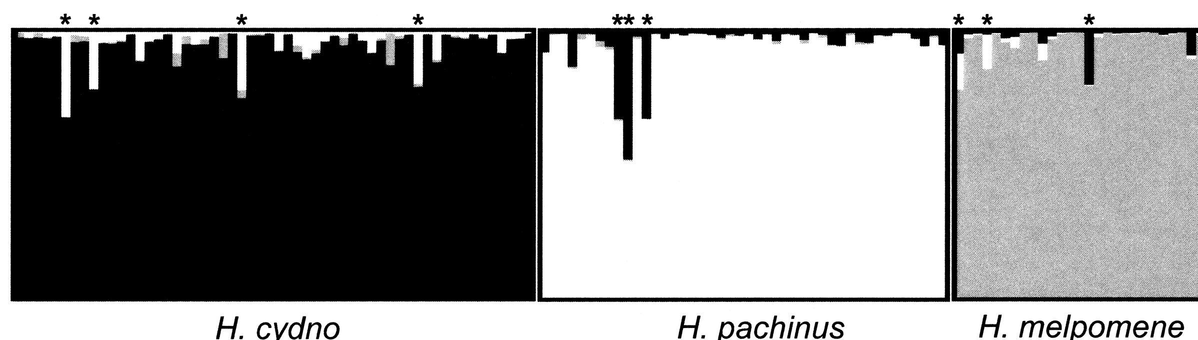

FIG. 2. Results of STRUCTURE admixture clustering. Each individual is represented by a narrow vertical column and the proportion of each of the three colors signifies the posterior mean proportion of ancestry from each of the three parental species. Black indicates proportion of genome from *Heliconius cydno*; white, from *H. pachinus*; and gray, from *H. melpomene*. Four *H. cydno*, three *H. pachinus*, and three *H. melpomene* (marked with asterisks) had probabilities of pure ancestry < 0.5. Individual order is as in the Appendix.

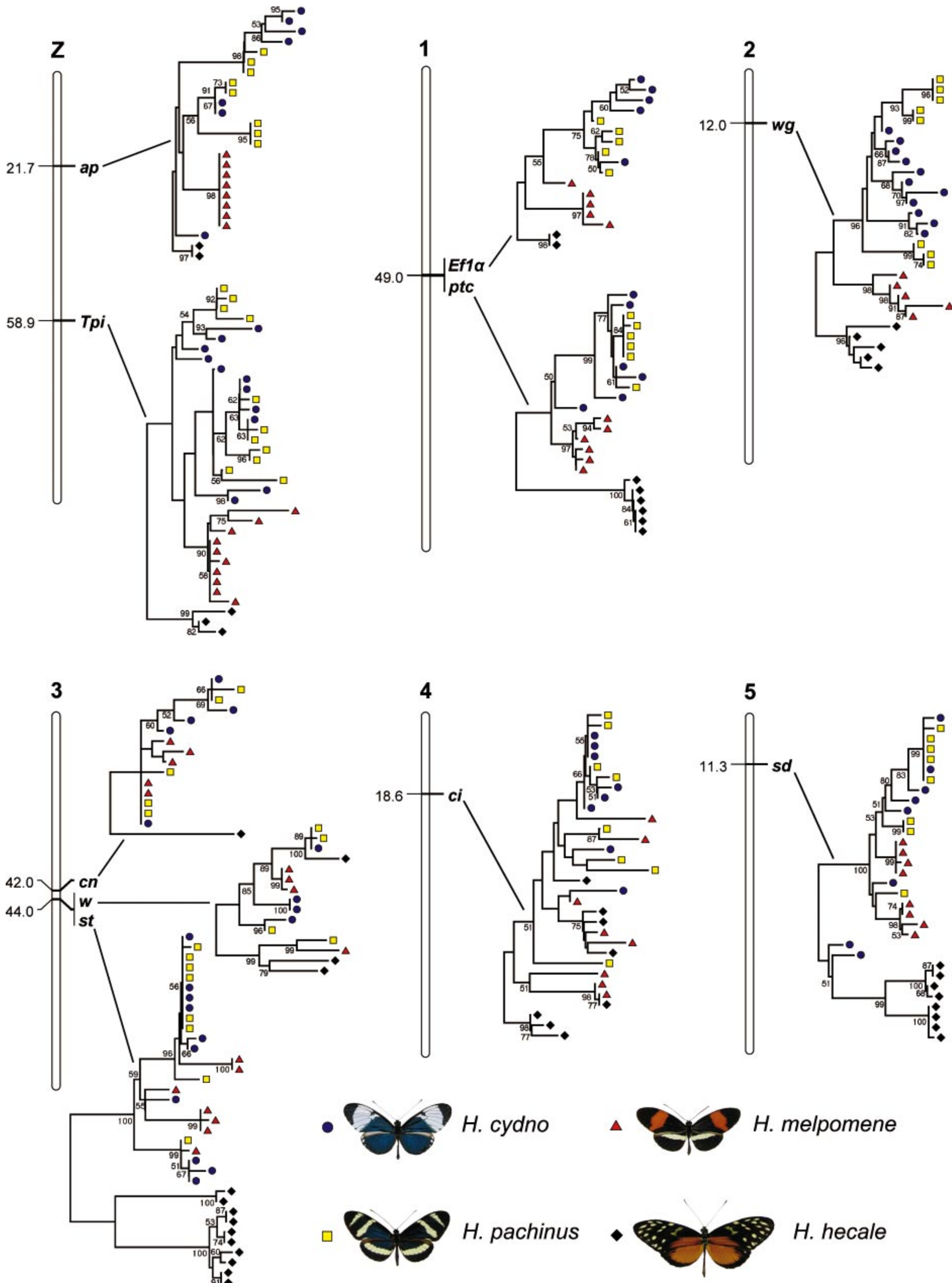

FIG. 3. Neighbor-joining gene trees for 15 loci on a *H. cydno* genetic map. Each locus is labeled with its position, in centimorgans, relative to the end of the linkage group. Terminal tree branches are labeled with species-specific colored markers according to the legend and nodes with 50% or greater bootstrap support are labeled.

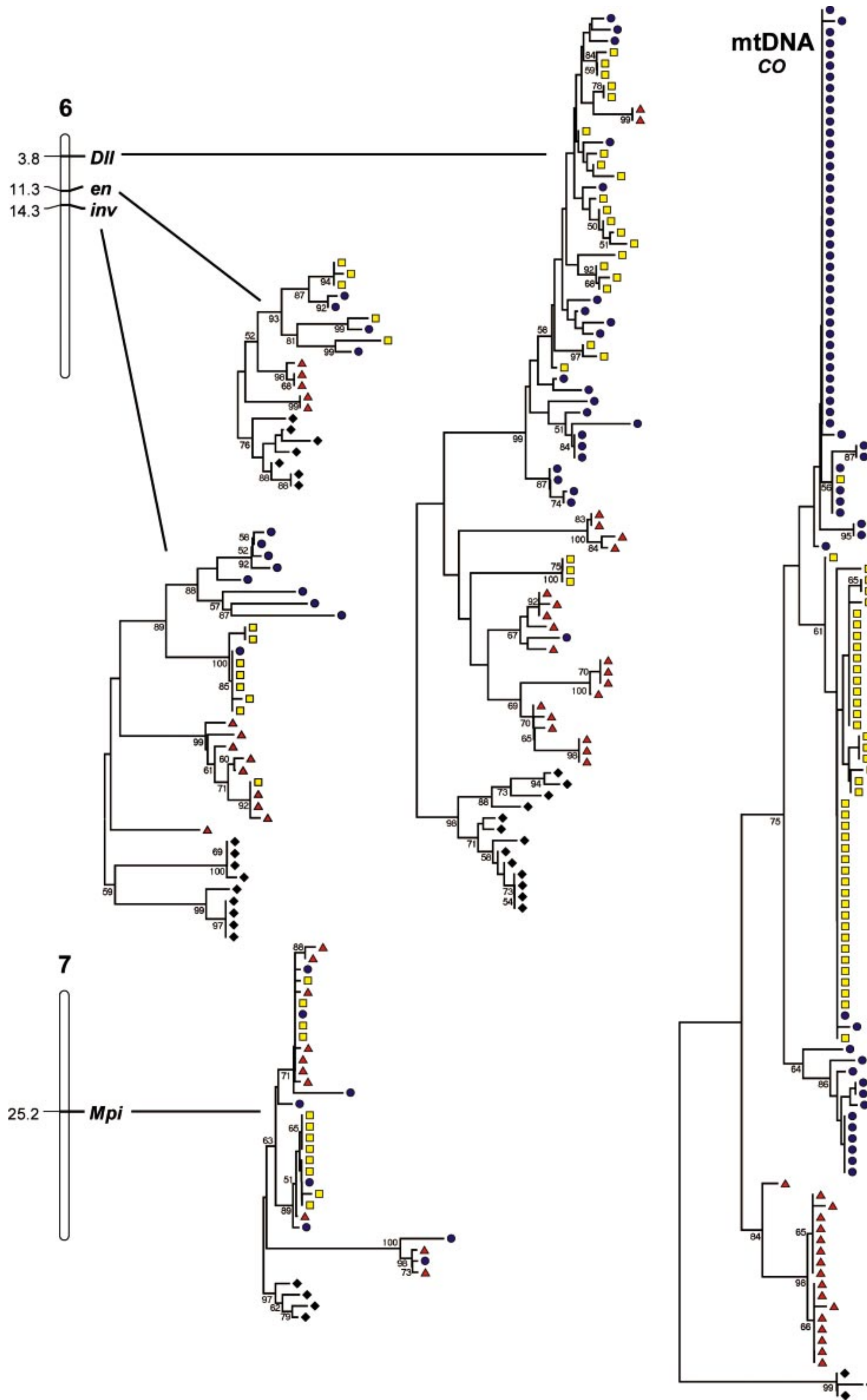

FIG. 3. Continued.

TABLE 2. Maximum-likelihood estimates (MLE) and 90% highest posterior density (HPD) intervals of IM model parameters; effective population size of species 1 (N_1), effective population size of species 2 (N_2), effective population size of ancestor (N_A), population migration rate from species 2 into species 1 ($2N_1m_1$), population migration rate from species 1 into species 2 ($2N_2m_2$), and time in years since species divergence (t).

Comparison	N_1	N_2	N_A	$2N_1m_1$	$2N_2m_2$	t
Heliconius cydno/*H. pachinus*						
MLE	5,197,000	1,539,000	3,942,000	4.326	0.502	543,000
Lower 90% HPD	3,088,000	672,000	25,000	2.644	0.060	274,000
Upper 90% HPD	8,461,000	2,535,000	50,191,000	7.146	1.570	9,424,000
H. cydno/*H. melpomene*						
MLE	4,323,000	1,849,000	2,951,000	0.000	0.294	939,000
Lower 90% HPD	3,133,000	1,076,000	23,000	0.000	0.116	464,000
Upper 90% HPD	6,062,000	2,864,000	33,328,000	0.454	0.737	8,201,000
H. pachinus/*H. melpomene*						
MLE	2,362,000	2,451,000	3,090,000	0.110	0.252	1,069,000
Lower 90% HPD	1,582,000	1,556,000	22,000	0.000	0.084	603,000
Upper 90% HPD	3,430,000	3,516,000	33,864,000	0.405	0.634	7,885,000

clustering with STRUCTURE correctly identified the three species and assigned all individuals to the appropriate group with posterior probabilities $\geq$ 0.95. Admixture clustering suggested multiple instances of mixed ancestry in all three species (Fig. 2). Eight *H. cydno*, four *H. pachinus*, and five *H. melpomene* had pure ancestry proportions less than 0.9. The individual with a hybrid phenotype was one of these, with a genome estimate of 87% *H. cydno*, 8% *H. pachinus*, and 5% *H. melpomene*. Additional analyses supported many apparent instances of mixed ancestry. Four *H. cydno*, three *H. pachinus*, and three *H. melpomene* specimens had population of origin genome proportion probability intervals that did not include one (Fig. 2). The *H. cydno* that exhibited phenotypic evidence of introgression was not one of these. To determine whether these possible instances of introgression were the result of recent hybridization, we assigned individuals to their respective species with high prior probability and estimated the posterior probability that each was, in fact, a member of another species or had a heterospecific ancestor within each of the last three generations. All misclassification probabilities, except for one (0.034), were 0.000. The four *H. cydno*, three *H. pachinus*, and three *H. melpomene* individuals identified previously all had probabilities of pure ancestry from the assigned species of less than 0.5. The probability of recent *H. pachinus* ancestry was small for the *H. cydno* that exhibited phenotypic evidence of introgression (0.006).

DNA Sequence Analyses

We analyzed 539 haplotypes from 15 loci comprising a total aligned length of 9343 bp (Table 1). Because most loci contained an intron, sequence variation was generally high (Table 1). Gene genealogies estimated with neighbor joining, parsimony, and Bayesian inference were highly concordant for all loci so only neighbor-joining trees are shown (Fig. 3). Across loci, the probability that haplotypes from a given species formed a monophyletic clade decreased with time since divergence. For instance, haplotypes from the outgroup, *H. hecale*, formed a well-supported clade for all loci except *ci* and *w*. *Heliconius melpomene* haplotypes also formed a monophyletic clade for a number of genes including *ap, CO, ptc, Tpi,* and *wg*. For others, *H. melpomene* haplotypes did not form an exclusive clade but clustered together and were distinguishable from those of other species, such as at *Ef1*α and *en.* Haplotypes from the closely related *H. cydno* and *H. pachinus* tended to cluster together but did not exhibit reciprocal monophyly. Identical haplotypes were commonly shared between species: *H. cydno* and *H. pachinus* shared haplotypes at eight loci (*ci, cn, CO, Mpi, sd, st, Tpi, w*); *H. cydno* and *H. melpomene* shared haplotypes at two loci (*cn, Mpi*); *H. pachinus* and *H. melpomene* shared haplotypes at three loci (*cn, inv, Mpi*); and *H. melpomene* and *H. hecale* shared a haplotype at *ci.*

To determine whether the shared DNA sequence variation among species was the result of introgression, we applied these data to the Isolation with Migration model using the program IM. For each pairwise comparison among *H. cydno*, *H. pachinus*, and *H. melpomene*, independent runs converged on the same marginal posterior probability distributions. The maximum-likelihood estimates and credibility intervals for these parameters were then converted into units of effective population size, population migration rate, and time in years since divergence (Table 2). In general, the results indicate that *H. cydno* has had an effective population size twice that of *H. pachinus* and *H. melpomene* and that *H. cydno* and *H. pachinus* diverged approximately 500,000 years ago with *H. melpomene* splitting from their common ancestor approximately one million years ago. Although the probability distribution for each of these demographic parameters had a clear maximum, the credibility intervals for ancestral population sizes and times since divergence were quite wide (Table 2). In terms of between species migration, the IM simulations yielded well-defined posterior distributions with clear maxima and narrow credibility intervals in all comparisons (Fig. 4). The results suggest extensive gene flow from *H. pachinus* into *H. cydno* ($2Nm$ = 4.326), and nonzero rates of introgression from *H. cydno* into *H. pachinus* and from *H. cydno* and *H. pachinus* into *H. melpomene* (Table 2).

For a more detailed look at introgression in the IM analyses, we measured the distribution of the number of introgression events for each locus as well as the distribution of

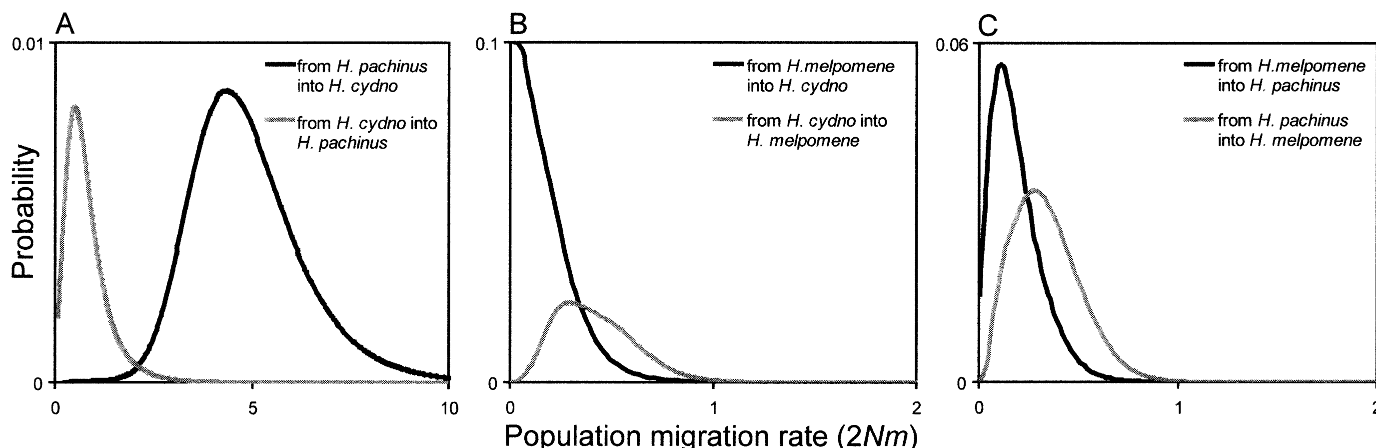

FIG. 4. Marginal posterior probability distributions for between-species population migration rates estimated with IM. Bidirectional introgression was estimated for each of three pairwise comparisons; (A) *Heliconius cydno* and *H. pachinus*, (B) *H. cydno* and *H. melpomene*, and (C) *H. pachinus* and *H. melpomene*. Note, the scale of the x-axis in A is different than B and C.

the average time of introgression over the course of the simulations (Table 3). Gene flow among all three species was not restricted to particular loci but rather, all 15 loci exhibited some evidence of introgression in each comparison.

DISCUSSION

When natural selection acts to maintain divergence between hybridizing species at a small number of loci, other portions of the genome can experience substantial interspecific gene flow. Here we have shown that three butterfly species that diverged and remain differentiated at five or six wing patterning loci due to selection for Müllerian mimicry are experiencing considerable interspecific gene flow. Multiple *H. melpomene*, *H. cydno*, and *H. pachinus* individuals exhibited evidence of mixed ancestry based on their AFLP genotypes and analyses of multilocus DNA sequence data indicate nonzero rates of historical introgression among all three species. These results seem to be at odds with the general rarity of distinguishable hybrids in the field. For instance, there is no defined hybrid zone between *H. cydno* and *H. pachinus*, and although individuals with recombinant wing patterns have been collected, they are rare. Furthermore, even though *H. melpomene* and *H. cydno* are broadly sympatric, distinguishable hybrids comprise only 0.1% of populations (Mallet et al. 1998). The frequency of hybrids between *H. melpomene* and *H. pachinus* is likely to be similar to that of *melpomene*/*cydno* hybrids, yet no individual with a recognizably hybrid wing pattern has ever been collected.

Whereas even rare hybridization could result in detectable introgression based on DNA sequence variation, our finding of recognizably admixed AFLP genotypes indicates that hybridization may be more common than collection records suggest. There are a number of reasons why gauging the prevalence of hybridization from numbers of individuals with recombinant phenotypes may bias our view of the frequency of hybridization. First, although wing patterns provide a clear indicator of very recent hybridization, their simple genetic basis allows evidence of hybrid ancestry to be lost very quickly. The differences in color pattern among *Heliconius* races and species, while visually striking, are controlled almost entirely by a small number of genes that generally act in a simple on/off ''switch'' fashion (Sheppard et al. 1985; Jiggins and McMillan 1997; Gilbert 2003; Naisbit et al. 2003). Hence, wing patterns provide a very limited set of diagnostic loci with which to judge the ancestry of an individual. Second, wing patterning loci often have dominant and recessive alternative alleles, and there are a variety of epistatic interactions among loci, both of which serve to conceal some of the potentially segregating wing pattern variation in hybrids. Add to this disruptive mimetic selection, imposed by predators, which likely removes obviously recombinant phenotypes quickly. The end result is that, within a few generations of initial hybridization, many individuals with hybrid ancestry are unlikely to be distinguishable based on phenotype alone. Our genetic data are consistent with this conclusion. For many of the individuals that had a high probability of recent mixed ancestry (Fig. 2), the results suggest that the heterospecific ancestor was likely to be a great-grandparent. Furthermore, a number of individuals exhibited evidence of introgression but had low probabilities or recent mixed ancestry, indicating hybridization more than three generations ago. As a whole, the results of these analyses suggest that introgression among hybridizing *Heliconius* species is common and, hybridization on a per individual basis may be relatively common as well.

Asymmetrical Gene Flow

Studies of hybrid zones regularly find evidence of asymmetric barriers to gene flow which allow more genes to pass in one direction than the other (Barton and Hewitt 1985). The directions of gene flow among *H. melpomene*, *H. cydno*, and *H. pachinus* are skewed. In particular, the IM results indicate that there is far greater introgression from *H. pachinus* into *H. cydno* than the reverse (Fig. 4, Table 2). Furthermore, although the migration rate credibility intervals overlap in both comparisons with *H. melpomene*, in each case introgression into *H. melpomene* is nonzero whereas introgression from *H. melpomene* into *H. cydno* or *H. pachinus* is not significantly different than zero (Table 2). Asymmetrical gene flow is expected given the genetics of wing patterning,

TABLE 3. Average number and timing of introgression events among *Heliconius cydno*, *H. pachinus*, and *H. melpomene* over the course of IM simulations.

	Locus	*ap*	*ci*	*cn*	*CO*	*Dll*	*Ef1α*	*en*	*inv*	*Mpi*	*ptc*	*sd*	*st*	*Tpi*	*w*	*wg*	Average
pachinus into *cydno*	Introgression	8	9	7	4	17	7	5	11	12	9	10	12	9	7	9	9.07
	Time (10^5 years)	3.61	3.51	4.60	1.58	2.90	4.41	3.75	4.83	6.44	5.30	5.16	4.55	1.82	5.35	3.37	4.08
cydno into *pachinus*	Introgression	4	6	4	2	6	4	3	5	6	4	6	5	2	5	4	4.00
	Time (10^5 years)	3.28	3.56	4.83	2.36	3.32	4.03	3.23	5.02	8.70	7.10	7.14	6.15	1.67	5.54	3.32	4.46
melpomene into *cydno*	Introgression	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0.13
	Time (10^5 years)	—	—	—	—	0.37	—	—	—	1.68	—	—	—	—	—	—	1.03
cydno into *melpomene*	Introgression	1	5	1	1	4	2	1	1	3	1	2	4	1	2	1	2.00
	Time (10^5 years)	5.29	2.50	3.49	5.62	3.57	4.10	6.28	7.18	1.35	5.95	2.91	3.32	5.05	1.85	5.54	4.00
melpomene into *pachinus*	Introgression	1	0	1	0	1	0	0	1	1	0	0	1	0	1	0	0.47
	Time (10^5 years)	4.54	—	2.88	—	5.01	—	—	3.94	1.46	—	—	3.94	—	2.56	—	2.36
pachinus into *melpomene*	Introgression	1	4	1	1	3	1	1	0	3	1	2	2	1	2	1	1.60
	Time (10^5 years)	6.59	2.48	4.30	7.61	4.30	9.58	7.85	—	2.09	9.43	7.61	4.38	4.77	2.96	9.51	5.96

the role this genetic control plays in shaping the appearance of hybrids, and the probability that hybrids of various phenotypes will survive and reproduce in different mimetic environments.

In general, *Heliconius* hybrids have low fitness because their recombinant wing patterns do not match either mimetic parent, leading to exaggerated rates of predation (Mallet and Barton 1989) and reduced mating success (Naisbit et al. 2001). However, due to just two color patterning loci, *cydno*/*pachinus* hybrids have wing patterns similar to pure *H. cydno*. A single locus with a dominant white and recessive yellow allele determines the base color of the forewing and a second locus with a dominant *H. cydno* ''shutter'' allele covers the dorsal hindwing with melanic scales (Gilbert 2003). The actions of these loci result in F_1 hybrids that, like *H. cydno*, display a white forewing and black hindwing.

The similarity between F_1 hybrids and *H. cydno* impacts the fitness of hybrids in two ways. First, hybrids should be protected from predators on the Caribbean drainage where the warning color pattern of white on blue/black wings is well-established by *H. cydno* and comimic *H. sapho*. On the Pacific drainage these same hybrids would likely be subject to very intense predation because the mimicry ring composed of *H. pachinus* and comimic *H. hewitsoni* offers no protection. Second, the primary cue used by males of both species to identify conspecific females is the color (white or yellow) of the forewing (Kronforst et al. 2006). Although hybrids are recognized as potential mates by *H. cydno*, they are not by *H. pachinus*. Thus, *cydno*/*pachinus* hybrids are likely to survive, successfully court or attract mates, and produce fit progeny only within the range of *H. cydno*. This conclusion is supported by the observation that the majority of phenotypically distinguishable *cydno*/*pachinus* hybrids that have been caught in the field, including the one collected as part of this study, have been found on the Caribbean drainage in areas adjacent to the Meseta Central.

In contrast to *cydno*/*pachinus* hybrids, females produced from matings between *H. melpomene* and either *H. cydno* or *H. pachinus* are sterile, making males the only route for interspecific gene flow. The largely unidirectional introgression from *H. cydno* and *H. pachinus* into *H. melpomene* is, again, likely a consequence of wing patterning genetics. F_1 hybrids from crosses involving *H. melpomene rosina* all possess the *H. melpomene* red forewing band, which behavioral data suggest is an important attractive mating cue for this species (Jiggins et al. 2004). Even though the mating success of F_1 hybrids is low (Naisbit et al. 2001), matings between F_1 males and pure *H. melpomene* females may be more likely because *H. melpomene* females recognize the red forewing band whereas *H. cydno* and *H. pachinus* females do not. This explanation has mixed empirical support; mate choice experiments have shown that *cydno*/*melpomene* F_1 males are slightly more likely to court and mate *H. melpomene* females than *H. cydno* females (Naisbit et al. 2001) but, based on field-caught hybrid phenotypes, F_1 males may be more likely to backcross to *H. cydno* in nature (Mallet et al. 2003). Future research on the genetic basis of mate preference and preference cues will help to identify the interactions that funnel gene flow from *H. cydno* and *H. pachinus* into *H. melpomene*.

Contrasting Heliconius Contact Zones

There appears to be more gene flow between *H. cydno* and *H. pachinus* than that which was discovered between *H. erato* and *H. himera* by Jiggins et al. (1997). This is somewhat surprising given that there is a definable hybrid zone between *H. himera* and *H. erato* and only occasional migrants between *H. cydno* and *H. pachinus*. Some of the difference may simply be the result of the analytical techniques used by the two studies. The large number of genetic markers, the individual-based clustering technique, and the multilocus genealogical data employed here are likely to expose evidence of introgression that would not have been apparent with population-based statistics derived from fewer markers. Although methodological differences make it difficult to compare patterns of nuclear gene flow, surveys of mtDNA variation provide a means of directly comparing the two contact zones. Jiggins et al. (1997) surveyed mtDNA haplotypes across the *erato/himera* hybrid zone and found four instances of mtDNA introgression from a total of 618 individuals. Allozyme data suggested three of the four were likely to be first-generation backcross hybrids. Here we identified three obvious instances of mtDNA introgression from a total of 98 *H. cydno* and *H. pachinus* individuals (Fig. 3) and all three represent stable introgression as opposed to recent hybridization. Although the data are limited, this comparison does suggest a difference between the two systems.

There are a number of biological reasons why the extent of introgression may differ between the two contact zones. First, unlike *cydno/pachinus* hybrids, F_1 hybrids between *H. erato* and *H. himera* look unique in comparison to both parental types (Jiggins et al. 1996; Jiggins and McMillan 1997) and thus are likely to experience extreme predation. Second, whereas ecological divergence between *H. cydno* and *H. pachinus* is limited to a shift in mimicry, *H. erato* and *H. himera* have also diverged in habitat specialization. *Heliconius erato* is widespread throughout secondary growth in wet regions of South and Central America and *H. himera* is restricted to the dry forests of southwester Ecuador and northern Peru. Furthermore, both species have adapted physiologically to their respective habitats, in terms of adult activity levels and larval development (Davison et al. 1999). Thus, an additional dimension of ecological divergence is layered on top of the already strong disruptive selection acting against hybrids. Hybrids between *H. erato* and *H. himera* are probably poorly adapted to both habitat types. As evidence of this, McMillan et al. (1997) showed that hybrids reared within the range of *H. himera* exhibited a positive relationship between developmental time and proportion of the genome derived from *H. erato*. The comparison of these two contact zones suggests that broad divergence in habitat specialization may be a critical step in reducing the permeability of the species boundary.

Significance

The findings of contemporary admixture and high historical rates of introgression among hybridizing *Heliconius* butterflies have three significant evolutionary implications. First, that these species remain distinct despite ongoing gene flow points to a central role for natural selection in the maintenance of species diversity. In the absence of strong selection, gene flow on the order of that detected here would quickly erode the species boundaries and drive these taxa into a single interbreeding unit. For *Heliconius* butterflies, this selection undoubtedly comes in the form of natural selection for Müllerian mimicry. Second, it is hypothesized that introgressive hybridization may play a causative role in adaptive radiation by supplying genetic variation upon which natural selection can act (Seehausen 2004). In *Heliconius* specifically, it appears that the occasional transfer of color patterning alleles between species has generated warning pattern diversity and fueled mimetic convergence (Gilbert 2003). In fact, one of the species studied here, *H. pachinus*, possesses a wing pattern phenotype that combines elements from the other two, *H. cydno* and *H. melpomene*, suggesting that it may have originated via hybridization (Gilbert 2003). Clearly, the significance of introgression as a source of genetic variation is dependent on the extent to which genes actually move among species. Here we have shown that this may be common in one active adaptive radiation. Finally, assortative mate preference, which serves as an additional form of reproductive isolation among interfertile *Heliconius* races and species (Jiggins et al. 2001, 2004; Kronforst 2004), varies geographically such that conspecific preference is enhanced in areas of sympatry (Jiggins et al. 2001; Kronforst 2004). All three species studied here exhibit evidence of strengthened mate preference in areas of interspecific contact (Kronforst 2004). Although such reproductive character displacement can result from a variety of evolutionary processes (Servedio and Noor 2003), our results lend support to the hypothesis that it is a consequence of natural selection against hybrids, a process known as reinforcement. The power of reinforcement to drive mate preference evolution is contingent on the frequency of hybridization—too much hybridization can result in gene flow sufficient to inhibit divergence whereas too little hybridization can limit the impact of reinforcement due to a lack of opportunities for selection to act against hybrids (Servedio and Noor 2003). Our results indicate that the three *Heliconius* species studied here hybridize frequently enough to leave recognizable evidence of admixture and introgression but not enough to erode species boundaries. An intermediate hybridization frequency such as this is particularly conducive to reinforcement (Servedio and Noor 2003).

ACKNOWLEDGMENTS

We thank U. Mueller for providing lab facilities, R. Reed for providing primers, and B. Wee for assisting with AFLP data analysis. We also thank K. Kronforst and A. Vega for assistance in the field, D. Falush for providing a copy of STRUCTURE 2.2, and reviewers for comments on the manuscript. Butterflies were collected under permits provided by Costa Rica's Ministerio del Ambiente y Energia. Funding was provided by the grants from the University of Texas Graduate Program in Zoology/Ecology, Evolution, and Behavior, a University of Texas Co-op Undergraduate Research Grant, an Organization for Tropical Studies' Graduate Research Fellowship, and National Science Foundation Grants DEB 0206613 and DEB 0415718.

Literature Cited

Barton, N. H. 2001. The role of hybridization in evolution. Mol. Ecol. 10:551–568.

Barton, N. H., and G. M. Hewitt. 1985. Analysis of hybrid zones. Annu. Rev. Ecol. Syst. 16:113–148.

Beltrán, M., C. D. Jiggins, V. Bull, M. Linares, J. Mallet, W. O. McMillan, and E. Bermingham. 2002. Phylogenetic discordance at the species boundary: comparative gene genealogies among *Heliconius* butterflies. Mol. Biol. Evol. 19:2176–2190.

Benson, W. W. 1972. Natural selection for Müllerian mimicry in *Heliconius erato* in Costa Rica. Science 176:936–939.

Brower, A. V. Z. 1994a. Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. Proc. Natl. Acad. Sci. USA 91:6491–6495.

———. 1994b. Phylogeny of *Heliconius* butterflies inferred from mitochondrial DNA sequences (Lepidoptera: Nymphalidae). Mol. Phylogenet. Evol. 3:159–174.

———. 1996. Parallel race formation and the evolution of mimicry in *Heliconius* butterflies: a phylogenetic hypothesis from mitochondrial DNA sequences. Evolution 50:195–221.

Brower, A. V. Z., and R. DeSalle. 1998. Patterns of mitochondrial versus nuclear DNA sequence divergence among nymphalid butterflies: the utility of *wingless* as a source of characters for phylogenetic inference. Insect Mol. Biol. 7:73–82.

Brower, A. V. Z., and M. G. Egan. 1997. Cladistic analysis of *Heliconius* butterflies and relatives (Nymphalidae: Heliconiiti): a revised phylogenetic position for *Eueides* based on sequences from mtDNA and a nuclear gene. Proc. R. Soc. Lond. B 264: 969–977.

Brown, Jr., K. S., P. M. Sheppard, and J. R. G. Turner. 1974. Quaternary refugia in South America: evidence from race formation in *Heliconius* butterflies. Proc. R. Soc. Lond. B 187: 369–378.

Brunetti, C. R., J. E. Selegue, A. Monteiro, V. French, P. M. Brakefield, and S. B. Carroll. 2001. The generation and diversification of butterfly eyespot color patterns. Curr. Biol. 11:1578–1585.

Burke, J. M., and M. L. Arnold. 2001. Genetics and the fitness of hybrids. Annu. Rev. Genetics 35:31–52.

Bush, G. L. 1994. Sympatric speciation in animals: new wine in old bottles. Trends Ecol. Evol. 9:285–288.

Butlin, R. 1987. Speciation by reinforcement. Trends Ecol. Evol. 2:8–12.

Carroll, S. B., J. Gates, D. N. Keyes, S. W. Paddock, G. E. F. Panganiban, J. E. Seleque, and J. A. Williams. 1994. Pattern formation and eyespot determination in butterfly wings. Science 265:109–114.

Cho, S., A. Mitchell, J. C. Regier, C. Mitter, R. W. Poole, T. P. Friedlander, and S. Zhao. 1995. A highly conserved nuclear gene for low-level phylogenetics: elongation factor-1α recovers morphology-based tree for heliothine moths. Mol. Biol. Evol. 12: 650–656.

Davison, A., W. O. McMillan, A. S. Griffin, C. D. Jiggins, and J. L. B. Mallet. 1999. Behavioural and physiological adaptation between two parapatric *Heliconius* species (Lepidoptera: Nymphalidae). Biotropica 31:661–668.

Emelianov, I., F. Marec, and J. Mallet. 2004. Genomic evidence for divergence with gene flow in host races of the larch budmoth. Proc. R. Soc. Lond. B 271:97–105.

Estrada, C., and C. D. Jiggins. 2002. Patterns of pollen feeding and habitat preference among *Heliconius* species. Ecol. Entomol. 27: 448–456.

Falush, D., M. Stephens, and J. K. Pritchard. 2003. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164:1567–1587.

Feder, J. L., S. B. Opp, B. Wlazlo, K. Reynolds, W. Go, and S. Spisak. 1994. Host fidelity is an effective premating barrier between sympatric races of the apple maggot fly. Proc. Natl. Acad. Sci. USA 91:7990–7994.

Flanagan, N. S., A. Tobler, A. Davison, O. G. Pybus, D. D. Kapan, S. Planas, M. Linares, D. Heckle, and W. O. McMillan. 2004. Historical demography of Müllerian mimicry in the neotropical *Heliconius* butterflies. Proc. Natl. Acad. Sci. USA 101: 9704–9709.

Gilbert, L. E. 2003. Adaptive novelty through introgression in *Heliconius* wing patterns: evidence for shared genetic ''tool box'' from synthetic hybrid zones and a theory of diversification. Pp. 281–318 *in* C. L. Boggs, W. B. Watt, and P. R. Ehrlich, eds. Ecology and evolution taking flight: butterflies as model systems. Univ. of Chicago Press, Chicago, IL.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41:95–98.

Hey, J., and R. Nielsen. 2004. Multilocus methods for estimating population sizes, migration rates and divergence time, with applications to the divergence of *Drosophila pseudoobscura* and *D. persimilis*. Genetics 167:747–760.

Higgins, D. G., and P. M. Sharp. 1988. CLUSTAL: a package for performing multiple sequence alignments on a microcomputer. Gene 73:237–244.

Hudson, R. R. 1985. The sampling distribution of linkage disequilibrium under an infinite allele model without selection. Genetics 109:611–631.

Hudson, R. R., and N. L. Kaplan. 1985. Statistical properties of the number of recombination events in the history of a sample of DNA sequences. Genetics 111:147–164.

Huelsenbeck, J. P., and F. Ronquist. 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics 17:754–755.

Jiggins, C. D., and W. O. McMillan. 1997. The genetic basis of an adaptive radiation: warning colour in two *Heliconius* species. Proc. R. Soc. Lond. B 246:1167–1175.

Jiggins, C. D., W. O. McMillan, W. Neukirchen, and J. Mallet. 1996. What can hybrid zones tell us about speciation? The case of *Heliconius erato* and *H. himera* (Lepidoptera: Nymphalidae). Biol. J. Linn. Soc. 59:221–242.

Jiggins, C. D., W. O. McMillan, P. King, and J. Mallet. 1997. The maintenance of species differences across a *Heliconius* hybrid zone. Heredity 79:495–505.

Jiggins, C. D., R. E. Naisbit, R. L. Coe, and J. Mallet. 2001. Reproductive isolation caused by colour pattern mimicry. Nature 411:302–305.

Jiggins, C. D., C. Estrada, and A. Rodrigues. 2004. Mimicry and the evolution of premating isolation in *Heliconius melpomene* Linnaeus. J. Evol. Biol. 17:680–691.

Kapan, D. D. 2001. Three-butterfly system provides a field test of Müllerian mimicry. Nature 409:338–340.

Keys, D. N., D. L. Lewis, J. E. Selegue, B. J. Pearson, L. V. Goodrich, R. L. Johnson, J. Gates, M. P. Scott, and S. B. Carroll. 1999. Recruitment of a hedgehog regulatory circuit in butterfly eyespot evolution. Science 283:532–534.

Kobayashi, N., K. Tamura, and T. Aotsuka. 1999. PCR error and molecular population genetics. Biochem. Genet. 37:317–321.

Kronforst, M. R. 2004. The role of hybridization in the evolution of *Heliconius* butterflies: species diversification, the evolution of reproductive isolation, and interspecific gene flow. Ph.D. diss., University of Texas, Austin, TX.

———. 2005. Primers for the amplification of nuclear introns in *Heliconius* butterflies. Mol. Ecol. Notes 5:158–162.

Kronforst, M. R., and T. H. Fleming. 2001. Lack of genetic differentiation among widely spaced subpopulations of a butterfly with home range behaviour. Heredity 86:243–250.

Kronforst, M. R., L. G. Young, D. D. Kapan, C. McNeely, R. J. O'Neill, and L. E. Gilbert. 2006. Linkage of butterfly mate preference and wing color preference cue at the genomic location of *wingless*. Proc. Natl. Acad. Sci. USA 103:6575–6580.

Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei. 2001. MEGA2: molecular evolutionary genetics analysis software. School of Life Sciences, Arizona State University, Tempe, AZ.

Mallet, J. 1993. Speciation, raciation, and color pattern evolution in *Heliconius* butterflies: evidence from hybrid zones. Pp. 226–260 *in* R. G. Harrison, ed. Hybrid zones and the evolutionary process. Oxford Univ. Press, New York.

———. 2005. Hybridization as an invasion of the genome. Trends Ecol. Evol. 20:229–237.

Mallet, J., and N. H. Barton. 1989. Strong natural selection in a warning color hybrid zone. Evolution 43:421–431.

Mallet, J., W. O. McMillan, and C. D. Jiggins. 1998. Mimicry and warning color at the boundary between races and species. Pp. 390–403 *in* D. J. Howard and S. H. Berlocher, eds. Endless forms: species and speciation. Oxford Univ. Press, New York.

Mallet, J., W. Neukirchen, and M. Linares. 2003. Wild-caught hybrids among *Heliconius* and *Eueides* species: a database. Available at: http://www.ucl.ac.uk/taxome/hyb/hybtab.html.

McMillan, W. O., C. D. Jiggins, and J. Mallet. 1997. What initiates speciation in passion-vine butterflies? Proc. Natl. Acad. Sci. USA 94:8628–8633.

Mueller, U. G., and L. L. Wolfenbarger. 1999. AFLP genotyping and fingerprinting. Trends Ecol. Evol. 14:389–394.

Naisbit, R. E., C. D. Jiggins, and J. Mallet. 2001. Disruptive sexual selection against hybrids contributes to speciation between *Heliconius cydno* and *Heliconius melpomene*. Proc. R. Soc. Lond. B 268:1–6.

Naisbit, R., C. D. Jiggins, M. Linares, C. Salazar, and J. Mallet. 2002. Hybrid sterility, Haldane's rule and speciation in *Heliconius cydno* and *H. melpomene*. Genetics 161:1517–1526.

Naisbit, R., C. D. Jiggins, and J. Mallet. 2003. Mimicry: developmental genes that contribute to speciation. Evol. Develop. 5: 269–280.

Nei, M. 1987. Molecular evolutionary genetics. Columbia Univ. Press, New York.

Nielsen, R., and J. Wakeley. 2001. Distinguishing migration from isolation. A Markov chain Monte Carlo approach. Genetics 158: 885–896.

Nijhout, H. F., G. A. Wray, and L. E. Gilbert. 1990. An analysis of the phenotypic effects of certain colour pattern genes in *Heliconius*. Biol. J. Linn. Soc. 40:357–372.

Ortíz-Barrientos, D., J. Reiland, J. Hey, and M. A. F. Noor. 2002. Recombination and the divergence of hybridizing species. Genetica 116:167–178.

Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945–959.

Reed, R. D., and L. E. Gilbert. 2004. Wing venation and distal-less expression in *Heliconius* butterfly wing pattern development. Dev. Genes Evol. 214:628–634.

Reed, R. D. and L. M. Nagy. 2005. Evolutionary redeployment of a biosynthetic module: expression of eye pigment genes *vermilion*, *cinnabar*, and *white* in butterfly wing development. Evol. Dev. 7:301–311.

Reed, R. D., and M. S. Serfas. 2004. Butterfly wing pattern evolution is associated with changes in a notch/distal-less temporal pattern formation process. Curr. Biol. 14:1159–1166.

Rozas, J., and R. Rozas. 1999. DnaSP version 3: An integrated program for molecular population genetics and molecular evolution analysis. Bioinformatics 15:174–175.

Schluter, D. 1998. Ecological causes of speciation. Pp. 114–129 *in* D. J. Howard and S. H. Berlocher, eds. Endless forms: species and speciation. Oxford Univ. Press, New York.

Seehausen, O. 2004. Hybridization and adaptive radiation. Trends Ecol. Evol. 19:198–207.

Servedio, M. R., and M. A. F. Noor. 2003. The role of reinforcement in speciation: theory and data. Annu. Rev. Ecol. Evol. Syst. 34: 339–364.

Sheppard, P. M., J. R. G. Turner, K. S. Brown, W. W. Benson, and M. C. Singer. 1985. Genetics and evolution of Müllerian mimicry in *Heliconius* butterflies. Philos. Trans. R. Soc. Lond. B 308: 433–613.

Simon, C., F. Frati, A. Beckenbach, B. Crespi, H. Liu, and P. Flook. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. 87: 651–702.

Smiley, J. 1978. Plant chemistry and the evolution of host specificity: new evidence for *Heliconius* and *Passiflora*. Science 201: 745–747.

Turner, J. R. G., and J. L. B. Mallet. 1996. Did forest islands drive the diversity of warningly coloured butterflies? Biotic drift and the shifting balance. Philos. Trans. Roy. Soc. London B 351: 835–845.

Turner, T. L., M. W. Hahn, and S. V. Nuzhdin. 2005. Genomic islands of speciation in *Anopheles gambiae*. PLoS Biol. 3:e285.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. Van De Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic. Acids Res. 23:4407–4414.

Won, Y. J., and J. Hey. 2005. Divergence population genetics of chimpanzees. Mol. Biol. Evol. 22:297–307.

Corresponding Editor: W. O. McMillan

Appendix

Sample information for individuals included in the AFLP admixture analyses. *Heliconius cydno* and *H. pachinus* individuals are listed in geographical order of collecting location, from closest to farthest from Costa Rica's Meseta Central. *Heliconius melpomene* individuals are listed in geographical order of collecting location from location 1, through the Meseta Central, to location 15. The bold sample exhibited phenotypic evidence of introgression (see text for details). See Figure 1 for the names and geographic relationships of the collecting locations.

H. cydno			*H. pachinus*			*H. melpomene*		
Sex	Year	Location	Sex	Year	Location	Sex	Year	Location
F	2000	12	M	2000	6	M	2002	1
M	2002	7	M	2000	6	M	2002	3
F	2002	7	M	2000	6	M	2002	3
M	2000	9	F	2000	6	M	2002	3
F	2000	9	M	2000	5	F	2002	5
M	2000	8	M	2000	5	F	2002	5
F	2000	8	M	2000	5	F	2002	5
F	2000	8	F	2000	5	F	2000	9
M	2002	8	F	2000	5	M	2000	8
M	2002	8	F	2000	5	M	2000	8
M	2002	8	F	2000	5	F	2000	8
M	2002	8	F	2000	5	M	2002	8
M	2002	8	M	2002	5	M	2002	8
M	2002	8	M	2002	5	M	2002	8
M	2002	8	M	2002	5	M	2002	8
M	2002	8	M	2002	5	M	2002	8
M	2002	8	M	2002	5	M	2002	8
M	**2002**	**8**	M	2002	5	M	2002	8
F	2002	8	M	2002	5	F	2002	8
F	2002	8	M	2002	5	F	2002	8
F	2002	8	M	2002	5	F	2002	8
F	2002	8	F	2002	5	F	2002	8
F	2002	8	F	2002	5	F	2002	8
F	2002	8	F	2002	5	M	2000	13
F	2002	8	F	2002	5	M	2000	13
M	2000	10	M	2000	4	M	2002	15
M	2000	10	M	2000	4	M	2002	15
M	2000	10	M	2000	3			
M	2000	11	M	2002	3			
M	2000	11	M	2002	3			
M	2000	11	F	2002	3			
M	2000	11	F	2002	3			
M	2000	11	F	2002	3			
M	2002	11	M	2000	2			
M	2002	11	M	2000	1			
F	2002	11	M	2002	1			
F	2002	11	M	2002	1			
F	2000	13	M	2002	1			
F	2000	13	M	2002	1			
M	2002	14	M	2002	1			
M	2002	14	M	2002	1			
M	2002	14	F	2002	1			
M	2002	15	F	2002	1			
M	2002	15	F	2002	1			
M	2002	15						
M	2002	15						
M	2002	15						
M	2002	15						
M	2002	15						
M	2002	15						
M	2002	15						
M	2002	15						
F	2002	15						
F	2002	15						
F	2002	15						
F	2002	15						