Evolution, 52(2), 1998, pp. 503–510

ESTIMATING THE MATING BEHAVIOR OF A PAIR OF HYBRIDIZING *HELICONIUS* SPECIES IN THE WILD

JAMES MALLET, W. OWEN MCMILLAN,[1] AND CHRIS D. JIGGINS
Galton Laboratory, Department of Biology, University College London, 4 Stephenson Way, London NW1 2HE, England

Abstract.—Premating isolation between incipient species is rarely studied in nature, even though mating tests in captivity may give an inaccurate picture of natural hybridization. We studied premating barriers between the warningly colored butterflies *Heliconius erato* and *H. himera* (Lepidoptera) in a narrow contact zone in Ecuador, where hybrids are found at low frequency. Eggs obtained from wild-mated females, supplemented with eggs and young larvae collected from the wild, were reared to adulthood. Adult color patterns of these progeny were then used to infer how their parents must have mated. Likelihood was used to estimate both the frequencies of potential parental genotypes from adult phenotypes collected in the wild, and the degree of assortative mating from the inferred parents. The frequencies of parental genotypes varied across the hybrid zone, but our statistical method allowed estimates of hybrid deficit and assortative mating to be integrated across all sites sampled. The best estimate of the frequency of F_1 and backcross hybrid adults in the center of the hybrid zone was 10%, with support limits (7.1%, 13.0%; support limits are asymptotically equivalent to 95% confidence limits). Mating was highly assortative: in the center of the hybrid zone the cross-mating probability between *H. erato* and *H. himera* was only 5% (0.3%, 21.4%). Wild hybrids themselves mated with both pure forms, and the probabilities that they mated in any direction were not significantly lower than those among conspecifics. These results are consistent with earlier laboratory studies on mate choice, and suggest that selection against hybrids must be strong to prevent formation of a hybrid swarm. Unfortunately, the wide support limits on mating behavior precluded a measure of the strength of selection from these data alone. Our statistical approach provides a useful general method for estimating mate choice in the wild.

Key words.—Generalized linear model, hybrid zones, interspecific hybridization, likelihood, mate choice, reproductive isolation, speciation.

Received June 20, 1997. Accepted November 12, 1997.

To understand the speciation of sexual taxa, we need to study how populations diverge by means of a reduction in "gene flow." Factors which reduce gene flow can be divided into two very different components: "premating isolation," actual gene flow prevention via assortative mating; and "postmating isolation," in which genes that have already flowed are selected against because of reduced hybrid fitness (including inviability, sterility, and other disruptive selection). Many of these traits are easy to study in captivity, but it is unclear how far laboratory results apply in the wild. In particular, assortative mating may be caused in part by habitat preferences that cannot be exerted in captivity: for example, in *Rhagoletis* fruit flies (Feder et al. 1994) and ducks (Gillham and Gillham 1996). Although lip service is often paid to the need to know about mating behavior in the wild, relatively few studies have been done. Some notable examples are work with birds (Moore and Buchanan 1985; P. R. Grant and Grant 1994; B. R. Grant and Grant 1996; Sætre et al. 1997), *Rhagoletis* fruit flies (Feder et al. 1994) and Orthoptera (Hewitt et al. 1987; Howard, in press).

We have been studying a hybrid zone between the warningly colored and mimetic butterflies *Heliconius erato* and *Heliconius himera* in southern Ecuador (Descimon and Mast de Maeght 1984; Jiggins et al. 1996). These parapatric taxa, which differ strongly in warning color pattern of the adult, are sometimes classed as species and sometimes as geographic races (Brown 1979; Brower 1996). We classify them as separate species because the frequency of hybrid phenotypes in the 5 km wide overlap zone is low: only about 10% of the population in the center of the zone are phenotypically hybrids, of which about half are F_1 hybrids between the pure species, the remainder being mainly backcrosses (Jiggins et al. 1996, 1997a; Mallet 1996; Mallet et al. 1998). This contrasts strongly with hybrid zones between geographic races of *Heliconius*, where color pattern genotypes are in Hardy-Weinberg equilibrium (Turner 1971; Mallet 1986a, 1993; Mallet et al. 1990). In areas of overlap, *H. erato* and *H. himera* fly and feed together, and their eggs are laid on the same *Passiflora* food-plants (Jiggins et al. 1996, 1997b). Morphological differences between these species and their hybrids are determined by two unlinked major switch-genes or gene-complexes affecting color pattern known from previous studies of hybrids between races of *H. erato* (Sheppard et al. 1985), together with a number of modifiers affecting minor pattern elements (Jiggins and McMillan 1997).

Arguably, the deficit of hybrid phenotypes in the *himera/erato* zone might apply only to color pattern. But this is not the case: there are fixed mtDNA RFLP differences across the zone, and even within the zone it is very rare to find mitochondrial genomes of the wrong species in "pure" color pattern phenotypes. Allozymes show a range of differences between the species; some loci have virtually fixed differences, others differ in allele frequency, and still others have very similar allele frequencies. The color pattern phenotypes in the hybrid zones, classified as pure *erato* (E), pure *himera* (H), F_1 hybrids, backcross to *erato* (BE), and backcross to *himera* (BH), are highly correlated with a multilocus allozyme hybrid index (Jiggins et al. 1997a). Presumably because most field-caught hybrids are recently produced F_1 or backcross hybrids rather than offspring of further crosses, the color pattern classification accurately reflects the entire genomic pattern.

Some potential causes for the hybrid deficit have been

---

[1] Present address: Departamento de Biología, Universidad de Puerto Rico, P.O. Box 23360, San Juan, Puerto Rico 00931-3360.

revealed in experiments. There was strong assortative mating in the laboratory: crosses between species were only about 11% as likely as matings within species (McMillan et al. 1997). F_1 hybrids, in contrast, mated readily in both directions as well as with other hybrids. Also, F_1, F_2, backcross, and further cross hybrids were as viable and fertile as the parent species (McMillan et al. 1997). This apparent absence of selection against hybrids in the laboratory creates a paradox. If 10% gene flow occurs in each generation and hybrids are fit, why doesn't a hybrid swarm eventually result that is similar to those typical of interracial hybrid zones within *H. erato*? The continued scarcity of hybrids is indirect evidence for ecologically mediated selection pressures caused by warning color and the abiotic environment (McMillan et al. 1997; Mallet et al., in press). Frequency-dependent predation, known to disfavor rare warning patterns in hybrid zones between races of *Heliconius* (Benson 1972; Mallet and Barton 1989), should select strongly against hybrids if they are scarce initially because of assortative mating. All known *himera/erato* hybrid zones occur on ecological gradients: *Heliconius himera* is found in the gallery forest of dry *Acacia* scrubland, while *H. erato* is found mainly in wetter forest (Mallet 1993; Jiggins et al. 1996). Host-choice experiments and distributions of larval food-plants, *Passiflora* species, show that host plant adaptation does not differ between the species (Jiggins et al. 1997b), but the tight associations of hybrid zones with aridity gradients suggest climatic factors may be more important. Developmental time, adult diurnal activity, and egg-laying rates do indeed differ between the species, while hybrids are intermediate (McMillan et al. 1997; Davison et al., unpubl.). Very likely, predation and climatic adaptations determine both the position of the hybrid zone and select strongly enough against hybrids to maintain separate genotypic clusters in the zone of overlap (McMillan et al. 1997; Mallet et al., in press).

However, our conclusions about strong ecological selection depend critically on the assumption that hybridization rates in the field are similar to low rates seen in the laboratory. Here, we test this assumption by estimating the level of assortative mating between *H. erato* and *H. himera* in wild populations. We determine the probable mates of wild-caught females by rearing their progeny. We then use likelihood to fit a frequency-dependent probability of cross-mating to the data. Our approach provides a general and powerful means of estimating gene flow in cases of natural hybridization.

MATERIALS AND METHODS

Mating Data

Between 1994 and 1996, we collected wild males and females of *H. erato, H. himera*, and hybrids from two transects through the hybrid zone: near Guayquichuma (sites 2–10) and near Chaguarpamba (sites 15–18) situated in Provincia de Loja, southern Ecuador. Exact locations and ecological details of these study sites are given in Jiggins et al. (1996), except for one new site, Quebrada de la Zarza (3°52′S, 79°37′W), situated between the two transects. We have no evidence for differences between the species in their catchability or microhabitat in areas of overlap; as many individuals were netted as possible without regard to phenotype, and we assume in our analysis that the collections at each site are random.

Females were collected alive and either were allowed to lay eggs in field cages, or eggs were obtained by "expression." To express an egg, the terminal abdominal segments of the female are manipulated and gently squeezed until a single fertilized egg is extruded. We have been able to express only a single fertile egg per female; subsequently expressed eggs are sterile, presumably because they have not been fertilized. This method was developed and previously used to study species limits in the "silvaniform" *Heliconius*, especially *H. numata* (Brown and Benson 1974). Egg expression was less effective in *H. erato* and *H. himera* than in other species; only 48% of expressions were successful compared with ~80% in *H. numata* (Mallet and M. Joron, unpubl. data). The field sex ratio in *H. erato* and *H. himera* at our sites was highly biased towards males, and the insects are anyway scarce and hard to capture in the steep, rugged terrain of the narrow zone of overlap. These factors greatly restricted the numbers of females from which we could rear progeny. We supplemented egg expressions with additional eggs and young larvae collected from *Passiflora* in the field. Larvae were reared to adulthood on a variety of *Passiflora* subgenus *Plectostemma* used by both species (Jiggins et al. 1997b). Because we suspected that strong selection stabilizes the low frequency of hybrids and the narrowness of the hybrid zone, it was important to exclude selection during rearing when estimating the hybridization frequency from the data. The egg-adult survival of all of these broods, reared with extreme care, was over 88%, with all deaths being attributable to human error. It is therefore unlikely that strong selection occurred during rearing (see also McMillan et al. 1997), so that reared offspring can be assumed to provide unbiased information on their parents' matings.

Heliconius erato females, like other *erato* group *Heliconius*, are virtually monandrous (Dunlap-Pianka et al. 1977; Boggs 1979). We have not been able to test whether there is sperm mixing or precedence for the sperm of the last male in these species; the monandry foiled our frequent attempts to obtain double matings with *erato* and *himera* males. In other Lepidoptera, including Nymphalidae, sperm precedence of the last mating is the rule (Labine 1966). Sperm precedence in ditrysian Lepidoptera may be due to structural peculiarities of the spermatophore and the separate mating and egg canals of the female reproductive system (Scoble 1992). It is therefore reasonable to assume that any assortativeness in our offspring is unlikely to result from "homogamy," that is, preferential fertilization or preferential production of "pure" zygotes from multiple matings as occurs in Orthoptera (Hewitt et al. 1987, 1989; Howard, in press) and *Drosophila* (Price 1997). *Heliconius erato* and its relatives are renowned for "pupal-mating," in which males sit on the pupa until the female starts to eclose, and mate her while she is freeing herself from the pupal exuvium and inflating her wings (Gilbert 1972; Deinert et al. 1994). However, we never observed pupal mating in our insectary cages (McMillan et al. 1997); males in our "male cage" ignored female pupae that had been reared in seminatural conditions as larvae and pupae within the cage; they mated only after female wing expansion.

Thus, pupal mating does not seem to be the rule in every population of *Heliconius erato* (see also Mallet 1986b).

From the adult phenotype of both female parent and offspring, together with knowledge of color pattern genetics (Jiggins and McMillan 1997), we determined the male parent's most likely color pattern genotype. We could also determine likely parental genotypes, although not their sexes, for eggs and larvae collected directly from the hybrid zone: for each hybrid larva resulting from parents of more than one kind, the mating was therefore scored as two reciprocal half-matings. For example, a backcross to *erato* (BE) offspring is assumed to have resulted from half a (male *erato* × female F_1) mating, and half a (female *erato* × male F_1) mating. There may, of course, be differences in assortative mating depending on which partner is female. However, with our limited data we were more interested in estimating F_1 production overall than in the details of any sexual asymmetry; in the analysis below we combine information across sexes.

Our identification of most likely parental genotypes will not be perfect. For instance, an apparently pure *himera* adult was expressed as an egg from a female collected from Quebrada de la Zarza whose phenotype was BH (i.e., F_1 × *himera*); the most likely mating is assumed to be BH female × *himera* male. If the mating had been the other way round, we would have scored this mating as a pure *himera* × *himera* mating because no evidence of hybridity would have been seen in either mother or offspring. However, our major interest is to compare the frequencies of *erato* × *himera* matings with all other types of matings. In any case, we found no evidence in the results that any matings involving hybrids are less likely than matings within each pure species; lumping these indistinguishable matings will therefore be unimportant. Cross-matings between pure types to produce easily identifiable F_1 offspring cannot, on the other hand, be underestimated. These matings might potentially be overestimated because rare backcross offspring can look similar to F_1 types. In practice, we obtained only one F_1 phenotype, which was expressed as an egg from a wild-caught *erato* female. This individual could have been the result of a backcross or further cross, but its male parent must have contained at least one copy of all of the color genes expressed in *himera*, that is, the latter must have been an F_1, BH, or H type, with H being much the most likely because of the rarity of hybrids. The problem is that, in a population with hybridization and backcrossing, there is actually no such thing as a "pure" species, or even "F_1" hybrids. The genotypic classification is merely a rule of thumb, one which is useful only because of the recency of most hybrid ancestries and because color pattern is highly predictive of an individual's entire genome (Jiggins et al. 1997a).

Mating Model and Likelihood Analysis

The probability of encounter between pure or hybrid types together with the probability of mating upon a given encounter combine to determine the overall probability that a given female is mated by a particular type of male in a given site. To make efficient use of the sparse data, we used a likelihood analysis to estimate both the frequencies of *erato*, hybrids and *himera* in each site (as surrogates for the probability that each female encounters males of these types) and the overall conditional probability of mating with these types across all sites simultaneously. Essentially we assume that a combination of gene flow between species and selection against hybrids has produced a stable distribution of parental genotypes in each site, and that these genotypes then mate nonrandomly to produce progeny distributions that can be estimated before selection via the egg expression and larval collection data. As far as we know, such an analysis has not been done before, but a general approach similar to ours may prove useful in other studies of hybridization between species as well as in studies of mate choice in polymorphic species.

The data consist of counts of males and females collected at each site and counts of inferred matings between particular kinds of males and females. Because of the scarcity of mating data on hybrids, we lump hybrid phenotypes of all types (BC, F_1, and BH) together as "hybrids" (F). Having done this, we estimate the scarcity of hybrids in each site via a hybrid deficit α analogous to the single-locus heterozygote deficit, F_{IS}. This model is not intended to indicate anything about how genotype frequencies are maintained; α is simply a useful measure of hybrid deficit that can be generalized across sites independently of the local frequency. Under this model, the frequencies of *erato*, hybrid, and *himera* genotypes (Q_E, Q_F, Q_H) within a particular site are:

$$Q_E = q_E^2 + \alpha q_E(1 - q_E),$$

$$Q_F = 2q_E(1 - q_E)(1 - \alpha),$$

$$Q_H = (1 - q_E)^2 + \alpha q_E(1 - q_E),$$

where the overall frequency of *erato* genomes in the site is q_E. For example, if $q_E = 0.25$ and $\alpha = 0.1$, then the frequency of the hybrids, Q_F, in the population would be $2 \times 0.25 \times 0.75 \times (1 - 0.1) = 0.3375$.

We next model the mating probability Ξ_{ij} that a female type i mates with a male type j within this site. A female of type i will encounter a male of type j with probability Q_j, and she mates with this male with conditional probability x_{ij}. If she does not mate at the first encounter, she will then again encounter and mate a male of a given type with the same probability. Thus, the mating model assumed for each female is a series of encounters followed by nonmatings until she eventually mates. Over all encounters, her mating probability with a type j male will then be:

$$\Xi_{ij} = \frac{x_{ij}Q_j}{\sum_k (x_{ik}Q_k)}$$

where k is any type of male. The conditional probability x_{ij} is measured relative to x_{EE} (the latter is set to 1 to simplify comparison).

In this study, there was no evidence for differences in either α or x_{ij} between sites, so the problem reduces to the need to estimate a single set of mating probabilities, x_{ij}, conditional on the hybrid deficit, α, overall, and on the frequencies of *erato* genotypes, q_E, at each site. In addition, we are most interested in cross-mating probabilities x_{EH} and x_{HE} compared with the intraspecific probability x_{EE} (the latter is set to 1, as already explained). We also found that x_{HH}, x_{EF}, x_{FE}, x_{HF}, x_{FF}, and x_{FH} were not statistically different from x_{EE}, and can

TABLE 1. Field collections of adult *Heliconius erato, H. himera,* and hybrids including all polymorphic sites mentioned by Jiggins et al. (1996) plus new individuals collected in 1996. Phenotypic classification: *erato* = pure *erato,* BE = backcross to *erato,* F_1 = first generation hybrids, BH = backcross to *himera, himera* = pure *himera.* Numbers given as females/males. Additional numbers of *erato* and *himera* for site 4 are of unknown sex, reported by Descimon and Mast de Maeght (1984). The frequency of *erato* genomes (q_E) and expected numbers of *erato,* hybrid (BE + F_1 + BH), and *himera* individuals, based on the maximum likelihood base model, are also given in parentheses under the observed numbers for each site.

Site	*erato* (expected)	BE	F_1 (expected − hybrids)	BH	*himera* (expected)	Total	q_E
2, Quebrada Naranjo	14/16 (30.04)	0/1	0/0 (0.31)	0/0	0/0 (0.65)	31	0.974
3, Guayquichuma	1/15 (16.03)	1/0	0/0 (0.30)	0/0	0/0 (0.66)	17	0.952
4, Guayquichuma glen	69/155+31 (253.05)	1/4	0/8 (15.65)	1/2	0/26+11 (39.30)	308	0.847
5	4/11 (16.17)	0/1	0/4 (3.43)	0/1	1/13 (15.40)	35	0.378
6, Las Orchideas	5/4 (10.29)	0/1	0/2 (2.86)	0/2	2/15 (17.85)	31	0.329
7	0/0 (0.29)	0/0	0/0 (0.09)	0/0	0/1 (0.63)	1	0.329
8	1/1 (2.05)	0/0	0/1 (0.72)	0/0	2/5 (7.23)	10	0.241
9, Escuela sin nombre	0/0 (0.65)	0/0	0/1 (0.31)	0/0	17/44 (61.04)	62	0.013
10, Río Inguna	0/1 (0.67)	0/0	0/0 (0.32)	0/0	7/51 (58.01)	59	0.014
Quebrada de la Zarza	0/0 (0.46)	0/0	0/0 (0.18)	1/0	0/2 (2.37)	3	0.182
15, Ombomba	34/47 (79.86)	0/1	1/2 (7.20)	0/0	2/20 (19.94)	107	0.780
16, Mizquillana	7/14 (19.76)	0/0	0/0 (3.78)	0/0	2/16 (15.47)	39	0.555
18, Olmedo North	1/1 (1.41)	0/0	0/0 (0.63)	0/0	6/16 (21.96)	24	0.072

also be set to 1. These simplifications considerably reduce the number of parameters that need to be estimated, and make efficient use of sparse data in each site.

These parameters (q_E for each population, α and x_{ij}) were estimated by means of likelihood. For each site, the support (the natural logarithm of the likelihood, $\log_e L$) for the parameters q_E and α is given by:

$$\log_e L\ (q_E, \alpha) = N_E \log_e Q_E + N_F \log_e Q_F + N_H \log_e Q_H$$

where N_E, N_F, and N_H are the observed numbers of *erato,* hybrids and *himera* from the population sample. Although our field collections showed a male-biased sex-ratio, in laboratory crosses the sex-ratio did not deviate significantly from 1:1 in either hybrid or pure broods (McMillan et al. 1997). We have therefore assumed for the purposes of phenotypic frequency estimation that there were no frequency differences between the sexes, so that N_E, N_F, and N_H represent the total numbers of both sexes caught in each site.

The support for mating parameters in a given site is given by:

$$\log_e L\ (x_{EE}, x_{EF}, \cdots x_{HH}) = n_{EE}\log_e \Xi_{EE} + n_{EF}\log_e \Xi_{EF} + \cdots + n_{HH}\log_e \Xi_{HH}$$

where n_{EF}, etc., are the numbers of *erato* females actually found to have mated hybrid males in the site, and so on. The overall support is then:

$$\log_e L\ (q_E, \alpha) + \log_e L\ (x_{EE}, x_{EF}, \cdots x_{HH})$$

for each site, and this sum is itself summed over sites to obtain the overall support across sites. The total support was maximized, giving maximum likelihood values of q_E for each site, and α, $x_{EF}, \cdots x_{HH}$ overall.

The difference in support ($\Delta\log_e L$) between hypotheses of different parameter values is used as a measure of inference (Edwards 1972). This difference is proportional to the "deviance" (D) in generalized linear models, and to the value of G in a standard G-test. $\Delta\log_e L$ can be compared with a χ^2 distribution because $G = D = 2\Delta\log_e L$ is distributed approximately as a χ^2 under the null hypothesis, where the degrees of freedom are the numbers of parameters allowed to vary. The support limits for a particular parameter (asymptotically equivalent to 95% confidence limits [Edwards 1972], shown in parentheses after the maximum likelihood estimate) are given by the values of that parameter for which $\Delta\log_e L$ drops to two units below the maximum along the ridge of maximum likelihood with respect to all other parameters. The support was found by using a simple computer program to calculate the likelihood of a given set of parameters. The support surface was explored by inputting parameter values by hand until the likelihood maxima were found. The performance of the method was checked by exploring the likelihood surfaces manually, and by comparing expected with observed values (e.g., see Table 1).

TABLE 2. Female parents and offspring obtained from egg expression and phenotype of individuals found as eggs or larvae.

Site	Found as eggs or larvae	Reared from wild females No. offspring (♀ parent → offspring)	Totals (expressed + eggs found)
2	1E		0 + 1
4	4E	19 (E → E), 2 (E → BE), 1 (E → F_1), 1 (F_1 → BH)	23 + 4
5	1BH	1 (E → E), 1 (H → H)	2 + 1
6	3H	2 (E → E)	2 + 3
7	1BH, 1BE		0 + 2
8	1H	1 (H → H)	1 + 1
9	1H		0 + 1
10	2H		0 + 2
Quebrada de la Zarza	—	1 (BH → H)	1 + 0
15	2E	5 (E → E), 1 (E → BE), 1 (H → H)	7 + 2
16	—	4 (E → E)	4 + 0
Totals	17	40	57

For a better studied hybrid zone, it might be possible to incorporate spatial information and cline theory in order to estimate local values of q_E more accurately than the site-by-site approach used here. However, the estimates of q_E for each site are "nuisance parameters" that do not strongly affect the parameters of interest, α or x_{ij}. We have tested the robustness of the estimates of α and x_{ij} by first setting all q_E to the value of their upper support limits to bias population frequencies upwards, and then at the values of their lower support limits as a method of instituting a downward bias. All maximum likelihood values of α, x_{EH} and x_{HE} so obtained are well within the support limits found using q_E at their maximum likelihood values. In any case, there is to our knowledge no appropriate cline theory for a partially hybridizing pair of species. We have therefore chosen the simpler and more conservative option of estimating q_E as though each site were independent.

The model used here is specific to the particular taxa under study, and would have to be modified for use with other species. For instance, in species with multiple mating and sperm mixing, the model would estimate overall premating isolation; including assortative mating and assortative fertilization. To unravel these two effects, one would have to study assortative fertilization experimentally with different kinds of matings. See also Hewitt et al. (1989) who performed an "analysis of deviance" to show that components due to first matings and to like matings were both significant.

RESULTS

In our analysis, we simultaneously estimated the phenotypic frequencies in each site and the mating behavior in a single analysis. Phenotypic frequencies are dealt with first, because they are fundamental to our model in which the pattern of mating depends on encounter frequency. A total of 727 field-collected individuals were used to estimate the frequencies of the species and their hybrids in each site. The numbers of individuals are shown in Table 1, together with the estimated frequencies q_E of *erato* at each site, and the expected numbers of *erato*, hybrids and *himera* estimated according to the maximum likelihood base model explained in detail below. It may be noticed that the maximum likelihood estimates of q_E are not exactly as predicted from the capture data alone (Table 1). For instance, at site 7, only a single adult was found, a *himera*, yet the frequency of *erato*, q_E, is estimated to be 0.329. This is because hybrid larvae were reared from there (Table 2), so there had to have been some *erato* genomes in this site among the parents of these offspring. The overall likelihood model allows this parental information to be incorporated appropriately. Overall, there is a strong hybrid deficit, with $\alpha = 0.804$ (support limits: 0.739, 0.858). This hybrid deficit is equivalent to a frequency of hybrids of $2 \times 0.5 \times 0.5 \times (1 - 0.804) \approx 9.8\%$ (7.1%, 13.0%) in the center of the hybrid zone where $q_E = 0.5$.

During the study, we collected 40 females from the hybrid zone for which egg expression and rearing of offspring was successful, and we reared an additional 17 individuals from young stages found in the wild. Table 2 shows the offspring obtained both from egg expression and from wild caught eggs and young larvae. The data used at each individual site can be demonstrated by means of the example of site 15 (Ombomba): here two *erato* (E) eggs were found in the wild, giving, together with the five *erato* offspring reared from *erato* mothers, seven E × E matings overall. The only two other matings at this site were one E × F_1 (because a BE was reared from an egg expressed from an E female) and one

TABLE 3. Matings inferred for all 57 offspring in Table 2. Figures outside parentheses are matings inferred on the basis of offspring reared from wild-caught females. Figures in parentheses are matings inferred from 17 egg or larval collections from the wild; in this case, where the parents clearly differed in genotype, the mating was assumed for the purposes of the analysis to have resulted from two half matings in each direction. For example, when a reared individual presented a phenotype classified as a backcross to *erato*, we could not tell which parent was the hybrid. We therefore scored this as half a mating of (female hybrid × male *erato*) and half a mating of (female *erato* × male hybrid). Matings at each site can be simply deduced from Table 2; for example, site 15 gives the following subtable: 5(+2), 1, 0; 0, 0, 0; 0, 0, 1. This table represents the summed matings over all sites. In the likelihood analysis, the inferred matings were used only with respect to the site frequencies Q_E, Q_F, and Q_H in their site of origin.

		Males		
		Pure *erato*	Hybrid	Pure *himera*
Females	Pure *erato*	31 (+7)	3 (+½)	1
	hybrid	0 (+½)	0	2 (+1)
	Pure *himera*	0	(+1)	3 (+7)

H × H. Table 3 gives matings inferred to have produced these progeny, summed across all sites. It is clear that there is assortative mating in the hybrid zone taken as a whole (Table 3). However, assortative mating would apparently occur across a hybrid zone even if mating were random within sites, because Table 3 combines information from sites with very different phenotype frequencies. This is why each female's mate choice must be analyzed only with respect to the frequencies of phenotypes (a surrogate for the probability of encounter with a particular male phenotype) collected in the site from which she was taken.

Inspection of Tables 2 and 3 reveals that we have very little data on matings between hybrids and other phenotypes. Therefore we estimate all five types of matings involving hybrids as a single conditional mating probability $x_F{}^* = x_{FE} = x_{FF} = x_{FH} = x_{EF} = x_{HF}$. We are interested in comparing the probability of hybrid matings $x_F{}^*$ and cross-mating (x_{EH} and x_{HE}) with the probability of matings within pure forms (x_{EE} and x_{HH}, which are set to 1), so we simplify the problem by estimating only three mating parameters, x_{EH}, x_{HE}, and $x_F{}^*$. The maximum likelihood values are then $x_{EH} = 0.084$, $x_{HE} = 0.000$, and $x_F{}^* = 0.964$. However, this model is not significantly different from a simpler model where hybrids mate with any phenotype with probability equivalent to that in intraspecific matings, that is, $x_F{}^* = x_{EE} = x_{HH} = 1$ ($\Delta\log_e L = 0.002$, 1 df, ns). Under this simpler model, the cross-mating probabilities and their support limits are $x_{EH} = 0.084$ (0.004, 0.424) and $x_{HE} = 0.000$ (0.000, 0.393). The maximum likelihood estimate of x_{HE} is zero because no female *himera* produced F_1 offspring. However, x_{EH} and x_{HE} are not significantly different ($\Delta\log_e L = 0.418$, 1 df, ns): setting the cross-mating probabilities to be equal gives $x_{EH} = x_{HE} = 0.055$ (0.003, 0.272); the overall best estimate of conditional cross-mating probability is then 5.5% of that for mating within species. This latter model is the "base model" referred to above, and used to generate the estimates of q_E and α, and the expected values of Table 1.

It might be argued that using the extra 17 wild-caught eggs and larvae to estimate mating frequencies is unjustified because some selection against hybrid larvae may already have occurred in the field. This is hopefully not the case, because the young larvae and eggs found would have experienced few of the environmental conditions that could lead to selection. In any case, restricting the analysis to the 40 progeny (see Tables 2, 3) for which we know the female parental phenotype makes little difference: we obtain a similar cross-mating probability with slightly broader support limits: $x_{EH} = x_{HE} = 0.073$ (0.004, 0.374).

DISCUSSION

In a natural hybrid zone, *Heliconius himera* and *erato* mate strongly assortatively, with a cross-mating probability of only about 5.5% (0.3%, 27.2%) of the probability of intraspecific mating in the center of the hybrid zone, equivalent to an actual cross-mating probability of 0.055/1.055 = 5.2% (0.3%, 21.4%). These results are consistent with laboratory experiments, where the equivalent cross-mating probability is about 10% (McMillan et al. 1997); the laboratory estimate falls well within the support limits of the field estimate and vice versa. Clearly, mating behavior forms a substantial barrier to gene flow between the two species, equivalent on a per-generation basis to an approximate 95% selection pressure against hybrids. Over a single generation, this is almost twice as strong as even a very strongly selected chromosomal translocation. Nonetheless, in the absence of any selection against hybrids, continued hybridization over a number of generations should eventually spawn a hybrid swarm in the overlap zone. Strong selection must be acting to prevent this.

In principle, an accurate measure of mating behavior in the hybrid zone should permit strong inferences to be made about the strength of selection against hybrids. Assuming equilibrium between gene flow and selection against hybrids, selection could be estimated by comparing the frequency of hybrids in the field with the frequency produced initially via natural hybridization. The frequency of hybrids estimated to exist in the center of the zone, about 9.8%, of which about half (5.2% of the total) are F_1 hybrids, is almost exactly accounted for by the approximate cross-mating frequency of 5.2% in each generation. The remainder of the hybrids are presumed backcrosses, and again they reach the approximate frequency expected for a single generation in the absence of selection (almost all F_1s should mate with pure types and should therefore just reproduce themselves as backcrosses). Yet, the absence of a hybrid swarm caused by multiple generations of intercrossing must indicate very strong selection of some kind. As mentioned in the introduction, two selection pressures against hybrids are likely: frequency-dependent selection for warning coloration and abiotic selection on either side of an aridity gradient centered in the hybrid zone (Jiggins et al. 1996; McMillan et al. 1997; Davison et al., unpubl. data). Selection of either kind may remove the "wrong" pure types on each side of the hybrid zone as well as hybrids, and competition between the species may enhance (or diminish) this environmental effect. In practice, the support limits are too broad for an estimate to be made. The upper support limit of 21.4% cross-mating frequency measured in the field could imply that selection against hybrids is as high as (21.4—5.2%)/21.4% = 76% (to give a 5.2% F_1 frequency); the lower bound would imply selection strongly favoring hybrids (leading to an unstable cline, which is known not to be the case).

If the selection is due to predation on rare phenotypes, a greater sex ratio distortion might be expected among the disfavored rarer forms. In warningly colored and mimetic butterflies the females are often more vulnerable (Ohsaki 1995). There is indeed a strong male bias among rare forms in this hybrid zone. At site 4 where we have the largest sample sizes, *himera* is rare compared with *erato*, and no females were caught among 26 *himera* (Table 1). There were only two females among 16 hybrids from the same site, whereas the commoner *erato* had 69 females among 224 individuals. Differences between the sex ratios of the phenotypes at this site are highly significant ($\Delta\log_e L = 9.97$, 2 df, equivalent to $P < 0.001$). There could be other explanations; it is possible that *H. himera* and hybrids differ from *H. erato* in their behavior so that *erato* females are more catchable. We need more data to test the tantalizing possibility of sex-biased predation, and mark-release studies of natural selection on adults would provide extremely valuable direct evidence of selection.

It is of interest to ask whether the strong mate choice we have found can be explained by reinforcement—the tendency for species that hybridize, but produce offspring of reduced fitness, to evolve assortative mating (Dobzhansky 1940; Butlin 1989). In the laboratory, strong assortative mating is found between individuals collected over 60 km from the hybrid zone, itself only 5 km wide; assortative mating is not just a feature of the zone of overlap (McMillan et al. 1997). These data do not completely rule out reinforcement, because the tendency to mate assortatively could have spread from the center of the hybrid zone to peripheral areas. However, our data provide no comfort to supporters of reinforcement as a general explanation of assortative mating.

This study uses a powerful statistical analysis to glean estimates of "premating isolation" in the wild from sparse field data. It is generally recognized that field studies are needed to assess barriers to gene flow, but in practice much of what we know about mate choice between species comes from highly unnatural laboratory experiments, especially using *Drosophila* (e.g., Coyne and Orr 1997). Our work demonstrates that analyses of mating behavior in a hybrid zone are feasible even in animal species that are hard to collect and study in the laboratory. While the precise model used will depend on the biology of the species studied, a general likelihood approach similar to ours should be valuable for estimating gene flow between other incipient species in the wild.

ACKNOWLEDGMENTS

We gratefully acknowledge that this work was carried out under the auspices of Ing. M. Moreno and G. Estévez at the Museo de Ciencias Naturales, Quito, with the permission of INEFAN. We thank M. Arias Diaz, M. del Carmen Avila, R. Carpio Ayala, J. Carpio Ayala, A. Davison, S. Dixon, A. Griffin, and P. Mallet for help with the plant care rearing and field work, F. Lopez and P. Lozano at the Fundación Arcoiris, P. Wilson, and J. and C. Hoffman for help with logistics. We thank the BBSRC for CDJ's studentship and for research funds, and an anonymous reviewer, C. Boggs, A. Davison, C.A.B. Smith, and Z. Yang for useful comments.

LITERATURE CITED

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

BOGGS, C. L. 1979. Resource allocation and reproductive strategies in several heliconiine butterfly species. Ph.D. diss., University of Texas, Austin, TX.

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

BROWN, K. S. 1979. Ecologia Geográfica e Evolução nas Florestas Neotropicais. Universidade Estadual de Campinas, Campinas, Brazil. Livre de Docencia.

BROWN, K. S., AND W. W. BENSON. 1974. Adaptive polymorphism associated with multiple Müllerian mimicry in *Heliconius numata* (Lepid.: Nymph.). Biotropica 6:205–228.

BUTLIN, R. 1989. Reinforcement of premating isolation. Pp. 158–179 *in* D. Otte and J. A. Endler, eds. Speciation and its consequences. Sinauer, Sunderland, MA.

COYNE, J. A., AND H. A. ORR. 1997. "Patterns of speciation in *Drosophila*" revisited. Evolution 51:295–303.

DEINERT, E. I., J. T. LONGINO, AND L. E. GILBERT. 1994. Mate competition in butterflies. Nature 370:23–24.

DESCIMON, H., AND J. MAST DE MAEGHT. 1984. Semispecies relationships between *Heliconius erato cyrbia* Godt. and *H. himera* Hew. in southwestern Ecuador. J. Res. Lepid. 22:229–239.

DOBZHANSKY, T. 1940. Speciation as a stage in evolutionary divergence. Am. Nat. 74:312–321.

DUNLAP-PIANKA, H. L., C. L. BOGGS, AND L. E. GILBERT. 1977. Ovarian dynamics in heliconiine butterflies: programmed senescence versus eternal youth. Science 197:487–490.

EDWARDS, A. W. F. 1972. Likelihood. Cambridge Univ. Press, Cambridge, U.K.

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. Nat. Acad. Sci. USA 91:7990–7994.

GILBERT, L. E. 1972. Pollen feeding and reproductive biology of *Heliconius* butterflies. Proc. Nat. Acad. Sci. USA 69:1403–1407.

GILLHAM, E., AND B. L. GILLHAM. 1996. Hybrid ducks. A contribution towards an inventory. Published by the authors, Wallington, Surrey, U.K.

GRANT, B. R., AND P. R. GRANT. 1996. Cultural inheritance of song and its role in the evolution of Darwin's finches. Evolution 50: 2471–2487.

GRANT, P. R., AND B. R. GRANT. 1994. Phenotypic and genetic effects of hybridization in Darwin's finches. Evolution 48:287–296.

HEWITT, G. M., R. A. NICHOLS, AND N. H. BARTON. 1987. Homogamy in a hybrid zone in the alpine grasshopper *Podisma pedestris*. Heredity 59:457–466.

HEWITT, G. M., P. MASON, AND R. A. NICHOLS. 1989. Sperm precedence and homogamy across a hybrid zone in the alpine grasshopper *Podisma pedestris*. Heredity 62:343–354.

HOWARD, D. J. In press. The evolution of barriers to fertilization between closely related organisms. *In* S. Berlocher and D. J. Howard, eds. Endless forms: species and speciation. Oxford Univ. Press, New York.

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 Biol. Sci. 264:1167–1175.

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. 1997a. The maintenance of species differences across a *Heliconius* hybrid zone. Heredity 79:495–505.

JIGGINS, C. D., W. O. MCMILLAN, AND J. L. B. MALLET. 1997b. Host plant adaptation has not played a role in the recent speciation of *Heliconius himera* and *Heliconius erato* (Lepidoptera: Nymphalidae). Ecol. Entomol. 22:361–365.

LABINE, P. A. 1966. The population biology of the butterfly *Euphydryas editha*. IV. Sperm precedence—a preliminary report. Evolution 20:580–586.

MALLET, J. 1986a. Hybrid zones in *Heliconius* butterflies in Panama, and the stability and movement of warning colour clines. Heredity 56:191–202.

———. 1986b. Dispersal and gene flow in a butterfly with home range behaviour: *Heliconius erato* (Lepidoptera: Nymphalidae). Oecologia (Berl.) 68:210–217.

———. 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.

———. 1996. The genetics of biological diversity: from varieties to species. Pp. 13–47 *in* K. J. Gaston, ed. Biodiversity: biology of numbers and difference. Blackwell, Oxford, U.K.

MALLET, J., AND N. H. BARTON. 1989. Strong natural selection in a warning color hybrid zone. Evolution 43:421–431.

MALLET, J., N. BARTON, G. LAMAS, J. SANTISTEBAN, M. MUEDAS, AND H. EELEY. 1990. Estimates of selection and gene flow from measures of cline width and linkage disequilibrium in *Heliconius* hybrid zones. Genetics 124:921–936.

MALLET, J., W. O. MCMILLAN, AND C. D. JIGGINS. In press. Mimicry and warning color at the boundary between races and species. *In* S. Berlocher and D. Howard, eds. Endless forms: species and speciation. Oxford Univ. Press, New York.

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

Moore, W. S., and D. Buchanan. 1985. Stability of the northern flicker hybrid zone in historical times: implications for adaptive speciation theory. Evolution 39:135–151.

Ohsaki, N. 1995. Preferential predation of female butterflies and the evolution of Batesian mimicry. Nature 378:173–175.

Price, C. S. C. 1997. Conspecific sperm precedence in *Drosophila*. Nature 388:663–666.

Sætre, G.-P., T. Moum, S. Bures, M. Král, M. Adamjan, and J. Moreno. 1997. A sexually selected character displacement in flycatchers reinforces premating isolation. Nature 387:589–592.

Scoble, M. J. 1992. The Lepidoptera: form, function and diversity. Oxford Univ. Press, London.

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

Turner, J. R. G. 1971. Two thousand generations of hybridization in a *Heliconius* butterfly. Evolution 25:471–482.

Corresponding Editor: T. Markow