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# PARALLEL RACE FORMATION AND THE EVOLUTION OF MIMICRY IN *HELICONIUS* BUTTERFLIES: A PHYLOGENETIC HYPOTHESIS FROM MITOCHONDRIAL DNA SEQUENCES

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*Abstract.*—Mimicry has been a fundamental focus of research since the birth of evolutionary biology yet rarely has been studied from a phylogenetic perspective beyond the simple recognition that mimics are not similar due to common descent. The difficulty of finding characters to discern relationships among closely related and convergent taxa has challenged systematists for more than a century. The phenotypic diversity of wing pattens among mimetic *Heliconius* adds an additional twist to the problem, because single species contain more than a dozen radically different-looking geographical races even though the mimetic advantage is theoretically highest when all individuals within and between species appear the same. Mitochondrial DNA (mtDNA) offers an independent way to address these issues. In this study, Cytochrome Oxidase I and II sequences from multiple, parallel races of *Heliconius erato* and *Heliconius melpomene* are examined, to estimate intraspecific phylogeny and gauge sequence divergence and ages of clades among races within each species. Although phenotypes of sympatric races exhibit remarkable concordance between the two species, the mitochondrial cladograms show that the species have not shared a common evolutionary history. *H. erato* exhibits a basal split between trans- and cis-Andean groups of races, whereas *H. melpomene* originates in the Guiana Shield. Diverse races in either species appear to have evolved within the last 200,000 yr, and convergent phenotypes have evolved independently within as well as between species. These results contradict prior theories of the evolution of mimicry based on analysis of wing-pattern genetics.

*Key words.*—Butterfly wing patterns, *Heliconius erato, Heliconius melpomene,* mimicry, phylogeny, mtDNA, Pleistocene refugium, vicariance biogeography.

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One of the most familiar examples of Müllerian mimicry occurs among the neotropical nymphalid butterflies in the genus *Heliconius* and closely allied genera. Four diverse, phenotypically divergent mimetic assemblages, each sharing a common, easy-to-recognize pattern, co-occur in Amazonia and elsewhere in the neotropics. These mimicry rings contain up to a dozen distinct heliconiine species (species in the traditional, biological sense of Mayr 1963) plus additional mimics from other lepidopteran groups. Members of each ring share an easy-to-recognize aposematic color pattern that advertises the butterflies' unpalatability. Unrelated species (even species from different nymphalid subfamilies) can be so similar in appearance that they are frequently sorted incorrectly by museum curators. On the wing, the various bearers of a convergent pattern are indistinguishable to collectors and (presumably) predators alike.

While *Heliconius* butterflies exhibit interspecific mimicry, many species also display diverse intraspecific geographical polymorphism (Fig. 1). A single biological species may contain more than 20 phenotypically differentiated, phylogenetically diagnosable allopatric races, which could be considered species under the phylogenetic species concept (Cracraft 1983; Nixon and Wheeler 1990; Davis and Nixon 1992) but will be treated in accord with the traditional taxonomy here. These races often precisely mimic the races of another species, both inhabiting a common geographical range (Figs. 2–3). This diversity confounded the systematic efforts of 19th-century collectors, who applied dozens of names to divergent geographical races and to the hybrids between them, which displayed a spectrum of recombinant wing patterns (e.g., Weymer 1893; Riffarth 1901; Stichel and Riffarth 1905; see references in Neustetter 1929). Although only 54 species of *Heliconius* (sensu Brower 1994a) are currently recognized (Brown 1981), more than 700 names have been applied to subspecies, races, aberrations, and interracial hybrid recombinant forms (Neustetter 1929).

In this century, researchers have determined that most of the phenotypic diversity in *Heliconius* is the result of intraspecific geographical differentiation and hybridization (Oberthür 1902; Eltringham 1916; Emsley 1964). The most dramatic example of this parallel race formation occurs between *Heliconius erato* and *Heliconius melpomene.* Although distantly related within the genus (Emsley 1965; Brown 1981; Brower 1994a), these two species share remarkable convergence of color patterns in their allopatrically or parapatrically distributed races, which range from southern Mexico to northern Argentina (Figs. 2–3). Phenotypes of each pair of races are monomorphic over most of their shared range, because strong normalizing selection is acting to maintain the aposematic color pattern. Local polymorphism in wing patterns occurs only where races abut and intraspecific hybridization occurs. Although there is apparently neither assortative mating nor intrinsic postzygotic barriers to gene flow between races (interracial hybrid individuals are completely viable and fertile [Turner 1971; Mallet 1989]), hybrid zones stay sharply defined because disproportionate predation on butterflies with unusual or unique phenotypes produces strong selection that counteracts gene flow, as has been shown by mark-release-recapture studies (Benson 1972; Mallet and Barton 1989).

Attempting to illuminate the ecological genetics of Müllerian mimicry, Sheppard and colleagues embarked on a mas-

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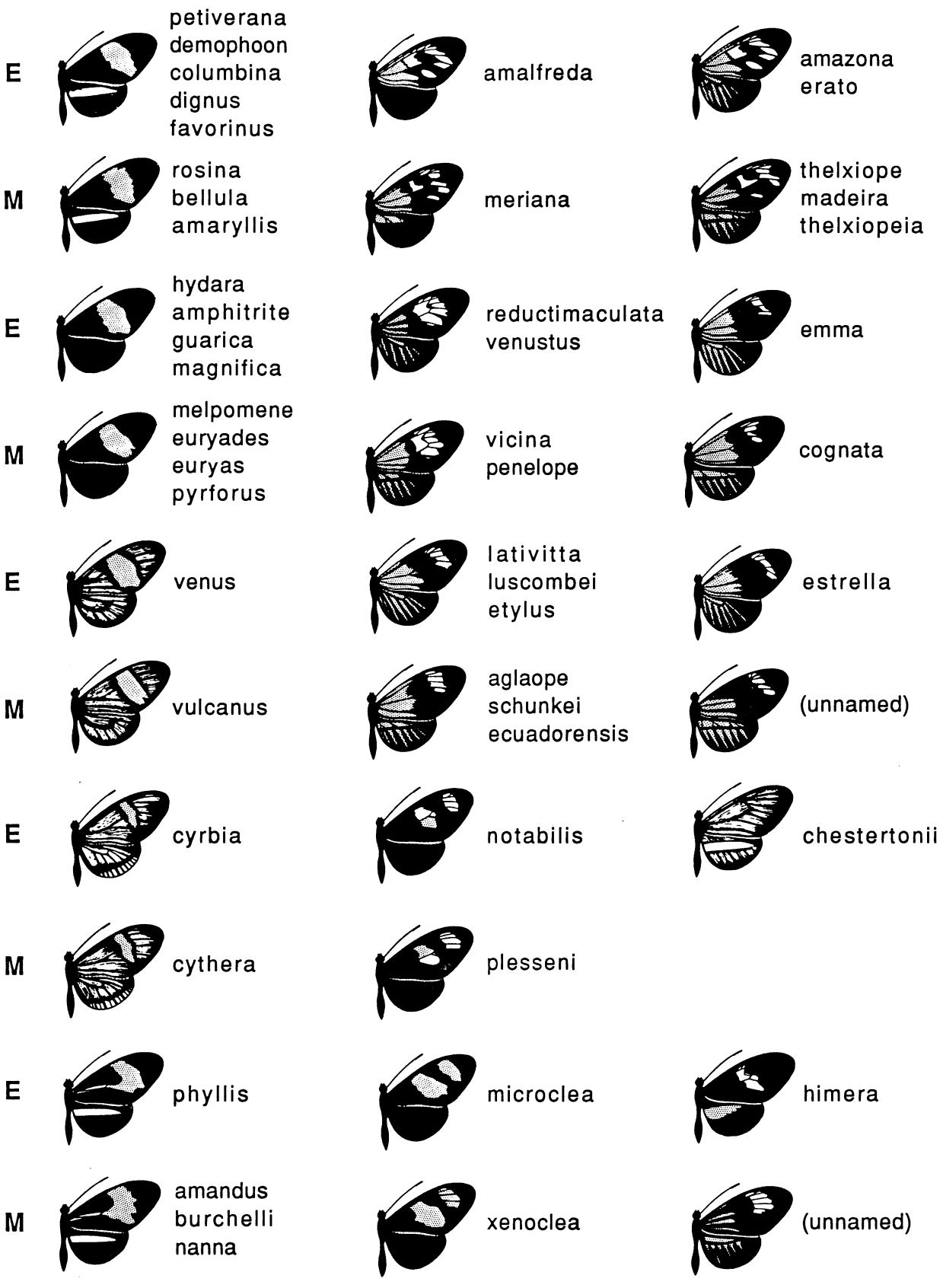
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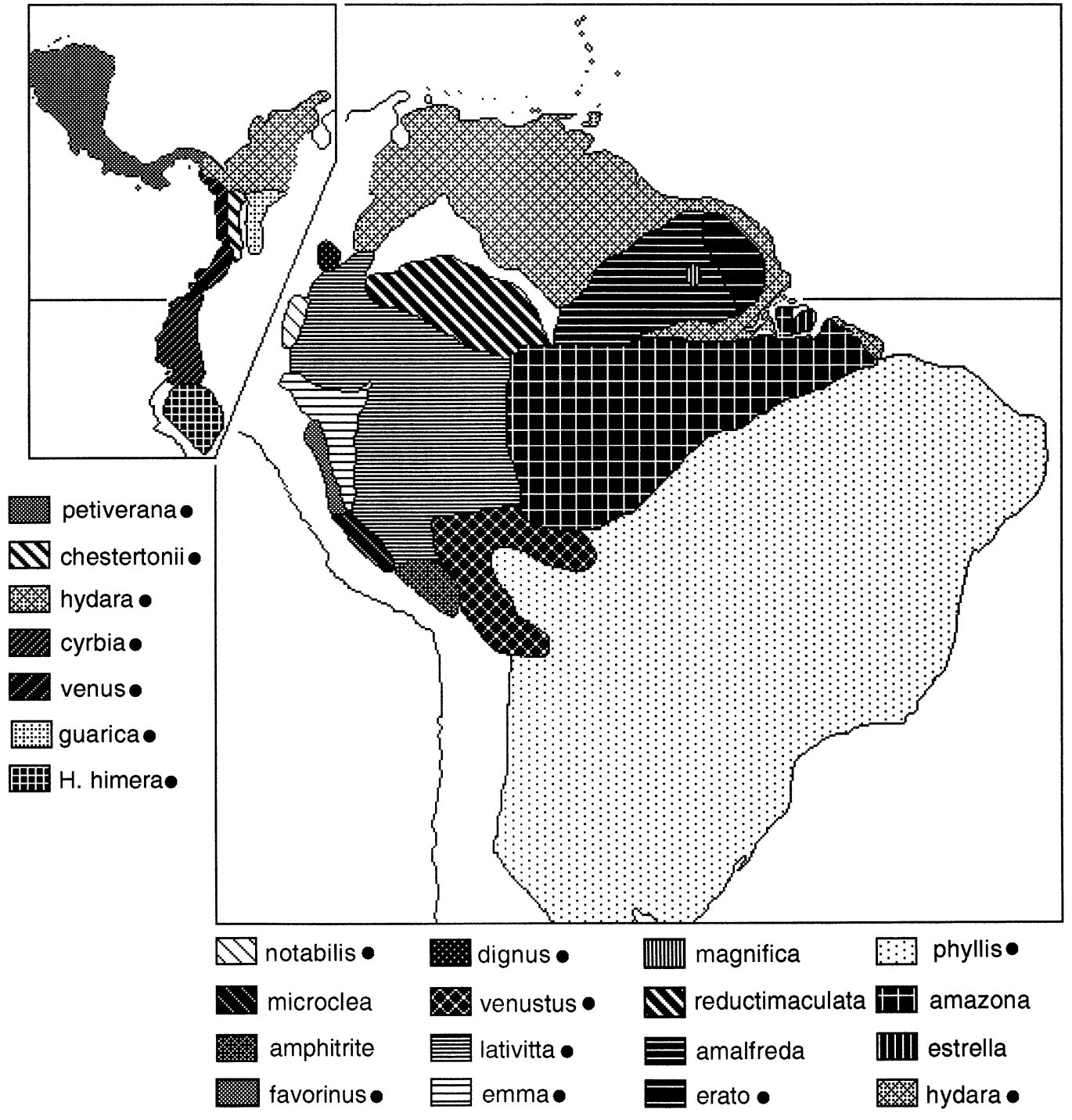


FIG. 2. Distribution of major phenotypic races in *Heliconius erato,* after Sheppard et al. (1985) and Brower (1994b). The ranges of the different races in both species were determined by those authors from extensive museum collection data. Allopatric populations with similar color patterns are considered to be distinct races. Western and Central American races are shown in the inset to emphasize the biogeographic disjunction between them and the eastern races (see text). To avoid excessive complexity, some minor parapatric races listed on Figure 1 are not shown as distinct from adjacent races.

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FIG. 1. Major phenotypes of geographical races of *Heliconius erato* and *Heliconius melpomene,* after Sheppard et al. (1985). Races are arranged in columns, paired according to mimetic associations. The E or M at the left identifies the species in each row. Colors are encoded as follows: black, black; stippled, red (pinkish in *notabilis/plesseni* and *cyrbia/cythera*); white, yellow (white in *cyrbia/cythera*); striated, irridescence (greenish in *chestertonii,* bluish in others). Some phenotypes are shared by more than one allopatric or parapatric race. *Heliconius erato chestertonii* has no *H. melpomene* mimic.

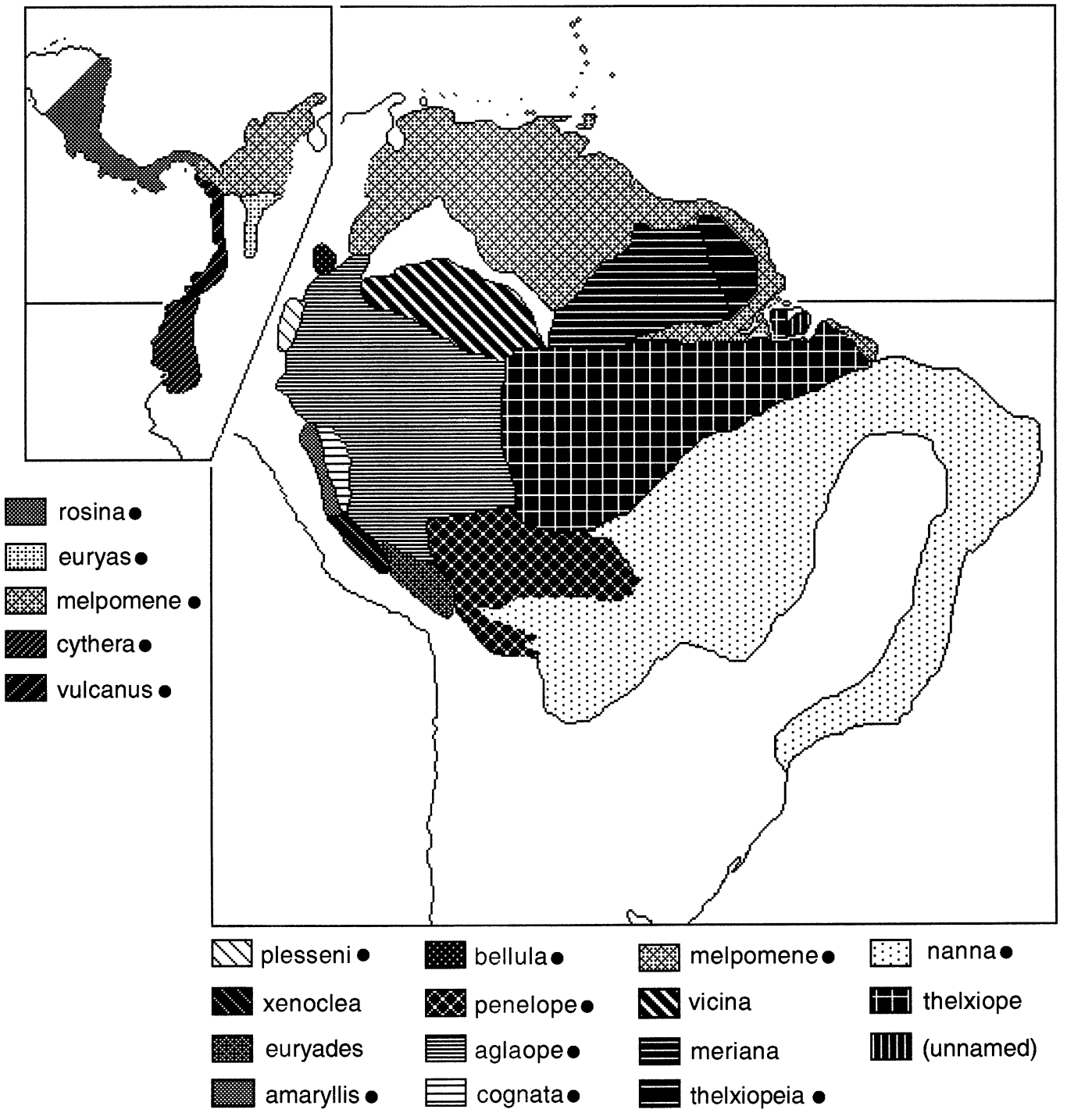


FIG. 3. Distribution of major phenotypic races in *Heliconius melpomene,* after Sheppard et al. (1985). Note the tight correspondence of racial boundaries between the two species (shared hatching patterns between Figs. 2–3 indicate similar phenotypes). Sympatric populations of *Heliconius erato* and *H. melpomene* always share a single mimetic phenotype, except in relatively narrow hybrid zones between races. As in Figure 2, some minor parapatric races listed on Figure 1 are not shown as distinct from adjacent races here.

sive analysis of wing-color polymorphism in *Heliconius* during the 1960s, conducting breeding experiments among selected races within each species. Over the next 25 yr (references in Sheppard et al. 1985), they identified nine independently assorting linkage groups for color-pattern characters in *H. erato* and seven groups in *H. melpomene* among the 21 chromosomes characteristic of both species (Suomalainen et al. 1971). Gilbert (in Nijhout et al. 1990; L. Gilbert, pers. comm., 1994) and Mallet (1989, 1993) continue to expand the genetic data base for both species. Different pattern elements (e.g., presence of a red patch on the forewing, presence of a yellow stripe on the hindwing) exhibit

straightforward dominance or semidominance, while additional "modifier genes" produce pleiotropic effects on size and shape of primary pattern elements. Many, but not all, linkage groups share apparently identical alleles in allopatric but phenotypically similar races in both species (the red forewing patch and yellow hindwing stripe, for instance, are apparently produced by the same alleles in both southeastern Brazilian and Central American races of each species; Sheppard et al. 1985). In some instances, however, similar pattern elements appear to be produced by different genetic mechanisms in different races (Mallet 1989). Between the two species, patterns are independently derived: the two species are far more similar to each other in outward appearance than either is to its more immediate sister taxa (Brower 1994a).

Sheppard, Turner, and colleagues hypothesized the relationships of races in each species by tracing genotypic evolution, according to several assumptions (Turner 1976; Sheppard et al. 1985). Alleles shared between races were assumed to be identical by descent, producing the same phenotypic characters due to common ancestry rather than convergence. In addition, character polarities were determined by postulating that recessive alleles are primitive to dominant alleles, based on the higher probability of establishment for dominant over recessive novel, advantageous alleles (Crow and Kimura 1970), a process Turner has referred to as "Haldane's Sieve" (Turner 1983). Turner used the alternate alleles of the multiple, independently segregating loci that produce wing patterns in *H. erato* and *H. melpomene* to construct parsimony networks of wing-pattern evolution in these two species (Fig.4). Although the genetic architecture underlying similar wing patterns in the two species is quite different (Sheppard et al. 1985, but see Nijhout et al. 1990), the branching order of these networks is similar, implying that the mimetic association between species has persisted since before the evolution of racial polymorphism within species and that strict coevolution (Janzen 1980) has occurred between them.

The evolutionary trees inferred by Sheppard et al. (1985) from these cladograms place the eastern Brazilian races close to the putative ancestor, with multiple offshoots leading to the Amazonian races, the Ecuadorian races, the Andean and northern coastal races, and the Central American races. The geographical distribution of the races suggests that newer, red rayed forms (E–H in Fig. 4) have arisen in the center of the species' range, in Amazonia, whereas the older alleles persist in multiple, allopatric populations at the periphery. Sheppard et al. argued that their interpretation is supported by the observation that close relatives of each species share some of the phenotypic characters produced by recessive alleles, implying that those traits are ancestral. However, they did not mention that the hypothetically derived phenotypes are also widespread among other members of the genus that participate in the *erato-melpomene* mimicry ring, complicating the assertion that similar patterns are shared among close relatives due to common descent. Nor did they refer to an explicit phylogenetic hypothesis when comparing patterns of hypothetical ancestors of each species with its respective sister taxa.

The origin of rampant intraspecific variation in butterflies protected by Müllerian mimicry presents an evolutionary paradox, as noted by Ford (1953) and Turner (1977). The advantage of sharing an aposematic color pattern is based on predators' ability to recognize the pattern, but their inability to discriminate among the different species displaying the pattern. Thus, the more widely an obvious, stereotypical pattern occurs across a group of unpalatable species, the more strongly will selection favor it. Why should butterflies participating in Müllerian mimicry rings exhibit numerous parapatric geographical races with radically different phenotypes, when theoretically predicted to show monomorphism across their distribution?

The failure of selection to produce phenotypic homogeneity among aposematic mimics and the intraspecific evolutionary hypothesis described above both strongly imply that divergence of the various races in each species occurred in allopatry. Vicariant fragmentation of, or dispersal and isolation from a widespread ancestral population would eliminate barriers to divergence imposed by stabilizing selection, allowing local populations to differentiate phenotypically due to genetic drift or selection to converge upon the pattern of a local mimetic complex. Once racial divergence had occurred in allopatry, the differences between adjacent races making secondary contact would be maintained by predation through frequency-dependent stabilizing selection against novel, nonmimetic hybrids (Mallet and Barton 1989). The Pleistocene refugium hypothesis (Haffer 1969; see Discussion) has been offered as a mechanism to explain the vicariant partitioning of ancestrally homogeneous *Heliconius* phenotypes, although selection in usually invoked to explain the rapid diversification of wing patterns once isolation is achieved (Brown et al. 1974; Turner 1983; Sheppard et al. 1985).

If racial divergence was driven or maintained in part by selection on wing patterns, phylogenetic and biogeographical relationships among races may be difficult to infer from that character system. The convergence of wing patterns between *H. erato* and *H. melpomene* races strongly suggests that similar wing patterns in disjunct conspecific races could likewise be at least in part a product of parallel, independent change. Thus, the most parsimonious cladograms of races derived from wing patterns may not reflect the underlying historical relationships of the butterfly populations that bear them. Our lack of an independent framework with which to assess the phenotypic data has made it impossible to draw conclusions about evolutionary rates and the relative roles of natural selection and genetic drift in the production of this diverse array of geographical variants. Not only are the wing patterns currently under intense selection (Mallet and Barton 1989), and thus unreliable for systematic analysis (Darwin 1859, pp. 414–415), but they also represent the physical manifestations of the evolutionary process we wish to explain.

An obvious means to break free from this circularity (or "bias"; sensu Maddison and Maddison 1992) is to frame an independent phylogenetic hypothesis of races in each species, based on characters unrelated to wing patterns. Such a separate systematic framework provides a means to test the assumptions of the Sheppard et al. (1985) model, as well as to examine further hypotheses about the evolution and geographical distribution of mimetic systems and the Pleistocene refugium model. Turner et al. (1979) and Mallet (pers. comm. 1988) have attempted to develop alternative data sets using

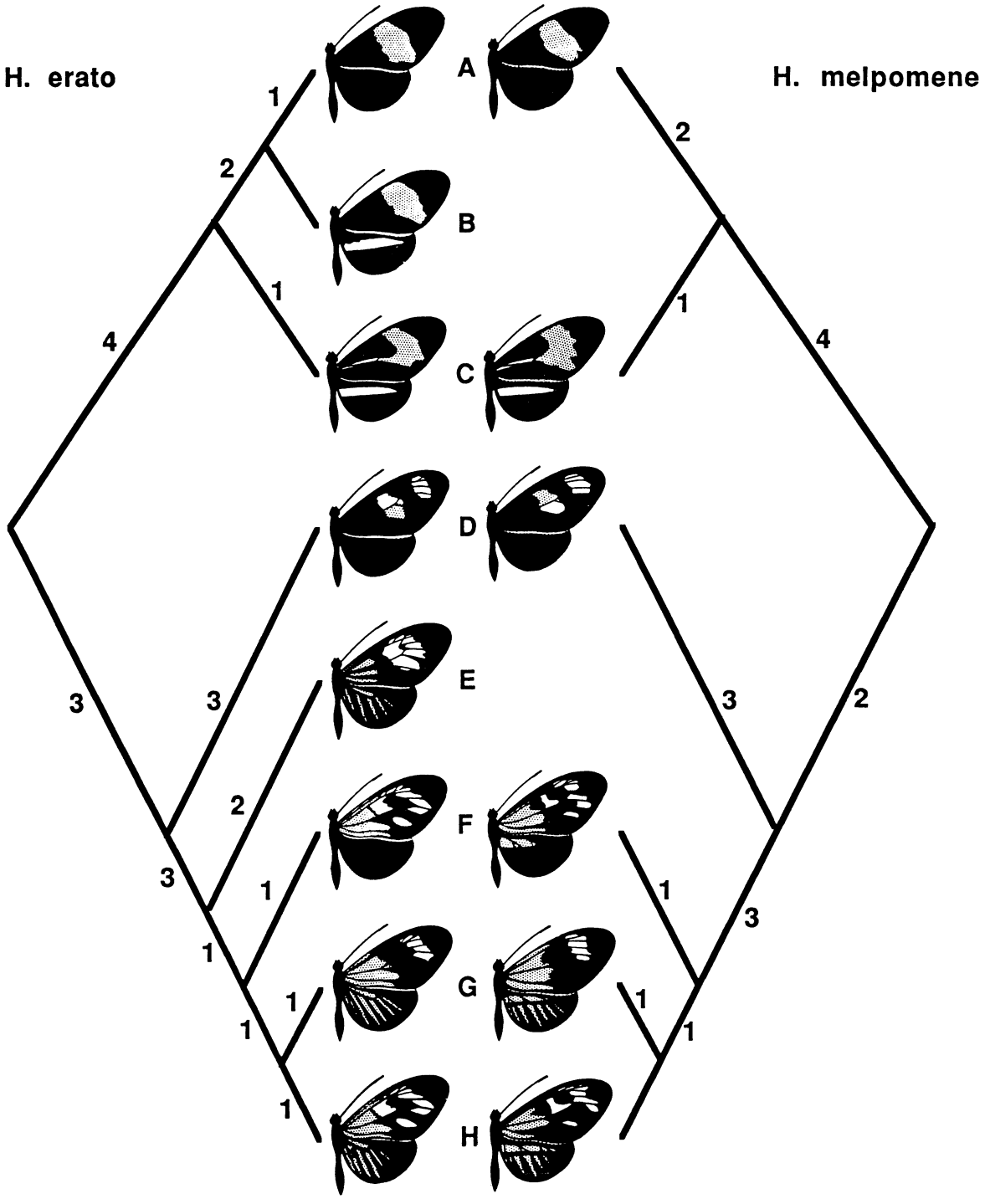


FIG. 4. Parsimony networks of wing-pattern alleles for *Heliconius erato* and *Heliconius melpomene*, proposed by Turner (1982, 1983) and Sheppard et al. (1985). Note the similarity between the two topologies. Numbers along the branches refer to the inferred number of phenotypic allele substitutions occurring on that branch. The hypothetical ancestor is thought to be most similar to C in its wing pattern and to bear only yellow markings. *Heliconius erato* races compared in these studies are: A, *hydara*; B, *petiverana*; C, *phyllis*; D, *notabilis*; E, *venustus*; F, *amalfreda*; G, *lativitta*; H, *amazona*. *Heliconius melpomene* races compared are A, *melpomene*; C, *nanna*; D, *plesseni*; F, *meriana*; G, *aglaope*; H, *thelxiope*.

allozymes but found a surprising lack of diagnostic differences between races for protein data, in contrast to the sharply differentiated phenotypic patterns. Although electromorph frequency shifts between populations and across hybrid zones exist for some loci, fixed differences are rare, and few intraspecific phylogenetic inferences may be made.

I have produced an independent phylogenetic hypothesis of races for each species, using mtDNA sequences. Mito-

chondrial DNA has become a standard tool for studying population structure and phylogeny in insects (DeSalle et al. 1986; Martin and Simon 1990; Beckenbach et al. 1993; Brown et al. 1994; Brower 1994a,b) and can reveal fixed differences between groups that exhibit protein electrophoretic allele frequency differences but no alternative fixation (Harrison et al. 1987). In addition, mtDNA is unlinked to nuclear genes, making it useful for estimating an independent phylogenetic framework to test hypotheses of nuclear gene evolution (i.e., wing patterns).

Assuming that relationships inferred from mtDNA reflect the evolutionary history of the butterfly populations (see discussion), my results allow me to address several questions left unanswered by studies of wing-pattern genetics. Within each species (1) are phenotypically similar but allopatric races within each species more closely related than geographically adjacent but phenotypically distinct races, as argued by Sheppard et al. (1985) and Mallet (1989); or are adjacent races more closely related, implying a vicariant biogeographical history and convergent evolution of wing patterns? If biogeography prevails, adjacent races should be more closely related to one another than to other disjunct races, even if those other races bear an identical phenotype. For example, the distributions of the phenotypically identical *H. erato petiverana* (Central America) and *H. erato favorinus* (Río Huallaga, eastern Peru) cannot be easily explained by vicariance if they are sister taxa.

(2) Within each species, is the biogeographical history implied by the mtDNA phylogeny consistent with the Pleistocene refugium model? If so, we expect to observe correspondence between mtDNA and phenotypic relationships, since divergence occurred during periods of severely reduced gene flow. Under refugium conditions, reduced population sizes might have increased the rate of fixation by genetic drift, speeding up the elimination of ancestral polymorphism at neutral loci. A periodic pattern of divergence reflecting synchronous isolation events during repeated cycles of glaciation (manifested as a series of star phylogenies—nodes with multiple, equal-length branches on the tree), would also support the refugium model (Benson 1982), even if strict phenotype-mtDNA haplotype correspondence were lacking. Alternatively, there is no reason to expect any correspondence of mtDNA haplotypes to phenotypic geographical patterns if parapatric differentiation has been predominant, because only those characters under selection (wing patterns) will have diverged along ecological gradients.

(3) Are the rates of sequence divergence compatible with Pleistocene-age subdivisions? Given the recency of the Pleistocene, branch lengths among closely related taxa should be very short. An mtDNA tree with a range of different branch lengths and a binary branching pattern is expected if local selection for ecological adaptation, acting independently in different geographical regions and on loci unlinked to mtDNA, is the causal agent for divergence.

(4) Between the two species, are the branching orders and amounts of sequence divergence in the mtDNA phylogenies of *H. erato* and *H. melpomene* races the same? Matched topologies and branch lengths imply strict coevolution (or shared vicariant history promoted by common evolutionary response to extrinsic events) between the two species, whereas discordant patterns suggest independent evolution and recent adaptive convergence between individual pairs of races.

## Materials and Methods

### *Sampling Strategy*

I sampled 49 individual *H. erato,* representing 14 phenotypic geographical races, and 35 individual *H. melpomene* specimens representing 13 races. Figure 5 shows the geographical distribution of sampling sites for these specimens, and Appendix Tables 1 and 2 list locality data. I failed to obtain material from several additional races in each species, but the samples I obtained cover the range of intraspecific diversity, both in terms of geographic and phenotypic extremes, in both species.

Appropriate outgroup taxa were selected on the basis of their close relationships to *H. erato* and *H. melpomene,* respectively, from another mtDNA sequence data set I compiled for interspecific phylogenetic comparisons (Brower 1994a). Outgroups for *H. erato* are *Heliconius sara, Heliconius clysonymus,* and *Heliconius telesiphe. Heliconius himera,* of uncertain specific status (Descimon and de Maeght 1983; J. Mallet, pers. comm., 1993) is also included in the *H. erato* analysis. Outgroups for *H. melpomene* are *Heliconius besckei* and *Heliconius hecale,* as well as seven specimens representing six taxa from the *Heliconius cydno* complex, traditionally considered to be *H. melpomene*'s sister species (Brown 1981; Brower, 1994a; but see below). Locality data for outgroup taxa are listed in Appendix Table 3. Neither ingroup nor outgroup taxa were constrained to be monophyletic in the analyses.

Because of the cost and labor-intensive nature of amplifying and sequencing DNA, I relied, in most cases, upon sampling a small number of individuals from each race. Most races in both data sets are represented by at least two individuals. To test for correspondence of mitochondrial haplotypes to phenotypic racial boundaries, I sampled a series of *H. erato* specimens along a transect in eastern Panama which crosses two intraspecific hybrid zones, where Mallet conducted a similar survey of phenotypic variation (Mallet 1986). To mitigate difficulties from introgression, other samples were collected as far from adjoining races as possible.

### *Preparation of Specimens*

The specimens were collected as adults, larvae, or pupae in the wild at various sites in Central and South America. Caterpillars and pupae were transported to the laboratory alive and reared to adulthood, while butterflies were transported alive, frozen in liquid nitrogen or on dry ice, or immersed in 100% EtOH. All preservation methods were effectively equivalent for the protocols employed in the study.

Genomic DNA was prepared from individual butterflies, using either thorax only; head and thorax; or head, thorax, and abdomen. The body parts were chilled with liquid nitrogen in a mortar and ground to a fine powder (specimens in EtOH were first blotted on a clean paper towel to remove excess liquid). The crushed sample was quickly transferred to a 1.5-ml Eppendorf tube with a spatula, and DNA was extracted via a version of the Harrison et al. (1987) SDS-

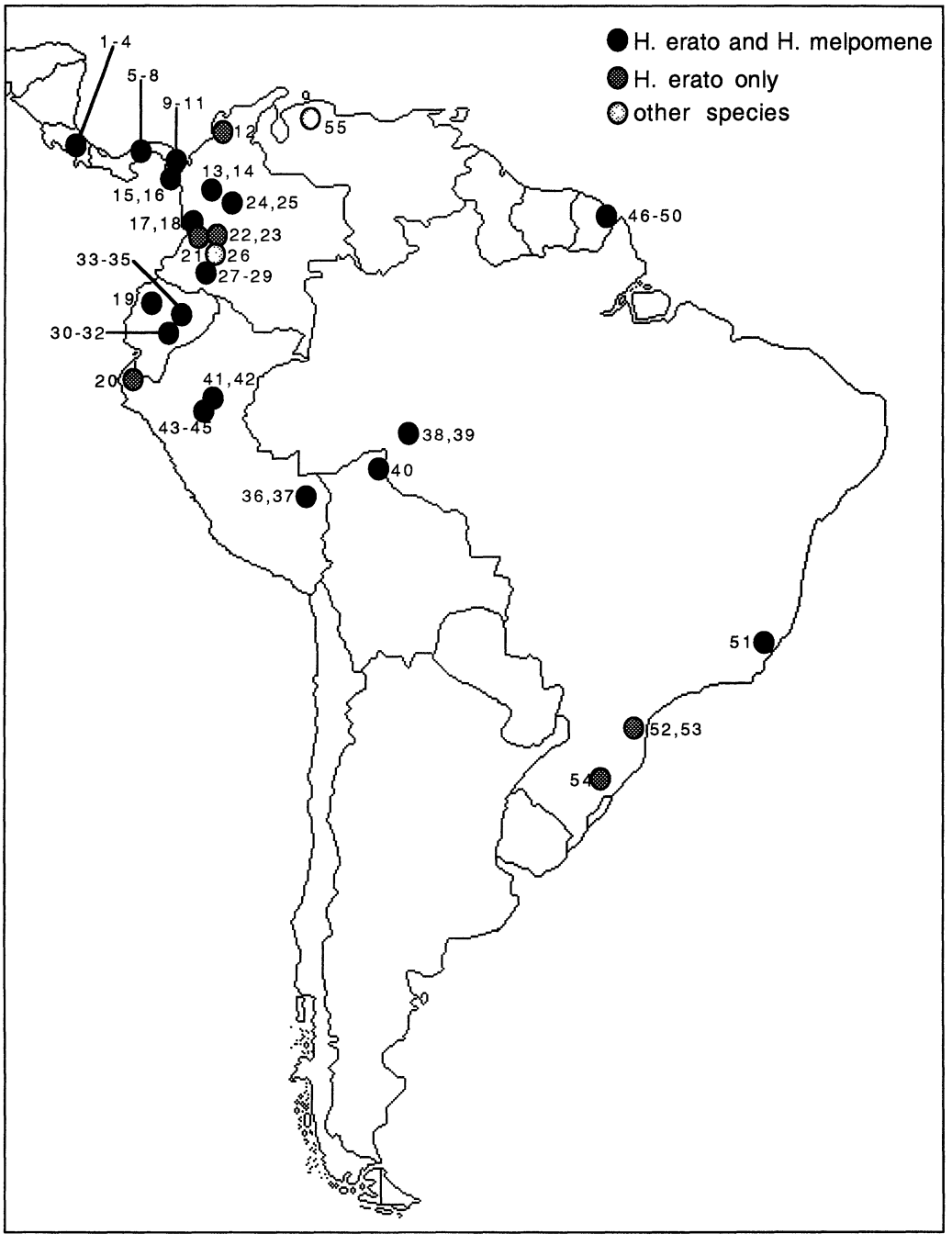


FIG. 5. Locations of sample sites for *Heliconius melpomene* and *Heliconius erato*. Black circles indicate sites where both species were taken; gray circles show sites for *H. erato* only, because no corresponding *H. melpomene* race exists or because no specimens were available. Lightly stippled circles are localities for other species examined in this study. Numbers adjacent to circles refer to specific collecting localities listed in Appendix Tables 1–3 and are also shown on the cladograms in Figures 6 and 8. Some circles represent more than one collecting locality within the range of a single pair of mimetic races.

phenol/chloroform method scaled-down to 1-ml aqueous volume, sometimes accompanied by Proteinase K and RNAse digestion, and omitting the DEPC step. Samples that retained contaminants that inhibited PCR amplification were subdivided, and 20% of the sample was reextracted as above, then further purified using Gene-Clean (Bio 101), which usually yielded amplifiable product. Specimens that repeatedly failed to amplify were replaced with a different individual, as redundant samples were available for most taxa. The wings, antennae, legs, and (when not ground up) abdomen of each specimen were preserved as dried voucher material, glued to a 1 × 3 cm card, and pinned in insect drawers, so wing patterns and other relevant characters could be easily examined. All voucher specimens and corresponding DNA sam-

ples were individually coded so they might be reassociated. The vouchers are deposited with the Cornell University Insect Collection (CUIC lot no. 1220). DNA samples are in the possession of the author.

### *PCR and Sequencing*

I studied a 942-bp region of mtDNA spanning the 3′ end of the cytochrome oxidase subunit I (COI) gene, the leucine tRNA gene, and almost all of the cytochrome oxidase subunit II (COII) gene (the region corresponds to bp 2800–3755 in the *Drosophila yakuba* sequence of Clary and Wolstenholme 1985). This fragment was chosen over the more variable A + T-rich region, because a preliminary study of that region revealed numerous insertion and deletion events among populations within a single race, which made sequence alignment difficult and phylogenetic analysis unreliable (A. Brower, unpubl. data).

The selected region was amplified from individual genomic DNA via PCR (Saiki et al. 1987; Kocher et al. 1989), using oligonucleotide primers designed from a comparison of *Heliconius* species with other insects (Clary and Wolstenholme 1985; Liu and Beckenbach 1992; for primer sequences and locations, see Brower 1994a). A Perkin-Elmer thermal cycler was used, with a cycling profile of (95°/1 min = 47°/1 min = 72°/2 min) × 35 cycles for double-stranded amplifications. Resulting double-stranded products were precipitated with 1/2 volume 7.5 M NaAC and 2 volumes EtOH, washed with 70% EtOH, and air dried. These fragments were reamplified in asymmetric reactions with one primer (Gyllensten and Erlich 1988) to produce single-stranded DNA, using approximately 15% of the double-stranded product as template [cycler profile (95°/1 min = 50°/1 min = 72°/2 min) × 30 cycles]. The single-stranded products were phenol/chloroform extracted, then precipitated, and washed as above. Clean, single-stranded DNA was sequenced using internal primers with $^{35}S$ (Biggin et al. 1983) and Sequenase version 2 (U.S. Biochemical) using the dideoxy-chain-termination procedure (Sanger et al. 1977) Sequences were visualized by autoradiography.

Sequences were entered and aligned with the GCG software package (Devereux et al. 1984). Because of the absence of insertion/deletion events and extremely low base-pair divergence, alignment by eye was straightforward. Although sequences were not systematically generated for both coding and anticoding strands of the entire fragment, no obvious discrepancies occurred in frequent regions of overlap between sense and antisense strand sequences. Regions containing compressions or hard-to-read areas were reamplified and sequenced from the complementary strand to mitigate ambiguity. For this type of study, it seems more appropriate to sequence another individual from the same race than to sequence both strands of the same individual. The integrity of the encoded protein message and the similarity of the sequences to one another between individuals offer ample confirmation that the sequences are read correctly (of course, "autapomorphies" resulting from error do not affect the phylogenetic analysis in any event).

### *Phylogenetic Analysis*

Phylogenetic analyses were performed with PAUP version 3.0s (Swofford 1990). Each unique haplotype was represented by a single individual to reduce computation time. This procedure does not change the topology of most parsimonious trees recovered in the searches. Because a large number of distinct sequences remained in each data set, the heuristic search option was used with TBR branch-swapping for 10 random stepwise addition replicates. No a priori assumptions were made with regard to rooting the trees: outgroup taxa were included simply as additional taxa in the analysis. Because the preponderance of informative characters fell at silent sites, equal weighting was used. Successive weighting (Farris 1969, 1988) was used to filter a subset from among the most-parsimonious trees found in the equally weighted searches, via the REWEIGHT option in PAUP (based on RC, best fit, base weight 1000).

## RESULTS

### *Heliconius erato*

The races of *H. erato* examined exhibit a high degree of overall similarity in their mtDNA sequences. Among the 49 specimens sequenced, there are 37 distinct haplotypes. The maximum uncorrected pairwise divergence between these is less than 4%. The data matrix contains 90 variable sites, of which 47 are phylogenetically informative within the species (Appendix Fig. 1). An additional 10 sites are shared by one of the *H. erato* specimens and one or more outgroup species, contributing homoplasy to the data set. Within *H. erato,* the COI segment is 12.1% variable (7.3% informative), whereas the COII gene is 9.2% variable (4.7% informative). The leucine tRNA gene has one informative site. Across the entire sequence, transitions are responsible for 79% of the informative sites. In protein-coding regions, 91% of informative sites fall at third codon positions. Only six sites display more than two alternative nucleotides; none display all four nucleotides.

In spite of the small amount of sequence variation evident, the changes that have occurred appear to be quite informative for phylogenetic analysis. The consistency index of the most-parsimonious trees is 0.53 excluding uninformative characters, a relatively high value for a data set containing so many taxa (Sanderson and Donoghue 1989). This suggests that, although intraspecific divergence is low, the observed differences occur in relatively unambiguous hierarchical patterns.

The strict consensus cladogram for the *H. erato* mtDNA data is shown in Figure 6. PAUP found 2094 shortest trees of 271 steps on the first of 10 heuristic search iterations with random addition. All nine subsequent searches terminated early when one of the same 271-step trees was encountered, again supporting the view that the resolved portions of the topology are unambiguously supported by the data (Maddison 1991).

All races of *H. erato* (including *H. himera*) form a monophyletic group with respect to the selected outgroups. The most basal node within *H. erato* represents a split between four entities: a group of five races (*cyrbia, guarica, hydara,*

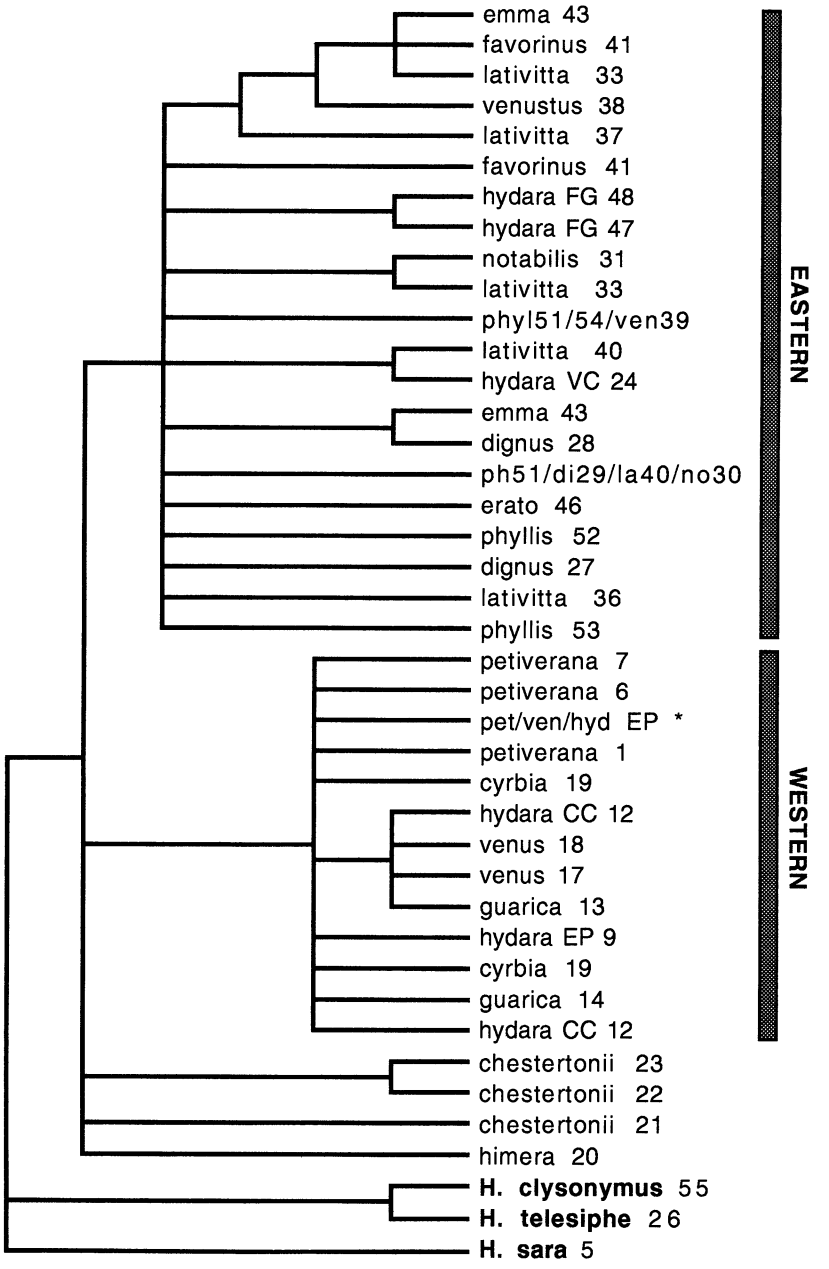


FIG. 6. Strict consensus of 2094 equally parsimonious minimum-length trees for the *H. erato* mtDNA sequence data. The trees are 271 steps long, with consistency indices of 0.532 (excluding uninformative sites). Individuals are numbered according to localities listed in Appendix Tables 1 and 3 and shown in Figure 5. The clade marked with the asterisk contains butterflies from localities 1, 5, 8, 10, 15, and 16. Note the broad correspondence of the topology with biogeography and the lack of monophyly of similar-looking but allopatric races.

*petiverana,* and *venus*) that occur west of the Andes, including the Cauca and Magdalena valleys in Colombia (the western clade); a group of nine races (*dignus, emma, erato, favorinus, hydara, lativitta, notabilis, phyllis,* and *venustus*) from Amazonia, the Guiana-Orinoco region, and southeastern Brazil (the eastern clade); *H. himera*; and the paraphyletic *Heliconius erato chestertonii. Heliconius himera* is a primarily allopatric semispecies (Descimon and de Maeght 1983) that occasionally hybridizes with *H. erato* in southwestern Ecuador, where it comes in contact with *Heliconius erato cyrbia. Heliconius erato chestertonii,* from the Cauca valley in Colombia (also west of the Andes) is basal and paraphyletic to other western *H. erato* races. The two large clades show some resolution but no further structure consistent with biogeography or racial boundaries. Some haplotypes are shared by multiple races: four allopatric races from Eastern Colombia, Eastern Ecuador, and southeastern Brazil share a common sequence, as do three races from Costa Rica and Panama.

Figure 7 is a phylogram of one of the 2094 most-parsimonious trees, selected arbitrarily from among 90 trees found

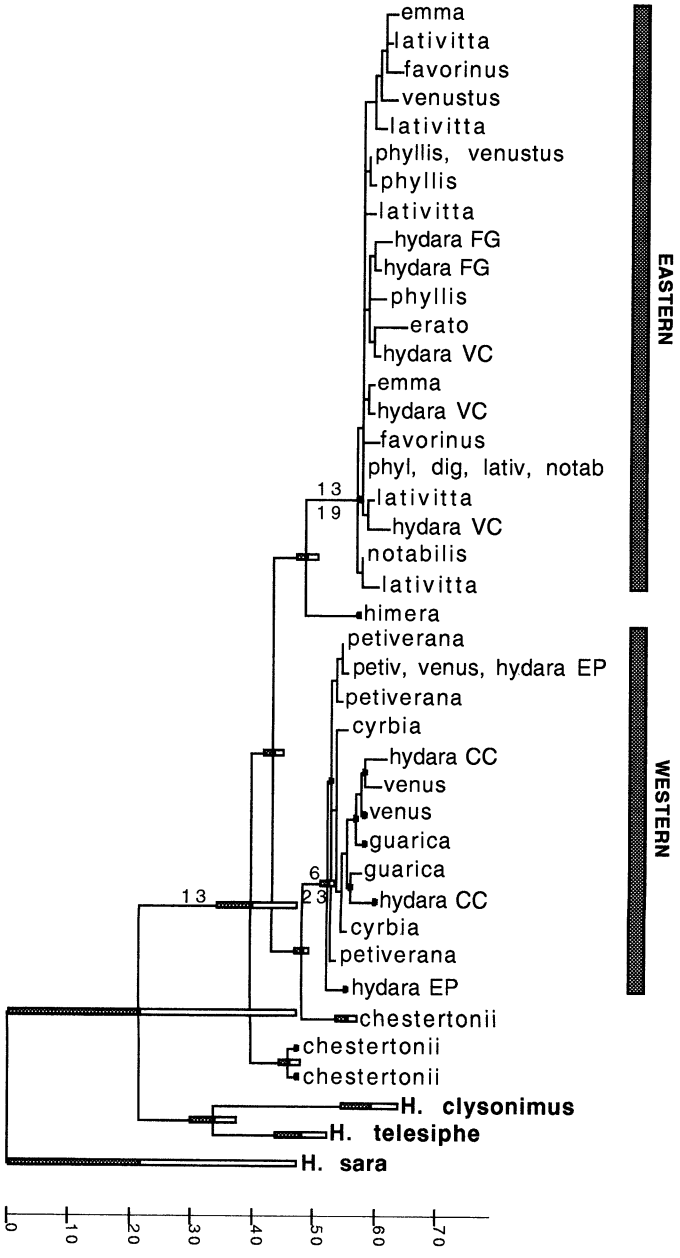


FIG. 7. One of the 2094 *Heliconius erato* trees, selected arbitrarily from among 90 trees, arrived at by successive weighting. The phylogram shows mean, minimum and maximum proportional branch lengths, and estimated Bremer support values at important nodes. Scale bar represents number of changes. See text for details.

by successive weighting. Mean branch lengths (with minimum-maximum bars) are shown, as determined by Maclade (in Maddison and Maddison 1992; polytomies randomly resolved; min-max-avg. number of changes averaged over all reconstructions for the selected tree). All the branches longer than a single step are resolved in the consensus of the southwestern trees. Numbers above branches are estimated Bremer support values (Bremer 1988; a.k.a. decay indices, Donoghue et al. 1992), indicating the number of steps longer the tree becomes when the clade above the branch becomes paraphyletic with respect to its sister clade (or polyphyletic, indicated by numbers beneath the branches). These values were determind by hand-swapping in Maclade, and lower values might be found by more intensive exploration of the tree space.

Most terminal branch lengths within the two main clades are extremely short: the 26 specimens in the eastern clade exhibit a maximum pairwise sequence divergence of 1.5% (mean = 0.6%; SD = 0.3%), whereas the western clade shows a maximum sequence divergence of 1.2% (mean = 0.5%; SD = 0.3%) among 20 individuals. *Heliconius e. chestertonii,* represented by three individuals in this study, exhibits greater intraracial sequence divergence (2.5%) between individuals from the east and west sides of the Cauca valley, Colombia (approximately 60 km apart) than there is interracial divergence within either of the large clades containing all the other races. The mean pairwise sequence divergences between the more divergent *chestertonii* individuals from the east side of the valley, and members of either large clade, are nearly equal (2.5%, east; 2.3%, west). In the tree shown, and the consensus of all the successive-weighted trees, this clade is sister to all other *H. erato races.* The western *H. e. chestertonii* individual is closer to the western clade and falls at its base in the successively weighted trees. *Heliconius himera* is most similar to the eastern clade (2.4%) and equally divergent from the western clade and *H. e. chestertonii* (2.9%). It is sister taxon to the eastern clade in all the successively weighted trees.

One race, *Heliconius erato hydara,* appears in both the eastern and western clades. *Heliconius e. hydara* has an apparently continuous distribution from Panama along the Caribbean coast to beyond the mouths of the Amazon (Brown 1979; Sheppard et al. 1985; Fig. 2). Individuals examined in this study from eastern Panama (EP) and Cartagena, Colombia (CC) fall in the western clade, whereas individuals from Villavicencio, Colombia (VC) and Guiana (FG) fall in the eastern clade, displaying the same major biogeographical disjunction (corresponding to the Andes) observed among the other races. Given that numerous allopatric races with similar phenotypes but different names (e.g., *dignus, petiverana,* and *favorinus*) are not sister taxa, it is likely that the *hydara* phenotype represents two or more distinct entities bearing the same wing pattern. Museum collections from along the northern coast of Colombia and Venezuela display a notable gap in localities corresponding to the Sierra de Perija, which may represent the vicariant barrier separating the populations (Brown 1979; Sheppard et al. 1985).

### *Heliconius melpomene*

As in *H. erato,* the sequences in the *H. melpomene* data set are very similar to one another. None of the 27 haplotypes exhibits more than 5% pairwise divergence from any other haplotype, including those from the *H. cydno* complex. Strict interpretation of intraspecific variation in *H. melpomene* is complicated by the apparent paraphyly of the species with respect to *H. cydno* and its close relatives, so summary statistics presented here include specimens of both species. The data contain 59 phylogenetically informative characters, among 83 variable positions (Appendix Fig. 2). The COI segment is 8.7% variable (6.8% informative), whereas COII

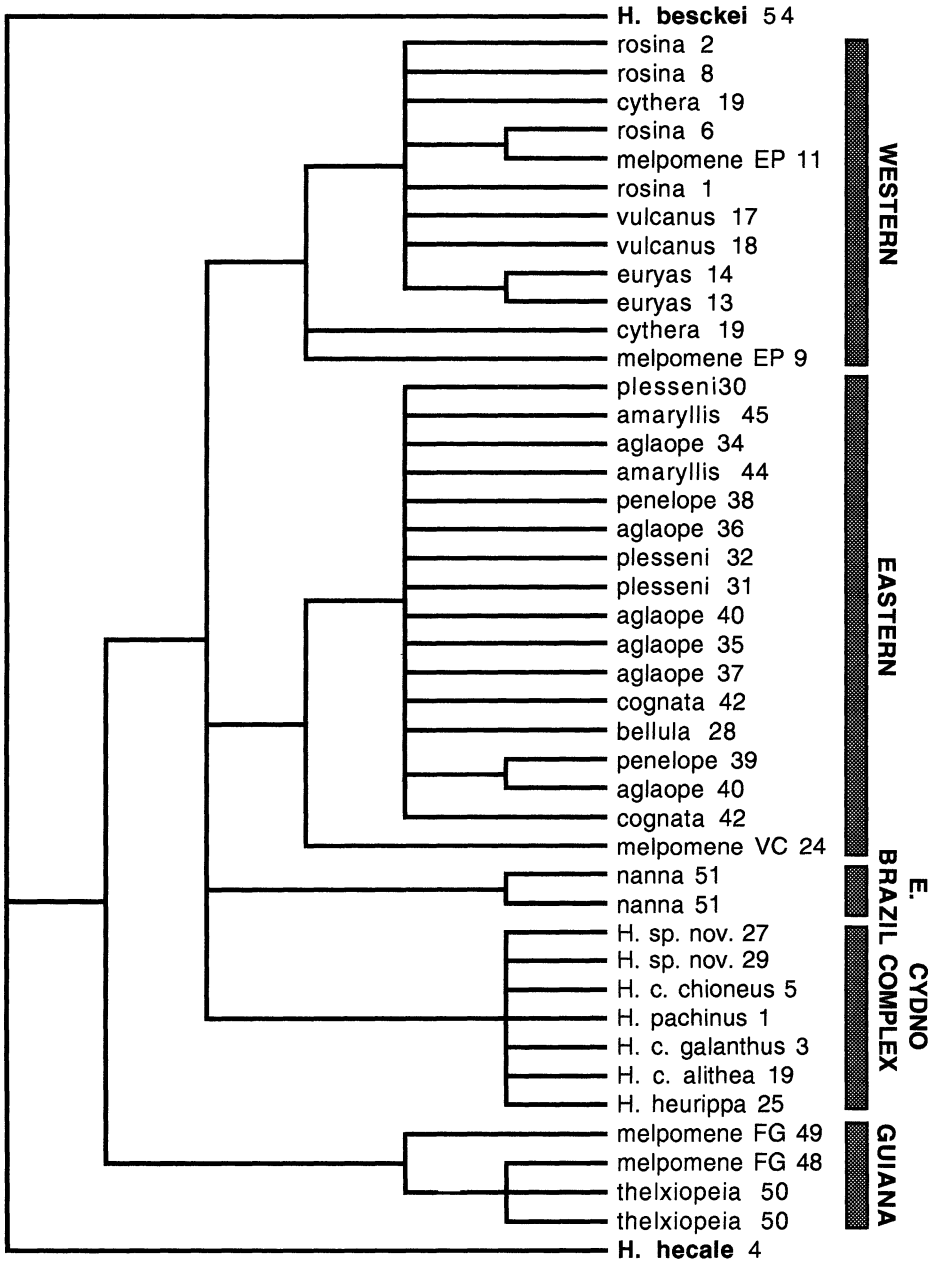


FIG. 8. Strict consensus of 2744 equally parsimonious minimum-length trees for the *Heliconius melpomene* mtDNA sequence data. The trees are 192 steps long, with consistency indices of 0.601 (excluding uninformative sites). Individuals are numbered according to localities listed in Appendix Tables 2–3. As in *Heliconius erato,* note the broad correspondence of the topology with biogeography and the lack of monophyly of similar but allopatric wing patterns.

is 9.8% variable (6.8% informative). The tRNA sequence is identical among all *melpomene* and *cydno* specimens examined. Transitions predominate again, comprising 68% of all variable sites. In the COI and COII coding regions, 79% of variable sites are at third positions. Seven sites display three alternative nucleotides, and one displays all four.

Although the *melpomene* data set contains fewer individuals (and fewer distinct haplotypes) than the *erato* data set, it shows greater phylogenetic resolution between groups of races. The C.I. of the most-parsimonious trees is 0.601, excluding uninformative characters, again falling above the Sanderson and Donoghue (1989) regression line.

The strict consensus of 2744 most-parsimonious trees for *H. melpomene* races is shown in Figure 8. All the trees were found on the first of 10 heuristic search iterations in each of two separate runs. The trees are 192 steps long. Although *H. cydno* and relatives were included as outgroups in the analysis, they emerge clearly nested among the various races of *H. melpomene.* Biogeographical and evolutionary implications of this unexpected result are discussed below. The two

additional outgroups, *H. besckei* and *H. hecale,* are representatives of the sister clade the *melpomene-cydno* clade, as determined by morphology (Brown 1981) and mtDNA (Brower 1994a), and here provide a plausible root for the *melpomene* and *cydno* topology. As in *H. erato,* the *melpomene-cydno* trees exhibit unambiguous geographical structure. The most basal *H. melpomene* clade contains all the individuals sampled from the two Guianan races (*melpomene* and *thelxiopeia*). The next node is a polytomy leading to four clades with relatively little internal resolution. These are a western clade containing five races (*cythera, euryas, melpomene, rosina,* and *vulcanus*) from Central America, northwestern Colombia, and the Pacific slope of the Andes; an upper Amazonian clade, including a specimen from Villavicencio, Colombia, in the Orinoco drainage, which contains seven races (*bellula, aglaope, amaryllis, cognata, melpomene, penelope,* and *plesseni*); two specimens from a single population in eastern Brazil (*nanna*); and the *H. cydno* clade. There is little further resolution within these clades.

Figure 9 is a phylogram representing one of 15 trees filtered by successive weighting from among the 2744 most-parsimonious trees found in the equal-weighted searches. Branch lengths and Bremer support values were determined as describe above for the *H. erato* phylogram (Fig. 7). Most of the internal branches in the large western and amazonian clades are only one or two steps long. Only the clade with *H. cydno* and its relatives exhibits multistep internal dichotomous branches, and even these are homoplasious and collapse to an unresolved *H. cydno* bush in the strict consensus of all equally parsimonious trees. The maximum sequence divergence within the western *H. melpomene* clade is 0.5%, (mean = 0.2%; SD = 0.1%) and within the upper Amazonian *H. melpomene* clade, 0.6% (mean = 0.4%; SD = 0.2%). Both values are substantially lower than the comparable values from the *H. erato* data set.

The named race *Heliconius melpomene melpomene* appears in three of the geographical clades identified here. As in its mimic *H. e. hydara,* these different mtDNA haplotypes probably represent phenotypically similar but genetically distinct geographical races, separated from one another by the Sierra de Perija, and perhaps the Colombian llanos. Another novelty is the apparent existence of an unrecognized *H. cydno* complex member which mimics *Heliconius melpomene bellula* and *Heliconius erato dignus,* from the upper Rio Putumayo in southeastern Colombia. One individual of *H. m. bellula* appears in the expected clade, with the other races that inhabit the headwaters of the Amazon at the feet of the Andes. Two other specimens (labeled "*H.* sp. nov." in Figs. 7–8) are nearly identical in wing pattern to *H. m. bellula* yet are sister taxa to *Heliconius heurippa,* a splinter species from the *H. cydno* complex, according to the mtDNA data. This relationship is borne out by detailed morphological analysis (Brower in press), and these butterflies are considered represent a new *H. cydno* splinter species and the first example of three-way mimetic convergence between the *H. erato, H. melpomene,* and *H. cydno* clades.

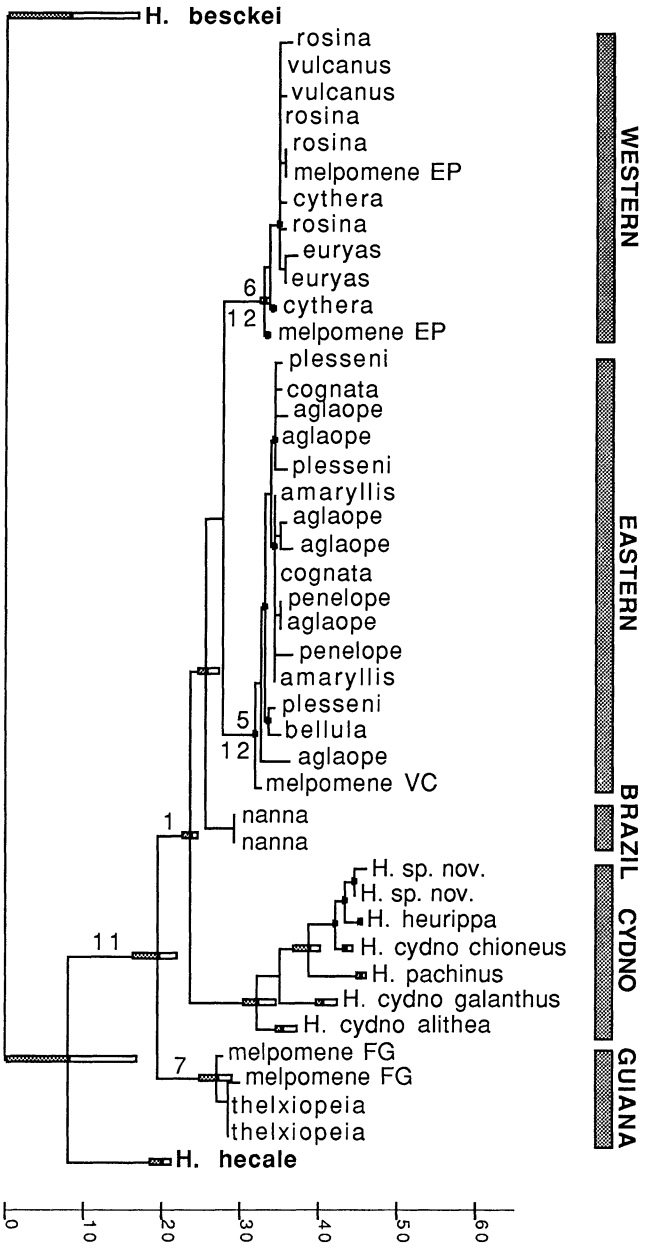


FIG. 9. One of the 2744 *Heliconius melpomene* trees selected from among 15 trees filtered by successive weighting. The phylogram shows mean, minimum and maximum proportional branch lengths and estimated Bremer support values at important nodes. Scale bar represents number of changes. See text for details. The eastern Brazilian clade can alternately be placed as sister to the *Heliconius cydno* group or as sister to the *H. cydno* + western + Amazonian clades, with only a single additional step.

## DISCUSSION

### *Phylogeny, Tokogeny, and Gene Genealogies*

Because the cladistic method relies on a strict branching topology, with no anastomosis of diverged taxa, phylogenetic analysis below the species level is inappropriate (Nixon and Wheeler 1990; but see Cracraft 1989). Thus, hybridizing phenotypic races experiencing gene flow and potential introgression of some alleles across racial boundaries could display anastomosing genealogical relationships and be intractable to cladistic analysis. The races of *H. erato* and *H. melpomene* examined in this study exhibit phenotypic monomorphism or, at most, minor polymorphic variation through-

out most of their ranges (Sheppard et al. 1985) and only exhibit extensive hybridization and mingling of phenotypic characters in relatively narrow hybrid zones where alternate races abut. Cracraft and Prum (1988) have successfully described a strongly analogous case in geographically differentiated Neotropical birds (which they consider to be phylogenetic species), using standard cladistic techniques with morphological data. Furthermore, because a clonally inherited marker is employed in this study, concerns about potentially tokogenetic relationships resulting from recombination are abrogated (Davis and Nixon 1992). Ambiguity in mtDNA cladograms can be problematical, but it cannot arise from failure to exhibit a branching pattern of descent, because all genealogies of mtDNA sequences exhibit phylogenetic evolution.

Of greater difficulty is the possibility that the gene tree produced from mtDNA does not correspond to the "true" tree of interracial relationships (i.e., the distribution of mitochondrial haplotypes may not reflect the actual history of the differentiated populations; Tajima 1983; Neigel and Avise 1986; Doyle 1992). In this study, the mtDNA phylogenies can be compared with geographical and phenotypic data, to examine concordance or lack thereof among the various character systems. First, there is no correspondence between the mtDNA cladograms and the hypothesized wing pattern parsimony trees (Fig. 4). There is a clear match of mtDNA relationships with large scale geographical patterns but no obvious match within those regions. The lack of geographical structure in mtDNA haplotype distributions within major regions, and broad distributions of some individual haplotypes across those regions, could be explained by convergent evolution of the mtDNA in different localities resulting from parallel selection or random drift. However, given the length of the sequences examined and the synonymous (and thus, apparently, neutral) nature of many of the changes occurring in them, neither selection on mtDNA nor coincident convergence by chance alone provides a compelling explanation for the observed distributions.

Two alternative historical phenomena may better explain the lack of resolution in mtDNA within major biogeographical regions. Introgression of mitochondrial haplotypes across phenotypic race boundaries could result in a lack of concordance between mtDNA phylogenies and phylogenies inferred from other character systems. Several studies (Ferris et al. 1983; DeSalle and Giddings 1986; Tegelström 1987) have demonstrated the penetration of mtDNA across recognized contact zones between taxa, resulting from hybridization and subsequent gene flow out of the contact zone (Barton and Jones 1983). The occurrence of introgression implies the prior existence of a genetic disjunction between adjacent populations: it is a product of secondary contact, observable only in contrast to a background of nonintrogressed characters. Given the apparent recency of divergence of mtDNA lineages (as inferred from low levels of sequence divergence), patterns of mtDNA distribution should exhibit at least some geographical structure (even if the patterns do not correspond to phenotypic hybrid zones) if introgression after secondary contact has taken place. The distribution of haplotypes within groups of races in both the *Heliconius* data sets shows no such ordering over vast geographical areas. Based on theoretical considerations (Barton 1983), Mallet (1993) argued that introgression of neutral alleles (or haplotypes) is resisted at coincident clines between adjacent races with alternatively fixed at multiple, coadapted loci (as observed in *Heliconius* wing patterns), even when the neutral and selected loci are unlinked. Although it is theoretically possible, given the rates of gene flow for these two species calculated by Mallet et al. (1990), to explain the observed mtDNA polymorphism by introgression, it seems improbable that neutral gene flow across multiple phenotypic hybrid zones could produce the observed lack of geographical structure across the entire Amazon basin.

A more parsimonious explanation of the lack of resolution is the retention of ancestral polymorphism in mtDNA haplotypes within major geographical clades. Absence of geographical structure in mtDNA might be expected if the different races had evolved so recently that shared neutral variants captured in alternative differentiated populations had not yet drifted to fixation. However, this explanation demands that differentiated wing patterns evolved concordantly between the two species at a more rapid rate than mtDNA and against the strong selective pressure to maintain a single, globally common pattern. Turner (1983) and Sheppard et al. (1985) argued that such divergence is extremely unlikely unless gene flow among populations was interrupted, allowing selection and/or drift to proceed independently within alternate local areas (Slatkin 1987). They invoked the controversial Pleistocene refugium hypothesis (Haffer 1969) as the mechanism promoting the inferred simultaneous local differentiation in their models of intraspecific wing-pattern diversification.

### *The Pleistocene Refugium Debate*

The distributions of many groups of neotropical organisms, including birds (Haffer 1974), lizards (Vanzolini and Williams 1970), plants (Prance 1973), and butterflies (Brown 1987), exhibit coincident boundaries between races, subspecies, and sister species (Simpson and Haffer 1978), yielding a generally congruent pattern of areas of endemism in the humid tropics of Central and South America. The theory of vicariance biogeography (Platnick and Nelson 1978) argues that shared distribution patterns among diverse clades of organisms are more parsimoniously explained by the occurrence of extrinsic geological or climatic events, which affected all the groups in the same way, than by intrinsic aspects of each group's history, which coincidentally produced parallel geographical patterns among all groups. These coincident distributions, or generalized tracks (Rosen 1975), may be used as evidence to infer the biogeographical history of a region. The similar distributions of multiple neotropical clades suggest that these taxa were subdivided by vicariant event(s) and, thus, that environmental changes affecting all of them equivalently have been responsible for current patterns of distribution.

Based on his compilation of distributional data for birds, Haffer (1969) inferred the following vicariant scenario for the Amazonian region. Periods of cooling and drying during the glaciation cycles of the Pleistocene caused the ancestrally homogeneous moist forest environment to contract repeatedly

into separate, isolated pockets, surrounded by xeric savannah. These moist areas acted as ecological refugia for organisms dependent on rain-forest conditions: small populations retreated into these buffered refuges, where they survived during environmentally inclement periods. Their numbers reduced by habitat loss, such populations were more susceptible to extinction or evolution by genetic drift and had an increased likelihood of divergence from conspecific populations isolated in other refugia. As the climate warmed during interglacial periods, the refugia expanded and melded together again, establishing secondary contact between the now differentiated populations. In its most ambitious form, the Pleistocene refugium hypothesis explains essentially all divergence among geographically separated populations of closely related taxa and, thus, is a major mechanism explaining the evolution of current tropical diversity (Haffer 1982).

Brown et al. (1974) argued that the paradox of rampant race formation in *H. erato* and *H. melpomene,* which has occurred in spite of stabilizing selection against novel phenotypes, offers evidence to support Haffer's (1969) Pleistocene refugium hypothesis. Numerous additional *Heliconius,* ithomiine, and troidine butterfly species share in the concordant distribution of parallel differentiated races, although they participate in multiple independent mimetic complexes (Brown 1981). Brown (1979, 1987) has identified and mapped 44 hypothetical neotropical paleoclimatic refugia on the basis of butterfly distributional data.

But the Pleistocene refugium hypothesis is not without critics. Endler ( 1977, 1982a,b) criticized the assumption of allopatric evolution in refugia because, he argued, the assembled data support only one of three criteria necessary to accept the strictly vicariant explanation for coincident distributions of neotropical organisms. Not only should more species occur in old refugia than surrounding regions, but contact zones between differentiated races or subspecies should occur midway between former refugia, because dispersal rates of conspecifics should be more or less equal. Furthermore, Endler stated that a correlation between dispersal ability and the width of contact zones should exist for differentiated populations expanding from adjacent refugia. The last two predictions are not borne out by the data from birds and butterflies that have been used to support the Pleistocene refugium hypothesis. Instead, Endler maintained that current ecological factors, such as transition zones between environmentally homogeneous areas, provide borders between adjacent races. He claimed that parapatric differentiation between these zones is a better explanation for the marked diversity of races observed in neotropical organisms.

Benson (1982) argued that adaptation to environmental conditions plays a more important role than historical factors in the current distribution of races in *H. erato* and *H. melpomene.* He sampled the two species extensively in several areas and observed contact zones located at environmental transitions. He suggested that selection for coloration contrasting strongly with the background, as described by Papageorgis (1975), maintains racial differences in different vegetational types. As pointed out by Mallet (1993), however, his hypothesis does not explain why habitat-selected phenotypes are not universally associated with a particular habitat type, nor why very different phenotypes occupy very similar habitats in different regions.

Turner (1982, 1983) staked out the middle ground in the selection-vicariance debate by pointing out that factors promoting phenotypic divergence among *Heliconius* races include not only genetic drift resulting from reduced population size (Pleistocene refugium-type divergence) and differential adaptation to local environmental conditions (Endlerian divergence) but also the shifting composition of the local Müllerian mimetic fauna. Müllerian mimics tend to converge on the "best protected" color pattern (degree of protection is a function of abundance and unpalatability; Marshall 1908; Fisher 1930); and because all *Heliconius* tested are apparently equally noxious (Brower 1984; Chai 1986), relative abundance must largely determine the trajectory of wing-pattern evolution in a given region. The differential faunal composition of forest patches produced by stochastic local extinction and population size fluctuation may result in rapid evolution to new stable mimetic pattern equilibria, whether forest patches are completely isolated from each other or not (Turner 1983).

Mallet (1993) argued that parapatric divergence resulting from differential selection and shifting balance processes (Wright 1977) is theoretically probable and thus at least as likely a cause as genetic drift and differential local adaptation in allopatry. He invoked models that make the traditional paradigm of allopatric divergence unnecessary and argued that the evidence for allopatry is weak and subject to alternative interpretations. He also contended that Turner's faunal drift model (1983) is unrealistic, based on the apparent absence of model species in many of the putative refugia. Although Mallet's lines of reasoning are generally convincing, he has not adequately addressed the fundamental observation of coincident distributions among geographically differentiated populations of numerous unrelated taxa. He shrugged off these data as sampling artifacts or explained them as a byproduct of coincident adaptations to a sharp ecotone. Given that many of the changes are in superficial characters such as color patterns, however, it is difficult to see how environmental gradients per se promote the spatially concordant divergence of mimicry complexes in butterflies and beak coloration in toucans.

Cracraft and Prum (1988) have convincingly argued for the greater parsimony of vicariant diversification models over Endler's (and Mallet's) omnipotent selection models to explain the evolution of neotropical bird clades. They found the distributions of toucan and parrot species to be compatible with extant barriers to dispersal, such as the Andes and major Amazonian river systems. Although often cited as opposing the Pleistocene refugium hypothesis (e.g., Capparella 1990), Cracraft and Prum see Quaternary cyclical climatic change as a viable, if poorly corroborated, explanation for at least some recent vicariance events. Data substantiating the causal climatic events leading to the isolation of refugia are both sparse and controversial (Colinvaux 1989; Flenley 1993): we need to obtain more direct evidence of Amazonian Pleistocene ecological change or stasis before firm conclusions may be drawn. Nevertheless, the reduction of gene flow by Pleistocene vicariance provides a compelling explanation for the biogeographical concordance of diversified races in numer-

ous unrelated neotropical taxa in areas not currently separated by obvious barriers to dispersal.

The widespread distributions of several mtDNA haplotypes in both *H. erato* and *H. melpomene* imply that severe population bottlenecks probably did not occur commonly in isolated populations of either species during glacial advances. However, because only long-term, drastic population size reduction is expected to result in the monophyly of all alleles within a population (Neigel and Avise 1986), the lack of geographical structure within the major clades casts doubt only upon the most extreme version of the Pleistocene refugium hypothesis (Haffer 1982). Nevertheless, the surprisingly similar branch lengths of haplotypes within unresolved clades, and the similarity of amounts of divergence both between clades within species and between comparable clades in *H. erato* and *H. melpomene,* suggest that extrinsic forces experienced in common, simultaneously, and recently, promoted the radiations of phenotypes in both species. Pleistocene climatic changes that disrupted community structure, as in Turner's (1983) model, could have released the two species from mimetic constraints and promoted concordant and rapid racial divergence, even in the absence of complete geographical isolation. Adjacent phenotypically differentiated races may result from very recent selective events and simply be too young to exhibit phylogenetic structure in nonselected characters.

### *Vicariance Biogeography, Divergence Dates, and Coevolution*

The main patterns of biogeographical affinity revealed by this study are not very surprising: Central America, the upper Amazonian region, and the Guiana shield have been recognized as possessing differential faunal compositions for over a century (Bates 1863). Although the biogeographical distributions of the various clades examined agree with traditional patterns of endemism, however, there is little correspondence in the historical area relationships between the two species (Fig. 10). Although the character incongruence between the area cladograms is not overwhelming (the mtDNA-based *H. melpomene* area tree can be rearranged to reflect the *H. erato* topology with only four extra steps; the converse takes 13), the consensus of these two area cladograms does not contain a single resolved node. As demonstrated by Cracraft (1988) among clades of Amazonian birds, the biogeography of the neotropical biota (as manifested in this study by phylogenetic hypotheses for two congeneric and mimetic butterfly species that one would expect to display similar patterns) is not satisfactorily explained by a single, simple vicariant history.

In *H. erato,* the fundamental biogeographical divisions occur between groups of races from opposite sides of the Andes and two other isolated western races (Fig. 6). Races from Guiana, southeastern Brazil, and the eastern foothills of the Andes form a single, undifferentiated clade. The distribution of haplotypes within the western clade is also relatively homogeneous. The comparatively high level of sequence divergence between the *H. e. chestertonii* haplotypes from opposite sides of the Cauca valley implies that this race is older than other *H. erato* races. Although western *H. e. chestertonii* and *Heliconius erato venus* come into contact in some low areas of the Sierra Occidental, Colombia, hybridization is rare (A. Brower, pers. obs.; Torres Nuñez and Takahashi 1983). Captured hybrid specimens have been all male, suggesting that heterogametic inviability (of females) may present at least a partial barrier to gene flow between these races. Like *H. e. chestertonii,* the sister taxon to eastern *H. erato* races, *H. himera,* occurs in a restricted area west of the Andes and is likewise partially genetically isolated from the adjacent *H. e. cyrbia* (Descimon and de Maeght 1983; J. Mallet, pers. comm., 1992). *Heliconius himera* also occurs in the upper Rio Marañon valley, east of the Andes (Descimon and Mast de Maeght 1983; Mallet 1993) and, given its position on the cladogram, may represent the source of the Amazonian radiation. If eastern and western populations of *H. himera* are contiguous and genetically undifferentiated, then the semispecies represents the only trans-Andean member of the *H. erato* clade.

Neither *H. himera* nor *H. e. chestertonii* shares its wing pattern with a parallel race of *H. melpomene* [*H. e. chestertonii* mimics the sympatric *Heliconius cydno gustavi* Staudinger; *H. himera* is not mimetic west of the Andes but may have a poor *H. melpomene* mimic in the Rio Marañon population (Sheppard et al. 1985)]. It is thus interesting to speculate that the mimetic association between *H. erato* and *H. melpomene* evolved after these two taxa had already diverged from the main *H. erato* lineage. Perhaps *H. e. chestertonii* was drawn into a mimetic association with *H. cydno* prior to the second invasion of the Pacific slope by the currently resident *H. melpomene* lineage.

The most basal clade in *H. melpomene* occurs in the Guiana shield. The divergence of the *H. cydno* complex from the *H. melpomene* stem may have resulted from an early vicariant event separating western and eastern clades. Although two large groups *H. melpomene* races are clearly subdivided by the Andes, subsequent reinvasion of Central America and the Pacific slope by *H. melpomene* must be invoked to explain the sympatric distributions of these races with the various *H. cydno* races and related forms that also occur there (Fig. 11). The greater sequence divergence among haplotypes sampled from the *H. cydno* clade implies their historical precedence over current resident *H. melpomene* in establishing differentiated populations west of the Andes.

I have compiled data to construct an evolutionary rate estimate for recently diverged (within the last 3 million yr) arthropod mtDNA sequences (Brower 1994b). Although the plot is based on a small number of data points, each of which is prone to many types of error, the data nevertheless exhibit a remarkably high coefficient of correlation ($R^2 = 0.986$), suggesting the existence of a clocklike rate of sequence evolution, at least for recently diverged taxa. Furthermore, this rate (approximately 1.1% per lineage per million yr) is very close to the 1% per-million-yr rate of initial mtDNA sequence evolution estimated by Brown et al. (1979, 1982) from comparisons between closely related primates.

This arthropod plot provides a preliminary calibration for the divergence dates of the various large clades examined in this study. Because there is so little phylogenetic structure within these clades, and because the little structure that occurs does not correspond to biogeography or phenotypic patterns,

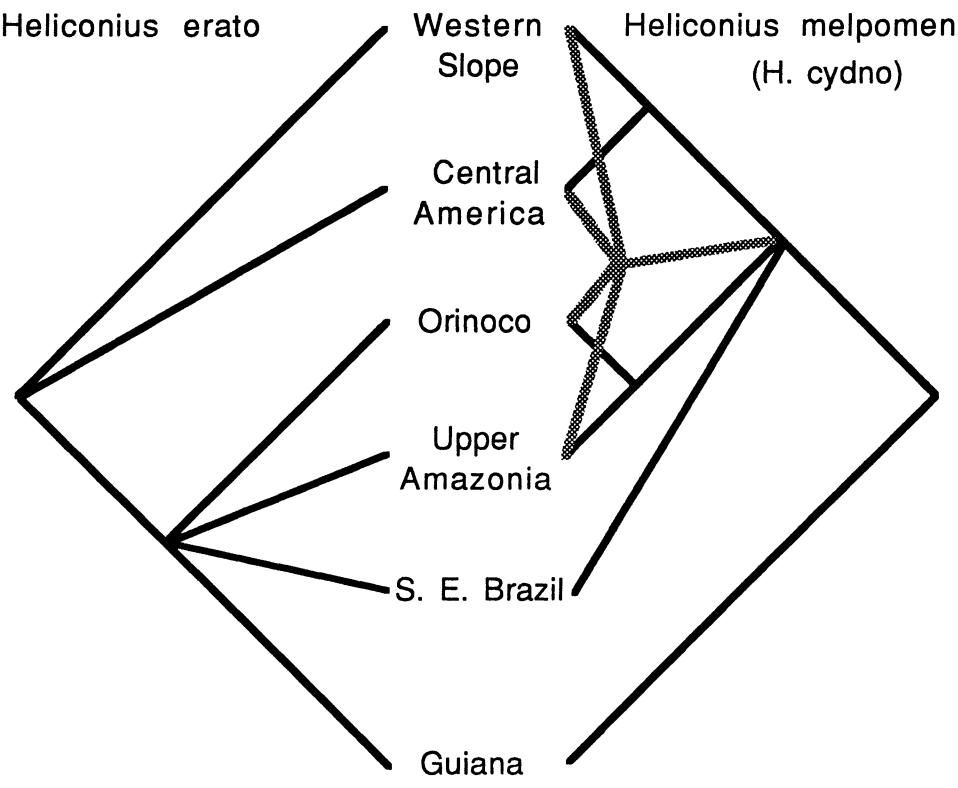


FIG. 10. Comparison of reduced area cladograms implied by distributions of *Heliconius erato* (left) and *Heliconius melpomene* (right) mtDNA strict consensus topologies. The gray line from the right side indicates the origination and distribution of *Heliconius cydno* and its relatives. Note that there are no area relationships shared in common between the two species.

divergence within a clade is estimated by the uncorrected mean pairwise sequence divergence of all individuals in that clade. By comparing this value to the plotted divergences of taxa with geologically inferred divergence dates, a rough date for the clade can be obtained. A more conservative estimate of dates might be based on maximum within-clade sequence divergence, but, because the overall differences among the sequences are so small, this would tend to overestimate time because the split by weighting in favor of haplotypes exhibiting greater divergence resulting from small stochastic variations in the substitution rate.

I estimated the age of divergence between the eastern and western *H. erato* clades to be approximately 1.5–2 mya, coinciding with or shortly following the beginning of the Pleistocene and with tectonic events that could have erected vicariant barriers in the northern Andes (van der Hammen 1974). The age of the respective radiations within the eastern and western *H. erato* clades (excluding *H. e. chestertonii*) was estimated to be approximately 150,000–200,000 yr (Brower 1994b). Based on the same plot, the eastern Andean *H. melpomene* radiation appears to be nearly the same age as, or slightly younger than, the eastern *H. erato* clade. The western *H. melpomene* radiation exhibits substantially less divergence and is thus estimated to be 65,000 yr old. If ancestral polymorphism were present in each clade at its genesis, these ages could be overestimated. In general, these date estimates are tentative and must be corroborated, both with additional geologically based divergence events to bolster the molecular-clock graph and with more refined estimates of genetic diversity within and between *Heliconius* races (Brower 1994b).

These divergence date estimates are at least in part consistent with Haffer's (Haffer 1969; Simpson and Haffer 1978) hypothesis that most geographically differentiated species are the result of Pleistocene climatic vicariant events, although probably not the most recent cycle of glaciation (21,000–13,000 ybp; van der Hammen 1974). Mitochondrial DNA divergences between allopatric populations of the South American mosquito *Anopheles nuneztovari* (J. Conn, pers. comm., 1994) are similar to those observed in *Heliconius,* suggesting that patterns (and rates) of genetic variation may be shared among diverse neotropical insect taxa, and supporting the vicariant model of divergence (Cracraft and Prum 1988). Molecular studies of neotopical birds (Capparella 1988; Hackett and Rosenberg 1990; Hackett 1993; Seutin et al. 1993) have shown that putatively closely related species or geographical races are often quite strongly differentiated at apparently neutrally evolving loci, suggesting ages older than the late Pleistocene. These authors date the divergences of allopatric populations variously, between 700,000 and 3 mya, contemporaneous with or before the onset of Pleistocene climatic cycles, dated at 800,000 years by Haffer (1974). The bird dates are thus generally older than the *Heliconius* dates, but it is difficult to decide whether the discrepancy is due to evolutionary differences, inappropriate comparison of nonhomlogous area relationships, or faulty estimation techniques. Birds have been thought to exhibit a slower rate of protein evolution (Avise et al. 1980; Gutiérrez et al. 1983)

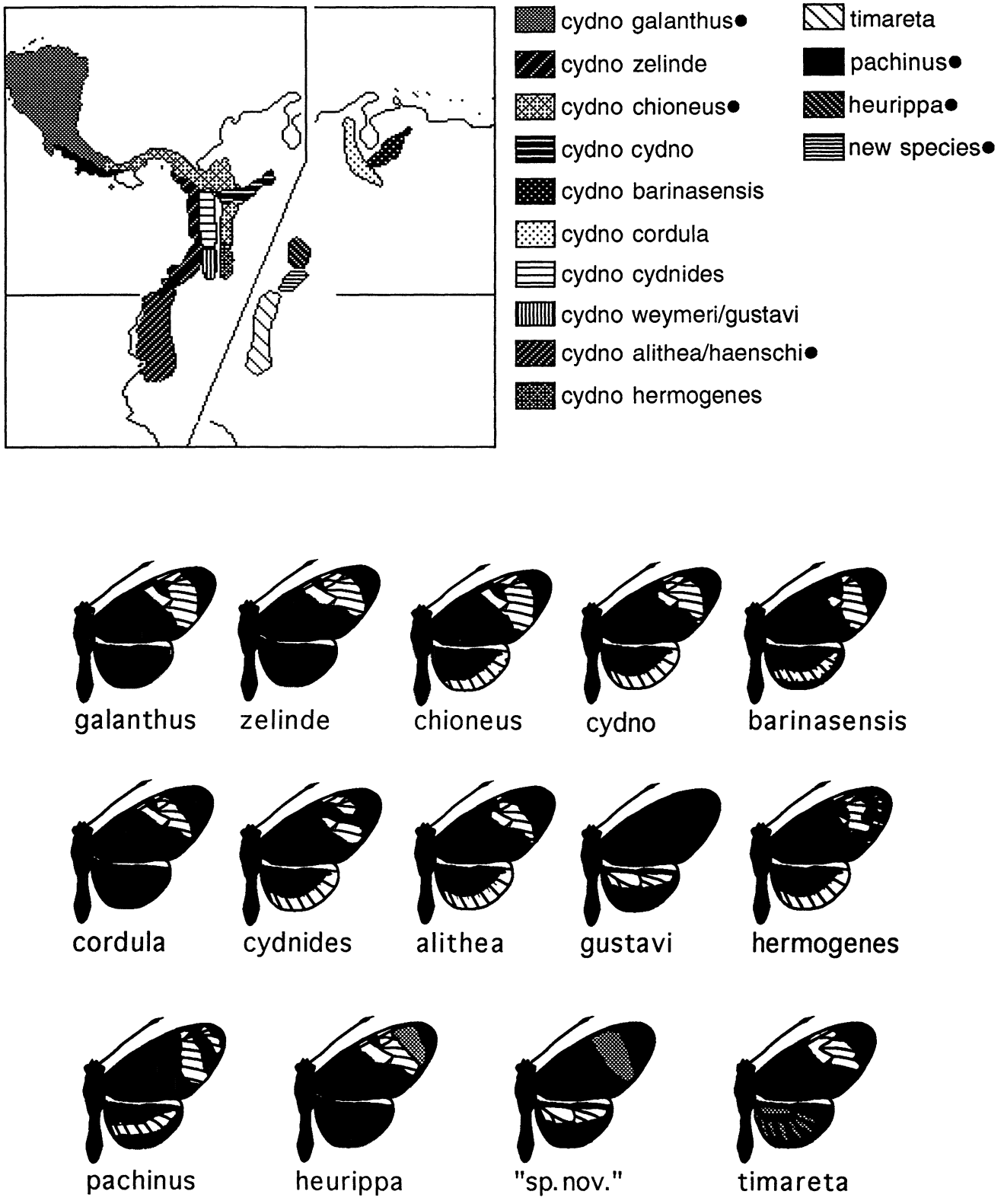


FIG. 11. Schematic distribution map and phenotypes of races of *Heliconius cydno*; closely related species *Heliconius heurippa, Heliconius pachinus,* and *Heliconius timareta*; and the "new species." Black dots represent taxa sampled in this study. As in the earlier maps, the races/species east and west of the Andes are separated for clarity. There is more extensive blending and local polymorphism among *H. cydno* races than is represented in this figure. Drawn from data and figures in Brown (1979).

and possibly a faster rate of mtDNA evolution (Bermingham et al. 1992) than other vertebrates. Additional data may bring dates and rates among taxa into greater accord and reveal a clearer picture of the biogeographical history of South America.

Although the haplotype distributions exhibit strong geographical stucture, the lack of topological concordance between the *H. erato* and *H. melpomene* mtDNA trees implies that, at least initially, the two species did not share a common biogeographical history. The basal *H. erato* clades diverge west of the Andes, whereas the first clade to split from the *H. melpomene* lineage occurs in Guiana. This lack of biogeographical congruence further suggests that mimicry between the species must have evolved several times independently, within separate biogeographical regions. The major radiations in the eastern and western clades took place recently, although probably not during the last glacial maximum. The evidence for or against the formation of refugia during these cool dry periods is scant, but the lack of concordance between mtDNA haplotype distributions and phenotypic racial boundaries suggests that severe population bottlenecks probably did not occur, as might be expected if populations were split up into small, isolated fragments of habitat for prolonged periods of time. There is no direct phylogenetic evidence supporting either parapatric or allopatric divergence of races, except between *H. e. chestertonii* and *H. e venus* and between *H. himera* and *H. e. cyrbia,* which are clearly in secondary contact, and may represent biologically discrete taxa. Nevertheless, strong natural selection acting on wing patterns has probably played an important role in the rapid diversification of phenotypes in these butterflies, because the unlinked mtDNA shows so little absolute divergence and no geographical structure corresponding to the boundaries between races. Additional phylogenetic data from nuclear loci may provide corroborative evidence for these conclusions for these two species. Parallel phylogenetic examinations of geographical variation in many other neotropical taxa will be essential to reveal general patterns of diversification in the neotropical biota, if they exist at all.

### *Evolution of Wing Pattern Diversity*

The distribution of wing patterns on the mtDNA trees of both species implies that the evolution of these patterns is not parsimonious, contrary to the models of Turner (1982, 1983) and Sheppard et al. (1985). Forcing similar phenotypes to be closest relatives makes the mitochondrial *H. erato* tree more than 100 steps longer and the *H. melpomene* tree more than 70 steps longer than the most-parsimonious trees found. Although resolution within major clades in each species is not high, it is nevertheless clear that at least some phenotypes (e.g., the *H. m. melpomene* pattern shared by three different clades) have arisen independently in each lineage, whereas others have either recurred or been maintained by selection while substantial mtDNA divergence was taking place. Given that races of *H. erato* and *H. melpomene* with red forewing bands (with or without the yellow stripe) share their pattern with no other unpalatable species abundant enough to act as Müllerian models, it is doubtful that the fluctuating dynamics of the local mimetic community have contributed to the maintenance of these patterns in multiple allopatric regions, unless the models have all gone extinct, which seems improbable (Mallet 1993). The forces driving initial pattern divergence among races thus remain obscure.

Sheppard et al. (1985) hypothesized that the ancestral patterns of both *H. erato* and *H. melpomene* were black, with yellow fore- and hindwing bands. According to reconstructions based on the mtDNA trees, the ancestral pattern of *H. erato* was either like that of *H. e. chestertonii* (black with a yellow hindwing band) or like that of *H. himera* (yellow forewing band, red hindwing band). The *H. e. chestertonii* pattern is mimicked by the sympatric *H. c. gustavi* in the upper Cauca valley (Fig. 11). These two races are the only *Heliconius* with an all-black forewing and have no additional models evident within their range. The *H. himera* pattern is nonmimetic and also occurs in two other closely related but allopatric *Heliconius* species, *H. clysonymus* and *Heliconius ricini.* The retention of this pattern in several species with no mimetic associations implies that it may be plesiomorphic and is thus a likely candidate for the *H. erato* ancestral pattern. However, bcause no other *H. erato* race bears the *H. himera* pattern, and because that pattern and the *H. e. chestertonii* pattern fall in opposite clades of Turner's wing-pattern allele parsimony cladogram, it is difficult to draw firm a hypothesis about the ancestral wing pattern.

In *H. melpomene,* reconstruction of pattern evolution is complicated by the apparent paraphyly of the species with respect to the *H. cydno* complex. *Heliconius cydno* is sympatric and reproductively isolated from *H. melpomene* over most of its range and is almost always a member of a different mimicry ring. *Heliconius cydno*'s mimics are in the *Heliconius sapho* and *Heliconius eleuchia* clade and share predominantly blue and white or blue and yellow patterns (Fig. 11). The participation in alternate mimicry complexes has been an important character separating *H. melpomene* and *H. cydno,* because they have been considered indistinguishable by genitalic characters (Eltringham 1916; but see figures in Emsley 1965). The presence of red coloration in *H. melpomene,* in particular, has been implicated as a courtship releaser in that species but not in *H. cydno* (Brown and Mielke 1972), suggesting the existence of a prezygotic barrier to gene exchange between the two species. The red forewing band of *H. heurippa* has thus been cited as evidence of its relationship to *H. melpomene* (Emsley 1965) or its hybrid origin from a *H. cydno* and *H. melpomene* cross (M. Linares, pers. comm.). With *H. heurippa* and the "sp. nov." I have recognized well nested within the *H. cydno* mtDNA clade, these hypotheses become untenable. The *melpomene*-red, *cydno*-no red distinction falls apart, unless a complex interspecific mtDNA introgression scenario is invoked. Although they may be crossed in the laboratory (Nijhout et al. 1990), wild-caught *H. melpomene-H. cydno* hybrids are unknown, except for a few specimens from northeastern Colombia (Ackery and Smiles 1976), which probably represent hybrids between *H. cydno* and *H. heurippa,* or the "sp. nov.," and not *H. melpomene* crosses at all. Furthermore, both of these taxa are sympatric with *H. melpomene* (the new form is a mimic of *H. m. bellula* and *H. e. dignus*), whereas neither of them is currently sympatric with a recognized race of *H. cydno.* Thus, recent hybrid origin of these red-bearing *H. cydno* relatives

is unlikely. The red pattern elements have probably evolved independently in the *H. cydno* complex or been retained in the clade since its divergence with *H. melpomene.*

Within *H. melpomene,* as in *H. erato,* similar wing patterns have evolved numerous times. The *H. m. melpomene* pattern (black with a red forewing patch) is basal in every clade where it occurs and may thus represent the plesiomorphic state. If this is so, then the yellow hindwing stripe must have evolved at least three times (and possibly four, if it is not a synapomorphy between *Heliconius melpomene amaryllis* and *H. m. bellula*). Because there is no single sister taxon to the *melpomene-cydno* clade (Brower 1994a), and because the color patterns exhibited in the sister clade are widely varying, no information about the ancestral color pattern can be made by outgroup comparison. Specimens from the basal Guiana clade exhibit both the red forewing patch pattern (*H. m. melpomene*) and the rayed pattern (*Heliconius melpomene thelxiopeia*), although the rayed specimens were collected at a site where *melpomene-thelxiopeia* hybrid individuals were present and may not contain *thelxiopeia* mtDNA.

In neither *H. melpomene* nor *H. erato* does there appear to be any clear evolutionary tendency toward the fixation of dominant wing pattern alleles. The observed phylogenetic distribution of wing patterns requires the multiple reappearance of some recessives (e.g., the hindwing yellow bar allele in *H. melpomene*), unless recessive alleles are preserved by natural selection in some areas and replaced in others. Some of these violations of "Haldane's Sieve" were noted in Sheppard et al. (1985) but ignored in their description of the model. Although that model provides a pleasing scenario, the dominance-driven evolution of wing patterns must be an oversimplification of the process by which the phenotypic diversity has arisen in these two species. Without additional data on the homology or nonhomology of alleles producing similar phenotypic effects in allopatric races, the selective maintenance versus multiple origins question remains open. If the developmental basis of pattern formation is relatively simple, as argued by Nijhout (1991), then perhaps patterns arise over and over again as a result of relatively minor changes in the timing of regulatory events. The propensity of *Heliconius* species to mimicry may be driven by the existence of a particularly finely tuned series of regulatory switches, operating on a relatively homogeneous developmental genetic template. Only finer genetic study of the underlying biochemical processes mediating wing-pattern development will enable the discrimination of truly homologous wing patterns from those derived in parallel resulting from natural selection.

To sum up the conclusions of the study in terms of the questions posed at the end of the introduction, the mitochondrial cladograms match biogeographical distributions better than the distribution of shared wing patterns in both species, thus failing to corroborate the wing-pattern parsimony hypotheses of Sheppard et al. (1985) and Mallet (1989). Because many races are poorly resolved by the mtDNA data, spatial relationships within major clades do not directly address the Pleistocene refugium hypothesis. However, similar, low levels of sequence divergence in both species are consistent with a Pleistocene origin of much of the observed phenotypic diversity. Branching order is not the same among *H. erato* races and *H. melpomene* races, especially when the paraphyly of *H. melpomene* with respect to *H. cydno* is considered, suggesting that strict coevolution (sensu Janzen 1980) between the two species has not always mediated their intraspecific diversification.

## Sequence Availabililty

Sequences representing each haplotype revealed in this study have been submitted to Genbank (Bilofsky and Burks 1988). Accession codes are U08472–U08507, U08516, U08518, U08523, U08524, U08526, and U08544 (*H. melpomene* and *H. cydno* races); U08560–U08594, U08530, and U08543 (*H. erato* races); U08510 (*H. besckei*); U08512 (*H. telesiphe*); U08520 (*H. hecale*); U08545 (*H. sara*); and U08558 (*H. clysonymus*).

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TABLE 1. Locality data of individual *Heliconius erato* specimens examined in this study. Locality numbers correspond to labels on Figures 5 and 6. Specimen codes correspond to individual voucher specimens. The list is alphabetical by race name. Starred specimens were reared from captive stock. *Heliconius erato* races not sampled include *microclea* Kaye (Rio Perené, Peru), *magnifica* Riffarth (Roraima region, Brazil), *amalfreda* Riffarth (Suriname), *estrella* Bates (Ilha do Marajó, Brazil), *amphitrite* Riffarth (Vilcanota, Peru), and *columbina* Staudinger (northern Rio Magdalena valley, Colombia).

| Race name | Locality number | Locality of origin | Code(s) |
|---|---|---|---|
| *chestertonii* Hewitson | 22 | La Moralia, Valle del Cauca, Colombia | C-10-1 |
| | 21 | old Cali-Buenavantura Road, km 26, Valle del Cauca, Colombia | C-21-2 |
| | 23 | Cauca valley, Colombia (locality unknown) | HCOL1* |
| *cyrbia* (Godart) | 19 | Tinalandia, Pichincha, Ecuador | E-4-7, E-9-2 |
| *dignus* Stichel | 27 | Pasto-Mocoa Road, km 130, Putumayo, Colombia | C-13-3 |
| | 28 | 1–3 km N. Mocoa, Putumayo, Colombia | C-14-7 |
| | 29 | 6 km N. Mocoa, Putumayo, Colombia | C-15-3 |
| *emma* Riffarth | 43 | Davidcillo, San Martín, Peru | JM1570, JM1571 |
| *erato* (Linnaeus) | 46 | Fourgassié, Guiana | G-3-2 |
| *favorinus* Hopffer | 41 | Rio Huallabamba near Juanjui, San Martín, Peru | JM1903, JM1904 |
| *guarica* Reakirt | 13 | 11 km E. la Peña, Cundinamarca, Colombia | C-7-7 |
| | 14 | Mariquita, Tolima, Colombia | C-9-1 |
| *hydara* (W) Hewitson† | 12 | Cartagena, Bolivar, Colombia | JB1, JB8 |
| | 9 | Cana, Darien, Panama | P-18-1 |
| | 10 | Puerto Obaldía, Comarca de San Blas, Panama | P-37-12, P-37-29 |
| *hydara* (E) Hewitson† | 24 | Villavicencio, Meta, Colombia | C-1-2 |
| | 47 | Pointe Macouria, Guiana | G-6-4 |
| | 48 | Route N-1, km 17, Guiana | G-22-1 |
| *lativitta* Butler | 33 | Archidona, Napo, Ecuador | E-3-1, E-3-2 |
| | 40 | Riberalta, Beni, Bolivia | RIB1, RIB35 |
| *luscombei* Lamas†† | 36 | Parque Nacional Manu, Madre de Dios, Peru | PA-10-1 |
| | 37 | Tambopata Preserve, Puerto Maldonado, Madre de Dios, Peru | TA-22-2 |
| *notabilis* Salvin & Godman | 30 | Rio Sucio, Puyo, Pastaza, Ecuador | E-5-1 |
| | 31 | Rio Puyo, Puyo, Pastaza, Ecuador | E-6-2 |
| *petiverana* Doubleday | 1 | Sirena, Parque Nacional Corcovado, Costa Rica | CR1, CR13 |
| | 5 | Pipeline Road, Parque Nacional Soberania, Panama | P-1-2, P-1-5 |
| | 6 | El Valle, Coclé, Panama | P-13-5 |
| | 7 | Punta San Blas, Comarca de San Blas, Panama | P-15-1 |
| | 8 | Loma del Naranjo, Lago Bayano, Panama | P-27-1 |
| *phyllis* (Fabricius) | 51 | Linhares, Espirito Santo, Brazil | B-1-31, B-1-32 |
| | 54 | Porto Alegre, Rio Grande do Sul, Brazil | B-2-1 |
| | 52 | Alta da Serra, Morretes, Curitiba, Brazil | B-3-1* |
| | 53 | Municipio de Quatro Barras, Paraná, Brazil | B-5-2 |
| *venus* Staudinger | 17 | old Cali-Buenavantura Road, km 32, Valle del Cauca, Colombia | C-22-5 |
| | 18 | old Cali-Buenavantura Road, km 39, Valle del Cauca, Colombia | C-23-1 |
| | 15 | Valle Alegre, Darien, Panama | P-35-9 |
| | 16 | 5 km E. Jaqué, Darien, Panama | P-36-11 |
| *venustus* Salvin | 38 | Cacaulandia, Rondonia, Brazil | RB005 |
| | 39 | Fazenda Rancho Grande, Rondonia, Brazil | RB111 |

† *Heliconius erato hydara* probably represents at least two distinct races. Specimens in the eastern and western clades are separated here (E = eastern clade; W = western clade).

†† *Heliconius erato luscombei* Lamas is very similar to *Heliconius erato lativitta* Butler, differing only slightly in the size of the forewing apical yellow patch. The two races are lumped as *lativitta* in this study.

TABLE 2. Locality data of individual *Heliconius melpomene* specimens examined in this study. Locality numbers correspond to labels in figures 5 and 8. Specimen codes correspond to individual voucher specimens. The list is alphabetical, by race name. Starred specimens were reared from captive stock. *Heliconius melpomene* races not included in the study include *xenoclea* Hewitson (Rio Perené, Peru), *euryades* Riffarth (Vilcanota, Peru), *pyroforus* Kaye (Roraima region, Brazil), and *meriana* Turner (Suriname).

| Race name | Locality number | Locality of origin | Code(s) |
|---|---|---|---|
| *aglaope* Felder and Felder | 34 | Puerto Misahuallí, Napo, Ecuador | E-7-1 |
| | 35 | Puerto Napo, Napo, Ecuador | E-8-1 |
| | 40 | Riberalta, Beni, Bolivia | RIB2, RIB32 |
| *amaryllis* Felder and Felder | 44 | Chazuta, San Martín, Peru | JM1558 |
| | 45 | Chumia, San Martín, Peru | JM1647 |
| *bellula* Stichel | 28 | 1–3 km N. Mocoa, Putumayo, Colombia | C-14-8 |
| *cognata* Riffarth | 42 | Rio Huallaga, 30 km NE Davidcillo, San Martín, Peru | JM1156, JM1162 |
| *cythera* Hewitson | 19 | Tinalandia, Pichincha, Ecuador | E-4-17, E-9-1 |
| *euryas* Boisduval | 13 | 11 km E. la Peña, Cundinamarca, Colombia | C-7-4 |
| | 14 | Mariquita, Tolima, Colombia | C-9-4 |
| *melpomene* (VC) (L.)† | 24 | Villavicencio, Meta, Colombia | C-1-12 |
| *melpomene* (FG) (L.)† | 49 | E. bank Mahury R., Rte. D6, Guiana | G-5-2 |
| | 48 | km 17, Rte. N1, Guiana | G-22-4 |
| *melpomene* (EP) (L.)† | 9 | Cana, Darien, Panama | P-19-3 |
| | 11 | Rio Ipetí, Pan-American Highway, Panama | P-33-3 |
| *nanna* Stichel | 51 | Linhares, Espirito Santo, Brazil | B-1-26, B-1-27 |
| *penelope* Staudinger | 38 | Cacaulandia, Rondonia, Brazil | RB004 |
| | 39 | Fazenda Rancho Grande, Rondonia, Brazil | RB127 |
| *plesseni* Riffarth | 30 | Rio Sucio, Puyo, Pastaza, Ecuador | E-5-7 |
| | 31 | Rio Puyo, Puyo, Ecuador | E-6-4 |
| | 32 | (Puyo-Shell region, Pastaza, Ecuador) | Gilbert15* |
| *rosina* Boisduval | 1 | Sirena, Parque Nacional Corcovado, Costa Rica | CRM1 |
| | 2 | Puerto Viejo, Heredia, Costa Rica | CRM2 |
| | 6 | El Valle, Coclé, Panama | P-3-3 |
| | 8 | Loma del Naranjo, Lago Bayano, Panama | P-27-4 |
| *schunkei* Lamas†† | 36 | Parque Nacional Manu, Madre de Dios, Peru | PA-6-3 |
| | 37 | Tambopata Preserve, Puerto Maldonado, Madre de Dios, Peru | TA-22-3 |
| *thelxiopeia* Staudinger | 50 | R. Comté Bridge, Rte. N2, Guiana | G-1-3, G-26-1 |
| *vulcanus* Butler | 17 | old Cali-Buenavantura Road, km 32, Valle del Cauca, Colombia | C-22-15 |
| | 18 | old Cali-Buenavantura Road, km 39, Valle del Cauca, Colombia | C-23-2 |

† *Heliconius melpomene melpomene* probably represents at least three distinct races. Specimens appearing in different clades are identified here (VC, Villavicencio, Colombia; FG, French Guiana; EP, eastern Panama).

†† *Heliconius melpomene schunkei* Lamas is very similar to *Heliconius melpomene aglaope* Felder and Felder, differing only slightly in the size of the forewing apical yellow patch. The two races are lumped as *aglaope* in this study.

TABLE 3. Locality data of individual outgroup *Heliconius* specimens examined in this study. Locality numbers refer to labels on Figures 5, 6, and 8. Specimen codes correspond to individual voucher specimens. The list is alphabetical, by species name (and race name, if relevant). Starred specimens were reared from captive stock.

| Race name | Locality number | Locality of origin | Code(s) |
|---|---|---|---|
| *H. besckei* Ménétriés | 54 | Porto Alegre, Rio Grande do Sul, Brazil | B-3-2* |
| *H. clysonymus* Latreille | 55 | Parque Nacional Yacambu, Venezuela | V2 |
| *H. cydno alithea* Hewitson | 19 | Tinalandia, Pichincha, Ecuador | E-1-2 |
| *H. cydno chioneus* Bates | 5 | Pipeline Road, Parque Nacional Soberania, Panama | P-1-3 |
| *H. cydno galanthus* Bates | 3 | Selva Verde, Heredia, Costa Rica | CRC1 |
| *H. hecale zuleika* Hewitson | 4 | Villa Colon, San Jose, Costa Rica | CRHEC1 |
| *H. heurippa* Hewitson | 25 | Villavicencio-Bogotá Road, km 98, Meta, Colombia | C-4-6 |
| *H. himera* Hewitson | 20 | Carretera Marginal km 23.5, Piura, Peru | JM578 |
| *H. pachinus* Salvin | 1 | Sirena, Parque Nacional Corcovado, Costa Rica | CRP1* |
| *H. sara* Fabricius | 5 | Pipeline Road, Parque Nacional Soberania, Panama | P-1-7 |
| *H. telesiphe* Doubleday | 26 | 62 km N. Mocoa, Putumayo, Colombia | C-17-1 |
| *H.* sp. nov.† | 27 | Pasto-Mocoa Road, km 130, Putumayo, Colombia | C-13-4 |
| | 29 | 6 km N. Mocoa, Putumayo, Colombia | C-15-4 |

† This is inferred to be the sister taxon to *H. heurippa* in this analysis. See text for details.

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                                                                                                                  11111
                  1111111122222222222333444445555666666666666777777777778888888888999999999000000
                  0133469900113336689289145790479013444667880114777799900346678890111456882222 4
Taxon             9509200329452585802875365720243216124254691093013614706080321438147708692589 9
-------------------------------------------------------------------------------------------------------------
Jm1570ge.Frg      ?TTACTAACACTGATCATTTGTTCTCACCTGCCTCTATTCTTTCTTCCCTCCCTTCGTACCCACCCCCTACAAATTC
Jm1903ge.Frg      CTTACTAACACTAATCATTTGTTCTTACTTGCTTCTATTCTTTCTTCCCTCCTTTCGTACCCACCCCCTACAAATTT
Rb005ge.Frg       CTTACTAACACTGATCATTTGTTCTCACCTGCTTCTATTCTTTCTTCCCTCCCTTCGTACCCACTCCCTGCAAATTT
P15-1ge.Frg       CTCACCATTACTAACTATCCGTTCCTACCTGTCTATACTTTTCTCTTCCTCATCTTGTGTTCGCCTTCCATAAATTT
P13-5ge.Frg       CTCACCATTACTAACTATCTGTTCCTACCTGTCTTTACTTTTCTCTTCCTCATCTTGTGTTCACCTTCCATAAATTT
P1-2ge.Frg        CTCACCATTACTAACTATCCGTTCCTACCTGTCTTTACTTTTCTCTTCCTCATCTTGTGTTCGCCTTCCATAAATTT
Cr1ge.Frg         CTCACCATTACTAACTATCCGTTCCTACCTGTCTTTACTTTTCTCTTCCTCATCTTGTGTTCACCTTCCATAAATTT
G22-1ge.Frg       CTTACTAACACTAATCATTTATTCTTACTTGCTTCTATTCTTTCTTCCCCCCTTTCGTACCTACCCCCTACAGATTT
E6-2ge.Frg        CTTGCTAACACTAATTATTTGTTCTTACCTGCTTCTATTCTTTCTTCCCTCCTTTCGTACCCACCCCCTACAAATTT
E3-1ge.Frg        CTTGCTAACACTAATTGTTTGTTCTTACCTGCTCCTATTCTTTCTTCCCTCCTTTCATACCCACCCCCTACAAATTT
B1-31ge.Frg       ??TACTAACACTAATCGTTTGTTCTTACCTGCTTCTATTCTTTCTTCCCTCCTTTCGTACCCACCCCCTACAAATTT
E4-7ge.Frg        CTCACCATTACTAACTATCTGTTCCTACCTGTCTTTACTTTTCTCTTCCTCATCTTGTGTTCGCCTTCCATAAATTT
P1-7ge.Frg        TTAATAAATATTATCTAATTATCTCTAATTATCTTCTTCATATTTTTTATAATTTTACTGTAATTAATAATGAATTT
Jb1ge.Frg         CTCACCATTACTACCTGTCTGTTCCTACCTATCTTTACTTTTCCATTCCTCATCTTGTATTCACCTTCCATAAATTT
Rib1ge.Frg        CTTACTAACACTAATCATTTGTTCTTGCCTGCTTCTATTCTTTCTTCCCTCCTTTCGTACCCACCCCCTACAAATTT
Hcol1ge.Frg       TTCACTGTCCCTATTCGTTTGTTCCTACCTGTCTTTACTCTTCTATTCCTCATTTCGTATCCACCTTTCATAAATCT
Jm1904ge.Frg      CTCACTAACACTGATCATTTGTTCTCACCTGCCTCTATCCCTTCTTCCCTCCCTTCGTACCCACCCCCTACAAATTT
E3-2ge.Frg        CTTACTAACACTGATCATTTGCTCTCACCTGCCTCTATTCTTTCTTCCCTCCCTTCGTACCCACCCCCTACAAATTT
P18-1ge.Frg       CACACCATTACTAACCATCTGTTCCTACCTGTCTTTACTTTTCTCTTCCTCATTTTGTGTTCACCTTCCGTAAATTT
Jm1571ge.Frg      CTTACTAACACTAATCATTTGTTCTTACCTGCTTCTATTCTTTCTTCCCTCCTTTCGTACCCACCCCCTACCAATTT
E9-2ge.Frg        CTCACCATTACTAACTGTCTGTTCCTACCTGTCTTTACTTTTCTCTTCCTCATCTTGTGTTCGCCTTCCATAAATTT
B1-32j.Frg        CTTACTAACACTAATCATTTGTTCTTACCTGCTTCTATTCTTTCTTCCCTCCTTTCGTACCCACCCCCTACAAATTT
G6-4ge.Frg        ??TACTAACACTAATCATTTGTTCTTACCTGCTTCTATTCTTTCTTCCCCCCTTTCGTACCCACCCCCTACAGATTT
C10-1ge.Frg       TTCACTGTCCCTATTTGTTTGTTCTTACCTGTCTTTACTCTTCTATTCCTCATTTCGTATCCACCTTTTATAAATCT
C23-1ge.Frg       CTCACCATTACTACCTATCTGTTCCTACCTGTCTTTACTTTTCTATTCCTCATCTTGTATTCGCCTTCCGTAAATTT
G3-2ge.Frg        CTAACTAACACTAATTATTTGTTCTTACCCGCTTCTATTCTTTCTTCCCTCCTTTCGTACCCACCTCCTACAGATTT
C9-1ge.Frg        ?TCACCATTACTAACTGTTTGTTCCTACCTGTCTTTACTTTTCTATTCCTCATCTTGTGTTCGCCTTCAATAAATTT
Ta22-2ge.Frg      CTTACTAACACTGATCATTTGTTCTCACCTGCTTCTATTCTTTCTTCCCTCCTTTCTTACCCACCCCCTACAAATTT
C14-7ge.Frg       ??TACTAACACTAATCATTTGTTCTTACCTGCTTCTATTCTTTCTTCCCTCCTTTCGTACCCACCCCCTACCAATTT
C1-2ge.Frg        CTTACTAACACTAATCATTTGTTCTTGCCTGCTTCTATTCTTTCTTCCCTCCTTTCTTACCCACCCCCTACAAATT?
C21-2ge.Frg       CTAATCATCACTAATTATCTGTTCCTACCCGTCTTTACTTTTCTCCCCCTCATCTTGTATCCACCTTCCACAAATT?
B3-1ge.Frg        CTTACTAACACTAATCACTTGTTCTTACCTGCTTCTATTCTTTCTTCCCTCCTTTCGTACCAACCCCCTACAGATTT
C22-5ge.Frg       CTCACCATTACTACCTGTCTGTTCCTACCTGTCTTTACTTTTCTATTCCTCATCTTGTATTCGCCTTCTATAAATTT
Jb8ge.Frg         CTCACCATTACTAACTGTCTGTTCCTACCTGTCTTTACTTTTTTATTCCTCATCTTATGTTCATCTTCCATAAATTT
C7-7ge.Frg        ?TCACCATTACTAACTGTCTGTTCCTACCTGTCTTTACTTTTCTATTCCTCATCTTGTATTCGCCTTCTATAAATTT
C13-3ge.Frg       ??TATTAACACTAATCATTTGTTCTTACCCGCTTCTATTCTTTCTTCCCTCCTTTCGTACCCACCCCCTACAGATTT
Pa10-1ge.Frg      ???ACTAACACTAATCATTTGTTCCTACCCGCTTCTATTCTTTCTTCCCTCCTTTCGTACCCACCCCCTACAAATTT
B5-2ge.Frg        CTTACTAACACTAATCGTTTGTTCTTACCTGCTTCTATTCTTTCTTCCCTCCTTTCGTACCCGCCCCCTACAAATTT
V2ge.Frg          TCTATTGATATCAACCATTTACTTTTATTTATTTTCTTTTTCCCTCTTATTTCTCTATGTTAAATCTTCGTAATCCC
C17-1ge.Frg       ?CTGAAAACACCAACCACTTACCTTTATCTGTTCTCTTTCTACTTTTTATTTTTCTGCTTTAAATATTCCTGATCCT
Jm578ge.Frg       ?TCACTAACATTAACTATTTATTCTTAACTGTTTTTACTCCTTTTTCCCTCCTTTTGTACCCATCTTCCACAAATTT
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APPENDIX FIG. 1. Informative sites in the *Heliconius erato* data set (including the outgroups) used in the cladistic analysis. Position numbers are listed at the top. Position 100 above corresponds to position 2800 in the *Drosophila yakuba* sequence (Clary and Wolstenholme 1985). Taxon codes are listed in Appendix Tables 1 and 3.

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                  1111112222222222234444455555566666777777788888888888899999999900000
                  1334890112223356890124500235722348111669900134566788901256677812234
Node              1092402453463835051389569768337616069152409608709214357605845962810
------------------------------------------------------------------------------------------
B3-2ge.Frg        CTATTATTTTCTCTCTATACCTTCATTGTCTTCTTTTTGCTTACCAATTTTAATATTTTTGATATCT
Crm2ge.Frg        TTTATACTCATACCTTTCTTTCCTACTATATTTTTTTTGTATTTTTACCGTGACATTTTTTATACTC
P27-4ge.Frg       TTTATACTCATACCTTTCTTTTCTACTATATTTTTTTTGTATTTTTACCGTGACATTTTTTATACTC
E5-7ge.Frg        TTTATATTTATACCCTTCTTATTTACTGCATTTTTTTTGTATATTTATTTTAATTTGTATTGCACTC
E9-1ge.Frg        TTTATACTCATACCTTTCTTTTCTACTATATTTTTTTTGTATATTTACCGTGACATTTTTTATACTC
Jm1647ge.Frg      TTTATATTTATACCTTTCTTATTTACTGCATTTTTTTTGTATATTTATTTTAATTTATATTGCACTC
E7-1ge.Frg        TTTATATTTATACCCTTCTTATTTACTGCATTTTTTTTGTATATTTATTTTAATTTATATTGCACTC
G5-2ge.Frg        ?TTATGTTCATACCCCTTTCTTCTGATGTATTCCTTTTATATATTCATTTTAGTACTTTTTATATTC
B1-26ge.Frg       TTTATATTCATATCTTTCTCTTTTATTGTATTTTTTTTGTATATTTATCCCAGTATCTTCTGTACTC
Jm1558ge.Frg      TTTATATTTATACCTTTCTTATTTACTGCATTTTTTTTGTATATTTATTTTAATTTATATTGCACTC
Rb004ge.Frg       TTTATATTTATACCTTTCTTATTTACTGCATTTTTTTTGTATATCTATTTTAATTTATTTTGCACTC
Pa6-3ge.Frg       TTTATATTTATACCTTTCTTATTTACTGCATCTTTTTTGTATATTTATTTTAATTTATATTGCACTC
G22-4ge.Frg       ?TTACGCTCATACCCCTTTCTTCTGATGTATTCCTTTTATATATTCATTTTAGTACTTTTTATATTC
P3-3ge.Frg        TTTATACTCATACCTTTCTTCTCTACTATATTTTTTTTGTATTTTTACCGTGACATTTTTTATACTC
Gil15ge.Frg       TTTATATTTATACCCTTCTTATTTACTGCATTTTTTTTGTGTATTTATTTTAATTTATGTTGCACTC
E6-4ge.Frg        TTTATATTTATACCCTTCTTATTTACTGCATTTTTTTTGTATATTTATTTTAATTTATATTGTACTC
G1-3ge.Frg        TTTACGCTCATACCCCTTTCTTCTGATGTATTCCTTTTATATATTCATTTTAGTACTTTTTATATTC
E4-17ge.Frg       TTTATACTCATACCTTTCTTTTTTACTATATTTTTTTTGTATTTTTACCGTGATATTTTTTATACTC
Crhec1ge.Frg      TTATTATTTTCTCTCTATATTTTCATTGTTTTTTTTATGCTTACTAATTTTTGTATTTTTTATATTT
B1-27ge.Frg       TTTATATTCATATCNTTCTCTTTTATTGTATTTTTTTTGTATATTTATCCCAGTATCTTCTGTACTC
Crm1ge.Frg        TTTATACTCATACCTTTCTTTTCTACTATATTTTTTTTGTATTTTTACCGTGACATTTTTTATACTC
Rib2ge.Frg        TTTATATTTATACCTTTTTTATTTACTGCGTCTTTTTTGTATATTTATTTTAATTTATATTGTATTC
P33-3ge.Frg       TTTATACTCATACCTTTCTTCTCTACTATATTTTTTTTGTATTTTTACCGTGACATTTTTTATACTC
E8-1ge.Frg        TTTATATTTATACCCTTCTTATTTACTGCATTTTTTTTGTATATTTATCTTATTTTATATTGCACTC
P19-3ge.Frg       TTTATACTCATACCTTTCTTTTCTACTGTATTTTTTTTGTATTTTTACCGTGATATTTTTTATACTC
Ta22-3ge.Frg      TTTATATTTATACCTTTCTTATTTACTGCATCTTTTTTGTATATTTATTTTAATTTATATTGCACTC
Jm1156ge.Frg      TTTATATTTATACCTTTCTTATTTACTGCATTTTTTTTGTATATTTATTTTAATTTATATTGCACTC
C14-8ge.Frg       TTTATATTTATACCCTTCTTATTTACTGCATTTTTTTTGTATATTTATTTTAATTTATATTATACTC
Rb127ge.Frg       TTTATATTTATACCTTTCTTATTTACTGCATTTTTTTTGTATATTTATTTTAATTTATATAGCACTC
Rib32ge.Frg       TTTATATTTATACCTTTCTTATTTACTGCATTTTTTTTGTATATTTATTTTAATTTATATAGCACTC
C22-15ge.Frg      TTTATACTCATACCTTTCTTTTCTACTATATTTTTTTTGTATTTTTACCGTGACATATTTTATACTC
G26-1ge.Frg       TTTACGCTCATACCCCTTTCTTCTGATGTATTCCTTTTATATATTCATTTTAGTACTTTTTATATTC
C13-4ge.Frg       ??TATATCCATACTTTTCTCACCTATCGCGCTTTTTACGTACATCTTTCTTGATTTACTTTGTGCTC
C23-2ge.Frg       ?TTATACTCATACCTTTCTTTTCTACTATATTTTTTTTGTATTTTTACCGTGACATTTTTTATACTC
C9-4ge.Frg        TTTATACTCATACCTTTCTTTTCTACTATATTTTTCTTGTATTTTTACCGTGACATTTTTTATACTC
C15-4ge.Frg       TCTATATCCATACTTTTCTCACTTATCGTGCTTTTTACGTACATCTTTCTTGATTTACTTTGTGCTC
C7-4ge.Frg        TTTATACTCATACCTTTCTTTTCTACTATATTTTTCTTGTATTTTTACCGTGACATTTTTTATACTC
C1-12ge.Frg       TTTATATTTATACCTTTCTTATTTACTGCATTTTTTTTGTATATTTATTTTAATATATATTGTACTC
Jm1162ge.Frg      TTTATATTTATACCCTTCTTATTTACTGCATTTTTTTTGTATATTTATTTTAATTTATATTACACTC
P1-3ge.Frg        TCTATATCCATACTTTCCTCTCTTATCGTACTTTTTACGTACATCTTTCTCAATTTACTTTGTGCTC
Crp1ge.Frg        CTTATATCCATACTTTTCTCACTTACCGTGCTTTTTCCGTATATCTTTCTCAATTTATTTTGTGTCC
Crc1ge.Frg        ?TTATATACTTACTCCACTCTCTTATCGTGCTTTCTACGTATATCTTTCTCAGTTTACTTTGTATCC
E1-2ge.Frg        ?CTATATCCTTACTTCACTCTCTTATCGTACTTTCTATGTATATCTATCTTAGTTTACTTTGTATCC
C4-6ge.Frg        TCTATATCCATACTTTCCTCATTTATCGTGCTTTTTACGTACATCTTTCTCGATTTACTTTGTGCTC
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APPENDIX FIG. 2. Informative sites in the *Heliconius melpomene* data set (including the outgroups) used in the cladistic analysis. Position numbers are listed at the top. Position 100 above corresponds to position 2800 in the *Drosophila yakuba* sequence (Clary and Wolstenholme 1985). Taxon codes are listed in Appendix Tables 2 and 3.