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Cladistic analysis of *Heliconius* butterflies and relatives (Nymphalidae: Heliconiiti): a revised phylogenetic position for *Eueides* based on sequences from mtDNA and a nuclear gene

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SUMMARY

A new phylogenetic hypothesis for *Heliconius* and related genera is presented, based on DNA sequence data from mtDNA combined with a region of the *wingless* gene. This study also adds eight new taxa to a previous cladistic hypothesis based on the mtDNA alone. Simultaneous phylogenetic analysis of the two gene regions together supports a topology largely in agreement with traditional views of heliconiine relationships based on morphology and suggests that the mtDNA support for the sister relationship between *Eueides* and *H. charithonia* is due to convergent evolution of homoplasious mtDNA sites.

1. INTRODUCTION

The systematics of *Heliconius* butterflies and related genera (subtribe Heliconiiti, *sensu* Harvey (1991)) have been studied by many authors for more than two centuries (indeed, the 'Heliconii' were one of Linnaeus's (1758) five original subgeneric groups within the single butterfly genus *Papilio*). Since Bates published his key insights about mimicry in 1862, an increasingly coherent picture of heliconiine systematics based on morphological characters has emerged. However, a clear understanding of relationships within the genus *Heliconius* has been complicated by rampant mimicry between species and geographical polymorphism within species, which together have produced a bewildering array of colourful aposematic wing patterns among members of the group (Riffarth 1901; Stichel & Riffarth 1905; Kaye 1907; Eltringham 1916; Neustetter 1929; Emsley 1965; Turner 1968; Brown 1972, 1981; Sheppard *et al.* 1985).

Traditional views of *Heliconius* phylogenetic relationships based on morphology (Emsley 1965; Brown 1981) were largely corroborated by cladograms produced from mitochondrial cytochrome c oxidase subunit 1 and 2 sequences (Brower 1994), with the exception of one important grouping: the mtDNA data implied that *Eueides*, the traditionally recognized sister-genus to *Heliconius*, sprang from the middle of the *Heliconius* clade considered by Benson *et al.* (1975) to be the most derived within the genus (figure 1). Although some morphological characters appear to contradict the mitochondrial result, Brower (1997) defended his preference for the mtDNA tree by questioning the explicit character support for the morphological topology and offering alternative parsimonious interpretations of character evolution for several of the putative synapomorphies supporting Brown's (1981) intuited phylogeny. Other *Heliconius* workers have expressed scepticism about the position of *Eueides* implied by the mtDNA (J. Mallet, personal communication; C. Penz, personal communication), but a formal analysis of morphological data that reflects the traditional view of *Heliconius* relationships remains unpublished to date. Thus, the mtDNA tree has represented the only explicit phylogenetic analysis for the group and, as such, the preferred hypothesis of relationships at present (Brower *et al.* 1996).

Simultaneous analysis of all relevant character data and choice of a topology by cladistic character congruence has been shown to be a theoretically sound and practically successful method for combining data from multiple sources (e.g. Miyamoto 1985; Kluge 1989; Barrett *et al.* 1991; Miller *et al.* 1997). We present here an analysis combining the previous mitochondrial sequences with new sequence data from the nuclear protein-coding gene *wingless*. Taxonomic representation is more complete in the current sample than in the previous study as well: four additional species of *Heliconius* (including the subgenus *Neruda*), one additional *Eueides* and three new outgroup taxa are included for both gene regions. The first cladistic hypothesis of relationships among the basal heliconiine genera is implied by the incorpora-

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Table 1. *Alphabetical list of new or different butterflies used in this study*

(Unless listed here, mtDNA and *wingless* were sequenced from the same individual listed in Brower (1994, table 1). Some duplicate conspecific individuals included in the 1994 analysis were deleted from the current matrix to enhance computational time.)

species	code	collection locality	sequence[a]
Agraulis vanillae (L.)	B-10-1	Brazil: Amazonas, Manacapurú	mt + wg
Dione glycera Felder & Felder	PE-7-5	Peru: Cuzco, Quebrada San Luis	mt + wg
Eueides aliphera (Godart)	P-11-5	Panama: Gatún Locks	wg
Eueides vibilia (Godart)	RB329	Brazil: Rondônia, Cacaulandia	mt + wg
Heliconius atthis Doubleday	E-4-4B	Ecuador: Pichincha, Tinalandia	wg
Heliconius erato (L.)	JB1	Colombia: Bolivar, Cartagena	wg
Heliconius hecuba Hewitson	E-10-2	Ecuador: Tungarahua, Rio Machay	mt + wg
Heliconius leucadia Bates	RB255	Brazil: Rondônia, Cacaulandia	wg
Heliconius timareta Hewitson	E-11-1	Ecuador: Pastaza, Shell	mt + wg
Heliconius tristero Brower	C-15-4	Colombia: Putumayo, Mocoa	mt + wg
Neruda metharme (Erichson)	RB302	Brazil: Rondônia, Cacaulandia	mt + wg
Philaethria dido (L.)	RB283	Brazil: Rondônia, Cacaulandia	wg
Speyeria cybele (Fabr.)	VA2	USA: Virginia, Blue Ridge	mt + wg

[a]Butterfly species sampled in Brower (1994), but for which a different individual was sequenced for *wingless* are indicated by 'wg'.

tion of a more distant outgroup at the root. The tree presented here supercedes that of Brower (1994) as our preferred hypothesis of heliconiine phylogenetic relationships.

2. MATERIALS AND METHODS

(a) *Taxon sampling*

Fifty-eight individual butterflies were sampled, representing 36 *Heliconius* species, six *Eueides* species and eight outgroup species. In most cases, the same individuals were used in this study as reported in Brower (1994). The eight species newly added to the analysis and alternate individuals of previously sampled species are listed in table 1. To assess the relationships among the basal Heliconiiti, *Speyeria* (from the heliconiine subtribe *Argynniti*) was included in the analysis as an outgroup.

(b) *Preparation of specimens*

Butterflies were netted in the field and preserved, either frozen in liquid nitrogen or immersed in 100% EtOH, until they could be prepared. The DNA isolation method followed a standard tris-EDTA lysis and phenol–chloroform extraction protocol. Details of the procedure are given in Brower (1994). Wing and abdomen vouchers of new material are preserved in the American Museum of Natural History, except for the voucher of *Heliconius tristero* (a holotype—see Brower (1996) for details), which is deposited in the Cornell University insect collection.

(c) *PCR, sequencing and alignment*

PCR for the mitochondrial COI-COII region used the primers and followed the procedures described in Brower (1994). PCR of a 370-base fragment of *wingless* used primers and procedures described in Brower & DeSalle (1997). All PCR amplifications were performed directly from genomic DNA. New sequences were generated by cycle sequencing with ABI Prism kits and automated sequencing on an ABI 373. *Wingless* sequences were generated from sense and antisense strands, but mtDNA sequences were sequenced with overlapping primers that did not always cover the entire 950 bp fragment in both directions. Alignments were performed by eye as there were few gaps in the mtDNA and none in the *wingless* sequences. Aligned data matrices in Nexus format are available from the American Museum web site (`http://research.amnh.org/molecular`) and individual sequences will be deposited with GenBank.

(d) *Phylogenetic analyses*

The theoretical basis for our choice of methodologies has been elaborated elsewhere (Brower *et al.* 1996; Miller *et al.* 1997). Characters (nucleotide sites) were weighted equally, with gaps scored as missing data for individual taxa within the matrix, and coded as presence/absence characters for all taxa at the end of the matrix. Cladograms were inferred from each gene region and from the combined data set by the maximum parsimony method using PAUP 3.1 (Swofford 1991). Successive approximations weighting (SAW; Farris (1969), implemented in PAUP) was used to select resolutions of polytomies from among most-parsimonious alternatives when multiple trees were discovered in equal-weighted searches. The initial weights were based on highest retention index values from the equal-weighted trees and, subsequently, on weights implied by the previous round of SAW. Heuristic searches were conducted by TBR-branch swapping in ten random-addition search iterations. Consistency indices (CI, excluding uninformative characters) and retention indices (RI) are presented. Branch lengths under alternate character optimizations were calculated in MacClade 3.0 (Maddison & Maddison 1992). Branch support (Bremer 1988, 1994; Davis 1995) for internal branches was assessed by anticonstraint searching with a single closest-addition TBR-branch swapping heuristic search. Significance of character congruence between the two gene regions was assessed with the ARN program (Farris *et al.* 1994).

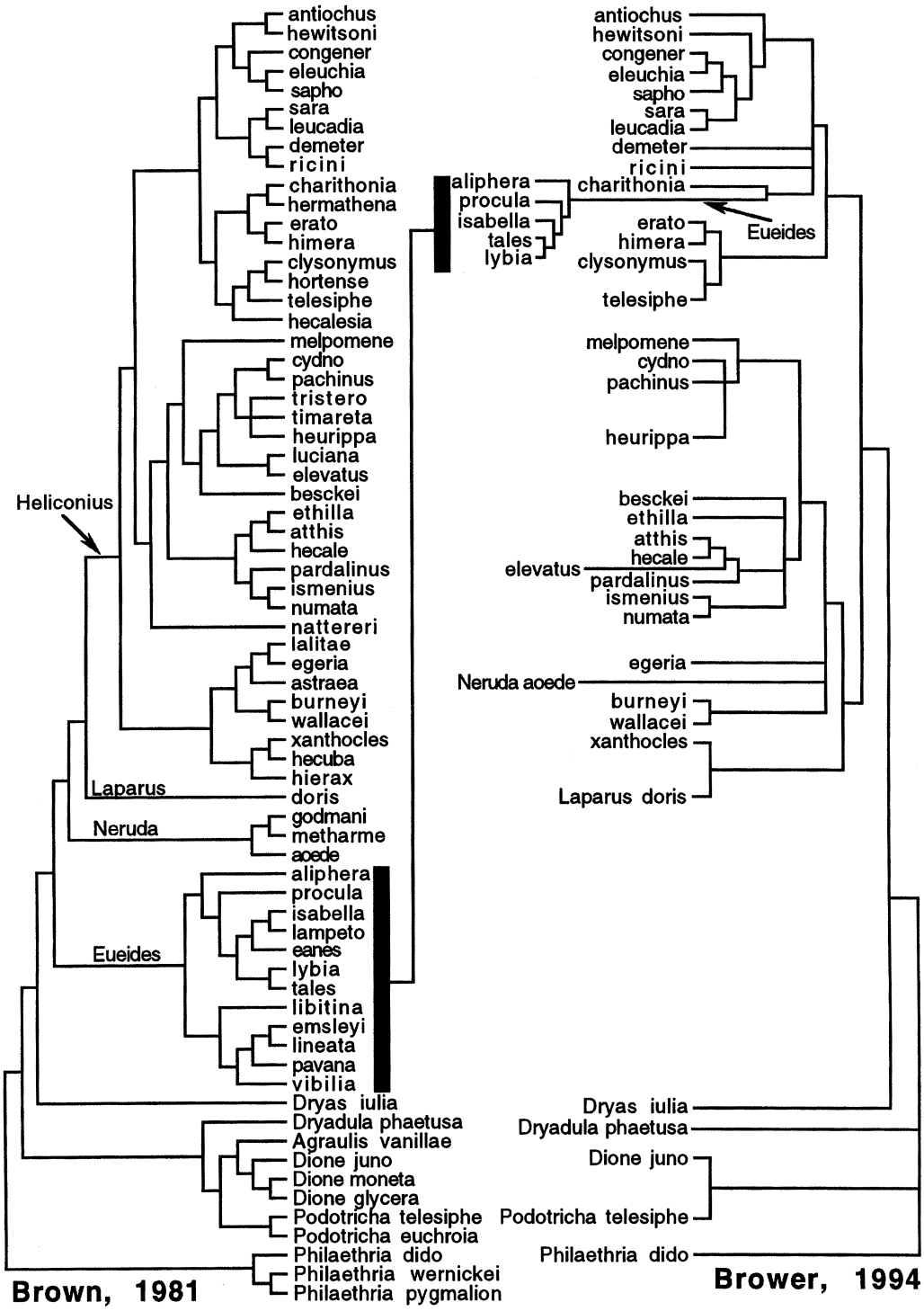

Figure 1. Prior hypotheses of heliconiine relationships: Brown's (1981) phylogeny, based on informal analysis of morphology and ecological characters (left), compared with Brower's (1994) strict consensus tree from cladistic analysis of mtDNA sequences for a subset of taxa (right). Notable topological incongruences are indicated by long branches in the mtDNA tree and the discrepancy between the trees for *Eueides* is further highlighted by the bars. The mtDNA tree is rooted with *Philaethria* here to improve comparability of basal taxa. *H. himera*, *H. tristero* and *H. lalitae* were not included in Brown (1981), but are included here based on subsequent work (Descimon & de Maeght 1983; Brower 1996; Brévignon 1996, respectively).

3. RESULTS

Eight new mtDNA sequences and a complete *Philaethria* sequence were added to the mtDNA matrix reported in Brower (1994). The mtDNA matrix analysed here contains 58 sequences with 950 aligned bases and five binary gap characters. Fifty-eight new *wingless* sequences in a matrix of 378 aligned characters. See Brower & DeSalle (1997) for details on comparative base composition and patterns of sequence divergence in these two gene regions at differing levels of phylogenetic divergence.

Separate analysis of the COI-COII sequences

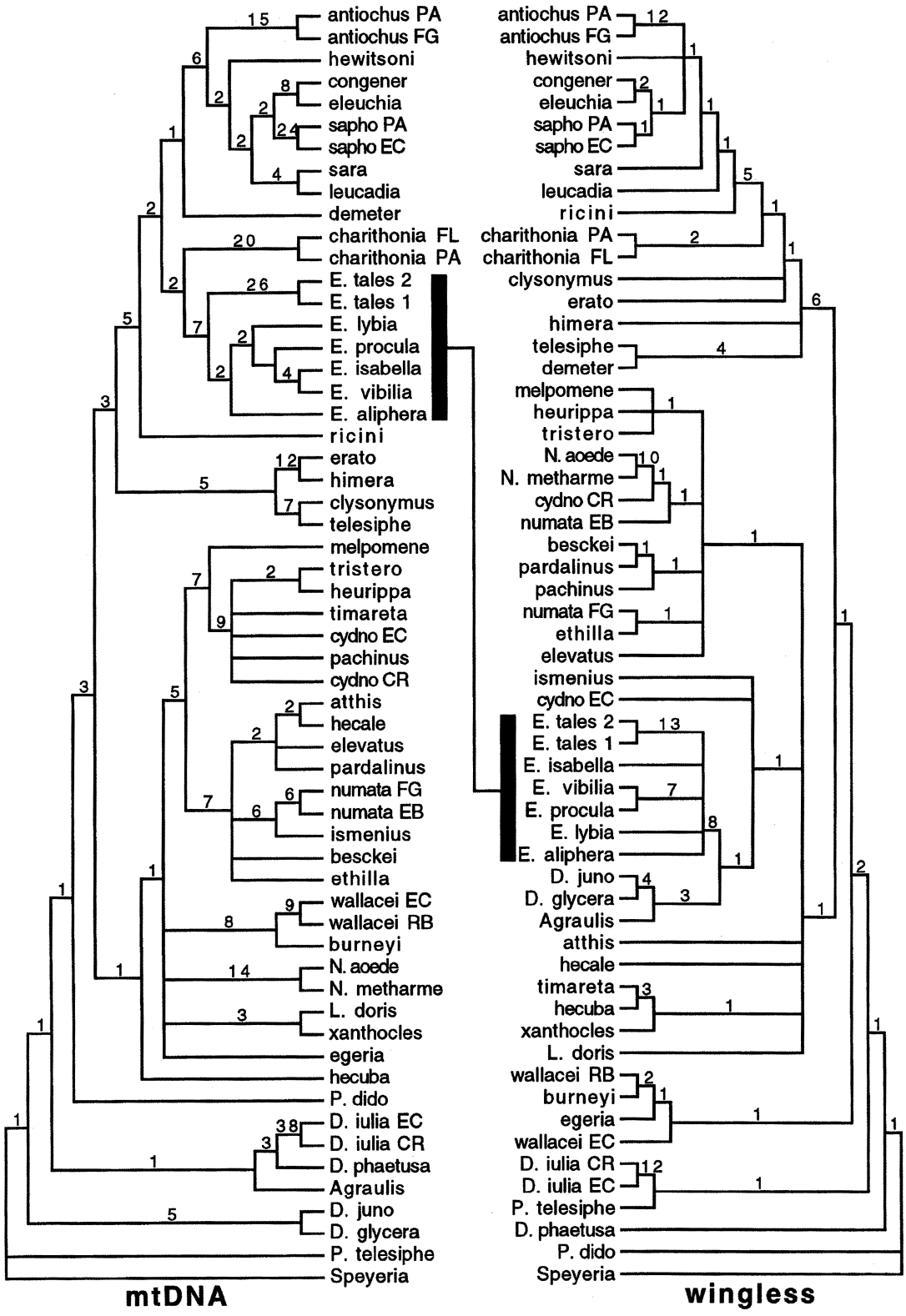

Figure 2. Cladistic relationships implied by separate analysis of COI-COII (left; strict consensus of 50 trees, length 1682, CI 0.291, RI 0.548) and *wingless* (right; strict consensus of 3575 trees, length 467, CI 0.400, RI 0.670). Branch support values are shown above branches. The disparate position of *Eueides* implied by the alternate analyses is highlighted by the bars.

yielded 50 equally parsimonious trees of 1682 steps, the strict consensus of which contains 46 resolved nodes (left-hand side of figure 2). Relationships implied by the mtDNA data exhibit no incongruence with the previously published mtDNA topology (Brower 1994), although relationships among the basal taxa are rearranged due to the rooting of the tree with *Speyeria* instead of *Dryas*. *Eueides* is still

Table 2. *Structure within and between the data from mtDNA and* wingless *gene regions*

(Numbers of variable and informative characters are indicated. Minimum length represents the lowest possible number of state changes per character, summed for all characters. Intrinsic homoplasy is the minimum number of additional steps required beyond the minimum length to hierarchically arrange the data. D homoplasy is the extra homoplasy contributed by combining the data in simultaneous analysis. ARN scores represent the proportion of jack-knifed random partitions resulting in trees as short, or shorter, than the observed most parsimonious trees, based on 999 samples.)

character source	number of characters	minimum length	number of trees	shortest tree discovered	intrinsic homoplasy	D homoplasy	ARN score
mtDNA	955 (424v; 292i)	580	50	1682	1102 (66%)		
wingless	378 (176v; 110i)	226	3575	467	241 (52%)		
combined	1333 (600v; 402i)	806	54	2212		63 (3%)	0.341 (N.S.)

nested within the derived, pupal-mating *Heliconius* clade, as sister to *H. charithonia*, supported by two steps of branch support.

The *wingless* data by themselves provided a less well-resolved picture of relationships (right-hand side of figure 2), yielding 3575 equally parsimonious cladograms. The strict consensus contains 38 resolved nodes, but many of these are not strongly supported, and some of the implied clades are strikingly at odds with any prior hypothesis of relationship. Neither of these two gene regions alone supports the monophyly of *Heliconius* with respect to *Eueides*. Although the ARN score (table 2) implies that the two partitions do not differ statistically in their hierarchical signal, topological congruence between trees from separate analyses offers little resolution beyond the grouping of conspecifics: aside from a few pairs of terminals, only the monophyly of *Eueides* and the *sara-sapho* group (table 3, clades 7 and 40, respectively) is supported by the strict consensus of topologies from the separate analyses.

The simultaneous analysis (SA) of data from both gene regions yields 54 equally parsimonious cladograms of 2212 steps. A phylogram representing one of these (chosen to show resolutions of polytomies favored by SAW) is shown in figure 3. Elements of both the mtDNA and the *wingless* topologies are preserved and although more trees were discovered than in the analysis of the mtDNA alone, the strict consensus SA tree is more fully resolved than that from the mtDNA. Further, the branch support for most individual nodes is increased substantially by the addition of the *wingless* data (table 3), implying underlying agreement between the data from the two genes. The major changes in the implied topology result from the combination of data sets rather than the addition of taxa: when the data from both genes are analysed without the new taxa, implied relationships among *Heliconius* and *Eueides* are almost entirely congruent with figure 3, except for one minor rearrangement within the *sara-sapho* group (data not shown). Thus, figure 3 represents our current preferred hypothesis of relationships among the species and genera of Heliconiiti.

4. DISCUSSION

(*a*) *Taxonomic implications*

The taxa added to the data set since 1994 occupy positions in figure 3 that concur with traditional hypotheses of heliconiine relationships. *Heliconius tristero* and *H. timareta* are part of the *H. cydno* clade and probably represent geographically differentiated populations or semi-species of *H. cydno* that parallel the tremendous intraspecific diversity of *H. erato* and *H. melpomene* (Brower 1996). *Heliconius hecuba*, a mimic of the ithomiine *Elzunia* where both occur together on the eastern slope of the Andes, appears to be among the most basal taxa in the genus. *Neruda metharme*, *Eueides vibilia* and *Dione glycera* are each uncontroversially associated with their respective congeners.

In the SA analysis (figure 3), the data support the monophyly of almost all traditional species groups and genera (with the exception of *Heliconius* in the narrow sense, excluding *Laparus* and *Neruda*). *Eueides* has moved from the unorthodox grouping within the 'derived' *erato* and *sara-sapho* groups implied by the mtDNA data alone (Brower 1994 and left-hand side of figure 2) to the position traditionally suggested by morphologists (e.g. Brown 1981) as sister taxon to *Heliconius*. The monophyly of *Heliconius* is quite well supported in the simultaneous analysis (branch support = 4; see Davis (1995) for a comparative study of branch-support measures); this pattern only emerges when the mtDNA and *wingless* data are combined.

The relationships among the basal genera are plausible, although the basal position of *Agraulis* as sister to all other Heliconiiti disagrees with traditional morphological classifications, which place it as sister to *Dione* (Michener 1942; Brown 1981). Although incorporation of additional outgroup taxa might change the position of the root, it is tempting to speculate about the symplesiomorphic status of the silver-spangled 'fritillary' wing pattern shared by *Speyeria* and these taxa. The clade composed of *Dryas*, *Dryadula* and *Philaethria* contrasts with Brown's

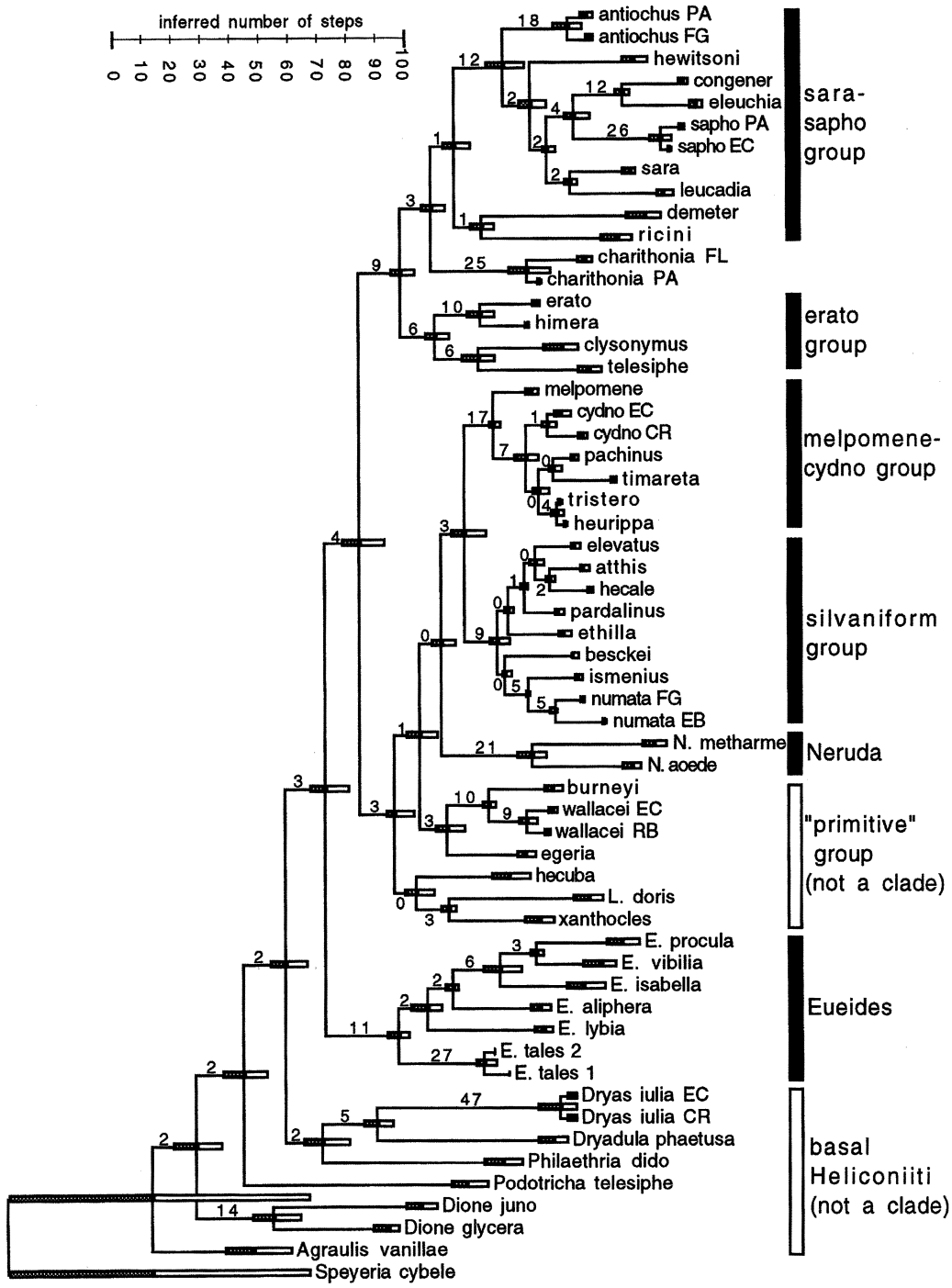

Figure 3. Phylogram for COI-COII and *wingless* data combined, the currently preferred hypothesis of relationships for Heliconiiti. This tree is one of 54 equally parsimonious cladograms of length 2212 (CI 0.304, RI 0.556). Bars on internal nodes reflect minimum, mean and maximum branch lengths under alternate character optimizations (scale at top). Branch support values are indicated adjacent to internal branches. Branches with a support value of zero are not resolved in the strict consensus tree.

(1981) view of *Philaethria* as the most primitive heliconiine genus, but is supported by morphological similarities in wing shape and pattern (A. Brower, personal observation).

(*b*) *Character support for position of* Eueides *and implications for weighting*

In the most parsimonious cladograms from the mtDNA data alone, nine characters support the sister taxon relationship of *Eueides* and *H. charithonia*, and the resultant paraphyly of *Heliconius* (table 4). Of these, seven are third-position transversions, one is a third-position transition and one is a first-position transition. All of these changes are silent. These sites are all relatively homoplastic (mean CI = 0.235, in contrast to the overall CI of 0.291 for the entire mtDNA data set).

In the most parsimonious trees from the combined

Table 3. *Estimated branch support for the most parsimonious tree from combined data (figure 3), from mtDNA alone (figure 2, left) and the difference between the two, showing the positive contribution of the wingless data to the preferred hypothesis of relationships*

(Nodes not supported by the mtDNA hypothesis are indicated with a dash and the difference in branch support is a minimum estimate. Clades supported by the topological congruence of separate analyses of mtDNA and *wingless* are indicated. A, all data; B, mtDNA; C, difference; D, topological congruence.)

node	A	B	C	D
1. *antiochus* PA + *antiochus* FG	18	15	+3	+
2. *congener* + *eleuchia*	12	8	+4	−
3. *sapho* PA + *sapho* EC	26	24	+2	+
4. 2 + 3	4	2	+2	−
5. *sara* + *leucadia*	2	4	−2	−
6. 4 + 5	2	2	0	−
7. 6 + *hewitsoni*	2	2	0	−
8. 1 + 7	12	6	+6	+
9. *ricini* + *demeter*	1	—	+1	−
10. 8 + 9	1	—	+1	−
11. *charithonia* FL + *charithonia* PA	25	20	+5	+
12. 10 + 11	3	—	+3	−
13. *erato* + *himera*	10	12	−2	−
14. *clysonymus* + *telesiphe*	6	7	−1	−
15. 13 + 14	6	5	+1	−
16. 12 + 15	9	—	+9	−
17. *cydno* EC + *cydno* CR	1	0	+1	−
18. *tristero* + *heurippa*	4	2	+2	−
19. *cydno* clade	7	9	−2	−
20. 19 + *melpomene*	17	7	+10	−
21. *atthis* + *hecale*	2	2	0	−
22. 21 + *elevatus* + *pardalinus*	2	1	+1	−
23. *numata* FG + *numata* EB	5	6	−1	−
24. 23 + *ismenius*	5	6	−1	−
25. *silvaniform* clade	9	7	+2	−
26. 20 + *silvaniform* clade	3	5	−2	−
27. *aoede* + *metharme*	21	14	+7	+
28. *wallacei* EC + *wallacei* RB	9	9	0	−
29. 28 + *burneyi*	10	8	+2	−
30. 29 + *egeria*	3	0	+3	−
31. 26 + 27 + 30	1	0	+1	−
32. *doris* + *xanthocles*	3	3	0	−
33. 31 + 32 + *hecuba*	3	1	+2	−
34. 16 + 33 (genus *Heliconius*)	4	—	+4	−
35. *E. procula* + *E. vibilia*	3	—	+3	−
36. 35 + *E. isabella*	6	6	0	−
37. 36 + *E. aliphera*	2	2	0	−
38. 37 + *E. lybia*	2	2	0	−
39. *E. tales* 1 + *E. tales* 2	27	26	+1	+
40. 38 + 39 (genus *Eueides*)	11	7	+4	+
41. 34 + 40 (*Heliconius* + *Eueides*)	3	3	0	−
42. *Dryas iulia* EC + *D. iulia* CR	47	38	+9	+
43. 42 + *Dryadula phaetusa*	5	3	+2	−
44. 43 + *Philaethria dido*	2	—	+2	−
45. 41 + 44	2	—	+2	−
46. 45 + *Podotricha telesiphe*	2	—	+2	−
47. *Dione juno* + *D. glycera*	14	5	+9	+
48. 46 + 47	2	—	+2	−

data set, the monophyly of *Heliconius* with *Eueides* as its sister-taxon is supported by six characters from each molecule (table 4). Ten of these are silent third-position changes, one is a non-synonymous third-position transversion and one is a non-synonymous first-position transition. The mean CI of these characters (0.254) is also relatively low compared to the CI for the entire tree (0.304), if slightly higher than that reported for the *H. charithonia* + *Eueides* clade above (but not significantly higher, Spjøtvoll–Stoline T' method).

The preponderance of A-T transversions in the COII sequences (nine of the 15 changes in the mtDNA at these nodes) reflects the strong A+T bias of insect mtDNA (Liu & Beckenbach 1992; Brower & DeSalle 1997). Although two non-synonymous changes in the mtDNA support the monophyly of *Heliconius* in SA, their low CIs indicate that these changes are far from uniquely derived. Indeed, both characters are variable within *Eueides* and *Heliconius*, requiring six and 11 steps on the most parsimonious cladograms. Note that character tracing and analysis of support for cladograms such as we present here is not possible using other commonly employed phylogenetic analytical methods, such as neighbour joining and maximum likelihood. We suggest that understanding of molecular character homology and evolution will be refined by using this sort of cladogram dissection, as it has in traditional systematics based on holomorphology (Hennig 1966).

(c) *Data congruence*

There has been considerable controversy over the question of whether to combine systematic data which imply alternate topologies or to keep them separate (reviewed in De Queiroz *et al.* 1995; Brower *et al.* 1996). This study corroborates the empirical findings of Miller *et al.* (1997) from josiine moths: first, that large data sets tend to be more intrinsically homoplasious within partitions than between partitions by as much as an order of magnitude; and second, that combining data partitions in a single analysis leads to vastly superior topological resolution and robustness than results from topological congruence approaches (e.g. Lanyon 1993; Miyamoto & Fitch 1995). In this study, phylogenetic noise and weak homoplastic signal appear to be cancelled out by complementary information from each data set. This is the result we would expect if homoplasy indeed occurred at random (albeit at high frequency) with respect to the underlying hierarchical pattern of relationships we are seeking to discover (Farris 1983). Although our preference for the most parsimonious topology from an explicit analysis of relevant data would not be swayed by a lack of topological congruence with prior informal phylogenetic hypotheses (Brower *et al.* 1996; Miller *et al.* 1997), it is gratifying that these apparently homoplastic and selectively neutral characters still retain sufficient hierarchical information to imply a phylogenetic hypothesis that

Table 4. *Characters supporting the monophyly of* Eueides + Heliconius charitonia *in the most parsimonious cladograms from mtDNA only and characters supporting monophyly of* Heliconius *to the exclusion of* Eueides *in the simultaneous analysis of both gene regions.*

mtDNA only – *Eueides* + *H. charithonia*						mtDNA + *wingless* – monophyly of *Heliconius*					
site[a]	change	position	V/I	amino acid	CI[b]	site	change	position	V/I	amino acid	CI
617	T → A	3rd	V	Ala	0.231	262	C → A	3rd	V	Phe → Leu	0.273
623	C → A	3rd	V	Pro	0.167	431	T → A	3rd	V	Pro	0.286
636	T → C	1st	I	Leu	0.250	636	C → T	3rd	I	Tyr	0.200
638	A → T	3rd	V	Leu	0.400	872	A → T	3rd	V	Ser	0.250
674	A → T	3rd	V	Thr	0.250	968	A → T	3rd	V	Gly	0.188
689	A → T	3rd	V	Gly	0.188	1017	A → G	1st	I	Ile → Val	0.167
761	A → T	3rd	V	Ser	0.222	1136	T → C	3rd	I	Leu	0.400
800	A → T	3rd	V	Val	0.231	1166	G → A	3rd	I	Leu	0.250
848	G → A	3rd	I	Val	0.176	1238	T → C	3rd	I	Asn	0.333
						1298	A → G	3rd	I	Pro	0.250
						1301	T → C	3rd	I	His	0.200
						1307	T → C	3rd	I	Pro	0.250

[a]Characters 101–1050 are mtDNA nucleotide sites, characters 1056–1483 are *wingless* nucleotide sites.
[b]Consistency index of individual characters on the most parsimonious topologies.

agrees with traditional notions of heliconiine relationships, even under the relatively simple analytical procedure of cladistic parsimony with equal character weights. We look forward to the opportunity to combine these sequence data with morphological data, which will further strengthen the robustness of the hypothesis of cladistic relationships and help to resolve long-standing questions about adaptive character evolution in *Heliconius* (e.g. Brower 1997).

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