Do pollen feeding, pupal-mating and larval gregariousness have a single origin in *Heliconius* butterflies? Inferences from multilocus DNA sequence data

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Received 22 November 2005; accepted for publication 4 November 2006

Phylogenetic information is useful in understanding the evolutionary history of adaptive traits. Here, we present a well-resolved phylogenetic hypothesis for *Heliconius* butterflies and related genera. We use this tree to investigate the evolution of three traits, pollen feeding, pupal-mating behaviour and larval gregariousness. Phylogenetic relationships among 60 Heliconiina species (86% of the subtribe) were inferred from partial DNA sequences of the mitochondrial genes cytochrome oxidase I, cytochrome oxidase II and 16S rRNA, and fragments of the nuclear genes elongation factor-1α, apterous, decapentaplegic and wingless (3834 bp in total). The results corroborate previous hypotheses based on sequence data in showing that *Heliconius* is paraphyletic, with *Laparus doris* and *Neruda* falling within the genus, demonstrating a single origin for pollen feeding but with a loss of the trait in *Neruda*. However, different genes are not congruent in their placement of *Neruda*; therefore, monophyly of the pollen feeding species cannot be ruled out. There is also a highly supported monophyletic ‘pupal-mating clade’ suggesting that pupal mating behaviour evolved only once in the Heliconiina. Additionally, we observed at least three independent origins for larval gregariousness from a solitary ancestor, showing that gregarious larval behaviour arose after warning coloration. © 2007 The Linnean Society of London, *Biological Journal of the Linnean Society, 2007, 92, 221–239. Additional keywords: Bayesian analysis – Ef1α – mimicry – mtDNA – parsimony – phylogeny.

INTRODUCTION

The widespread availability of molecular sequence information has greatly facilitated the inference of phylogenetic relationships between species. These phylogenetic hypotheses have been used to investigate the history of ecological and morphological traits (Mitter & Brooks, 1983; Sillén-Tullberg, 1988; Wannorp et al., 1990; Miller & Wenzel, 1996; Maddison & Maddison, 1997). In particular, they have facilitated tests of whether unusual characteristics of particular taxa have arisen through convergent evolution or from a single origin (Miller, Brower & DeSalle, 1997; Mitter & Brooks, 1983). In addition, complete species level phylogenetic hypotheses are being increasingly used to investigate factors associated with species diversification. A phylogenetic tree provides evidence on the relative rate of lineage splitting among clades, and can therefore be used to test whether particular traits are associated with higher or lower rates of species formation (Mitter, Farrell &
Wiegmann, 1988; Barraclough, Harvey & Nee, 1995; Barraclough, Hogan & Vogler, 1999; Barraclough & Nee, 2001). Species-level phylogenetic hypotheses can therefore be highly informative, especially in taxa that have been the object of extensive ecological and evolutionary study.

**UNUSUAL ECOLOGICAL AND BEHAVIOURAL TRAITS IN HELICONIUS**

The genus *Heliconius* or passion-vine butterflies, together with the closely-related genera *Laparus*, *Eueides*, and *Neruda*, are one of the best-known groups of Neotropical butterflies, and have been important in studies of ecological processes such as coevolution between insects and plants (Brown, 1981). These derived members of the subtribe Heliconiina have undergone rapid speciation and divergence, while also exhibiting impressive mimetic convergence in wing patterns. Additionally, *Heliconius* butterflies have two traits that may have facilitated rapid adaptive radiation, pollen feeding and pupal-mating behaviour (Gilbert, 1991).

Most adult lepidopterans feed on fluid resources such as nectar, decomposing animals and fruit, and dung. However, Gilbert (1972) showed that *Heliconius* butterflies collect pollen for its nutritive value, rather than as an indirect result of visits for nectar as had previously been assumed. The butterflies collect and accumulate large loads of pollen and the production of abundant saliva helps keep pollen attached to the proboscis, which can gently masticate the pollen load for long periods, allowing butterflies to obtain amino acids (Gilbert, 1972). Amino acids assimilated from pollen increase egg production and enable a long adult life span of up to 6 months (Gilbert, 1972; Boggs, Smiley & Gilbert, 1981; Mallet, McMillan & Jiggins, 1998). In addition, pollen can provide nitrogen and precursors for synthesis of cyanogenic glycosides that may increase the concentration of defensive chemicals in adult butterflies (Cardoso, 2001; Nahrstedt & Davies, 1981).

Morphological studies have revealed no unique structures among the species that use pollen in their diets (Penz & Krenn, 2000; Krenn, Zulka & Gatschnegg, 2001). However, there are a combination of features that assist collection and processing of pollen. For example, *Laparus* and *Heliconius* have the second segment of the labial palpi cylindrical rather than club-shaped as in the rest of the Heliconiina. Penz (1999) suggested that narrow labial-palpi help *Heliconius* and *Laparus* to keep pollen attached to their proboscis. Behavioural modifications are also important: pollen-feeding species manipulate *Lantana* flowers faster and more thoroughly compared to nonpollen feeding relatives (Krenn & Penz, 1998).

A second unusual trait found in some *Heliconius* species is a unique mating behaviour known as ‘pupal-mating’. Males of certain species search larval food plants for female pupae. The males then sit on the pupae a day before emergence, and mating occurs the next morning, before the female has completely eclosed (Gilbert, 1976; Deinert, Longino & Gilbert, 1994). Various kinds of pupal-mating occur scattered across several insect orders (Thorhill & Alcock, 1993); in passion-vine butterflies, almost half the *Heliconius* species (42%) are pupal-maters (Gilbert, 1991). It has long been thought that pupal-mating has a single origin within *Heliconius*, without subsequent loss. However, previous data do not provide strong statistical support for monophyly of the pupal-mating group (Brower, 1997; Beltrán et al., 2002).

Gilbert (1991) suggested that pupal-mating might play an important role in the radiation of *Heliconius*, as well as in the packing of *Heliconius* species into local habitats. Pupal-mating might enhance the possibility of intrageneric mimicry because, in most cases, each mimetic species pair consists of a pupal-mating and a nonpupal-mating species. The strikingly different mating tactics of these groups could allow phenotypically identical species to occupy the same habitats without mate recognition errors. Second, this mating tactic may influence host-plant specialization, as it has been suggested that pupal-mating species may displace other heliconiines from their hosts by interference competition (Gilbert, 1991). Males of these species sit on, attempt to mate with, and disrupt eclosion of other *Heliconius* species of both mating types. This aggressive behaviour may prevent other heliconiine species from evolving preference for host plants used by pupal-mating species.

Additionally, virtually all larvae in the Heliconiina subtribe are warnedly coloured to some degree and almost 50% of *Heliconius* species deposit their eggs in clusters with associated larval gregariousness (Brown, 1981). Sillén-Tullberg (1988) proposed that aggregation among butterfly larvae arises after the evolution of unpalatability, because gregariousness ought to be disadvantageous for palatable organisms that live in exposed habitats and are relatively immobile. By contrast, gregariousness can be advantageous for unpalatable organisms because the predator avoids prey after a few encounters. Sillén-Tullberg (1988) tested this idea among several groups of butterflies, including the Heliconiina. Using the phylogeny of Brown (1981), she inferred five cases of independent evolution of gregariousness and four reversals to solitary living for the Neotropical heliconines, all of them evolving after warning coloration.

Recent phylogenetic analyses (Brower, 1994a; Penz, 1999) have led to disagreement over the phylogenetic
relationships between the heliconiine butterflies. Therefore, a more complete phylogeny is needed to investigate the evolution of pollen feeding, pupal mating, and larval gregariousness.

**Systematics of Heliconius butterflies**

In the last 60 years, seven major studies have addressed the systematics of the passion-vine butterflies or Heliconiina (Michener, 1942; Emsley, 1963, 1965; Brown, 1981; Brower, 1994a; Brower & Egan, 1997; Penz, 1999) (Fig. 1). Current taxonomy places the ‘passion vine butterflies’ as a subtribe, Heliconiina, within the tribe Heliconiini. This tribe includes various other Asian genera, as well as the neotropical genera considered here. The Heliconiini are placed in the nymphaline subfamily Heliconiinae,

![Phylogenetic Analysis of Heliconius](image-url)
which also includes the Argynnini or fritillaries, and the Acræini (Lamas et al., 2004).

The revisions of Michener (1942) and Emsley (1963, 1965) (Fig. 1A) included species in Heliconius that are currently classified in the genera Eueides Hübner, Laparus Billberg & Neruda Turner. Turner (1976) formally recognized three subgenera, Neruda, Laparus, and Eueides, as distinct from Heliconius (sensa stricto). Neruda is characterized by a distinct wing shape, particularly the broad triangular forewings with very extensive friction patches in the male, although the females have wings of more typical shape for Heliconius. Other characters are the lack of scoli on the head of the larva, pupal morphology, and short antennae in the adult. Turner (1976) also considered Laparus sufficiently distinct to be a candidate for generic rank, in particular due to the pupae, which lack the gold spots and flanges and well developed antennal spines of other species. In addition, Laparus has a marked colour polymorphism as an adult, is the only species with marked morphological polymorphism as a pupa, and is the only species, apart from Neruda metharme, to produce blue colour not by iridescence but by laying white scales over black (Turner, 1976).

Brown (1981) considered Heliconius (s.l.) to consist of four separate genera: Eueides (12 species), Neruda (three species), Laparus (one species), and Heliconius (38 species) (Fig. 1B), following Turner (1976), and used characters to justify monophyly of his species groupings. However, neither he nor any of the earlier commentators performed any formal phylogenetic analysis. Cethosia, an Old World heliconiine genus, was used to root the tree and place Agraulis, Dione, Podotricha, Dryadula, and Dryas as a group paraphyletic or ‘basal’ to Heliconius (s.l.). As in Cethosia, in the ‘basal’ group, the wing venation of the discal cell of the hind wing is open. These ‘open cell’ heliconiines are generally fast flying to avoid predation and are relatively edible (Brower, 1995). In addition, their highly dispersive populations are associated with open sunny habitats, where they visit specialized butterfly pollinated flowers with short corollas and large floral displays (e.g. Lantana) (Gilbert, 1991).

The remaining genera Eueides, Neruda, Heliconius, and Laparus (i.e. Heliconius (s.l.)), were termed the ‘advanced genera’ and are the most diverse in terms of numbers of species. All of these possess a closed discal cell (Brown, 1981). Their wing patterns differ from the general nymphaline ground plan by great simplification and loss of many elements, as well as by the appearance of several novel mimetic patterns (Nijhout, 1991). The ‘closed-cell’ genera, Eueides, Neruda, Heliconius, and Laparus are relatively unpalatable, aposematic, and slow flying. Heliconius and Laparus also feed on pollen from specialized butterfly pollinated flowers such as Psiguria (Gilbert, 1991). Within Heliconius, Brown used the absence of a signum on the female bursa copulatrix as a character to define the pupal-mating group (erato + sara sapho group; Fig. 1B).

Recent contributions (Brown, 1994a; Brower & Egan, 1997; Penz, 1999) have proposed new phylogenetic hypotheses for passion-vine butterflies. All these analyses employed formal analyses using parsimony or weighted parsimony analysis. Brower (1994a) presented a cladogram based on parsimony, with successive approximations weighting, for 35 species of Heliconius and the related genera Eueides, Laparus, and Neruda, based on mtDNA sequences from cytochrome oxidase subunits I and II (950 bp of CoI and 950 bp of CoII) (Fig. 1C). The data supported most traditionally recognized species groups and also the monophyly of the four closed-cell genera with respect to other heliconiine outgroups. However, in Brower’s phylogeny Heliconius (s.s.) was made paraplyetic by the internal placement of Eueides, Laparus, and Neruda. Most surprisingly Eueides was nested within the Heliconius pupal-mating group.

Three years later Brower & Egan (1997) added a short nuclear protein-coding sequence from the gene wingless (wg, 375 bp) to the mtDNA and this led to a revision of the position of Eueides. Neither of these two gene regions alone supported the monophyly of Heliconius with respect to Eueides but simultaneous parsimony analysis supported a topology largely in agreement with traditional views of heliconiine relationships based on morphology, in which Eueides is basal to Heliconius, Neruda, and Laparus. However, Heliconius remained paraplyetic because Neruda and Laparus still branched internally to the genus (Fig. 1D). These results suggested that pollen-feeding behaviour evolved in the common ancestor of Laparus and Heliconius and was subsequently lost in an ancestor of Neruda.

Most recently Penz (1999) proposed a higher-level phylogeny for the passion-vine butterflies based on 146 morphological characters from early stages and adults. She analysed 24 exemplar species representing the ten currently accepted genera of Heliconiina. The phylogeny derived from the combined analysis of character sets gathered from different life stages supported the monophyly of all genera but differed in topology from previous hypotheses (Fig. 1E). In particular, unlike the molecular hypotheses, Heliconius was monophyletic with respect to Laparus, Eueides, and Neruda, a grouping supported by three pupal morphology characters. Penz (1999) and Penz & Peggie (2003) suggested that pollen-feeding behaviour either evolved independently in Laparus and the
ancestor of Heliconius, or evolved in the common ancestor of the genera Laparus, Neruda, Eueides, and Heliconius but was subsequently lost by the ancestor of Neruda and Eueides.

**CONFLICT BETWEEN PHYLOGENIES**

In summary, the current phylogenetic hypotheses are in conflict with one another, in particular with regard to the relationships among the genera Heliconius, Eueides, Neruda, and Laparus. Three features might contribute to this conflict: taxon sampling, number of informative characters, and methods of phylogenetic inference (Brower, DeSalle & Vogler, 1996).

Sampling selected species in each higher taxon can result in erroneous hypotheses of character state homology that lower accuracy of phylogenetic inference. Simulations have shown that using species as terminal taxa gives the most accurate trees under almost all conditions, often by a large margin (Wiens, 1998). Therefore, the broad species sampling is a positive aspect of the DNA analysis by Brower (1994a) and Brower & Egan (1997), in contrast with the morphological analysis of Penz (1999) where just one species per genus was sampled.

In molecular systematics the inference of phylogenies can benefit from a combination of data sets that evolve at different rates (Huelsenbeck et al., 2001). The study by Brower & Egan (1997) clarified the position of Eueides by including the slower evolving nuclear gene wg (Brower & DeSalle, 1998). However, the number of characters informative for the basal branches of the Heliconiina remains low, due to saturation at third positions in CoI and CoII (Brower, 1996a) and short wg sequences (375 bp). Resolution of relationships could improve from addition of more nuclear gene sequences.

Finally, previous species-level phylogenetic analyses of the heliconiines have all used maximum parsimony (MP), although recent work suggests that model-based approaches such as maximum likelihood and Bayesian methods commonly outperform MP with difficult phylogenetic data sets (Huelsenbeck et al., 2001). It would therefore benefit our understanding of heliconiine systematics to apply modern model-based methods to the analysis of molecular data.

**IMPLICATIONS FOR THE EVOLUTION OF KEY TRAITS**

These conflicts and uncertainties in the phylogenetic hypotheses for the heliconiine species have implications for our understanding of the evolution of the traits discussed above. To establish a useful robust phylogenetic hypothesis for the heliconiines, it would be helpful to add more taxa, more molecular data, and to compare the results from different methods of phylogenetic inference. The principal goal of the present study was to construct a species level phylogeny using more data from mitochondrial DNA and exons of nuclear genes, and include more taxa. This phylogenetic hypothesis was then used to address the following questions. Is Heliconius monophyletic? How many times has pollen feeding arisen in the Heliconius group? What are the relationships within major clades of Heliconius? Is the pupal-mating group monophyletic? How many times has larval gregariousness evolved in the group?

**MATERIAL AND METHODS**

**SAMPLING METHODS**

We sampled 122 individual butterflies, representing 38 Heliconius, ten Eueides, and ten outgroup species (see Supplementary Material, Table S1). According to the classification of Lamas (1998) and Lamas et al. (2004), only 11 species of Heliconiina are missing from the study: four species of Heliconius (Heliconius astreae, Heliconius latitae, Heliconius tristero and Heliconius luciana), one Neruda (Neruda godmani), one rare Eueides (Eueides emsleyi), and five outgroup heliconiines (Podotricha judith, Philaethria constantinoi, Philaethria ostara, Philaethria pygmaliaon, and Philaethria wernickei) (see Supplementary Material, Table S1). To evaluate relationships between basal Heliconiina, we included Castilia perilla (Nymphalidae: Nymphalinae: Melitaeini: Phyciodina) as an outgroup. Butterflies collected for the study were preserved in liquid nitrogen and are stored in the Smithsonian Tropical Research Institute in Panama. Wings of voucher specimens are preserved in glassine envelopes (images are available at http://www.heliconius.org). From each individual, one-sixth of the thorax was used and the genomic DNA was extracted using the DNeasy Kit (Qiagen) following the manufacturer’s recommended protocols. Samples from different prior collections were obtained as DNA aliquots.

**MITOCHONDRIAL DNA**

Two mitochondrial DNA regions were used: first, a region of cytochrome oxidase (Co), spanning cytochrome oxidase subunits I (CoI), the mitochondrial gene for leucine transfer RNA gene (tRNA-leu), cytochrome oxidase subunits II (CoII); and second, the region coding for 16S ribosomal RNA (16S). Both regions have been used to explore phylogenetic relationships in insects (DeSalle, 1992; Brower, 1994a, b; Caterino & Sperling, 1999; Smith, Kambhampati & Armstrong, 2002), although here we use 1611 bp of CoI + CoII compared to Brower's 950 bp. Two different
sampling strategies were followed: for CoI + CoII at least two individuals per species were sequenced and for 16S just 12 individuals were sequenced in order to check the relationships within Heliconius (811 Heliconius melpomene rosina, 346 Heliconius numata, 8560 Heliconius burneyi, 8549 Heliconius hecuba, 846 Laparus doris, 8569 Neruda aede, 440 Heliconius erato hydara, 8537 Heliconius clysonymus, 842 Heliconius clysonymus eleuchia, 8037 Heliconius clysonymus, 8548 Heliconius numata, Heliconius melpomene rosina, to check the relationships within Heliconius by the Owen McMillan laboratory in Puerto Rico and were isolated in Drosophila in 1995, and were sequenced by GenBank accession no. X03240) was used as a reference. The clean template obtained was sequenced in a 10 μL cycle sequence reaction mixture containing 1 μL BigDye, 0.3 × buffer, 2 mM primer, and 2 μL of template. The cycle profile was 96 °C for 30 s, then 96 °C for 10 s, 50 °C for 15 s, and 60 °C for 4 min for 30 cycles. This product was cleaned by precipitation using 37.5 μL of 70% EtOH and 0.5 mM MgCl₂. The samples were re-suspended in 4 μL of a 5:0.12 deionized formamide: crystal solution, denatured at 85 °C for 2 min and loaded into 5.5% acrylamide gels. Gels were run on BaseStation (MJ Research) for 3 h.

The additional mitochondrial region used was the 16S. This region was amplified using 16Sar1 5'-CCC GCC TGT TTA TCA AAA ACA T-3' and Ins16Sar 5'-CCC TCC GGT TTG AAC TCA GAT C-3'. Primers were obtained by modifying those of Palumbi (1996) to improve amplification in Lepidoptera. The identity of this region was confirmed by comparison with Eresia burchellii (GenBank accession no. AF188861). Double-stranded DNA was synthesized in 10-μL reactions containing 2 μL of genomic DNA, 1 × buffer, 1 mM MgCl₂, 0.8 mM dNTPs, 0.5 mM of each primer, and 0.05 μL of Taq polymerase. DNA was amplified using the following step-cycle profile: 94 °C for 5 min, 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min for 34 cycles. These products were sequenced as described for CoI and CoII.

**Nuclear loci**

Four nuclear loci were used, elongation factor-1α (Eflα), apertuous (ap), decapentaplegic (dpp) and wingless (wg). Eflα is a key factor in protein synthesis playing a central role in protein chain elongation (Bischoff et al., 2002). This gene has been used in many phylogenetic studies and the results have demonstrated informativeness of synonymous nucleotide substitutions up to divergences of 60 myr (Cho et al., 1995; Mitchell et al., 1997; Reed & Sperling, 1999). The genes ap and dpp are involved in wing development in Drosophila and were isolated in Heliconius by the Owen McMillan laboratory in Puerto Rico (Jiggins et al., 2005; Tobler et al., 2005), but there is no report of their phylogenetic utility. The sampling for Eflα was the same as CoI and CoII, and the sampling for ap and dpp was the same as 16S (just 12 individuals representing the major clades in Heliconius). In addition, wg sequences were included in the analysis although not for the same individuals. Sequences of wg were loaded from Brower’s GenBank accessions AF014126 to AF014135, and AF169869 to AF169921.

The Eflα region was initially amplified and sequenced from genomic DNA using a mix of primers from Papilio (Efl-1) (Reed & Sperling, 1999) and bumble bees (F2-rev) (Walldorf & Hovemann, 1990). The primers were situated at position 15 (Efl-1) and 955 (F2-rev) of Papilio glaucus (GenBank accession no. AF044826). Then, initial Heliconius sequences were aligned and Heliconius specific primers were designed to amplify the region consistently using genomic DNA extracts. The specific primers designed were Efl-H-5-LAG GAA GGC CAG GAA ATG-3' and Efl-H-r 5'-CCT TGA CRG ACA GTG TTC TT-3'. DNA was amplified using the step cycle profile described for 16S and sequenced as for the mitochondrial region.

The other two nuclear genes sequenced were ap and dpp. The gene ap was amplified using primers ap-f35 5'-TGA ATC CTG AAT ACC TGG AGA-3' and ap-r224 5'-GGACC ATA CCT GAA CCC-3' and dpp using dpp-f34 5'-AGA GAA GGC CAG GAC ACA CTG-3' and dpp-r327 5'-GAG GAA AGT TGC GTA GGA AC-3' (Jiggins et al., 2005; Tobler et al., 2005). The identities of the regions were verified by aligning with Precis coenia GenBank accession no. L42140 and L42141, respectively. The products from ap and dpp were sequenced as described above.

**ALIGNMENT AND PHYLOGENETIC ANALYSES**

Chromatograms were edited and base calls checked using SEQUENCER, version 4.1 (Gene Codes Corporation, Inc). The protein-coding mtDNA and nuclear DNA sequences were checked for reading-frame errors and unexpected stop codons by translating the nucleotide sequences to peptides using MacClade, version 4.0 (Maddison & Maddison, 1997). Maximum likelihood models of sequence evolution for each gene were estimated using ModelTest, version 3.04 (Posada & Crandall, 1998). Bayesian analysis run in MrBayes (Huelsenbeck & Ronquist, 2001) was used to infer the phylogeny based on the best-fit model selected by ModelTest. Model parameter values were estimated for each gene separately in the combined analysis. Four chains were run simultaneously, each Markov chain was started from a random tree and run for one million generations, sampling a tree every 100
generations. The log-likelihood scores of sample points were plotted against generation time to determine when the chain became stationary. All sample points prior to reaching stationarity (2000 trees) were discarded as burn-in samples. Data remaining after discarding burn-in samples were used to generate a majority rule consensus tree, where percentage of samples recovering any particular clade represented the posterior probability of that clade (Huelsenbeck & Ronquist, 2001). Probabilities ≥ 95% were considered indicative of significant support. Branch lengths of the consensus tree were estimated by maximum likelihood. Although model-based methods are preferable, we also present MP analyses to facilitate comparison with previous work. MP trees were obtained using PAUP*, version 4.0b8 (Swofford, 2000) in an equal weighted heuristic search with tree-bisection-reconnection (TBR) branch swapping. The consensus tree was calculated using majority rule. Bootstrap (1000 replicates, heuristic search TBR branch swapping) was used to assess support for each node.

The Incongruence for Length Difference test (ILD; Farris et al., 1994) implemented by PAUP* was used to test incongruence between the different partitions [e.g. Col/ColII versus Ef1α; mtDNA (Col, tRNA-leu, ColII, 16S) versus nuclear (Ef1α, ap, dpp, wg); Col versus ap; Ef1α versus ap, etc.]. This test was applied to a matrix including the 12 individuals sequenced for Col, ColII, Ef1α, ap, and dpp adding wg sequences of GenBank for these species. Additionally, to test specific hypotheses, alternative a priori scenarios were compared using the method of Shimodaira & Hasegawa (Shimodaira & Hasegawa, 1999; Goldman, Anderson & Rodrigo, 2000) and implemented using PAUP*, version 4.0b8. For each genus (i.e. Heliconius, Laparus, Neruda, Eueides), two or three topologies were compared in the same test. To generate trees for each scenario, the topology shown in Figure 3 was modified using MacClade (Maddison & Maddison, 1997). Finally, to establish the relative sequence of the evolution of gregariousness among the heliconinones, data on egg-laying habits and larval sociality (Brown & Benson, 1977; Brown, 1981; J. Mallet, pers. observ.) were mapped on onto our phylogeny using parsimony implemented in MacClade (Maddison & Maddison, 1997). The outgroup character state was considered as unknown. To resolve equivocal ancestral states we compared results using ACCTRAN (accelerated changes) and DELTRAN (delayed changes) optimizations.

RESULTS

CHARACTERIZATION OF THE NUCLEOTIDE DATA

The final nucleotide data set contained 3834 positions (2119 mitochondrial, 1716 nuclear), translating to 1083 amino acids (511 mitochondrial, 572 nuclear). The individual sequences are available as GenBank accession numbers in the Supplementary material (Table S1) and the alignment of full data are available at http://www.heliconius.org.

For mitochondrial DNA (mtDNA) 1611 bp were obtained from the Col + ColII region including nucleotides and gaps. These represent 822 bp of Col corresponding to position 2191–3009 of the D. yakuba sequence (X03240), the complete tRNA-leu gene (78 bp) and 711 bp representing the entire ColI coding sequence, matching positions 3012–3077 and 3083–3766 in D. yakuba, respectively. For 16S ribosomal RNA 512 bp were amplified corresponding to positions 26–541 in E. burchelli (AF186861). Length variation was concentrated in tRNA-leu and in 16S. At the beginning of tRNA-leu, an insertion of 12 bp was found in one individual of H. demeter (STRI-B-8563) whereas 7 bp of the same insertion was shared by Heliconius charithonia, Heliconius peruvianus, Heliconius ricini, and the second individual of H. demeter (STRI-B-8562). Another 3 bp insertion was observed at position 71 in Heliconius ismenius. In the 16S region, a total of 29 gaps were found located between positions 51–63, 241–280, and 337–351. Additionally, codon deletions were found. In Col the third codon of the alignment, corresponding to amino acid position #243 in D. yakuba, X03240), was deleted in some Eueides species (Eueides lineata, E. vibilia, Eueides lybia, Eueides aliphera, Eueides isabella, and Eueides tales). There was another codon deletion in H. ismenius just before the Col stop codon. In ColII, three closely adjacent codon deletions were observed at amino acid position #126 in Dryadula phaetusa, #127 in H. sara and at position #129 in D. iulia.

The nuclear genes Ef1α 876 bp, ap 195 bp and dpp 270 bp were aligned with P. glaucus (GenBank accession no. AF044826) at positions 50–925, P. coenia (L42140) at positions 193–387, and P. coenia (L42141) at positions 145–414, respectively. Only dpp showed length variation with respect to the reference sequence, a codon deletion at position 196 of P. coenia (L42141) was observed in Heliconius cydno chioneus, H. numata and H. burneyi.

Patterns of genetic variability for mitochondrial and nuclear regions are shown in Tables 1 and 2, and models of sequence evolution for the same regions are described in Table 3.

CONGRUENCE TEST

ILD tests between mitochondrial data (Col + tRNA-leu + ColII + 16S) versus nuclear data (Ef1α + ap + dpp + wg) provided no evidence for incongruence based on nucleotides (P = 0.08), or between amino acid
PHYLOGENETIC ANALYSES

Our phylogenetic hypothesis for the Heliconiinae included more species and more phylogenetic information than previous studies. Nine new species were added to those used by Brower & Egan (1997): five Heliconius (Heliconius nattereri, Heliconius hierax, Heliconius hecalesia, H. peruvianus, and Heliconius hermathena); four Eueides (Eueides lampeto, Eueides pavana, E. lineata, Eueides heliconioides), and one outgroup species (Dione moneta). Following Lamas et al. (2004), the species included represent 36 of 40 Heliconius species (90%), and 60 of 69 (86%) of the species in the subtribe Heliconiina. One of the missing species is H. tristero, recently described by Brower (1996b); however, this species is very close to and/or a hybrid of H. melpomene and H. cydno. The remaining missing species H. astraea, H. laitata, and H. luciana, were difficult to obtain as they are restricted to small areas of Brazil, French Guiana, and Venezuela, respectively; they probably belong to the 'primitive' Heliconius group (Figs 2, 3; Brown, 1981). Additionally, three new nuclear regions were studied for this subtribe Ef1α (876 bp), ap (195 bp) and dpp (270 bp), and 659 bp were added to the 950 bp Col + Coll region reported by Brower (1994a, b) and Brower & Egan (1997) for Heliconius.

Topologies for individual data sets are shown in Figure 2A (mtDNA), Figure 2B (Ef1α), and Figure 2C (ap, dpp and ug) and the combined hypothesis using all genes is shown in Figure 3. Phylogenetic resolution was somewhat weaker at nuclear loci compared with the mtDNA. For example, mtDNA and Ef1α showed a monophyletic clade that included the sister clades cydno-melpomene and the silvaniforms but, in Ef1α, there was no resolution of species relationships within that clade (Fig. 3A, B). However resolution increased for clades in which species are more distantly related such as sarala/sapho and erato.

The data also produced well-resolved relationships among genera (Fig. 3). Heliconius, Leparbus, and Neruda formed a well-supported monophyletic clade with Eueides basal to this group, in agreement with traditional relationships and prior molecular hypotheses (Brown, 1981; Brower & Egan, 1997). Leparbus fell in a well-supported clade with H. hierax, Heliconius wallacei, and H. hecuba. Also, Neruda fell within Heliconius closely related to cydno/melpomene and the silvaniform group.

CHARACTER MAPPING AND TOPOLOGY COMPARISONS

Systematic pollen feeding has been observed in both Heliconius and Leparbus species that have been studied in the wild, but is not seen in Eueides, Neruda, or other genera (Gilbert, 1972). Thus, our phylogenetic hypothesis implies a single origin for

sequence partitions (P = 0.23). Comparisons within mtDNA did not show any incongruence either (e.g. Col versus CoII, P = 0.18; CoI + CoII versus 16S, P = 0.40), and neither did mtDNA versus individual partitions of nuclear genes. Within nuclear genes, only one comparison showed significant incongruence, Ef1α versus ug (P = 0.01) and it was the only significant test out of 18 comparisons in total. Therefore, there was no strong evidence for significant incongruence between data sets and total data were used to calculate a combined evidence phylogenetic hypothesis.

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Table 1. Nucleotide variability over genes and codon position. The values were calculated for the whole data set in Co (Col + tRNA-leu + ColI) and Eflα. CI, consistency index; RI, retention index

<table>
<thead>
<tr>
<th></th>
<th>All sites</th>
<th>Codon position 1</th>
<th>Codon position 2</th>
<th>Codon position 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of characters</td>
<td>1611</td>
<td>511</td>
<td>511</td>
<td>511</td>
</tr>
<tr>
<td>Number of invariants</td>
<td>955</td>
<td>375</td>
<td>454</td>
<td>66</td>
</tr>
<tr>
<td>Number variable</td>
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<td>136</td>
<td>57</td>
<td>445</td>
</tr>
<tr>
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<td>587</td>
<td>106</td>
<td>32</td>
<td>417</td>
</tr>
<tr>
<td>Tree length</td>
<td>3751</td>
<td>460</td>
<td>109</td>
<td>3086</td>
</tr>
<tr>
<td>CI</td>
<td>0.262</td>
<td>0.361</td>
<td>0.596</td>
<td>0.236</td>
</tr>
<tr>
<td>RI</td>
<td>0.715</td>
<td>0.797</td>
<td>0.799</td>
<td>0.704</td>
</tr>
<tr>
<td>Eflα</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of characters</td>
<td>876</td>
<td>292</td>
<td>292</td>
<td>292</td>
</tr>
<tr>
<td>Number of invariants</td>
<td>615</td>
<td>259</td>
<td>271</td>
<td>84</td>
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<tr>
<td>Number variable</td>
<td>261</td>
<td>33</td>
<td>20</td>
<td>208</td>
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<tr>
<td>Number of invariants</td>
<td>186</td>
<td>12</td>
<td>5</td>
<td>169</td>
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<tr>
<td>Tree length</td>
<td>734</td>
<td>53</td>
<td>34</td>
<td>647</td>
</tr>
<tr>
<td>CI</td>
<td>0.47</td>
<td>0.66</td>
<td>0.67</td>
<td>0.44</td>
</tr>
<tr>
<td>RI</td>
<td>0.83</td>
<td>0.83</td>
<td>0.6</td>
<td>0.83</td>
</tr>
</tbody>
</table>

Table 2. Nucleotide variability for the additional genes sequenced just for 12 species representing the major clades. CI, consistency index; RI, retention index

<table>
<thead>
<tr>
<th>Gene</th>
<th>16S</th>
<th>ap</th>
<th>dpp</th>
<th>wg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of characters</td>
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<td>195</td>
<td>270</td>
<td>375</td>
</tr>
<tr>
<td>Number of invariants</td>
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<td>Number variable</td>
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<td>94</td>
</tr>
<tr>
<td>Number of invariants</td>
<td>38</td>
<td>15</td>
<td>27</td>
<td>34</td>
</tr>
<tr>
<td>Tree length</td>
<td>155</td>
<td>54</td>
<td>86</td>
<td>157</td>
</tr>
<tr>
<td>CI</td>
<td>0.748</td>
<td>0.722</td>
<td>0.837</td>
<td>0.669</td>
</tr>
<tr>
<td>RI</td>
<td>0.426</td>
<td>0.423</td>
<td>0.745</td>
<td>0.212</td>
</tr>
</tbody>
</table>

Pupal mating behaviour has been studied in H. erato and Heliconius hewitsoni and observed in other members of the erato and sarasapho groups (Deinert et al., 1994). Previous authors have inferred that all members of these clades are pupal mating, although mating behaviour has not been documented in some of the rarer species. However, pupal mating has never been observed in heliconiines outside this clade so we can infer a single origin in the common ancestor of these groups. Monophyly of this clade was highly supported by Bayesian and MP analysis (Fig. 3, black heart).

For comparison, we carried out a re-analysis of the mtDNA data of Brower (1994a), in which Eueides clustered with H. charithonia, making the pupal mating clade paraphyletic. An ML tree reconstructed using the mtDNA data of Brower (1994a), based on the general-time-reversible time model of nucleotide substitution (GTR + Σ +I) (Yang, 1994), showed Eueides basal to Heliconius. Similarly, Bayesian analysis of the same data set showed strong support for placing Eueides basal to Heliconius. Nonetheless, even in our larger mtDNA data set, the method of Shimodaira & Hasegawa (Shimodaira & Hasegawa, 1999) still could not rule out the possibility that Neruda was basal to Heliconius (mtDNA, P = 0.507; nuclear, P = 0.365; combined, P = 0.329).
Table 3. Best supported models of molecular evolution and estimated parameter values for the different data sets

<table>
<thead>
<tr>
<th>Data set</th>
<th>Col + CoII</th>
<th>16S</th>
<th>Ef/1α</th>
<th>ap</th>
<th>dpp</th>
<th>wg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>GTR + I + G</td>
<td>F81 + G</td>
<td>GTR + I + G</td>
<td>K2P + G</td>
<td>K2P + G</td>
<td>TrNef + G</td>
</tr>
<tr>
<td>Base frequencies</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.374</td>
<td>0.4421</td>
<td>0.28</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>C</td>
<td>0.1081</td>
<td>0.0649</td>
<td>0.2444</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>G</td>
<td>0.0647</td>
<td>0.1234</td>
<td>0.2447</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>T</td>
<td>0.4532</td>
<td>0.3697</td>
<td>0.2313</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Substitution model</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tr/tv ratio</td>
<td>All equal rates</td>
<td>1.9162</td>
<td>2.1976</td>
<td>6.2038</td>
<td>12.7904</td>
<td></td>
</tr>
<tr>
<td>[C-T]</td>
<td>27.3268</td>
<td>1.6729</td>
<td>1.7038</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tv [A-C]</td>
<td>2.9031</td>
<td>3.3225</td>
<td>1.7038</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[A-T]</td>
<td>1.7001</td>
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<td>1</td>
<td></td>
<td></td>
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<tr>
<td>[C-G]</td>
<td>2.8548</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[G-T]</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invariable sites</td>
<td>0.5001</td>
<td>0.543</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gamma parameter</td>
<td>0.5187</td>
<td>0.0876</td>
<td>0.0639</td>
<td>0.2419</td>
<td>0.3561</td>
<td></td>
</tr>
</tbody>
</table>

GTR, six-parameter general time reversible model of nucleotide substitution (Yang, 1994); TrNef, model of Tamura & Nei (1993); F81, model of Felsenstein (1981); K2P, two-parameter model of Kimura (1980); I, invariable sites; G, gamma parameter.

Figure 3. Bayesian phylogenetic hypothesis for heliconiine species based on combined mitochondrial (Co and 16S) and nuclear data (Ef/1α, dpp, ap and wg). Only one individual per species was used and the wg sequences included were from GenBank. Branch lengths were estimated using maximum likelihood. Values above branches show Bayesian probabilities and those below show parsimony bootstrap support for the equivalent node, after 1000 replicates. Branches without support were not found in the maximum parsimony bootstrap consensus tree. P, Panama; E, Ecuador; G, French Guiana; C, Colombia; Pe, Peru. Black clubs indicate the gain of pollen feeding behaviour, black hearts indicate the gain of pupal-mating. White clubs and hearts represent loss of the same traits, respectively.

Eueides was part of the pupal mating group (mtDNA, $P = 0.266$; nuclear, $P = 0.017$; combined, $P = 0.005$). However, this hypothesis was a significantly worse fit to our data based on either nuclear DNA or combined evidence data.

Our phylogeny suggested at least three independent origins for larval gregariousness from a solitary ancestor (Fig. 4). Resolving the equivocal branches, ACCTRAN (accelerated changes) showed three origins with four reversals to solitary living. By contrast, DELTRAN (delayed changes) showed seven independent origins of gregariousness. Two of the possible reversals to solitary living, in *E. lampeto* and *D. glycera*, show low branch support in our phylogenetic hypothesis and must therefore be treated with caution.

**DISCUSSION**

The phylogenetic hypothesis from combined evidence (Fig. 3) largely agrees with that of Brower & Egan (1997). Of 25 nodes at or above the species level, 23 are concordant including the position of the genera *Eueides*, *Neruda* and *L. doris*. The position of *L. doris* as a member of *Heliconius* was well supported. The position of *Neruda* within *Heliconius* was independently supported by nuclear and mtDNA data (Fig. 2), but cannot be considered unequivocal because topology tests failed to rule out the hypothesis that *Neruda* is sister to *Heliconius*. The most probable hypothesis therefore is that pollen feeding arose once but was subsequently lost in *Neruda*. Nonetheless, we cannot reject a more parsimonious single-origin no-loss hypothesis of pollen feeding arising in a sister taxon to *Neruda*, which went on to diversify into present day *Laparus* and *Heliconius*.

Morphological studies have shown no obvious structural adaptations to feeding on pollen (Krenn & Penz, 1998), implying that this is largely a behavioural adaptation. It is perhaps surprising, therefore, that it is such a phylogenetically conserved trait being, as far as we know, unique in the Lepidoptera. For the species that do feed on pollen, it may be such an...
PHYLOGENETIC ANALYSIS OF HELICONIUS

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0.01 substitutions/site

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Figure 4. The evolution of larval sociality among the heliconiines. Boxes at branch tips indicate known larval behaviour, with 'intermediate' indicating species that generally lay solitary eggs but occasionally clump a few eggs together. For a species to be classified as gregarious, a minimum of ten eggs must be included in the range of egg or larval aggregation sizes. The outgroup character state was considered as unknown.

advantageous ecological strategy that is unlikely to be lost and is associated with the greater species diversity of the genus *Heliconius* as compared to related genera (Gilbert, 1991).

The combined results provided strong support for a monophyletic 'pupal-mating' clade, demonstrating that this unusual mating strategy has evolved only once in the group. This is therefore consistent with the previous argument that this trait has played an important role in the phylogenetic expansion of *Heliconius*, as well as in the packing of *Heliconius* species into local habitats (Gilbert, 1991). Our results contrast with the first published molecular phylogeny of *Heliconius*, reconstructed using parsimony, which showed a surprising placement of the genus *Eueides* within the pupal mating clade of *Heliconius* (Brower, 1994a). Bayesian reanalysis of the same data suggests that this was an artefact of parsimonious interpretation of homoplastic character states, perhaps due to 'long-branch attraction': fast-evolving sites in the mitochondrial *Col* gene happen to show convergent evolution between the *H. erato* group and the genus *Eueides*, but these are outweighed in the Bayesian analysis by information from putatively slower and more informative sites. In a model-based analysis, the likelihood of homoplasy is taken into account and inferred rapidly evolving sites are downweighted, leading to more realistic phylogenetic reconstruction concordant with results from other, slower-evolving genes.

Our character mapping of larval behaviour showed at least three independent origins for gregariousness in the *Heliconiini*. Depending on the character optimization methods used, we show between three and seven independent origins and between four and zero subsequent reversions to solitary living. Nonetheless, our results clearly support the hypothesis of Sillén-Tullberg (1988) proposing that gregariousness arose multiple times subsequent to the evolution of strong unpalatability.

**RELATIONSHIPS IN THE 'PUPAL-MATING CLADE'**

The 'pupal-mating clade' includes *sara/sapho*, *erato/himera* and *H. charithonia* groups (Fig. 3; Brown, 1981; Brower, 1994a; Brower & Egan, 1997). The
**RELATIONSHIPS IN THE MELPOMENE/CYNDO AND SILVANIFORM GROUP**

The *melpomene/cyndo* group and the silvaniform complex consist of a rapidly radiating group of species with little differentiation at nuclear loci. The combined analysis reveals two monophyletic groups, *melpomene/cyndo* and the silvaniforms, both with a posterior probability support of 1.0 (Fig. 3). This result is mostly due to information from mtDNA (Fig. 2A) because *Efiα* has little informative variation (Fig. 2B).

In the *melpomene/cyndo* group, races of *H. melpomene* cluster into two different clades. *Heliconius melpomene* races from west of the easternmost Andean chain in Colombia clustered with the *H. cydno* clade, whereas races of *H. melpomene* from east of the Andes were clustered with *H. melpomene* from French Guiana (Brown, 1996b; Flanagan et al., 2004). *Heliconius cyno* appeared paraphyletic with respect to *Heliconius heurippa*, *Heliconius pachinus*, and *Heliconius timareta* (Fig. 2A). Brower (1994a, 1996a) and Lamas (1998) suggested that *H. heurippa*, *H. tristero*, *H. pachinus*, and *H. timareta* might represent well-differentiated races of *H. cydno* rather than distinct species, because they are parapatric or allopatric. Clearly, these taxa are close; however, analyses of genitalia, allozymes, random amplification of polymorphic DNAs, and mating behaviour show that *H. heurippa* is a good species (Beltrán, 1999; Salazar et al., 2005; Mavarez et al., 2006).

The composition of the silvaniform complex agrees with Brower (1994a) (Fig. 3), but the exact topology differed. The *H. numata* + *H. ismenius* and *Heliconius attthis* + *Heliconius hecale* species pairs were the only nodes in agreement with Brower & Egan (1997). It has been considered that *Heliconius ethilla* is a sister to *H. attthis*, but here *H. ethilla* clustered with *H. nat-tereri*, one of the new species included. *Heliconius attthis* and *H. hecale* are sympatric in Ecuador and it is possible that their sister species relationship could be a result of recent gene exchange. Additionally, it is clear that *Heliconius elevatus* and *Heliconius besckei* are part of this complex rather than in the *melpomene/cyndo* group as proposed by Brown (1981). Most of the silvaniforms have a typical ‘tiger’ colour pattern and Brower (1994a, 1997) proposed that the ‘postman’ pattern (red forewing patches and yellow hindwing stripes on a black background) of *H. besckei* might be the ancestral colour pattern of this clade. This idea is supported here because *H. besckei* is placed basal as sister to the silvaniforms.

**PARAPHyletic taxa**

Paraphyly was observed at several different levels. Paraphyly of species relative to their sisters was observed in the *melpomene/cyndo* group, *H. melpomene* was paraphyletic with respect to a clade that includes *H. cydno* and related species. In the *erato* group, *H. erato* was paraphyletic with respect to *H. himera* and *H. hermathena* (Fig. 2A, B; Brower,
1994a, b; Brower & Egan, 1997; Flanagan et al., 2004). Second, at the genus level, Heliconius was paraphyletic with respect to Laparus and Neruda (Fig. 3).

At the species level, this paraphyly is expected due to hybridization and recent speciation. Many wild hybrids between H. cydno and H. melpomene (Mallet et al., 2006) and H. erato and H. kimera (Jiggins et al., 1996; Mallet et al., 2006) have been found, and it is known that these species have strong but incomplete reproductive isolation (McMillan, Jiggins & Mallet, 1997; Naisbit et al., 2002). There is also evidence of introgression of DNA sequences between these two species in nature (Bull et al., 2006; Kronforst et al., 2006) for this reason, and because ancestral polymorphisms may persist after speciation, phylogenies of recently evolved species, which may still exchange genes, are inevitably difficult to resolve and likely to produce paraphyletic taxa, even in cases where the initial split was a simple bifurcation. Paraphyletic patterns for the closely-related species were observed where a number of races for each species were included in the present study. This paraphyly might be observed in more pairs of sister species if more geographical populations were sequenced because approximately 33% of Heliconius species hybridize in the wild (Mallet, 2005; Mallet et al., 2006).

At the genus level, it is clear that L. doris is part of Heliconius, suggesting only a single origin for pollen feeding in the Heliconius group. Laparus doris was suggested as a different genus by Turner (1976), in part due to the marked colour polymorphism as an adult (red, yellow and the unique blue or green ray pattern in hindwing). It is the only species within Heliconius with morphological polymorphism as a pupa, and the pupa do not have the gold spots and flanges and well developed antennal spines of other species. Also, it is the only species apart from N. metharme, to produce blue colour not by iridescence, but by laying white scales over black (Turner, 1976). However, these morphological traits to support the generic status of Laparus (Turner, 1976; and see above) may not be good characters for phylogenetic analysis. Colour patterns are known to evolve rapidly, and pupal characters may be derived adaptations to gregarious larval ecology. Neruda was also defined as a subgenus by Turner (1976), due to its short antennae, wing shape, and pupal morphology. In particular, the broad triangular forewings with extensive friction patches of the male are very distinctive, although the females have wings of more normal shape for the genus Heliconius. Additionally, the Neruda larva does not have scoli on the head, as do other Heliconius species. Again, these may be rapidly evolving characters perhaps due to sexual selection and therefore misleading. We have here retained traditional nomenclature, but it is likely that the genus Laparus, at least, should be subsumed within Heliconius.

CONCLUSIONS

The Heliconiina have become an important group in the understanding of evolutionary biology, in topics as diverse as coevolution, mimicry, behavioural ecology, hybrid zones, and speciation. Overall, there is a good concordance of the molecular hypothesis presented here with previous molecular phylogenies in this group of butterflies. However, the inclusion of more species and the addition of more sequence information has clarified some relationships within the Heliconiina. The hypothesis shows that Heliconius as currently defined is not monophyletic because L. doris, and possibly Neruda, fall within the genus. These results suggest that pollen-feeding behaviour evolved only once in the common ancestor of Laparus and Heliconius. Pollen-feeding may have been lost subsequently by the ancestor of Neruda, although the addition of more genetic data might clarify further the position of Neruda. The results provided strong support for exclusion of Eueides from Heliconius and for a monophyletic ‘pupal-mating clade’ including the eratos/saral/sapho groups. Furthermore, we show that our revised phylogeny supports the hypothesis that gregariousness arose subsequent to the evolution of warning coloration (Sillén-Tullberg, 1988). This phylogenetic hypothesis can now be used to test further hypotheses regarding evolutionary patterns of rapid diversification and character evolution across the subtribe Heliconiina.

ACKNOWLEDGEMENTS

We would like to thank the Autoridad Nacional del Ambiente in Panama, Instituto de Ciencias Naturales in Peru, and the Ministerio del Ambiente in Ecuador for permission to collect butterflies; Gerardo Lamas for valuable identification of specimens; Keith Willmott and Carla Penz for sharing samples; and Maribel Gonzalez, Nimiaidina Gomez, and Oris Sanjur for help in the laboratory. This work was funded by the Smithsonian Tropical Research Institute (Panama), an Overseas Research Scheme Award (UK) and Bogue Fellowship (University College London UK) awarded to M.B. and a Royal Society University Research Fellowship to C.J.

REFERENCES


SUPPLEMENTARY MATERIAL

The following material is available for this article online:

Table S1. Heliconiina included in the study. ID numbers are STRI collection numbers and should be prefixed by ‘stri-b’ for individuals belonging to different collections (i.e. = A. Brower).

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1095-8312.2007.00830.x
(This link will take you to the article abstract).

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