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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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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. *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; Wanntorp *et al*., 1990; Miller & Wenzel, 1995; 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 &

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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 & Davis, 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 (Thornhill & 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 warningly 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 heliconiines, all of them evolving after warning coloration.

Recent phylogenetic analyses (Brower, 1994a; Penz, 1999) have led to disagreement over the phylogenetic

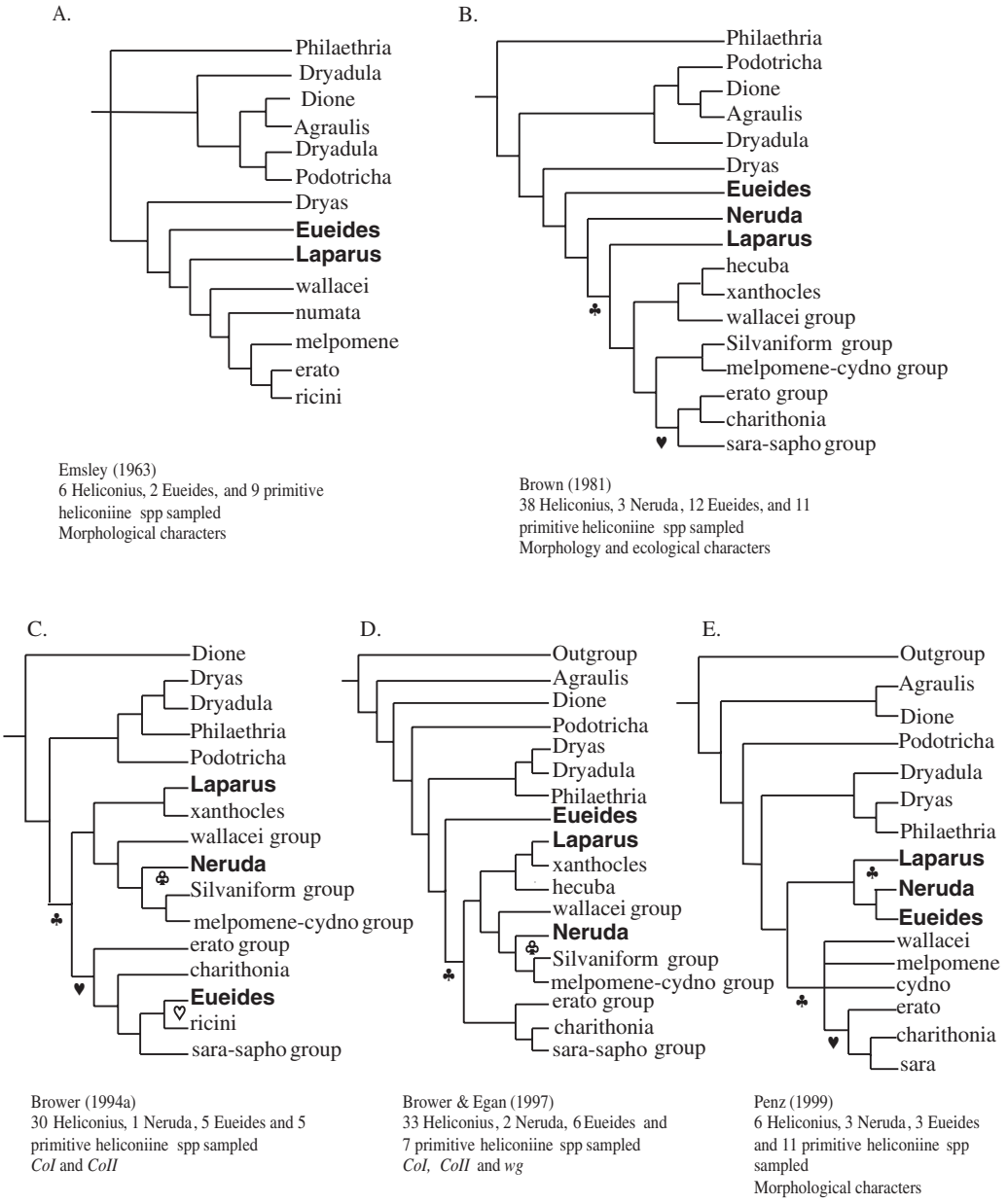


Figure 1. Summary of major phylogenetic hypotheses for heliconiine butterflies published in the last 60 years. Note that the genus name is omitted for *Heliconius*. A, Emsley (1963). B, Brown (1981). C, Brower (1994a). D, Brower & Egan (1997). E, Penz (1999). Black clubs indicate the gain of pollen feeding behaviour, black hearts indicate the gain of pupal-mating. White clubs and hearts represent loss of the same traits, respectively. *Eueides*, *Laparus*, and *Neruda* are shown in bold for clarity.

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,

which also includes the Argynnini or fritillaries, and the Acraeini (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* (*sensu 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 unspecialized 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 (Brower, 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 paraphyletic 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 paraphyletic 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 us 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 astraea*, *Heliconius lalitae*, *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 pygmalion*, 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.

Molecular regions and sequencing methods

Mitochondrial DNA

Two mitochondrial DNA regions were used: first, a region of *cytochrome oxidase* (*Co*), spanning *cytochrome oxidase subunit I* (*CoI*), the mitochondrial gene for leucine transfer RNA gene (tRNA-leu), *cytochrome oxidase subunit 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 aoede*, 440 *Heliconius erato hydara*, 8037 *Heliconius clysonymus*, 842 *Heliconius clysonymus eleuchia*, 8562 *Heliconius demeter*, 320 *Eueides vibilia*, and 293 *Dryas iulia*).

The mitochondrial *CoI* + *CoII* region was amplified using primers and protocols described previously (Beltrán *et al*., 2002). A *Drosophila yakuba* sequence (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 $MgCl_2$. The samples were re-suspended in 4 μL of a 5 : 0.12 deionized formamide: crystal violet 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 *16S*ar1 5′-CCC GCC TGT TTA TCA AAA ACA T-3′ and Ins*16S*ar 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. AF186861). Double-stranded DNA was synthesized in 10-μL reactions containing 2 μL of genomic DNA, 1 × buffer, 1 mM $MgCl_2$, 0.8 mM dNTPs, 0.5 mM of each primer, and 0.05 μ μL^{-1} of Qiagen 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-1*α (*Ef1*α), *apterous* (*ap*), *decapentaplegic* (*dpp*) and *wingless* (*wg*). *Ef1*α is a key factor in protein synthesis playing a central role in protein chain elongation (Bischoff *et al*., 2002). This gene has been using 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 *Ef1*α 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 AY090135, UO08554, AF014126 to AF014135, and AF169869 to AF169921).

The *Ef1*α region was initially amplified and sequenced from genomic DNA using a mix of primers from *Papilio* (*Ef1*–5) (Reed & Sperling, 1999) and bumble bees (F2-rev) (Walldorf & Hovemann, 1990). The primers were situated at position 15 (*Ef1*–5) 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 *Ef1*-H-f 5′-GAG AAG GAA GCC CAG GAA AT-3′ and *Ef1*-H-r 5′-CCT TGA CRG ACA CGT TCT 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′-GGA ACC ATA CCT GTA AAA CCC-3′ and *dpp* using *dpp*-f34 5′-AGA GAA CGT GGC GAG ACA CTG-3′ and *dpp*-r327 5′-GAG GAA AGT TGC GTA GGA ACG-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 SEQUENCHER, 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. *CoI*/*CoII* versus *Ef1α*; mtDNA (*CoI*, tRNA-leu, *CoII*, *16S*) versus nuclear (*Ef1α*, *ap*, *dpp*, *wg*); *CoI* versus ap; *Ef1α* versus *ap*, etc.]. This test was applied to a matrix including the 12 individuals sequenced for *CoI*, *CoII*, *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 heliconiines, 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.

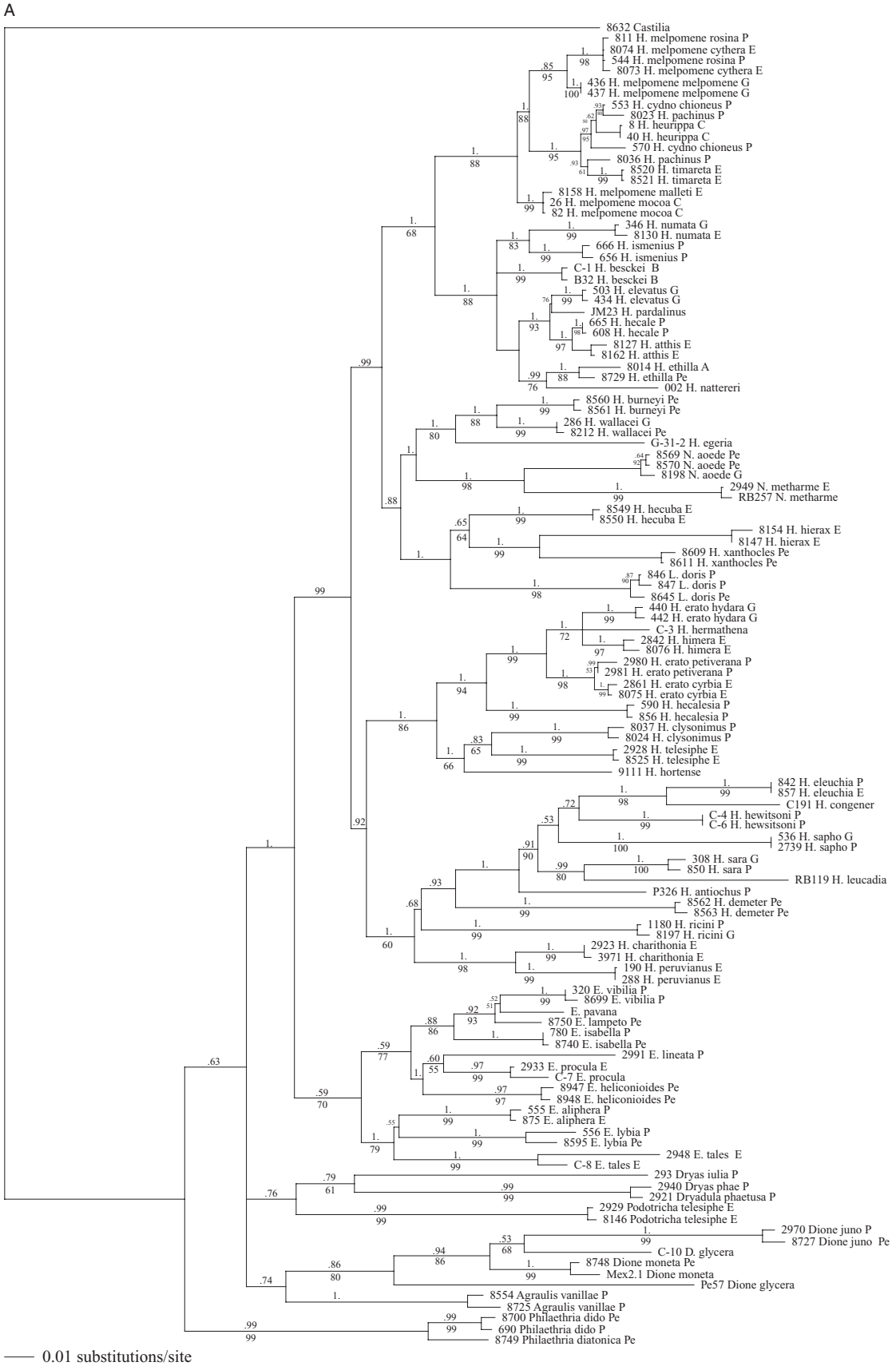
For mitochondrial DNA (mtDNA) 1611 bp were obtained from the *CoI* + *CoII* region including nucleotides and gaps. These represent 822 bp of *CoI* corresponding to position 2191–3009 of the *D. yakuba* sequence (X03240), the complete *tRNA-leu* gene (78 bp) and 711 bp representing the entire *CoII* coding 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. burchellii* (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 337–3511. Additionally, codon deletions were found. In *CoI* 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 *CoI* stop codon. In *CoII*, 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* (LA42141) 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* (LA42141) 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 (*CoI* + *tRNA-leu* + *CoII* + *16S*) versus nuclear data (*Ef1α* + *ap* + *dpp* + *wg*) provided no evidence for incongruence based on nucleotides ($P = 0.08$), or between amino acid


A
8632 Castilia
811 H. melpomene rosina P
8074 H. melpomene cythera E
544 H. melpomene rosina P
8073 H. melpomene cythera E
436 H. melpomene melpomene G
437 H. melpomene melpomene G
553 H. cydno chioneus P
8023 H. pachinus P
8 H. heurippa C
40 H. heurippa C
570 H. cydno chioneus P
8036 H. pachinus P
8520 H. timareta E
8521 H. timareta E
8158 H. melpomene malleti E
26 H. melpomene mocoa C
82 H. melpomene mocoa C
346 H. numata G
8130 H. numata E
666 H. ismenius P
656 H. ismenius P
C-1 H. besckei B
B32 H. besckei B
503 H. elevatus G
434 H. elevatus G
JM23 H. pardalinus
665 H. hecale P
608 H. hecale P
8127 H. atthis E
8162 H. atthis E
8014 H. ethilla A
8729 H. ethilla Pe
002 H. nattereri
8560 H. burneyi Pe
8561 H. burneyi Pe
286 H. wallacei G
8212 H. wallacei Pe
G-31-2 H. egeria
8569 N. aoede Pe
8570 N. aoede Pe
8198 N. aoede G
2949 N. metharme E
RB257 N. metharme
8549 H. hecuba E
8550 H. hecuba E
8154 H. hierax E
8147 H. hierax E
8609 H. xanthocles Pe
8611 H. xanthocles Pe
846 L. doris P
847 L. doris P
8645 L. doris Pe
440 H. erato hydara G
442 H. erato hydara G
C-3 H. hermathena
2842 H. himera E
8076 H. himera E
2980 H. erato petiverana P
2981 H. erato petiverana P
2861 H. erato cyrbia E
8075 H. erato cyrbia E
590 H. hecalesia P
856 H. hecalesia P
8037 H. clysonimus P
8024 H. clysonimus P
2928 H. telesiphe E
8525 H. telesiphe E
9111 H. hortense
842 H. eleuchia P
857 H. eleuchia E
C191 H. congener
C-4 H. hewitsoni P
C-6 H. hewitsoni P
536 H. sapho G
2739 H. sapho P
308 H. sara G
850 H. sara P
RB119 H. leucadia
P326 H. antiochus P
8562 H. demeter Pe
8563 H. demeter Pe
1180 H. ricini P
8197 H. ricini G
2923 H. charithonia E
3971 H. charithonia E
190 H. peruvianus E
288 H. peruvianus E
320 E. vibilia P
8699 E. vibilia P
E. pavana
8750 E. lampeto Pe
780 E. isabella P
8740 E. isabella Pe
2991 E. lineata P
2933 E. procula E
C-7 E. procula
8947 E. heliconioides Pe
8948 E. heliconioides Pe
555 E. aliphera P
875 E. aliphera E
556 E. lybia P
8595 E. lybia Pe
2948 E. tales E
C-8 E. tales E
293 Dryas iulia P
2940 Dryas phae P
2921 Dryadula phaetusa P
2929 Podotricha telesiphe E
8146 Podotricha telesiphe E
2970 Dione juno P
8727 Dione juno Pe
C-10 D. glycera
8748 Dione moneta Pe
Mex2.1 Dione moneta
Pe57 Dione glycera
8554 Agraulis vanillae P
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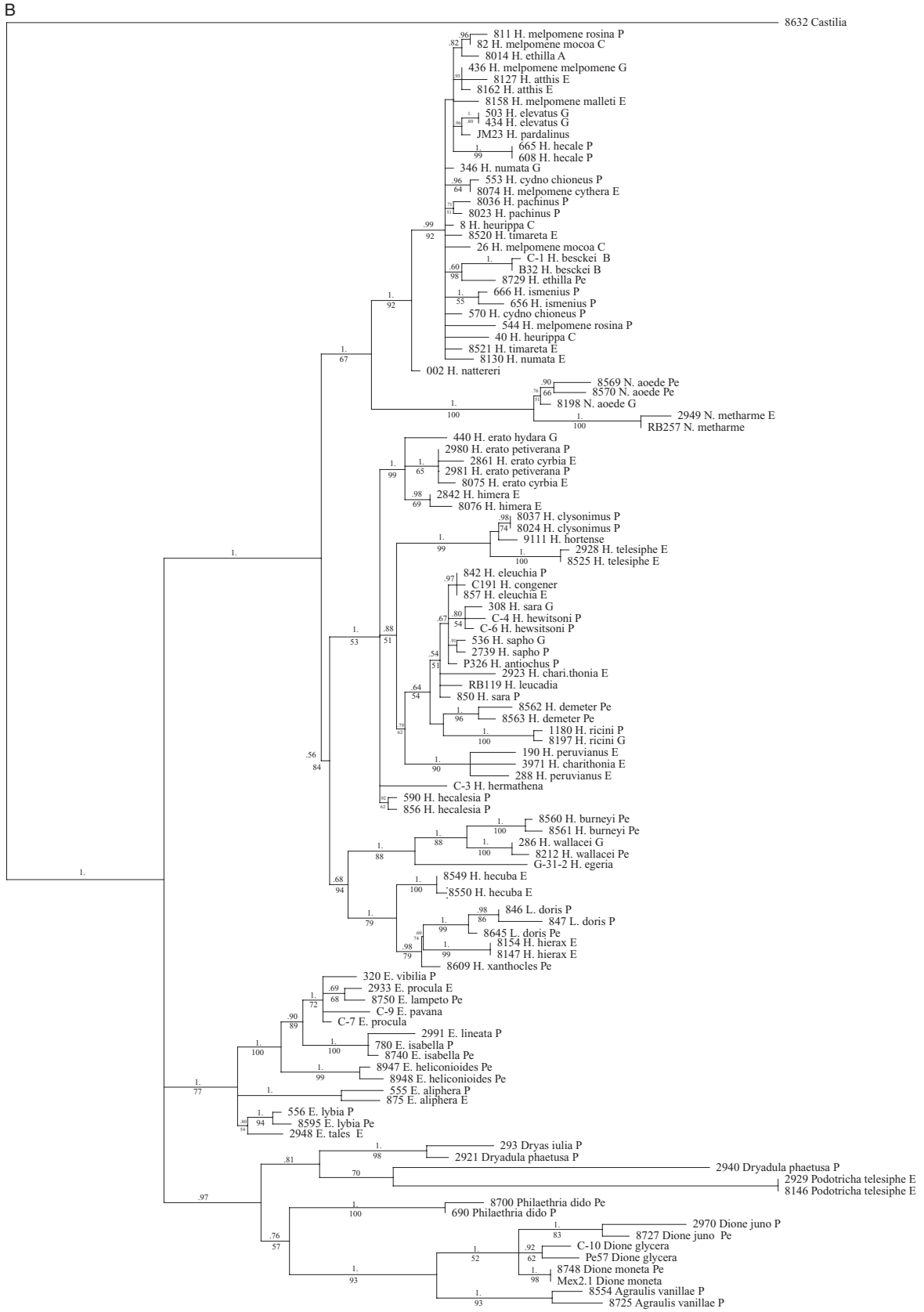
B
8632 Castilia
811 H. melpomene rosina P
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8037 H. clysonimus P
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842 H. eleuchia P
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2739 H. sapho P
P326 H. antiochus P
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Mex2.1 Dione moneta
8554 Agraulis vanillae P
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0.005 substitutions/site

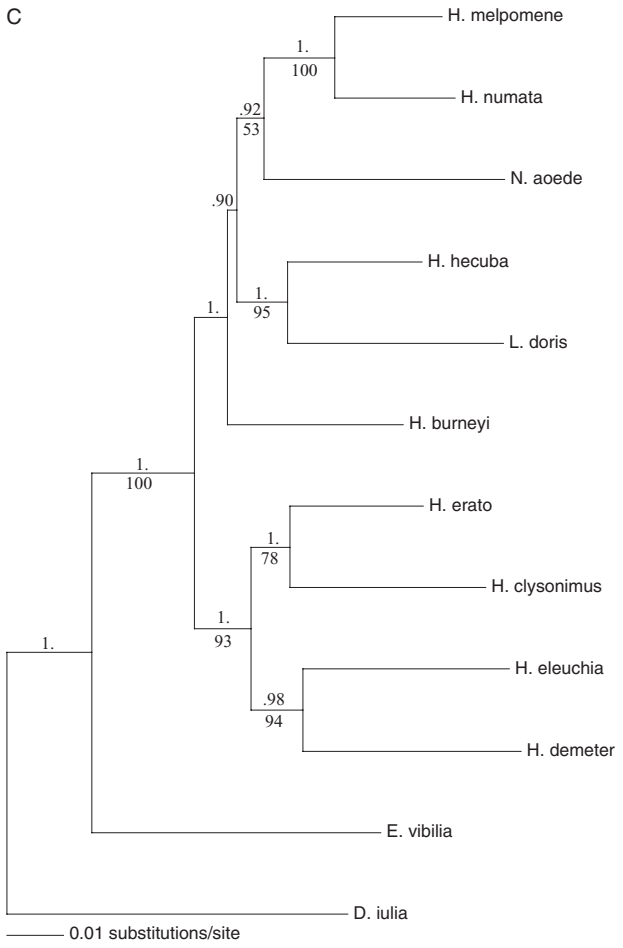


Figure 2. Bayesian phylogenies for heliconiine species based on separate data partitions for mtDNA (*Co* and *16S*) and nuclear data. A, mtDNA. B, *Elongation factor-1α* (*Ef1α*). C, *Co*, *16S*, *Ef1α*, *dpp*, *ap*, and *wg* for12 species representing the major clades within *Heliconius* and *Dryas* as a root. 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.

sequence partitions ($P = 0.23$). Comparisons within mtDNA did not show any incongruence either (e.g. *CoI* 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 *wg* ($P = 0.01$) 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.

Phylogenetic analyses

Our phylogenetic hypothesis for the Heliconiina 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. lalitae*, 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 *CoI* + *CoII* 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 *wg*) 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 *sara/sapho* and *erato*.

The data also produced well- resolved relationships among genera (Fig. 3). *Heliconius*, *Laparus*, 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). *Laparus* 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 *Laparus* 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

Table 1. Nucleotide variability over genes and codon position. The values were calculated for the whole data set in *Co* (*CoI* + tRNA-leu + *CoII*) and *Ef1α*. CI, consistency index; RI, retention index

	All sites	Codon position 1	Codon position 2	Codon position 3
Co				
Number of characters	1611	511	511	511
Number of invariants	955	375	454	66
Number variable	656	136	57	445
Number of informatives	587	106	32	417
Tree length	3751	460	109	3086
CI	0.262	0.361	0.596	0.236
RI	0.715	0.797	0.799	0.704
Ef1α				
Number of characters	876	292	292	292
Number of invariants	615	259	271	84
Number variable	261	33	20	208
Number of informatives	186	12	5	169
Tree length	734	53	34	647
CI	0.47	0.66	0.67	0.44
RI	0.83	0.83	0.6	0.83

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

Gene	*16S*	*ap*	*dpp*	*wg*
Number of characters	512	195	270	375
Number of invariants	413	163	211	281
Number variable	99	32	59	94
Number of informatives	38	15	27	34
Tree length	155	54	86	157
CI	0.748	0.722	0.837	0.669
RI	0.426	0.423	0.745	0.212

pollen feeding behaviour in *Heliconius* (Fig. 3, black clover), including *Heliconius* and *Laparus*, with a loss of this character in *Neruda* (Fig. 3, white clover). To test this hypothesis, various alternative topologies were compared using the method of Shimodaira & Hasegawa (Shimodaira & Hasegawa, 1999). The results showed that a tree constrained to have both *L. doris* and *Neruda* basal to *Heliconius*, was a significantly worse fit both for mtDNA and nuclear DNA alone and also for combined evidence ($P < 0.001$). When *Laparus* alone was forced to be basal to *Heliconius*, the resulting tree was a worse fit to the data based on combined evidence ($P = 0.039$). By contrast, even combined evidence could not exclude the possibility that *Neruda* was basal to *Heliconius* (mtDNA, $P = 0.507$; nuclear, $P = 0.365$; combined, $P = 0.329$). This might be a result of the different placements suggested by different genes: mtDNA placed *Neruda* inside the *Heliconius* 'primitive' group (Fig. 2A), whereas nuclear data from *Ef1α*, *ap*, *dpp*, and *wg* placed *Neruda* basal to the silvaniforms + *cydno/melpomene* group (Fig. 2B, C).

Pupal mating behaviour has been studied in *H. erato* and *Heliconius hewitsoni* and observed in other members of the *erato* and *sara/sapho* 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 heliconiiines 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 + Γ +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

Table 3. Best supported models of molecular evolution and estimated parameter values for the different data sets

Data set	*CoI + CoII*	*16S*	*Ef1α*	*ap*	*dpp*	*wg*
Model	GTR + I + G	F81 + G	GTR + I + G	K2P + G	K2P + G	TrNef + G
Base frequencies						
A	0.374	0.4421	0.28	0.25	0.25	0.25
C	0.1081	0.0649	0.244	0.25	0.25	0.25
G	0.0647	0.1234	0.2447	0.25	0.25	0.25
T	0.4532	0.3697	0.2313	0.25	0.25	0.25
Substitution model		All equal rates				
Tr/tv ratio				1.9162	2.1976	
Tr [A-G]	15.2471		6.2591			6.2038
[C-T]	27.3268		13.4501			12.7904
Tv [A-C]	2.9031		1.6729			1
[A-T]	1.7001		3.3225			1
[C-G]	2.8548		1.7038			1
[G-T]	1		1			1
Invariable sites	0.5001	0	0.543	0	0	0
Gamma parameter	0.5187	0.1236	0.8076	0.0639	0.2419	0.3561

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 (*Ef1α*, *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.

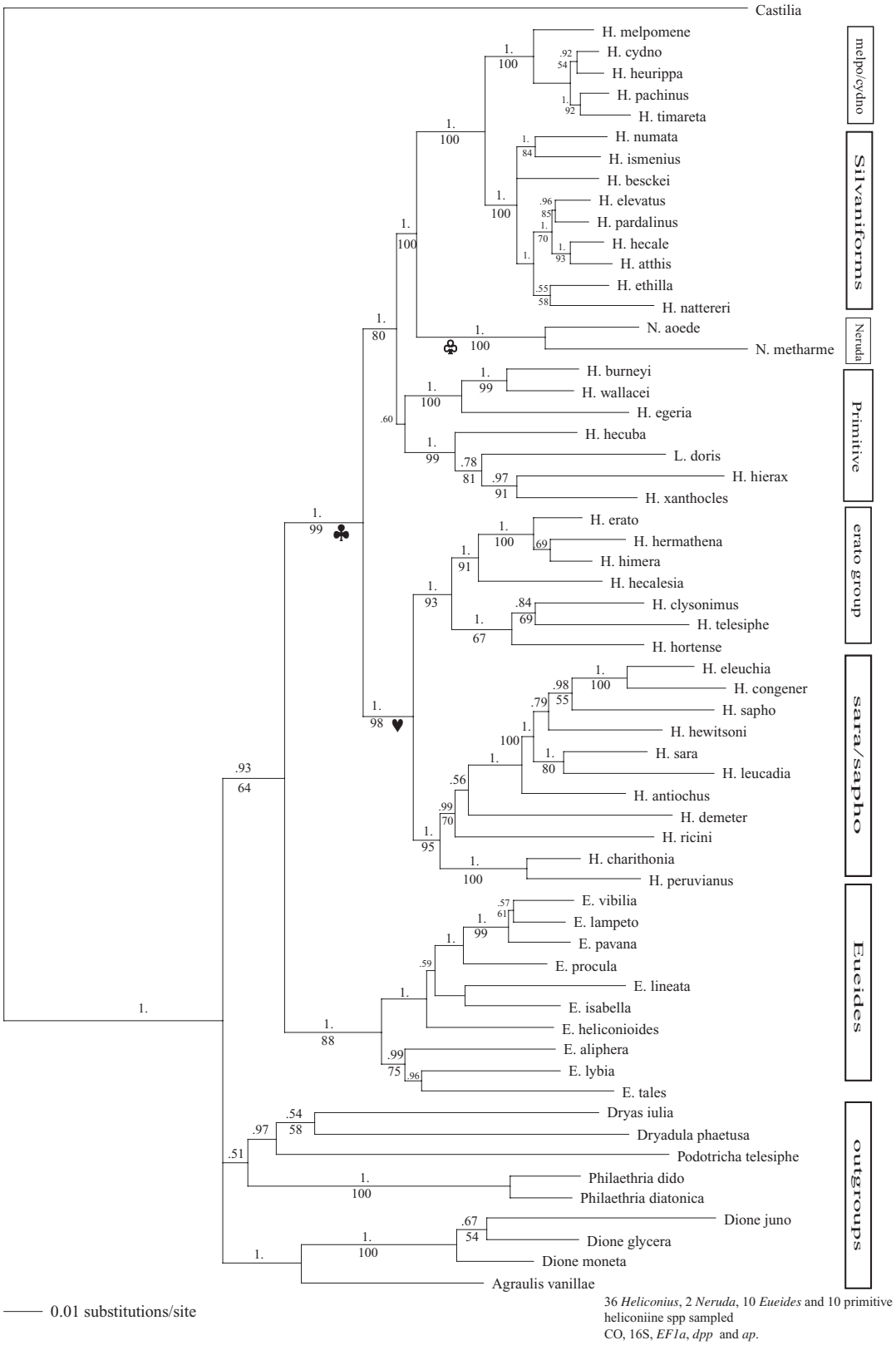
Eueides was part of the pupal mating group (mtDNA, $P = 0.266$; nuclear, $P = 0.017$; combined, $P = 0.005$). However, this hypothesis was a significantly worse fit to our data based on either nuclear DNA or combined evidence data.

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


Castilia
H. melpomene
H. cydno
H. heurippa
H. pachinus
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H. hewitsoni
H. sara
H. leucadia
H. antiochus
H. demeter
H. ricini
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H. peruvianus
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E. lampeto
E. pavana
E. procula
E. lineata
E. isabella
E. heliconioides
E. aliphera
E. lybia
E. tales
Dryas iulia
Dryadula phaetusa
Podotricha telesiphe
Philaethria dido
Philaethria diatonica
Dione juno
Dione glycera
Dione moneta
Agraulis vanillae
melpo/cydno
Silvaniforms
Neruda
Primitive
erato group
sara/sapho
Eueides
outgroups
0.01 substitutions/site
36 *Heliconius*, 2 *Neruda*, 10 *Eueides* and 10 primitive heliconiine spp sampled
CO, 16S, *EF1a*, *dpp* and *ap*.

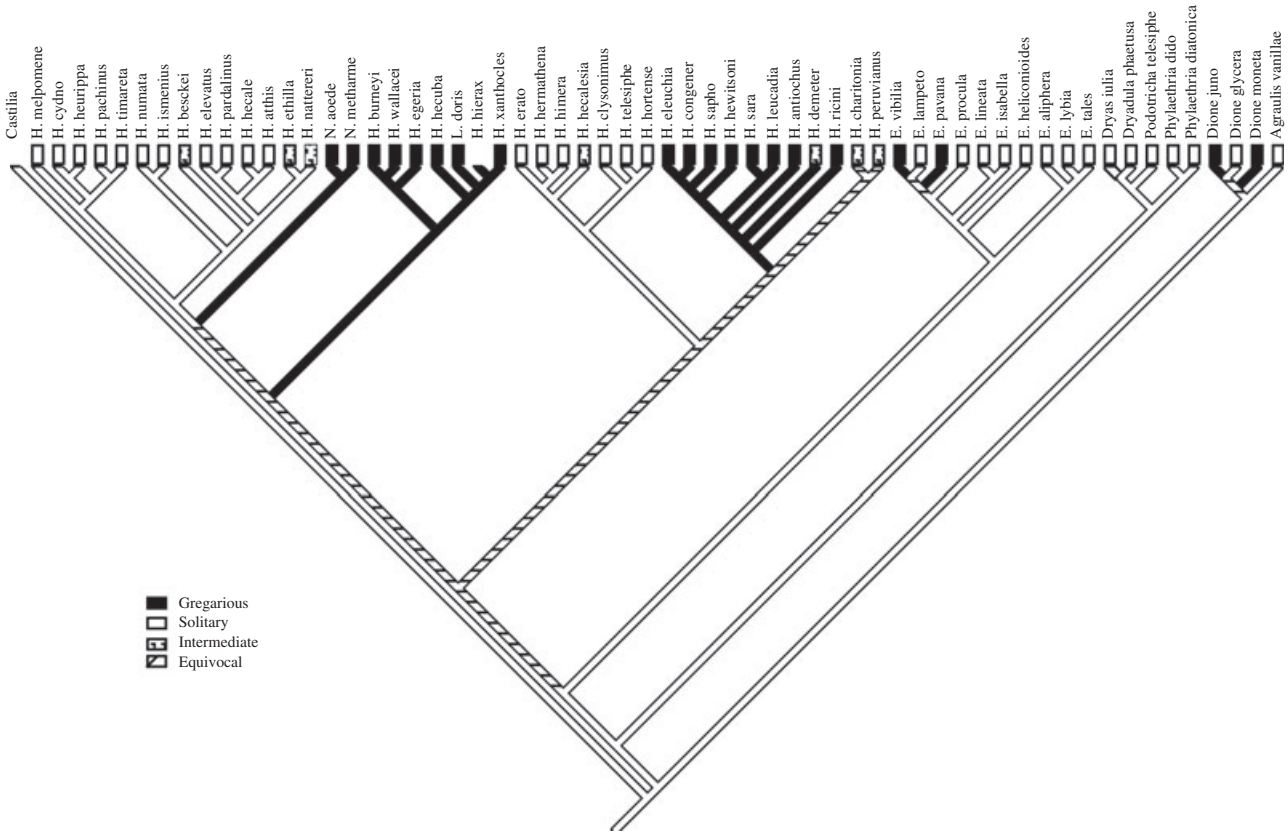

Figure 4. The evolution of larval sociality among the heliconiines. Boxes at branch tips indicate known larval behaviour, with 'intermediate' indicating species that generally lay solitary eggs but occasionally clump a few eggs together. For a species to be classified as gregarious, a minimum of ten 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 *CoI* 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 down-weighted, 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

sara/sapho clade had the largest sister species genetic distances in *Heliconius*, suggesting that species such as *H. eleuchia*, *Heliconius congener*, and *H. sapho* are relatively ancient. In this clade, the topology remained largely unchanged compared with the previous hypothesis (Brower & Egan, 1997), and the additional newly elevated species *H. peruvianus* was placed as sister to *H. charithonia* as expected (Jiggins & Davies, 1998).

In the *erato* clade, three additional species were included, *H. hermathena*, *H. hecalesia*, and *Heliconius hortense*. The placements of *H. hermathena* and *H. hortense* were different to those previously suggested (Brown, 1981). *Heliconius hermathena* is restricted to certain nonforest habitats in the Brazilian Amazon. One of its four subspecies *Heliconius hermathena vereatta*, is mimetic of sympatric *Heliconius melpomene melpomene* and *H. e. hydara* and is very restricted geographically (Brown & Benson, 1977). The other three are nonmimetic, little differentiated and apparently widespread but the populations are patchy and in low densities. Their wing colour pattern is black, yellow, and red; the forewing is black with red, resembling *H. m. melpomene* and *H. e. hydara*, whereas the hindwing is black with yellow bars and spots, resembling *H. charithonia*. Brown & Benson (1977) suggested, based on adult morphology and pupal-mating behaviour, that *H. hermathena* is closely related to *H. erato* and *H. charithonia*. However, the pupae lack the derived characters of long pupal head appendages, suggesting the species is relatively primitive, near to the *melpomene* group, in which all the members show shortened head appendages. The results shown here demonstrate that *H. hermathena* is a member of the 'pupal-mating clade', but with some discordance between nuclear and mtDNA results. The nuclear data (*Ef1*α) show *H. hermathena* basal to the 'pupal-mating clade' (Fig. 2B), whereas mtDNA data place *H. hermathena* as a sister to *H. erato* (Fig. 2A). Perhaps *H. hermathena* is a basal member of this clade, which would explaining the unusual pupal morphology shared with *H. melpomene*, but has acquired red colour pattern elements and mtDNA haplotypes via recent hybridization with *H. erato*. Occasional presumptive hybrids between the widely separated species *H. charithonia* and *H. erato*, and between *H. clysonymus* or *H. hortense* and *H. hecalesia* are known (Mallet, Neukirchen & Linares, 2006).

Relationships in the *melpomene/cydno* and silvaniform group

The *melpomene/cydno* 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/cydno* 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 *Ef1*α has little informative variation (Fig. 2B).

In the *melpomene/cydno* 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. m. melpomene* from French Guiana (Brower, 1996b; Flanagan *et al*., 2004). *Heliconius cydno* 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; Mavárez *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 atthis* + *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. atthis*, but here *H. ethilla* clustered with *H. nattereri*, one of the new species included. *Heliconius atthis* 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/cydno* 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/cydno* 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, 1996b; 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. himera* (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 *erato/sara/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.

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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).

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