Towards a better understanding of the higher systematics of Nymphalidae (Lepidoptera: Papilionoidea)

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Abstract

Research on the molecular systematics of higher taxa in the butterfly family Nymphalidae (Lepidoptera) is only just beginning. Outgroup selection is difficult at the moment due to the lack of consensus on the basal relationships of the major groups in Nymphalidae. We identify four major clades in the Nymphalidae based on a cladistic analysis of one mitochondrial gene sequence (COI, 1450 bp) and two nuclear gene sequences (EF-1α, 1064 bp, and wingless, 412–415 bp) from 54 exemplar species sampled from all currently recognized subfamilies. The COI data set was found to be highly incongruent with the nuclear data sets and a Partitioned Bremer Support analysis shows that the COI data set largely undermines support for most clades. Transitions at the third codon positions of the COI data set were highly saturated, but analyzing the combined data set with the COI third positions removed did not change the results. The major clades we found are termed the danaine clade (including Danainae), the satyrine clade (including Charaxinae, Satyrinae, Calinaginae, and Morphinae), the heliconine clade (including Heliconiinae and Limenitidinae excluding Biblidini, Cyrestini, Pseudergolini, and Coeini) and the nymphaline clade (including Nymphalinae, Apaturinae, and Coeini, Cyrestini, Pseudergolini, and Biblidini from Limenitidinae). The heliconine and nymphaline clades are sister groups, while the most parsimonious explanation for the combined data set places the danaine clade as the most basal large group of Nymphalidae. Our results give one of the strongest hypotheses for the subfamilial relationships within Nymphalidae. We were able to resolve the polyphyletic nature of Limenitidinae, which we recommend to be split into three subfamilies: Limenitidinae, Biblidinae, and Cyrestinae. The tribe Coeini belongs in Nymphalinae.

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1. Introduction

The higher systematics of Nymphalidae (Lepidoptera) has long been a matter of contention (e.g., Ackery, 1984; Ackery et al., 1999; de Jong et al., 1996; Ehrlich, 1958; Harvey, 1991), even though members of the family are extremely well-known by evolutionary biologists and even lay people (e.g., the Monarch Danaus plexippus, the longwings Heliconius spp. and the brilliant blue Morpho butterflies). Within the family, a few groups have been consistently recognized and their circumscriptions have remained relatively stable (e.g., Libytheinae, Apaturinae, Charaxinae, Calinaginae, Melitaeini, Heliconiina, and Acraeina), while the concepts of other taxa have expanded and contracted with each new publication (e.g., Nymphalinae, Limenitidinae, Satyrinae, Morphinae, and Danainae). Moreover, our understanding of the relationships of the variously recognized higher taxa have also changed with each new publication, depending on the data gathered and the analytical methods used (see e.g., Brower, 2000; de Jong et al., 1996; Ehrlich, 1958; Freitas, 1999). Consistent in all of these studies is a lack of support (either character or statistical) for the more basal nodes.

The most recent classification of Nymphalidae is by Ackery et al. (1999), who largely follow Harvey’s (1991) classification with some subfamilies combined (e.g., Brassolinae is placed within Morphinae and Ithomiinae within Danainae). We follow Ackery et al.’s (1999) classification in this paper. Several hypotheses have been proposed on the relationships of some of the subfamilies.
based on adult and larval morphology. DeVries et al. (1985) placed Satyrinae, Morphinae, Charaxinae, and Apaturinae together, to the exclusion of Danainae and Nymphalinae. de Jong et al. (1996) note that Morphinae, Calinaginae, and Satyrinae tend to group together as do Nymphalinae and Heliconiinae. Harvey (1991) notes that the subfamily Limenitidinae is an unnatural assemblage, but retained it “for convenience only,” as did Ackery et al. (1999). Recently, Freitas (1999) found six major groups in the Nymphalidae based on a large data set of both adult and larval morphology. The groups found correspond to (sensu Ackery et al., 1999) Libytheinae, Danainae, Nymphalinae + Heliconiinae, Limenitidinae excluding Biblidini, Biblidini on its own, and finally a large group with the rest of the subfamilies.

Molecular evidence points to some intriguing patterns that require confirmation. Brower (2000) found that Biblidini (sensu Harvey, 1991) and Apaturinae may be associated and that both of these may be related to Nymphalinae. However, Brower’s (2000) strict consensus tree does not offer any new insights into the relationships of the subfamilies recognized here. Brower (2000) did produce a well-resolved tree by applying successive approximations weighting (Farris, 1969) to his data set, but such methods have been shown to possibly lead to unreliable results when used with molecular data sets (Källersjö et al., 1999).

Several groups within Nymphalidae have remained unplaced entirely. The enigmatic genus Calinaga, with only eight species, has traditionally been placed in its own subfamily Calinaginae. The closest relatives of Calinaga have never been clear, and various authors have allied the genus with different subfamilies. Most often Calinaginae has been allied with the members of the so-called satyrine assemblage (Satyrinae and Morphinae), based on one morphological feature of the adult wing (Ehrlich, 1958). The cladistic analysis of Freitas (1999) places Calinaginae close to the Morphinae. Previous published molecular studies of the Nymphalidae have not sampled Calinaga. Two other groups, the tribes Cyrestini and Pseudergolini, have never been placed in any of the existing subfamilies with comfort. Most often they are placed in Limenitidinae (which has had the status of a “convenient” subfamily for a while), though occasionally they are placed in Apaturinae (e.g., Parsons, 1999).

The identification of the major clades in Nymphalidae and the resolution of their relationships is imperative for the meaningful study of evolutionary processes in this otherwise extremely well-studied group (Boggs et al., 2003). Work on the molecular systematics of various groups within Nymphalidae is only just beginning to take off and researchers are faced with the problem of selecting suitable outgroups to test the monophyly of their study groups and to root their phylogenetic trees. Outgroups that are too distantly related to the ingroup may affect the results of analyses through such problems as long branch attraction (Felsenstein, 1978; Wheeler, 1990). In this paper we aim to identify the major clades in Nymphalidae and to clarify the relationships of the major clades using sequence data from three genes (one mitochondrial and two nuclear).

2. Material and methods

In order to resolve the relationships of the major lineages in the family Nymphalidae, we have generated sequences of three genes from 54 exemplar species of all 10 subfamilies of Nymphalidae recognized by Ackery et al. (1999). For subfamilies known to be nonmonophyletic (e.g., Limenitidinae) we sampled species from the different clades found by Brower (2000). The sampled species and their currently recognized subfamilies are shown in Table 1. When possible, we sampled species from at least two different tribes for each subfamily. The bias in sampling of Nymphalidae reflects our current work on this group and our need to identify suitable outgroups to test the monophyly of Nymphalinae.

We extracted DNA mainly from one or two legs of freshly frozen or dried butterflies using QIAgen’s DNAEasy extraction kit. DNA of Erebia palarica was extracted from the thorax and of Lasionimata megera from the abdomen. The spread voucher specimens can be viewed at http://www.zoologi.su.se/research/wahlberg/. For each of the specimens we sequenced 1450 bp of the cytochrome oxidase subunit I gene (COI), 1064 bp of the Elongation Factor-1α gene (EF-1α), and 412–415 bp of the wingless gene. Primers for COI were taken from Wahlberg and Zimmermann (2000), for EF-1α from Monteiro and Pierce (2001) and for wingless from Brower and DeSalle (1998). We performed all PCRs in a 20 μl reaction volume. The cycling profile for COI and wingless was 95 °C for 5 min, 35 cycles of 94 °C for 30 s, 47 °C for 30 s, 72 °C for 1 min 30 s, and a final extension period of 72 °C for 10 min. The cycling profile for EF-1α was 95 °C for 7 min, 35 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and a final extension period of 72 °C for 10 min. For COI and wingless, the PCR primers were also used for sequencing, while in EF-1α, we used an internal primer (EFmid 5'– CAA TAG CRC CRA TTT TGT-3') in addition to the PCR primers for sequencing. Sequencing was done with a Beckman–Coulter CEQ2000 capillary sequencer. We checked the resulting chromatograms using the program BioEdit (Hall, 1999) and aligned the sequences by eye. The sequences are available on GenBank (accession numbers in Table 1).

We investigated various properties of the sequences using the program DAMBE (Xia and Xie, 2001), such as the proportions of transitions and transversions at the three codon positions and pairwise distances of all taxa.
for the three gene sequences. We tested the potential incongruence of the three data sets using the incongruence length difference (ILD) test of Farris et al. (1994), as implemented in the program Winclada (Nixon, 2002). We tested each pairwise combination using 1000 replicates of two random additions and tree bisection-reconnection (TBR) branch swapping. We searched for the most parsimonious cladograms from the equally weighted strict consensus. We searched for the most similar sequences from the equally weighted strict consensus. We searched for the most similar sequences from the equally weighted strict consensus.
weighted and unordered data matrix consisting of 54 taxa using a heuristic search algorithm in the program NONA 2.0 (Goloboff, 1998). The heuristic searches were conducted with 100–1000 random addition replicates using TBR branch swapping with up to 20 trees held during each step. We did this for each gene separately and for all three genes combined.

We evaluated the robustness of the clades in the resulting cladograms using bootstrap analyses (Felsenstein, 1985) and Bremer support (Bremer, 1988; Bremer, 1994). We calculated bootstrap values from 1000 pseudoreplicates with 10 random additions per pseudoreplicate. We used the program TreeRot (Sorensen, 1999) in conjunction with PAUP* (Swofford, 1998) to calculate Bremer support values. We assessed the contribution of each gene to the Bremer support values of the combined analyses using Partitioned Bremer Support (PBS) (Baker and DeSalle, 1997; Baker et al., 1998) using the program TreeRot (Sorensen, 1999). When discussing our results, we will refer to the support values as either giving weak, moderate, good or strong support. Delimiting such qualitative classes is an inherently subjective and thus, for this study, we define weak support as Bremer support values of 1–2 (bootstrap values 50–63%), moderate support as values between 3 and 5 (bootstrap values 64–75%), good support as values between 6 and 10 (bootstrap values 76–88%), and strong support as values >10 (bootstrap values 89–100%).

Rooting our resulting trees proved to be difficult, most likely due to long branch attraction (see Wheeler, 1990). Preliminary tests using a species of Pieridae and a species of Lycaenidae as outgroups showed that the pierid grouped with *Vindula* and the lycaenid grouped with the two Danainae in all analyses, without affecting the rest of the topology (shown in Section 3). We thus chose to root the trees obtained from the parsimony analysis of unrooted trees with *Libythea*, for which morphological and molecular evidence points to a sister group relationship with the rest of Nymphalidae (Ackery et al., 1999; Brower, 2000; de Jong et al., 1996; Ehrlich, 1958; Freitas, 1999; Martin and Pashley, 1992).

3. Results

3.1. General properties of sequences

The full data set consisted of 2929 aligned nucleotide sites with almost no missing data. We were unable to sequence the second half of the EF-1α gene in *Ammosia*, and thus half of the EF-1α data set is coded as missing data for this taxon. In addition, we downloaded the wingless sequence for *Actinote stratonice* from GenBank, where it was placed by Brower and DeSalle (1998). Aligning COI and EF-1α did not require any indels, while in wingless the four species of Melitaenii had a 3bp insertion (as found by Brower, 2000). Of the 1450 bp sequenced for COI, 671 sites were variable and of these 542 were parsimony informative. The respective numbers for EF-1α are 1064 bp, 412 variable and 344 parsimony informative, and for wingless, 412–415 bp, 223 variable and 178 parsimony informative sites. COI has a strong AT bias (71% on average), as is common in insect mitochondrial genomes (DeSalle et al., 1987; Simon et al., 1994), while the two nuclear genes have an almost equal proportion of the four nucleotides. Uncorrected pairwise differences ranged between 6 and 16% (average 12.7%) for the COI sequences, between 3 and 16% (average 11.1%) for the EF-1α sequences, and between 3.5 and 29.5% (average 16.8%) for the wingless sequences.

The ratio between transitions and transversions showed quite different patterns in all three gene sequences when comparing the third codon position with the first and second (Fig. 1). As expected, the third position divergences were much higher than the first and second position divergences. The two nuclear gene sequences had similar patterns of divergence against the transition/transversion ratio (Fig. 1). In both of the sequences, the transition/transversion ratio of the first and second positions is mostly within the range of variation of the third position (in EF-1α there are 224 comparisons in which there are zero inferred transversions in the first two codon positions, while in wingless there are 26 such comparisons). Saturation of transitions in relation to transversions at the third position does not appear to be a problem in the two nuclear gene sequences. In contrast, the transition/transversion ratio in the COI sequences showed a marked difference in pattern. The ratio was less than 1 in almost all comparisons of the third position, suggesting that transitions were saturated, which can lead to high levels of homoplasy (Simon et al., 1994). The comparisons of first and second positions all had a transition/transversion ratio of more than 1 (3.4 on average), suggesting that saturation of transitions was not a problem at these positions. To investigate the effects of the high saturation of transitions in the COI third positions on our phylogenetic analyses, we performed all analyses of the COI data set with and without the third positions.

3.2. Results of the phylogenetic analyses

There was significant incongruence between the mitochondrial and the two nuclear genes (ILD for COI vs EF-1α, *P* = 0.001; ILD for COI vs wingless, *P* = 0.008), but not between the two nuclear genes (ILD for EF-1α vs wingless, *P* = 0.073). Removing the third codon positions from the COI data set did not make the mitochondrial data set more congruent with the nuclear data sets (ILD for COI no third vs EF-1α, *P* = 0.001; ILD for COI no third vs wingless, *P* = 0.035). The incongruence
is clearly seen when comparing the most parsimonious cladograms found for each gene separately (Fig. 2a–d). Consistent in the results of all separate analyses is the weak or no support for most nodes in the cladograms. This is the result of too few phylogenetically informative characters in the individual data sets given our sparse taxon sampling. The COI data set with the third codon positions included yielded a strict consensus tree that is unresolved at the deeper nodes (Fig. 2a), which might be expected given the high levels of saturation of transitions (Fig. 1). Removing the third codon positions produces a more resolved consensus tree (Fig. 2b), though the recovered clades are not very consistent with current taxonomy. The EF-1α data set yields a well resolved consensus tree (Fig. 2c), with many of the currently recognized subfamilies forming monophyletic units (Limenitidinae, Satyrinae, and Morphinae are para- or polyphyletic). The wingless data set produces a less resolved consensus tree with only Danainae, Nymphalinae, Heliconiinae, and Apaturinae forming monophyletic units (Fig. 2d).

Cladistic analysis of the equally weighted, combined data set yielded three equally parsimonious trees (Fig. 3). The strict consensus tree has four major clades which we have termed the danaine, satyrine, heliconine, and nymphaline clades. Removing the third codon position from the COI data set and combining it with the other two data sets yielded two equally parsimonious trees (Fig. 4). The strict consensus of the two trees differs from Fig. 3 only at the tips, the deeper nodes are identical and the same four major clades are recovered. Henceforth, we will only consider the results presented in Figs. 3 and 4 in more detail.

All subfamilies fall within one of the major clades recovered, except Limenitidinae, which forms a polyphyletic assemblage in two of the major clades (the heliconine and nymphaline clades). The danaine clade is particularly well supported with Bremer support >20 steps and bootstrap values >98% regardless of the combined data set used. The three genera in the danaine clade all belong to the subfamily Danainae. The satyrine clade has good Bremer and strong bootstrap support regardless of the data set used. Representatives of the subfamilies Satyrinae, Morphinae, Charaxinae, and Calinaginae all occur in the satyrine clade. The monophyly of Satyrinae and Morphinae are unresolved with the entire data set, while both are polyphyletic according to the data set with COI third positions removed, though the relationships of the representatives of the two subfamilies have weak or no support (Fig. 4). The two representatives of Charaxinae form a strongly supported monophyletic group with Calinaginae as the sister subfamily, albeit with weak Bremer support.

The heliconiine clade has good Bremer and moderate bootstrap support with the entire data set (Fig. 3) and the support increases with the removal of the COI third codon positions (Fig. 4). The heliconiine clade contains representatives of the subfamily Heliconiinae and representatives of the tribe Limenitidini (sensu Harvey, 1991). Heliconiinae has weak (entire data set) or moderate (COI third positions removed) Bremer support as...
Fig. 2. The results of cladistic analyses of the data sets of the three genes on their own. Numbers given above branches are Bremer support values and numbers below the branch are bootstrap values for the node to the right of the number. Consistency index (CI) and Retention index (RI) are calculated with informative characters only. The two letters in parentheses after each taxon name is the currently recognized subfamily abbreviated as follows: Lb, Libytheinae; Da, Danaeinae; Sa, Satyrinae; Mo, Morphinae; Ca, Calminagiae; Ch, Charaxinae; Lm, Limenitidinae; He, Heliconinae; Ap, Apaturinae; and Ny, Nymphalinae. (a) COI gene, strict consensus of seven trees, length 5127 steps, CI 0.20, and RI 0.28. (b) COI gene with third codon positions removed, strict consensus of 12 trees, length 986 steps, CI 0.21, and RI 0.40. (c) EF-1α gene, strict consensus of four trees, length 2804 steps, CI 0.21, and RI 0.39. (d) Wingless gene, strict consensus of 222 trees, length 1539 steps, CI 0.22, and RI 0.46.
a monophyletic group. The tribe Limenitidini has strong support regardless of the data set.

The final major clade, the nymphaline clade has weak Bremer and no bootstrap support with the entire data set and the support increases slightly to moderate Bremer and weak bootstrap support with the removal of the COI third positions. The nymphaline clade contains representatives of the subfamilies Nymphalinae, Apaturinae, and the tribes Biblidini, Coeini, Cyrestini, and Pseudergolini (all four tribes are generally included in the subfamily Limenitidinae). Apaturinae and Biblidini have good to strong support as monophyletic groups regardless of data set used. Nymphalinae is polyphyletic, although the clade containing *Colobura* (in the tribe Coeini, generally included in Limenitidinae) and representatives of Nymphalinae excluding *Amnosia* (placed in Nymphalinae by Harvey, 1991) has good support regardless of data set used. *Amnosia* forms a monophyletic group with *Stibochiona* with moderate good support, and indeed prior to Harvey’s revision, both were placed in the tribe Pseudergolini. *Amnosia, Stibochiona,* and *Cyrestis* (the latter in the tribe Cyrestini) form a monophyletic group with weak to moderate Bremer support and no bootstrap support.

Since some of the traditionally circumscribed subfamilies are polyphyletic in our analyses, in particular Limenitidinae, Morphinae, Satyrinae, and Nymphalinae, we have investigated the effect of forcing these
subfamilies to be monophyletic on the length of the resulting most parsimonious trees. Forcing the traditionally held (albeit “for convenience only”) concept of Limenitidinae to be monophyletic resulted in trees 48 steps longer with the entire data set and 43 steps longer with the COI third positions removed than the most parsimonious unconstrained trees. Since *Colobura* occurs within Nymphalinae with good support, we forced Limenitidinae without *Colobura* to be monophyletic, resulting in trees 24 and 21 steps (for entire and COI third positions removed, respectively) longer. The tribe Biblidini has sometimes been considered to be subfamily of its own, thus we forced representatives of the tribes Limenitidini, Cyrestini, and Pseudergolini (including *Amnosia*) to be monophyletic, resulting in trees 17 and 12 steps (for entire and COI third positions removed, respectively) longer. The subfamily Morphinae is monophyletic in one of the most parsimonious trees from the entire data set, and for the data set with COI third positions removed, forcing Morphinae to be monophyletic resulted in trees two steps longer than the most parsimonious unconstrained trees. Forcing Satyrinae to be monophyletic gave trees six steps longer with the entire data set and three steps longer with the removal of the COI third positions. And finally forcing *Amnosia* to be within Nymphalinae resulted in trees 23 steps longer with the entire dataset and 17 steps longer for the data set with COI third positions removed.

Fig. 4. Strict consensus of two equally parsimonious trees from the combined data set of all three genes with the third codon position removed from the COI data set (length 5444, CI 0.21, and RI 0.40). Numbers given above branches are Bremer support and bootstrap values, respectively, for the node to the right of the number. Numbers below the branches are the contribution of the COI, EF-1α, and wingless data sets, respectively, to the Bremer support value of the combined analysis (i.e., the results of the Partitioned Bremer Support analysis). To the right of the tree are the informal names of the major clades discussed in the text. Abbreviations of subfamilies as in Fig. 2.
The relationships of the four major clades have only weak support with the entire data set, but the sister group relationship of the heliconiine and nymphaline clades receive good Bremer support and moderate bootstrap support with the removal of the COI third positions. The position of the satyrine clade remains poorly supported though.

### 3.3. Results of Partitioned Bremer Support analyses

The PBS results suggest that the greatest source of conflict is the COI data set. With the entire data set, the COI data set is in conflict with 24 of the 49 nodes of the consensus tree (Fig. 3). The respective numbers for the EF-1α and wingless data sets are 10 and 12. With the removal of the COI third positions, the number of conflicting nodes for the COI data set does not change (25 of 51 nodes), while the number of conflicting nodes for EF-1α increases (17 of 51 nodes) and decreases for wingless (seven nodes) (Fig. 4). However, the number of nodes where any data set conflicts strongly (>5 steps) decreases from 15 to 2 with the removal of the COI third positions. Indeed, with the entire data set, the COI partition conflicts strongly with 14 nodes, most of which are the deeper nodes in the consensus tree. The COI partition with the third positions removed is still in conflict with many of the deeper nodes but the conflict is not as strong.

The EF-1α data partition has a strong effect on our results. It is clear that the structure of both of the combined data trees is mainly due to the EF-1α data set (compare Fig. 2c with Figs. 3 and 4). PBS analysis shows that the EF-1α partition compensates at all the nodes that show strong conflict with the entire COI partition leading to positive and at some nodes good Bremer support. With the removal of the COI third positions, the average support for each node given by the EF-1α partition drops from 7.2 to 2.3. This is mainly due to the noisy nature of the COI third positions which greatly increase the length of any trees found when searching with anticonstraints. This is amply illustrated by Figs. 3 and 4, where the removal of the COI third position results in a reduction of almost half the number of steps required to explain the data in the most parsimonious way. Also, the consistency index of the COI data set decreases from 0.19 to 0.27, and the retention index increases from 0.26 to 0.35. These results suggest that the EF-1α sequence is very suitable in resolving the relationships of species at the level of divergence exhibited by members of the family Nymphalidae. Indeed its phylogenetic signal is so strong that the highly homoplasic COI data set is unable to overcome it.

The wingless data partition agrees with the EF-1α partition in general, despite significant incongruence. Removal of the COI third positions increases the effect of the wingless partition on the results, as seen by the increase in the average support for each node from 3.8 to 5.2. Thus, despite the shortness and high variability, wingless is also very useful at the level of divergence exhibited by Nymphalidae when used in conjunction with another more conserved sequence. Analyzing just the two nuclear genes combined results in eight equally parsimonious trees, of which the strict consensus is essentially identical to Fig. 4 (the clade containing Stichophthalma, Manataria, and Melanitis is unresolved, as are the relationships of Biblidini, Apaturinae, and Nymphalinae).

### 4. Discussion

We have identified four major clades in the family Nymphalidae that can be termed the danaine clade (which includes Danainae sensu Ackery et al., 1999), the satyrine clade (including Charaxinae, Satyrinae, Calinaginae, and Morphinae), the heliconiine clade (including Heliconiinae and Limenitidini sensu Harvey, 1991), and the nymphaline clade (including Nymphalinae, Apaturinae, Coeini, Biblidini, Cyrestini, and Pseudergolini). The major clades shown in Figs. 3 and 4 are one of the strongest hypotheses of subfamilial relationships presented to date. Our most parsimonious cladograms resolve the relationships of these four major clades, though the basal nodes do not receive support in the combined analysis of all three genes, due to the incongruence of the COI data set with the nuclear data sets (Figs. 3 and 4). The removal of the COI third positions from the combined data set gives good support to the sister group relationship of the heliconiine and nymphaline clades (Fig. 4).

Our results confirm and strengthen some of the unexpected results of earlier studies. The polyphyly of Limenitidinae (sensu Harvey, 1991) is clearly supported by our data set as shown in Figs. 3 and 4. The tribe Coeini (represented by Colobura) belongs to the subfamily Nymphalinae as has been found in previous molecular (Brower, 2000) and morphological studies (Freitas, 1999). On the other hand, Anmosia clearly does not belong in the subfamily Nymphalinae as suggested by Harvey (1991), but rather is more related to Pseudergolini, which is where it was placed prior to Harvey's revision. Biblidini (represented by Hammadryas, Sevenia, Nica, Eurytela, Ariadne, and Catonephele in our study) does not belong to Limenitidinae, but should be considered a subfamily of its own, the Biblidinae. The monophyly of Biblidini (sensu Harvey, 1991) has not been questioned and is supported by a unique morphological synapomorphy, the male hypandrium.

The tribes Cyrestini (represented by Cyrestis) and Pseudergolini (Stichochiona and Anmosia) appear to be sister groups and also do not belong to Limenitidinae.
The three taxa form a monophyletic unit that does not appear to be associated with any of the other subfamilies very strongly and should thus be also considered a subfamily of its own, the Cyrestinae. Harvey (1991) placed one genus (*Dicorrhagia*, not sampled in this study), that is usually considered to be in Pseudergolini, into Cyrestini, and he expressed doubt about the monophyly of Pseudergolini. Of the four genera usually placed in Pseudergolini, we have not sampled two (the previously mentioned *Dicorrhagia* and *Pseudergolis*). Whether these two genera group with *Stibochiona* and *Amnoseis* or with *Cyrestis* remains to be investigated.

The basal positions of Limenitidini, Cyrestini, Pseudergolini, and Biblidini in the heliconine and nymphaline clades suggests that these tribes have been placed together earlier based on symplesiomorphies. Our results suggest that great care must be taken when coding morphological characters in these groups in order to avoid having more symplesiomorphies than potential synapomorphies, which might cause these tribes to group together in a cladistic analysis.

Our results also confirm the association of Apaturinae with the Nymphalinae as has been found by Weller et al. (1996) and Brower (2000). The close relationships of the Nymphalinae, Apaturinae and Biblidini, or Limenitidini and Heliconiinae have never been suggested in morphological studies (e.g., de Jong et al., 1996; Freitas, 1999; Harvey, 1991). Both of these relationships were recovered by Brower (2000) after successive approximations weighting was used on the data matrix. We now confirm the relationships with an increased number of characters without the need to reweight the characters.

We found Satyrinae and Morphinae to be polyphyletic as did Brower (2000). However, our analyses suggest that it is premature to change the circumscriptions of these two families, as only a few steps were required to make the subfamilies monophyletic. More sampling of taxa will resolve this question, especially for Satyrinae which contains over 1700 species.

We show quite convincingly that Calinaginae is part of the satyrine clade, as has been indicated by several morphological studies, though never explicated. Ehrlich (1958) in his description of the family Nymphalidae points out several morphological features of *Calina* that ally it with the morphines and satyrines, yet in his phenogram of the relationships of nymphalid subfamilies, he does not ally Calinaginae with any group in the Nymphalidae (in Ehrlich’s diagram of relationships the names Calinaginae and Charaxinae are transposed). Similarly, de Jong et al. (1996) ally Calinaginae with Charaxinae, even though all of their cladistic analyses of morphological characters, coded for exemplar species of butterflies (Papilionoidea), place *Calina* as the sister to *Morpho* (Morphinae). In our study, the combined data set suggests that Calinaginae is the sister group to Charaxinae, though this position has weak to moderate support (Fig. 4).

Our placement of Charaxinae in the satyrine clade is somewhat of a surprise, as usually these fast and showy insects are allied with Nymphalinae or Apaturinae. However, it is quite clear that Charaxinae and Apaturinae are similar due to convergence. In both subfamilies the adults have thick bodies and sharp wings, and both subfamilies are renowned for their flight speed and life in the canopies of trees.

Our results differ somewhat from the results of a recent morphological study (Freitas, 1999), which found the Nymphalinae and Heliconiinae to be sister groups. In our study these two subfamilies are not immediate sister groups, but both are part of two major clades which are most likely to be sister (Figs. 3 and 4). Interestingly, our cladograms suggest that larval spines (scoli) have evolved once in the ancestor of the heliconine and nymphaline clades and have been lost secondarily in the Apaturinae. Yet the larval spines of Heliconiinae are not thought to be homologous with the larval spines of Nymphalinae (Freitas, pers. comm.; Harvey, 1991). This question clearly needs further investigation.

Our study suggests that the number of currently recognized subfamilies in Nymphalidae are too few. The tribe Biblidini should be raised to subfamily status without a doubt and quite likely the tribes Cyrestini and Pseudergolini need to be placed in their own subfamily (Cyrestinae would have precedence over Pseudergolini). Limenitidinae should be restricted to the genera and subtribes placed in the tribe Limenitidini by Harvey (1991). We have sampled all the groups that have previously been unplaced in Nymphalidae or have changed places often and thus do not anticipate any further new subfamilies to be necessary. A revised classification of the subfamilies and tribes of Nymphalidae based on our results is given in Appendix A.

Our study sampled fewer species than a previous molecular study of the Nymphalidae (Brower, 2000). However, our inclusion of the EF-1α and COI data sets has allowed us to confidently identify four major groups in Nymphalidae. We believe that further sampling will not remove these groups, though the internal relationships of these groups will change. The relationships between the four groups is not entirely clear with our data sets, though the sister group relationship of the heliconine and nymphaline clades receives good support with the removal of the COI third positions from the combined data set. Whether danaines are the most basal large group of Nymphalidae (Freitas, 1999) or the sister group to the satyrine clade (Brower, 2000; Weller et al., 1996) remains to be discovered with more character data or more taxon sampling.

COI has been shown to be an excellent gene for generic and species level studies in Lepidoptera (Caterino...
and Sperling, 1999; Wahlberg et al., 2003; Wahlberg and Zimmermann, 2000), while EF-1α has been proposed as a good source for characters to resolve deeper divergences (Mitchell et al., 1997). Wingless has shown itself to be of great utility at intermediate levels (Brower, 2000; Brower and DeSalle, 1998; Nylin et al., 2001). Our study shows that with sparse sampling of a higher level taxon, one should concentrate on slowly evolving nuclear genes, rather than quickly evolving mitochondrial genes. With increased sampling the phylogenetic information in quickly evolving genes can be recovered (e.g., Källersjö et al., 1998), though what is a sufficient level of sampling for quickly evolving genes is not clear at the moment.

Based on our results, it is now possible to choose the correct outgroups for higher level molecular systematic research on the Nymphalidae. Previous work (e.g., Ackery, 1984; Ackery et al., 1999; de Jong et al., 1996; Ehrlich, 1958; Harvey, 1991) has not allowed unambiguous choice of outgroups for molecular work, hence our wide range sampling to find suitable outgroups for our work on the subfamilies Nymphalinae and Satyrinae. It is now clear that we must sample Apaturinae and Biblidini (sensu Harvey, 1991) more intensively in order to discover which group is the sister group to Nymphalinae, as well as include samples of Calinaginiae and Charaxinae in our study of the Satyrinae. Our results also allow for more focussed research in the future. In our opinion, the two most important areas of research in the higher systematics of Nymphalidae are the resolution of the internal relationships of the major clades and the scrutiny of the monophyly of the nymphaline clade.

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Appendix A

A revised higher classification of Nymphalidae. Tribes according to Harvey (1991). A full list including authors, years and genera within tribes can be viewed at http://www.zoologi.su.se/research/wahlberg/.

NYMPHALIDAE
Libytheinae
Danainae
Danaini
Tellervini
Ithomiini
Charaxinae
Charaxini
Euxanthini
Prothoini
Pallini
Preponini
Anaeini
Morphinae
Morphini
Amathusiini
Brassolini
Satyrinae
Haeterini
Melanitini
Elymniini
Eritini
Ragadiini
Satyrini
Calinaginiae
Heliconiinae
Pardopsidini
Acræini
Heliconiini
Argynnini
Limenitidinae
Limenitidini
Neptidini
Parthenini
Euthaliini
Cyrestinae
Cyrestini
Pseudergolini
Biblidinae
Biblidini
Eurytelini
Catonephelini
Ageronini
Epiphilini
Dynamini
Callicorini
Apaturinae
Nymphalinae
Nymphalini
Coeini
Kallimini
Melitaeini
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