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ZOOLOGISKA
INSTITUTIONEN
Stockholms Universitet S-106 91 Stockholm

HIGHER LEVEL PHYLOGENY OF SATYRINAE BUTTERFLIES (LEPIDOPTERA: NYMPHALIDAE) BASED ON DNA SEQUENCE DATA: A PRELIMINARY STUDY

Författare: Carlos Peña Bieberach
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Higher level phylogeny of Satyrinae butterflies (Lepidoptera: Nymphalidae) based on DNA sequence data: A preliminary study

Författarens namn: **Carlos Peña Bieberach**

Handledare: **Niklas Wahlberg**

Forskningsämne: **Ekologi**

Granskningskommitté: **Sören Nylin**
Kjell Arne Johanson

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A preliminary study

Carlos Peña Bieberach

Department of Zoology, Stockholm University

Abstract

The subfamily Satyrinae is the most diverse group in Nymphalidae (about 2000 species of worldwide distribution). This diversity is not reflected in the number of studies on the systematics of the group. I have used 3090 base pairs of DNA from the mitochondrial gene COI and the nuclear genes *EF-1* and *wingless* for 93 Satyrinae taxa and 16 outgroups in order to test the monophyly of the subfamily and elucidate patterns of relationships of its major lineages. In a combined analysis, the data sets support an almost fully resolved topology, which recovered Satyrinae as polyphyletic, and several of its currently recognized tribes and subtribes as monophyletic clades. The most noteworthy findings are that *Bia* has a strongly supported relationship with Brassolini; *Manataria* is closely related to Melanitini; *Palaeonympha* should belong to Euptychiina; *Oressinoma*, *Orsotriaena* and *Coenonympha* group with the Hypocystina; and Miller's (1968) subdivisions of Parargina correspond actually to distantly related lineages. These three genes used in a combined analysis prove very useful in resolving relationships of Satyrinae at the subtribal and tribal levels. More sampling of the taxa most related to Satyrinae as well as extensive sampling of genera in the tribes and subtribes for this group is critical to test the monophyly of the subfamily and establish a stronger basis for future evolutionary studies.

Introduction

Butterflies are one of the most studied and best known groups of organisms. The great amount of information published on this group of insects includes a variety of topics in ecology, evolutionary biology and conservation biology (e.g. Boggs *et al*., 2003). However, the phylogenetic relationships of butterfly taxa are still poorly known. Several areas of study in comparative biology (namely evolution of host plant preferences, mimicry, behavior, etc) depend on phylogenetic hypotheses to have a strong basis for research. Despite some efforts to elucidate the higher level relationships in the family Nymphalidae (Wahlberg *et al*., 2003a; Freitas and Brown, 2004), we still have almost no understanding about the patterns of relationships within the most diverse group of butterflies belonging to Nymphalidae, the subfamily Satyrinae (about 2000 species of worldwide distribution). The diversity of the group is not reflected in the number of studies on the systematics of the group, in fact the latest study to encompass the whole group is Miller's (1968) outdated work, who followed an orthogenetic criterion for his hypothesis of the Satyrinae phylogeny.

The group Satyrinae has been a matter of confusion. The taxonomic status and even the taxa forming what we consider now as Satyrinae has been changing since long ago. One of the first modern attempts to classify the butterflies was the work by Ehrlich (1958), who considered Satyrinae as a subfamily of Nymphalidae, being related to Morphinae and Calinaginae. Later, Ehrlich and Ehrlich (1967) used a quantitative phenetic approach to propose a scheme of classification retaining the same taxonomic status for Satyrinae. Then Miller (1968), without any explanation, considered the group as having the family rank “Satyridae”. Miller proposed new family level groupings and used those that were already proposed to classify the entire group, considering Brassolinae (including *Bia*, *Antirrhea*, *Caerois* and *Melanitis* therein) as members of his Satyridae. DeVries *et al*., (1985) used a cladistic analysis of mainly immature stages characters to show that Miller's Antirrhini should be removed into the Morphinae stating that the Biini of Miller (*Bia*) is of uncertain position and that Melanitini should remain in the Satyrinae. Harvey (1991), in his classification scheme, based especially on immature stages, treated Satyrinae as a subfamily again, removed the Brassolinae from the Satyridae of Miller, to be a subfamily on its

own inside Nymphalidae, removed Miller's Antirrhini into Morphinae (as claimed by DeVries *et al*., 1985), and left *Bia* in Satyrinae. For the classification of tribes and subtribes, Harvey largely followed Miller's scheme, but downranking his subfamilies and tribes to tribes and subtribes respectively (Harvey, 1991). The last classification of butterflies is by Ackery *et al*. (1999) that made some changes in Harvey's (1991) classification but entirely followed his conception of Satyrinae.

After these rearrangements, some level of consensus in placing the satyrine butterflies as a subfamily was reached. The resolution of the major lineages in Satyrinae was the next logic step in order to have a strong basis for posterior evolutionary studies. The study by Viloria (1998; 2003) was one of the first efforts to address this subject. Viloria (2003), according to a cladistic and biogeographic study of American and Australian satyrine butterflies, proposed that good part of the genera considered in Pronophilina should belong to Erebiina and Hypocystina. The rest of the works trying to uncover the relationships of satyrine butterflies are some studies on species (Monteiro and Pierce, 2001) and genus level relationships (Martin *et al*., 2000; Torres *et al*., 2001). Martin *et al*. (2000) examined the phylogeny of some satyrine genera distributed in Europe, concluding that *Aphantopus hyperantus* should be removed from Coenonymphina into Maniolina.

Except through the study of Viloria (2003), we have almost no knowledge about the phylogenetic relationships of the major lineages of Satyrinae. Since a robust phylogenetic hypothesis is crucial for integrating natural groups in our classification schemes, identifying the major lineages and resolving the relationships of the satyrine butterflies is a critical matter to accomplish. At the moment, our current classification of Satyrinae relies on the work of Miller (1968) for the most part.

It is amply acknowledged that phylogenies are the backbones for comparative studies. We need to identify sister groups before we can study the evolution of host plant use, and its possible consequences on the diversification of the taxa in this highly diverse subfamily. Phylogenies are also critical for biogeographic studies, since a match between sister taxa and allopatric distributions may be evidence of vicariant events. Also, the information generated by biogeography in conjunction with phylogenies and geological data will allow us to establish

timings of diversification, and in the end, will help us to reconstruct the evolutionary history of the subfamily.

For these reasons, the aim of this study is to test the monophyly of Satyrinae, elucidate patterns of relationships of the major groups (tribes and subtribes) and provide evidence on the relationships among these lineages by using a cladistic analysis based on molecular data. The resulting phylogeny will be a first step in understanding the diversification of this globally successful subfamily. In this study, I follow Ackery *et al*.'s (1999) classification for families and subfamilies and Miller's (1968) classification for the groups in Satyrinae as modified by Harvey (1991).

Systematics

To date, there are three methodological approaches to infer phylogenies: the cladistic, the phenetic (proposed originally for morphological data) and the modelling approach. These three methods differ in the way they search for the phylogenetic tree and test the robustness of the resulting cladogram, but most importantly is that they differ in philosophical principles. The cladistic framework uses the maximum parsimony criterion to select the tree that implies the minimum amount of evolutionary change (evolutionary steps) required to explain a given data set (Farris, 1970; Swofford *et al*., 1996). The main method in the phenetic approach is Neighbor-joining (NJ), which calculates the distance between each pair of taxa to construct a tree by joining pairs of taxa that have most similar sequence data, minimizing the sum of branch lengths in the resulting tree (Saitou and Nei, 1987). There are two principal methods in the modelling approach: Maximum likelihood (ML) and Bayesian analysis. In the ML method, the preferred tree is the one which is found to be the most likely to result by using a pre-established model of the evolutionary process (Swofford *et al*., 1996). The Bayesian analysis is used to apply the same models within a Bayesian statistical framework (e.g. to find out the probabilities of a X group for being monophyletic) (Lewis, 2001). In this way it is possible to deal with large data sets and complex models in a reasonably short time (which would be computationally too expensive for the ML approach).

To select from any of these approaches, it is necessary to analyse the method that presents

an adequate philosophical framework, and avoid weakness in theory and practice. The NJ was proposed as a method to produce a unique final tree (which is not necessarily the minimum-evolution tree) considered as the "correct tree" (Saitou and Nei, 1987). But the single tree given may depend on the order of the taxa in the data matrix (Farris *et al*., 1996). The results of the modelling methods depend on the assumed model of evolution, thus inadequate models (models that poorly fit the data) may result in erroneous inferences (Bollback, 2002). In addition to the problem of choosing the right model to use for inferring phylogenies, some models need to be constructed after examining the patterns of evolution which are the result of inferring phylogenies. Thus, if a product of inferring phylogenies is used to infer phylogenies, we are likely to fall in circular thinking.

In this study, I chose the cladistic framework since it bases the search of phylogenies by ordering the shared characters (synapomorphies) into a nested hierarchy (Kitching *et al*., 1998) mirroring the process of evolution.

Material and Methods

To resolve the relationships of the lineages in the subfamily Satyrinae, I have obtained DNA sequences of three genes from 93 exemplar species of 15 subtribes included in 4 tribes recognized by Harvey (1991) and some taxa of uncertain position as *Manataria* and *Palaeonympha*. Table 1 shows the sampled species in their current taxonomic classification.

I extracted DNA from two legs of the butterflies, freshly conserved in 96% alcohol using QIAgen's DNEasy extraction kit. For each species, I sequenced 1450 bp of the cytochrome oxidase subunit I gene (COI) from the mitochondrial genome, and 1240 bp of the *Elongation Factor-1* gene (*EF-1*), and 400 bp of the *wingless* gene, from the nuclear genome. Part of the sequences for the data sets were taken from Wahlberg *et al*. (2003b). The sequences from COI and *EF-1* for *Hallelesis asochis* were taken from Monteiro and Pierce (2001). The primers for the COI were taken from Wahlberg and Zimmerman (2000), for *EF-1* (primers ef51.9 and efrcM4) from Monteiro and Pierce (2001) and for *wingless* from Brower and DeSalle (1998). Additional

primers taken from Cho *et al*. (1995) were used for *EF-1* sequences, Starsky (sense: 5'-CAC ATY AAC ATT GTC GTS ATY GG-3') and Luke (antisense: 5'-CAT RTT GTC KCC GTG CCA KCC-3'), another primer taken from Reed and Sperling (1999), Cho (sense: 5'-GTC ACC ATC ATY GAC GC-3') and Verdi (courtesy from F. Sperling's lab) (antisense: 5'-GAT ACC AGT CTC AAC TCT TCC-3'). Voucher specimens are deposited at the Department of Entomology, Museo de Historia Natural, Universidad Nacional Mayor de San Marcos, Peru; and at the Department of Zoology, Stockholm University, Sweden.

The PCR reactions were performed in a 20 l volume. The reaction cycle profile for COI was 95ºC for 5 min, 34 cycles of 94ºC for 30s, 47ºC for 30s, 72ºC for 1 min 30s, and a final extension period of 72ºC for 10 min. The reaction cycle profile for primers Starksy-Luke and Cho-Verdi was 95ºC for 7 min, 34 cycles of 95ºC for 30s, 55ºC for 30s, 72ºC for 2 min, an extension period of 72ºC for 10 min and a final one of 20ºC for 10s. The reaction cycle profile for primers ef51.9-efrcM4 and the *wingless* gene was 95ºC for 5 min, 39 cycles of 95ºC for 1 min, 51ºC for 1 min, 70ºC for 1 min 30s and a final extension period of 72ºC for 7 min. The PCR primers used for sequencing were the same in case of *EF-1* and *wingless*, while in COI I used an internal primer designed by N. Wahlberg (Patty 5'-ACW GTW GGW GGA TTA ACW GG-3') in addition to the PCR primers for sequencing. The sequencing of the PCR products was done with a Beckman-Coulter CEQ8000 capillary sequencer. The resulting chromatograms were checked by using the program BioEdit (Hall, 1999) and the sequences were aligned by eye. The sequences will be submitted to GenBank, eventually.

The complete data set consisted of 109 taxa (including 16 outgroups) and 3090 nucleotide base pairs. All the characters were treated as unordered and equally weighted. The resulting data matrix was analyzed according to a cladistic framework by performing a heuristic search with 1000 random addition replicates using TBR branch swapping with up to 200 trees held during each step in the program PAUP* (Swofford, 1998). The same procedure was applied for each gene separately and for all the three genes combined. Some taxa were not included in the separate analysis of the *wingless* data set since it was not possible to get sequences for them: i.e. *Pharneuptychia*, *Hallelesis* and *Hypocysta*.

I evaluated the robustness of the clades in the resulting cladograms by using bootstrap

analyses (Felsenstein, 1985) and Bremer support (Bremer, 1988; 1994). I calculated bootstrap values from 1000 pseudoreplicates with 10 random additions per pseudoreplicate in PAUP*(Swofford, 1998). I used the program TreeRot (Sorensen, 1999) in conjunction with PAUP* (Swofford, 1998) to calculate Bremer support values. I assessed the contribution of each gene data set to the total Bremer support in the combined analyses by using Partioned Bremer Support (PBS) (Gatesy *et al*., 1999) using the program TreeRot (Sorensen, 1999) in conjunction with PAUP* (Swofford, 1998). In the results and discussion sections, I will refer to the support as weak for 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%).

I decided to root the resulting networks with *Libythea* because of the wide consensus in regarding this taxon as sister group to the rest of Nymphalidae (e.g. Ehrlich, 1958; Ackery *et al*., 1999; Brower, 2000). Additional outgroups including taxa from the "satyroid" (*sensu* Freitas and Brown, 2004) subfamilies were used to test the monophyly of Satyrinae.

Results

General properties of sequences

The full data set consisted of 3090 aligned nucleotide sites without any indels. I was not able to amplify the *wingless* gene for *Pharneuptychia* and *Hypocysta*. The COI and *EF-1* sequences for *Hallelesis* were taken from Monteiro and Pierce (2001) and it was not possible to get samples for this genus to sequence the *wingless* gene. Of the 1450 bp sequenced for COI, 780 sites were variable and of these 612 were parsimony informative. The respective numbers for *EF-1* are 1240 bp, 567 variable and 427 parsimony informative, and for *wingless*, 400 bp, 257 variable and 195 parsimony informative sites.

Results of the phylogenetic analyses

In the separate analyses for each gene data set, the COI sequences produced a partially

resolved strict consensus tree (Fig. 1). The strict consensus recovered many of the currently recognized taxonomic groups as monophyletic clades, among them: Haeterini, Mycalesina (without *Orsotriaena*), Satyrina, Euptychiina (without *Oressinoma*), and Maniolina (+ *Aphantopus*). Although the COI gene on its own yielded well resolved intra- tribal and subtribal relationships, it was not able to resolve the deeper nodes of ancient origin (Fig. 1). The *EF-1* data set yielded a strict consensus tree partially resolved in some agreement with the current taxonomic classification (Fig. 2). Several groups were recovered as monophyletic clades, among them: Brassolini (+ *Bia*), Haeterini, Melanitini (+ *Manataria*), Mycalesina (without *Orsotriaena*), Satyrina, Euptychiina (with *Amphidecta* but without *Oressinoma*), Maniolina (+ *Aphantopus*) and Ypthimina. The *EF-1* data lacks resolution for more recent relationships as most of the intra-tribal and subtribal relationships are polytomies and was unable to recover the deeper nodes in the strict consensus (Fig. 2).

The *wingless* data set produced an almost fully resolved strict consensus tree with some deeper nodes unresolved (Fig. 3). The relationships in the strict consensus are poorly consistent with current taxonomy, having some plesiomorphic outgroups nested in derived positions within the satyrine ingroup (e.g. *Danaus* and *Heliconius*). Few groups are recovered as monophyletic entities Brassolini (+ *Bia*), Haeterini, Melanitini (+ *Manataria*), Mycalesina (without *Orsotriaena*), Satyrina and Hypocystina (without *Zipaetis*) (Fig. 3).

The cladistic analysis of the combined data sets with the characters equally weighted produced four equally parsimonious cladograms. The strict consensus in Fig. 4 shows the relationships among the resulting major clades of the satyrine butterflies as well as relationships with the outgroups selected for this study. For this data set, the Morphinae and the Satyrinae appear as polyphyletic groups, grouping together in a clade with good Bremer and weak bootstrap support, and standing apart from other "satyroid" (*sensu* Freitas and Brown, 2004) butterflies is the Charaxinae.

According to our current picture of Satyrinae, it appears as a polyphyletic assemblage, with some representatives, having been considered as the most "primitive" satyrines (Miller, 1968), grouping with a tribe of the Morphinae, the Amathusiini. The "Amathusiini" clade has moderate Bremer and no bootstrap support and, in addition to the Amathusiini, includes the

satyrine subtribes Elymniina, Zetherina, and representatives of the Lethina (*Neorina* and *Ethope* in this study). *Neorina* and *Ethope* form a monophyletic clade with the Zetherina having strong Bremer and bootstrap support values (>30 steps; 100%), but Zetherina (*Penthema* and *Zethera* in this study) is recovered as polyphyletic since *Penthema* and *Zethera* group with *Neorina* and *Ethope* respectively, with strong Bremer and bootstrap support. *Neorina* and *Ethope* + Zetherina form a monophyletic group with Amathusiini with good Bremer and no bootstrap support. The Elymniina is monophyletic and appears as sister of the polyphyletic Zetherina + Amathusiini with moderate Bremer and no bootstrap support.

The next derived clade is the "morphine" clade, formed by the Morphinae tribes: Morphini, Antirrhini and Brassolini with good Bremer and weak bootstrap support. *Bia* (the satyrine Biini) is basal in the Brassolini clade with strong Bremer and bootstrap support. The "morphine" clade (the Amathusiini appears excluded) is recovered as sister to the proper "satyrine" clade, which includes all the satyrine representatives in this study but Elymniina, Zetherina, and *Neorina* and *Ethope* (as stated above).

The "satyrine" clade is recovered with moderate Bremer and no bootstrap support and includes two major clades, encompassing and recovering as monophyletic entities some of the currently recognized tribal and subtribal satyrine categories (Harvey, 1991). One of these clades is formed by two monophyletic groups, both with strong Bremer support - the Haeterini with strong bootstrap support and, the Palaeotropical and Austro-Oriental Melanitini + the Neotropical genus of uncertain position *Manataria* with moderate bootstrap support. The other clade is somewhat robust with strong Bremer and moderate bootstrap support, and includes the largely recognized as "non-primitive" satyrine butterflies.

Basal within the "advanced" satyrine butterflies is a robust monophyletic group with strong Bremer and bootstrap support formed by some members of the Lethina - *Pararge*, *Lopinga* and *Lasiommata* - which correspond with one of Miller's (1968) subdivisions of his "Lethini", his *Pararge*-series. Mycalesina is recovered as a polyphyletic group since *Orsotriaena* is placed among the Hypocystina. However, Mycalesina without *Orsotriaena* is a very cohesive clade with strong Bremer and bootstrap support (20 steps; 99%). Miller's *Lethe*-series (represented by *Lethe* and *Neope* in this study) appears as basal in a clade containing Mycalesina, with moderate Bremer

and weak bootstrap support. The remaining major clade is the Satyrini, recovered as a clade with moderate Bremer and no bootstrap support. Satyrini would have been monophyletic if it were not including one of the members of Mycalesina (*Orsotriaena*).

Within the Satyrini clade, three clades can be identified, the Hypocystina, the Euptychiina and a clade composed by the Satyrina, Pronophilina, Erebiina, Maniolina, Melanargiina and the Ypthimina. The Hypocystina clade, including *Orsotriaena*, Coenonymphina (*Coenonympha* in this study) and *Oressinoma*, is recovered as a clade with moderate Bremer and no bootstrap support. *Orsotriaena* appears as sister to the Indian genus *Zipaetis* with strong Bremer and moderate bootstrap support. A surprising result is the inclusion of the Neotropical euptychiine genus *Oressinoma* within Hypocystina, appearing as sister to *Hypocysta* with moderate Bremer and no bootstrap support. Euptychiina, including the pronophiline *Amphidecta* and without *Oressinoma*, is recovered as a robust clade with strong Bremer and bootstrap support (18 steps; 98%). Another surprising result is the inclusion of the Oriental genus *Palaeonympha* (thus far of uncertain position) nested inside the cohesive Euptychiina which is a group entirely restricted to the Americas. Interestingly, Miller (1968) was not sure whether *Palaeonympha* belongs to either Euptychiina or Ypthimina. Sister to Euptychiina is the clade composed by Satyrina, Pronophilina, Erebiina, Maniolina, Melanargiina and Ypthimina.

Ypthimina is recovered as monophyletic with moderate Bremer and no bootstrap support, being sister to Melanargiina with weak Bremer and no bootstrap support. Satyrina is recovered as monophyletic with strong Bremer and bootstrap support (>30 steps; 100%) and as sister of Melanargiina + Ypthimina with weak Bremer and no bootstrap support. Erebiina (represented by *Erebia* in this study) appears nested within Maniolina with weak Bremer and no bootstrap support.

The clade Maniolina without *Erebia* and *Cercyonis* is robust with strong Bremer and bootstrap support (18 steps; 100%). The subtribal relationships in this clade are weakly supported by Bremer and no bootstrap support values. The Melanargiina + Ypthimina + Satyrina clade is grouping with one group of Pronophilina members in a clade with weak Bremer and no bootstrap support, and Erebiina + Maniolina are grouping with the rest of the Pronophilina representatives in a second minor clade also with weak Bremer and no bootstrap support.

Results of the Partitioned Bremer Support

For the data set in this study, the major source of conflict is the *wingless* partition. In the combined analysis, the *wingless* data set is conflicting in 70 of the 102 nodes of the strict consensus tree, while the conflicting nodes are 10 and 40 for the COI and the *EF-1* respectively (Fig. 4). The *wingless* partition conflicts in both, deep and shallow nodes in the consensus tree, and in some nodes is ambiguous (PBS = 0).

The COI partition is responsible for almost all of the deeper nodes in the combined analysis, conflicting in only one node (Fig. 4). The partition values for COI are high enough to overcome the conflicting signal of the *EF-1* and even the more conflicting *wingless*.

It is obvious that the main source of signal in the combined analysis comes from the COI and *EF-1* data sets since most of the groups recovered in the combined analysis are also recovered by COI and *EF-1* in their respective consensus trees during the separate analyses (compare Figs. 1 and 2 with Fig. 4). However, the COI and *EF-1* data together produced a poorly resolved topology which recovers part of the deeper nodes present in the combined analysis of the three genes. Thus, despite being in conflict in most of the nodes in the combined analyses, the *wingless* data set has some degree of phylogenetic information, contributing positively to several nodes in both deeper and shallow relationships when used in combination with the two other genes.

Discussion

To date, this study includes the most extensive sampling of satyrine butterflies in an attempt to uncover the phylogenetic relationships under a quantitative approach, including 15 subtribes in 4 tribes of the currently recognized satyrine classification (Harvey, 1991). Although some taxa, that might represent major lineages in the subfamily, were not included in the analyses, this study produced significant results on the relationships of the Satyrinae butterflies.

The results of the combined analysis of the three genes provide evidence for the

monophyly of several of the current tribes and subtribes recognized in Satyrinae, namely Haeterini, Satyrini, Satyrina, Maniolina (including *Aphantopus*). This study also suggests new interesting relationships of taxa long considered of uncertain affinities (i.e. *Manataria* and *Palaeonympha*).

However, this study recovers the Satyrinae and Morphinae as polyphyletic since Elymniina and Zetherina tend to group with the Amathusiini. More sampling of the tribes in the Morphinae is needed to clear up these situations because too few taxa of the tribes in the Morphinae were used for this study.

Since the subtribe Erebiina is represented by only one genus herein, it is not possible to test its monophyly. Again, more sampling will provide additional information to test its phylogenetic status and also to resolve its intersubtribal relationships which are weakly supported in this data set.

Elymniina and Melanitini are monophyletic (Fig. 4), which is expected because *Elymnias* and *Elymniopsis* are considered synonyms by some authors (but see Lewis, 1974), and some species of *Gnophodes* are sometimes included in the genus *Melanitis* (Larsen, 1991). The close relationship between *Manataria* and Melanitini is new and well supported by the data. *Gnophodes* species do not have much activity during the day (Larsen, 1991) and *Melanitis* has crepuscular habits flying at dusk and dawn (Larsen, 1991; Braby, 2000), while the same crepuscular activity is recorded for *Manataria* (DeVries, 1987; Stevenson and Huber, 2000). Moreover, *Manataria* has the unusual behavior of roosting in tree holes or shaded areas along forest trails in Costa Rica, in groups up to 80 individuals (Stevenson and Huber, 2000; Murillo and Nishida, 2004). Interestingly, Larsen (1991) reports that *Gnophodes* species also form small congregations in forest trails. The close relationship between *Manataria* and Melanitini in a monophyletic clade (Fig. 4) suggests that this similar behaviour may be due to a common origin. Whether these similarities are synapomorphies or just convergence needs further investigation, since some other "satyroid" taxa are also crepuscular (e.g. *Caligo* in Brassolini and *Taygetis* in Satyrinae). Miller and Miller (1997) suggested a close affinity among *Manataria* and *Aeropetes*-series of Parargina, but that relationship is supported by very weak morphological evidence, and needs to be tested with molecular data.

There is strong evidence for a polyphyly of Zetherina, which includes *Neorina* and *Ethope* in a monophyletic clade in this study (Fig. 4). It is very likely that additional taxa will not change this grouping since it is very strongly supported by Bremer and bootstrap values. Elymniina and the polyphyletic Zetherina tend to group with the Amathusiini, though this is not very well supported. Interestingly, Elymniina members feed on palms (Arecaceae) as larvae as some species of the Amathusiini, also *Neorina* may be feeding on palms (Ackery, 1988). Very few satyrine butterflies feed on Arecaceae plants, eg. some Haeterini (*Dulcedo*; DeVries, 1987).

The position of *Bia* (the Biini) in a monophyletic clade with Brassolini (Fig. 4) is supported by their common characteristics in immature stages (Freitas *et al*., 2002). This relation was also found by Brower (2000) and is congruent with the successive weighting analysis tree of morphological data of Freitas & Brown (2004). Thus, the hypothesis for *Bia* to belong to the Brassolini has gained strong support.

The traditionally recognized tribe Haeterini and subtribe Satyrina are recovered as very cohesive entities, and additional characters, I believe, are not likely to modify their monophyletic status. In the same fashion, "non-primitive" satyrine butterflies as a clade (which includes the Mycalesina, Satyrini and some members of Parargina, *Pararge* and *Lethe* series) has good support and may remain after addition of more data.

Euptychiina and Mycalesina also appear as robust monophyletic groups that are very well supported. However, some genera traditionally associated with these subtribes are clearly not related to them, e.g. *Orsotriaena* is not grouping with Mycalesina and *Oressinoma* is not grouping with Euptychiina. In addition, *Amphidecta* is clearly within Euptychiina, though it is traditionally associated with Pronophilina.

The monophyly of Mycalesina without *Orsotriaena* is quite surprising. *Orsotriaena* is largely recognized as closely related to *Mycalesis* (Parsons, 1999; Braby, 2000) mainly due to adult morphology. But, larval and pupal morphology of *Mycalesis* and *Orsotriaena* are strikingly different. If *Orsotriaena* is not related to the other Mycalesina as evidenced by my results and morphological differences of immature stages, the similar adult morphology may be merely superficial homoplasy. Moreover, the veins of *Orsotriaena* are not inflated as they are in *Mycalesis* (Parsons, 1999). On the other hand, the position of *Oressinoma*, far away from

Euptychiina is not so surprising. *Oressinoma* has a much differentiated adult morphology, some authors being inclined to consider *Oressinoma* as an aberrant genus (Miller, 1968). Unfortunately, nothing is known about the immature stages or details of the natural history of this enigmatic genus.

Another surprising result is the inclusion of the oriental genus *Palaeonympha* inside Euptychiina. This association is very robust and will remain stable with the addition of more data. If this hypothesis is corroborated through a comparative study of *Palaeonympha* and the Euptychiina, the former would the only euptychiine taxon distributed outside the Americas. More interestingly is the fact that, in this data set, *Palaeonympha* appears related to specimens from the Southeastern Atlantic forests in Brazil. Miller (1968) was not willing to place *Palaeonympha* in Euptychiina because of the disjunct distribution of this taxon. The inclusion of the pronophiline genus *Amphidecta* inside Euptychiina is a robust hypothesis here. DeVries (1987) did not consider *Amphidecta* in Pronophilina, and according to morphological data, the position of *Amphidecta* is somewhat uncertain (Freitas, pers. com.)

This study corroborates the placement of *Aphantopus* from the Coenonymphina into Maniolina (Martin *et al*., 2000), although *Cercyonis* appears related to *Erebia* but with weak support. More sampling of the taxa in Erebiina is needed since only *Erebia* was used as the single representative in this investigation.

In this study, Hypocystina appears as a monophyletic clade, including *Orsotriaena*, *Oressinoma* and *Coenonympha*, though the relationships are weakly supported. Inside the Hypocystina, the mycalesine *Orsotriaena* and *Zipaetis* appear as sister taxa with very strong support. This implies a very recent divergence of *Orsotriaena* and *Zipaetis*. As stated above, the relationships of *Orsotriaena* and *Coenonympha* need further investigation. This would imply a larger distribution of Hypocystina, including the Neotropics and the Palaeartic region.

Members of Miller's *Pararge*-series (*Pararge*, *Lasiommata* and *Lopinga*) form a cohesive monophyletic clade. Miller's other subdivisions of Parargina form independent clades in congruence with each of Miller's series. Obviously each of Miller's sections represent very different lineages, *Neorina*-series group with Zetherina, *Lethe*-series group with Mycalesina, *Pararge*-series on its own. These results suggest that Parargina (*sensu* Miller) should no longer be

used.

The large Pronophilina is recovered as polyphyletic in a clade with Maniolina, Satyrina, Ypthimina and Melanargiina, with weak support for the relationships. Apparently, these results are in conflict with Viloria's (2003) hypothesis of a great part of the Pronophilina belonging to Hypocystina and Erebiina. More sampling of Northern and Southern pronophiline as well as Erebiina taxa will be needed to produce the necessary phylogenetic evidence to support or contradict Viloria's hypothesis.

The COI has been very useful for uncovering relationships at the generic and specific level (Wahlberg *et al*., 2003b; Caterino and Sperling, 1999) due to its property of a fast-evolving gene. However, in this study, the COI gene carries most of the phylogenetic signal of the Satyrinae relationships. This is congruent with Källersjö *et al*. (1998) statement of it being possible to recover phylogenetic information from such a gene as it is provided that extensive sampling is achieved. The *EF-1* is informative for resolving deep divergences as found by Mitchell *et al*. (1997). Despite the level of homoplasy provided by the *wingless* gene, it is clear that it proves useful for resolving relationships when used in combination with the two other genes.

What to do next?

This study clearly represents the first step towards understanding the evolutionary history of satyrines. I have identified well-supported major lineages of the butterflies and uncovered some surprising relationships. However, the relationships of the major lineages are not well-supported, making any attempt to study the biogeography or timing of diversification premature.

It is clear now that we need to sample more data, i.e. morphology and other genes, as well as sampling more extensively the “primitive” satyrine butterflies and analyse them in a systematic study including as many taxa of the other related “satyroid” groups as possible, which means more sampling of the tribes in the Morphinae mainly. After this is accomplished, we will have the certainty whether Satyrinae is indeed either a monophyletic or polyphyletic assemblage.

We know now that the more “derived” satyrine butterflies, that form the main number of species in the subfamily, form a well supported monophyletic clade, and we can choose the right outgroups for resolving the phylogenetic relationships at the subtribal level.

This study generates very interesting questions. If *Palaeonympha* belongs to Euptychiina, what was the evolutionary process and the timing of divergence that permitted such disjunct distribution? Is it the product of a wide distribution of Euptychiina through a North American connection? We need to include in our analyses more euptychiine genera to discover which of the euptychiine lineages *Palaeonympha* is related to, in order to propose a possible evolutionary history for such a disjunct relationship.

If *Orsotriaena* and *Zipaetis* are sister taxa, why do they look so different? And why does *Orsotriaena* resemble so much Mycalesina species? Is it that *Orsotriaena* has maintained a plesiomorphic external appearance? Or the similarities are the product of mimicry using Mycalesina species as models? If so, which species is acting as a model?

The fact that the "primitive" satyrine butterflies seem to be the most basal taxa in the subfamily Satyrinae, and most of them feed on palms as larvae while the other highly diverse satyrine clade (the most derived) feeds on grasses, leads us to ask whether is feeding on grasses a character that has assisted in the diversification of the derived Satyrinae?

In conclusion, more data and taxon sampling of critical groups will provide the basis to answer these questions and clear the path for future research on these and other interesting subjects in the evolutionary biology of the subfamily Satyrinae.

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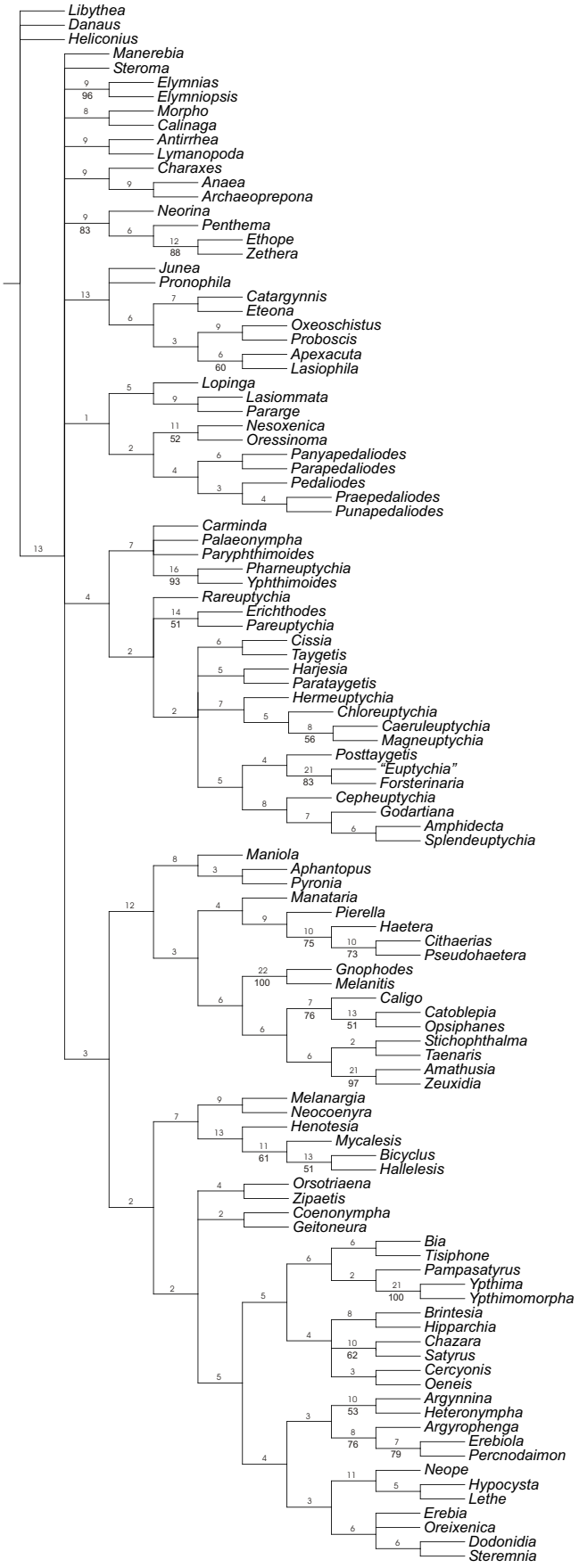
List of ilustrations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Junea
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Chazara
Satyrus
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Oeneis
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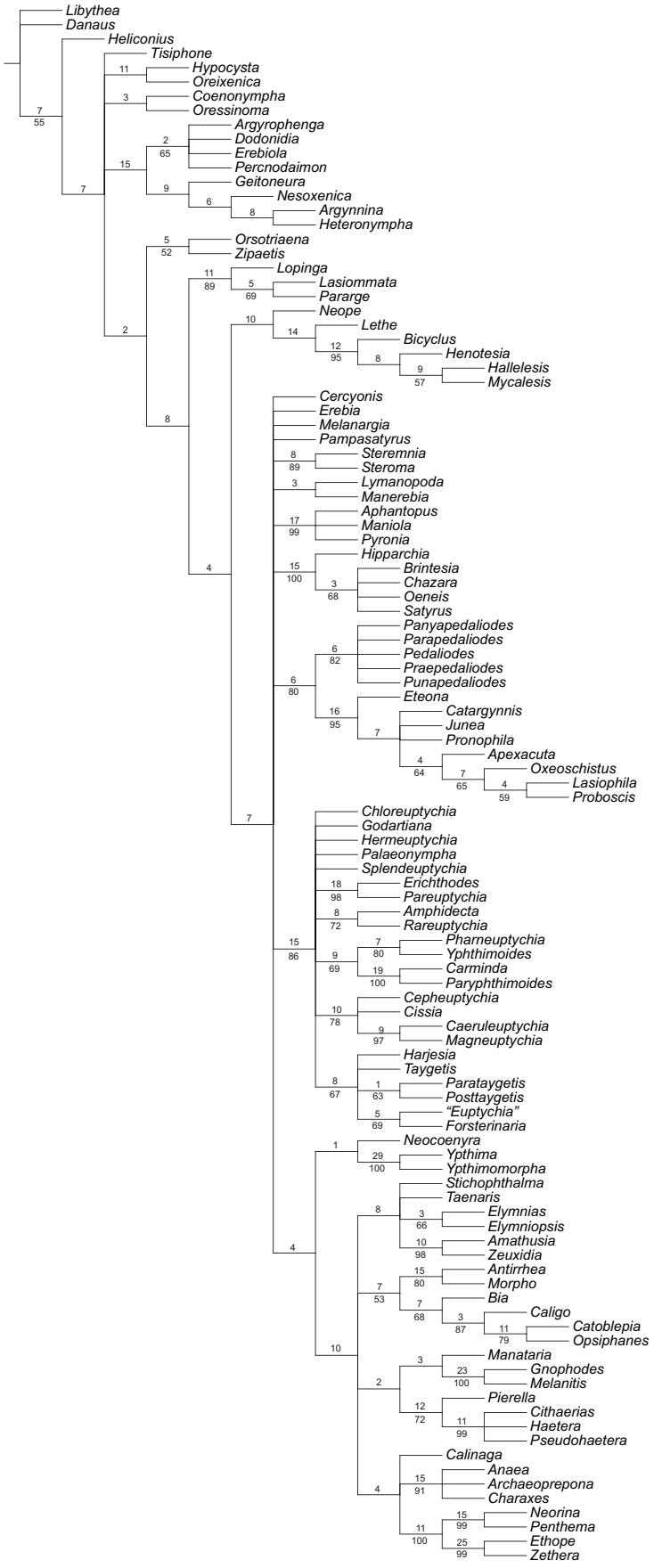
Oreixenica
Dodonidia
Steremnia

Fig. 1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Penthema
Ethope
Zethera

Fig. 2

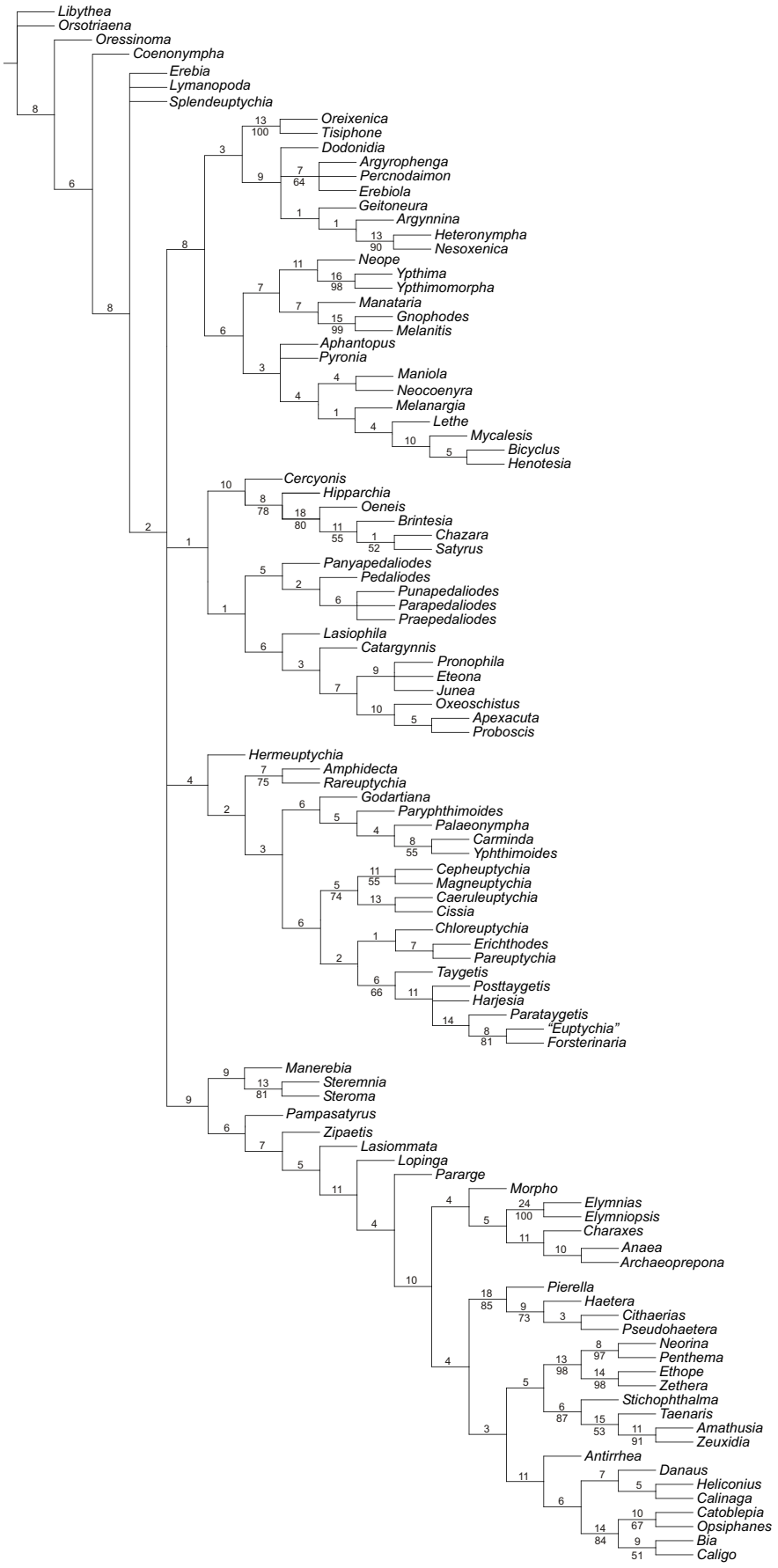
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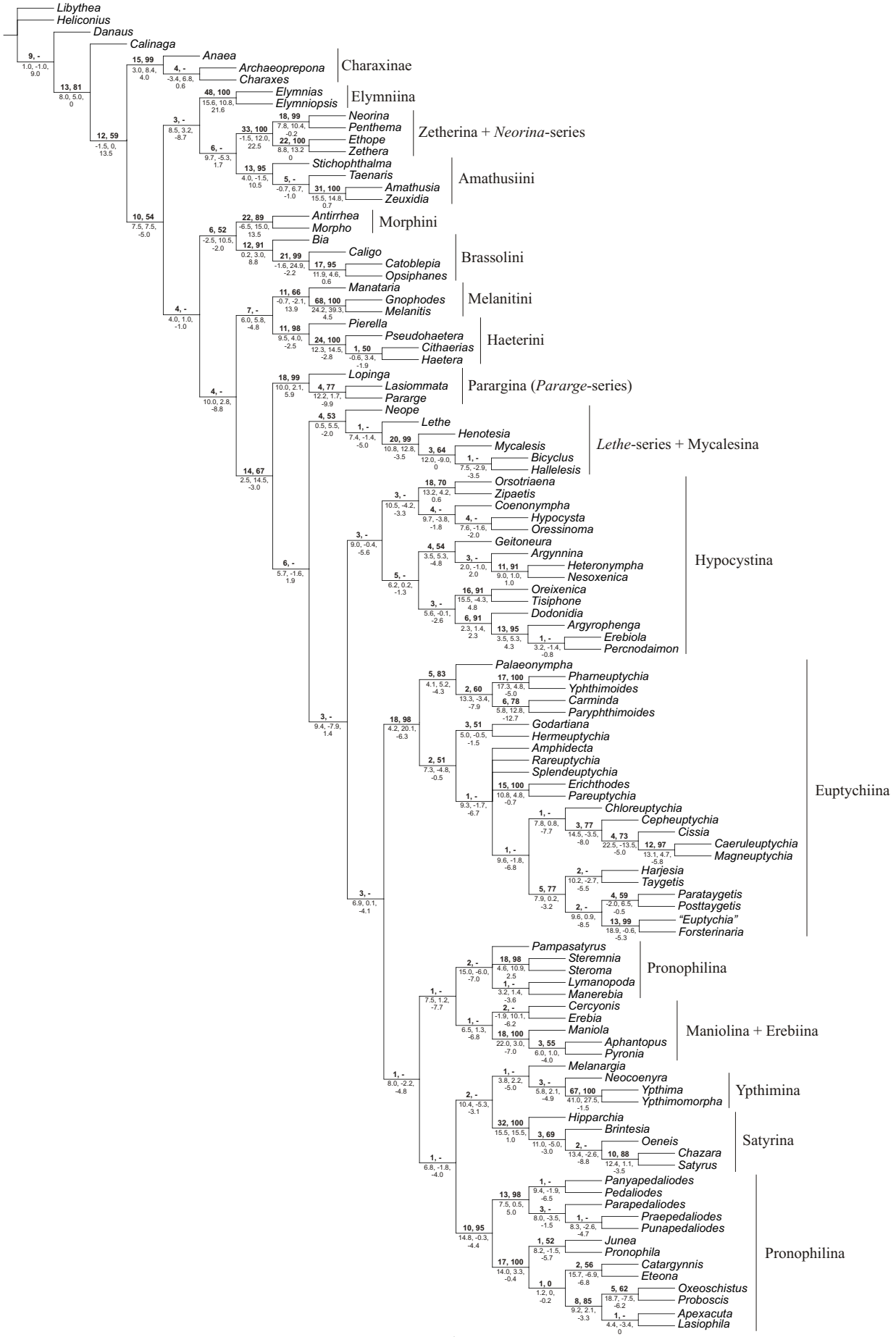
Fig. 3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Neorina
Penthema
Ethope
Zethera
Zetherina + Neorina-series
Stichophthalma
Taenaris
Amathusia
Zeuxidia
Amathusiini
Antirrhea
Morpho
Morphini
Bia
Caligo
Catoblepia
Opsiphanes
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Melanitis
Melanitini
Pierella
Pseudohaetera
Cithaerias
Haetera
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Lopinga
Lasiommata
Pararge
Parargina (Pararge-series)
Neope
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Bicyclus
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Orsotriaena
Zipaetis
Coenonympha
Hypocysta
Oressinoma
Geitoneura
Argynnina
Heteronympha
Nesoxenica
Oreixenica
Tisiphone
Dodonidia
Argyrophenga
Erebiola
Percnodaimon
Hypocystina
Palaeonympha
Pharneuptychia
Yphthimoides
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Paryphthimoides
Godartiana
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Amphidecta
Rareuptychia
Splendeuptychia
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Harjesia
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Parataygetis
Posttaygetis
"Euptychia"
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Pronophilina
Cercyonis
Erebia
Maniola
Aphantopus
Pyronia
Maniolina + Erebiina
Melanargia
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Punapedaliodes
Junea
Pronophila
Catargynnis
Eteona
Oxeoschistus
Proboscis
Apexacuta
Lasiophila
Pronophilina

Fig. 4

Table 1
Species from which the COI, *EF-1* , and *Wingless* genes were sequenced. The subfamily follows Ackery et al. (1999), tribes and subtribes follow Harvey (1991).

Subfamily	Tribe	Subtribe	Species	Source of specimen
Libytheinae			*Libythea celtis*	Spain: Barcelona
Danainae	Danaini		*Danaus plexippus*	Portugal: Madeira, Monte
Heliconiinae	Heliconiini		*Heliconius hecale*	Stratford Butterfly Farm, UK
Calinaginae			*Calinaga buddha*	Stratford Butterfly Farm, UK
Charaxinae	Charaxini		*Charaxes castor*	Stratford Butterfly Farm, UK
Charaxinae	Preponini		*Archaeoprepona demophon*	Stratford Butterfly Farm, UK
Charaxinae	Anaeini		*Anaea* sp.	Stratford Butterfly Farm, UK
Morphinae	Morphini	Morphina	*Morpho peleides*	Stratford Butterfly Farm, UK
Satyrinae	Morphini	Antirrheina	*Antirrhea miltiades*	Costa Rica: Area de Conservación Guanacaste
Satyrinae	Amathusiini		*Amathusia phidippus*	Indonesia: Bali
Satyrinae	Amathusiini		*Stichophthalma howqua*	Taiwan
Satyrinae	Amathusiini		*Taenaris bioculatus*	Indonesia: West Papua, Sorong Island
Satyrinae	Amathusiini		*Zeuxidia dohrni*	Indonesia: Java, Bandung
Satyrinae	Brassolini		*Caligo telamonius*	Stratford Butterfly Farm, UK
Satyrinae	Brassolini		*Catoblepia orgetorix*	Costa Rica: Area de Conservación Guanacaste
Satyrinae	Brassolini		*Opsiphanes quiteria*	Costa Rica: Area de Conservación Guanacaste
Satyrinae	Brassolini		*Bia actorion*	Peru: Loreto, Yurimaguas
Satyrinae	Haeterini		*Cithaerias aurorina*	Peru: Loreto, Rio Paiwa
Satyrinae	Haeterini		*Haetera piera*	Peru: Loreto, Rio Paiwa
Satyrinae	Haeterini		*Pierella lamia*	Peru: Loreto, Rio Paiwa
Satyrinae	Haeterini		*Pseudohaetera hypaesia*	Peru: Junin
Satyrinae	Melanitini		*Gnophodes chelys*	Uganda: Kibale National Park
Satyrinae	Melanitini		*Melanitis leda*	Australia: Queensland, Cairns
Satyrinae	Elymniini	Parargina	*Ethope noirei*	Vietnam
Satyrinae	Elymniini	Parargina	*Lasiommata megera*	Sweden: Stockholm
Satyrinae	Elymniini	Parargina	*Lethe minerva*	Indonesia: Bali
Satyrinae	Elymniini	Parargina	*Lopinga achine*	Sweden
Satyrinae	Elymniini	Parargina	*Neope bremeri*	Taiwan
Satyrinae	Elymniini	Parargina	*Neorina* sp.	Indonesia: West Java, Bodogol
Satyrinae	Elymniini	Parargina	*Pararge aegeria*	France: Aude, Villegly
Satyrinae	Elymniini	Zetherina	*Penthema darlisa*	Vietnam: Cuc Phong National Park
Satyrinae	Elymniini	Zetherina	*Zethera* –	–
Satyrinae	Elymniini	Elymniina	*Elymnias casiphone*	Indonesia: Bali
Satyrinae	Elymniini	Elymniina	*Elymniopsis bammakoo*	Ghana
Satyrinae	Elymniini	Mycalesina	*Bicyclus anynana*	Zimbabwe: Harare
Satyrinae	Elymniini	Mycalesina	*Hallelesis asochis*	--
Satyrinae	Elymniini	Mycalesina	*Henotesia simonsii*	Zimbabwe
Satyrinae	Elymniini	Mycalesina	*Mycalesis* sp.	--
Satyrinae	Elymniini	Mycalesina	*Orsotriaena medus*	Bangladesh
Satyrinae	Satyrini	Coenonymphina	*Aphantopus hyperantus*	Sweden: Stockholm
Satyrinae	Satyrini	Coenonymphina	*Coenonympha pamphilus*	--
Satyrinae	Satyrini	Erebiina	*Erebia palarica*	Spain
Satyrinae	Satyrini	Euptychiina	*Caeruleuptychia lobelia*	Peru: Madre de Dios
Satyrinae	Satyrini	Euptychiina	*Carminda griseldis*	Brazil: Minas Gerais, Extrema
Satyrinae	Satyrini	Euptychiina	*Cepheuptychia* sp. n.	Peru: Madre de Dios
Satyrinae	Satyrini	Euptychiina	*Chloreuptychia* sp.	Peru: Madre de Dios
Satyrinae	Satyrini	Euptychiina	*Cissia* sp.	Brazil
Satyrinae	Satyrini	Euptychiina	*Erichthodes antonina*	Peru: Madre de Dios
Satyrinae	Satyrini	Euptychiina	*"Euptychia" pronophila*	Brazil: Minas Gerais, Extrema
Satyrinae	Satyrini	Euptychiina	*Forsterinaria boliviana*	Peru: Junin
Satyrinae	Satyrini	Euptychiina	*Godartiana muscosa*	Brazil: São Paulo, Serra do Japi
Satyrinae	Satyrini	Euptychiina	*Harjesia blanda*	Peru: Madre de Dios
Satyrinae	Satyrini	Euptychiina	*Hermeuptychia hermes*	Brazil: Minas Gerais, Extrema
Satyrinae	Satyrini	Euptychiina	*Magneuptychia* sp. n.	Peru: Madre de Dios
Satyrinae	Satyrini	Euptychiina	*Oressinoma typhla*	Peru: Junin
Satyrinae	Satyrini	Euptychiina	*Parataygetis albinotata*	Peru: Junin
Satyrinae	Satyrini	Euptychiina	*Pareuptychia hesionides*	Peru: Madre de Dios
Satyrinae	Satyrini	Euptychiina	*Paryphthimoides* sp.	Brazil: São Paulo, Atibaia
Satyrinae	Satyrini	Euptychiina	*Pharneuptychia* sp.	Brazil: Minas Gerais, Extrema
Satyrinae	Satyrini	Euptychiina	*Posttaygetis rectifasciata*	Brazil: Acre
Satyrinae	Satyrini	Euptychiina	*Rareuptychia clio*	Brazil: São Paulo, Campinas
Satyrinae	Satyrini	Euptychiina	*Splendeuptychia itonis*	Peru: Madre de Dios
Satyrinae	Satyrini	Euptychiina	*Taygetis laches*	Brazil: São Paulo, Campinas
Satyrinae	Satyrini	Euptychiina	*Yphthimoides castrensis*	Brazil: São Paulo, Aguas da Prata
Satyrinae	Satyrini	Hypocystina	*Argynnina cyrila*	Ra121
Satyrinae	Satyrini	Hypocystina	*Argyrophenga antipodium*	New Zealand
Satyrinae	Satyrini	Hypocystina	*Dodonidia helmsi*	New Zealand
Satyrinae	Satyrini	Hypocystina	*Erebiola butleri*	New Zealand
Satyrinae	Satyrini	Hypocystina	*Geitoneura klugii*	Ra54
Satyrinae	Satyrini	Hypocystina	*Heteronympha merope*	Australia: ACT, Cook
Satyrinae	Satyrini	Hypocystina	*Hypocysta pseudirius*	S128
Satyrinae	Satyrini	Hypocystina	*Nesoxenica leprea*	Ra61

Continuation Table 1

Subfamily	Tribe	Subtribe	Species	Source of specimen
Satyrinae	Satyrini	Hypocystina	*Oreixenica lathoniella*	Ra165
Satyrinae	Satyrini	Hypocystina	*Percnodaimon merula*	New Zealand
Satyrinae	Satyrini	Hypocystina	*Tisiphone abeona*	S104
Satyrinae	Satyrini	Hypocystina	*Zipaetis saitis*	India
Satyrinae	Satyrini	Maniolina	*Cercyonis pegala*	USA: Oregon
Satyrinae	Satyrini	Maniolina	*Maniola jurtina*	Spain: St. Climent
Satyrinae	Satyrini	Maniolina	*Pyronia cecilia*	France: Aude, Villegly
Satyrinae	Satyrini	Melanargiina	*Melanargia galathea*	France: Aude, Cabrespine
Satyrinae	Satyrini	Pronophilina	*Amphidecta callioma*	Brazil: Acre
Satyrinae	Satyrini	Pronophilina	*Apexacuta astoreth*	Peru: Apurimac
Satyrinae	Satyrini	Pronophilina	*Catargynnis schreineri*	Brazil: Minas Gerais, Extrema
Satyrinae	Satyrini	Pronophilina	*Eteona tisiphone*	Brazil: Minas Gerais, Extrema
Satyrinae	Satyrini	Pronophilina	*Junea doraete*	Peru: Pasco
Satyrinae	Satyrini	Pronophilina	*Lasiophila cirta*	Peru: Junin
Satyrinae	Satyrini	Pronophilina	*Lymanopoda rana*	Peru: Junin
Satyrinae	Satyrini	Pronophilina	*Manerebia cyclopina*	Peru: Junin
Satyrinae	Satyrini	Pronophilina	*Oxeoschistus leucospilus*	Peru: Junin
Satyrinae	Satyrini	Pronophilina	*Pampasatyrus gyrtone*	Brazil: São Paulo, Campos do Jordão
Satyrinae	Satyrini	Pronophilina	*Panyapedaliodes drymaea*	Peru: Apurimac
Satyrinae	Satyrini	Pronophilina	*Parapedaliodes parepa*	Peru: Lima
Satyrinae	Satyrini	Pronophilina	*Pedaliodes* sp.	Peru: Apurimac
Satyrinae	Satyrini	Pronophilina	*Praepedaliodes phanias*	Brazil: Minas Gerais, Extrema
Satyrinae	Satyrini	Pronophilina	*Proboscis propylea*	Peru: Pasco
Satyrinae	Satyrini	Pronophilina	*Pronophila thelebe*	Peru: Junin
Satyrinae	Satyrini	Pronophilina	*Punapedaliodes flavopunctata*	Peru: Pasco
Satyrinae	Satyrini	Pronophilina	*Steremnia umbracina*	Peru: Huanuco
Satyrinae	Satyrini	Pronophilina	*Steroma modesta*	Peru: Junin
Satyrinae	Satyrini	Satyrina	*Hipparchia semele*	Sweden
Satyrinae	Satyrini	Satyrina	*Brintesia circe*	France: Aude, Villegly
Satyrinae	Satyrini	Satyrina	*Chazara briseis*	Greece
Satyrinae	Satyrini	Satyrina	*Oeneis jutta*	Sweden
Satyrinae	Satyrini	Satyrina	*Satyrus actaea*	France: Aude, Villegly
Satyrinae	Satyrini	Ypthimina	*Neocoenyra petersi*	Tanzania
Satyrinae	Satyrini	Ypthimina	*Ypthima nymias*	Indonesia: Central Sulawesi, Palolo Valley
Satyrinae	Satyrini	Ypthimina	*Ypthimomorpha itonia*	Zambia: Ikelenge
Satyrinae	Satyrini	-uncertain-	*Palaeonympha opalina*	Taiwan: Pingtung, Wutai Shiang
Satyrinae	-uncertain-		*Manataria maculata*	Costa Rica