MOLECULAR SYSTEMATICS OF THE BUTTERFLY TRIBE PREPONINI (NYMPHALIDAE: CHARAXINAE)

By

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To my family: Rosa, Ana and Juan

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(NYMPHALIDAE: CHARAXINAE)

By

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The first complete species-level phylogeny for the nymphalid butterfly tribe Preponini is presented. In this molecular phylogenetic reconstruction three genes were used, comprising two mitochondrial genes, Cytochrome Oxidase subunits I and II (COI and COII), and the nuclear gene, Elongation Factor – 1 alpha (EF-1α). Of the three different methodologies that were employed to reconstruct the phylogeny, ML (Maximum Likelihood) provided the highest resolution.

This phylogenetic approach allowed me to explore taxonomic limits at different levels. The position of *Anaeomorpha splendida* Rothschild, 1984 was examined to test tribal limits, with the result that maintaining monophyly of the tribe requires *Anaeomorpha*'s exclusion. However, the placement of *Anaeomorpha* within the subfamily Charaxinae is still unresolved, thus a more intensive higher-level phylogeny is required to accurately identify *Anaeomorpha*'s taxonomic position. A second goal was to investigate generic boundaries, in particular the relationship of *Noreppa* Rydon, 1971 to *Archaeoprepona* Fruhstorfer, 1915 and *Agrias* Doubleday, [1845] to *Prepona* Boisduval, 1836. In the first case, I synonymize *Noreppa* with *Archaeoprepona* **syn. nov.** since the phylogenetic placement of the former within the latter has high branch support. This

revised taxonomy is further supported by morphological data (Marconato 2008), since the same synapomorphies that were formerly used to define *Archaeoprepona* remain useful after the inclusion of *Noreppa*. For *Agrias* and *Prepona* my results also suggest that these genera should be synonymized. I recommend this merging since *Agrias* is placed within *Prepona* and the morphological characters currently used to define these genera show that the same synapomorphies used to identify *Prepona* apply to *Agrias* as well. However, it is worth noting that other taxonomic modifications can solve the paraphyly of *Prepona* as currently conceived. I therefore suggest a detailed morphological study is needed to identify whether there are potential synapomorphies that would support such alternative taxonomic modifications. The use of an additional genetic marker would also be useful to identify and explore the closest relatives to *Agrias* and provide corroborative evidence before making taxonomic changes. Additionally, I suggest that the description of a new genus for *Prepona pheridamas* (Cramer, 1777) should be considered, supported by its basal taxonomic placement and differences in morphology in comparison with other *Prepona* + *Agrias* (e.g. lack of conspicuous ventral hindwing submarginal eyespots and differences in genitalic characters). To determine whether a new description is the best option, I propose to include an additional marker to increase node support regarding the relationship of *Prepona pheridamas* to remaining *Prepona* + *Agrias*.

This study also revealed a number of potential taxonomic issues at lower levels that definitely merit further attention. These include the relationship of *Archaeoprepona amphimachus* (Fabricius, 1775) to *A. meander* (Cramer, 1775), *Agrias claudina* (Godart, [1824]) to *A. narcissus* Staudinger, [1885], broad divergence within *Prepona deiphile*

(Godart, [1824]) that might merit recognition as two or more species, and the paraphyly of *Prepona pylene* Hewitson, [1854].

Finally, in addition to examining tribal and generic limits, this study also explored the potential for DNA sequence data to clarify species boundaries within the *Prepona laertes* (Hübner, [1811]) group. Preliminary results do not falsify the hypothesis that *Prepona laertes* is a polymorphic species with a widespread distribution, since no correspondence between morphology and molecular data was found, and based on the premise proposed by Hebert et al. (2003b) that a 3% genetic divergence is useful for delimiting species. However, the use of such fixed thresholds has been criticized (e.g. Meyer and Paulay 2005), thus suggesting the need for alternative approaches. Nonetheless, when the observed genetic divergences for *Prepona laertes* were compared to the 9% mean interspecific divergence found for nine preponine species based on the COI sequence data used in the species-level phylogeny, it is evidenced that divergence within *P. laertes* is still minimal, even in comparison with sister species of *Prepona* only (4.6 % mean divergence for pair wise comparisons of three species pairs). Nevertheless, there are long branches characterizing several clusters, which might indicate increased genetic distance between such groups, hence the possible presence of cryptic species. However, before drawing any conclusions, it is important to increase sampling effort to rule out artifacts from a lack of sampling. Therefore, I recommend that additional data is required before robust conclusions concerning species identity can be reached. These data should include a broader geographic sampling and a higher number of individuals to help identify stable clusters that can be examined to identify reliable, diagnostic morphological characters.

CHAPTER 1
INTRODUCTION

The Subfamily Charaxinae

The nymphalid butterfly subfamily Charaxinae contains approximately 400 species within 28 genera (Ackery et al., 1999; Chacón and Montero, 2007) and is distributed throughout the world's tropical regions. Placed within the satyrine group (Wahlberg et al., 2009), the spectacular wing patterns of many charaxines have made them highly popular among collectors (DeVries, 1987). These butterflies are characterized by a robust body, short and thick proboscis, long palpi (DeVries, 1987), and a rapid flight (Chacón and Montero, 2007). Adults inhabit the canopy and are typically seen only when descending to feed on rotten fruit, dung or carrion (DeVries, 1987). Most species have a brightly colored dorsal surface, whereas the ventral surface is cryptic, resembling dead leaves (Chacón and Montero, 2007; DeVries, 1987). The subfamily includes 5 recognized tribes (Wahlberg et al., 2009), Prothoini, Pallini, Charaxini, Anaeini, and Preponini, with the last two occurring only in the neotropical region.

The Tribe Preponini

The charaxine tribe Preponini, containing 22 currently recognized species, is endemic to the neotropics, where the highest species richness can be found in the Amazon basin (DeVries, 1987). A number of species possess narrow ranges, such as *Agrias hewitsonius* H. W. Bates, 1860 and *Prepona werneri* Hering & Hopp, 1925. The brilliant dorsal wing coloration reaches its peak in the genus *Agrias*, the species of which are of high commercial value (Neild, 1996). This bright dorsal coloration merits research, because of its high variability (e.g. as shown by Austin (2009) in *Agrias claudina* and *A. amydon* Hewitson, [1854]), its putative involvement in mimetic rings

between *Agrias* and *Callicore* Hübner [1819] (Descimon, 1976), and because of its presumed function in sexual signaling. Preponine butterflies have been the focus of numerous studies in many topics, including physiology (Berthier, 2005), ecological studies (Devries and Walla, 2001) and immature stage biology (Furtado, 2001; Muyshondt, 1974; Salazar, 1999). However, despite the fact that these spectacular butterflies have been the subject of various taxonomic treatments (Johnson and Descimon, 1988, 1989; Llorente-Bousquets et al., 1992), there still remain major questions to be addressed in terms of both generic- and species-level systematics. Recent studies that have included only a handful of representatives have found problems with defining the tribe, the genera *Prepona* and *Archaeoprepona*, and in delimiting some of the most common species (Freitas and Brown, 2004; Marconato, 2008; Wahlberg et al., 2009). A large part of the problem is the relative morphological homogeneity among species, providing few characters of taxonomic value. Hence, analysis from the molecular perspective seems promising for the group. However, before beginning any systematic study it is important to review the current and historical taxonomy of the group to provide a broad knowledge about its classification and thus develop appropriate methodologies.

Historical Taxonomy

The first species descriptions of preponines were published in the mid-18th century. All species described at that time were placed within *Papilio*, and are now included within the genus *Archaeoprepona*; *A. demophon* (Linnaeus, 1758), *A. amphimachus* (Fabricius, 1775), *A. meander* (Cramer, 1775) and *A. licomedes* (Cramer, 1777). Preponine species are distributed within five genera (Figure 1-1), with the first member of *Archaeoprepona* described by Linnaeus in 1758. The genus *Prepona* was

introduced in 1836 by Boisduval, followed by Doubleday's introduction of *Agrias* in 1845, a genus that only differs from the former in wing pattern and minor variations in the antennae and genitalia according to Fruhstorfer (1916). Later, in 1894, *Anaeomorpha* was established by Rothschild, positioning it as a genus that “stands mid way between *Anaea* Hübn. and *Prepona* Boisd.”, followed by Fruhstorfer's *Archaeoprepona* in 1915. Finally, *Noreppa* was introduced by Rydon in 1971 based on the eyes possessing ‘hairs’, and on differences in both genitalia and mid- and hind- tibia and tarsi (Neild, 1996).

Since the introduction of the various preponine genera the taxonomy of Preponini has been stable. However, a detailed study of the group’s taxonomy is lacking. Several studies in butterfly systematics have included preponine representatives, which serve as stepping-stones for further research and for resolving taxonomic conflict within the group.

Current Taxonomy

Existing phylogenetic studies of Preponini have been done only as part of higher-level phylogenetic studies of butterflies. Brower (2000), based on partial sequences of the nuclear gene *wingless*, assessed the phylogenetic relationships among the tribes of the family Nymphalidae. Even though he included Preponini, just one representative of the tribe was taken into account, *Prepona* sp*.* The most parsimonious cladogram yielded a good resolution for the subfamily Charaxinae, placing the tribe Preponini sister to all other charaxines. In a later work carried out by Freitas and Brown (2004), a phylogenetic hypothesis of the family Nymphalidae was proposed based on morphological characters of each of the life stages. However, this generic-level phylogeny only had one representative of Preponini, *Archaeoprepona chalciope*

(Hübner, [1823]). Marconato (2008) presented a phylogeny of the subfamily Charaxinae based on morphological characters, including the tribe Preponini, which was represented by 13 species. She concluded that maintaining the monophyly of the tribe requires that *Anaeomorpha splendida* be excluded from the group, which agrees with the suggestion made by Peña and Wahlberg (2008) and Wahlberg et al. (2009) that this species is more closely related to the tribe Anaeini. Wahlberg et al. (2009) proposed a phylogenetic hypothesis of 400 genera of butterflies, which included one species of four out of the five recognized genera for the tribe, and provided more support for Marconato's findings.

Scope of the Project

Further progress in preponine taxonomy requires a comprehensive approach, in terms of both taxa and characters. According to Mayr (1963) the use of independent data sets, such as morphological and molecular data, has been considered important for reconstructing patterns of relatedness at both higher and lower taxonomic levels. Baker et al. (1998) showed that using both molecular and morphological data sets for a wide range of taxonomic groups, including plants, insects, reptiles and primates, had a positive effect on the combined analysis as showed by partitioned Bremer support. On the other hand, independent data sets of genomic origin (i.e. different genes) can also be used to obtain more robust results as shown by Rokas et al. (2003) when combining 106 gene regions of eight yeast species. Finally, Miller et al. (1997) and Wahlberg et al. (2005) showed the usefulness of combining independent data sets in phylogenetic analyses of Lepidoptera in particular.

To date, in butterflies, morphology has been key in classification (Ackery et al., 1999) at different taxonomic levels. The most intensively studied characters include

wing venation, color pattern and genitalia. Wing venation has been extensively used in the classification of insects at the order and family level (Combes and Daniel, 2003), and is also widely employed in the Lepidoptera (DeVries, 1987), at family (Triplehorn and Johnson, 2005), genus (LeCrom et al., 2004), and species levels (Elias et al., 2007). However, in many cases, venation characters should be reinforced with other morphological characters when delimiting groups (Albrecht and Kaila, 1997). For butterfly species identification, color patterns are virtually always useful, and the use of color plates for identification is almost universal (e.g. Bravo et al., 2009; DeVries, 1987; Neild, 1996). Color patterns may also be converted into standard identification keys (e.g. LeCrom et al., 2004). However, as noted by Bickford et al. (2007), in some cases morphology might not provide reliable characters, for example due to strong selection for intra- or interspecific sexual signaling, and/or selection against character divergence.

In Preponini, wing venation might not be a helpful character in distinguishing genera since it is uniform across several genera (Fruhstorfer (1916) recalling Schatz), such as *Prepona* and *Agrias*. Instead, Fruhstorfer (1916) suggested that the color of the hindwing anal margin androconial tufts could be used to identify the two main groups in the tribe (further supported by differences in their genitalia), namely the genera *Archaeoprepona* and *Prepona* + *Agrias*. However, he acknowledged the potential limitation of such coloration characters, since at least some South American species show geographic variation in the color of their androconial tufts. The limitation of this character was also discussed by Marconato (2008).

Another morphological character which might not reliably distinguish sibling species in Preponini is color pattern, because it apparently shows high variation within

species, as shown by Austin (2009) for *Agrias* (noted earlier by Fruhstorfer (1916)), and as noted by Neild (1996) for *Prepona*. At the generic level, color pattern is the main character used to distinguish *Prepona* and *Agrias* (Fruhstorfer (1916), despite the fact that genitalia show no consistent differences between the genera. *Prepona praeneste* Hewitson, 1859 exemplifies a species intermediate in wing pattern between the two genera *Agrias* and *Prepona*, and indeed Furtado (2008) demonstrated hybridization between the two genera. The close relationship between *Agrias* and *Prepona* was discussed by Marconato (2008), who suggested that *Agrias* represented an unusually colorful group of *Prepona*.

At higher levels, although both morphological (as evidenced above) and molecular characters suggest problems with preponine taxonomy (Marconato, 2008; Wahlberg et al., 2009), existing studies have not been sufficiently taxonomically comprehensive to resolve these. Therefore, my goal was to use molecular sequence data to attempt to resolve major taxonomic issues in the Preponini group through the first species-level phylogenetic approach. Furthermore, the molecular phylogenetic results can be compared with previous phylogenies, both molecular and morphological, to identify areas of congruence or conflict, and to gain a better understanding of character evolution. The resulting phylogeny will be an initial step to a possible future revision of genera within the tribe, in particular *Agrias* and *Prepona*. To explore the potential of molecular characters to help in resolving species complexes, a cryptic species group will also be the focus of my research to improve our knowledge of the potential diversity and taxonomy of the group.

Specifically my aims are:

1. Define the limits of the tribe Preponini, in particular examining the taxonomic position of *Anaeomorpha splendida.*

2. Test the monophyly of all preponine genera.

3. Assess species limits within the *Prepona laertes* group.

Figure 1-1. Representatives of each genus of the tribe Preponini. From upper left towards lower right: *Agrias*, *Anaeomorpha*, *Archaeoprepona*, *Prepona*, and *Noreppa.* Coloration pattern on the left side corresponds to the dorsal view, the right side corresponds to the ventral view. Photographs by Pablo Sebastián Padrón.

CHAPTER 2
MOLECULAR PHYLOGENY OF THE TRIBE PREPONINI

Introduction

Preponine species have been included in several recent systematic studies, but most of these have concentrated on higher-level taxonomic issues. However, Marconato (2008) focused her cladistic analysis on the subfamily Charaxinae, thus providing a finer resolution of the phylogenetic relationships within the group. She highlighted several key points in the topology that should be further studied: i) the relationship of *Anaeomorpha splendida* to other Preponini, with implications for tribal limits, and ii) the relationships of *Noreppa* and *Agrias* to other genera, with implications for generic classification.

Tribal Limits

The representation of preponine butterflies in phylogenetic studies has increased over time. Freitas and Brown (2004) proposed a phylogeny of the family Nymphalidae, including only one species of Preponini, *Archaeoprepona chalciope*. In both of their phylogenetic reconstructions, this species appeared as a sister species to the charaxine tribe Anaeini. Later, Peña et al. (2006) included *Archaeoprepona demophon* as the only representative of Preponini in their molecular study of Satyrinae. In the topology presented for the combined dataset of three genes *Archaeoprepona demophon* appeared as sister to *Hypna clytemnestra* (Cramer, 1777), within the tribe Anaeini, with the latter taxon appearing as a paraphyletic group. Brower's (2000) phylogenetic study of the Nymphalidae based on the nuclear gene *wingless* placed the only included representative of the tribe Preponini, *Prepona* sp., within a polytomy with members of the tribes Charaxini and Anaeini. After successive approximations weighting the

resolution improved, leaving *Prepona* sp*.* as sister species to a group formed by the tribes Charaxini and Anaeini. Later, Peña and colleagues (2008) found no support for the monophyly of the tribe in a study that included the genera *Agrias, Prepona, Archaeoprepona* and *Anaeomorpha.* In the Bayesian Inference topology the monotypic genus *Anaeomorpha* appeared as sister to the only representative of the African tribe Pallini, with *Anaeomorpha* + Pallini being the sister group to the tribe Anaeini. Consistent with Peña et al.'s (2008) results were those of Wahlberg et al. (2009), who reconstructed the phylogeny for approximately 74% of recognized Nymphalidae genera based on 10 genes and 235 morphological characters, also omitting the genus *Noreppa.* The maximum likelihood analysis showed *Anaeomorpha* as sister to Anaeini, whereas *Agrias, Prepona,* and *Archaeoprepona* formed a monophyletic group. Lastly, Marconato (2008) developed the only phylogenetic study containing representatives of all five Preponini genera. In her cladistic analysis the most parsimonious tree placed *Anaeomorpha* within a polytomy with Anaeini representatives. After successive approximations weighting, *Anaeomorpha* was again placed outside Preponini, and as sister species to the monophyletic Anaeini.

Preponini Genera

As described above for Preponini tribal limits, generic limits within the tribe have also been debated, and even though morphology has been used to distinguish Preponini genera (Figure 1-1) since their descriptions, some relationships remain problematic. The identification of *Prepona* and *Archaeoprepona* is straightforward and based on four principal characters (Johnson and Descimon, 1988). These characters were also analyzed by Marconato (2008), but while they distinguished *Archaeoprepona*

from *Prepona* they failed to differentiate in some instances among *Noreppa*, *Anaeomorpha* and *Agrias* (see below).

The first character discussed by Johnson and Descimon (1988) for identification of preponine genera is the modified male scent scales, or androconia. In *Archaeoprepona* and *Noreppa* these patches of long, hair-like scales are diffuse and the hairs flexible, located along the anal margin of the dorsal hindwing. In contrast, *Prepona* and *Agrias* possess well-defined and rigid brush-like setae located in the same position. In addition to the morphology, the coloration of the androconial scales can be used to identify each genus, being dark in *Archaeoprepona* and yellow in *Prepona* and *Agrias* (Marconato, 2008). Marconato (2008) suggested the presence of androconial scales of this form as a synapomorphic character for the tribe Preponini, except for the genus *Anaeomorpha* which lacks this character.

ii) Small and undifferentiated ventral hindwing submarginal eyespots in the genera *Archaeoprepona* and *Noreppa* in comparison with the large eyespots present in *Prepona*. This character was divided into two characters by Marconato (2008), who suggested that having eyespots in Rs-M1 and Cu1-Cu2 equal in size and shape to other marginal hindwing eyespots was plesiomorphic, thus the conspicuous eyespots in *Prepona* can be considered synapomorphic. However, *Prepona pheridamas* lacks conspicuous eyespots, sharing the ancestral characteristic of *Archaeoprepona*. Marconato (2008) also addressed the size of the marginal ventral hindwing eyespots in the genera *Archaeoprepona* and *Agrias*, suggesting that the plesiomorphic character state was small eyespots in *Archaeoprepona* (with the exception of *Archaeoprepona chalciope* which lacks eyespots) in contrast to larger eyespots as in the genus *Agrias*.

Even though she did not address the state of this character in *Noreppa*, I would suggest that this genus also possesses the plesiomorphic character state.

Other diagnostic characters are found in the male genitalia (iii), which is characterized by a flat gnathos lacking spines and with the genitalic capsule being more 'robust' in *Archaeoprepona*, whereas in *Prepona* the gnathos is rod-like with the presence of spines and the genitalia are more 'slender' overall. In Marconato's (2008) morphological study she scored variation within the gnathos as two independent characters. First, the degree of sclerotization was found to differ between *Archaeoprepona* and *Prepona*, being heavily sclerotized in the former (also including *Noreppa*) and moderately sclerotized in the latter (including *Agrias*). Lack of sclerotization was found to be the plesiomorphic character state. The second character addressed was the morphology of the gnathos. She suggested four character states in which the lack of texture (either spines or striations) was considered plesiomorphic. For this character the *Archaeoprepona* + *Noreppa* clade possessed striations (including a reversal in *Archaeoprepona amphimachus*) whereas the *Prepona* + *Agrias* clade possessed a high number of spines. Interestingly, the gnathos of *Prepona pheridamas* possessed several spines, and this species appeared as basal to *Prepona* + *Agrias* (Marconato, 2008).

The last character proposed by Johnson and Descimon (1988) refers to the female sterigma, which is 'Y'-shaped in *Prepona* and circular in *Archaeoprepona*. Marconato (2008) also addressed this character but proposed three character states. The plesiomorphic character state was found to be the 'Y'-shaped fused lamella antevaginalis found in the *Agrias amydon* clade only. *Archaeoprepona* possessed the

apomorphic character state, which is V-shaped. Finally, *Noreppa* possessed the autopomorphy of having the fused lamella antevaginalis U-shaped. For this character the genus *Prepona* was not scored.

Noreppa, even though easily differentiated from the above genera (*Archaeoprepona* and *Prepona*) due to the presence of orange spots near the dorsal hindwing margin (Neild, 1996), appeared within *Archaeoprepona* in Marconato's (2008) study. As explained above, for some characters this genus is not differentiated from *Archaeoprepona*, such as androconial shape and color, size and location of marginal hindwing eyespots, and gnathos sclerotization and texture (male genitalia), thus suggesting that *Noreppa* should perhaps be synonymized with *Archaeoprepona*.

Overall, Marconato's (2008) morphological study, which included 6 members of *Prepona* and *Agrias*, found that *Agrias amydon* and *Agrias claudina* appeared within *Prepona*. Furtado (2008) provided additional evidence for the close relationship between *Agrias* and *Prepona* by successfully pairing a female *Agrias claudina godmani* Fruhstorfer, 1895 with a male of *Prepona omphale* (Hübner, [1819]) (currently *Prepona laertes* (Hübner, [1811]) according to Lamas, 2004).

In summary, studies to date have shown that more comprehensive work is needed to define the tribal and generic limits in Preponini. A more thorough study of the tribe's phylogenetic relationships should help to choose taxonomic solutions depending on the phylogeny of included taxa. Hall and Harvey's (2001) cladistic analysis of the genera *Juditha* Hemming, 1964, *Lemonias* Hübner, [1807], *Thisbe* Hübner, [1819] and *Uraneis* H. W. Bates, 1868 (Lepidoptera: Riodinidae: Nymphidiini) is a good example of the several possible relationships which may be found among taxa within genera, and of the

potential solutions to disentangle the results. Since monophyletic clusters are required in a phylogenetic classification, they overcame paraphyly and polyphyly by using synonymy in some cases as well as describing a new genus. In the present study, I use a comprehensive molecular phylogenetic approach to suggest the most robust and appropriate taxonomy for the tribe Preponini.

Materials and Methods

Sample Acquisition and Origin

To maximize the number of species and specimens for this study I carried out fieldwork in Colombia, and contacted collaborators in other neotropical countries. Fieldwork involved sampling in two seasons, from May-July 2010 and two weeks in the month of December 2010. Colombia promised to be a useful country for sampling because it comprises different habitats and biogeographic regions, with a correspondingly high number of representatives of the tribe; 19 species out of 22 according to Lamas (2004). I sampled several regions, including the two interandean valleys (Cauca and Magdalena), Pacific coast – Chocó, Llanos/Plains, and Amazonian rainforest (Figure 2-1). To collect preponine butterflies I used baited traps that were hung from the canopy and left there for a minimum of six days. They were freshly baited with rotten fish or fermenting fruit every 48 h, and were checked every day for the whole collecting period. Butterflies were stored inside glassine envelopes for transport, with two legs removed and placed in 96% EtOH, and finally spread in the laboratory.

In addition, I received recently collected material, both papered and pinned, from collaborators in Mexico, Honduras, Germany, United Kingdom, Venezuela, and Ecuador. I also obtained some tissue samples from the collection at the McGuire Center for Lepidoptera and Biodiversity, Gainesville, FL, which included specimens from Costa

Rica and Brazil, as well as from visits to the Smithsonian Institution National Museum, Washington D.C., and Museo de Zoología Alfonso L. Herrera at Universidad Nacional Autónoma de México, México D.F.

Gene Regions

For the molecular phylogeny of Preponini I sequenced two mitochondrial genes, COI and COII, and one nuclear gene, EF-1α. Multi-locus analyses provide increased support for phylogenetic hypotheses (Brito and Edwards, 2009; Liu et al., 2008; Wahlberg and Wheat, 2008), and these genes have proven of value in phylogenetic studies of butterflies (Hundsdoerfer et al., 2009; Kandul et al., 2004; Silva-Brandão et al., 2005).

DNA Extraction and Amplification

I extracted DNA from two legs either i) removed from freshly killed specimens and stored in 96% EtOH, or ii) recently removed from dried specimens stored in glassine envelopes or spread. I extracted DNA from butterfly tissue using the Qiagen DNEasy Extraction Kit and following their protocol. For old tissue samples the extraction protocol was modified following Iudica et al. (2001). Modifications included prolonged digestion with proteinase K for approximately 36 – 48 h, Ph test of the DNA sample before adsorption on the QIAGEN column (pH higher than 7.0 decreases adsorption), and final elution in 50ul of elution buffer.

I used standard Polymerase Chain Reaction (PCR) to amplify the target genes. The COI barcode region of approximately 650bp in length was amplified following Hebert et al.'s (2004a) PCR protocol with modifications and using the primers LEPF1 (f) and LEPR1 (r) also as described by these authors. In cases where the previous primers were not successful the primers LCO (f) and HCO (r) were used (Folmer et al., 1994).

The COII mitochondrial gene of approximately 1000bp in length was amplified following Hillis et al.'s (1996) protocol, and using the primers George (f) and Eva (r) (Monteiro and Pierce, 2001). Finally, the nuclear gene EF-1α of approximately 1000bp in length was amplified following Hillis et al.'s (1996) protocol and the primers EF44 (f) and EFrcM4 (r) (Monteiro and Pierce, 2001) (primer names and sequences available in table 2-1). The PCR mix used was the same for all gene regions, which is described in table 2-2. PCR protocols for each gene are detailed in table 2-3.

DNA Sequencing and Alignment

Custom DNA Sequencing from both strands of each gene was carried out by University of Florida's Interdisciplinary Center for Biotechnology Research Sanger Sequencing Group. Geneious v5.3 (Drummond et al., 2010) was used to manually edit both strands of each gene, with the resulting sequences aligned using ClustalW (Larkin et al., 2007) and a consensus sequence produced. The final sequence obtained for COI was 618bp in length, 897bp for COII, and 955bp for EF-1α. Subsequently, I constructed a matrix combining these three loci for a total length of 2470bp.

Phylogenetic Analyses

The complete data set for the three genes was analyzed under the Maximum Parsimony (MP) optimality criterion using PAUP* 4.0 (Swofford, 2003).The data were also analyzed by Maximum Likelihood (ML) using Randomized Axelerated Maximum Likelihood BlackBox (RAxML BlackBox) (Stamatakis, 2006; Stamatakis et al., 2008) and under the Bayesian Inference (BI) criterion using Mr. Bayes 3.1 (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003), both implemented in the Cipres Portal

(http://www.phylo.org/). To edit the resulting trees I used the program FigTree v1.3.1 (Rambaut, 2006-2009).

Maximum parsimony (MP)

Phylogenetic tree estimation was carried out by a heuristic search in which characters were equally weighted and gaps were treated as missing data, using the tree-bisection-reconnection (TBR) branch swapping algorithm and a simple stepwise addition. A strict consensus tree was estimated when multiple equally parsimonious trees were obtained, followed by a bootstrap analysis (Felsenstein, 1985) as a measure of branch support and reliability of the resulting tree (Hall, 2008). This analysis involved a full heuristic search including 1000 replicates and retaining groups with frequency ≥ 50%.

Maximum likelihood (ML)

The ML analysis involved a partitioned analysis in which the sequence evolution model for each gene was selected using the program jModelTest (Posada and Crandall, 1998). The model GTR + G was chosen for all data sets. The phylogenetic tree estimate was followed by rapid bootstrap analysis (Stamatakis et al., 2008) consisting of 1000 replicates.

Bayesian inference (BI)

The model of sequence evolution chosen by jModelTest (Posada and Crandall, 1998) was GTR + G for the whole data set of 2470bp as well as for each individual gene. BI analyses involved both a partitioned and a non-partitioned analysis. Four Markov Chain Monte Carlo simulations of 200,000 generations sampling every 100 generations with a burn-in of 25% of generations were executed. A majority rule consensus tree with branch posterior probabilities was constructed for both analyses.

Results and Discussion

A total of 43 individuals from all 22 species recognized in the tribe by Lamas (2004) were included in this study. From this group a total of 23 individuals possessed the complete gene dataset, only two genes were successfully amplified for nine individuals, and the remaining eight individuals had DNA data for just one gene. The outgroup species represented the charaxine tribe Anaeini, and the subfamilies Brassolinae and Satyrinae for a total of 11 species from which only two had an incomplete gene dataset (lacking sequence for EF-1α).

The ML tree constructed for the combined data of the three genes is shown in Figure 2-2. The topology produced by the ML analysis showed the best resolution for the group. However, MP (including bootstrap analysis) and BI analyses were also carried out, and even though the topologies were not well resolved they highly supported several nodes which are denoted by * and $^{+}$ respectively in Figure 2-2.

The ML analysis was designed to test two major issues of Preponini systematics. The first issue is the monophyly of the tribe, particularly the placement of *Anaeomorpha splendida* (top right Figure 1-1). Rothschild, in 1984, suggested that *Anaeomorpha splendida* was related to the tribe Anaeini, and other authors, based on phylogenetic studies, have come to similar conclusions (Wahlberg et al., 2009) or shown a relationship to the tribe Pallini (Peña and Wahlberg, 2008). Recently, Marconato's (2008) study left *Anaeomorpha* as a member of Preponini, despite the fact that *Anaeomorpha splendida* does not possess androconial tufts which are considered a synapomorphy for Preponini according to her analysis. Her results showed *Anaeomorpha* to be placed in a polytomy with representatives of Pallini, Anaeini,

Preponini and Charaxini, though after successive approximations weighting it was placed as sister to Anaeini.

The results from the ML analysis support the suggestion that *Anaeomorpha splendida* is misplaced within the tribe Preponini, and thus the monophyly of the latter requires *Anaeomorpha*'s exclusion. The clade formed by both Ecuadorian specimens shows high bootstrap and posterior probability values and is placed as sister clade to Anaeini + Preponini; however, this position does not have significant branch support. Contrary to what was proposed by Wahlberg et al. (2009) and Marconato (2008), these results show that *Anaeomorpha*, instead of being more closely related to the tribe Anaeini, may actually fall outside the current boundaries of the tribe Anaeini, a result consistent with Peña and Wahlberg's (2008) suggestion that *Anaeomorpha* is more closely related to the tribe Pallini. Therefore, I suggest that *Anaeomopha splendida* should be excluded from Preponini and placed in an *incertae sedis* until its placement within the charaxines is further analyzed. Future work in this aspect should include representatives from other tribes, particularly from Pallini. In addition, in order to increase the node support, I would suggest broadening the gene pool, by adding another nuclear gene, such as CAD, IDH or RpS5, which are useful markers in this kind of study (Wahlberg, pers. comm.).

The second objective of this analysis was to test the monophyly of the Preponini genera. In particular, two main issues involving preponine genera were suggested as requiring further research by Marconato (2008): i) the placement of *Noreppa chromus* (Guérin-Méneville, [1844]) with respect to the genus *Archaeoprepona* and ii) relationship of *Agrias* with the genus *Prepona.* Escalante et al. (2010) further

investigated these issues but focused only on Mexican preponines. Therefore the present study, besides representing a broader geographic dimension, includes all preponine species, which increases the accuracy and adds robustness to the results.

The first issue regarding Preponini generic classification involves the potential placement of *Noreppa* within *Archaeoprepona*. In Figure 2-2 it is clear that *Noreppa chromus* is placed within the genus *Archaeoprepona,* and is sister to *A. licomedes*. Its well-supported position, and the relatively low differentiation among other *Archaeoprepona* species suggest that *Noreppa* should be considered as a representative of *Archaeoprepona*. Additional support for this merging can be found in Marconato's (2008) morphology-based phylogeny. According to her results, the synapomorphies that characterize *Archaeoprepona* also occur in *Noreppa*. Thus, even though there are other taxonomic modifications that could solve this conflict, for instance maintaining *Noreppa* and describing additional genera for other monophyletic groups, the genetic evidence coupled with the morphological data suggest that placing *Noreppa* within *Archaeoprepona* is the best solution. Therefore, I here synonymize the genera *Noreppa* **syn. nov.** and *Archaeoprepona*.

Also of interest within the *Archaeoprepona* cluster is that individuals of *A. amphimachus* and *A. meander* form a mixed group, and even though the MP, ML and BI node support are not significant, the ML analysis suggests that these species should be further studied. Attempting to complete the full set of genes for the individuals analyzed here should be a priority and could prove helpful in improving node support. The potential merging of both species was suggested by Escalante et al. (2010), who used COI barcode data to assess the phylogenetic relationships among Mexican

preponines. According to their results and due to the low genetic distance between both species they propose their merging. However, they only included one individual from *Archaeoprepona meander,* thus yielding a result that could potentially be an artifact of the lack of samples. Further research on this topic should be attempted in the future, including more individuals from different subspecies, in order to disentangle the real evolutionary relationships among these taxa.

I also highlight the need for additional genetic data for the species *Archaeoprepona demophoon* (Hübner, [1814]). My results show that *Archaeoprepona demophoon* is sister to remaining species of *Archaeoprepona* clade, but the low node support and fact that only one individual was included (which lacked EF-1α data), suggest that this placement may require confirmation My results contrast to Marconatos's (2008) cladistic analysis in which *Archaeoprepona chalciope* was sister to remaining *Archaeoprepona*, followed by *A. demophon* and then *A. demophoon* as sister species to the group formed by *A. licomedes* + (*A. chromus* + *A. amphimachus*). Therefore, broadening the phylogenetic breadth in terms of individuals from *Archaeoprepona demophoon* and DNA data might prove to be useful in corroborating my current results.

Moreover, a remaining issue to be considered regarding the former genus *Noreppa* involves the status of the Colombian taxon *priene* (Hewitson, 1859), currently treated as a subspecies of *A. chromus* but formerly regarded as a distinct species. I had only a single individual available for study, and I was not successful in obtaining DNA data. Therefore, future analyses should also aim to address this issue.

The second issue involving preponine generic classification regards the relationship between the genera *Agrias* and *Prepona.* Several important facts are apparent in Figure 2-2. Firstly, *Agrias* representatives form a well-defined clade within *Prepona*, although the nearest relatives of *Agrias* are still unclear since significant MP bootstrap support and BI posterior probability are lacking. In contrast, the internal nodes of the *Agrias* clade have significant support. This cluster is composed by two major subdivisions, one of which is the clade formed by *Agrias narcissus, A. claudina,* and *A. aedon* Hewitson, 1848. This clade suggests that *Agrias claudina* is not monophyletic, with both individuals of *Agrias claudina lugens* Staudinger, 1886 split, with one of them showing a higher affinity to *A. narcissus*. Increasing the phylogenetic scope to test for experimental error would be useful to confirm this result, since including just one individual from a particular species in a species-level study could yield inaccurate results (Funk, 1999). In particular, more individuals of *Agrias narcissus* with a full set of genes should be included, since the only individual of *A. narcissus* included lacked EF-1α data, and one individual of *Agrias claudina lugens* lacked COI data. If *Agrias claudina* continues to appear as a paraphyletic species, this may reflect ancient polymorphism (Avise and Ball, 1990) or introgression resulting from interspecific hybridization (Sota and Vogler, 2001). The successful pairing of a male of *Prepona* with a female *Agrias* by Furtado (2008) suggests that interspecific mating between *A. narcissus* and *A. claudina* is certainly possible.

The second clade within *Agrias* is formed by *Agrias hewitsonius* and *A. amydon*, a group for which all the methodologies yielded significant node support. Even though the ML analysis proved to be useful in clarifying the relationships within the genus

Agrias, several species were represented by just one individual (e.g. *A. aedon*) and some individuals lacked DNA data. Improving both of these aspects would yield more solid results in future studies.

Moving towards deeper nodes in the topology, the *Agrias* clade is placed as sister to the group formed by *Prepona praeneste* and *P. deiphile neoterpe* Honrath, 1884. *Prepona deiphile neoterpe* is the sister species to *P. praeneste,* a clade supported by high bootstrap and posterior probability values. However, *Prepona deiphile* appears as polyphyletic since individuals from a different subspecies, *P. deiphile ibarra* Beutelspacher, 1982, group with *P. pylene* thus forming a group sister to *Agrias* + (*P. praeneste* + *P. deiphile neoterpe*). These results suggest that *P. deiphile* might constitute two different species. However, the node support is very low for all three methodologies, suggesting the use of additional markers to increase support for these findings. Similarly, *Prepona pylene* appears as paraphyletic, a result that despite having high node support merits further study by adding more specimens as well as more markers. The results shown here suggest that both subspecies might be independent entities, potentially different species.

The remaining species of the genus *Prepona*, namely *P. werneri, P. laertes, P. dexamenus* Hopffer, 1874*,* and *P. pheridamas* appear as well established groups within the *Prepona* + *Agrias* cluster. Even though these four species have high bootstrap node support, the inclusion of more individuals of *P. laertes* might increase the posterior probability values to ≥ 0.95 as in the case of *P. werneri, P. dexamenus*, and *P. pheridamas*. The star in Figure 2-2 denotes the presence of splitting that has very small branch length (3×10^{-6}) thus appearing as a polytomy.

The ML topology clearly shows that *Prepona* as currently conceived is paraphyletic, and even though there are several possible taxonomic solutions, one of these seems by far the most appropriate since it requires fewer taxonomic modifications and is more reasonable in view of the current classification of other charaxine genera. I recommend that *Agrias* should be synonymized with *Prepona* since, despite the fact that *Agrias* species form a well-defined cluster, they are placed well within the *Prepona* clade. Another possible taxonomic solution would be expanding the currently conceived *Agrias* to include the taxa *deiphile*, *praeneste*, *pylene* and *werneri*, a clade supported by high ML bootstrap values, in addition to describing new genera for the highly supported taxa *dexamenus* and *pheridamas*, and keeping *Prepona* as a monotypic genus (*Prepona laertes* as its single representative). Nevertheless, when combining the available morphological and genetic information, the synonymy of *Agrias* seems the most taxonomically reasonable solution. This is due to the fact that the morphological synapomorphies that define *Prepona*, with the exception of *Prepona pheridamas* (discussed below), can also be used to define *Agrias* according to Marconato's (2008) cladistic analysis.

Description of a new genus for *Prepona pheridamas* should also be considered, since its morphology (e.g. lack of conspicuous eye spots on the ventral hindwing and structure of the gnathos in the male genitalia) differs from *Prepona* and its placement is basal to the *Prepona* + *Agrias* clade. These results also support Marconato's findings based on morphological data. The gnathos morphology (i.e. possessing several spines) of *Prepona pheridamas* is unique (Marconato (2008), and thus would provide an autopomorphy for a new genus. However, I would also suggest a more detailed

morphological study addressing additional possible characters that could be used to better define a new genus.

In conclusion, before a more detailed morphological study aiming to identify synapomorphic characters for the potential putative new genera (e.g. for the taxon *dexamenus*) is available, I would suggest that species from *Agrias* and *Prepona* should be considered as congeneric. In addition, and as suggested above for other cases, I think that adding an extra nuclear marker would be useful to provide corroborating evidence prior to taking such a taxonomic step.

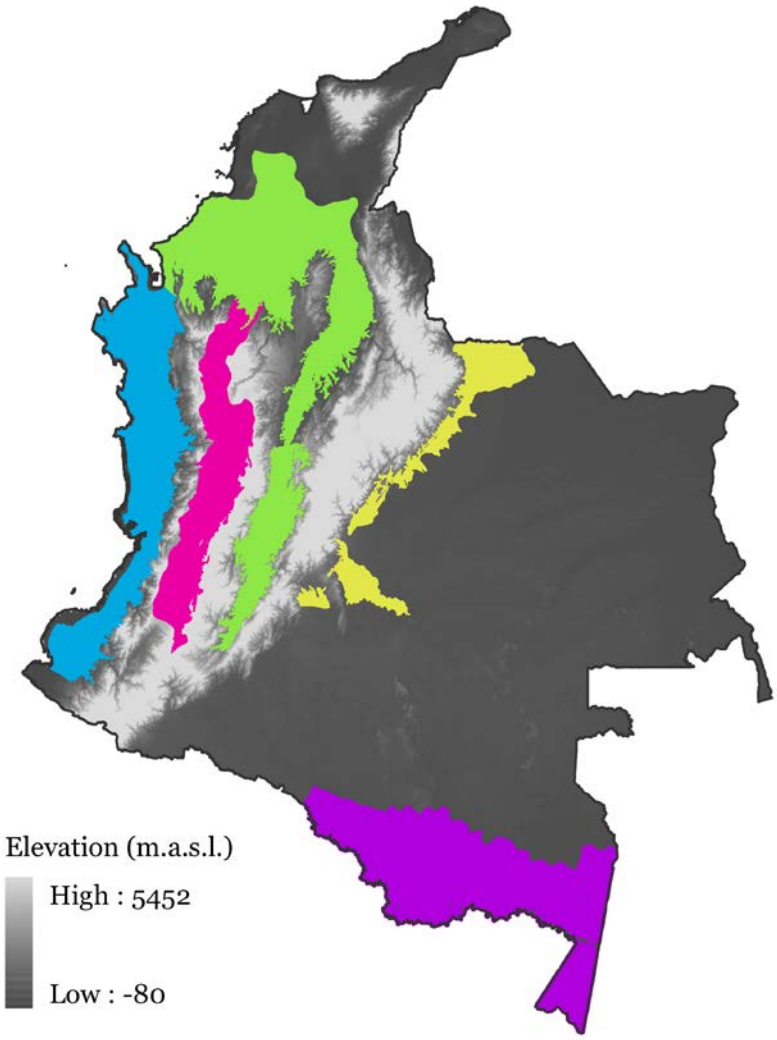

Figure 2-1. Geographic regions sampled in Colombia. Pacific Coast – Chocó Region (blue), Interandean Valley, Cauca (pink) and Magdalena (green), Llanos/Plains (yellow), and Amazon Rainforest (purple).

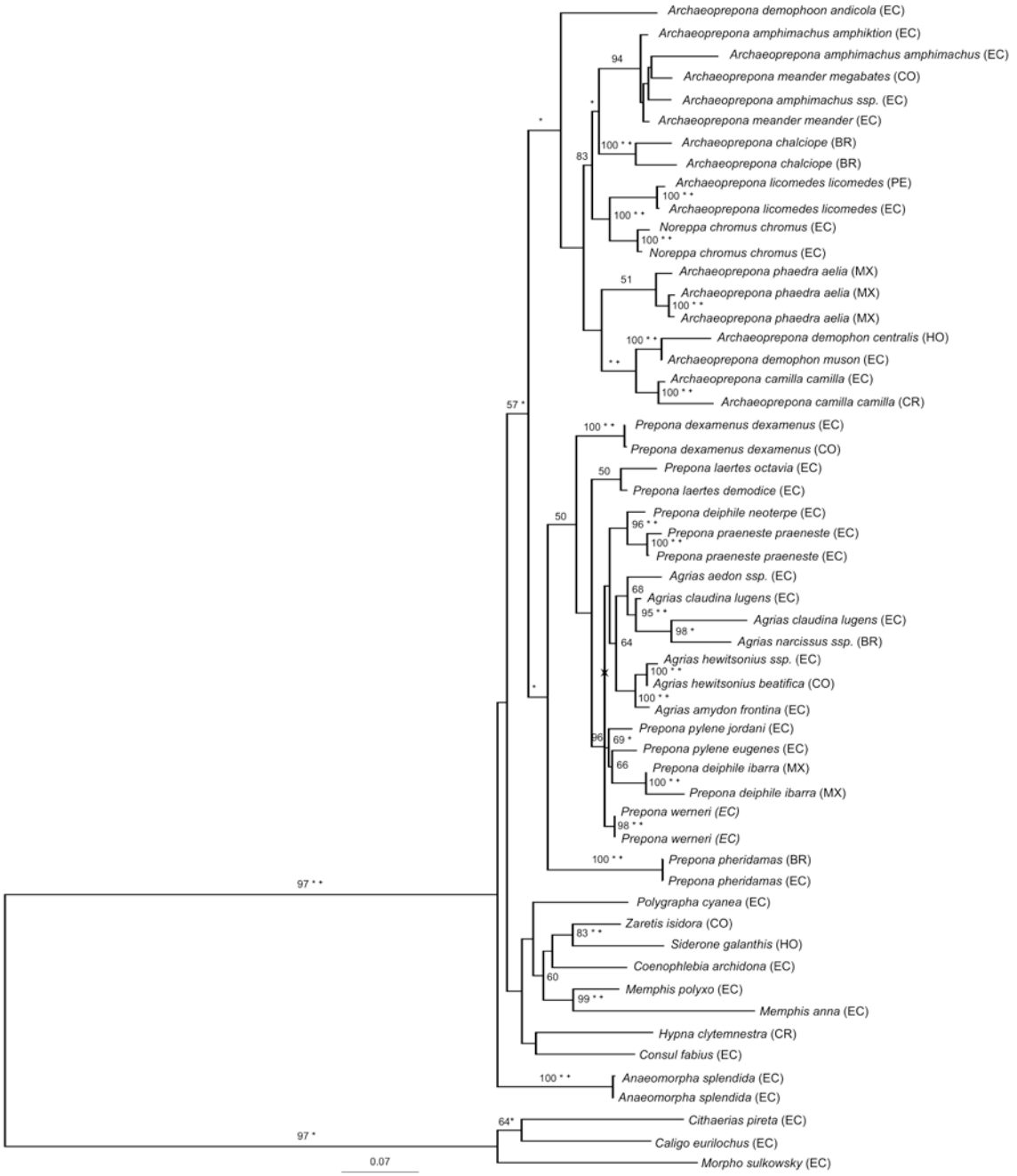

Figure 2-2. ML inferred tree for the combined data set of COI, COII and EF-1α genes. Numbers above branches correspond to rapid bootstrap values $\geq$ 50% from the ML analysis. Asterisks (*) correspond to nodes with > 50% bootstrap support from the MP analysis and sum symbols (+) represent nodes with ≥ 0.95 posterior probability in the BI analysis. Star symbol denotes a splitting of branches.

Table 2-1. Primers used for DNA amplification. Both primers are shown in the 5' – 3' direction.

Gene	Primers Forward	Reverse
COI (Hebert et al., 2004a) (Folmer et al., 1994)	LEP-F1 ATTCAACCAATCATAAAGATAT LCO TAAACTTCAGGGTGACCAAAA AATCA	LEP-R1 TAAACTTCTGGATGTCCAAAAA HCO GGTCAACAAATCATAAAGATATTGG
COII (Monteiro and Pierce, 2001)	George ATACCTCGACGTTATTCAGA	Eva GAGACCATTACTTGCTTTCAGTCATCT
EF-1α (Monteiro and Pierce, 2001)	EF44 GCYGARCGYGARCGTGGTATYAC	EFrcM4 ACAGCVACKGTYTGYCTCATRTC

Table 2-2. PCR mix protocol used for DNA amplification. The same protocol was used for the three genes. Asterisks indicate that in some cases the amount of DNA was increased to 4ul in which case the amount of ddH_2O was decreased to 10.6ul.

Substance	Volume (ul)
Buffer 10x	2
dNTP's (2.5uM)	0.8
$MgCl_2$ (50mM)	0.8
Primer Forward	0.4
Primer Reverse	0.4
ddH_2O	12.6*
Taq Platinum	1unit/ul
DNA	2*
Total	20

Table 2-3. PCR cycles for DNA amplification. Asterisks indicate that each cycle has three different steps: DNA denaturation, primer annealing, and DNA elongation, each with its specific time and temperature.

Gene	Number of cycles	Time (seconds)	Temperature (Celsius)
COI	1	60	94.0
	5*	60	94.0
		30	45.0
		40	72.0
	35*	30	94.0
		40	51.0
		40	72.0
	1	300	72.0
COII and EF-1α	35*	60	95.0
		60	58.0
		60	72.0
	1	300	72.0

CHAPTER 3
SPECIES LIMITS WITHIN *PREPONA LAERTES*

Introduction

Prepona laertes Group

Preponini species-level taxonomy has traditionally been a highly debated topic. The most recent Neotropical butterfly checklist (Lamas, 2004) recognized twenty-two species in the tribe, within five genera: *Prepona* containing seven species, *Archaeoprepona* with eight, *Agrias* with five, and *Noreppa* and *Anaeomorpha* both monotypic. Neild (1996) stated that there are approximately nine species within *Archaeoprepona*, and two within *Noreppa*, but remained uncertain about the number of species in *Prepona* and *Agrias*. His treatment of *Prepona* was based on his "own observations in the field and in collections", but his study was limited to Venezuela and environs. He suggested that the real number of species in *Agrias* was likely to lie between four to nine, as previously stated by Barselou (1983) and D'Abrera (1987), respectively.

Table 3-1 summarizes various taxonomic proposals for each of the Preponini genera over time. It is evident that these proposals are remarkably variable, in particular for the genus *Prepona*, in a large part due to the taxonomy of the *Prepona laertes* group. The variation in color pattern within this species group has resulted in an extraordinary number of taxonomic descriptions since the beginning of the twentieth century, when LeMoult (1932 in Vane-Wright, 1974) proposed approximately 70 new names. Neild (1996) recalled personal communication from Lamas that species within the *Prepona laertes* complex should be considered one polymorphic species, including *P. omphale* and *P. laertes* that were formerly divided by Fruhstorfer (1916). This

treatment was reflected in the latest checklist by Lamas (2004), who suggested four subspecies for the complex which comprises the former species known as *Prepona omphale, P. joiceyi, P. pseudojoiceyi, P. philipponi, P. rothschildi, P. lilianae,* and *P. pseudomphale* (all described by LeMoult, 1932 with the exception of *P. omphale*), amongst others. Neild (1996), however, considered *Prepona philipponi*, from the Orinoco, Amazon, Guianas, and southern Brazil, and *P. pseudomphale*, from Venezuela, Guianas and the Amazon, to be species distinct from the widespread *P. laertes*. Polymorphism is commonly seen in other butterfly groups such as Nymphalinae and Heliconiinae, and is often the result of mimicry (Joron et al., 1999; Linares, 1996).

Studies to date have been based on qualitative consideration of ecology, geographic distribution and morphology.Neild (1996) identified morphological characters from wing pattern and androconial tufts for *Prepona laertes* as well as for *P. philipponi* and *P. pseudomphale*. According to Neild, *Prepona laertes* is characterized by yellow to yellow-orange androconial tufts compared to the brownish-red coloration of *P. pseudomphale* and brownish-orange in *P. philipponi*. This character, however, is somewhat variable and its assessment in some specimens is very subjective. Neild also gave wing characters that he suggested were useful in identification of these three putative species. The shape of the black line located in the median section on the ventral hindwing is a helpful character, which is straight or slightly curved in *Prepona philipponi*, in contrast to curved and irregular in *P. pseudomphale* and curved and irregular to a lesser extent in *P. laertes.* In addition, *Prepona philipponi* hindwing coloration shows a sharp contrast between the creamy-ochre portions of the wing, a contrast that is not so strong in the remaining two species. To separate males of *P.*

pseudomphale and *P. laertes* it is useful to examine ventral hindwing cells M2-Rs in which there is a creamy colored space between the postdiscal black line and the ochre portion of the wing that is absent in *P. laertes*. Regarding females, Neild (pers. comm.) confirmed that they are very difficult to identify. Despite this, he suggested useful characters for the *Prepona laertes* complex females from Venezuela and the Guianas, such as the presence of blue coloration in spaces four, five and six in the dorsal forewing of *P. philipponi* females, a coloration that is absent in females of *P. pseudomphale*. Since such morphological characters vary from region to region and are somewhat subjective, an independent approach, such as molecular study, could provide an important new set of data to help disentangle the relationships and species limits within the *Prepona laertes* group.

DNA Barcoding

DNA 'barcoding' is a novel method that uses a short, easily amplified gene region to universally identify species. Identification can be achieved by either assigning an unknown sequence to a particular species by comparing the sequence against a matrix of knowns or by hypothesizing the discovery of cryptic species. Proponents of the technique point out several problems of traditional taxonomic methods that can be reduced by using molecular methods. For example, morphology might be of limited value in identifying species when phenotypic plasticity is present or, on the contrary, when phenotypic characters are invariant (e.g. cryptic species) (fully described by Hebert et al., 2003b). In addition, the current use of morphological keys, which is common in traditional taxonomic revisions, requires significant knowledge of the organisms and sometimes can be limiting in terms of gender or life stage (Hebert et al., 2003b).

DNA barcoding, which has set itself the target of ‘identifying global biodiversity’, has been the center of ongoing debate. The short segment of mtDNA (Cytochrome Oxidase subunit I (COXI or COI) in the case of animals) from individuals of interest is analyzed and compared to a mtDNA matrix based on distance methods, yielding information about sequence variation within the sequence pool (Hebert et al., 2003b). For the DNA barcode approach to work, either all species must be reciprocally monophyletic (Wiens, 2002), or intraspecific variation must be lower than interspecific divergence (Meyer and Paulay, 2005), the so-called barcoding gap (Figure 3-1). For species diagnosis, Hebert et al. (2004b) proposed the 10x rule in which the average sequence divergence between sequence clusters should be greater than ten times divergence within clusters. Other, somewhat arbitrary thresholds may also be chosen (e.g. Meyer and Paulay, 2005), such as mean interspecific divergence, but such thresholds can lead to errors (Meier et al., 2008). This issue is critical according to Wiemers and Fiedler (2007), who did not find a barcoding gap in *Agrodiaetus* Hübner, 1822 (Lepidoptera: Lycaenidae), a group in which interspecific divergences were as low as 0%, and in which many closely related species showed low interspecific divergence yielding an overlap of 18% in COI sequence divergence when intra and interspecific sequences were compared.

DNA barcoding might assist in two main areas of systematic biology (Sites and Marshall, 2003; Wiens, 2007): i) discover and describe new species, and ii) assess their phylogenetic relationships. However, the utility of DNA barcoding in terms of these two major goals remains unresolved, with conflicting opinions generating a continuing debate. Concerns about sequence variation and how to interpret them to delimit

species, the distance methods employed, substitution rates of mtDNA, exhaustive sampling including broader areas and sister species, and the need for *a priori* hypotheses are examples of the non-agonist side of the coin (e.g. Brower, 2006; Meyer and Paulay, 2005; Moritz and Cicero, 2004; Prendini, 2005; Will and Rubinoff, 2004) that need to be taken into account when using the technique.

Regardless of these criticisms, DNA barcodes help in the quest of finding at least some species boundaries and their identification (Hebert and Gregory, 2005), even if they might not provide sufficient evidence for species description (but see Brower, 2010). The majority of researchers suggest that a comprehensive, integrated data analysis (fully described by Will et al., 2005) is the best approach, requiring independent data sets (Baker et al., 1998; Mayr, 1963; Miller et al., 1997; Rokas et al., 2003; Wahlberg et al., 2005), their combination (Ferguson, 2002; Rubinoff and Holland, 2005), and the development of innovative concepts and applications such as the taxonomic circle (DeSalle et al., 2005), in which the interactions between data sets is assessed to reveal new species. However, in some situations non-molecular data sets such as morphology might not be helpful in systematic research, as pointed out by Bickford and colleagues (2007). Where morphology is suspected to be intraspecifically variable, the DNA barcoding technique is a powerful tool (Elias et al., 2007; Magnacca and Brown, 2010). Nevertheless, attempting to obtain a more comprehensive matrix should not be left to one side.

There is ever-increasing research on DNA barcoding, and its achievements in a wide group of organisms have been well documented. The purpose of this review is to synthesize the information available on Lepidoptera, and some other groups of

arthropods. I aim to address the two main goals of the method by examining specific cases in arthropods, butterflies, and moths. In these sections I will also focus on important critiques of the technique and how they have been assessed in studies of Lepidoptera. Some important applications of the technique will be reviewed. Subsequently, I will address further insights into how to use the technique efficiently in the case of *Prepona laertes*. Finally, I will discuss several difficulties associated with and alternatives to this molecular tool.

Species identification

One of the main goals of DNA barcoding is to assist in species identification. As explained in the previous section, the mechanism by which COI sequences from unidentified organisms can be linked to particular taxa requires a data matrix containing DNA data from a pool of individuals from the same and other species, to which the unknown sequence can be compared and matched using distance methods that compare sequence variation (Hebert et al., 2003b). Importantly, as comprehensive a matrix as possible is required to obtain accurate results (Ekrem et al., 2007; Little and Stevenson, 2007). Brower (2006) debated whether DNA barcoding could permit species identification. He stated that instead of concluding that a sequence from an unknown taxon belongs to 'X' species, "one can only say" that the sequence is more similar to another than to the remaining sequences in the matrix, because one is relying on % of sequence variation. The fact that DNA barcoding relies on distance methods to generate an identification was examined by Little and Stevenson (2007). They compared different algorithms when assessing gymnosperm identification, and their results showed contrasting results depending on the algorithm used. They showed that hierarchical clustering methods such as Neighbor Joining (NJ), Parsimony and

Maximum Likelihood performed the worst; these rely on comparing known and unknown sequences in a database, and finding a topology after their alignment. On the other hand, similarity methods addressed such as BLAST performed better; these methods yield a similarity and probability score after comparing the unknown sequence against an un-aligned matrix using a pair-wise partial alignment. Regardless of these findings, clustering methods such as NJ have been and continue to be widely used with barcode sequence data to identify unknown sequences, at least in Lepidoptera (e.g. Hebert et al., 2004a).

Despite the continuing debate regarding the benefits and disadvantages that DNA barcoding yields, it has been shown to be a fair technique to approach species identification at different levels. When introducing the barcoding concept, Hebert et al. (2003a) evaluated identification of unknowns at phyla, order (insect), and species level. This was achieved by gathering a data set of 100 sequences (one per species), 100 sequences (one per family), and 200 sequences (closely related species) respectively. The resulting NJ topology based on the analysis of amino acids coded by COI for seven phyla showed mixed results; there was good resolution for some groups but not for others (e.g. Arthropoda vs. Mollusca respectively). At the ordinal level there was better resolution, with all orders monophyletic except Coleoptera. Also, they assessed COI sequence variation and tested assignations of unknowns to three taxonomic categories, with a high rate of success; 100% at the ordinal and species level and 96.4% at phylum level.

Within Lepidoptera there has been extensive work done on DNA barcoding, partly because this group supposedly poses a rigorous test for the technique because of its

high diversity and the low sequence divergence amongst families (Hebert et al., 2003a). Lepidoptera contains almost 160,000 described species and is amongst the five most speciose insect orders (Gullan, 2004). Studies have evaluated biodiversity under the barcode perspective, and some assess specific criticisms of the method. Janzen et al. (2005) found the DNA barcoding technique to be successful in identifying members of the complex and diverse Lepidoptera fauna of the Area de Conservación Guanacaste, Costa Rica. Hajibabaei et al. (2006) provided similar results for the same Costa Rican region, in which approximately 98% of 521 species belonging to the families Hesperiidae, Sphingidae, and Saturniidae were successfully identified. Later, Elias et al. (2007) tested the technique's performance in a diverse group of butterflies with a solid taxonomy based on morphology. They showed that the barcoding technique might not be as effective as previously stated, at least in complex tropical faunas, with only 44 of 57 species identified based on molecular data. The number successfully identified decreased when intra- and interspecific variation increased due to inclusion of geographically distant samples.

Since the technique relies on clustering sequences according to their resemblance to each other, monophyly of species would seem to be important. However, Lukhtanov et al. (2009) suggested that monophyly was not required for species identification in a sample of Palearctic butterflies. They found that the separation of monophyletic groups from paraphyletic groups was highly supported by bootstrap analysis, and that such groups "form non-overlapping clusters". Secondly, if a cluster is formed by monophyletic and paraphyletic parts (i.e. two species), one can still identify a diagnostic barcode sequence for the paraphyletic part since it shares

synapomorphies with the monophyletic part and at the same time lacks the synapomorphies present in the monophyletic remainder. The monophyletic portion would have a barcode sequence characterized by the synapomorphies shared with the paraphyletic part plus its own synapomorphies.

Despite the fact that several authors have suggested that broader geographic sampling is needed in order to increase interspecific and intraspecific variation (e.g. Meyer and Paulay, 2005), Lukhtanov et al. (2009) found that the success of identifying species was not drastically reduced when the geographic scale was broadened. They studied approximately 70% of the central Asian butterfly fauna comprising 353 species, 370 populations, and 880 COI sequences, which they compared using the Kimura 2-parameter yielding a NJ tree further analyzed by bootstrap testing. They explicitly addressed the influence on identification success of including geographically separated populations by analyzing the genetic distance between pairs of closely related species when: i) including one population, and ii) including additional populations. Their results show that increasing the geographic breadth did not reduce the success in identifying unknowns because increasing intraspecific variation “does not ‘fill’ the interspecies hiatus”.

Other evaluations (e.g. Prendini, 2005) of the barcoding approach (e.g. Barrett and Hebert, 2005; Hebert et al., 2004b) have noted that barcoding studies typically exclude close relatives (usually allopatric) and therefore miscalculate error rates (Meyer and Paulay, 2005). Meyer and Paulay (2005) tackled the importance of geographic variation and the distribution of species, suggesting that both could pose particular problems to the method. They called attention to the fact that comprehensive sampling needs to be

achieved for DNA barcoding to be successful, in terms of increasing: i) interspecific variation through an extensive geographic survey, and ii) intraspecific variation by means of increasing the individuals analyzed. In their study of cowries (Gastropoda, Cypraeidae) they show proof of the importance of a broad geographic sampling. When assessing a regionally restricted fauna the 'barcoding gap' proved to be higher (lower error rates), but as an artifact of the exclusion of allopatric sister taxa. However, they acknowledge that each model organism might have different geographic scales in which error reduction occurs, thus the extent of a "comprehensive sampling" will depend on the organism assessed.

Species delimitation

Another main goal of DNA barcoding is to provide an important tool to help in studies of species delimitation as an initial approach (Hebert et al., 2004a). Sometimes research focused on identifying sequences of unknown individuals can evolve into studies involving cryptic species complexes or delimiting species. In such cases the results obtained from a "routine" identification process might reveal a cluster of unknowns within the matrix where divergence among them is lower than with respect to remaining clusters. As explained in the first section, the implementation of thresholds is a useful approach when using the technique to analyze sequence data for species delimitation. Here, contrary to the case of species identification, in species delimitation I believe that it is crucial that putative species are monophyletic.

Several studies regarding this topic and involving Lepidoptera have been published, such as Hebert et al. (2003b). This study, in addition to providing more support for the technique's success in Lepidoptera by being able to differentiate closely related species of butterflies and moths, provided an estimate of the mean divergence

value for pairs of closely related species in the order, namely 6.6%. Later, Burns et al. (2008) demonstrated the utility of DNA barcoding when assessing cryptic species complexes in Costa Rican Hesperiidae by providing evidence for four cryptic species within *Perichares philetes* (Gmelin, [1790]). However, contrasting results regarding the discrimination of closely related species of Lepidoptera have also been published (e.g. Kaila and Stahls, 2006).

As well as showing success for the technique in Lepidoptera, Hebert and colleagues (2004a) provided a controversial example of how DNA barcoding can help in assessing cryptic species complexes in a study of the skipper butterfly *Astraptes fulgerator* (Walch, 1775). Here they combined their genetic results with ecology and natural history traits to provide a strong case for the existence of cryptic species, suggesting that the former *A. fulgerator* comprised a group of 10 cryptic species. However, several critiques of this work have been published since, particularly Brower (2006), who presented a rigorous evaluation of the paper by reanalyzing the data by means of other methods. He called attention to three main elements of Hebert et al.'s work, which in his opinion, needed to be re-evaluated. First he mentioned the fact that clade support was not assessed, thus we cannot be certain about the stability of the groups. Second, he analyzed DNA sequence data variation using other methods such as population aggregation analysis and cladistic haplotype analysis to corroborate the topology provided by Hebert et al. (2004a). He found some contradictions between these results and those originally presented. Third, he noted that no out-group was used, which would be needed to make an evolutionary calibration of the data. Finally,

he suggested that a broader sampling effort would be needed to incorporate all possible mtDNA haplotypes corresponding to *Astraptes fulgerator* before drawing conclusions.

It is difficult to establish a fixed amount of genetic divergence at which different clusters should be considered different species. In my opinion, the amount of divergence needed to treat clusters as different species depends on several factors, such as the group's inherent characteristics for genetic divergence. For example, Meyer and Paulay (2005) noted that marine gastropods tended not contain allopatric sister taxa, whereas the opposite was usually true for terrestrial gastropods, where dispersal among regions was much more limited. Hebert et al. (2003a; 2003b) found low sequence divergence in the COI region in the phylum Cnidaria, and suggested that this might be partially explained by the fact that their mitochondria possess an excision repair mechanism that is absent in other groups.

Since increasing geographic sampling tends to increase divergence within species clusters, the threshold divergence for species recognition should depend on the extent of geographic sampling. In general, broader geographic sampling would require lower divergence between clusters to qualify for species recognition. On the contrary, narrower geographic sampling would demand more divergent clusters. Additionally, where possible, information other than DNA sequence divergence should be used in making taxonomic decisions. For example, Furtado (2008) suggested that two Preponini genera, *Agrias* and *Prepona*, should be combined since their species were able to hybridize.

Applications of DNA barcoding

DNA barcoding can be used to address other biological questions in addition to species discovery and identification. Hajibabaei et al. (2007) assessed the potential use

of DNA barcode regions and related techniques such as DNA microarrays for monitoring biodiversity in mammals. They noted the importance of scale in determining which method of study is most appropriate. Large scale studies would benefit from COI barcoding because this sequence-based approach can discover unknown haplotypes by comparing the unknown sequence data to previously identified organisms. With DNA microarrays, however, with a broad scale of study the appearance of new haplotypes might complicate the analysis because this technique requires previous knowledge of target sequences to be analyzed. Darling and Blum (2007) reviewed the use of DNA barcoding for identifying and monitoring invasive species. They highlighted the value of barcoding when addressing unknown samples but also emphasized the potential difficulties with more complex scenarios such as surveying overall diversity in a specific area. In those complex cases they suggested that using other methodologies (e.g. DNA fingerprinting) would be more appropriate. Smith et al. (2006) tested the use of DNA barcoding to address the cryptic species complex of parasitoid flies reared from lepidopteran caterpillars in Costa Rica, and advocated the use of this method in developing strategies of biological control in related groups. Cywinska et al. (2006) and Rivera and Currie (2009) successfully applied DNA barcoding in medical studies.

Some studies have implications for biodiversity conservation in natural areas where biodiversity is impacted, either by anthropogenic factors or by non-native fauna displacing native species. Other conservation approaches using DNA barcoding are common, although the goals are not always clear (Moritz 1994). Petit et al. (1998) used genetic markers to assess allelic richness and thus determine how best to protect a population of the argan tree (*Argania spinosa* (L.) Skeel) in Morocco. A number of other

studies on the usefulness of genetic markers in biodiversity conservation and monitoring have been published for different organisms (e.g. DeSalle, 2006; Féral, 2002; Gaudeul et al., 2000; Rubinoff, 2006; Schwartz et al., 2007). Within Lepidoptera, Gompert et al. (2006) used genetic data (mtDNA) in conservation and management of the Karner blue butterfly, *Lycaeides melissa* (W. H. Edwards, 1873), but highlighted the need to complement the results obtained with different markers for the reliable diagnosis of conservation units.

COI barcoding: difficulties and alternatives

DNA barcoding has been the subject of much research, yielding insights into the dynamism of species, their relationships with each other and their boundaries. However, research has also created awareness of the limitations and difficulties involved with the technique, not only because of theoretical and empirical issues discussed above, but also because of external factors. Examples of some of the latter difficulties include: i) heteroplasmy, ii) pseudogenes, and iii) *Wolbachia*, Hertig, 1936.

i) Heteroplasmy can be defined as an organism having more than one mitochondrial DNA haplotype (Magnacca and Brown, 2010); thus species showing heteroplasmy might be equivocally identified or remain unknown. Magnacca and Brown (2010) tested the effect of heteroplasmy on the accuracy of DNA barcoding for species identification of bees in the genus *Hylaeus* Fabricius, 1793. They found that approximately 43% of *a priori* morphological species sampled were heteroplasmic. DNA barcoding identified 100% of homoplasmic species, but only 71% of heteroplasmic species. However, they also noted that success in identifying heteroplasmic species could be improved (in this case from 71% to 86%) by treating polymorphisms as character states.

This study also found the presence of pseudogenes (ii). Pseudogenes (numts) are DNA sequences that resemble functional genes but are not expressed (Li et al., 1981). Hence, the presence of numts in the organisms being tested either for identification or for species delimitation can yield equivocal results due to co-amplification of the pseudogene with the mitochondrial DNA functional gene. Numts have been studied in several organisms, including insects. Bensasson et al. (2000) showed that migration of mitochondrial DNA and its assimilation in the nucleus is frequent in grasshoppers, a phenomenon further examined by Bensasson et al. (2001) and reviewed by Timmis et al. (2004). Song et al. (2008) evaluated the effect of numts on DNA barcoding success in two different lineages of arthropods. They found that numts were co-amplified with the COI gene. In grasshoppers, their analysis found 17 clusters based on a threshold of 3% sequence divergence, which means that 13 more species were 'detected' in addition to the four currently recognized. As a conclusion, they suggest future research is needed to detect the possible presence of numts in order to avoid overestimating the number of species or compromising their correct identification. Sorenson and Quinn (1998) reviewed different techniques used to detect numts. First, one could clone the PCR products followed by sequencing. Also, the use of internal primers to the original sequence can be used to differentiate between both sets of sequences. The use of restriction endonucleases to digest one of the copies is another approach that can be used. In addition, one can use mRNA of the gene product to obtain a cDNA copy of the expressed mtDNA. Finally, the use of the southern blot technique using the DNA isolated against a purified mtDNA probe is a more straightforward way to detect numts.

Another feature that might jeopardize studies based on mitochondrial DNA is the presence of the maternally inherited endosymbiont *Wolbachia* (iii). This bacterial endosymbiont is found in many arthropod lineages and involved in reproductive alterations such as cytoplasmic incompatibility and parthenogenesis (Hoy, 2003). Its evolution and phylogenetic relations have been analyzed (e.g. Werren et al., 1995b; Zhou et al., 1998), as well as several of its biological aspects such as its ability to transfer horizontally by means of parasitism (Dedeine et al., 2005; Heath et al., 1999), its capability of transferring its genetic material to the host's DNA (Kondo et al., 2002), its potential advantage to its host against viral infections (Hedges et al., 2008), and its possible benefit against insect disease transmission (Brownstein et al., 2003).

Some studies have evaluated the involvement of *Wolbachia* in insects, for instance its effect on *Drosophila* Fallén, 1823 (Shoemaker et al., 2004), and *Solenopsis* Westwood, 1840 (Shoemaker et al., 2003) mtDNA evolution. Other studies have assessed its taxonomic distribution (e.g. Werren et al., 1995a; Werren and Windsor, 2000), while others have studied its role in barcoding approaches. Hurst and Jiggins (2005) addressed *Wolbachia* effects on phylogeography, phylogeny and barcoding studies, and concluded that a *Wolbachia* infection might lead to a mistaken inference of evolutionary history. Similarly, Narita et al. (2006) examined the prevalence of *Wolbachia* in the butterfly *Eurema hecabe* (Linnaeus, 1764) populations in Japan. Their results suggest that there are two sibling species within the recognized *Eurema hecabe*. These two forms were supported by nuclear and biological data. On the contrary, the mtDNA data did not support the former results due to the infection of the populations with *Wolbachia*. A later study by Whitworth et al. (2007) in the genus *Protocalliphora*

Hough, 1899 (Diptera: Calliphoridae) found evidence for the possible introgression of *Wolbachia* genetic material, which introduced error and suggested that these flies were not monophyletic. Lastly, Linares et al. (2009) found *Wolbachia* infections in their study of Madagascar butterflies, and showed that the use of generalized primers can enhance the amplification of *Wolbachia*'s DNA sequences, hence suggesting the parallel use of *Wolbachia* discovery methods coupled with mtDNA barcoding techniques.

Nonetheless, if the above issues prove difficult to control for in a study group, there are alternatives to COI mtDNA barcoding that have been developed and used to address the same goals. Hajibabaei et al. (2007) used DNA microarrays to identify and assess mammalian species, including felines, canines, bats, rodents, and primates among others, as explained previously. Even though the methodology is entirely unrelated to barcoding, involving hybridization of sample DNA with DNA probes, it has been shown to be useful for similar purposes. Furthermore, they also showed the functionality of the species-level marker cytochrome b for the same goal.

Similarly, alternative DNA regions have been used such as the ribosomal DNA (rDNA) Internal Transcribed spacer 2 (ITS2), which has a nuclear genome origin. Yao et al. (2010) suggested this molecular marker as a universal DNA region for plant and animal barcoding based on certain characteristics such as the possibility of developing universal primers that match its conserved regions. Much research has been done in this parallel field of DNA barcoding. It includes a broad array of taxonomic groups such as nematodes (Clapp et al., 2000), mites (Ben-David et al., 2007), flatworms (Prasad et al., 2009), fig wasps (Li et al., 2010), and Lycaenidae butterflies (Wiemers et al., 2009). The latter work involves comparison of the COI mitochondrial marker and ITS2 nuclear

marker, in terms of their efficiency in species identification. The nuclear marker proved difficult to align (Wiemers et al., 2009) but inferring its secondary structure (i.e. translation of the genetic code into pseudo-proteins) has proven to be effective when addressing lower taxonomic levels (Coleman, 2003). Wiemers et al. (2009) illustrated the performance of both markers, and showed that both genes are comparable in their phylogenetic signal, thus advocating the use of a nuclear marker in part to overcome the inherent drawbacks that mtDNA barcoding presents (e.g. low mtDNA variation due to incomplete lineage sorting).

Further studies have been done in Lepidoptera regarding the availability and utility of other markers for species-level phylogenetics research. Wilson (2010) evaluated the usefulness of the DNA barcode region in molecular systematics in Lepidoptera in comparison with other markers including EF-1α, WG, 18S, CAD, and DDC. He tested the performance of the COI barcode region at different taxonomic levels based on three criteria: i) practicality in terms of universality and sequence quality (reliability of the marker to produce sequences with low percentage of ambiguous bases), ii) phylogenetic utility regarding informative sites, and iii) accuracy of phylogenetic signal. For the first criterion, the COI region proved to be 100% successful in the 24 families surveyed as well as of high sequence quality for the same families. For the second criterion, the COI region showed good performance across taxonomic levels in relation to variable sites, parsimony informative sites, and minimum number of state changes, and was the most appropriate marker at lower taxonomic levels. These results support the use of this marker in studies involving different genera, such as the present study of Preponini. Lastly, for the third criterion, COI was found to have similar

phylogenetic signal to nuclear markers at lower taxonomic levels. However, the lowest category studied was genus, thus we lack information regarding the performance of COI in comparison with other markers at lower, species levels.

Using DNA barcoding effectively: the *Prepona laertes* case.

Several outcomes are possible in using DNA barcoding to examine the taxonomy of the *Prepona laertes* group. Firstly, the nine different phenotypes treated previously as species might prove to be reciprocally monophyletic, providing strong evidence that they are valid taxa. Alternatively, there may be no, or limited, differentiation among the phenotypes, consistent with the Lamas (2004) hypothesis of a single species. Most likely, however, some phenotypes will prove to be reciprocally monophyletic, others paraphyletic or polyphyletic, and still others undifferentiated.

I aim to test the hypothesis proposed by Lamas (2004) in which *Prepona laertes* corresponds to one polymorphic species. Testing this hypothesis requires analyzing as many individuals and subspecies as possible, comprising several formerly recognized species such as *P. philipponi*, and *P. pseudomphale.* Even though changes in coloration patterns can lead to speciation in butterflies through reproductive isolation (Jiggins et al., 2001; Jiggins et al., 2006; Lukhtanov et al., 2005), an abrupt character change could be more likely to have an effect on reproductive isolation through, for instance, mate recognition. Still, hybridization can occur (Mallet et al., 2007), sometimes giving rise to new lineages (Mavarez et al., 2006). On the other hand, despite the fact that coloration changes can enhance reproductive isolation, sympatric speciation can arise with coloration stasis (Jiggins et al., 2006), which suggests that not all variation in coloration implies speciation events. Therefore variation in coloration in butterflies may

not indicate speciation events but represent polymorphism, in which different morphs can be found throughout a geographic distribution.

Generally the characters that separate putative *Prepona laertes* group species are slight; they involve subtle changes from one putative species to other. Despite the fact that such wing pattern characters have been used to infer species, their presence might not imply speciation events, meaning that the variation seen can be attributed to intraspecific variation rather than to species-specific features. For instance the main difference between *P. pseudojoiceyi* and *P. joiceyi* is the presence of a hook-like termination of the light blue band on the forewing in *P. pseudojoiceyi*, whereas in *P. joiceyi* the band ends abruptly (D'Abrera, 1987). Hence, such difference in coloration can be indicative of intraspecific variation instead of being a defining character of each species.

If the Lamas hypothesis is correct, I predict all the individuals analyzed will have low divergence in the COI mitochondrial gene sequence, in comparison with inter-specific divergence for other well-defined Preponini species, independently of their phenotype. Additionally, I predict that individuals from the same putative species will not form clusters and/or will lack reciprocal monophyly (either for sympatric or allopatric species (Figure 3-2a)). On the other hand, clustering of barcode sequences would suggest the existence of additional species within *Prepona laertes* in which reciprocal monophyly of sympatric forms such as *P. pseudomphale* and *P. philipponi* would be strong evidence for ranking them as separate species (Figure 3-2b). If the clusters belong to allopatric forms (e.g. *P. rothschildi* and *P. philipponi*), the amount of

divergence between the clusters should be quantified to decide whether the clusters merit species or subspecies status (Figure 3-2c).

Materials and Methods

Sample Acquisition and Origin

As described in the previous chapter, in order to maximize the number of individuals of *Prepona laertes* I carried out fieldwork in Colombia, and contacted collaborators in other countries, which provided tissue or COI sequence data (table 3-2). In addition to those samples, I searched for specimens in the collection of the McGuire Center for Lepidoptera and Biodiversity, as well as recently dead specimens from the Butterfly Rainforest at the Florida Museum of Natural History. Furthermore, I searched the GenBank (http://www.ncbi.nlm.nih.gov/genbank/) repository to obtain sequences from other countries (table 3-2).

DNA Extraction and Amplification

DNA was extracted from two butterfly legs previously stored in 96% EtOH or recently removed from dried specimens. I used the Qiagen DNEasy Extraction Kit and followed their protocol. However, some modifications were employed (see materials and methods in Chapter 2). In this case only the COI barcode region was amplified, following the protocol and primers discussed in the Materials and methods section of Chapter 2.

DNA Sequencing and Alignment

DNA sequencing from only one strand of the COI barcoding region was carried out at the University of Florida by the University of Florida's Interdisciplinary Center for Biotechnology Research Sanger Sequencing Group. I used Geneious v5.3 (Drummond et al., 2010) to edit and align the sequences. I constructed two matrices, one of 618bp

corresponding to 24 individuals that I processed. I created the second matrix after searching the Genbank database and complementing my dataset with additional sequences provided by D. Janzen (table 3-2). A total of 97 COI sequences composed this second matrix for a total length of 612bp.

Cluster Analysis

I used Neighbor Joining (NJ) (Saitou and Nei, 1987) to produce a cluster diagram in which the sequences in the dataset are grouped based on similarity (i.e. genetic distances). Genetic distances were estimated by the Kimura 2-parameter model (K2P) (Kimura, 1980) with equal rates of variation. The outgroup species used in this analysis corresponds to COI sequence data for two individuals processed for the phylogenetic analysis, *Prepona pylene eugenes* H. W. Bates, 1865 and *P. dexamenus dexamenus* Hopffer, 1874.

Results and Discussion

The resulting topologies corresponding to both datasets are shown in Figures 3-3 and 3-4. The COI sequence data I processed represented seven neotropical countries: Mexico, Honduras, El Salvador, Colombia, Venezuela, Ecuador and Brazil, for a total of 24 individuals. Despite the fact that all individuals analyzed belong to *Prepona laertes* according to Lamas (2004), they possess differences in their morphology, particularly in their coloration pattern. These characters have been used to define several species that currently are considered synonyms to *Prepona laertes*. Phenotypes representing *Prepona philipponi* and *P. pseudomphale*, as defined by Neild (1996), were included in this analysis and are considered in both figures as different species to denote the differences in their morphology. Additionally, different geographical forms of *Prepona laertes* in reference to the Andes mountain range were represented in this analysis;

according to the Lamas taxonomy, these forms represent different subspecies, *P. l. octavia* Fruhstorfer, 1905 representing populations located to the west of the Andes and *P. l. demodice* (Godart, [1824]) to the east. Moreover, another morph is denoted in the analysis as *Prepona laertes* ssp1, corresponding to an unpublished classification of Colombian charaxines by J. F. LeCrom (Le Crom, pers. comm.). Yellow androconial tufts and the presence of light and dark blue dorsal bands characterize this putative new subspecies.

The results show that there are no clear clusters of COI barcode sequences corresponding either to variations in coloration pattern (i.e. *Prepona philipponi*, *P. pseudomphale*) or geographical origin in reference to the Andes (Figures 3-3, 3-4). Nonetheless there is a cluster of sequences regarding geographic region denoted by node 1. These sequences belong to individuals from Colombia and Venezuela and represent different morphological entities, including differences in androconial tufts coloration (orange in *P. pseudomphale* and yellow in *P. laertes*) and presence/absence of dark blue band, for instance present in *P. laertes* ssp1 and absent in *P. l. laertes* (Hübner, [1811]). Even though the origin of the samples is close (from the perspective of the entire geographic distribution represented in the analysis) this mixture of forms complicates the analysis since no correspondence is found between morphology and genetic data. Furthermore, these individuals are embedded in a polytomy within the outgroup species which obscures their real placement and their relation to the remaining barcode groups.

Cluster number 2 represents individuals from diverse regions as well as of different phenotypes, while the most diverse cluster in terms of countries represented is

cluster 3. These two are well-defined clusters with approximately 2% divergence. Nevertheless, this amount of genetic divergence is less than 3% suggested by Hebert et al. (2004b) to be a useful threshold to identify species. Hence, these clusters do not reach the amount of genetic divergence considered by some to be sufficient for species status, in addition to the fact that they do not correspond to sympatric groups with consistent morphological differences. The use of a fixed threshold of genetic divergence for recognizing species status within all groups has been widely criticized, and perhaps, these results would have provided evidence against their use if correspondence between morphological variations and genetic clusters had been found, coupled with a < 3% genetic divergence. An alternative approach to the use of fixed thresholds is comparing the divergence found for *Prepona laertes*' clusters to the mean genetic divergence that characterizes other *Prepona* and *Agrias* species. I used the COI sequence data of nine *Prepona* and *Agrias* species included in the species-level phylogeny to quantify the mean interspecific divergence and found that amount to be ≈ 9%. This suggests that the genetic divergence found for *Prepona laertes* is minimal compared to other preponine species. Then, I addressed the mean interspecific divergence between sister species of the *Prepona* and *Agrias* group and found that such divergence is ≈ 4.6 %, a result that further suggests that the genetic divergence seen in *Prepona laertes* is low in comparison to other preponine species.

In terms of reciprocal monophyly, clusters 2 and 3 are reciprocally monophyletic, though they represent a wide variety of forms and a diverse geographic origin, which complicates the interpretation of the results since allopatric and sympatric forms are mixed together within the clusters. Similarly, there are two clear groups within cluster 3.

One corresponds to the Mexican + El Salvador samples, the other represents Colombian and Ecuadorian samples. Even though the Mexico + El Salvador cluster correspond to one morph, *Prepona laertes octavia*, the second group represents different localities and contains different morphological variations, including *P. l. octavia*. These clusters are also characterized by < 3% of genetic divergence.

The results so far do not falsify the hypothesis proposed by Lamas (2004), in which he states that the former *Prepona philipponi* and *P. pseudomphale* are polymorphic forms of *P. laertes*. However, the degree of divergence among individuals is also high, suggesting an alternative interpretation that multiple cryptic species lie within *P. laertes* that will only become clear through additional sampling.

Figure 3-4 corresponds to all available COI data for *Prepona laertes*. This analysis included the data I processed plus additional samples from Mexico, Costa Rica and Panama. The Mexican samples are identified as *Prepona laertes octavia* according to the latest checklist (Lamas, 2004). Similarly, the Costa Rican samples are identified under the same subspecies, which according to D. Janzen (pers. comm.) should be considered two different species, which he denotes as *Prepona demodice* 1 and *P. demodice* 2 (a nomenclature not adopted here). His suggestion is based on ongoing research into not only COI barcode sequence data, but also natural history and ecology.

Interestingly, there are two clusters that are mainly formed by Costa Rican samples, as noted by Janzen (Figure 3-4). However, there are several important issues to be addressed regarding my results. First, all Janzen's samples come from one locality in Costa Rica, and such single region studies have prompted a debate regarding the minimum sufficient geographic breadth of the samples and level of sampling effort

(Brower, 2000; Meyer and Paulay, 2005). However, samples from other countries such as Ecuador, Panama, Honduras and Colombia also fall within the Costa Rican samples, showing that the Costa Rican clusters obtained by Janzen appear to remain stable with addition of samples from other countries, thus surpassing possible geographic barriers. Incomplete lineage sorting or retained ancient polymorphism might explain the pattern seen, so the inclusion of more individuals from throughout the geographic distribution could prove helpful in corroborating such apparent structure. Additionally, the *Prepona laertes* study could benefit from the inclusion of an additional marker aimed to test whether the COI sequence data are yielding accurate results.

My results show that increasing geographic breadth might change the clustering pattern (Figure 3-4). Escalante et al. (2010) found *Prepona laertes octavia* to be monophyletic based on four COI sequences amplified from butterflies collected in the states of Guerrero and Veracruz, but when I used those sequences in my analysis and included the additional Mexican sample I processed (collected in the state Tabasco), this monophyletic cluster disappeared. These results suggest that increasing the geographic dimension in the *Prepona laertes* group indeed has an effect on the results, even though the geographic expansion was not apparently great (Tabasco state borders with Veracruz). Together, these results highlight the need for broad geographic sampling in future studies of *Prepona* phylogeography.

The most basal *Prepona laertes* samples form two clear clusters (denoted with numbers 1 and 2 in Figure 3-4) which are characterized by long branch lengths. Each cluster is represented by a diverse group of individuals, each possessing differences in their coloration patterns (denoted in the figure as different species), as well as from

different geographical origins. Additional samples from as many localities and morphs should be included in the future in order to increase the COI sequence variation and help to clarify the results. For instance, including more samples from the same locality could be useful to reduce the observed long branch lengths, which might help resolve the relationships, for example, of the Colombian specimens (node 1 in Figures 3-3, 3-4). Including additional data is important, since the current results which indicate substantial differentiation might change with inclusion of additional individuals, showing apparent divergence to be continuous rather than discontinuous. For example, in the case of Costa Rican specimens we would have observed a similar result as for the Colombian specimens if the analysis had only included a couple of specimens, such as those labeled with an asterisk.

Based on the COI sequence data presented here, it is still unclear if there is a cryptic species complex within *Prepona laertes.* Despite the fact that some COI sequences form distinctive clusters (e.g. nodes 1 and 2 in Figure 3-4), the genetic distances in terms of branch lengths are high for some individuals and negligible for others, which might potentially be an artifact of the lack of sampling. Thus, thorough geographic sampling and the inclusion of more individuals from the same localities could help disentangle the still unresolved taxonomy of the *Prepona laertes* group.

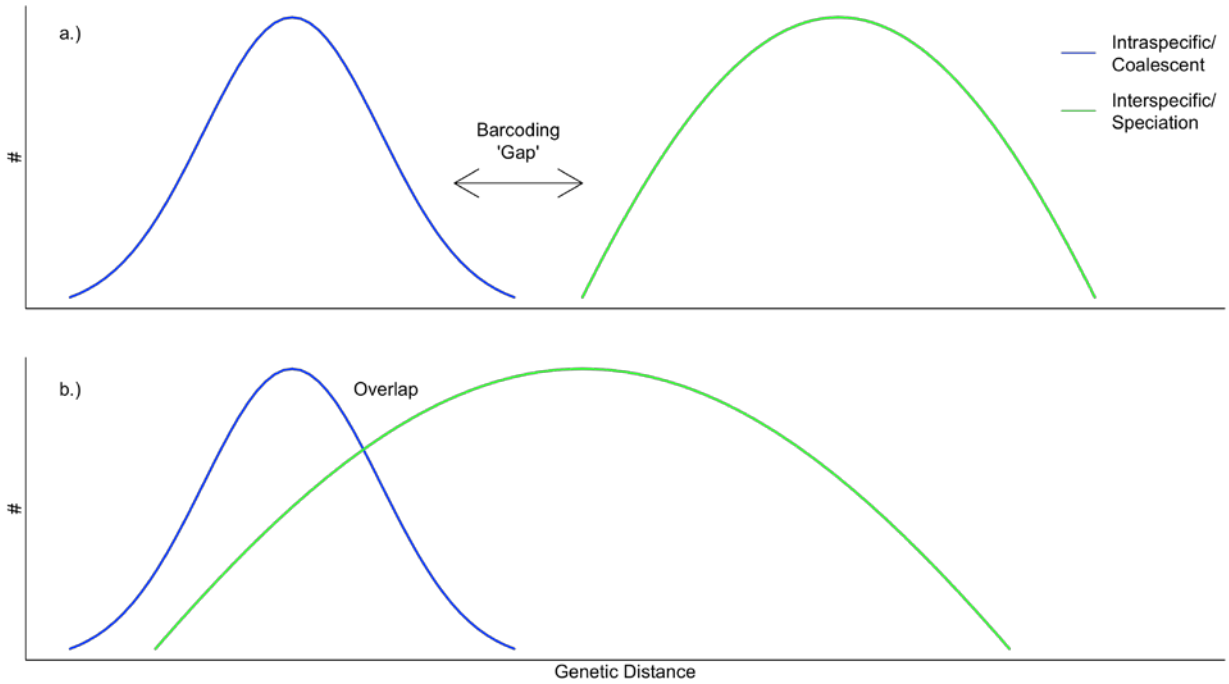

Figure 3-1. The DNA ‘barcode gap’. a. Presence of a barcode gap. b. Absence of a gap.

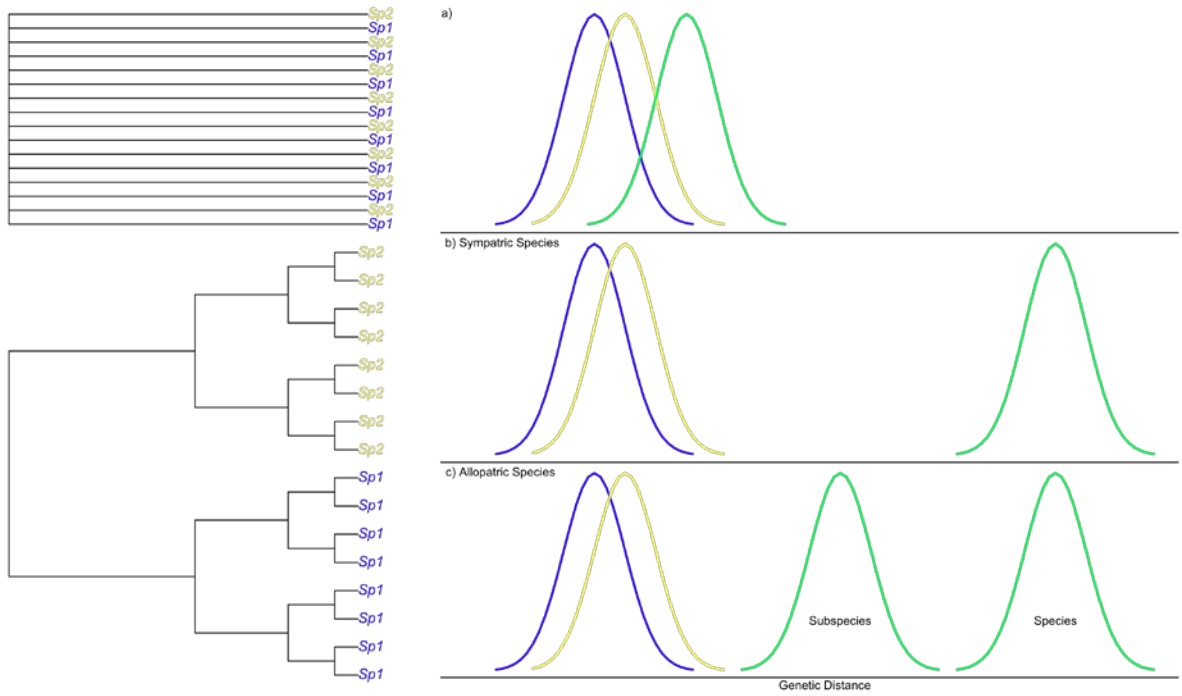

Figure 3-2. Possible outcomes for the *Prepona laertes* analysis. Blue curve corresponds to intraspecific comparisons of genetic distances of species 1, yellow line to intraspecific comparisons of genetic distances of species 2, and green line to interspecific comparisons of genetic distances for species 1 and 2. Only two "putative species" are shown for simplicity. a) Lack of barcoding gap and monophyletic clustering, b) Illustration of topology and barcode gap when sympatric forms are involved and c) when allopatric forms are involved.

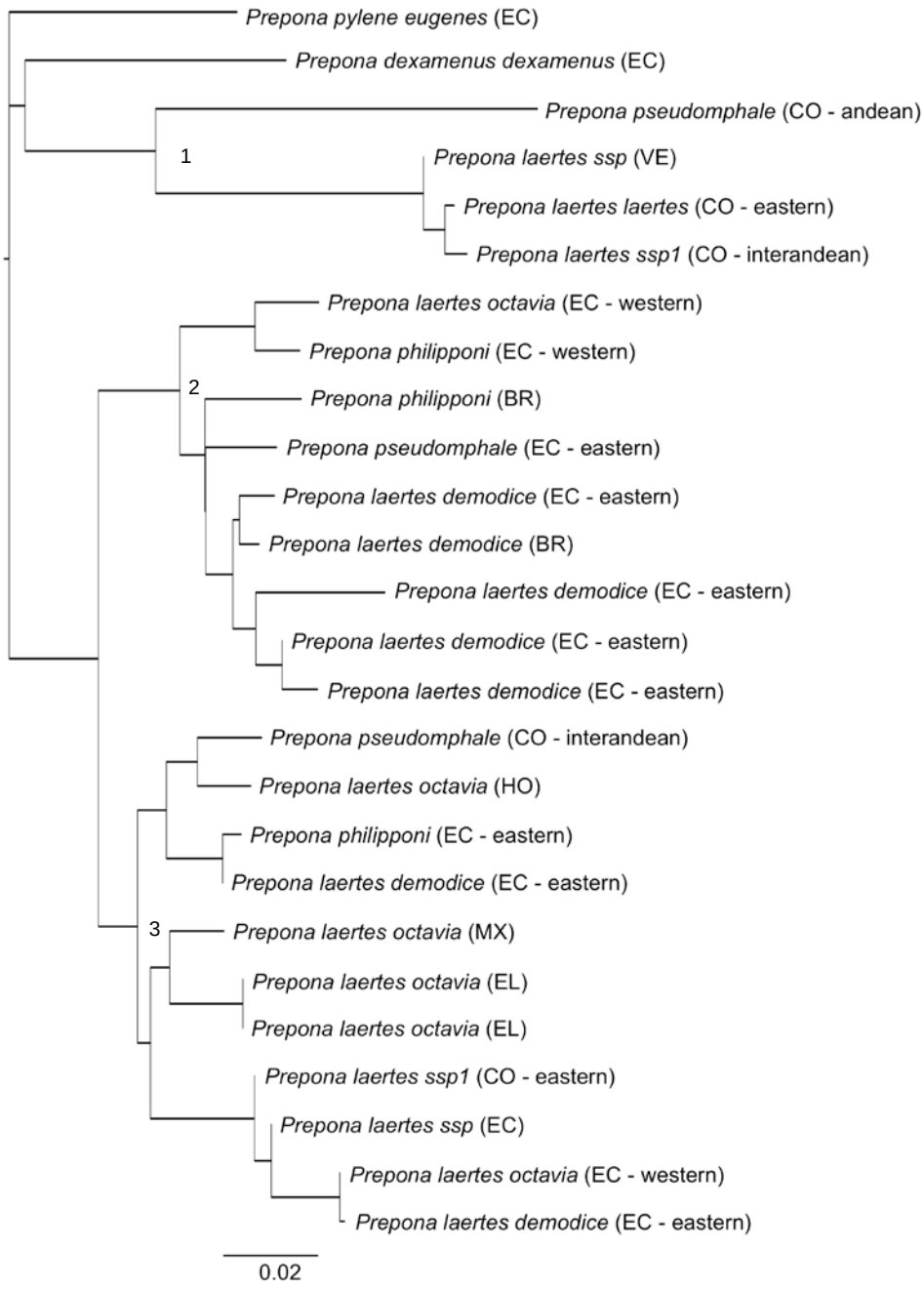

Figure 3-3. NJ tree for the *Prepona laertes* complex. It includes 24 COI gene sequences from different putative species, which are currently considered within *Prepona laertes*, such as *P. philipponi*, and *P. pseudomphale*. Text in parenthesis corresponds to the country of origin as follows: MX: Mexico, HO: Honduras, EL: El Salvador, CO: Colombia, EC: Ecuador and BR: Brazil, and the location in relation to the Andes mountain range. Scale bar represents genetic distance.

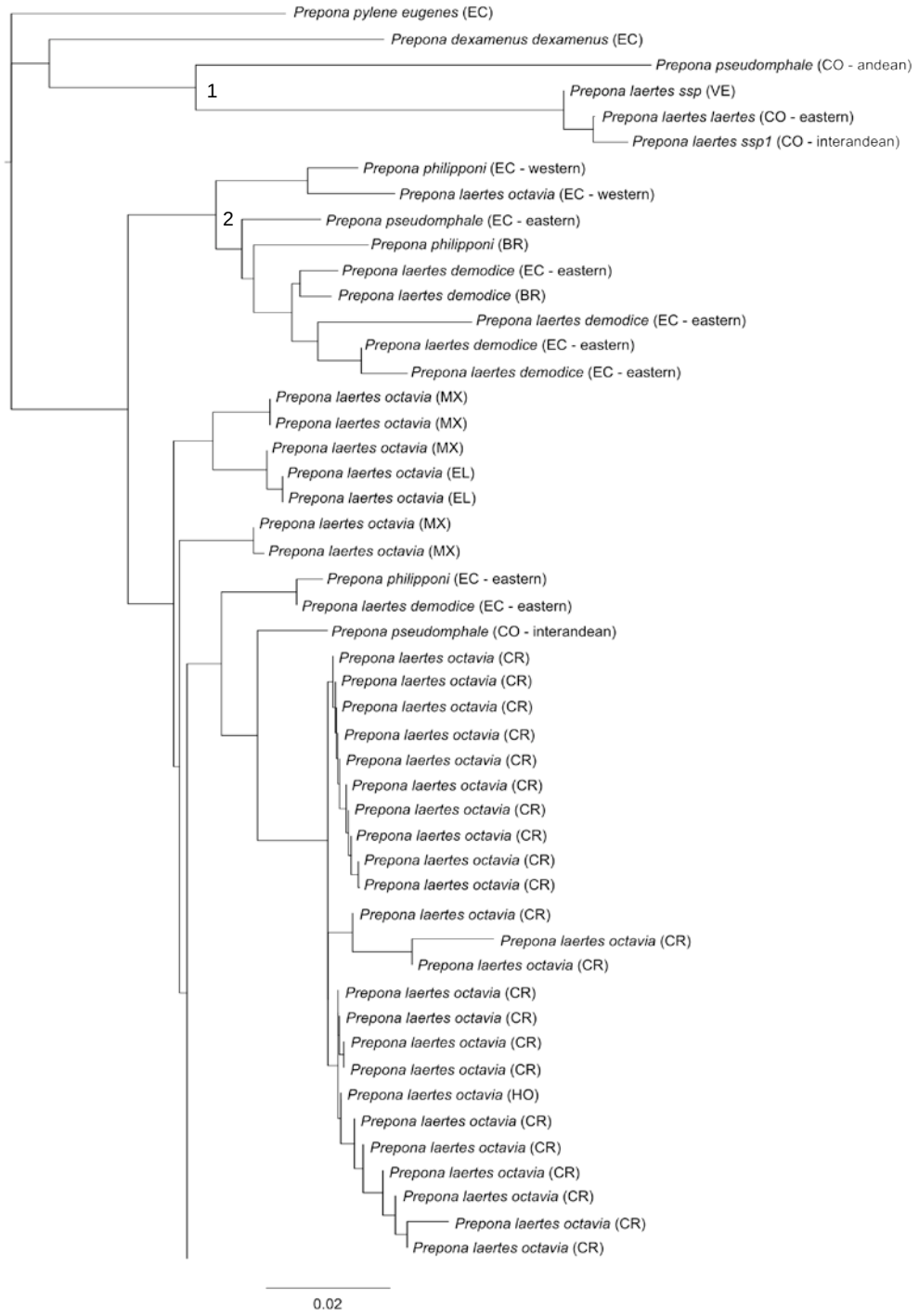

Figure 3-4. NJ tree of all available data for *Prepona laertes*. Text in parenthesis follows explanation in Figure 3-3 adding CR: Costa Rica and PA: Panama as a countries of sample origin. Scale bar represents genetic distance.

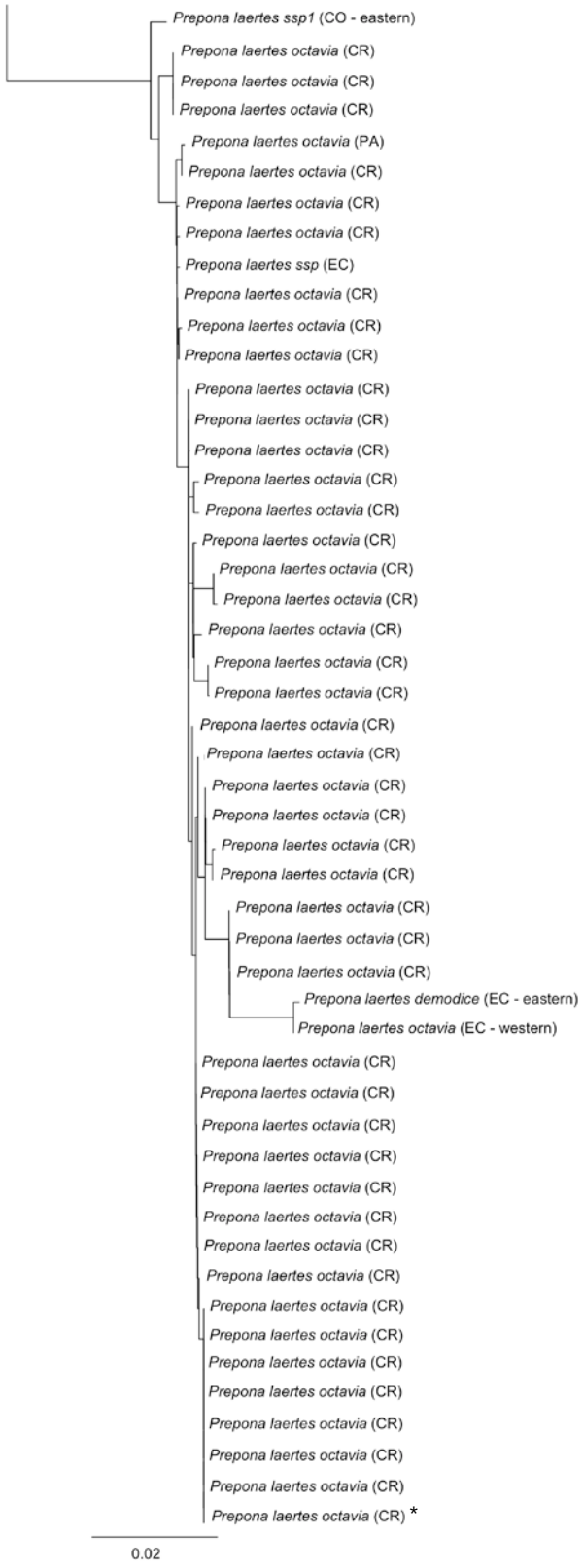

Figure 3-4. Continued.

Table 3-1. Historical classifications of the tribe Preponini.

Author	Genus *Prepona*	*Agrias*	*Archaeoprepona*	*Anaeomorpha*	*Noreppa*
Fruhstorfer (in Seitz 1907-1924)	14	7	10	1	n/a
Vane-Wright (1974)	9	n/a	n/a	n/a	n/a
Barselou (1983)	n/a	4	n/a	n/a	n/a
Spaeth (in D' Abrera 1987)	n/a	9	n/a	n/a	n/a
D' Abrera (1987)	21	n/a	5	1	2
Neild (1996)	n/a	4 - 9	≈ 9	n/a	2
Lamas (2004)	7	5	8	1	1

Table 3-2. Specimen codes for COI sequence data used in the *Prepona laertes* study. Asterisk denotes Genbank accession numbers when available.

Taxa	Specimen Code	Author
Prepona laertes octavia	HQ025040*	Escalante et al. 2010
	HQ025041*	Escalante et al. 2010
	HM888287*	Escalante et al. 2010
	HM888286*	Escalante et al. 2010
	HM416506*	International Barcode of Life (iBOL), 2010
	GU334298*	Janzen and Hajibabaei, 2009
	GU334299*	Janzen and Hajibabaei, 2009
	GU334300*	Janzen and Hajibabaei, 2009
	BCIBT154-09	
	MHAAC001-06	
	MHAAC003-06	
	MHAAC004-06	
	MHAAC005-06	
	MHAAC006-06	
	MHAAC007-06	
	MHAAC008-06	
	MHAAC009-06	
	MHAAC010-06	
	MHAAC011-06	
	MHAAC012-06	
	MHAAC013-06	
	MHAAC014-06	
	MHAAC015-06	Unpublished - Janzen (iBOL)
	MHAAC016-06	
	MHAAC017-06	
	MHAAC018-06	
	MHAAC019-06	
	MHAAC020-06	
	MHAAC021-06	
	MHAAC022-06	
	MHAAC023-06	
	MHAAC024-06	
	MHAAC025-06	
	MHAAC026-06	
	MHAAC027-06	
	MHAAC028-06	
	MHAAC029-06	

Table 3-2. Continued.

Prepona laertes octavia	MHAAC030-06	Unpublished - Janzen (iBOL)
	MHAAC031-06	
	MHAAC032-06	
	MHAAC033-06	
	MHAAC034-06	
	MHAAC035-06	
	MHAAC036-06	
	MHAAC037-06	
	MHAAC038-06	
	MHAAC039-06	
	MHAAC040-06	
	MHAAC041-06	
	MHAAC042-06	
	MHAAC043-06	
	MHAAC044-06	
	MHAAC045-06	
	MHAAC046-06	
	MHAAC047-06	
	MHAAC048-06	
	MHAAC049-06	
	MHAAC050-06	
	MHAAC051-06	
	MHAAC326-07	
	MHAAC352-07	
	MHMXB880-06	
	MHMXG633-07	
	MHMXG634-07	
	MHMXG636-07	
	MHMXG643-07	
	MHMXS246-08	
	MHMXS257-08	
	MHMXY742-09	
	MHMXY743-09	
	MHMXY744-09	
	MHMYB035-09	
	MHMYB036-09	

CHAPTER 4
CONCLUSIONS

This phylogenetic study has suggested that several taxonomic modifications are required within the Preponini. They include modifications at different taxonomic levels, from tribal limits to species boundaries. More specifically, I recommend the exclusion of *Anaeomorpha splendida* from the tribe Preponini into an *incertae sedis* section. The genetic data used in this study provide strong evidence that *Anaeomorpha* is misplaced within the Preponini, but the true relationship of *Anaeomorpha* to other members of the subfamily Charaxinae is still inconclusive. The inclusion of additional markers and a broader taxonomic sampling would prove to be useful in determining the correct placement of *Anaeomorpha*, either as a member of an existing tribe, or perhaps as a new tribe.

Regarding generic modifications, the genus *Noreppa* should be synonymized with *Archaeoprepona* **syn. nov**. The combined results from genetic and morphological data (Marconato, 2008) support such merging, since the ML inferred tree shows that *Noreppa chromus* is embedded in the *Archaeoprepona* clade, and the synapomorphies used to define *Archaeoprepona* can also be used to identify *Noreppa*. I also suggest, according to the available data to date (i.e. genetic and morphological), that *Agrias* should probably be synonymized with *Prepona*, despite the fact that other taxonomic solutions are possible. Although *Agrias* proved to be a well-defined monophyletic group, it is placed well within the *Prepona* clade. Nonetheless, the bootstrap support and posterior probabilities are not particularly high. This leads me to conclude that additional markers would be required to yield more robust results before making a definite change, which could perhaps clarify *Agrias*' closest relatives, thus providing more insight into the

relationships among members of this clade. In addition, I would encourage a detailed morphological study that attempts to identify synapomorphies that could perhaps support other taxonomic modifications (e.g. expanding *Agrias* to include *Prepona deiphile*, *P. werneri*, *P. praeneste*, and *P. pylene*). Also, I suggest that describing a new genus for the taxon *pheridamas* should be considerd, since, besides being basal to the *Prepona* + *Agrias* clade, it has distinctive morphology (i.e. coloration pattern and genitalia), and lacks morphological synapomorphies of remaining *Prepona*. In this case it would also be helpful to include an additional marker to increase the node support regarding *Prepona pheridamas*' relationship to the remaining *Prepona* + *Agrias* species.

This phylogenetic study of Preponini, besides allowing me to address my specific questions, has also indicated several taxonomic issues involving preponine species that require further study. It is clear that the relationships among taxa included within *Archaeoprepona amphimachus* and *A. meander* deserve more research. Similarly, the phylogenetic relationships among the different subspecies of *Prepona deiphile* should be further investigated, since here they appear to be independently evolving lineages and do not form a monophyletic group. In addition, the relationship between *Agrias claudina* and *A. narcissus* merits further research; the inclusion of more individuals representing different subspecies and from different geographical origins might prove useful in defining their taxonomy. Likewise, broadening the taxonomic breadth of *Prepona pylene* samples would likely shed light on its true taxonomic status. Only two individuals were included in this analysis, both belonging to the same species according to Lamas (2004) but not to other researchers (e.g. Willmott, pers. comm.). Therefore, future study should include other forms of *Prepona pylene*.

Within the *Prepona laertes* group, the hypothesis that *Prepona laertes* is a widespread polymorphic species as proposed by Lamas (2004) was not falsified by the COI barcode data. Even though distinctive clusters of COI barcode sequences were obtained, they did not show sufficient genetic divergence to consider them different species according to Hebert et al. (2003b). Additionally, when such divergence was compared to the mean genetic distances found for other preponine species, ≈ 9%, it is clear that the divergence found within *Prepona laertes* is low, even when addressing only the mean interspecific divergence between sister species (4.6 % for three species pairs). This conclusion is further supported by the lack of consistency in morphological characters within clusters. In spite of this, the tendency for clusters to have some geographic signal, such as the group formed by Colombian and Venezuelan specimens, deserves further attention. The NJ trees constructed using the COI barcode data suggest that further sampling is required to better represent genetic variation and clarify results. My results also suggest that, contrary to what has been proposed previously, increasing the geographic scope of the study does appear to have an effect on the results. Therefore, I recommend thorough geographic sampling and inclusion of as many individuals as possible in future studies of the *Prepona laertes* group and of other Preponini species.

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BIOGRAPHICAL SKETCH

Elena Ortiz Acevedo is a young biologist broadly interested in ecology and evolution, using diurnal butterflies as a model to understand the mechanisms that generate and maintain species diversity. She is particularly interested in understanding the causes of speciation and diversification, and as a consequence she has lately focused her work in phylogenetics and species delimitation. She graduated in 2007 from Universidad de los Andes in Bogotá, Colombia, where butterflies first attracted her attention during her undergraduate thesis. For this work, she compared butterfly diversity in two riparian forests in the eastern foothills of the eastern cordillera in Colombia. She worked in the field over several months, after which her interest in butterfly diversity led her to continue in the pursuit of knowledge about Colombian butterflies.

After graduating, she worked for the Tropical Andean Butterfly Diversity Project, digitizing the information available in the butterfly collection of the Instituto de Genetica at Universidad de los Andes, allowing her to increase her knowledge about the diversity and distribution of Colombian butterflies, as well as improving her skills in managing large amounts of data and information. She volunteered for the museum for natural history at Universidad de los Andes where she discovered her passion for biological collections and learned about curatorial techniques. She has also written several grants, of which one of the first was funded by the Tropical Andean Diversity Project to compare butterfly communities in certified and non-certified shade coffee plantations. She executed this project from field design to data analysis, and a resulting paper will be submitted in 2011 to the journal Conservation Biology. In addition to these two projects, she has attended two workshops and field courses about butterflies. At the first

course she attended as a student, and at the second she was invited as an organizer, as a result of her experience in organizing logistics and knowledge about Colombian butterflies.

In 2009, she became a master's student at the Department of Entomology and Nematology and McGuire Center for Lepidoptera and Biodiversity, at the Florida Museum of Natural History at the University of Florida. There, she became interested in the butterfly tribe Preponini, an interest which drove her to propose and execute a project in which she aimed to resolve the phylogenetic relationships of the tribe and clarify the number of species in the group. During her master's, she additionally worked as a Research Assistant in the McGuire Center's butterfly collections, where she further improved her knowledge about neotropical butterflies and also curatorial techniques of biological collections. These experiences have maintained her interest in butterfly research, improved her skills in planning and executing research projects, enhanced her abilities for critical thinking and data analysis, and finally have increased her commitment to working with biological collections, a commitment which she hopes to keep developing for many years.