Phylogenetic Relationships and Character Evolution of the Neotropical Butterfly Genus Hamadryas (Nymphalidae: Biblidinae)

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Phylogenetic Relationships and Character Evolution of the Neotropical Butterfly Genus *
Hamadryas* (Nymphalidae: Biblidinae)

A Dissertation

Submitted to the Graduate Faculty of the
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in
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Dedicado a mi familia,
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Abstract

The butterflies in the genus *Hamadryas* are popular and noticeable representatives of the Neotropical Lepidoptera fauna. After a thorough taxonomic revision, 20 species were acknowledged within the genus, however no hypothesis of their phylogenetic relationship was proposed. The present dissertation provides a step further into the understanding of this fascinating group of butterflies not only by proposing the first phylogenetic hypothesis for the genus based on morphological and molecular data, but also by exploring for the first time in a group of butterflies the potential effect of venation associated with a specific behaviour on wing shape. Furthermore, this dissertation provides testable evolutionary hypotheses about the pattern of change for some of their most interesting natural history characters such as sound production and sexual dimorphism. The dissertation is organized in three chapters that can be visualized as manuscripts ready for publication; the first of these being already published (Garzón-Orduña, 2012).

Character change, Monophyly, Synapomorphy, Sound Production, Sexual Dimorphism, Neotropical Lepidoptera, Morphometrics, Phylogenetic Analysis.
Introduction

During the past 30 years phylogenies have become essential tools in almost all research lines of biology. They are used to identify evolutionary significant units (Moritz, 1995), to prioritize conservation areas based on their phylogenetic richness (Vane Wright, 1991), to detect sale of meat from protected species (Baker et al., 1996), to uncover the processes of speciation (Avise, 2000), to predict the effect of biodiversity loss on biomass production (Cadotte et al., 2008) and last but not least, phylogenies are considered the basis to achieve a natural classification (e.g., Hennig, 1968; Farris, 1983).

Studies focusing on the origin of morphological diversity (Willmott, 2003), population dynamics (Brower, 1994) and biogeography of Neotropical butterflies (Wahlberg and Freitas, 2007) greatly benefited from the consideration of the phylogenetic relationships among the focal organisms. In particular, phylogenetic hypotheses have been used to investigate the history of ecological and morphological characters and whether they were originated through convergent evolution or from a single, common event (e.g. Jiggins et al., 2006; Beltran et al., 2007). For example, Sillén-Tullberg (1988) and Tullberg and Hunter (1996) studied the evolution of larval gregariousness in the presence of warning coloration and in the presence of physical or chemical defense against predators. Based on the phylogenies of 10 different groups of butterflies, it was found that the evolution of warning coloration in larvae (an indirect measure of unpalatability) preceded that of gregariousness (Sillén-Tullberg, 1988). The study by Tullberg and Hunter (1996) considered separate origins (to avoid problems with phylogenetic dependence) of warning colors, defense against predators, and gregariousness. That study concluded that gregariousness is more likely to evolve in
lineages with physical or chemical defense and in branches with warning coloration, confirming the results from Sillén-Tullberg (1988). Studies of this type highlight the importance of phylogenetic hypotheses to our understanding of character evolution (see Coddington et al., 1997 for an example outside Lepidoptera).

Given that phylogenies can be used to infer the direction of character change and character associations, this dissertation aimed to propose a phylogeny for Hamadryas that could be used to address important aspects of their biology such as sound production and sexual dimorphism. Hamadryas are noticeable representatives of the Neotropical Lepidoptera fauna and males produce audible sounds during aerial interactions (Otero, 1988). Yack et al. (2000) reported peak frequencies of 13-15 KHz for H. feronia, a much higher sound frequency than that reported for other butterflies e.g. Heliconius cydno (1200 Hz, Medina and Mankin 2004). The clickling sound produced by Hamadryas has fascinated scientist since the 19th century including Darwin (1874) who, after hearing them in Brazil, pondered if sound had a role during courtship. Sound production involves thickened veins at the apical portion of the discal cell of the forewings that strike at the end of the upstroke (Otero, 1990). In addition, the deformation of the wings from a straight to concave form also produces sound (Yack et al., 2000). Not all species in Hamadryas produce sound, and those reported to do so are: H. februa, H. feronia, H. fornas, H. belladonna, H. amphinome, H. epinome, H. ipphthime, H. guatemalena, H. amphichloe, H. arinome (DeVries, 1983; Jenkins, 1983; Otero, 1988; Otero, 1990; Marini-Filho and Benson, 2010). The importance of sound production in this genus lays on the suggestion that sound is used for sexual recognition (Marini-Filho and Benson, 2010), and although it is unknown whether there is interspecific variation in the sound, sound can be under sexual selection in which case it could act as a prezygotic reproductive isolation mechanism.
Sexual dimorphism is a common condition in butterflies, and it varies within and between genera, and also at higher level groups. In *Hamadryas*, strong sexual dimorphism is present in *H. belladonna*, *H. laodamia*, *H. arete*, and *H. velutina*, and involves two features, wing shape and wing color. The remaining species of *Hamadryas* are monomorphic. In the dimorphic species *H. laodamia*, *H. arete*, and *H. velutina*, the sexes can be differentiated by the presence of a submarginal white band and almost straight forewing margin in the females, while the males exhibit a disorganized set of white maculae and a convex forewing margin. Given that the present work led to the proposal of a phylogenetic hypothesis for *Hamadryas*, sexual dimorphism can be investigated within an evolutionary context.

In the first chapter, morphological characters were used to establish if *Hamadryas* corresponded to a monophyletic group and to infer how species were related. This phylogeny was then used to determine the pattern of character change of sound production and sexual dimorphism. Although the morphological data provided a resolved phylogenetic hypothesis, some internal nodes were weakly supported. Therefore, the addition of DNA sequence data offered the opportunity to investigate a source of characters independent from morphology, and to increase the number of characters to be used for phylogeny reconstruction.

It has been suggested that congruence between different types of data is an indirect measure of robustness in phylogenetic analysis (Miyamoto and Fitch, 1995) therefore the inclusion of different sources of evidence allows us to test the support that each type of evidence provides for certain groups (Goloboff et al., 2008). Mitochondrial and nuclear DNA provide information about the phylogenetic relationships among taxa at different
temporal scales (Avise, 2000). In order to cover divergences at different levels, in the second chapter sequencing data from one mitochondrial and four nuclear markers were added to the previous morphological matrix. The molecular data was also analyzed independently and these results were compared to the results with morphology. The new phylogenetic hypothesis based on the combination of morphological and DNA sequence data was used to reassess inferences about the evolution of sound production and to establish the timing of the species divergence inside Hamadryas.

Chapter 3 explores whether changes in venation associated with sound production are correlated with different wing shapes. The production of sound in Hamadryas occurs during aerial interactions, these interactions include spiral flight and chases (Otero, 1988; Yack et al., 2000). Because wing morphology has been shown to be associated with flight behavior (e.g., DeVries et al., 2010), species of Hamadryas that produce sound might possess morphological attributes that are distinctive from others that do not.

Several ecological characters present in Hamadryas await more comprehensive field observations and have yet to be studied under an historical context; for example, unpalatability, aposematism and oviposition behavior. As larvae, Hamadryas feed mainly on Dalechampia and Tragia (DeVries, 1987), both belonging to the family Euphorbiaceae. Euphorbiaceae is widely known to contain a large variety of phytotoxins, examples of this is the presence of triterpenes and steroids in leaves of Dalechampia pernambucensis (Araújo et al., 2007), and terpenoid resins in the flowers of almost all the species in this genus (Armbruster et al., 1997). Chai (1986; 1988) showed that certain species of Hamadryas are unpalatable as adults. In feeding experiments in which different butterfly species were offered to captive rufous-tailed jacamars, H. amphinome and H. laodamia, were rejected by the birds and found to be unpalatable while H. feronia and H. iphthime
were readily consumed. Although *H. amphinome* is similar in dorsal coloration to other *Hamadryas* species (i.e. *H. feronia*), the ventral side exhibits a red and black color pattern. Individuals of *H. feronia* (a species that lives in sympatry with *H. amphinome* but that is palatable and does not exhibit the black and red pattern in the underside) in which the underside coloration pattern of *H. amphinome* had been artificially painted were rejected (Chai, 1988). Furthermore, individuals of *H. amphinome* to which the red-black color pattern was artificially covered were initially attacked and rejected after some handling. Chai’s results show that the ventral pattern in the hindwing of *H. amphinome* is aposematic and that birds interpret as a signal of unpalatability. It is still not clear the source from which species like *H. amphinome* are obtaining their chemical protection. As adults, *Hamadryas* as most Biblidinae, feed mainly on rotten fruit and tree sap (DeVries, 1987; Jenkins, 1983), so the host plant is the most reasonable source. However, it is unclear why not other species that feed on the same host plant are also unpalatable.

Based on descriptions of their life cycle, it is known that the females of some species such as *H. arete, H. februa, H. guatemalena* lay eggs singly (Muyshondt & Muyshondt, 1975a,b; Young, 1974) while the female of other species such as *H. amphinome* and *H. fornax* lay eggs in strings, in which case the larvae exhibits gregarious habits (Muyshondt & Muyshondt, 1975). Laying eggs singly represents the generalized condition outside *Hamadryas*. The modification to lay eggs in strings could be associated to unpalatability and therefore it should be analyzed in together with unpalatability and gregariousness (Sillén-Tullberg, 1988).

Given its small size, its interesting natural history and its taxonomic stability, *Hamadryas* proved to be a valuable focal group for my studies. *Hamadryas*’ interesting ecological traits begged questions about their origin, pattern of variation and the direction of change along
the diversification of the genus, but the lack of a phylogenetic hypothesis had limited the utility of these traits beyond descriptions. The phylogenetic hypothesis proposed here could be used to provide testable hypotheses about the evolution of sound production and sexual dimorphism and, in concert with quantitative data, it also showed an association between venation patterns and wing shape. In the future the proposed phylogenetic hypothesis can be used to study biogeographical patterns regarding the diversification of the genus in the Neotropics or the evolution of the above mentioned natural history characters.
References


Jiggins, C. D., Mallarino, R., Willmott, K., Bermingham 2006 The phylogenetic pattern of speciation and wing pattern change in Neotropical Ithomia butterflies (Lepidoptera: Nymphalidae). Evolution 60:1454-1466.


Chapter 1. Phylogenetic Evidence for Loss of Sound Production and a Shift in Sexual Recognition Signals in *Hamadryas* Butterflies (Nymphalidae: Biblidinae)

Abstract:

The Neotropical butterfly genus *Hamadryas* Hübner comprises 20 species that exhibit an intriguing variation in their natural history traits. Although revised in 1983, no phylogenetic hypothesis was presented: the first phylogenetic hypothesis is estimated here based on 93 characters and including species from the three other genera in the tribe Ageroniini. The phylogeny is used to test the monophyly of the genus, establish the sister group of *Hamadryas* and identify its apomorphies. The tree allows the inference of patterns of character change in sound production and sexual dimorphism. Implied weights show that *Hamadryas* is monophyletic and corroborate *Ectima* Doubleday as a sister genus. Previously suggested subgenera for *Hamadryas* were non-monophyletic, with the exception of the *laodamia* clade, supported by the presence of a complete sterigma. Sound production is inferred to be a derived condition in *Hamadryas* that has been lost in the *laodamia* clade. This, plus the presence of androconial organs and sexual dimorphism in the *laodamia* clade, suggests a shift in sexual recognition signalling. Furthermore, the phylogeny indicates that the colour pattern of males in the *laodamia* clade is novel, supporting a Darwinian origin of sexual dimorphism.
Introduction

The species of *Hamadryas* Hübner are medium-sized Neotropical nymphalids belonging to the subfamily Biblidinae and the tribe Ageroniini. These butterflies are recognized by their distinctive spotted “calico” dorsal wing pattern (Figs. 1 and 2) and erratic flight (e.g., Young & Borkin, 1985). As adults they feed on rotten fruits and typically rest head-down on tree trunks with their wings spread (Fruhstorfer, 1916). *Hamadryas* are known for the audible clicking sound made by the males during flight (Godman & Salvin, 1883; Otero, 1988); hence their common names of “crackers” in English, “matracas” or “rechinadoras” in Spanish and, “estaladeiras” in Portuguese.

Although species of *Hamadryas* are relatively homogenous in their morphology (Jenkins, 1983), they vary in natural history traits such as sound production, and sexual dimorphism. Males of some species can produce sound; in the field, individuals perform aerial interactions accompanied usually by an audible clicking. Production of sound by these butterflies mesmerized many naturalists who were intrigued by the mechanism and location of the sound production organ (Darwin, 1871; Swinton, 1877; Godman & Salvin, 1883; Hampson, 1892; Fruhstorfer, 1916). Sound production involves thickened veins at the distal portion of the forewing discal cell (Fig. 3) with sound produced in two ways: by these veins striking at the end of the upstroke, and also by the deformation of the wing membranes (straight to concave) during flight which allows the production of sound by individual wings (Otero, 1990; Yack *et al.*, 2000). Sexual dimorphism is a common condition in Biblidinae, for example all species of *Epiphile* Doubleday and many species of *Catonephele* Hübner are sexually dimorphic. Although most species of *Hamadryas* are monomorphic, marked sexual dimorphism occurs in a few species (e.g., *H. laodamia* Cramer, 1777; Fig. 3E, F). The variation found in these natural history traits within *Hamadryas* begs the question about their origin and their modifications. A robust and resolved phylogenetic hypothesis will improve our understanding of character evolution and allow the evolutionary biology of *Hamadryas* to be disentangled.

Four generic names have been used for species placed currently in *Hamadryas*. Hübner (1806) described *Hamadryas* for *Papilio amphinome* Linnaeus, and *Ageronia* for *Papilio chloe* Stoll (Hübner, 1819). Lacodaire (1833) erected *Peridromia* for *Papilio arethusa* Cramer, and Felder (1861) created *Amphichlora* from *Papilio feronia* Linnaeus. Based
on wing venation, Godman & Salvin (1883) moved three of the four species originally placed in *Ageronia* by Hübner (1816) to *Peridromia*. In their arrangement *Peridromia* included the type species of *Hamadryas, Papilio amphinome* plus 12 other species. Godman & Salvin (1883) also listed four other species in *Ageronia* (one of which was new), which after this included seven species. The use of the name *Hamadryas* in a taxonomic context was negligible for the first part of the twentieth century (Bouton, 1962; Lamas *et al.*, 1995) and all subsequent authors used the name *Ageronia*. Accordingly, the name *Hamadryas* was not used by Fruhstorfer (1916), and although his species groups followed the same arrangement proposed by Godman & Salvin (1883), he classified all species in two species groups within *Ageronia*, the *Ageronia* and *Peridromia* groups. Jenkins (1983) grouped all species within *Hamadryas*, and synonymized two-thirds of the previously described species and subspecies names. Although Hemming (1967) recognized all four generic names (*Hamadryas, Ageronia, Peridromia* and *Amphichlora*) as valid genera, currently *Hamadryas* is the only valid name used in reference to the 20 species Jenkins maintained inside the genus (Jenkins, 1983; Lamas, 2004), whereas *Ageronia, Peridromia* and *Amphichlora* are treated as junior synonyms (Lamas, 2004).

Given its taxonomic history, some species groups have been maintained inside *Hamadryas*. Based on wing venation and male genitalia, Jenkins (1983) divided the genus into three species groups (vaguely suggested as subgenera) that agree mostly with earlier arrangements by Godman & Salvin (1883) and Fruhstorfer (1916) (left and middle columns in Table 1 respectively). These species groups were (right column in Table 1): the *februa* group which corresponds to the subgenus *Ageronia* (seven species), the *feronia* group equivalent to the subgenus *Hamadryas* (ten species); and the *laodamia* group corresponding to the subgenus *Peridromia* (three species). Although Jenkins (1983) presented the only comprehensive taxonomic study for *Hamadryas* to date, no phylogenetic hypothesis of species relationships was provided and the monophyly of putative species groups was untested.

Although inferring species relationships is the immediate outcome of phylogenetics, the phylogeny also allows the study of ecological characters and their variation under an historical context. Many examples of such studies have come from arthropods (Kuntner & Coddington, 2009; see Miller & Wenzel, 1995 for a review). For example, butterflies exhibit interesting life histories, and placing them in a phylogenetic framework has
improved our understanding of mimicry (Brower, 1995; 1997; Jiggins et al., 2006; Elias et al., 2008; Oliver & Prudic, 2010) and sexual dimorphism (Kunte, 2008).

Here I use morphology and wing colour data to provide the first species-level phylogenetic hypothesis for Hamadryas to: (1) test the monophyly of Hamadryas and identify its apomorphies, (2) infer the sister group of genus, (3) test the validity of suggested subgenera as monophyletic units, and (4) determine if selected natural history traits resulted from common ancestry or convergent evolution.

Material and Methods

Taxon Sampling

This study includes 19 of the 20 Hamadryas species. Except for the female of H. belladonna Bates, and female H. albicornis Staudinger, male and female specimens were obtained from the following collections: The Milwaukee Public Museum (MPM); Florida Museum of Natural History McGuire Center for Lepidoptera and Biodiversity (FLMNH); American Museum of Natural History (AMNH); Smithsonian Institution, National Museum of Natural History (NMNH); DeVries Collection (PJD); and Natural History Museum of Los Angeles County (LACM). Taxonomic determinations followed Jenkins' (1983) revision. Examined specimens are listed in Table S1.

Batesia hypochlora Felder & Felder, Panacea prola Doubleday, P. divalis Bates (sensu Hill et al., 2002), and all the four species of Ectima Doubleday were used as outgroups. These three genera together with Hamadryas comprise the tribe Ageroninii, which is considered monophyletic based on the most recent phylogeny of Nymphalidae (Wahlberg et al., 2009). Batesia hypochlora was used to root the tree; this results in Panacea Godman & Salvin appearing as sister group of Hamadryas plus Ectima; however, a sister relationship between Batesia and Panacea has been shown previously (Hill et al., 2002; Wahlberg et al. 2009).

Characters

Leg and genitalia dissections were made following standard procedures using a 10% KOH solution, and were kept in a 3:1 solution of 70% ethanol and glycerol. Examination of characters and drawings were conducted using a stereo-microscope equipped with a
camera lucida. Terminology for external morphology and genitalia follows Kristensen (2003), and homologies of wing pattern elements (Fig. 1E) follow Nijhout (1991). The matrix includes 93 characters (88 informative): characters 1–15 refer to venation (Figs. 1 and 3), wing shape and androconia; characters 16–49 describe wing colour (Figs. 1 and 2), characters 50–92 concern male and female genitalia (Figs. 4, 5 and 6) and one character (char. 93) describes oviposition patterns. Information about the patterns of oviposition was taken from the literature. The character list, matrix and literature records of the patterns of oviposition are in Tables S2, S3 and S4 respectively. Abbreviations used throughout the text are: forewing (FW), dorsal forewing (DFW), ventral forewing (VFW), hindwing (HW), dorsal hindwing (DHW) and ventral hindwing (VHW).

**Cladistic analysis**

The matrix was analyzed under Equal Weights (hereafter EW) and Implied Weights (hereafter IW) (Goloboff, 1993) in TNT 1.1 (Goloboff *et al*., 2008a). EW remains as the traditional approach used in systematics and as such in this study EW was used for exploratory purposes. Under IW I explored a wide range of IW concavity values ($k=1$–100). Regardless of the weighting scheme, tree searches included 500 replicates of Random Addition Sequence, holding 10 trees per replication, TBR for branch swapping and 90 iterations of Ratchet (Nixon, 1999). After the search, branches of length zero were collapsed and duplicate trees discarded (coll rule 4; condense; unique;). All characters were considered unordered, although making multistate characters additive was explored. When dealing with polymorphisms TNT treats the states as either/or and thus only adds a step when the ancestral state is not included within the polymorphism.

Three measures of group support were calculated: the Absolute Bremer support (ABS, Bremer, 1994) which measures the total amount of favourable evidence, the Relative Bremer support (RFD, Goloboff & Farris, 2001) which provides an estimate based on the amount of evidence in favour and against each node, and Symmetric Resampling (SR) or Symmetrical Jackknife which uses the same probability for character deletion and character inclusion thus eliminating the influence of weighting against homoplasy (Goloboff *et al*., 2003).

The Bremer support values (ABS and RFD) were calculated by retaining up to 3000 trees with different suboptimal values from 0.2–4 steps longer than the optimal, and
running 500 replications of Wagner while keeping all the trees of each replication. The
relative Bremer support is calculated as the Relative Fit Difference, RFD, between two
trees. If RFD is 0, the amount of evidence supporting the group equals the amount of
evidence contradicting the group, and if RFD is 1, the group is entirely uncontradicted.
SR was conducted by generating 1000 pseudo-replicates of the matrix and the results
expressed in differences of group frequencies, GC (for Group present/Contradictory)
values instead of straight group frequencies. Using GC provides the advantage of
knowing the support of groups with low resample values (with less than 50%), which are
otherwise collapsed under the standard calculation of frequencies (Goloboff et al., 2003).
GC represents the difference between the frequency of the group in question and the
frequency of its most frequent contradictory group (Goloboff et al., 2003). A GC value of
-1 indicates maximum contradiction, GC=0 indicates indifference and GC=1 represents
complete support.

The trees were explored and edited with MacClade 4.08 (Maddison & Maddison, 2005).
MacClade was used also to reconstruct the ancestral state of selected natural history
traits and to optimize the minimum number of changes for these traits under traditional
parsimony.

Results
EW found 10 equally parsimonious trees of 260 steps, the strict consensus of which is
shown in Figure 7A. In contrast, IW concavity values from \( k=2 \) to \( k=100 \) or higher found
one single most parsimonious tree (MPT) (Fig. 7B). This tree, together with the
optimization of all the unambiguous transformations and branch support, is shown in
Figure 8. This tree corresponds to one of the 10 MPTs under equal weights. Making
multistate characters additive had no effect on the topology under either analysis. Here
the optimal solution found by IW is preferred because IW assigns weights to characters
according to their reliability along the search of the topology (weighting against their
homoplasy) instead of assuming a priori that all characters bear the same importance as
evidence of phylogenetic relationships. Furthermore, IW has been shown empirically to
produce more stable hypotheses than EW and to improve jackknife frequencies,
especially in morphological data (Goloboff et al., 2008b). Below I describe the optimal
tree under IW and present the apomorphies for selected groups of taxa (nodes A–E in
Figure 8). The names used throughout the text for some groups of species are not
intended to have any taxonomic value; they are used only to facilitate the description of the results.

**The position of Hamadryas within the Ageroniini**

*Ectima* is the sister group of *Hamadryas* (see also Wahlberg *et al.*, 2009), a relationship supported by three synapomorphies and two homoplasious characters (1:1 and 81:1 Fig. 4P; RFD=0.74, ABS=0.37, GC=0.97). The synapomorphies of *Ectima* plus *Hamadryas* are: internal side of the base of valva with a projection that smoothes into an internal folding (char. 77:1 Fig. 5M), juxta slightly sclerotized and not projected posteriorly (char. 79:1 Fig. 5N), and the presence of signa (91:1 Fig. 6H).

**Monophyly and apomorphies of Hamadryas**

*Hamadryas* is monophyletic as indicated by three apomorphies and four homoplastic character changes (chars. 7:2; 32:1 Fig. 1F, Fig. 2C-E; 70:0). The apomorphies of *Hamadryas* are: DFW spot at R3, R4 white (char. 26:0 Fig. 2B–F), hypandrium with lateral edges projected into elongated rami (char. 51:1 Fig. 4A, C, D), and ductus seminalis connecting very near to the corpus bursa (char. 89:0 Fig. 6E). The monophyly of *Hamadryas* has a RFD of 0.16, an ABS of 0.17 and a GC value of 0.68.

**Species relationships**

In *Hamadryas* the first split in the tree corresponds to a single species branch (Fig. 8 branch labeled A): *H. atlantis* Bates. This is one of the most distinctive species in the genus, as demonstrated by the considerable number of character changes on its branch. Two colour pattern and three genitalia characters separate *H. atlantis* from other *Hamadryas*. In *H. atlantis* the DFW band inside the discal cell between elements *c* and *d* is green as in species of *Panacea* (20:3), and the the antrum is entirely membranous (88:0) unlike all other species of *Hamadryas* in which the dorsal side of the antrum has a small sclerotized plate (88:1 Fig. 6E).

After the split of *H. atlantis* there is a clade composed of *H. chloe* and *H. albicornis* (Fig. 8 branch labeled B). This clade is supported by two apomorphies, three homoplastic character changes and has a RFD of 0.27, an ABS of 0.22, and a GC of 0.89. The apomorphies are: HW CuA₂ vein noticeably longer than CuA₁ and 1A+2A (15:1 Fig. 2A), and DHW pattern element *e* formed by red scales (36:0 Fig. 2A–D). *Hamadryas alicia*
(Fig. 8 branch labeled C) is the second single species branch in the topology and appears as the sister taxon to the remaining species. This relationship is supported by four apomorphies: a vestigial spot comprised by blue scales in the proximal portion of the DFW band between pattern elements c and d (22:1 Fig. 1F; Fig. 2B–F), the presence of DHW pattern element k (48:1 Fig. 1F; Fig. 2C, E), the presence of short and fine setae along the rami (59:0 Fig. 4G which are long and thick in H. atlantis, H. chloe, and H. albicornis), and a triangular base of the saccus (82:1 Fig. 5N; 82:0 Fig. 5O), in contrast to a squared base present in H. atlantis, H. chloe, and H. albicornis. The most important characters that differentiate H. alicia from the rest of the Hamadryas are: colour of the distal band to pattern element e which is blue in H. alicia (31:0 Fig. 3A; 31:1 Fig. 3C) and white in the rest of the species, the posterior edge of the hypandrium is short in H. alicia (52:0 Fig. 4A; 52:1 Fig. 4F), which is extended considerably in the rest of the species; and finally the shape of the ductus bursa, which is shortened in H. alicia (90:0 Fig. 6G) and pear-shaped or elongated in the rest of Hamadryas (Fig. 6G, H).

Clade D (Fig. 8 branch labeled D) is comprised by H. februa, H. amphichloe Boisduval, H. glauconome Bates, and H. julitta Fruhstorfer. This clade is supported by two apomorphies and three homoplastic character changes (7:0, 29:0, 30:1). The apomorphies are presence of an ocellus on the dorsal side of forewing in cell R₃ (27:1 Fig. 2F, Fig. 2B, D, E), and internal ring of the pattern element h white (42:2 Fig. 1F). This is a well-supported clade (RFD= 0.55, ABS= 0.21, and GC=0.83). Clade E is supported by two apomorphies and three homoplastic character changes (5:1, 6:1, 21:1); four are structural and one pertains to wing colour. The synapomorphies are: forewing veins R and Rs₁ stalked in males (3:1 Fig. 1A, C), and FW veins Rs₁, Rs₂+Rs₃+Rs₄, Rs₃+Rs₄+M₁ and M₁-M₂ swollen in the males (4:1 Fig. 3B), this last character changes in the laodamia clade (below). Within Hamadryas, the transformation of M₁ arising from the same point as Rs₂+₃+₄ (5:0 Fig. 3A) to M₁ arising at midpoint between Rs₂+₃+₄ and M₂ (5:1 Fig. 3B) is unique and constant to this clade. However, this character does not appear as a unique apomorphy because state ‘1’ is present also in Batesia hypochlora. Clade E has low support (RFD=0.03, ABS=0.02, GC=0.08). This group includes all the species in Hamadryas that possess all or some of the venation components for sound production. Nested within clade E there is the only species group proposed by Jenkins (1983) that was found to be monophyletic, the laodamia clade Together with H. laodamia this clade also includes H. arete Doubleday, and H. velutina
Bates. It is supported by ten apomorphies (4:2, 8:1, 9:2, 10:1, 13:1, 28:3, 43:1, 45:4, 53:0, 87:1) and nine homoplastic character changes; it has a RBS of 99, and SR of 100. Three of the most interesting apomorphies of this clade are: FW veins Rs₁-Rs₂+Rs₃+Rs₄, R₃+R₄-M₁ and M₁-M₂ thick but not swollen in the males (4:2 Fig. 3C), ostium bursa extending into the seventh sternite (87:1 Fig. 6C; 87:0 Fig. 6B) and the presence of androconial scales (10:1 Fig. 1C).

Pattern of character change: sound production
The production of sound involves four venation characters (3–6) present in the FW of males (Figs. 1 and 3). When the minimum number of unambiguous transformations of these characters is traced onto the topology (Table 2), only character six requires an extra step. Based on the distribution of these four characters, sound production evolved once and was lost once at the node subtending *H. laodamia* and its relatives. In Figure 7B dark boxes show the species with venation suitable for sound production.

Pattern of character change: sexual dimorphism
Here, species were considered sexually dimorphic when the differences between male and female lead to an unambiguous visual sex determination. Sexual dimorphism (SD) in *Hamadryas* is obvious and affects two features: wing shape and colour pattern. In some species males and females differ in wing shape only, in others the organization of the white bands in the DFW (maculae) varies between the sexes, and there are also some species in which SD involves changes in both features. Because wing shape and presence/absence of DFW maculae are encoded in the data matrix, separating presence or absence of sexual dimorphism would be redundant. Figure 7B represents the variation of SD across the topology in wing shape (third column) and organization of the DFW maculae (right column). Forewing shape is sexually dimorphic in two instances: in *Ectima* (except for *E. iona* Doubleday) and in the laodamia clade. In both cases the FW distal margin is modified into a convex shape departing from the more generalized form of *Hamadryas* in which males and females both show a slightly concave (almost straight) wing margin. SD of the wing margin in *Ectima* and the laodamia clade differs, however, since in *Ectima* the females exhibit a concave margin whereas in the laodamia clade this feature is found in the male sex. The pattern of organization in the DFW maculae is sexually dimorphic in *H. belladonna* and in the laodamia clade. In the males of *H. belladonna* the DFW maculae are
disjointed, which is the generalized condition in the other (monomorphic) *Hamadryas*, whereas the DFW maculae in the males of the laodamia clade are reduced to small iridescent blue spots. The females of *H. amphinome*, *H. arinome* Lucas, and *H. belladonna* have DFW elongated maculae that are almost organized into a diagonal postmedial band, in the females of the laodamia clade the DFW maculae are fully aligned to form a similar band.

**Discussion**

*Species groups within Hamadryas*

Although the phylogenetic hypothesis proposed here supports some of the species affinities suggested by Jenkins (1983), the only species group supported is the laodamia clade (*Peridromia*). This clade is the most morphologically distinctive group of *Hamadryas*, as these three species are the only ones in the genus that exhibit conspicuous sexual dimorphism, male scent organs, and also unpalatability at least in *H. laodamia* (Chai, 1990). Members of this group have the gnathos conspicuously elongated, and these are the only species that posses a complete female sterigma (with lamellae ante and postvaginalis), deviating noticeably from the rest of the genus.

Attempts to separate the species now grouped in *Hamadryas* into different genera, subgenera or species groups were based initially in the variation of one venation character (char. 3, Fig. 1A, B; Godman & Salvin, 1883; Fruhstorfer, 1916) and later, on the combination of a few genitalia and venation characters (Jenkins, 1983). All abovementioned authors noticed that males of some species exhibited a different state of character 3 than their conspecific females. Accordingly, Godman & Salvin (1883) included in *Ageronia* all the species in which both male and female have the FW veins R and Rs$_1$ separated, and placed the species in which the males exhibit veins R and Rs$_1$ stalked in *Peridromia*. Fruhstorfer (1916) followed the same organization but, unlike Godman & Salvin (1883), he classified all species under *Ageronia*, which was then partitioned in species groups.

Although the diagnosis of *Peridromia sensu* Godman & Salvin (1883) was correct, *H. alicia* was included erroneously in this group. *Hamadryas alicia* does not exhibit the venation character used to define *Peridromia* and it does not share the most recent
common ancestor of the other members of *Peridromia* (Fig. 8). Jenkins (1983) split *Peridromia* into the *feronia* and *laodamia* species groups due to the morphological departure of *H. laodamia* and relatives, but the diagnosis of the *feronia* group lacked unique characters. Furthermore, he did not consider that the two groups could be nested (the *laodamia* inside the *feronia* species group as has been shown here). Granting them the same rank (e.g. subgenera), would render the *feronia* species group non-monophyletic (Fig. 8). Finally, this study found no apomorphies to support a common origin of the species placed in *Ageronia sensu* Godman & Salvin (1883). Moreover, the main character state used to define *Ageronia* is sympleiomorphous.

The data set in this study included a set of different character systems. Venation and other features of the wing provided 15 characters, wing colour included 33 characters, and genitalia accounted for 42 characters; three data partitions. Not one of these partitions alone included enough informative characters to resolve the relationships among species or species groups (results not shown).

*Sound production*

Published records suggest that 10 species produce cracking sounds, however only eight species have all the venation components required for sound production (left-most column in Fig. 7B). Otero (1990) noted that the sound production venation was present in *H. feronia*, but absent in *H. februa*. However, Monge-Nájera & Hernandez (1991) and Jenkins (1983) reported individuals of *H. februa* producing sounds in the field. Recently Marini-Filho & Benson (2010) showed that *H. februa* does not produce sound based on hand tests (Otero, 1990) and observations of caged individuals. Jenkins (1983) also reported sound production in *H. amphichloe* but this species lacks the suitable venation. Given the fast and erratic flight of *Hamadryas* and that species that do not produce sound fly together with those that do, field observations could be based on erroneous identifications. However, a so-far undiscovered mechanism of sound production in these species cannot be eliminated.

The context in which sound production occurs varies within butterflies. For example, Kane (1982) described acoustic signals in *Pharneuptychia nr. pharnabazos* in the presence of mates and or food. More recently, female *Heliconius cydno* were observed producing audible wing clicks during interactions with conspecifics during the day and at
roosting time (Medina Hay-Roe & Mankin, 2004). After some debate about the purpose of sound in *Hamadryas* (Darwin, 1871; Monge-Nájera *et al.*, 1998), three studies seem to have led to a consensus supporting the use of sound as an aid in sexual recognition (Otero, 1988, 1990; Marini-Filho & Benson, 2010). All behavioral studies agree that sound occurs during aerial encounters. These encounters can occur between two (or more) males or between a male and a female, and in both cases sound is produced. However, in male-male interactions sound is produced continually, while in male-female interactions sound is produced only during the initial phase of the pursuit (Marini-Filho & Benson, 2010). This observation led Marini-Filho & Benson (2010) to suggest that in male-female encounters, once sexual recognition occurs there is a shift of behaviour from sexual recognition to courtship, which is accompanied by the cessation of sound production.

The pattern of species relationships presented here has interesting implications for the evolution of sound production in *Hamadryas*. First, it suggests that sound production is a derived condition that evolved only once, but it also suggests that sound production was lost in the laodamia clade. The loss of sound production is accompanied by two other transformations: the presence of male scent organs (not present in any other species in the genus) and the presence of sexual dimorphism in wing shape and colour pattern (Fig. 7B). The congruence of these characters suggests that sound production cues have been replaced by visual and olfactory signals in *H. laodamia, H. arete* and *H. velutina*. If sound production is used as an aid for sexual recognition as has been suggested, sexual dimorphism could have replaced sound production in the species of the laodamia clade (Marini-Filho & Benson, 2010). The presence of androconial scales could further facilitate species recognition. Given that the three transformations occur at the node of *H. laodamia* and its relatives, it is impossible to know the sequence of the transformations, but the potential association of these three characters deserves further investigation. This result reinforces the importance of knowledge of phylogenetic (cladistic) relationships in the study of character evolution, without which unexpected losses cannot be assessed reliably.

**Sexual dimorphism**

The main hypotheses about the origin of sexual dimorphism (SD) date back to Darwin (1871) and Wallace (1889), with a third and more recent hypothesis proposed by
Silberglied (1984). Silberglied suggested that since brilliant colour patterns of males could work as signals of communication (e.g. recognition of other males, advertisement of his own sex), SD could originate from interactions between males. Although it would have been interesting to discuss the SD of *Hamadryas* in terms of Silberglied’s hypothesis, we know very little about the aerial interactions between males and almost nothing about these interactions in the sexually dimorphic species. Therefore I restrict the discussion to Wallace’s and Darwin’s hypotheses, which seem more appropriate given our data.

According to Darwin’s view, SD was the result of sexual selection based on female preference for specific male attributes. Over time female choice causes the deviation of male phenotype from the ancestral pattern. According to Wallace, however, SD could also result from natural selection acting on female traits. He suggested that females could evolve a protective coloration (camouflage or mimicry) and would therefore deviate from the ancestral condition to obtain a fitness benefit. Both hypotheses have received some support from butterflies, and are not mutually exclusive. The origin of SD through sexual selection has been demonstrated in *Bicyclus anynana* (Robertson & Monteiro, 2005) and in *Hypolimnas bolina* (Kemp, 2007) and SD due to female-limited mimicry was shown in a number of *Papilio* species (Kunte, 2008). The phylogeny together with the distribution of SD in *Hamadryas* suggests that the colour pattern of females in the laodamia clade is ancestral (although modified towards the alignment of the DFW maculae into an almost straight diagonal band), and that the male colour pattern is the novel condition. Although this study does not provide direct evidence of sexual selection, these results support Darwin’s model for the origin of SD.

**Closing remarks**

Although the optimal tree presented here was resilient to different strengths of the concavity function, overall the topology has very low support. The GC values show moderate support for some internal nodes and for some species groups, but it shows indifference particularly for clade E. Although *Hamadryas* exhibits variation in ecological traits, otherwise it is rather uniform in terms of structures such as genitalia. This translates into a lack of informative characters, reflected here by low values of ABS. Phylogenetic studies of *Hamadryas* could be improved in the following ways: (1) with a
comparative morphological study of early stages, in the hope that they would provide additional informative characters as has been the case for other groups (e.g. Penz & Peggie, 2003), and (2) by the addition of molecular markers which will offer a larger source of characters.

Based on the phylogeny, my study identifies a shift in the signals used for sexual recognition inside *Hamadryas* (from sound to sexual dimorphism and androconial scales), but the aids used for sexual recognition prior to the appearance of sound remain undetermined. Although aerial interactions (in the form of spiral flights) are present in all species, there is some evidence suggesting that these interactions might not be so significant in the sexual recognition for the species that produce sound (D. Otero personal communication). More field observations and cage experiment will be crucial to determine if this is the case.
Acknowledgments

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References


Agronomia, Universidad Central de Venezuela, Maracay.


Table 1.1 Composition of Hamadryas species groups suggested in the past.

<table>
<thead>
<tr>
<th>Hamadryas Species groups</th>
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Laodamia Species Group:
laodamia
arete
velutina
Table 1.2 Evidence for clade E. The theoretical minimum number of steps is shown together with the number of extra steps in implied weights (IW) topology. Synapomorphies are in bold case. The numbers of extra steps were calculated inside *Hamadryas* only and were obtained from the unambiguous optimization of each character using MacClade 4.08 (Maddison & Maddison, 2005).

<table>
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Appendix 1.1 Examined material with repository collection in parentheses. Dissected specimens are labeled with an asterisk and dissection numbers are provided. Abbreviations: M: male; F: female; AMNH: American Museum of Natural History; FLMNH: Florida Museum of Natural History, McGuire Center for Lepidoptera and biodiversity; LACM: Los Angeles County Museum; MPM: Milwaukee Public Museum; PJD: Phil DeVries Private Collection; NMNH: Smithsonian Institute National Museum of Natural History.

Hamadryas:

**H. albicornis**

**H. alicia**
1F*. Solimões, IX. Dissection 07-34 by I. Garzón (UFLMNH).

**H. amphichloe**

**H. amphinome**

**H. arete**
1M. Brazil, São Paulo (MPM); 1M*. Brazil, São Paulo. Araçatuba Dissection 07-32 by I. Garzón (UFLMNH).

**H. arinome**
1M*. Peru, Madre de Dios, Los amigos Research Center, 250m. Dissection 09-17 by I. Garzón (PJD); 1M*. Peru, South America. Dissection 10-16 by I. Garzón (MPM); 1M*. Ecuador, Sucumbios, Garza Cocha. Dissection 98-22 by R. Hill (PJD).

**H. atlantis**

**H. belladonna**

**H. chloe**

**H. epinome**

**H. februa**

**H. feronia**

**H. fornax**

**H. guatemalena**

**H. glauconome**

**H. iphthime**
1F*. Mexico, Chiapas. Dissection 07-36 by I. Garzón (MPM).

**H. julitta**

**H. laodamia**

**H. velutina**

Out groups:

**Batesia hypochlora**
1F*. Ecuador, Sucumbios, Garza Cocha. Dissection 00-01 by R. Hill (PJD).

**Ectima erycinoideas**

**E. iona**

**E. lirides**

**E. thecla**

**Panacea prola**

**P. divalis**
Appendix 1.2 Character List.

1. White scales on antennae: (0) absent, (1) present.
2. Female, foreleg tibial spines: (0) absent, (1) present.

Venation and wings characters

3. Male FW, veins R and Rs\(_1\) (Jenkins, 1983): (0) separated, (1) share a common stem. Fig. 2 A–C.
   Note: Regardless of the species females have R and Rs\(_1\) arising separately, however a few females of *H. iphthime* and *H. formax* had R and Rs\(_1\) arising from a single point.

4. In males FW, Rs\(_1\)-Rs\(_2\), Rs\(_2\)-M\(_1\), and M\(_1\)-M\(_2\): (0) same width as other veins, (1) fully swollen, (2) thick but not swollen. Fig. 1 A–C.
   Note: This corresponds to the sound production “organ” described by Otero (1990). The females of *H. epinome*, *H. iphthime* and *H. formax* have thick veins but not swollen. State 2 implies that the veins are thicker than in the species scored with state 0 but they are not swollen as in the species scored with state 1.

5. In males, FW vein M\(_1\): (0) arising from the same point as Rs\(_2\), (1) arising at mid point length between Rs\(_2\) and M\(_2\). Fig. 1 A–C.
   Note: all species were sexually monomorphic for this character, except for *H. formax* in which there are females where M\(_1\) and Rs\(_2\) arise from the same point.

6. In males, FW vein M\(_2\): (0) mildly curved towards M\(_3\), (1) conspicuously curved towards M\(_3\). Fig. 1 A–C.
   Note: There are a few modifications associated with the presence of the sound production organ, such us the M\(_1\) arising from a different point than Rs and a bowed M\(_2\), however this last modification is not present in *H. arete*, *H. laodamia* and *H. velutina*. Generally, females have a straight M\(_2\) regardless of the species (females of *H. epinome* have a bowed M\(_2\) and females of *H. iphthime* and *H. formax* are polymorphic for this character).

7. In males FW, crossvein M\(_2\)-M\(_3\) (Jenkins, 1983): (0) joins the M\(_3\)-CuA\(_1\) fork, (1) joins the Cu\(_1\)-Cu\(_2\) cross vein, (2) joins M\(_3\). Fig. 2 A, B.
   Note: *H. iphthime* and *H. epinome* are sexually dimorphic for this character, with the females having state 0.

8. In males, FW anal margin (modified from Jenkins, 1983): (0) straight, (1) convex. Fig. 2 A, C.

9. Males and females distal margin of FW: (0) mildly concave, (1) sexually dimorphic, females’ margin convex, males’ margin almost straight, (2) sexually dimorphic, females’ margin almost straight and males’ margin convex. Fig. 2 C.

10. Androconial scales on VFW surface from Cu\(_2\) to the anal margin: (0) absent, (1) present. Fig. 2 C.

11. VFW extension of the androconial scale patch: (0) patch on and above Cu\(_2\), (1) patch does not reach Cu\(_2\). Fig. 2 C.

12. Androconial scales in the costal margin of DHW surface: (0) absent, (1) present. Fig. 2 D.
13. Androconial scales on DHW surface from Sc until M₂: (0) absent, (1) present. Fig. 2 D.
14. Colour of androconial scales on DHW surface from Sc until M₂: (0) dark brown/black, (1) light brown.
15. HW Cu₂ vein: (0) as long as Cu₁, (1) noticeable longer than Cu₁ and 1A+2A. Fig. 3 A, B.
16. VHW, predominant coloration: (0) black, (1) red/orange, (2) brick, (3) yellow, (4) mustard, (5) gold-brown, (6) flax, (7) beige, (8) chalk. Fig. 1 G; Fig. 3 A–D, F. Note: the ventral colour of *P. divalis* is not as bright as the one of *P. prola*, however they are coded with the same state since there is not doubt about the monophyly of *Panacea* and to avoid adding homoplasy to the character.
17. When VHW predominant coloration is gold-brown, flax, beige or chalk, pattern elements: (0) opaque brown, (1) copper/iridescent brown. Fig. 2 G; Fig. 2 A, B.
18. Ventral coloration of thorax: (0) red, (1) mustard, (2) brown, (3) flax, (4) chalk.
19. When ventral colour of thorax is red: (0) completely red, (1) red patches on a brown background.

*The next characters are based on Nijhout’s (1991: fig. 2.17) nomenclature, and refer to the dorsal wing pattern.*

20. Colour of scales in DFW band inside discal cell between elements c and d: (0) red to brick, (1) orange, (2) black (indistinguishable from background), (3) green, (4) brown. Fig. 2 G; Fig. 3 A–F. Note: although there is variation in the tone of this band due to age and some intraspecific variation (*H. iphtime* is coded polymorphic), very few individuals of *H. feronia*, *H. guatemalena* or *H. fornax* had an orange band, and no individuals of *H. februa*, *H. epinome*, *H. glauconome* and *H. amphichloe* had a red band. This character was coded in the females for *H. glauconome* and *H. laodamia*, *H. arete* and *H. velutina*.

21. In species where DFW band inside discal cell between elements c and d is distinguishable from background, this band: (0) reaches the coastal margin, (1) vestigial, the band does not reach the coastal margin of the discal cell. Fig. 2 G; Fig. 3 A, B, D.
22. Spot of blue scales in the proximal portion of the DFW band between elements c and d: (0) absent, (1)vestigial, (2) well developed. Fig. 2 F; Fig. 3 B–F.
23. DFW band between elements c and d bordered by: (0) black/dark scales, (1) blue scales. Fig. 3 A, D.
24. In females, colour of DFW discal cell band between pattern elements d and e: (0) white, (1) blue, (2) iridescent green, (3) beige/light brown. Fig. 2 F; Fig. 3 C–E. Note: individuals of *H. chloe* vary from a whiter band to a darker band, but there are still white scales inside the band. This band also varies in *H. amphinome* from a light blue to an iridescent green band, however blue is the most common colour. *H. glauconome* this band is brown, although it is covered by scattered gray scales.

25. In females, DFW pattern element e: (0) composed of dark scales, (1) composed of (brown) light scales, (2) composed of blue scales, (3) composed of red scales. Fig. 2 F; Fig. 3 A–E.
26. DFW band/spot at R₃, R₄: (0) white, (1) blue, (2) absent. Fig. 3 B–F.
27. In females, DFW ocellus at R₃, R₄: (0) absent, (1) present. Fig. 2 F, G; Fig. 3 B, D, E.
28. In females, DFW R₄, R₅: (0) absent, (1) vestigial ocellus, (2) ocellus fully present, (3) only pupil visible. Fig. 2 F, G; Fig. 3 A, B.
29. In females DFW band/spot between R₅ and M₁: (0) cream, (1) white, (2) blue, (3) absent. Fig. 3 B, D, F.
30. DFW in females, width of space between margin of the discal cell and m₁-m₂ band: (0) narrow, (1) wide, (2) no space, reaching distal margin of discal cell. Fig. 2 F; Fig. 3 B–E.
31. In females, DFW M₂ cell. Colour of next distal band to pattern element e: (0) blue, (1) white, (2) brown, (3) green. Fig. 3 A, C.
32. DFW in female’s M₂ cell. Shape of band distal to pattern element e: (0) entire, elongated towards distal margin of wing and pattern element f not visible, (1) split by pattern element f, proximal band oval. Fig. 2 F; Fig. 3 C–E.
33. In species where DFW band in the M₂ cell is entire. Inner margin of postmedian diagonal band: (0) straight, (1) irregular. Fig. 3 E.
34. In species where DFW band at M₂ is entire. Postmedian diagonal band extends: (0) from R₃+R₄+R₅ to Cu₄₂, (1) from coastal margin to Cu₄₂, (2) from R₃+R₄+R₅ to Cu₄₁, (3) from coastal margin to 1A+2A. Fig. 3 C, E.
35. DHW in females, pattern element d: (0) center of d composed of light scales, (1) composed of dark scales only, (2) center of d composed of red scales. Fig. 2 F; Fig. 3 A–D.
36. DHW in females, colour of the distal edge of discal cell (pattern element e): (0) red, (1) light, (2) dark brown. Fig. 1 F; Fig. 3 A–D.
37. DHW in females, proximal portion of f: (0) scattered, (1) continuous. Fig. 3 A, B.
38. DHW shape of pattern element f: (0) not continuous across the wing (broken), (1) continuous across the wing and narrowed into an almost straight line, (2) slightly continuous across the wing but dislocated (irregular line). Fig. 2 F; Fig. 3 A, C, D.
39. When adjacent edges do not match, width of DHW pattern element f: (0) wide, (1) intermediate, (2) thin. Fig. 2 F; Fig. 3 B–D.
   Note: next characters about the border ocelli were coded based on ocellus number 6 in Fig. 2 E.
40. DHW pattern element h (border ocelli): (0) absent, (1) present. Fig. 3 A, C–E.
   Note: Although Panacea does have border ocelli, the ocelli are reduced and do not exhibit as many elements as do the ocelli in Ectima or Hamadryas. This is even more obvious in P. prola and P. divalis in which the ocelli are vestigial and therefore I had to code the next characters as inapplicable.
41. DHW pattern element h (border ocelli): (0) with internal ring, (1) without internal ring. Fig. 2 F; Fig 3 A–D.
   Note: H. feronia has a very small almost vestigial internal ring only visible through the stereoscope.
42. DHW. Pattern element h (border ocelli) in species with internal ring. Colour of internal ring: (0) greenish, (1) blue, (2) white. Fig. 2 F; Fig. 3 A.
43. Most external ring of DHW pattern element h: (0) as a complete circle, (1) as an incomplete circle. Fig. 3 C–F.
44. DHW of males. In species where external ring of pattern element h is incomplete, proximal portion of the external ring present only in ocellus: (0) 4-5-6, (1) 5-6. Fig. 3 F.
Composition of DHW ocellus 2 (Rs cell): (0) complete ocellus, (1) blurred ocellus, (2) only the most external ring present (empty ocellus), (3) external ring and a pupil present, (4) pupil only. Fig. 1 G; Fig. 3 A-D, F.

Composition of DHW ocellus 4 (M3 cell): (0) ocellus present, (1) empty ocellus. Fig. 1 F; Fig. 3 A.

DHW ocellus 7 (Cu2 cell): (0) pupil present, (1) empty ocellus. Fig. 2 G; Fig. 3 A–D.

DHW pattern element k: (0) absent, (1) present. Fig. 2 F; Fig. 3 C, E.

DHW j pattern element: (0) both lines continuous, (1) both lines broken, (2) only one line visible and broken, (3) two lines, joined in the middle of the cell. Fig. 2 F; Fig. 3 A, C, D, F.

Note: Element j is composed of two lines of scales in some species or only one broad line in others. There is some intraspecific variation in this character but the differences between species hold. *H. laodamia*, *H. velutina* and *H. arete* have a wide broken band at the edges of each cell, I believe that could be the result of filling up the empty space between both lines present in *H. fornax* and hence my codification.

Hypandrium
The term hypandrium has been traditionally used in reference to the modified 8th sternite present in the males of Biblidinae. It is in this same sense that I am using it in this paper.

Anterior edge of hypandrium: (0) slightly extended anteriorly, (1) conspicuously extended anteriorly reaching internally almost the mid length of the abdomen. Fig. 4 B, C, D.

Lateral edges of posterior margin of hypandrium: (0) composed of tooth-like serrations, (1) projected into elongated rami. Fig. 4 A, C, D.

In species with rami. In ventral view, extension of posterior edge of hypandrium: (0) to a small degree from the base of rami, (1) considerably beyond the base of rami. Fig. 4 A, F.

In species with hypandrium extended beyond the base of rami, posterior margin of hypandrium: (0) rounded, (1) squared. Fig. 4 E-G.

When squared, posterior margin of hypandrium: (0) moderately straight, (1) conspicuously irregular (wavy). Fig. 4 F-H.

Macrochaete setae on hypandrium: (0) absent, (1) present. Fig. 4 F, G.

Note: Jenkins (1983) refers to these setae as spines; these setae arise from a socket, and are pointy, heavily sclerotized and restricted to the hypandrium. I am calling them macrochaetes to distinguish them from other type of setae, although whether they are mechanoreceptors or not is unknown.

Location of macrochaete setae on hypandrium: (0) present only at the base of rami, (1) present on lateroposterior margin and some setae reaching the posterior margin of the sternite. Fig. 4 E-H.

Macrochaete setae at the posterior margin of hypandrium (base of rami): (0) as long as the macrochaete at the tip of rami, (1) smaller. Fig. 4 E, G.

Setae on lateral surface of rami: (0) absent, (1) present but few, (2) present in large numbers. Fig. 4 A, G.

When present, setae on external side of rami: (0) short and fine, (1) long and thick. Fig. 4 A.

Macrochaete setae along the rami: (0) absent, (1) present. Fig. 4 E.

Note: These setae are very long and curved in *H. chloe.*
61. Tip of rami: (0) rounded, (1) pointed. Fig. 4 I.

**Male Genitalia**

62. In dorsal view, anterior edge of tegumen: (0) approximately squared, (1) rounded, (2) elongated. Fig. 4 J-N.
63. In dorsal view, constriction of tegumen at the point of attachment with gnathos: (0) small, (1) large. Fig. 4 K-M.
64. In lateral view, dorsal outline of uncus: (0) curved, (1) angled. Fig. 5 A, B.
65. Length of setae on the basal section of uncus: (0) short, (1) medium, (2) long. Fig. 5 A-C.
66. Long setae in the distal portion of uncus: (0) absent, (1) present. Fig. 5 C.
67. Posterior portion of uncus (tip): (0) width smoothly decreases towards the tip, (1) width decreases conspicuously at the tip (giving the tip the appearance of a claw). Fig. 5 A, B.
68. Latero-anterior margin of vinculum: (0) straight, (1) extended anteriorly. Fig. 5 D, E.
69. In ventral view, length of gnathos: (0) not longer than broad, (1) longer than broad. Fig. 5 F-H.
Note: This character refers to the general appearance of the complete gnathos, the proximal and distal portions.
70. In lateral view gnathos arms (modified from Jenkins, 1983): (0) thin, (1) wide.
71. In lateral view, ventral projection of distal portions of gnathos: (0) absent, (1) present. Fig. 5 I, J.
Note: in lateral view, the distal portion of gnathos is extended ventrally.
72. In ventral view, length of the distal processes of gnathos, from the point where they are fused to the tip: (0) short, (1) elongated. Fig. 5 F-H.
73. In dorsal view, species with distal portion of gnathos elongated, width of posterior portions: (0) broad, (1) medium, (2) thin. Fig. 5 H.
74. In ventral view, sclerotization of the distal portions of gnathos: (0) complete (all the distal portion of gnathos is fully sclerotized), (1) interrupted (only the sides of the distal portions are sclerotized). Fig. 5 F, H.
75. In lateral view, coastal edge of valva: (0) straight, (1) with a coastal projection at mid point. Fig. 5 D, E.
76. In lateral view, distal portion of valva: (0) excavated or straight, (1) projected. Fig. 5 K-M.
77. In ventrolateral view, internal side of base of valva. (0) with a projection extended ventrally, (1) with a projection that smoothes into an internal folding of the valva. Fig. 5 N, O.
78. In ventral view, internal outline of valva: (0) with a projection close to the base of the valvae, (1) with a projection at mid length. Fig. 5 D, E, P, Q.
79. In dorso-lateral view, juxta: (0) heavily sclerotized and projected posteriorly, (1) slightly sclerotized and not projected. Fig. 5 P, Q.
80. Setae on phallus shaft: (0) absent, (1) present. Fig. 5 R.
81. Cornuti: (0) absent, (1) present. Fig. 5 R.
82. In ventral view, base of saccus: (0) squared, (1) triangular. Fig. 5 P, Q.
Female genitalia

83. Posterior edges of abdominal 7th sternite: (0) straight, (1) slightly projected posteriorly. Fig. 6 B, C.
Note: In H. chloe the 7th sternite seem to had been extended posteriorly, it looks extended a little bit in H. atlantis too, and it has small sclerotizations at the lateral edges. State 1 is developed in a greater degree in species of Myscelia, Temenis, and Nica.

84. Membranous pocket between the sclerotized portions of the abdominal segments 7th and 8th: (0) absent, (1) present. Fig. 6 A, B.
85. 8th sternite: (0) free, (1) fused to the 7th sternite. Fig. 6 A–D.
86. Lamella antevaginalis: (0) absent, (1) present. Fig. 6 B–D.
87. Ostium bursa: (0) free, (1) contained into the 7th sternite, (2) contained into the 8th sternite. Fig. 6 C, D.
88. In dorsal view, heavily sclerotized plate on dorsal portion of antrum: (0) absent, (1) present. Fig. 6 E.
89. Ductus seminalis connecting to ductus bursa (char 52 in Hill et al., 2002): (0) very near of corpus bursa, (1) far from corpus bursa, and near ostium bursa. Fig. 6 F, J.
90. Shape of corpus bursa: (0) short and wide, (1) rounded, (2) cone shaped (narrow near ductus bursa), (3) pear shaped. Fig. 6 G–J.
91. Signa: (0) absent, (1) present. Fig. 6 F, G, I.
92. Shape of signa: (0) elongated, (1) two spine-shaped invaginations. Fig. 6 G–I.

Natural history characters

93. Oviposition pattern: (0) eggs laid singly, (1) eggs laid in clusters.
Note: this character is based on literature descriptions of life cycles (References in Appendix 4).
### Appendix 1.3 Morphological data matrix

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Continuation of the morphological data matrix

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Appendix 1.4 Published records of the patterns of oviposition.

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<th>Species</th>
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<th>Reference</th>
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<td>Batesia hypochlora</td>
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<td>DeVries et al., 2000.</td>
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<td>Hamadryas amphinome</td>
<td>cluster</td>
<td>Muyschondt &amp; Muyschondt, 1975; Muller 1886 and d’Almeida, 1922 in Jenkins, 1983.</td>
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<tr>
<td>H. arete</td>
<td>singly</td>
<td>Muller 1886 in Jenkins 1983.</td>
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<td>H. februa</td>
<td>singly</td>
<td>d’Almeida, 1922 in Jenkins, 1983; Young, 1974; Muyschondt &amp; Muyschondt, 1975a.</td>
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<td>H. fornax</td>
<td>cluster</td>
<td>Muller 1886 in Jenkins, 1983.</td>
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<tr>
<td>H. guatemalena</td>
<td>mostly singly, two eggs at the most</td>
<td>Muyschondt &amp; Muyschondt, 1975b.</td>
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<tr>
<td>H. iphthime</td>
<td>singly</td>
<td>Muller, 1886 in Jenkins, 1983.</td>
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Figure 1.1 Wing venation characters and wing colour characters used in this study. (A), (B), and (C) represent the three patterns of forewing venation; (C) and (D) show the location of scent organs in the fore and hind wing respectively; (E) shows a map of the pattern elements used in this study (following Nijhout, 1991); (F) and (G) are the female and male of *H. glauconome* respectively with wing colour characters labeled. Dorsal side on the left, ventral side on the right, scale bar=1 cm.
Figure 1.2 Wing colour characters used in this study plotted on selected species of *Hamadryas*. Dorsal side on the left, ventral side on the right, scale bar=1 cm. Featured species: (A) *H. chloe*, (B) *H. iphthime*, (C) *H. amphinome*, (D) *H. fornax*, (E) female *H. laodamia* and (F) male *H. laodamia*. 
Figure 1.3 The three patterns of male forewing venation found in *Hamadryas* indicating the location of the sound organ and some of the characters used in this study. (A) forewing of *H. glauconome*; (B) forewing of *H. iphthime*; (C) forewing of *H. laodamia*; scale bar=1 cm.
Figure 1.4 Characters from male genitalia used in this study. Scale bar= 1mm. (A) hypandrium of *H. chloe* in ventral view, lateral view of the hypandrium below; (B) hypandrium of *Panacea prola* in ventral view, lateral view below; (C) hypandrium of *Ectima thecla* in ventral view, lateral view above; (D) hypandrium of *Batesia hypochlora*; (E) hypandrium of *H. velutina* in ventral view; (F) hypandrium of *H. februa* in ventral view; (G) hypandrium of *H. epinome* in ventral view; (H) hypandrium of *H. arinome* in ventral view; (I) Tip of rami: (a) *H. atlantis*, (b) *H. arete*, (c) *H. alicia*, (d) *H. februa*, (e) *H. albicornis*; (J) tegumen of *Batesia hypochlora* in dorsal view, (K) tegumen of *H. arete* in dorsal view, (L) tegumen of *H. chloe* in dorsal view, (M) tegumen of *H. fornax* in dorsal view, (N) tegumen of *Ectima thecla* in dorsal view.
Figure 1.5 Continuation of male characters used in this study. Scale bar = 1mm. (A) Uncus of *H. alicia* in lateral view; (B) uncus of *H. glauconome* in lateral view; (C) lateral view of the genitalia of *H. albicornis*; (D) lateral view of the genitalia of *H. atlantis*; (E) lateral view of the genitalia of *H. fornax*; (F) gnathos of *H. velutina* in ventral view; (G) gnathos of *H. arinome* in ventral view; (H) gnathos of *H. alicia* in ventral view; (I) diagram of the genitalia of *H. amphinome* in lateral view; (J) diagram of the genitalia of *H. feronia* in lateral view; (K) valva of *H. amphinome* in lateral view; (L) valva of *H. guatemalena* in lateral view; (M) valva of *H. chloe* in lateral view; (N) diagram of internal side of the valva in species of *Panacea* and *Batesia*; (O) diagram of internal side of the valva in species of *Hamadryas*; (P) genitalia of *H. amphinome* in ventral view; (Q) genitalia of *Batesia hypochlora* in ventral view; (R) phallos of *H. arete* in ventral view.
Figure 1.6 Characters from female genitalia used in this study. Scale bar=1 mm. (A) sterigma of *Ectima erycinoides*; (B) sterigma of *H. chloe* in ventral view; (C) sterigma of *H. amphinome* in ventral view; (D) sterigma of *H. arete* in ventral view; (E) sclerotized plate in antrum of *H. arete* in ventral view; (F) bursa copulatrix of *Panacea prola*; (G) bursa copulatrix of *H. februa*; (H) bursa copulatrix of *H. arinome*; (I) bursa copulatrix of *H. chloe*; (J) bursa copulatrix of *H. atlantis*. 
Figure 1.7 (A) Strict consensus of the 10 MPT obtained under equal weights. (B) Unique MPT found under implied weights.
Figure 1.8 Most parsimonious tree under implied weights (k=2-100) with unambiguous optimization of all the characters. Support values are shown above (GC from symmetrical resampling) and below (relative Bremer support, RFD and absolute Bremer support, ABS) branches.
Chapter 2. Phylogenetic analysis of *Hamadryas* (Nymphalidae: Biblidinae) based on the combined analysis of morphological and molecular data.

Abstract:

A phylogeny for the Neotropical butterfly genus *Hamadryas* based on the combination of morphological and molecular data is presented. This new phylogenetic hypothesis is based on the combination of a morphological matrix, one mitochondrial (COI) and four nuclear markers (CAD, RpS5, EF1a and Wingless). Results from analyses of the new molecular evidence are compared to a previously published morphological phylogeny. The combination of molecular data and the analysis of the complete data set support the monophyly of *Hamadryas* and species affinities suggested by the morphological data. The addition of DNA sequences to the morphological matrix helped define species groups for which no morphological synapomorphies were found. Though resolved the combined evidence tree shows low resample values particularly among species groups whose relationships were characterized by short internodes. Partitioned Bremer Support showed that RpS5, EF1a and Wingless were in conflict with almost all the nodes in the preferred tree. Divergence estimates suggest that *Hamadryas* evolved at the end of the Oligocene and that short internodes with low support corresponded to rapid divergences. A reassessment about the pattern of character change for sound production is presented and discussed.
Introduction

Hamadryas Hübner is one of the most popular and easily recognized groups of Neotropical butterflies (Fruhstorfer, 1916; DeVries, 1987). This is due to noticeable, widespread species such as H. feronia and H. amphinome which occupy a wide variety of habitats (Muyschondt and Muyschondt, 1975a,b), plus the ability of males in some species to produce audible sound while in flight (Godman and Salvin, 1883). Sound is produced in two ways: the contact between swollen veins located at the distal edge of the forewing discal cell at the end of the upstroke (Otero, 1990), or by the deformation of a particular region of the forewing, in which case each wing produces sound independently (Yack et al., 2000). Eight of the twenty species in the genus possess this particular venation and it is believed only these are capable of producing sound (Marini-Filho and Benson, 2010; Garzon-Orduña, 2012). Observations in the field (Otero, 1988; Yack et al., 2000) and cage experiments (Marini-Filho and Benson, 2010) suggested sound is used as a mechanism for sexual recognition.

The two most important contributions to the systematics of this genus used complementary approaches. The taxonomic revision by Jenkins (1983) pooled all species under Hamadryas (in the past, species in Hamadryas were segregated in four genera, see Garzón-Orduña, 2012 for a historical review), restructured species definitions by synonymizing nearly 100 names (recognizing only 20 species, see also Lamas, 2004) and suggested the existence of three subgenera. Based on this taxonomic framework, Garzón-Orduña (2012) presented the first phylogenetic hypothesis for Hamadryas using 93 characters mostly from genitalia and wing color (Fig. 1). The phylogeny was used to test whether the subgenera recognized by Jenkins (1983) constituted natural groups, and to study the pattern of character change in sound production and sexual dimorphism. Morphology supported the monophyly of Hamadryas, and only one of the previously suggested subgenera was found to be monophyletic (the clade composed of H. laodamia, H. arete and H. velutina; Fig. 1). The optimization of sound production, sexual dimorphism and presence of androconial scales onto the phylogeny had important implications for the evolution of male-male and male-female interactions. Garzón-Orduña (2012) showed that male sound production is a derived condition that evolved once and was lost once in Hamadryas, and that the loss was congruent with the appearance of sexual dimorphism and androconial scales. These associated changes suggest a shift in the main signal used for sexual recognition from acoustic to visual plus chemical cues.
The advantages of including multiple, independent sources of data in phylogenetic studies are widely acknowledged (Gatesy et al., 1999; Baker and Gatesy, 2002; Wahlberg et al., 2005). For example, by adding two genes to an original matrix based on morphology and one gene (Nylin et al., 2001), Wahlberg and Nylin (2003) were able to resolve conflicting relationships among species of *Polygonia*. Furthermore, relationships based on the larger dataset had higher support than the previous hypothesis. These findings are in agreement with other studies that used either parsimony or model-based analyses and spanned through various taxonomic levels (e.g. Miller et al., 1997; Giannini & Simmons, 2005; Lopardo et al., 2011).

The main advantage of combining characters from different sources in a simultaneous analysis relies on the power of their congruence given that there is only one tree of life (Kluge, 1989; Gatesy et al., 1999). Phylogenetic signal is expected to increase with the addition of characters, thus overriding the potential misleading effect of stochastic noise. Furthermore, because the support of a group depends on the amount of evidence favoring it relative to the amount of evidence against it, the interaction between characters is a decisive factor in the estimation of the degree of support (Goloboff et al., 2003). The inclusion of as much evidence as possible is therefore an effective approach to estimate the reliability of novel or traditionally accepted groups. The use of measurements of character conflict does not oppose the combination of all the evidence (Kluge, 1989), however they are not used to exclude incongruent data, but to identify the source of conflicting signals (e.g. ILD Mickevich and Farris, 1981; Farris et al., 1994a and the PBS Baker and DeSalle, 1997). Although the previously proposed morphology-based phylogeny for *Hamadryas* was well resolved (Garzón-Orduña, 2012; Fig. 1) and the topology was resilient to different values of the concavity constant under implied weights, several nodes had low support implying character conflict. Thus, phylogenetic studies of this genus can potentially benefit from consideration of multiple, independent data sources.

This study provides a phylogeny for *Hamadryas* using multiple, independent data sources. In our study new DNA sequence data for one mitochondrial and four nuclear markers, was added to a morphological matrix from Garzón-Orduña (2012), and aims to compare the phylogenetic signal provided by molecular and morphological data, as well as to revise previous inferences about the evolution of sound production.
Materials and Methods

Specimens
This study used field collected and preserved specimens from museum or personal collections. The samples were either conserved in 96-100% ethanol or dried. A total 40 specimens representing 17 of the 20 species of Hamadryas were sequenced. Sequences of five outgroup species were obtained from GeneBank (http://www.ncbi.nlm.nih.gov/genbank/). Table 1 lists the collection locality and GeneBank accession number for all specimens.

Gene selection
One mitochondrial (cytochrome oxidase subunit I, COI) and four nuclear genes (Ribosomal protein S5, RpS5; Carbamoylphosphate synthase domain protein, CAD; Wingless, WG; and elongation factor 1α, EF1a) were selected for this study. These markers were chosen because they are highly variable (Wahlberg and Wheat, 2008) and were phylogenetically informative at the species level for several butterflies groups (Silva-Brandao et al., 2008; Jiggins et al., 2010; Penz et al., 2012). Table 2 lists the primers used and their source.

DNA extraction and sequencing
One or two legs from each specimen were used for DNA extraction. Genomic DNA was extracted using DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). Gene amplification followed a standard polymerase chain reaction (PCR). PCR’s were in a 20 µl volumn and included 1µl of DNA extract. The master mix initially contained, per sample: 12.5 µl of dH2O, 2 µl of 10x buffer, 2 µl of MgCl2, 1 µl of forward primer, 1 µl of the reverse primer, 0.4 µl dNTP, 0.1 µl Taq polymerase, and 1 µl of the DNA extract, following the protocols published in Wahlberg and Wheat (2008); in the final stages of the study the master mix contained, 12.5 µl of OneTaq™ 2x MM (New England Biolabs, Ipswich, MA, USA), 0.5 µl of the forward primer, 0.5 µl of the reverse primer and 5.5 µl of water, per sample. The general thermocycler profile included: denaturation at 95 °C for 5 minutes, followed by 40 cycles of 94 °C for 30 seconds, 50 °C for 30 seconds (annealing temperature, see below), 72 °C for 1 minute and 30 seconds, and a final extension at 72 °C for 10 minutes. To obtain a single band of the targeted PCR product, the annealing temperature was adjusted depending on the primer used (nuclear primers required higher temperatures) and the quality of DNA.

PCR products were purified with ExoSAP-IT (usb ®). The master mix for the sequencing reaction included per sample 1 µl of BigDye® Terminator v.3.1 (Applied Biosystems), 1.5 µl of
BigDye® Terminator 5X sequencing buffer, 3 µl of water and 1.5 µl of the primer. Sequencing was conducted in both directions (forward and reverse) for all the samples. The sequencing reaction included 3 µl of the purified PCR product and 7 µl of the sequencing reaction master mix. Sequencing was carried out on an ABI 3130 XL Genetic Analyzer (Applied Biosystems) at the University of New Orleans.

**Sequence edition and alignment**

Chromatogram evaluation, editing, and assemblage were conducted using Geneious 5 (Drummond et al., 2011). All sequences were subjected to a search in BLAST (implemented by the National Center for biotechnology Information (NCBI) website http://www.ncbi.nlm.nih.gov) against the GenBank nucleotide database to check for contamination and to confirm the targeted marker. Heterozygous positions (positions with two peaks of the same height) in the nuclear genes were coded following the IUPAC ambiguity code. Gene partitions were aligned in MUSCLE (Edgar, 2004) from Geneious under default settings, and the alignment did not contain gaps.

**Phylogenetic Analysis**

A previous morphological matrix (Garzon-Orduña, 2012) was modified to include *Panacea regina* and exclude taxa for which molecular sequences were not available. Excluded taxa were: *Ectima erycinoides*, *E. lyrides*, *E. iona*, *Panacea divalis*, *P. prola* (all outgroups), *Hamadryas albicornis* and *H. arete*. *Hamadryas rosandra* was not available for study. The morphological matrix (morphology partition hereafter) and the DNA sequences from the five molecular markers (molecular partition hereafter) were concatenated using SequenceMatrix 1.78 (Vaidya et al., 2011), and the combined matrix was exported as TNT (Goloboff et al., 2008), Phylip and Nexus formats.

Exploratory analyses of individual genes were conducted to examine resolution levels, and to determine which nodes were consistently recovered. Results from analyses of the complete data set are favored because this increases explanatory power, maximizes character independence and allows the emergence of secondary signal or hidden support (Kluge, 1989; Nixon and Carpenter, 1996; Baker and DeSalle, 1997). The combined matrix includes 93 characters from morphology (85 informative) and 4576 bp from DNA sequence (878 informative). The matrix was analyzed under Parsimony in TNT (Goloboff et al., 2008), Bayesian Inference (BI) in Mr.Bayes version 3.1.2 (Huelsenbeck and Ronquist, 2001) and Maximum Likelihood (ML) in RaxML (Stamatakis et al., 2008). All characters were unordered.
Parsimony analyses explored Equal Weights (EW) and Extended Implied Weights (EIW), which aimed at minimizing the effect of homoplasy over phylogenetic signal (command `xpiwe`). Under this command, character sets (e.g. genes) are weighted using their average homoplasy (P. Goloboff personal communication). Additionally, because characters with missing entries cannot have as much homoplasy as observed characters (and therefore they receive a high fit), the option `piwe(*)` was included in the EIW analysis to determine the weights based on the number of missing entries present in the set (P. Goloboff personal communication). Under EIW several values of the concavity constant were explored K=1-50. Parsimony searches included 500 replicates of Random Addition Sequence holding 10 trees per replication, TBR for branch swapping and 90 iterations of Ratchet (Nixon, 1999) (`mult: replic 500 tbr hold 10 ratchet`). After the search, zero length branches were collapsed and duplicate trees discarded (`coll rule 4; condense; unique;`).

Maximum Likelihood and Bayesian searches used GTR+Γ as the model of molecular substitutions. The morphological partition was analyzed under the “standard discrete model” (Lewis, 2001). State frequencies and substitution rates were estimated in Mr. Bayes v. 3.1.2. Nucleotide frequencies, substitution rates, shape of the gamma distribution, the proportion of invariable sites, and overall rate of evolution were allowed to vary among partitions (`unlink statefreq=(all) revmat=(all) shape=(all) pinvar=(all)`). Four chains of Markov Monte Carlo (MCMC) were run for 2.500.000 generations, sampling every thousand generation. The first 25% of the sampled trees were discarded as ‘burn in’, and the lnL probability plot was checked for stationary in TRACER v1.5 (Rambaut and Drummond, 2007).

Branch support in parsimony was assessed using Symmetric Jackknife resample (SJ) and Partitioned Bremer Support (PBS, Baker and DeSalle 1997, Gatesy et al., 1999) in TNT. The results from the SJ are expressed in differences of group frequencies (GC) (Goloboff et al., 2003) instead of straight group frequencies. PBS was calculated using a script written by Carlos Peña (pbsup.run at [http://www.zmuc.dk/public/phylogeny/tnt/scripts/](http://www.zmuc.dk/public/phylogeny/tnt/scripts/)). Posterior probabilities (PP) and Bootstrap support are provided for the BI and ML trees. Trees were edited using FigTree version 1.3.1 (Rambaut, 2006-2009 [http://tree.bio.ed.ac.uk/software/figtree/](http://tree.bio.ed.ac.uk/software/figtree/)) and Adobe Photoshop.

**Divergence time estimation**

The combined data set was used to estimate divergence times for *Hamadryas* under a relaxed molecular clock (Drummond et al., 2006) in BEAST ver. 1.7 (Drummond et al., 2012). Two
calibration estimates taken from Wahlberg et al., (2009a) were used as priors under normal distributions. The age of the most recent common ancestor (MRCA) of Ageroninii (Batesia, Panacea, Ectima and Hamadryas) was set to 33.88 Ma with a standard deviation of 2.1; the second calibration corresponded to the age of the MRCA of Hamadryas and its sister genus Ectima, which was set to 26.67 Ma with 2.5 standard deviation.

Unlinked GTR models of nucleotide substitution and gamma-rate heterogeneity were used for each gene while the simple substitution model was used for the morphological partition. A single relaxed molecular clock using the uncorrelated log-normal model was applied to the data set with the speciation tree prior set to a birth-death process. The MCMC analyses included 50 million generations (10% burn in) sampling parameters every 1000 steps, to ensure an effective samples size (ESS) of over 100. The log and tree files from six independent runs were combined in LogCombiner ver. 1.5.2 included in the BEAST package. Tracer ver. 1.4.1 (Rambaut and Drummond, 2007) was used to examine the ESS of the different parameters and to define the ‘burn in’. TreeAnnotator ver. 1.5.2 (in the BEAST package) was used to conduct the ‘burn in’ and to generate a maximum clade credibility topology of all the sampled trees. Finally, FigTree ver. 1.3.1 was used to visualize the topology.

Results

**Molecular Partition**

DNA markers analyzed independently produced many equally parsimonious solutions (results not shown) except for CAD that produced only three. These solutions differed on the relative position between groups of species after the branching of H. chloe and H. atlantis. As a result, strict consensus trees were poorly resolved for all markers. Under ML and BI optimal solutions were characterized by having very short branches between species and between species groups. With the exception of H. arinome and H. februa, all specimens from species with more than one sample clustered together in all individual gene analyses.

Parsimony analysis under EW produced two most parsimonious trees with alternative resolutions between specimens of H. fornax. EIW under values of the concavity constant from $k=7-20$ found one optimal tree that was identical to one of the two EW trees. This tree with the relationships within H. fornax collapsed is shown in Fig. 2a, where numbers above and below branches represent the GC values from SJ resample and the number of nucleotide
substitutions, respectively. Parsimony analyses of the DNA partition support the monophyly of *Hamadryas* and split the genera in seven lineages (color-coded in Fig. 2a and thereafter), five of which are species groups and two are single species lineages. *Hamadryas chloe* and *H. atlantis* constitute early splits followed by the *feronia* and *amphinome*-clades, and an assemblage including the *fornax*, and *laodamia* plus *februa*-clades.

Both model-based methods found similar topologies (Fig. 2b), which differed from that recovered by parsimony (Fig. 2a). While BI yielded the same seven lineages supported by parsimony, the ML analysis did not recover the *fornax*-clade, and *H. epinome* plus *H. iphthime* grouped with the *amphinome*-clade (see gray branches in Fig. 2b). Parsimony and BI topologies differed in the position of the *laodamia*-clade, which is sister to the *februa*-clade in Fig. 2a but splits off earlier than the *februa*-clade in Fig. 2b. The position of the *fornax*-clade also differs between these two topologies; it is sister to the *februa* plus *laodamia*-clades under parsimony (Fig. 2a) but it groups with the *amphinome*-clade under BI.

**Combined evidence**

Parsimony analysis under EW and ELW (with k>30) produced the same optimal topology (Fig. 3). Lower values of the concavity constant (more weight against homoplasy) found a tree similar to Fig. 3 except that *H. fornax*, and *H. epinome* plus *H. iphthime* are recovered as a clade (gray branches in Fig. 3). BI found a topology slightly different from the one obtained with parsimony, in which *H. fornax*, *H. epinome* and *H. iphthime* as a clade are sister taxa to the *amphinome* and the *laodamia* clades (Fig. 4).

Under both parsimony and BI the combined data tree has the same seven lineages recovered by the molecular partition alone (compare Fig. 2a and 3-4), but excludes *H. alicia* from the *laodamia* clade, splitting *Hamadryas* into eight lineages. Topologies based on the DNA partition and the combined data differed mainly in the position of the *laodamia* clade and the *fornax* clade. Based on the DNA partition the *laodamia* clade was either sister to the *februa* clade (parsimony) or branched off earlier, after the *feronia* clade (BI and ML). In contrast, in the combined data tree the *laodamia* clade is sister to the *amphinome* clade. Under parsimony the combined data generally does not support *H. epinome*, *H. iphthime* and *H. fornax* as a clade (except for low values of k under ELW). Instead, it suggests that *H. iphthime* and *H. epinome* are more closely related to the *februa* clade than to *H. fornax*. 

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Examination of the characters supporting the topology in Fig. 3 shows relatively low congruence between data sets. For example, 12 nucleotide substitutions and 5 ambiguous morphological changes support the clade resulting from the exclusion of the *feronia* clade (open circle in Fig. 3). The sister relationship between the *laodamia* clade and the *amphinome* clade is supported by 4 nucleotide substitutions and 9 morphological characters (characters 1:0, 18:0, 24:1, 25:0, 30:2, 32:0, 41:1, 57:0, 90:3). The *amphinome* clade is supported by 33 nucleotides and four morphological changes (characters 38:0, 48:2, 68:0, and 71:0). Finally, the *fornax* clade is supported only by molecular data (6 nucleotides changes; compare Fig. 2.1, 2.2 and 2.3). All the morphological characters referenced here and in the next sections are described in Table 3.

Resample values (1000 replicates) of the combined data matrix were generally low. The recovered tree from resample (not shown) resembles Fig. 2.3 but the relationship between some species groups are collapsed, particularly after the *feronia* clade. One is the sister relationship between the *februa* clade and the *fornax* clade which was not recovered after resample. GC values of the recovered groups are shown above the branches in Fig. 2.3. Partitioned Bremer Support (PBS) values for these major clades are shown in Fig. 2.4. PBS values show that the clade resulting from the exclusion of the *feronia* clade (open circle in Fig. 2.4) has positive support from COI, CAD and morphology, but it is in conflict with the signal provided by the other nuclear markers (Ef1a, RPS5, WG). The sister relationship among the *laodamia* and *amphinome* clades is supported by CAD and morphology, but contradicted by other molecular markers (black circle in Fig. 2.4). The relationship of *H. fornax*, *H. iphthime* and *H. epinome* (as a clade or not) to the *februa* clade is supported by COI and morphology only, and is in conflict with the other markers (gray circle in Fig. 2.4). RPS5, EF1a, and Wingless were consistently in conflict with the signal provided by COI, CAD and morphology and had negative values for 21 of 41 nodes, in contrast, the signal provided by the morphology was not in conflict with any of the nodes in the tree. Furthermore CAD agreed with the morphology in 25 of 41 nodes, and in 11 nodes with the signal provided by COI.

**Divergence times**

Estimated divergence times for *Hamadryas* are depicted on a maximum credibility tree in Fig. 2.5, and posterior probabilities are indicated above branches. These estimations suggest the genus originated at the end of the Oligocene, approximately 23.63 Ma ago (95% credibility interval 20-27 Ma) and the majority of the speciation events occurred during the second half of the Miocene.
Discussion

**DNA versus morphology**

Analyses of DNA alone supported the monophyly of *Hamadryas* as well as most of the sister-level relationships inferred with morphology (Fig. 2.1). Analyzed independently both sources of data support the phylogenetic affinities of *H. februa* with *H. amphichloe*, *H. glauconome* and *H. julitta*; the sister relationship of *H. feronia* and *H. guatemalena*; and the phylogenetic affinities between *H. laodamia* and *H. velutina* (*H. arete* is not included in the molecular analysis). DNA also provided support for relationships not recovered by morphology, such as *H. amphinome*, *H. arinome*, and *H. belladonna* as a monophyletic group, and *H. ipthime* and *H. epinome* as sister taxa.

The placement of some species is incongruent between data sets. For example, in contrast to morphology DNA supported *H. chloe* and not *H. atlantis* as the first split within *Hamadryas*. Although DNA places *H. alicia* as sister species of *H. laodamia* and relatives, this placement is based only on an incomplete COI fragment. There are other important discrepancies between both data sets mainly involving the relative relationships between species groups. Based on morphology *H. feronia* and *H. guatemalena* are derived species, nested within clade S in Fig. 2.1. This clade includes all the species that have some or all the venation components required for sound production (Garzón-Orduña, 2012). The placement of *H. feronia* and *H. guatemalena* within clade S was supported by one morphological synapomorphy (character 22:2) and four homoplastic transformations (characters 35:1, 36:2, 45:2, 56:1), four of these were color characters and one regarded the location of macrochaeta in the hypandrium (character 56; see Garzón-Orduña, 2012). In contrast, DNA places *H. feronia* and *H. guatemalena* as the first split after *H. atlantis*, an earlier origin than what is implied by morphology. This result was obtained in all the analyses that include the DNA data, and in the RPS5 and CAD gene trees.

DNA and morphology also disagreed with regard to the relationships among some species groups. The molecular partition did not support a close relationship between *H. amphinome* and relatives plus *H. laodamia* and relatives. This clade was supported in the morphological phylogeny by 5 apomorphies (characters 20:2, 24:1, 45:3, 54:1, 90:3) and 6 homoplastic transformations (1:0, 25:0, 30:2, 32:0, 49:2, 60:0) and had high resample values (Garzón-Orduña, 2012). Instead, DNA data suggested two alternative positions of the clade of *H.*
Laodamia and H. velutina (depending on whether parsimony or model-based methods are used), neither of which is congruent with morphology alone (Garzón-Orduña, 2012 and Fig. 2.1).

The molecular partition was moderately sensitive to the perturbations made with resampling. Resample values for the relationships between species groups were intermediate to low, particularly in the case of short internal branches (Fig. 2.2b). The morphological study also yielded low resample values between species groups (Garzon-Orduña, 2012). Short internal branches of a DNA-based tree and scarce character support of a morphology-based tree are an indication of little divergence between clades and could be a result of rapid speciation (e.g. Ober and Heider, 2010). It is therefore evident that neither data set alone provides robust phylogenetic signal for deeper nodes of the Hamadryas tree.

**Combined Data**

This study provides a fully resolved species level phylogenetic hypothesis for Hamadryas based on the combination of all available characters. Two important and unexpected findings of our study are: (1) H. feronia and H. guatemalena are basal species in comparison to the position suggested by morphology; the combined data suggest these two species constitute a sister group to the rest of Hamadryas after the split of H. alicia; (2) H. februa and relatives are nested within a clade of sound-producing Hamadryas implying a loss of sound production in this species group.

Although well resolved, short branches of combined data tree have low support, and these correspond to relationships among species groups within the genus. In particular, it is not clear whether H. fornax, H. iphthime and H. epinome are sister taxa to H. februa and relatives. This result was obtained only under Parsimony and it is lost after resampling; meanwhile BI and EIW with low values of the concavity constant (penalizing homoplasy harder) suggest that they are sister to the amphinome plus laodamia clade. The absence of resampling support in Parsimony suggests that their position might change with the addition of more evidence. Similar to other species level phylogenetic studies on butterflies, this study found low support at the intermediate parts of the tree, while obtaining strong support for clades near the tips (see Monteiro and Pierce, 2001; Silva-Brandao et al., 2008).

Partitioned Bremer support showed interesting conflicts in the interaction between mitochondrial and nuclear DNA markers. Here, three (WG, Rps5, Ef1a) of the four nuclear markers were consistently in conflict with the combined data topology; the exception was CAD, which was in
agreement with COI and morphology in several nodes. Wahlberg et al. (2009b) found strong
conflict between the signal provided by mitochondrial (COI and ND1) and nuclear markers (EF1-
a, WG, GAPDH and RPS5). Our results are in agreement with their findings even though that
study did not include CAD. One explanation for incongruence particularly between mtDNA and
nDNA is that of recent divergence, in which case slow evolving genes have not had enough
time to sort (e.g., McCracken & Sorenson, 2005). As mentioned in the results single gene
reconstructions of these markers show low resolution suggesting ambiguity in the data, however
low the signal provided by these markers, it seems to be incongruent from the signal provided
by COI, CAD and morphology. Taken together, the conflict among nuclear and mitochondrial
markers, the presence of short internal branches in the combined analyses and the lack of
morphological characters supporting relationships between species groups seem to suggest
that some clades in Hamadryas diverged rapidly. For example, given the divergence times
estimated here, the fornax, amphinome and laodamia clades, plus their MRCA (circled numbers
in Fig. 2.5), seemed to have diversified within a time frame of two million years in concordance
with rapid divergence (e.g. Cronn et al., 2002; Satler et al., 2011). The unexpected congruence
of CAD to COI and morphology suggest that this gene might be evolving faster than other
commonly used nuclear genes, and we therefore recommend its inclusion in future studies
involving species level phylogenetic inference.

Sometimes ambiguous or contradictory data sets produce a robust phylogenetic signal upon
their combination (Lee, 2009). This unexpected result, due to the interaction between
characters, is known as ‘hidden support’ (Gatesy et al., 1999). Although in the combined matrix
the morphological data represents a significantly smaller fraction of the informative characters
(apt. 9%), in some instances the phylogenetic signal from morphology exceeded the signal
provided by DNA. For example, all the DNA-based analyses suggested that the laodamia clade
was closely related to the februa clade. In contrast, EW, EIW and BI analyses of the combined
evidence supported the relationship suggested by morphology: i.e., that H. laodamia and
relatives are a sister clade to H. amphinome and relatives. The emergence of this relationship
upon the combination of data sets implies that there was indeed support in the molecular
partition.

The phylogeny provided in this study can be used to reconstruct the biogeographical history of
Hamadryas and to test biogeographical hypotheses regarding its diversification. Some of the
above mentioned limitations in the current phylogeny of Hamadryas could be addressed by
using longer fragments and faster evolving genes, as well as completing the sequences for species containing many missing entries as it is the case of *H. alicia* in this study.

**Implications for the evolution of sound production**

In *Hamadryas* males that produce sound exhibit five modifications of the FW venation (Fig. 2.6). The modifications are: (1) R1 and R2 veins stalked (character 3:1 in Garzón-Orduña, 2012); (2) M1 rising independently from R3, R4-R5 (character 5:1); (3) M2 conspicuously bowed (character 6:1); (4) Cross vein at the posterior edge of discal cell swollen (character 4:1) (5) M2-M3 cross vein joins the cu1-cu2 cross vein (character 7:1).

Trees based on the combined data (Fig. 3 and 4) suggest a different scenario for the evolution of sound production than proposed by Garzón-Orduña (2012). Based on combined data, these five changes in venation appear earlier in the phylogeny (compare Fig. 2.3 and 2.4 to Fig. 2.1). The venation for sound production is inferred to have evolved after *H. alicia* branched off, at the common ancestor of *H. feronia* plus *H. guatemalena* and the rest of *Hamadryas* (Fig. 2.3, 2.4). More importantly the tree based on combined data suggests two losses of the venation required for sound production: one in which the five characters reverse at the node of *H. februa* and relatives (a complete reversal to the plesiomorphic venation) and another in which only two of the five characters reverse at the node of the *laodamia* clade (resulting in a third and intermediate venation pattern).

Although both reversals imply the loss of sound production, they are different. Males of *H. laodamia* and *H. velutina* reversed in two of the five characters: FW vein M₂ is almost straight (6:0) and the cross vein at the posterior edge of discal cell is thicker than in other species but not swollen (4:2). Additionally, the loss of sound production in these species concurred with the evolution of scent scales (androconia) and sexual dimorphism (SD) which was interpreted as a switch in sexual recognition signals from sound to visual and scent cues (Garzon-Orduña, 2012). The loss of sound production in the clade of *H. februa* and relatives is more difficult to explain because it implies the reversal of all five characters. Based on field observations of interactions between males and females of *H. feronia*, Otero (1988) concluded that spiral flights have little importance, in the sexual recognition of this species (for which the production of sound is predominant); this in contrast to the interactions of *H. februa*, in which spiral flights are the signature response of males to chases during aerial interactions (Otero, 1988, Otero Pers. communication).
As mentioned above *H. laodamia* and relatives have three of the five characters required for sound production. The presence of an intermediate venation in a clade that does not produce sounds suggests that changes in characters 6 and/or 4 are critical to the production of sound. Indeed Otero’s (1990) ablation experiments on *H. feronia* showed that if the crossvein at the apical part of the discal cell is removed sound production ceases. His experiment, although extreme, represents the only available analogy between states 0 and 1 of character 4, and highlights the importance of this character for the production of sound.

**Divergence times and the diversification of Hamadryas in the Neotropics**

The times of divergence estimated here suggested that the major linages within *Hamadryas* formed at the end of the Miocene, while most sister species split during the Pleistocene (Fig. 2.5). This estimation is congruent with the diversification of other Neotropical butterfly genera such as *Morpho* (Penz et al., 2012), and it is also congruent with major geographical and climatic changes in the Neotropics such as the uplifting of the Andes and the shift in the drainage of the Amazon Basin eastwards (Hoorn and Wesselingh, 2010). This congruence might be indication of a common response to these events; biogeographical analyses could be used to assess the existence of a general pattern across taxa.

Individuals of *H. februa* did not group together in the phylogenetic analysis (Fig. 2.2 and 2.3), and diverged at different times (Fig. 2.5). Moreover, accounts about the ability of *H. februa* to produce sound are incongruent. Several authors reported sound production in populations of *H. februa* from Brazil (Jenkins, 1983), Costa Rica (DeVries, 1983; Monje-Najera and Hernandez, 1991), El Salvador (Muyshondt and Muyshondt, 1975b) and Mexico (Ross, 1963). In contrast, Otero (1988) and Marini-Filho and Benson (2010) both tested the ability of *H. februa* to make sound in populations from Venezuela and five populations from Brazil respectively, and found that none of the specimens tested were capable of producing sound. In concert, the phylogeny proposed here and the contradictory records of sound production suggest the possibility that the taxon currently identified as *H. februa* (following Jenkins, 1983) is composed of more than one lineage.
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References


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<td>X</td>
<td>X*</td>
<td>X</td>
<td>X</td>
<td></td>
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<tr>
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<td>X</td>
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<td>H. guatemalena</td>
<td>ADW02</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td></td>
<td>Starcco. Rio Grande City, Fort Ringold, Texas, US</td>
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<td>H. iphithone</td>
<td>UJ07</td>
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<td>H. laodamia</td>
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<td>X</td>
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<td><strong>Out-groups</strong></td>
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<td>Ectima thecla</td>
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<td>GQ864762</td>
<td>GQ864586</td>
<td>GQ865429</td>
<td>GQ864451</td>
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<td>Batesia hypochlora</td>
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<td>GQ864619</td>
<td>GQ864743</td>
<td>GQ864837</td>
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<td>Panacea Regina</td>
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<td>AY788702</td>
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<td>AY788464</td>
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<td>Eunica viola</td>
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<td>GQ864767</td>
<td>GQ864861</td>
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<td>GQ864455</td>
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<td><strong>Length of marker (bp)</strong></td>
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<td>1470</td>
<td>1240</td>
<td>613</td>
<td>403</td>
<td>4576 bp</td>
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* Incomplete fragment
Table 2.2 List of the primers used for PCR and sequencing reactions and their source.

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<tr>
<th>Marker</th>
<th>Sequence</th>
<th>Source</th>
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<td>LCO (f)</td>
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<td>Folmer et al., 1994</td>
</tr>
<tr>
<td>HCO (r)</td>
<td>T AAA CTT CAG GGT GAC CAA AAA ATC A</td>
<td>Folmer et al., 1994</td>
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<td>Jerry (f)</td>
<td>C AAC AYT TAT TTT GAT TTT TTG G</td>
<td>Simon et al., 1994</td>
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<tr>
<td>Pat (r)</td>
<td>A TCC ATT ACA TAT AAT CTG CCA TA</td>
<td>Simon et al., 1994</td>
</tr>
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<td><strong>Wingless</strong></td>
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<td>LepG1 (f)</td>
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<td>Brower and DeSalle, 1998</td>
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<td>LepG2 (r)</td>
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<td><strong>Rps5</strong></td>
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<td>Wahlberg and Wheat, 2008</td>
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<td>HybrpS5degR</td>
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<td><strong>EF1-alpha</strong></td>
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<td>Cho et al., 1995</td>
</tr>
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<td>Monica</td>
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<td>HybAlF</td>
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<td>Cho et al., 1995</td>
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<td>HybEFrM4</td>
<td>A CAG CVA CKG TYT GYC TCA TRT C</td>
<td>Cho et al., 1995</td>
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Table 2.3 Morphological characters from Garzon-Orduña (2012) referenced in this study.

<table>
<thead>
<tr>
<th>Character</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>White scales on antennae: (0) absent, (1) present</td>
</tr>
<tr>
<td>3</td>
<td>In males, FW veins R and Rs1 (Jenkins, 1983): (0) separated, (1) share a common stem</td>
</tr>
<tr>
<td>4</td>
<td>In males, FW Rs-Rs2+3+4, Rs2+3+4-M1, and M1-M2: (0) same width as other veins, (1) fully swollen, (2) thick but not swollen</td>
</tr>
<tr>
<td>5</td>
<td>In males, FW vein M1: (0) arising from the same point as Rs2+3+4; (1) arising at midpoint length between Rs2+3+4 and M2</td>
</tr>
<tr>
<td>6</td>
<td>In males, FW vein M2: (0) midly curved towards M3, (1) conspicuously curved towards M3</td>
</tr>
<tr>
<td>7</td>
<td>In males, FW crossvein M2-M3 (Jenkins, 1983): (0) joins the M3-cuA1 fork, (1) joins the Cu1-Cu2 crossvein, (2) joins M3</td>
</tr>
<tr>
<td>18</td>
<td>Ventral coloration of thorax: (0) red, (1) mustard, (2) brown, (3) flax, (4) chalk</td>
</tr>
<tr>
<td>22</td>
<td>Spot of blue scales in the proximal portion of the DFW band between elements c and d: (0) absent, (1) present</td>
</tr>
<tr>
<td>24</td>
<td>In females, colour of DFW discal cell band between pattern element d and e: (0) white, (1) blue, (2) iridescent green, (3) beige/light brown</td>
</tr>
<tr>
<td>25</td>
<td>In females, DFW pattern element e: (0) composed of dark scales, (1) composed of (brown) light scales, (2) composed of blue, (3) composed of red scales</td>
</tr>
<tr>
<td>30</td>
<td>DFW in females, width of space between margin of the discal cell and m1-m2 band: (0) narrow, (1) wide, (2) no space, reaching distal margin of discal cell</td>
</tr>
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<td>32</td>
<td>DFW in M2 cell in females, shape of band distal to pattern element e: (0) entire, elongated towards distal margin of wing and pattern element f not visible, (1) split by pattern element f, proximale band oval</td>
</tr>
<tr>
<td>35</td>
<td>DHW in females, pattern element d: (0) centre of element d composed of light scales, (1) element d composed of dark scales only, (2) centre of element d composed of red scales</td>
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<td>36</td>
<td>DHW in females, colour of the distal edge of discal cell (pattern element e): (0) red, (1) light, (2) dark brown</td>
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<td>41</td>
<td>DHW pattern element h (border ocelli): (0) with internal ring, (1) without internal ring</td>
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<td>45</td>
<td>Composition of DHW ocellus 2 (Rs cell): (0) complete ocellus, (1) blurred ocellus, (2) only the most external ring present (empty ocellus), (3) external ring and a pupil present, (4) pupil only</td>
</tr>
<tr>
<td>56</td>
<td>Location of macrochaete setae on hypandrium: (0) present only at the base of rami, (1) present on lateroposterior margin and some setae reaching the posterior margin of the sternite</td>
</tr>
<tr>
<td>57</td>
<td>Macrochaete setae at the posterior margin of hypandrium (base of rami): (0) as long as the macrochaete at the tip of rami, (1) smaller</td>
</tr>
<tr>
<td>58</td>
<td>Setae on lateral surface of rami: (0) absent, (1) present but few, (2) present in large numbers</td>
</tr>
<tr>
<td>62</td>
<td>In dorsal view, anterior edge of tegumen: (0) approximatively squared, (1) rounded, (2) elongated</td>
</tr>
<tr>
<td>90</td>
<td>Shape of ductus bursa: (0) short and wide, (1) rounded, (2) cone shaped (narrow near ductus bursa), (3) pear shaped</td>
</tr>
</tbody>
</table>
Figure 2.1 Phylogeny of *Hamadryas* based on 93 morphological characters from Garzón-Orduña (2012). Origin and loss of sound production (S and LS, respectively) are labeled on the tree at the corresponding branches. Jenkins (1983) species groups are highlighted in gray.
Figure 2.2 DNA-based *Hamadryas* phylogeny. (A) Parsimony results, numbers above and below the branches represent GC values from symmetrical resample and the number of nucleotide substitutions respectively. (B) Bayesian inference and Maximum Likelihood analysis (gray inset), numbers above and below branches represent posterior probabilities and bootstrap values respectively. Gray branches represent the alternative relationship of the *fornax* clade to the *amphinome* clade found under ML.
Figure 2.3 Combined evidence topology found under EW and EIW under concavity constant $k=7-50$. Numbers above branches represent GC values from symmetrical resample. The gray inset represents the only difference between this tree and alternative solutions under $k$ values $<30$. 
Figure 2.4 Partitioned Bremer support values for the nodes of the optimal tree obtained with Parsimony using the combined evidence.
Figure 2.5 Maximum credibility tree found with BEAST. This is the same topology as the majority rule consensus found with Mr. Bayes. Values above branches represent posterior probabilities and bars on nodes represent 95% credibility intervals.
Figure 2.6 Schematic drawings of three venation character combinations found in *Hamadryas*. The implied optimization of these patterns based on the combined evidence tree is shown in Fig. 2.3. Pattern A corresponds to the venation of species known to produce sound based on hand tests and field observations. Pattern B represents the venation of most species that do not produce sound, dotted vein in B represents an alternative state of character 7 present only in *H. chloe* and *H. atlantis*. Pattern C is present in *H. laodamia*, *H. velutina* and *H. arete* (the latter was not included in this study).
Chapter 3. Using Geometric Morphometrics to study correlated character changes: sound production and wing shape of Hamadryas butterflies (Nymphalidae: Biblidinae)

Abstract:
This study aims to assess whether species of Hamadryas that possess distinctive venation patterns, one of which is associated with sound production, have different wing shape. Using landmark based geometric morphometrics, we demonstrated that the three observed venation patterns correspond to significantly different wing morphology for both males and females. Furthermore within each venation group we found significant differences in the wing shapes of males and females. Since some of the females of sound-producing species possess intermediate states of a few venation components associated with sound production, genetic correlation between sexes is offered as a potential explanation for the significant difference in female wing shape found among the three venation groups. However, the expression of the venation characters in these females was intermediate, allowing for wing shape sexual dimorphism. Finally, a permutation test of the procrustes landmark coordinates onto the phylogeny indicated that wing shape in Hamadryas contained phylogenetic signal.
Introduction
The relationship between morphological variation and natural history can be a powerful explanatory tool in evolutionary biology. Although in many cases phylogenetic relatedness is the main reason for the similarities between species, different life histories can lead to phenotypic divergence between closely related species (Chai and Srygley, 1990; Elias et al., 2008; Wiklund, 2003). As a consequence, anatomical designs can be viewed as the byproduct of a tradeoff between phylogeny and ecology.

Insect wing shape is one of the best-documented examples of the interaction between phylogeny and ecology (e.g. Bai et al., 2011; Outomuro and Johansson, 2011). Flight is used for foraging, mate location and predator avoidance, hence it might be expected that wing morphology would be conserved among closely related species. Nonetheless, there are many instances in which wing shape has been found to vary within groups of closely related species, and also between individuals of the same species (e.g., Breuker et al., 2007a; Bots et al., 2009).

Variation in wing shape can be correlated with behavioral diversification or with fluctuating environmental conditions (e.g., Outomuro and Johansson, 2011). In male butterflies, perching versus patrolling mate location behavior has influenced the evolution of distinctive wing shapes among other anatomical traits (Wickman, 1992; Wiklund, 2003). Wickman (1992) showed that in 44 temperate species, males of perching species had higher aspect ratios, higher wing loading and larger thorax/body mass ratios than patrolling species (Wickman, 1992). DeVries et al. (2010) showed that vertical stratification and flight behavior influenced wing morphology in *Morpho* butterflies (Nymphalidae: Satyrinae). Males in the *M. hecuba* group predominantly use gliding flight to patrol at the canopy level while female activities span over the canopy and understory. In all other species both sexes employ flapping flight in the mid to understory. The switch to gliding flight at the canopy level is associated with a significant increase in wing centroid and aspect ratio for males, but not females in the *M. hecuba* group, suggesting that selection operates differently between sexes (DeVries et al., 2010). Despite their recent association to mountainous landscapes, variation in forewing morphology of the potato moth *Tecia solanivora* (Gelechiidae) was found to be associated with altitude in the Ecuadorian highlands (Hernandez et al., 2010). Moths at higher elevations had slender forewings than those at lower altitudes. Slender wings are thought to compensate for the functional constraints of flight at higher altitudes hence contributing to the invasion success of this species (Hernandez et al., 2010). Overall, these studies demonstrate that different natural history
attributes can influence rapid changes in wing shape at intraspecific and interspecific levels, as well as between sexes, illustrating the potential of correlated character changes on explaining morphological divergence.

Because males and females use flight for distinctive behavioral roles, sexual dimorphism in wing shape is not uncommon in butterflies (Betts and Wooton, 1988; Breuker et al., 2007b; DeVries et al., 2010; Benitez et al., 2011). For example, Pararge aegeria (Nymphalidae: Satyrinae) females have lower wing aspect ratio than males. Furthermore, the sexes also differ in flight muscle allocation and other aspects of flight morphology (Van Dyck and Wiklund, 2002) shown to result in sex-related differences in flight performance (Berwaerts et al., 2006).

Variation in the forewing shape is associated to dispersal in females, not males of Melitaea cinxia (Nymphalidae: Nymphalinae) although the sexes did not differ in dispersal rates. The wings of dispersing females were more rounded than the wings of local individuals (Breuker et al., 2007a). Therefore sexes might respond differentially respond to selection when facing similar environmental conditions.

The 20 species in the butterfly genus Hamadryas Hübner vary in wing shape and color, and four of these species are sexually dimorphic (Jenkins, 1983). Males of eight species produce an audible sound during aerial interactions by means of thickened veins at the edge of the forewing discal cell that get in contact at the end of the upstroke, and also by the deformation of the wing membrane (Yack et al., 2000) allowing the production of sound with only one wing (Otero, 1990). Sound production involves a few other modifications in the wing venation of males, some of these modifications are absent in their coespecific females as well as in both sexes of species that do not produce sound. Based on the combination of characters states of five venation characters of the male forewing (characters 3-7 in Garzón-Orduña, 2012) three venation patterns can be identified within Hamadryas, these and the description of each character is shown in Fig. 1. Sound is produced during aerial interactions that occur during exploratory flights usually involving a patrolling (always a male) and a perched individual (male or female), which after the chase engage in spiral flights (Yack et al., 2000; Otero, 1988). Given the nature of these interactions, it seems that sexual selection has played a role in the evolution of sound production and flight behavior in these butterflies.

Despite the importance of venation in wing deformation during flight (Combes and Daniel, 2005; Tanaka and Shimoyama, 2010), the effect of wing venation on wing shape has received little
attention. Some studies have highlighted the variation of the forewing distal outline (concave, straight, or convex; e.g. *Opoptera*, see illustrations in Penz, 2009), and the non-homologous hind wing tails present in various groups within Papilionoidea (Kristensen, 2003). However these cases involve elongation or shortening of wing veins that are predictably correlated with modifications of the wing contour. The effects of different venation patterns at the medial area of the wing (such as cross veins and forks) on wing shape are less obvious.

Here we use geometric morphometrics to ask whether modifications in wing venation in sound-producing *Hamadryas* are associated with changes in forewing shape. The analyses focus on two questions: (1) Did changes in venation associated with sound production affect the forewing shape of male and female *Hamadryas*? If the evolution of sound production affected wing shape and given that only males produce sounds, we expected that only in males, will species group according to the three venation patterns in a morphological space and these groups will differ significantly in their wing shape. (2) Do male and female *Hamadryas* differ in wing shape?. Sexual dimorphism in wing shape is expected to occur only in species in which males produce sound.

**Methods**

**Specimens**

This study includes 356 individuals from 19 species of *Hamadryas* (Table 1, Appendix 1). Males and females were photographed using a Canon G9 digital camera and a tripod; a grid marked at 5 mm intervals was used as a scale and was placed under one side of the body of each specimen. The majority of the samples were photographed at Florida Museum of Natural History; the rest were obtained on loan from different institutions (Milwaukee Public Museum, National Museum of Natural History – Smithsonian Institution, American Museum of Natural History). Table 1 shows the number of specimens per species included in the analyses, sex and their a priori group assignment (see below).

**Group assignment**

The combination of character states that constitute each venation pattern with the corresponding states for males and females can be found in Table 2 (see also Fig. 1). Species of *Hamadryas* composing each venation pattern were assigned to the following groups:
Group A: amphinome, arinome, belladonna, epinome, iphthime, feronia, fornax and guatemalena; Group B: albicornis, alicia, amphichloe, atlantis, chloe, februa, glauconome, and julitta; and Group C: arete, laodamia, and velutina.

The 356 individuals were assigned a priori to one of three groups according to their venation pattern, and the number of specimens assigned to each group is shown in Table 1. Although females of some species exhibit a slightly different venation pattern from that of their corresponding males (Table 2, asterisks in Fig. 1), for the purposes of the analyses, such females were assigned to the same group as their conspecific males.

Quantification of wing shape

Geometric morphometrics (GM) offers a powerful approach to quantify wing shape variation in insects (Gidaszewski et al., 2009; Breuker et al., 2010; Bai et al., 2011). By comparing landmarks across species, GM allows for an objective examination of the complete variation in shape and its analysis under different statistical approaches (Klingenberg, 2011). For example, GM has been used to study the effects of eyespot shape on wing shape in Bicyclus anynana (Monteiro et al., 1997), to test whether variation in wing shape in P. aegeria is environmentally determined (Breuker et al., 2010), and to determine sexual dimorphism in moths (Benitez et al., 2011). GM was therefore selected as a means of assessing differences in wing shape within Hamadryas.

Ten landmarks were positioned and digitized on the dorsal side of the forewing in TpsDig 2.12 (Rolf, 2008; Fig. 2). Eight landmarks (landmarks 2-9) were located where the vein meets the edge of the wing (following Breuker et al., 2010), landmark 1 was located proximally on the wing at the base of the discal cell, and landmark 10 was placed on the coastal margin by projecting a straight line from the crossvein located at the anterior, distal edge of the discal cell. None of the landmarks corresponded to the venation characters involved in sound production.

Variation in shape was quantified using GM methods based on generalized least squares Procrustes superimposition methods (Rohlf and Slice, 1990) in MorphoJ 1.0 (Klingenberg, 2011) and tpsRelw 1.49 (Rohlf, 2010). Procrustes methods analyze shape by superimposing the configuration of landmarks of two or more individuals to achieve an overall best fit, eliminating variation in scaling and rotation and preserving only shape as a variable. Procrustes distances (pd) were used to summarize shape differences between the average shapes of
groups. In GM the pd are measured as the squared root of the sum of the squared distances between corresponding landmarks of two optimally aligned configurations (Klingenberg and Monteiro, 2005). To make the visualization of the wing deformation, transformation grids were scaled to a factor of 10.

**Statistical Analyses**
Differences in wing shape in a morphospace were visualized by conducting a relative warp analysis (RWA) in TpsRelw v.1.49 (Rolf, 2003). RWA generates shapes variables (also called partial warps) to describe the variation among specimens within a sample in terms of their variance in the parameters of a fitted function, which is analogous to a principal components analysis. The variance is expressed relative to a bending energy matrix (Rolf, 1993). Thin-plate spline deformation grids were generated along the relative warp ordination plot to make the visualization of shape differences easier. This analysis was conducted only for males, plotting males and females made the visualization of the results impossible.

A Canonical Variates Analysis (CVA) and a Procrustes ANOVA in MorphoJ 1.0 (Klingenberg, 2011) were conducted in order to determine if there were significant differences in wing shape between venation patterns for both males and females, and between sexes within each venation group. In the CVA statistical significance was determined by applying 10000 permutations, CVA finds the canonical variates (axes) that maximize the difference between groups relative to the variation within groups of the landmarks configuration. In the ANOVA the three venation patterns and sex were used as classifiers and added as an individual effect.

**Phylogenetic signal**
To reconstruct the evolutionary history of wing shape for male *Hamadryas*, generalized procrustes superimposition of the landmark configuration was mapped onto the genus phylogeny (Garzón-Orduña et al. in prep., Chapter 2) under weighted squared-change parsimony (Maddison, 1991). The formalization of squared change parsimony in MorphoJ uses the generalized least-squares method to find the values for the internal nodes so that the sum of squared changes along the branches is minimized over the phylogeny (Klingenberg and Gidaszewski, 2010). The implied morphological changes along the phylogeny, were obtained by setting the hypothetical common ancestor of all *Hamadryas* as a starting node and the featured nodes as targets nodes in MorphoJ.
The null hypothesis of complete absence of phylogenetic signal was examined with a permutation test in MorphoJ. In this test the shape means among the species are permutated (10000 times) and mapped onto the phylogeny under weighted squared change parsimony (weighting by branch lengths). The empirical P-values are obtained as the proportion of the permutations in which the tree lengths were equal or shorter than the ones obtained with the original data.

Results

Relative Warp analysis

Figure 3 shows a scatterplot of the scores of 197 *Hamadryas* males for the first two relative warps. Groups of species based on each venation pattern are shown as different colors, Group A is shown in blue, and Groups B and C in orange and fuchsia, respectively. Deformation grids for selected specimens are shown at the corners of the plot; points in the deformation grids correspond to landmarks. Deformation grids can be interpreted as representative of typical shapes found in each quadrant of the morphospace relative to the average shape (center of plot, not shown). Most of the variation among specimens (relative to bending energy) is along the first relative warp axis (RW1, 64%); the second axis (RW2) explained only 14% of the variation. Along RW1 the major differences between samples at each end occur at landmarks 1, 4, 5, 6 and 10. The longest vectors (not shown) for RW1 show displacements of landmarks 4, 5 and 6 outward to the distal wing margin under positive scores (in which case the wing margin is convex) and inward under negative scores (wing margin concave). Landmark 10 moves proximally under positives values (in which case the wing looks round) and distally under negative scores (resulting in an elongated wing). The negative quadrant of RW1 and RW2 was almost exclusively represented by species of Group A, and species of Group C were all found at the most extreme right portion of RW1. Specimens of Group B showed the largest dispersion along RW1 and were present at both positive and negative scores along this axis. Specimens of Group A showed the largest dispersion along RW2, some of which were close to specimens in Group B. In sum, the wing shapes of Group A and Group B were morphologically more similar to each other than to that of Group C (Fig. 1 and 3).

Canonical Variates analysis and ANOVA: males

Species groups A, B and C differed significantly in the shape of their forewing. Group A differed significantly from Group B (pd=0.043, \( P<0.0001 \)) and Group C (pd=0.11, \( P<0.0001 \)). Groups B and C also differed significantly from each other (pd=0.083, \( P<0.0001 \)). Canonical Variant 1
(CV1) explained 72.2% of the shape differences between the groups and Canonical Variant 2 (CV2) explained the remainder 27.7%. Figure 4 shows a scatterplot of CV1 against CV2 revealing that Group A (blue) can be clearly distinguished from B (orange) and C (Fuschia).

The major morphological differences between the three groups along the first and second canonical variates are shown as deformation grids in Fig. 5a and 5b respectively. Changes along the first canonical axis implied movements of landmarks 4, 5, 6 inward, resulting in a concave forewing distal edge. Landmarks 10 and 9 also have high magnitudes and their direction suggest an extension of the coastal margin of the wing. The second canonical axis implies movements of landmarks 2, 3, 4, 5 and 10 outward the wing, resulting in a rounder and broader wing.

Finally ANOVA was used to test if the three groups differ in wing shape. Similarly to CVA, the results of the ANOVA also indicated that the type of venation has significant effect on male wing shape (df=32, Pillai’s trace=1.57 P<.0001).

Canonical Variates analysis and ANOVA: females
As in the males, the three venation groups also differed significantly for females (Group A versus B: pd=0.0141, P=0.001; Group A versus C: pd=0.07, P<.0001; Group B versus C: pd=0.0635, P<.0001). Canonical Variant 1 (CV1) explained 81.2% of the shape differences between the groups, while the Canonical Variant 2 (CV2) explained only 18.7%. Figure 6 shows a scatterplot of CV1 against CV2 featuring the position of the three groups in the canonical space. There are however three noticeable differences with respect to the results from the male-only analysis. First, in the female data set the Procrustes distances between the groups are smaller. Second, the dispersion of data points within the groups is wider for females than for males (Fig. 6). Finally, the separation of the groups in the CVA is smaller for the female data set than for the males, and this is particularly evident between Groups A and B (Fig. 6).

Deformation grids along the first and second canonical variates featured in Fig. 7a and 7b respectively. These represent major morphological differences between the three groups found for the female data set. Along CV1, changes in the wing shape of females corresponded to the displacement of the same landmarks as in the males, particularly landmarks 5, 6, 7 and 10, which displaced in the same direction as in males but with less magnitude (Fig. 7a). Changes
along CV2 (Fig. 7b) included the displacement of landmark 10 towards the base of the wing, and landmarks 3, 4, 5 and 6 outward the wing.

Similarly to what was done for the males an ANOVA was conducted to test for differences in shape between the three groups. The results indicated that the type of venation have significant effect on the female wing shape (df=32, Pillai’s trace=1.29 $P<.0001$).

**Sexual dimorphism**

The wing shape of males and females within each group differed significantly according to the CVA (Group A: f-m pd=0.042 $P<.0001$; Group B: f-m pd=0.012 $P=0.02$; Group C: f-m pd=0.037 $P<.0001$) and to the Procrustes ANOVA (Group A: df=16, Pillai’s trace= 0.66 $P<.0001$; Group B: df=16, Pillai’s trace= 0.39 $P<.0001$; Group C: df=16, Pillai’s trace= 0.78 $P<.0001$). These results indicate the existence of wing shape sexual dimorphism in *Hamadryas*.

**Phylogenetic signal**

Figure 8 shows transformations in wing shape (scaled to a factor of 10) along the phylogeny as deformations grids for the main clades. Results from the permutation test indicated that only 0.007% of the simulated trees were of equal length or shorter than the observed tree length, demonstrating that wing shape in male *Hamadryas* contains strong phylogenetic signal.

**Discussion**

This study examined the variation in forewing shape in three groups of *Hamadryas* that differ in venation. In the case of males, using CVA and ANOVA we found significant differences in wing shape between the groups of species possessing distinctive venation. Ordination plots showed that the three groups were well separated along the first CV1 (Fig. 4). Species and specimens of Group A were at the positive extreme of this axis and those of Group C at the negative extreme, while those of Group B lay in the middle. These results show significant variation in the forewing shape in male *Hamadryas*, and that there seems to be an association between forewing shape and venation pattern.

According to the RWA (Fig. 3) and the deformation grids of the CVA (Fig. 5a, b), differences in wing shape were consistently associated with the displacement of specific landmarks. Landmarks located at midlength of the distal wing margin such as 5 and 6, and also landmark 10 on the coastal margin, displaced inward and outward respectively. These landmarks
exhibited a larger magnitude of change in species within Group A and their direction of displacement was also congruent among other species in this group. This is evident when the changes in wing were mapped onto the phylogeny (Fig. 8). The main differences among the three venation patterns were all related to the location in the wing where sound is produced. These characters occur at the apex (characters 2, 3, 4; Fig. 1) and distal edge (character 5, Fig. 1) of the discal cell. These observations are congruent with the displacement of the above-mentioned landmarks, suggesting an association between wing shape and wing venation.

Although only male *Hamadryas* produce sound, we found significant differences in wing shape of females from the three groups defined by the male venation patterns (Fig. 6). Furthermore, the deformation grid along the first canonical variate showed that the major changes in female wing shape corresponded accurately to the changes implied by the CV1 of males (compare Fig. 5a to Fig. 7a). Since females do not produce sound and do not possess all of the venation components for sound production, we did not expect to find significant differences in wing shape. Nonetheless, females of species in Groups A and C exhibited intermediate states in some venation characters involved in sound production (Fig. 1, Table 2), characters 2, 3 and 4 for Group A, and character 3 for Group C. Furthermore, females of *H. epinome, H. iphthime* and *H. fornax*, assigned to Group A, were polymorphic for character 5 (Fig. 1). Genetic correlation between sexes in venation and wing shape is a possible explanation for this finding. In his study of the effects of mating behavior on male morphology Wickman (1992) predicted that only male design would be affected by mating system. Nevertheless, he found that the male mating system affected females in the same variables and in the same direction as males, suggesting genetic correlation between the sexes. Since sound is used for sexual recognition, it is likely that venation suitable for sound production is under strong selection. In such cases, if the characters show high genetic correlation (as it is the case of venation in butterflies) females may express such characters to a lesser degree even if they are useless (Lande, 1987). If genetic correlation is a valid explanation for our results, the effect of venation on female wing shape is expected to disappear when the covariance between sexes is removed (as in Wickman, 1992).

 Females of the three venation groups showed distinctive wing shapes, and the modifications in wing shape were congruent with those of the males. However, differences between groups were smaller. Although our results suggest that venation is affecting the females' wing shape in a similar manner (by the displacement of the same landmarks) as in males, it is happening to a lesser degree. Resulting in wing shape sexual dimorphism, among males and females within
each venation group. Flight mediates activities such as foraging, predator evasion, male-male and male-female interactions, and search for oviposition sites (Wiklund, 2003). If selection operates to maximize flight efficiency and minimize energy expenditure as suggested by DeVries et al. (2010), finding that males and females have slightly different wing shape is not surprising.

This study found strong phylogenetic signal in the landmark data, and the permutation test indicated that the phylogenetic signal for wing shape is statistically significant. The null hypothesis of this test is the complete absence of any phylogenetic signal and its rejection indicates that wing shape of closely related species tend to be more similar to each other than to distantly related taxa (see Klingenberg and Gidaszewski, 2010). This result supports the RWA and CVA analyses, given that closely related species tended to be near each other in morphometric space (Fig. 3, 4, 6). Phylogenetic signal in the landmark data suggests that differences in wing shape could be due to common ancestry. Since both wing shape and venation are distributed in the same way in the phylogeny, by extension venation might also be associated with phylogeny (see Garzón-Orduña, 2012, Chapter 1 and 2). Finally, it is possible that other factors also affected the evolution of wing shape to a lesser degree, the potential effect of behavior (e.g. aerial interactions) or other anatomical features (e.g. patches of scent scales) cannot be ignored.

Production of sound in Hamadryas depends entirely on venation (including the deformation of the wing membrane around these veins) and occurs specifically during aerial interactions (spiral flights and chases). In this chapter we have shown that distinctive patterns of wing venation in Hamadryas also correspond to different wing shapes, which is a first step towards the formulation of other questions regarding correlated character changes. An obvious one is to test whether these differences in wing shape affect flight performance and if so, to what extent. This is particularly interesting because it seems that aerial interactions are a less important component of the sexual recognition of species that make sound than for those that do not (Otero, 1988).
Acknowledgements

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Table 3.1 Combination of characters states of each group (venation pattern) for male and female *Hamadryas*. Group A includes the species of *Hamadryas* that produce sound, species in Groups B and C do not produce sound. The asterisks refer to notes at specific characters in Figure 1.

<table>
<thead>
<tr>
<th>Character/Venation pattern</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
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<tbody>
<tr>
<td></td>
<td>M</td>
<td>F</td>
<td>M</td>
</tr>
<tr>
<td>Character 1</td>
<td>1</td>
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<td>0</td>
</tr>
<tr>
<td>Character 2</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Character 3</td>
<td>1 1*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Character 4</td>
<td>1 1*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Character 5</td>
<td>1 01*</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 3.2 Number of specimens per species, sex and venation groups. A: sound venation, B: no sound venation pattern I, C: no sound venation pattern II.

<table>
<thead>
<tr>
<th>Species</th>
<th>M</th>
<th>F</th>
<th>Total per species</th>
<th>A</th>
<th>B</th>
<th>C</th>
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</tr>
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<td>10</td>
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</tr>
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<td>H. iphthime</td>
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<td>159</td>
<td>356</td>
<td>185</td>
<td>122</td>
<td>49</td>
</tr>
</tbody>
</table>
Figure 3.1 Description of the three venation patterns found in *Hamadryas*. Venation characters are plotted in the same color across the different patterns to facilitate the comparison. Group A corresponds to the species of *Hamadryas* that produce sound. Species in Groups B and C do not produce sound.

Character 1. In males, FW veins R and Rs$_1$ (Jenkins, 1983): (0) separated, (1) share a common stem.
Note: females of all species exhibit character state 0.

Character 2. In males, FW Rs-Rs$_{2+3+4}$, Rs$_{2+3+4}-$M$_1$, and M$_1$-M$_2$: (0) same width as other veins, (1) fully swollen, (2) thick but not swollen.
Note: The coespecific females of males *Hamadryas* with venation pattern A have these crossveins thick but not swollen. They are however thicker than in the coespecific females of males with venation pattern B or C.

Character 3. In males, FW vein M$_1$: (0) arising from the same point as Rs$_{2+3+4}$; (1) arising at midpoint length between Rs$_{2+3+4}$ and M$_2$.
Note: *The coespecific females of males *Hamadryas* with venation pattern A and C have M$_1$ arising distally from Rs$_{2+3+4}$ however not as distal as in the males. Coespecific females of males *Hamadryas* with venation pattern B exhibit character state 0.

Character 4. In males, FW vein M$_2$: (0) mildly curved towards M$_3$, (1) conspicuously curved towards M$_3$.
Note: *The coespecific females of males *Hamadryas* with venation pattern A have M$_2$ bowed towards M$_3$ however to a lesser degree than in their coespecific males.

Character 5. In males, FW crossvein M$_2$-M$_3$ (Jenkins, 1983): (0) joins the M$_5$-CuA$_1$ fork, (1) joins the Cu$_1$-Cu$_2$ crossvein, (2) joins M$_3$.
Note: females of all the species show the same state as their coespecific males (*some females of *H. iphtheim*, *H. epinome* and *H. formax* show state 0). Character state 2 is exclusive of *H. chloe*, *H. albicornis* and *H. atlantis*. 
Figure 3.2 Position of the landmarks in an exemplar specimen of *Hamadryas*. None of the landmarks corresponded to the venation components involved in sound production.
Figure 3.3 Scatterplot of the Relative Warp Analysis. Deformation curves of extreme individuals are featured to visualize the location of the major wing deformations across the morphospace.
Figure 3.4 Results of the Canonical variates analysis for the males. Dots are label according to the three species Groups.
Figure 3.5 Deformation grids of Canonical Variate 1 (a) and Canonical Variate 2, (b) for the males. Transformation grids are scaled to a factor of 10.
Figure 3.6 Results of the Canonical variates analysis for the females. Dots are labeled according to the three patterns of venation.
Figure 3.7 Deformation grids of Canonical Variate 1 (a) and Canonical Variate 2, (b) for the females. Transformation grids are scaled to a factor of 10.
Figure 3.8 Deformation of wing shape along the phylogeny of *Hamadryas*. The featured deformations are the changes in the wing shape using the node in green as the start node and the corresponding node in black as the targeted node. The names of the terminals are colored according to their venation pattern. Transformation grids are scaled to a factor of 10.
Appendix 3.1 List of specimens photographed and used in the landmark analysis.

**H. albicornis**
Males: Rio Huallaga, Peru (AMNH).

**H. alicia**
Males: Tocantins, Solimoes, Brazil (UFLMNH); Sao Paulo de Olivenca, Amazonas, Brazil (UFLMNH); Rio Solimoes, Brazil (UFLMNH).
Females: Sao Paulo de Olivenca, Amazonas, Brazil (UFLMNH).

**H. amphichloe**
Males: Rio Coca, Napo, Ecuador, (UFLMNH); San Bernardo, Ecuador (USNMNH); Delta Amacuro, Venezuela (UFLMNH); Zulia, Machiques, Venezuela (UFLMNH); La Altagracia, Dominican Republic (UFLMNH); La Vega, Dominican Republic (UFLMNH); Guanica Forest, Puerto Rico (UFLMNH); Dominican Republic (UFLMNH).
Females: Guayaquil, Ecuador, (USNMNH); Rio Coca, Napo, Ecuador, (UFLMNH); Nopales, Ecuador (UFLMNH); Nopales, Ecuador (UFLMNH); Guanica Forest, Puerto Rico (UFLMNH); Port au Prince, Haiti (UFLMNH); Dominican Republic (UFLMNH); Dominican Republic (UFLMNH); La Vega, Dominican Republic (UFLMNH); La Altagracia, Dominican Republic, (UFLMNH); La Vega, Dominican Republic (UFLMNH); Ecuador, Guayaquil (USNMNH).

**H. amphinome**
Males: Pichincha, Ecuador (UFLMNH); Palenque, Chiapas, Mexico (MPM); Obidos, Para, Brazil (MPM); Guatemala, guazacapan (UFLMNH); El Salvador, San Salvador (UFLMNH); Ecuador, Napo, Puerto Misahuallli (UFLMNH); Misiones, Argentina (UFLMNH); Madre de Dios, Cerro Pantiacolla, Peru (UFLMNH); Colima, Colima, Mexico (UFLMNH); Pichincha, Ecuador (UFLMNH); Prov. Topo, Tungurahua, Ecuador (UFLMNH); Puerto Cabello, Carabobo, Venezuela (UFLMNH); Colima, Colima, Mexico (UFLMNH); Palmar, Manabi, Ecuador (UFLMNH); Pichincha, Ecuador (UFLMNH); E. Santo, Brazil (UFLMNH).
Females: La Crespa, Manabi, Ecuador (UFLMNH); Veracruz, Mexico (MPM); Itaci, Sao Paulo, Brazil (MPM); Los amigos research center, Madre de Dios, Peru (PJDVries. Personal collection); Gatamaco, Veracruz, Mexico (UFLMNH); Hacienda el rodeo, Costa Rica (UFLMNH); Italtuba, Rio Tapajos (UFLMNH); Linares, Espiritu Santo, Brazil (UFLMNH); Junin, Santipo, Peru (UFLMNH); Colima, Colima, Mexico (UFLMNH); Palmar, Manabi, Ecuador (UFLMNH); Pichincha, Ecuador (UFLMNH); E. Santo, Brazil (UFLMNH).

**H. arete**
Males: Sao Paulo, Brazil (MPM); South America, Brazil (MPM); Aracastubo, sao Paulo, Brazil (UFLMNH); Mendes, S. Paulo, Brazil (UFLMNH); Mendes, S. Paulo, Brazil (UFLMNH); Mendes, S. Paulo, Brazil; Paraguay (UFLMNH).
Females: E. Santo, Linhares, Brazil (UFLMNH).

**H. arinome**
Males: Prov. Sucumbios, Garza Cocha, Ecuador (PJDVries personal collection); South America, Peru (UFLMNH); Buena Vista, dept. Santa Cruz, Bolivia (UFLMNH); Beni, Bolivia, (UFLMNH); La Fria, Tachira, Venezuela (UFLMNH); French Guyana (UFLMNH); Sao Paulo de Olivenca, Amazonas, Brazil (UFLMNH); Obidos, Para, Brazil (UFLMNH); Tingo Maria, Peru (UFLMNH); Espirito Santo, Linhares, Brazil (UFLMNH); Tingo Maria, Peru (UFLMNH).
Females: Prov. Sucumbios, Garza Cocha, Ecuador (PJDVries personal collection); Buena Vista, dept. Santa Cruz, Bolivia (UFLMNH); French Guyana (UFLMNH); Obidos, Amazonas, Brazil (UFLMNH); Obidos, Para, Brazil (UFLMNH); Tingo Maria, Peru (UFLMNH); Espirito Santo, Linhares, Brazil (UFLMNH).
**H. atlantis**
Males: Acahuizotla, Mexico (UFLMNH); Morelos, Rancho Viejo, Mexico (UFLMNH); Gro. Acahuizotla (UFLMNH); Sinaloa, Mexico (UFLMNH); No Locality data Spec#992 7039 Kent Wilson Collection 1993 (UFLMNH); Sonora, Mexico (UFLMNH).
Females: Caño de Lobos, Morelos, Mexico (UFLMNH); Guatemala (UFLMNH); San Luis Potosi, Mexico (UFLMNH); Guadalajara, Mexico (UFLMNH).

**H. belladonna**
Males: Perú (UFLMNH); Rio Pichis, Huanuco, Peru (UFLMNH); Sao Paulo de Olivenca, Amazonas, Brazil (UFLMNH); Loreto, Peru (UFLMNH); Sao Paulo, Amazonas (UFLMNH); Huanuco, Rio Pichis, Peru (UFLMNH); Pasco, Rio Palcazu, Peru (UFLMNH); Huanuco, Rio Pichis, Peru (UFLMNH); Huanuco, Rio Pichis, Peru (UFLMNH); Pasco, Rio Palcazu, Peru (UFLMNH); Loreto, Iquitos, Peru (UFLMNH); Rio Palcazu, Peru (UFLMNH); Loreto, Iquitos, Peru (UFLMNH); Loreto, Iquitos, Peru (UFLMNH).

**H. chloe**
Males: Tingo Maria, Peru (Purchased); Garza Cocha, Prov. Sucumbios, Ecuador (PJDVries personal collection); Huanuco, Peru (UFLMNH); Caucalandia, Rondonia, Brazil (UFLMNH); Caucalandia, Rondonia, Brazil (UFLMNH); French Guyana (UFLMNH); Rondonia, Brazil (UFLMNH); Rondonia, Brazil (UFLMNH); S. Arquimes, Rondonia, Brazil (UFLMNH); Females: Recife, Pernambuco, Brazil (UFLMNH); Tingo Maria, Peru (Purchased); Obidos, Para, Brazil (UFLMNH); S. Arquimes, Rondonia, Brazil (UFLMNH); S. Arquimes, Rondonia, Brazil (UFLMNH); S. Arquimes, Rondonia, Brazil (UFLMNH); Tingo Maria, Peru (UFLMNH).

**H. epinome**
Males: Ituzaingo, Argentina (UFLMNH); Sao Bento do sul, Santa Catarina, Brazil (UFLMNH); Nova Friburgo, Rio do Janeiro, Brazil (UFLMNH); Urundel, Salta, Argentina (UFLMNH); Sao Bento do sul, Santa Catarina, Brazil (UFLMNH); Col. Independencia, Paraguay (UFLMNH); Salta, Urudel, Argentina (UFLMNH); Salta, Urudel, Argentina (UFLMNH); Salta, Urudel, Argentina (UFLMNH); Sao Paulo, Araras, Brazil (UFLMNH); Misiones, El dorado, Argentina (UFLMNH); Rondonia, Brazil (UFLMNH); Sao Lua do Parana, Parana, Brazil (UFLMNH).
Females: Ituzaingo, Argentina (UFLMNH); Sao Bento do sul, Santa Catarina, Brazil (UFLMNH); Sao Bento do sul, Santa Catarina, Brazil (UFLMNH); Ituzaingo, Argentina (UFLMNH); Sao Bento do sul, Santa Catarina, Brazil (UFLMNH); Encarnacion, Paraguay (UFLMNH); E. Santo, Baixo Guandu (UFLMNH); S. Anna, Misiones, Argentina (UFLMNH).

**H. februa**
Males: Pinhal, Brazil (MPM); Ituzaingo, Argentina (UFLMNH); Encarnacion, Paraguay (UFLMNH); Canal Zone, Panama (UFLMNH); Delta Amacuro, Venezuela (UFLMNH); Arroyos, Paraguay (UFLMNH); Sao Bento do sul, Santa Catarina, Brazil (UFLMNH); M. Grosso, Brazil (UFLMNH); Nicaragua (UFLMNH); La Libertad, El Salvador (UFLMNH); La Libertad, El Salvador (UFLMNH); San Cristobal, Guatemala (UFLMNH); San Cristobal, Guatemala (UFLMNH); Rondonia, Brazil (UFLMNH).
Females: Managua, Nicaragua (MPM); Pinhal, Brazil (UFLMNH); Sonora, Mexico (UFLMNH); Gualima de alajuela, Costa Rica (UFLMNH); Arroyos, Paraguay (UFLMNH); Encarnacion, Paraguay (UFLMNH); Canal Zone, Panama (UFLMNH); Colon, Panama (UFLMNH); Canal Zone, Farfan, Panama (UFLMNH); Sao Paulo, Santa do Paranaiba, Brazil (UFLMNH); E. Santo Baixo Guandu, Brazil (UFLMNH); E. Santo Baixo Guandu, Brazil (UFLMNH); Oxaca, Mexico (UFLMNH); Piste, Yucatan, Mexico (UFLMNH); Sinaloa, Mexico (UFLMNH); La Libertad, El Salvador (UFLMNH); La Libertad, El Salvador (UFLMNH); Mazatlan, Mexico (UFLMNH).
H. feronia
Males: Tingo Maria, Peru (UFLMNH); Pichincha Providence, Ecuador (UFLMNH); San Miguel de los Bancos, Ecuador (UFLMNH); East Ecuador (MPM); Garza Cocha, Prov. Sucumbios, Ecuador (PJDVries personal collection); Chiapas, Mexico (UFLMNH); Oxaca, Mexico (UFLMNH); Oxaca, Chiltepec, Mexico (UFLMNH); Mapastepec, Mexico (UFLMNH); Darien, Panama (UFLMNH); Pomeroon, Guyana (UFLMNH); Tachira, La fria, Venezuela (UFLMNH); Zulia, Maquines, Venezuela (UFLMNH); Zulia, Machiques, Venezuela (UFLMNH); Mader de Dios, Peru (UFLMNH); Junin, Peru (UFLMNH); Itaituba, Rio Tapajos, Amazonas, Brazil (UFLMNH); Huanuco, Peru (UFLMNH); Monagas, Venezuela (UFLMNH); San Jeronimo, Chiaapas, Mexico (UFLMNH); Cordoba, veracruz, Mexico (UFLMNH); Rio Maizito, Manabi, Ecuador (UFLMNH); Oxaca, Mexico (UFLMNH); Tinalandia, Ecuador (UFLMNH).
Females: Satipo, Junin, Peru (UFLMNH); Pichincha Providence, Ecuador (UFLMNH); Colombia (MPM); Ocoyoacac, Mexico (UFLMNH); East Brazil (MPM); Oxaca, Mexico (UFLMNH); Chiapas, Palestina, Mexico (UFLMNH); Atenas, Costa Rica (UFLMNH); Puerto de la Cruz, Costa Rica (UFLMNH); Canal Zone, Piña, Panama (UFLMNH); Valle del Cauca, Colombia (UFLMNH); Beni, Bolivia (UFLMNH); Guanabara, Brazil (UFLMNH); Rio Mai, Brazil (UFLMNH); Monagas, Venezuela (UFLMNH); Rondonia, Brazil (UFLMNH); Pichincha, Ecuador (UFLMNH); Monagas, Venezuela (UFLMNH); Rondonia, Brazil (UFLMNH).

H. fornax
Males: South America, Peru (MPM); Satipo, Peru (UFLMNH); Satipo, Peru (UFLMNH); Satipo, Junin, Peru (UFLMNH); Presidio, Veracruz, Mexico (UFLMNH); Villavicencio, Colombia (UFLMNH); Darien, Panama (UFLMNH); Presidio, Veracruz, Mexico (UFLMNH); Tierra Blanca, Mexico (UFLMNH); Allutiquia (UFLMNH); Veracruz, Fortin de las Flores, Mexico (UFLMNH); Las Yungas, Bolivia (UFLMNH); Tingo Maria, Peru (UFLMNH); Tierra blanca, Mexico (UFLMNH).
Females: Santa Catarina, Brazil (MPM); Presidio, Veracruz, Mexico (UFLMNH); Sao Luiz do Para, Curitiba, Brazil (UFLMNH); Veracruz, Fortin de las Flores, Mexico (UFLMNH); Santa Catarina, Sao Bento do Sul (UFLMNH); Sao Paulo, Campinas, Brazil (UFLMNH); Santa Catarina, Sao Bento do Sul (UFLMNH); Santa Catarina, Sao Bento do Sul (UFLMNH); Santa Catarina, Sao Bento do Sul (UFLMNH); Santa Catarina, Sao Bento do Sul (UFLMNH).

H. glauconome
Males: Palo Verde, Costa Rica (Collected by the author); Managua, Nicaragua (MPM); Chiapas, Mexico (UFLMNH); Morelos, Rancho Viejo, Mexico (UFLMNH); Morelos, Rancho Viejo, Mexico (UFLMNH); Acapulco, Mexico (UFLMNH); La Libertad, El Salvador (UFLMNH); Jutiapa, Guatemala (UFLMNH); Sonora, Alamos, Mexico (UFLMNH); Guantanamo, Cuba (UFLMNH); Managua, Nicaragua (UFLMNH); Atene, Costa Rica (UFLMNH).
Females: Palo Verde, Costa Rica (Collected by the author); Finca El Refugio, Ahuachapan, El Salvador (purchased); El Carrizal, Veracruz, Mexico (UFLMNH); Gro. Coastal area, Mexico (UFLMNH); S. Jalapa, Mexico (UFLMNH); Tierra Blanca, Veracruz, Mexico (UFLMNH); Jutiapa, Guatemala (UFLMNH); Colima, Colima, Mexico (UFLMNH); Gro. Coastal area, Mexico (UFLMNH); Mexico (UFLMNH); Ocoyoacac, Mexico (UFLMNH); San Francisco, Veracruz, Mexico (UFLMNH).

H. guatemalena
Males: Hacienda Palo Verde, Costa Rica (Collected by the author); Parque sta. Rosa, Guanacaste, Costa Rica (UFLMNH); Nayarit, Mexico (UFLMNH); Colima, Colima, Mexico (UFLMNH); Petatlan, Mexico (UFLMNH); Sinaloa, Mexico (UFLMNH); Petatlan, Mexico (UFLMNH); La Libertad, El Salvador (UFLMNH); Atenas, Costa Rica (UFLMNH); La Libertad, El Salvador (UFLMNH); Oxaca, Mexico (UFLMNH).
Females: Mexico (USNMNH); El Sol, San Luis Potosi, Mexico, (UFLMNH); Colima, Colima, Mexico, (UFLMNH); Chiapas, Mexico (UFLMNH); Nicaragua, (UFLMNH); La Libertad, El Salvador (UFLMNH); La Libertad, El Salvador (UFLMNH); xxx, (UFLMNH); Oxaca, Mexico (UFLMNH).
H. iphthime
Males: Huanuco, Peru (UFLMNH); Huanuco, Peru (UFLMNH); Chiapas, palenque, Mexico (UFLMNH); Oxaca, Mexico (UFLMNH); El Sol San Luis Potosí, Mexico (UFLMNH); Chiapas, Mexico (UFLMNH); Tingo Maria, Peru (UFLMNH); Huanuco, Peru (UFLMNH); E. Santo, Linhares, Brazil (UFLMNH); Tingo Maria, Peru (UFLMNH).
Females: Chiapas, Mexico (UFLMNH); Oxaca, Mexico (UFLMNH); Oxaca, Mexico (UFLMNH); Oxaca, Tuxtepec, Mexico (UFLMNH); Yucatan, Mexico (UFLMNH); E. Santo, Brazil (UFLMNH); E. Santo, Brazil (UFLMNH).

H. julitta
Males: Piste, Yucatan, Mexico (UFLMNH); Piste, Yucatan, Mexico (UFLMNH); Piste, Yucatan, Mexico; Piste, Yucatan, Mexico (UFLMNH); Piste, Yucatan, Mexico (UFLMNH).
Females: Yucatan, Mexico (UFLMNH); Piste, Yucatan, Mexico (UFLMNH); Piste, Yucatan, Mexico (UFLMNH); Piste, Yucatan, Mexico (UFLMNH); Piste, Yucatan, Mexico (UFLMNH); Merida, Yucatan, Mexico (UFLMNH); X-can, Mexico (UFLMNH); Piste, Yucatan, Mexico (UFLMNH); Piste, Yucatan, Mexico (UFLMNH); Piste, Yucatan, Mexico (UFLMNH); Piste, Yucatan, Mexico (UFLMNH); Piste, Yucatan, Mexico (UFLMNH).

H. laodamia
Males: Peru, Tingo Maria (UFLMNH); Prov. Sucumbios, Garza Cocha, Ecuador (UFLMNH); Oxaca, Mexico (UFLMNH); Catemaco, Playa Azul, Veracruz, Mexico (UFLMNH); Dos Amantes, Veracruz, Mexico (UFLMNH); Chiapas, Mexico (UFLMNH); OAX. Tuxtepec, Mexico (UFLMNH); Mayan Indian Ruins, Chiapas, Mexico (UFLMNH); Huanuco, Tingo Maria, Peru (UFLMNH); Huanuco, Tingo Maria, Peru (UFLMNH); S. Ariquemes, Rondonia, Brazil (UFLMNH); St. Ann's Trinidad (UFLMNH); Rio Mai, Brazil (UFLMNH); Baixo Guandu, E. Santo, Brazil (UFLMNH); Tingo Maria, Peru (UFLMNH); Satipo, Junin, Peru (UFLMNH).
Females: Itacoatiara, Amazonas, Brazil (UFLMNH); Huanuco, Tingo Maria, Peru (UFLMNH); Madre de Dios, Peru (UFLMNH); Baixo Guandu, E. Santo, Brazil (UFLMNH); Tingo Maria, Peru (UFLMNH); Tingo Maria, Peru (UFLMNH); Tingo Maria, Peru (UFLMNH); Satipo, Junin, Peru (UFLMNH); Heredia, Costa Rica (UFLMNH); Oxaca, Mexico (UFLMNH).

H. velutina
Males: Loreto, Peru (UFLMNH); S. Ariquemes, Rondonia, Brazil (UFLMNH); S. Ariquemes, Rondonia, Brazil (UFLMNH); Obidos, Para, Brazil (UFLMNH); Obidos, Para, Brazil (UFLMNH); Obidos, Para, Brazil, (UFLMNH); Loreto, Peru (UFLMNH); Iquitos, Loreto, Peru, (UFLMNH).
Females: Obidos, Para, Brazil (UFLMNH); Obidos, Para, Brazil (UFLMNH); Obidos, Para, Brazil, (UFLMNH); Obidos, Para, Brazil, (UFLMNH); Obidos, Para, Brazil, (UFLMNH).
Vita

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