UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DE BIOLOGIA

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"DELIMITANDO ESPÉCIES: CONTRIBUIÇÃO DE MARCADORES MORFOLÓGICOS E MOLECULARES PARA A COMPREENSÃO DO GÊNERO HERMEUPTYCHIA FORSTER (NYMPHALIDAE: SATYRINAE: EUPTYCHIINA)"

Dissertação apresentada ao Instituto de Biologia para obtenção do Título de Mestre em Ecologia.

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Co-Orientador: Prof. Dr. André Victor Lucci Freitas

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"Is, then, the species a part of the 'order of nature', or part of the order-loving mind?" (Dobzhansky 1935)

"In short, we shall have to treat species in the same manner as those naturalists treat genera, who admit that genera are merely artificial combinations made for convenience. This may not be a cheering prospect; but we shall at least be freed from the vain search for the undiscovered and undiscoverable essence of the term species." (Darwin 1959)

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ABREVIAÇÕES UTILIZADAS

mtDNA	mitochondrial DNA
nDNA	nuclear DNA
cox 1	subunit I of the citochrome c oxidase gene
nad6	subunit 6 of the nicotinamide adenine dinucleotide dehydrogenase gene
RpS5	subunit 5 of the ribosomal protein
EDTA	Ethylenediamine tetra acetic acid
PCR	Polymerase Chain Reaction
DMSO	Dimetilsulfoxid
NJ	Neighbor-Joining
BI	Bayesian Inference
MP	Maximum Parsimony analysis
PBS	Partitioned Bremer Support
RI	Retention Index
CI	Consistence Index
Conf.I.	Confidence Interval
LR	Likelihood Ratio
MEFLG	Museo Entomológico Francisco Luis Gallego, Medellín, Colombia
MHNSM	Museo de Historia Natural, Universidad Nacional Mayor de San Marcos, Lima, Peru
FLMNH	Florida Museum of Natural History, Gainesville, FL, USA
MZUEC	Museu de Zoologia da Universidade Estadual de Campinas - UNICAMP
BMNH	The Natural History Museum, London, England
ZMB	Zoologische Museum Berlin (Museum für Naturkunde, Zoological Collection), Berlin, Germany
AVFL (collection)	André Freitas' collection at Unicamp
NSP (collection)	Author's collection, testimonial material will be deposited in MZUEC
CO	Colombia
EC	Ecuador
BR	Brazil
US	United States

RESUMO

O gênero *Hermeuptychia* Forster (Nymphalidae, Satyrinae, Euptychiina) está amplamente distribuído no continente Americano, desde a Argentina até o sul dos Estados Unidos. O gênero foi anteriormente considerado um complexo de espécies, e atualmente são reconhecidas oito espécies . Todas as espécies possuem um padrão alar muito parecido, o que compromete a identificação taxonômica correta. Em adição, a posição filogenética do gênero dentro da subtribo Euptychiina permanece incerta. Para o presente estudo foram obtidos espécimens de 45 localidades de cinco países, com maior ênfase em uma amostragem no Brasil. Três marcadores moleculares, dois do DNA mitocondrial (*cox1* 5' e *nad6*) e um do DNA nuclear (RpS5) foram utilizados para gerar hipóteses filogenéticas (Máxima Parcimônia e Inferência Bayesiana), para delimitar espécies, e para gerar estimativas de tempo de divergência e distribuição ancestral. Adicionalmente, o desempenho da região anterior da *cox1* como '*barcode* – código de barras*'* para delimitar as espécies de *Hermeuptychia* foi testado. Além disso, análise morfológica da genitália masculina foi empregada para a delimitação e identificação de espécies. Os indivíduos amostrados agruparam-se em dez clados nas análises moleculares, correspondendo a sete espécies reconhecidas mais *H. gisella,* que havia sido anteriormente sinonimizada com *H. cucullina*. A análise morfológica dos indivíduos possibilitou o estabelecimento de caracteres diagnósticos para todas as espécies de *Hermeuptychia* – incluindo *H. cucullina,* que não está presente nas análises moleculares – e concordou com os agrupamentos obtidos através das análises moleculares. As relações filogenéticas entre as espécies de *Hermeuptychia* permanecem incertas, possivelmente devido a um padrão de evolução rápida, descrito anteriormente para outros Satyrini. Entretanto, dois grupos de espécies-irmãs podem ser identificados, *H. pimpla* + *H. harmonia*, e *H. gisella* + *H. fallax*, ambos sustentados por ocorrência simpátrica. Em adição, *H. gisella* e *H. fallax* parecem apresentar um isolamento reprodutivo incompleto, com formação de híbridos. Algumas espécies de *Hermeuptychia* estão distribuídas amplamente, como *H. atalanta, H. hermes* e *H. gisella*, sendo que *H. atalanta* é a espécie mais comum encontrada no Brasil. *H. fallax* é uma espécie restrita a Mata Atlântica; *H. pimpla* e *H. harmonia* são espécies restritas à região andina, encontradas em altitudes moderadas; *H. maimoune* pode ser encontrada na região andina e no sul da Amazônia brasileira, correspondendo a duas espécies crípticas; *H. cucullina* foi encontrada no centro-oeste brasileiro e na região andina, e é a espécie mais rara de *Hermeuptychia*; e *H. sosybius* pode ser encontrada do sul dos Estados Unidos até a região norte da Colômbia. O gênero diversificou-se de seu grupo-irmão a cerca de 8,2 milhões de anos (mya), a diversificação das espécies ocorreu entre 3,5 e 1,4 mya, e a distribuição ancestral estimada é a cordilheira dos Andes. Apenas com a região 'barcode' e análise de distância usando Neighbor-Joining, foi possível separar as espécies de *Hermeuptychia* com uma taxa de 2% de erro. O limite entre as distâncias intra e interespecíficas estimado fica em torno de 2% de divergência genética.

PALAVRAS-CHAVE: Delimitação de espécies, marcadores moleculares, Nymphalidae, hibridização, Lepidoptera.

ABSTRACT

The *Hermeuptychia* genus Forster (Nymphalidae: Satyrinae: Euptychiina) is widely distributed in the American continent, from Argentina to South United States. Previously considered a species complex, the genus presents eight valid species taxa at the moment. Wing pattern is very similar in all *Hermeuptychia* species resulting in difficult and prone to error taxonomic identification. Additionally its position within the subtribe Euptichiina remains uncertain. Samples from 45 locations in five countries, with major emphasis in Brazilian territory sampling, were obtained for the present study. Three molecular markers, two from mitochondrial DNA (*cox1* 5' and *nad6*) and one from nuclear DNA (RpS5), were used to generate phylogenetic hypothesis (Maximum Parsimony and Bayesian Inference), to delimit species, and to estimate divergence time and ancestral distributions. The 'barcode' region performance (*cox1* 5') was tested for *Hermeuptychia* species. Male genitalia morphology was also used to identify and delimitate species. Sampled individuals are grouped in ten molecular clusters, corresponding to seven valid species and *H. gisella*, previously synonymized to *H. cucullina*. Morphological analysis of individuals revealed morphological diagnose traits to identify all *Hermeuptychia* species, including *H. cucullina*, which is not present in the molecular analysis, and was congruent with molecular analysis. Phylogenetic relationships among *Hermeuptychia* species remain unresolved due to a possible rapid evolutionary pattern common to Satyrini. However, two pairs of sister species could be identified: *H. pimpla* + *H. harmonia* and *H. gisella* + *H. fallax*, both sympatric. Additionally, *H. gisella* + *H. fallax* present incomplete reproductive isolation, with hybrids. Some *Hermeuptychia* are widely distributed as *H. atalanta, H. hermes*, and *H. gisella*, and *H. atalanta* is the most common species found in Brazil. *H. fallax* is restricted to Atlantic Forest; *H. pimpla* and *H. harmonia* are restricted to Andes region, at moderately high altitudes; *H. maimoune* is found in the Andine and in the Amazonian regions, corresponding to two cryptic species; *H. cucullina* is the rarest *Hermeuptychia* and was found in Central Brazil and in Andes; and *H. sosybius* is the only *Hermeuptychia* found in North America, been present from South United States to north Colombia. The genus diverged from its sister group around 8.2 my, species diversification occurred between 3.5 and 1.4 my and the ancestral estimate distribution is Andine region. Only the 'barcode' region was able to identify each *Hermeuptychia* species, with an 2% error rate and the molecular threshold for intra and interspecific distance was around 2% of genetic divergence.

KEYWORDS: Species delimitation, Molecular markers, Nymphalidae, Hybridization, Lepidoptera

INTRODUÇÃO GERAL

1.1 O conceito de espécie

O conceito de espécie é uma das grandes questões da história da ciência. Desde a publicação do primeiro e mais influente livro de Darwin (1859), biólogos e naturalistas têm debatido exaustivamente o assunto sem chegar a um consenso (Wilkings 2011). Entretanto, como definir espécies, e as fronteiras entre elas, sem tocar nesse delicado assunto?

Essa discussão é relevante para qualquer trabalho em ecologia ou taxonomia, mas nem sempre recebe o devido cuidado. É necessário decidir previamente por uma definição de espécie quando o trabalho implica a separação de organismos em grupos, e muitas vezes esse cuidado não existe por parte de ecólogos e taxonomistas ao estabelecerem seus métodos de trabalho. Entretanto, o conceito por trás da classificação escolhida pode ter implicações importantes especialmente em trabalhos de riqueza de espécies ou de macroecologia (Isaac *et al.* 2004).

Em seu livro "Evolução", Ridley (1996) discute sete conceitos de espécies, o mesmo número apresentado em um apêndice contendo críticas aos conceitos no livro de Coyne & Orr (2004). Mayden (1997) vai ainda mais longe, enumerando 24 conceitos em seu capítulo e, finalmente, Wilkings (2011) enumera 27 definições de espécie. Todos esses conceitos podem ser divididos em duas categorias (como proposto por Hey 2001 e por Coyne & Orr, 2004): conceitos que pressupõem um processo evolutivo e conceitos que não pressupõem nenhum processo. Na primeira categoria encontramos o conceito biológico de espécie, o conceito ecológico e o conceito filogenético de espécie, entre outros. Na segunda categoria encontram-se os conceitos nominais de espécie, baseados em formas identificação das espécies pelos taxonomistas. Dentro dessa segunda categoria encontra-se a visão de Darwin, também defendida atualmente por Mallet (1995), com algumas alterações necessárias para o acréscimo do conhecimento atual de genética e filogenia.

Um conceito nominalista traz vantagens ao trabalho do taxonomista (Mallet 1995), sendo que a maior delas é a liberdade para a definição de espécie baseada em padrões de convergência morfológica e genética, sem uma preocupação com os pressupostos de modelos evolutivos (por exemplo, isolamento reprodutivo). A outra grande vantagem é justamente a capacidade de estudar os padrões evolutivos da especiação sem que um argumento circular seja invocado (Mallet 1995).

Mas e o papel do modelo de especiação? Recentemente Mallet *et al.* (2007) trabalharam com hibridização natural e artificial entre espécies de heliconiine para definir o papel do isolamento

reprodutivo na especiação. O trabalho inclui um total de 22 cruzamentos entre 22 espécies, totalizando 161 híbridos gerados. Além dos cruzamentos, o artigo traz uma árvore filogenética das espécies estudadas construída com DNA mitocondrial. Quando comparados o número de híbridos produzidos (em escala logarítmica), com a distância genética média entre as espécies parentais, foi obtida uma reta de inclinação negativa ($p < 0,05$). É interessante notar que foram incluídos nessa análise híbridos intraespecíficos (variedades) e interespecíficos, e que os dados sugerem um limite específico em torno de 2% de divergência genética, sendo que a partir desse marco o número de híbridos produzidos cai drasticamente. Esse trabalho sugere que híbridos são raros em números de indivíduos (0,05%), mas que o processo é comum entre as espécies desse grupo de borboletas (cerca de 26-29% das espécies apresentam híbridos). Essas descobertas são condizentes com uma visão do isolamento reprodutivo como um subproduto da divergência genética e da especiação, ao contrário da visão tradicional do conceito biológico de espécie, em que o isolamento é a causa da especiação.

Diante do exposto acima, e com o atual conhecimento nesse campo de estudo, parece razoável utilizar um conceito de espécie que seja independente de um modelo de especiação e que torne mais fácil a tarefa de identificação dos taxonomistas. O presente estudo pretende aplicar o conceito nominal de espécie, proposto inicialmente por Darwin e alterado por Mallet (1995), para permitir que se acrescente o uso das ferramentas moleculares na identificação de espécies e consequentemente no estudo de suas fronteiras.

1.2 Uso de marcadores moleculares como auxiliares na resolução de problemas taxonômicos.

Ao longo das últimas décadas o uso de sequências de DNA e RNA acrescentou-se à taxonomia clássica, auxiliando na definição e delimitação de espécies (DeSalle *et al.* 2005; Vogler & Monaghan 2006; Silva-Brandão *et al.* 2009). Embora o uso da taxonomia molecular (*DNA taxonomy*) tenha recebido muitas críticas (Will & Rubinoff 2004; Wheeler 2005), o uso de marcadores moleculares é recomendado como uma ferramenta adicional (Miller *et al.* 1997; Silva-Brandão *et al.* 2009), uma vez que pode trazer luz a casos em que a morfologia é de difícil análise (Pfenninger *et al.* 2007, Damm *et al.* 2010) e ainda pode ser utilizada como uma ferramenta extra para testar os resultados obtidos com morfologia e biogeografia (DeSalle *et al.* 2005). Para uma análise taxonômica molecular adequada, é preciso empregar amostragens amplas, diversas regiões do DNA, análises rigorosas e integração entre os resultados moleculares e evidências da morfologia e biogeografia (Brower 2006). Marcadores

moleculares também podem servir para identificar táxons que precisem de revisão taxonômica e auxiliar na escolha de amostras para a realização de tais estudos (Hajibabaei *et al.* 2007).

O uso de um marcador molecular padronizado pode trazer a vantagem de possibilitar um acúmulo maior de informações e a comparação entre diferentes estudos em grupos taxonômicos próximos ou distantes. O DNA mitocondrial tem sido muito utilizado devido à sua fácil amplificação e sequenciamento, pequeno tamanho relativo, alta taxa de mutações e ao seu arranjo conservado de genes, o que permite o desenvolvimento de inicializadores universais que possam ser utilizados em uma ampla gama de grupos taxonômicos (Freeland 2006). Além disso, o seu modo de herança, geralmente materna e sem recombinação, permite a identificação de linhagens gênicas de maneira mais direta do que o DNA nuclear (veja exceções em: Gyllensten *et al.* 1991; Kvist *et al.* 2003 para herança paterna; Ladoukakis & Zouros 2001; Burzynski *et al.* 2003; Tsaousis *et al.* 2005 para recombinação em mitocôndrias). Entretanto o uso de mais de um marcador pode acrescentar informações cruciais a um estudo taxonômico (Damm *et al.* 2010), e o uso de marcadores de origem mitocondrial e nuclear pode proporcionar a comparação desses dados revelando ou esclarecendo casos de hibridização (Wahlberg *et al.* 2009). O tamanho da região amostrada também é importante, uma vez que regiões menores apresentam menor variação podendo falhar em diferenciar espécies muito próximas ou com baixa variabilidade (Burns 2007). A escolha dos marcadores empregados e da amostragem pode definir o sucesso ou o fracasso de um esforço de sistemática molecular.

A região anterior da subunidade I da *citocromo oxidase* (*cox* 1), que foi proposta por Hebert (2003a, 2003b) como um código de barras (‘barcode’) molecular para identificar as espécies de animais, tem sido amplamente utilizada em diversos grupos, tornando seus resultados altamente comparáveis (Silva-Brandão *et al.* 2009). Além disso, trata-se de uma região adequada para trabalhar grupos de espécies próximas devido à sua baixa variabilidade intraespecífica e alta variabilidade interespecífica (Avise 2004). Além dessa região de mtDNA, Cameron e Whiting (2008), comparando diferentes genomas mitocondriais de Lepidoptera, sugerem a utilização das subunidades da nicotinamida adenina dinucleotídeo desidrogenase (*nad*) para a separação de grupos de espécies irmãs. Wahlberg e Wheat (2008) propõem 10 genes para o estudo de filogenias, sendo nove deles nucleares. Dentre os genes nucleares propostos, aquele que apresentou maior divergência entre espécies próximas foi a subunidade 5 da proteína ribossomal (RpS5) (Wahlberg, com. pess.), o que o torna, portanto, o mais recomendável dentre os genes estudados por eles para avaliação de padrões sistemáticos de espécies de um mesmo gênero.

1.3 O gênero *Hermeuptychia* Forster

O gênero *Hermeuptychia* foi descrito por Forster em 1964, tendo como tipo *Euptychia hermes* (Fabricius, 1775). Forster reuniu no gênero algumas espécies de coloração marrom homogênea que estavam antes incluídas nos gêneros *Euptychia* e *Neonympha*. A diagnose do gênero foi feita com base em caracteres da genitália masculina, que apresenta valvas retilíneas e alongadas, além de um edeago geralmente longo e fino. Forster examinou principalmente indivíduos provenientes da Bolívia, e na sua descrição original do gênero incluiu nove espécies: *H. hermes* (Fabricius, 1775), *H. gisella* (Hayward, 1957), *H. sosybius* (Fabricius, 1793), *H. fallax* (Felder & Felder, 1862), *H. pimpla* (Felder & Felder, 1862), *H. harmonia* (Butler, 1867), *H. cucullina* (Weymer, 1911), *H.calixta* (Butler, 1877) e *H. narapa* (Schaus, 1902). Neste trabalho, Forster incluiu ilustrações das genitálias masculinas das oito primeiras espécies (sendo que em pelo menos uma delas, *H. hermes*, Forster dissecou o tipo). Recentemente, Lamas (2004) reconheceu os oito primeiros táxons como pertencentes ao gênero *Hermeuptychia*, transferiu *H. narapa* para o gênero *Erichtodes,* incluiu duas novas espécies, *H. atalanta* (Buler 1867) e *H. maimoune* (Butler 1870), e sinonimizou *H. gisella* com *H. cucullina,* e *H. calixta* com *H. harmonia*.

Forster (1964) ressalta a dificuldade de identificação das espécies do gênero baseada apenas no padrão alar, e recomenda que sejam utilizados caracteres de genitália masculina, a mesma recomendação feita por Anken (1995). A grande variação apresentada em padrões alares levou a uma grande quantidade de descrições de espécies ou subespécies novas na literatura (Lamas 2004 apresenta 12 sinônimos às espécies do gênero), não correspondendo necessariamente a novas espécies de fato.

Devido à dificuldade na identificação das espécies do gênero e à ausência de uma revisão adequada, na maioria das listas de espécies publicadas para o Brasil *Hermeuptychia* é representado principalmente pelo nome *Hermeuptychia hermes* (Cardoso 1949; Brown & Mielke 1968; Brown 1992; Motta 2002; Iserhard & Romanowski 2004; Silva *et al.* 2007; Paz *et al.* 2008; Pinheiro *et al.* 2008; Sackis & Morais 2008; Vasconcelos *et al.* 2009 Iserhard *et al.* 2010; Silva *et al.* 2010;), sendo que em poucas listas aparecem também citações de *H. fallax*, *H. harmonia* (Emery *et al.* 2006), *H. calixta* (Brown & Mielke 1967), e *H. cucullina* (Anken 1995). No entanto, estes nomes são pouco confiáveis dada a falta de conhecimento taxonômico das espécies de *Hermeuptychia.*

1.4. Objetivos

A presente dissertação está organizada como capítulo único que permeia questões relacionadas à diferenciação de unidades taxonômicas dentro de um gênero de borboleta comum na região

Neotropical, explorando assuntos que incluem: 1. A caracterização dos limites de espécies dentro do gênero *Hermeuptychia* com base em marcadores morfológicos e moleculares, aplicando o conceito nominal de espécie, proposto inicialmente por Darwin (1859) e alterado por Mallet (1995) para permitir que se acrescente o uso das ferramentas moleculares na identificação de espécies e consequentemente no estudo de suas fronteiras. 2. A avaliação da validade da região proposta como 'barcode' molecular como ferramenta taxonômica para identificar e delimitar espécies em *Hermeuptychia.* 3. O conhecimento dos padrões de distribuição das diferentes espécies reconhecidas para *Hermeuptychia,* com base nos resultados obtidos.

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CAPÍTULO ÚNICO

DELIMITING SPECIES: MORPHOLOGICAL AND MOLECULAR MARKERS CONTRIBUTION FOR THE UNDERSTANDING OF THE GENUS *HERMEUPTYCHIA* FORSTER (NYMPHALIDAE: SATYRINAE: EUPTYCHIINA)

1. INTRODUCTION

In a world that loses biodiversity at an unprecedented rate (Western 1992) taxonomic efforts are of great importance to make conservation decisions based on reliable data (Savage 1995). Molecular-based approaches to taxonomy could provide the acceleration needed in the process of identifying new species (Pons *et al.* 2006) and help to resolve taxonomic problems in poorly known groups (Damm *et al.* 2010). However, taxonomic decisions should be carefully taken to avoid taxonomic inflation (Isaac *et al.* 2004), thus evidence from various sources must be available to justify a new species status (DeSalle *et al.* 2005).

Successfully applied effort for molecular systematics studies must be based on combined evidence from mitochondrial and nuclear DNA in order to avoid the effect of introgression and differences between gene trees and species trees (Vogler & Monaghan 2006; Wahlberg *et al.* 2009b). A broad sampling across the species distribution is also desirable, so the discontinuities or clusters in the molecular data due to spatial distance and incomplete sampling will be not inferred as different species or gene pools (Vogler & Monaghan 2006). Furthermore, to test the results of a molecular systematics approach, or to identify new species, classical taxonomic information must be available, since without it incongruence between gene trees and traditional classifications may be wrongly concluded (Brower *et al.* 1996), rendering species paraphyletic or polyphyletic more frequently than it ought to be (Vogler & Monaghan 2006). Funk and Omland (2003) described that approximately 23% of known species present some degree of polyphyly or paraphyly when molecular identification is compared to morphological taxonomic units. If absence of monophyly is a common trait, identification of species based only on DNA taxonomy could be wrong at the same rate as absence of monophyly is found, because it does not uses morphological characters that could be compared with the type for the species. However, Brower *et al.* (1996) and Vogler and Monaghan (2006) argument that this may not be a result of species lack of monophyly, but a result of poor identification tools, or subjective diagnose characters (Brower 2006).

Hebert *et al.* (2003b) proposed that the 5' portion of the mtDNA gene *cox1*, composed of approximately 660 base pairs, could be used as a tag for species diagnose and delimitation (known as a 'barcode') for animals. Moreover, the 'barcode' region was also proposed as a tool for revealing new undescribed species (Matz & Nielsen 2005), but this approach is far more controversial (DeSalle 2006). The idea of a standard gene region working as a flag for each species, allowing specific identification

without the need of a taxonomic knowledge, is not new (Sperling 2003) but it is genial in itself (Hebert *et al.* 2003a; Hebert *et al.* 2003b). If it really works is still a valid question, although, for Lepidoptera, recent reviews have showed great potential for the use of the proposed 'barcode' region as an identification tool and also as a systematic aid (Silva-Brandão *et al.* 2009). A more recent approach suggests that 'barcodes' could be used as a first survey of taxa for both phylogenetic and population genetics studies, for a first insight into intra and interspecific diversity or as a comparative tool in studies of genetic diversity between species or ecological settings (Hajibabaei *et al.* 2007).

Recently, the use of the 'barcode' region has outnumbered the use of other mitochondrial regions in studies with animals, including many Lepidoptera (Silva-Brandão *et al.* 2009). Nonetheless, availability of complete mitochondrial genomes of several species of Lepidoptera (Cameron & Whiting 2008; Yang *et al.* 2009) has allowed the evaluation of new genes for studies of closely related species. For example, Cameron and Whiting (2008) suggested that the subunits of the nicotinamide adenine dinucleotide dehydrogenase (NADH) may be very useful in phylogenetic studies of closely related species in Lepidoptera.

Until recently, nuclear gene regions with sufficient variability for use in phylogenetics studies of congeneric species, and thus also for species boundaries delimitations, were scarce (Sperling 2003) and the most commonly used nDNA regions (wingless and elongation factor genes) were too slow-evolving for applying in closely related species studies. Wahlberg and Wheat (2008) described six previously unused gene regions suited for phylogenetic use in butterflies. Among them, the RpS5 region showed enough variation among congeneric species to be informative in phylogenetic studies at low taxonomic levels (Wahlberg, pers. comm.)

Butterflies of the family Nymphalidae are a very charismatic and appealing group of insects, with a long history as models for numerous evolutionary and ecological studies (Boggs *et al.* 2003), such as for studies on selection (Ford 1964), speciation (Jiggins *et al.* 2001), species boundaries (Mallet *et al.* 2007), coevolution (Brower 1996) and hybrid zones (Dasmahapatra *et al.* 2002). The nymphalid subfamily Satyrinae is one of the most diverse groups of butterflies (Peña *et al.* 2006), although most of its biodiversity is still poorly known and many species are still unidescribed (Freitas *et al.* 2010). Wahlberg *et al.* (2009a) defined nine tribes within the Satyrinae. Five of which are present in the Neotropics (Haeterini, Amathusini, Morphini, Brassolini and Satyrini), a region that harbors the greatest taxonomic diversity in the subfamily. The Neotropical Satyrini is divided in two subtribes, Euptychiina and Pronophilina, both defined by few morphological traits and geographical distribution

(Miller 1968). Recent studies have failed to define Euptychiina as a monophyletic group (Murray & Prowell 2005; Peña *et al.* 2006; Peña & Wahlberg 2008; Peña *et al.* 2010; Peña *et al.* 2011), and the main problem seems to be the position and polyphyly of the genus *Euptychia* Hübner (Peña *et al.* 2010; Peña *et al.* 2011). Aside from that, there are five consistent clades, named after the most species rich genera included in each of them: *Megisto, Hermeuptychia, Taygetis, Pareuptychia* and *Splendeuptychia*. The *Hermeuptychia* clade is composed of six genera, including the genus *Hermeuptychia* Forster (Peña *et al.* 2010).

The original description of *Hermeuptychia* was based on the morphology of male genitalia, in a comprehensive approach to clarify the confusion in the previously broad genus *Euptychia* (Forster 1964). Since then, no new efforts have been made to clarify the genus, its species relationships or its species identification parameters. Currently the two-century-old descriptions are of little help; many types are mutilated and/or are lost (eg. *H. atalanta, H. calixta, H. pimpla, H. fallax*, previously known to be in BMNH), making the drawings of Forster (1964) the only registers for morphology of male genitalia.

Due to the poor taxonomic knowledge of the group, species identification in *Hermeuptychia* has been done based on the highly variable wing patterns combined with geographical distribution. As a result, many specimens have been wrongly assigned to *Hermeuptychia hermes*, the type species of the genus, leading this species to be documented as the most common and widespread of the genus across its assumed broad range from south US to Argentina.

In the present study, we combined molecular markers, morphology (mainly male genitalia) and a phylogenetic approach to define the species boundaries in the genus *Hermeuptychia*. We discuss possible conflict between nuclear and mitochondrial genes and classic morphological approaches to identify and delimitate species. We also provide new identification tools and propose new distribution ranges for *Hermeuptychia* species based on revised data.

2. Material and Methods

2.1. Sampling

A total of 189 individuals of *Hermeuptychia* were sampled from 45 localities in the Americas (a table of samples data is attached as Att.1). All individuals were identified to genus level based on morphological wing characters. These butterflies are small, with dark brown body and red eyes. The

dorsal wings are entirely dark brown with no conspicuous markings. The ventral wings are crossed by two dark brown lines that extend from the costal part of the forewing to the end of the hindwing. A small incomplete dark brown line is found between these markings in both fore and hindwings. Hindwings present five to six ocelli, black surrounded by beige scales, with variable shape and size, sometimes with a white pupil. Ventral wings back color is dark brown to beige; scales are easy to remove hence the color intensity can be age sensitive.

At least one leg of each specimen was preserved in DMSO buffer (0,25M EDTA; pH 7,5: 20% dimetilsulfoxid, saturated with NaCl – Feinstein 2004) for further DNA extraction. Males had the posterior half of the abdomen cut for morphological analysis of genitalia and the remaining of the body was kept as testimonial material. Colombian samples are deposited in the Museu Entomológico Francisco Luis Gallego (MEFLG - Universidad Nacional de Colombia - Medellín), Ecuadorian samples are deposited in Florida Museum of Natural History - USA (FLMNH), Brazilian samples and US samples are deposited either in Freitas collection at Unicamp or in the author collection. Testimonial material will be deposited in the Museu de Zoologia da Universidade Estadual de Campinas (MZUEC).

2.2 Molecular techniques

The total genomic DNA was extracted using two different protocols. Samples collected during 2007-2008 used fenol-chloroform protocol (modified from - Infante-Vargas & Azeredo-Espin 1995), and for those collected during 2009-2010 the Invisorb Spin Tissue Mini Kit protocol was used. Samples were stored in -20˚C.

2.2.1. Polymerase Chain Reactions (PCRs) and Sequencing

Three different DNA regions were investigated, two mitochondrial (660 bp of the 5' portion of the cytochrome oxidase subunit I gene - *cox1* 5'; and 439 bp of the subunit 6 of the nicotinamide adenine dinucleotide dehydrogenase - *nad6*) and one nuclear (610 bp of the subunit 5 of the ribosomal protein - RpS5). Primers to amplify each region are presented in Table 1. PCRs were standardized for a final volume of 50 µL. *Cox1* 5' amplification reactions contained 20mM of Tris-HCl, 50 mM of KCl, 3mM of $MgCl_2$, 0.5 mg of BSA/ml, 0.2 mM of each primer, 4 nM of each nucleotide base and 1u of Taq DNA-Polimerase (Invitrogen). For *nad6* amplification the $MgCl_2$ concentration in the final reaction mixture was increased to 5mM and we added 0.5% DMSO to the reactions. For RpS5 amplification

$MgCl_2$ concentration was 10mM and it was also added 0.5% DMSO. The programs used follow protocols described in Silva-Brandão *et al* (2008a) for *cox1* 5', Silva-Brandão *et al* (*in press*) for *nad6* and Wahlberg & Wheat (2008) for RpS5.

An aliquot of PCR product was analyzed by electrophoresis through 1% agarose gel stained with ethidium bromide. PCR products were purified by using ExoSAP-IT (GE Healthcare), and then sequenced by ABI Prism BigDye Kit protocol in an ABI 3700 automated sequencer, with forward primers used for amplifications. RpS5 forward and reverse primers were used for sequencing this region. Sequences were analyzed with the program FinchTV 1.4.0 (Geospiza Inc.), and aligned manually using Se-Al v2.0a11 (Rambaut 2002).

Table1. Primers used to amplify the DNA of specimens of *Hermeuptychia.*

GENE	PRIMER	F/R	POSITION, (3')	SEQUENCE (5'→3')
cox1 5'	LCO1490[A]	F	1490[B]	GGTCAACAAATCATAAAGATATTGG
	Nancy-mod[C]	R	2192[B]	CCTGGTAAAATTAAAATATAAACTTC
nad6	tPro-J10090[D]	F	10090[E]	ATCWATAATCTCCAAAATTAT
	ND6-N10624[D]	R	10624[E]	GGNCCATAAAAAATATTWGT
RpS5	HybrpS5degF[F]	F		ATGGCNGARGRAAYTGGAAYGA
	HybrpS5degR[F]	R		CGGTTRGAYTTRGCAACACG

[A] Folmer *et al* 1994; [B] relative position in relation to *Drosophila yakuba* mitochondrial genome (Clary & Wolstenholme, 1985); [C] Silva-Brandão *et al* 2005; [D] Silva-Brandão *et al* (*in press*); [E] Relative position to *Manduca sexta* mitochondrial genome (Silva-Brandão *et al. in press*); [F] Wahlberg & Wheat, 2008. Degenerated primers: N = A, C, T or G; W = A or T; R = A or G; Y = C or T.

2.2.2 Molecular analyses

Outgroups

Eight species were chosen for use as outgroup based on Peña *et al.* (2010): *Godartiana muscosa* (NW127-08), a species that did not group in any clade within Euptychiina; and the seven species belonging to the *Hermeuptychia* clade: *Pharneuptychia innocentia* (CP12-06)*, Zischkaia pacarus* (CP14-02)*, Splendeuptychia boliviensis* (CP02-48)*, S. itonis* (CP02-44)*, Rareuptychia clio* (CP01-23)*, Amphidecta callioma* (NW126-21)*,* and *Euptychia ordinata* (CP01-14). GenBank accession numbers and sample data are attached (Att 2).

2.2.2.1 Initial evaluation of genetic variability

All following analyses were developed using sequences of the 'barcode' region *cox1* 5' from 189 specimens of *Hermeuptychia* sampled throughout the overall distribution of the genus, hereafter called "'barcode' data" (Att. 1).

Descriptive measures

Mean genetic distances were calculated under the MEGA5 program (Tamura *et al.* 2011), and standard error values were obtained by a bootstrap procedure (with 2,000 replicates). Analysis was performed using J-C model (Jukes & Cantor 1969), and all positions containing gaps and missing data were eliminated. The nucleotide substitution model was chosen following Nei and Kumar (2000) recommendation. According to them, for closely related sequences ($d \leq 0.25$ for p distance) all models perform similarly, and therefore the least complex model should be applied to obtain smaller variance. A complementary table for genetic distance based on K2P model (Kimura 1980) can be found in Att 3, since this model is suggested in the original BOLD protocol (Ratnasingham & Hebert 2007).

Distance tree

Distance trees were estimated with the program MEGA v.5 (Tamura *et al.* 2011). Analysis was performed using Neighbor-Joining (NJ) criterion under the K2P model (Kimura, 1980) of nucleotide substitution and also under J-C (Jukes & Cantor 1969) model, and the support of each branch was determined using a nonparametric bootstrapping procedure (Felsenstein, 1985), with 2,000 replicates.

Bayesian Inference

The program MrModeltest v. 2.0 (Nylander 2004) was used to determine the available substitution model with the best fit to this region. The best fit model was found to be the most complex available, i.e., the GTR + G + I [General Time-Reversible model (Rodrígues *et al.* 1990), with gamma distribution (G) and with proportion of invariable sites (I)]. It has been noted that the G shape parameter and the I parameter are highly correlated and are considered to be pathological when estimated together (Ren *et al.* 2005), thus Bayesian Inference analyses (Huelsenbeck & Ronquist 2001; Huelsenbeck *et al.* 2001; Huelsenbeck *et al.* 2002) were carried out for the combined dataset under the GTR + G model, under the program MrBayes (Huelsenbeck & Ronquist 2001). Eight simultaneous

chains were conducted for $20.0x10^6$ generations, sampling trees every 1,000 cycles for a total of 40,000 sampled trees. The first 10,000 trees were discarded as ‘‘burn in’’. *Godartiana muscosa* was used as outgroup

2.2.2.2 Species delimitation analyses

Species delimitation analyses were performed using the concatenated datasets of *cox1*, *nad6* and RpS5 regions of selected *Hermeuptychia* individuals, hereafter called “concatenated data”. Samples were chosen from highly supported clusters obtained by BI analysis of ‘barcode’ data (above), trying to maximize the variability included in the final concatenated matrix and to preserve geographical and genetic diversity representativeness. Male genitalia of four to ten individuals within each clade were analyzed (details below).

Descriptive measures and distance trees

The descriptive measures were calculated as mentioned above, only under the J-C model (Jukes & Cantor 1969) of nucleotide substitution rates. NJ trees were calculated for *cox1*, *nad6* and RpS5 regions separately, for comparative purposes, following the same protocol described above.

Phylogenetic hypotheses

Maximum parsimony (MP) analyses were performed using the New Technology Search implemented in TNT, employing all four methods - ratchet, tree-fusing, tree-drifting and sectorial (Goloboff 1999), followed by traditional search using tree-bisection-reconnection (TBR) branch swapping, with all characters equally weighted. A strict consensus tree was computed whenever multiple equally parsimonious trees were obtained. The stability of each branch was determinate using the nonparametric bootstrapping procedure (Felsenstein 1985), with 1,000 replicates and 100 random taxon additions. Consistence index (CI) and retention index (RI) were calculated with the program Winclada (Nixon 1999). Bremer support and partitioned Bremer support values (to obtain the contribution of each data set to the Bremer support values of the combined analysis; reviewed in Brower 2006) were calculated using the scripting feature of TNT (Peña et al. 2006). The analysis was conducted with 100 random taxon addition replicates, TBR branch swapping and 100 trees held in each replicate.

The Bayesian Inference was obtained using the same procedure described above. The best fit models found were the GTR + G + I [General Time-Reversible model (Rodrígues *et al.* 1990), with gamma distribution (G) and with proportion of invariable sites (I)] for *cox* 1 5'; GTR + G for *nad6* and SYM + G for RpS5.

Estimative of divergence times

Concatenated data of *cox1* 5', *nad6* and RpS5 was used to estimate divergence times for *Hermeuptychia* groups. Species-level phylogeny was generated using a Bayesian uncorrelated lognormal relaxed clock model in Beast v. 1.6.1 (Drummond & Rambaut 2007). The dataset was partitioned in three regions, corresponding to the two mitochondrial and one nuclear region. Each region followed a GTR + G model of substitution implemented in Beast, and two Markov chain Monte Carlo were run for 80,000,000 generations, using Yule speciation model, sampling trees every 10,000 generations, and burn-in of 1,600 trees. A time constraint was used to date the divergence of *Hermeuptychia* from its sister-group (11.8195 ± 1.84 SE My), based on a recent calibration for the subtribe Euptychiina (Peña *et al.* 2010). The posterior distribution of trees was summarized with TreeAnnotator v. 1.4.7 (Drummond & Rambaut 2007), computing the maximum clade credibility tree with average branch lengths.

Sequence-based species delimitation analyses

As an alternative method for species delimitation, the statistical model GMYC proposed by Pons *et al.* (2006) was applied to predict species boundaries, as described by Papadopoulou *et al.* (2008), using combined datasets. First, a fully resolved tree with branch lengths was inferred in Beast v. 1.6.1 (Drummond & Rambaut 2007), as above, with no time constraints and no outgroup. GMYC model was run on this tree as implemented in R statistical package using the SPLITS package (available at CRAN repository) and single and multiple threshold models.

Biogeographical analysis

First, a fully resolved tree with branch lengths was inferred in Beast v. 1.6.1 (Drummond & Rambaut 2007), as above, with no time constraints and no outgroup. Ancestral distributions of *Hermeuptychia* were estimated using the S-DIVA program (Yu *et al.* 2010a). The S-DIVA program is based on DIVA and reconstructs the ancestral distribution in a phylogeny by optimizing a three-

dimensional cost matrix, in which extinctions and dispersals "cost" more than vicariance (Ronquist 1997; Lamm & Redelings 2009). However, the S-DIVA program is built to implement the methods of Nylander *et al.* (2008) and Harris and Xiang (2009), and determinates statistical support for ancestral range reconstructions using the S-DIVA value (Yu *et al.* 2010b). In S-DIVA, the frequencies of an ancestral range at a node in ancestral reconstructions are averaged over all trees and each alternative ancestral range at a node is weighted by the frequency of the node occurring or by some other measure of support for the node.

Species distributions were divided into nine regions. Colombian and Ecuadorian samples were divided into oriental, occidental and central Andes, being the latter only present in North Colombia, where Andes mountain are divided into two or three mountain chains. US samples are together in another region, and Brazilian samples are divided into six groups according to vegetation types and distance: Minas Gerais (shrubland), São Paulo and Rio Grande do Sul (both Atlantic forest, but separated by long distance), Central Brazil (Cerrado savanna), South Amazon (Amazon Forest), and Northeast Brazil (Caatinga savanna).

Minimum spanning network analyses

The minimum spanning network was calculated under the program TCS 1.21 (Clement *et al.* 2000), which shows how haplotypes are connected to each other, using all three molecular regions separately, following the program's manual recommendation. The program estimates gene genealogies from DNA sequences with statistical parsimony analysis (Templeton *et al.* 1992), and the connection of two haplotypes is limited by a probability of parsimony for DNA pairwise differences lower than 0.95.

2.3 Morphological study

Wing pattern

Ventral and dorsal wings were photographed and their pattern was compared to the original descriptions, and also to individuals belonging to the same molecular clusters. All previously described taxa were studied in details (type data table attached Att. 4).

Male genitalia

Four to ten males were selected from each group revealed by the molecular analyses for morphological studies. Dissections were made following standard techniques (as in Willmott & Freitas

2006). Morphological terms for genitalia followed Klots (1956). Samples were photographed under stereoscopic microscope and stored in glycerol. Morphological characters were marked and measured in the pictures, and relative measures were taken for various genitalia parts (e.g. *valvae* and its parts*, tegumen, eadeagus, saccus*). Presence of lobes, teeth and appendices were also used as morphological characters. Results were compared to Forster (1964) and Hayward (1957) (both with genitalia illustrations) and to the specimens from the Museo de Historia Natural, Universidad Nacional Mayor de San Marcos - Peru (MHNSM). Colombian samples were dissected and photographed (genitalia and wings) by Mario Alejandro Marin Uribe from MEFLG, and Ecuadorian samples were dissected and photographed by Luisa Motta in the FLMNH.

A species identification key based on genitalia morphology was produced for further use in *Hermeuptychia* species identification. For this purpose we included three samples of *H. cucullina,* two from MEFLG collected in Peru.

2.4 Species distribution.

Distribution maps were constructed with the program DIVA-GIS v 7.1.6 (Hijamans 2009), using all specimens of *Hermeuptychia* identified at specific level either by genitalia morphology or molecular 'barcode' clusters.

3. RESULTS

3.1 Molecular analyses

3.1.1 Initial evaluation of genetic variability

Descriptive measures

The 'barcode' data matrix is composed by 197 specimens, 189 belonging to the genus *Hermeuptychia* and eight species to outgroups. Considering only *Hermeuptychia* specimens, from a total of 660 base pairs, 249 are variable sites and 73 parsimony informative sites. Average interspecific genetic distance is 5.0% (range, 2.4% and 7.2%, corresponding to genetic distances between *H. sosybius* and *H. gisella,* and *H. fallax* and *H. maimoune* (group A)*,* respectively). Minimum and maximum genetic distance within species are 0.7% and 1.7%, corresponding to *H. sosybius* and *H. fallax,* respectively, with average genetic distance within species 0.8% (Table 2). Distributions of genetic distances within and between species are shown in Fig. 1a. *H. maimoune* is separated in two

different groups corresponding to different geographical ranges: group A can be found in the Amazon basin, and oriental Andes; and group B can be found in occidental Andes region. The mean distance within *H. maimoune* is 2.1% (± 0.4%), and distance between *H. maimoune* groups is 3.5%.

Table 2. Mean genetic distances among groups based on J-C model of nucleotide substitution for 'barcode' region**.** Values above diagonal are standard error (obtained by bootstrap with 2,000 repetitions). Last column shows within groups mean genetic distance ± standard error.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**WITHIN GROUPS**
1 *H. atalanta*	-	0.010	0.006	0.008	0.009	0.008	0.009	0.009	0.007	0.009 ± 0.002
2 *H. fallax*	0.063	-	0.008	0.011	0.011	0.010	0.009	0.010	0.009	0.009 ± 0.002
3 *H. gisella*	0.032	0.051	-	0.008	0.009	0.006	0.007	0.008	0.005	0.010 ± 0.002
4 *H. maimoune* A	0.052	0.072	0.045	-	0.007	0.008	0.009	0.009	0.008	0.012 ± 0.003
5 *H. maimoune* B	0.055	0.070	0.050	0.035	-	0.009	0.011	0.010	0.009	0
6 *H. hermes*	0.044	0.069	0.034	0.048	0.054	-	0.009	0.009	0.007	0.010 ± 0.003
7 *H. pimpla*	0.055	0.060	0.043	0.055	0.070	0.054	-	0.007	0.008	0.011 ± 0.003
8 *H. harmonia*	0.050	0.069	0.045	0.058	0.064	0.053	0.043	-	0.008	0.010 ± 0.003
9 *H. sosybius*	0.035	0.053	0.024	0.043	0.047	0.039	0.042	0.045	-	0.007 ± 0.002

Distance tree

Both N-J trees, estimated under the K2P and J-C model of nucleotide substitutions were equal, even in bootstrap support for the species groups, therefore only the K2P tree is presented in Fig 2. N-J distance tree for 'barcode' data presented ten well supported groups. Seven groups were identified as *H. harmonia, H. pimpla, H. atalanta, H. hermes, H. fallax, H. sosybius* and *H. gisella*, based on the morphology of male genitalia. *H. maimoune* was not present in Forster (1964) and thus was identified based on the wing pattern of the type specimen. This group, however, showed up divided in two main clades related do geographical occurrence.

The individuals MT14 and CO09 are females, and because of this, it was not possible to assign them to any recognized species, thus they will be named *H. sp1* hereafter. Individuals RS26 and RS28, formed a cluster with *H. sosybius*, although with low bootstrap support. Due to uncertainty on their

classification, these samples will be named *H. sp2* hereafter. Finally, individual L03 is included with low support into the *H. fallax* cluster. Individual CO46 appears as part of the outgroup, and because of this it did not group with any other species.

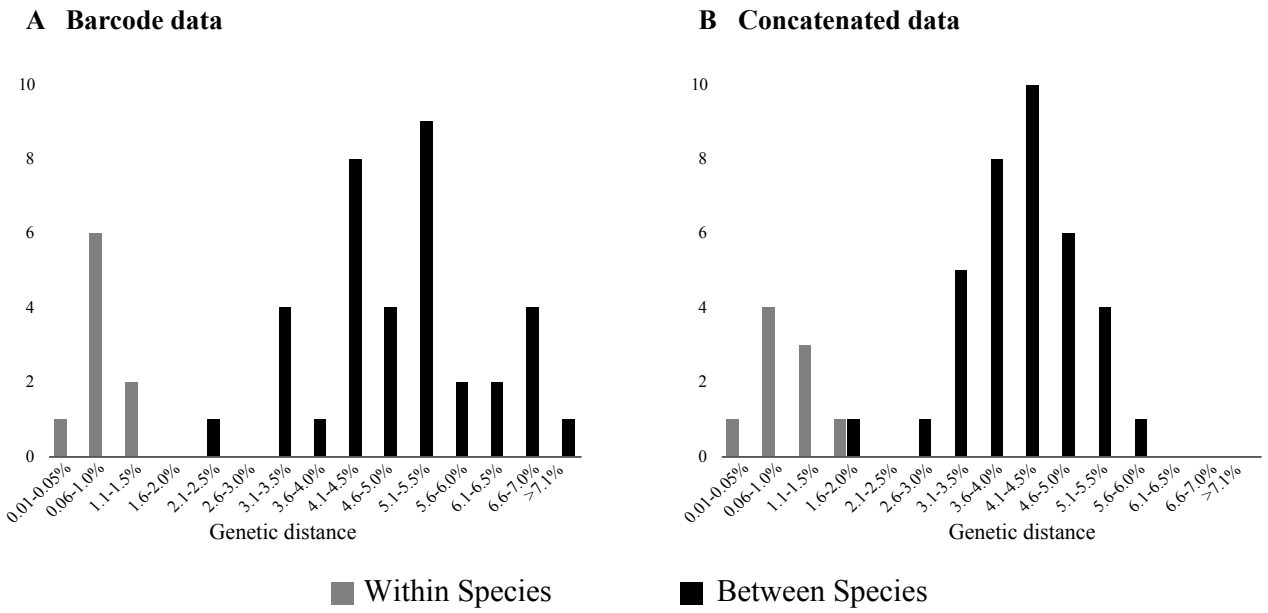

Figure 1. Graphic representation of within and between species genetic distances for **A.** 'barcode' and **B.** concatenated data sets of *cox1* 5', *nad6*, and RpS5.

Bayesian Inference

Tree obtained by BI shows *Hermeuptychia* as monophyletic, with individual CO46 as the most external group (Fig. 3). *H. fallax* is sister to all other *Hermeuptychia*. *H. harmonia* and *H. pimpla* are sister species, and all other species clades relationships are not well supported. However, all species have high posterior probability values (> 95), with the exception of *H. fallax* (0,89). Individual L05 is part of *H. gisella.*

3.1.2 Species delimitation analyses

Descriptive measures

The concatenated data matrix is composed by 82 specimens, 74 belonging to the genus *Hermeuptychia* and eight species to outgroups. Considering only *Hermeuptychia* specimens, from a total of 1,709 base pairs, 360 are variable sites and 260 parsimony informative sites (Table 3).

Table 3. Comparative descriptive data of combined datasets of *cox1*, *nad6* and RpS5.

DNA REGION	BASE PAIRS	VARIABLE NUCLEOTIDE SITES (%)	PARSIMONY INFORMATIVE SITES (%)	VARIABLE AMINO ACID SITES (%)
cox1 5'	660	151 (22)	121 (18)	12 (5)
nad6	439	93 (21)	81 (18)	31 (21)
RpS5	610	115 (19)	57 (9)	5 (2)

Mean overall genetic distance was 3.8% (± 0.3%) S.E (with a total of 1,375 valid base pairs in the matrix). Minimum and maximum interspecific genetic distance were 2.7% and 6.1%, corresponding to genetic distances between *H. harmonia* and *H. pimpla,* and *H. fallax* and *H. hermes*, respectively. Average genetic distances among species was 4.1%. Distance between *H. maimoune* groups A and B was 2.0%, and distance within *H. maimoune* was 1.5% (± 0.2%). Minimum and maximum genetic distance within species were 0.6% and 1.7%, corresponding to *H. hermes* and *H. fallax*, respectively. Average genetic distance within species was 0.9% (Table 4). Distributions of genetic distances within and between species are shown in Fig. 1b.

Distance trees

All species clades were equally recovered in all distance trees obtained with distinct datasets. Distance tree obtained with *nad6* sequences is unique in showing more incongruencies than the other trees when compared to the tree obtained with 'barcode' data (Fig. 4). Specimen EQ20 appears as part of the *H. hermes* clade, CO38 appears inside *H. pimpla*, CO46 appears inside *H. sosybius*, EQ05 is inside *H. gisella*, and the group composed by CO09 and MT14 is part of *H. gisella*. It is interesting to note that these incongruencies do not appear in any other tree, so these individuals can be considered as part of their most common-found clades. This is also the only single gene tree in which *H. fallax* is a monophyletic group, with high bootstrap value (100) and short interior branches.

In the RpS5 distance tree (Fig. 5) bootstrap values for the species clades were generally lower than the values found in the other gene trees, and several species did not form supported groups. This is the case for *H. atalanta* (bootstrap value 12) and *H. maimoune* (28). *H. harmonia* appears as sister to all other species in *Hermeuptychia*, and *H. pimpla* is part of *H. harmonia* clade. *H. fallax* was not monophyletic and samples L03 and L05 appear as part of the *H. gisella* clade.

Table 4. Mean genetic distances among groups based on J-C model of nucleotide substitution, for concatenated data of *cox1*, *nad6* and RpS5. Values above diagonal are standard error (obtained by bootstrap with 2,000 replicates). Last column shows within groups mean genetic distance ± standard error.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**WITHIN GROUPS**
1 *H. atalanta*	-	0.005	0.004	0.005	0.005	0.005	0.005	0.005	0.005	0.010 ± 0.001
2 *H. fallax*	0.049	-	0.005	0.006	0.006	0.006	0.006	0.006	0.005	0.017 ± 0.003
3 *H. gisella*	0.033	0.043	-	0.005	0.005	0.005	0.005	0.005	0.004	0.012 ± 0.002
4 *H. maimoune* A	0.038	0.053	0.035	-	0.003	0.005	0.005	0.005	0.004	0.011 ± 0.002
5 *H. maimoune* B	0.038	0.051	0.038	0.020	-	0.005	0.006	0.006	0.005	0
6 *H. hermes*	0.041	0.061	0.044	0.041	0.043	-	0.005	0.005	0.005	0.006 ± 0.001
7 H. pimpla	0.046	0.052	0.042	0.042	0.047	0.047	-	0.003	0.004	0.014 ± 0.002
8 *H. harmonia*	0.042	0.051	0.039	0.042	0.043	0.047	0.027	-	0.005	0.007 ± 0.001
9 *H. sosybius*	0.036	0.047	0.033	0.032	0.032	0.040	0.040	0.037	-	0.006 ± 0.001

Phylogenetic hypotheses

The combined dataset produced 20,000 equally parsimonious cladograms with length of 1,318 steps (CI= 48 and RI= 76). Consensus tree is shown in Fig. 7. *Hermeuptychia* is monophyletic, with the specimen CO46 from Puerto Berrio-CO sister to all species of *Hermeuptychia*. *H. sosybius* is sister to the other species of the genus and the interior nodes of this group are poorly resolved. *H. pimpla* is monophyletic, and *H. harmonia* clade does not have strong support and is collapsed, thus rendering impossible to determine its monophyly or paraphyly. All other species groups are monophyletic, although *H. gisella* does not present strong bootstrap support (43%). Specimens L03 and L05 are the most external individuals within it the monophyletic *H. fallax* clade.

The Partitioned Bremer Support analysis (Table 5) showed that *nad6* region is the source of most incongruences among molecular datasets. Its spurious results should be the reason why *H. harmonia* and *H. pimpla* clade has low support. On the other hand, it is also the main reason for *H. fallax* clear monophyly. The RpS5 region gives high Bremer support to *H. hermes* and to the *Hermeuptychia* genus itself, and the *cox1* region gives the major part of the support to species clades, with the exception of *H. fallax*.

As found in MP consensus tree, the genus *Hermeuptychia* is monophyletic and CO46 is sister to all known species in the tree obtained by BI (Fig 8). It does not show any incongruence with the morphological data, and all species clades are well supported and monophyletic. *H. sosybius* appears as a sister group to the other species of the genus. The relationship between *H. maimoune* and *H. hermes* is not well resolved and shows low values of posterior probability. These two species are sister group to *H. atalanta.* Together, these three species are sister to a clade composed by *H.pimpla* +*H. harmonia* and *H. fallax* + *H. gisella.*

Estimates of divergence times

Bayesian tree obtained with Beast (v 1.6.1, Drummond & Rambaut 2007) recovered the same groups as the other analyses (Fig. 9). Individual CO46 is not part of *Hermeuptychia* being sister to the outgroups; all the remaining samples are monophyletic and the genus has good support (0.97 posterior probability). Tree topology is a little different from the BI, with the clade *H. harmonia* + *H. pimpla* as sister to the other *Hermeuptychia.* Excluding samples from Peña (2010), all species were recovered as well supported monophyletic groups with moderate to high posterior probability values (Fig. 10). As in the other phylogenetic analyses, *H. fallax* and *H. gisella* appears as sister species, as well *H. harmonia* and *H. pimpla.* Other relationships between *Hermeuptychia* species do not have strong support.

The estimated time of the initial divergence of the species of *Hermeuptychia* is 8.2 ± 3.6 My. The divergence between *H. fallax* and *H. gisella* is 4.5 ± 3.0 My, with *H. fallax* clade origin at 2.44 ± 2.1 My and *H. gisella* at 3.5 ± 2.6 My. The divergence between *H. harmonia* and *H. pimpla* is dated to 4.8 ± 2.0 My, with *H. harmonia* clade origin at 2.8 ± 2.4 My and *H. pimpla* at 2.3 ± 2.6 My. The species with older origin dates are *H. gisella* and *H. atalanta* (3.3 ± 2.4 My), and the most recent species are *H. sosybius* (1.7 ± 2.0) and *H. hermes* (1.4 ± 1.5). *H. maimoune* divergence occurred at 2.7 ± 2.2 My, with group A divergence around 1.81 my, and group B around 0.23 my.

Sequence-based species delimitation analyses

GMYC model recovered five clusters (Conf.I. = 3-20; LR = 4.7597; $p = 0.1902$) using the single model (threshold time = -0.0234) (Fig. 9b), corresponding to A. *H. atalanta* grouped with MT14 + CO09; B. the sister species *H. harmonia* + *H. pimpla*; C. *H. sosybius* together with RS26 +RS28 and *H. maimoune*; D. *H. hermes* alone; and E. the sister species *H. gisella* and *H. fallax* (Fig. 9a). Although the grouping is interesting, since sister species tended to be joined together, the analysis failed to

distinguish *Hermeuptychia* species, and the threshold found was not significant. Multiple threshold model did not improve analysis (p = 0.4741).

Table 5. Partitioned Bremer support for the three gene regions

CLADE	*COX1*	*NAD6*	RPS5	BREMER SUPPORT
Hermeuptychia + CO46	13.1	3	-8.1	8.00
Hermeuptychia	18.1	-0.5	10.4	28.00
H. sosybius	1.7	0.5	0.8	3.00
H. sp2	10.1	9.5	2.4	22.00
H. harmonia + *H. pimpla*	16.5	-18.9	6.4	4.00
H. harmonia	4.4	-2.7	-1.6	0.10
H. pimpla	16.5	-18.9	6.4	4.00
H. fallax	1.6	15	-4.6	12.00
H. gisella	0.3	0.9	0.4	1.60
H. gisella + *H. fallax*	1.5	1	-0.5	2.00
H. sp1	13.8	7.3	2.9	24.00
H. hermes	28.8	-23.4	15.7	21.10
H. maimoune	13.4	-2.4	-2.1	8.90
H. maimoune group A	1.8	1.2	-2.1	0.90
H. maimoune group B	8.7	3.1	-0.8	11.00
H. atalanta	1.6	1.7	1.8	5.10

Biogeographical analysis

The ancestral distribution estimated for *Hermeuptychia* is Occidental Andes (Fig. 10). The S-DIVA analysis also recovered a single most probable ancestral distribution for most species within *Hermeuptychia*. The ancestral geographical range for *H. harmonia, H. pimpla* and *H. maimoune* is suggested to be in the Andes; South US is suggested as ancestral distribution for *H. sosybius,* and São Paulo-BR for *H. fallax*. For *H. hermes*, all possible combinations of internal branches distributions are equally possible, also indicating a widespread ancestral distribution.

Minimum spanning network analyses

The minimum spanning network analyses (Figs 11-13) present different connectivity for the species according to the gene region used, what reflects different mutation rates. Analysis based on RpS5 and *cox1* show 46 haplotypes and maximum length of 11 steps. The *nad6* analysis shows 44 haplotypes and maximum length of eight steps. The RpS5 network (Fig. 13) presents the most connected network, with *H. hermes* and *H. fallax* appearing isolated from the main network. Although species are connected, which shows comparatively low mutation rates for this region, all species are segregated within this network allowing the discrimination among them. The analysis based on *cox1* 5' region (Fig 11) presents a different arrangement, with *H. sosybius* and *H. gisella* joined together in one network. *H. atalanta*, *H. maimoune*, *H. fallax,* and *H. harmonia* have disconnected networks. The network obtained with *nad6* sequences (Fig. 12) shows almost all species separated in different networks and without haplotype discontinuities. The exceptions are *H. harmonia* and *H. pimpla,* separated by the maximum distance of eight steps, and *H. gisella,* with one of its haplotypes disconnected from the main network.

In the *cox1* 5' network, *H. atalanta* has three haplotypes (A7, A8 and A9) forming a separated network, but with one additional step, these samples are connected in the main network. It is even more intriguing when we compare individual MG12 (haplotype A3) with MG11 (haplotype A7) and MG21 (haplotype A08), since they are all sympatric with disconnected *cox1* haplotypes. Remaining species are isolated, with the exception of *H. sosybius* and *H. gisella*. These species are into the same network but have haplotypes at least seven mutational steps apart.

The minimum spanning networks show almost the same incongruencies found in the distance trees. The individual CO12, that appears within the *H. atalanta* clade in the distance tree obtained with *cox1* 5' region (Fig. 2), appears as part of the *H. sosybius* and *H. gisella* network obtained with the same molecular region (Fig. 11). It is separated from individual EUA04 by 11 mutational steps. Individuals L03 and L05 share RpS5 haplotypes with L01 and M13, and although L05 is only one mutational step apart from L01 in the *cox1* network, L03 is at least 10 mutational steps distant from the closest *H. gisella* haplotype (GH8).

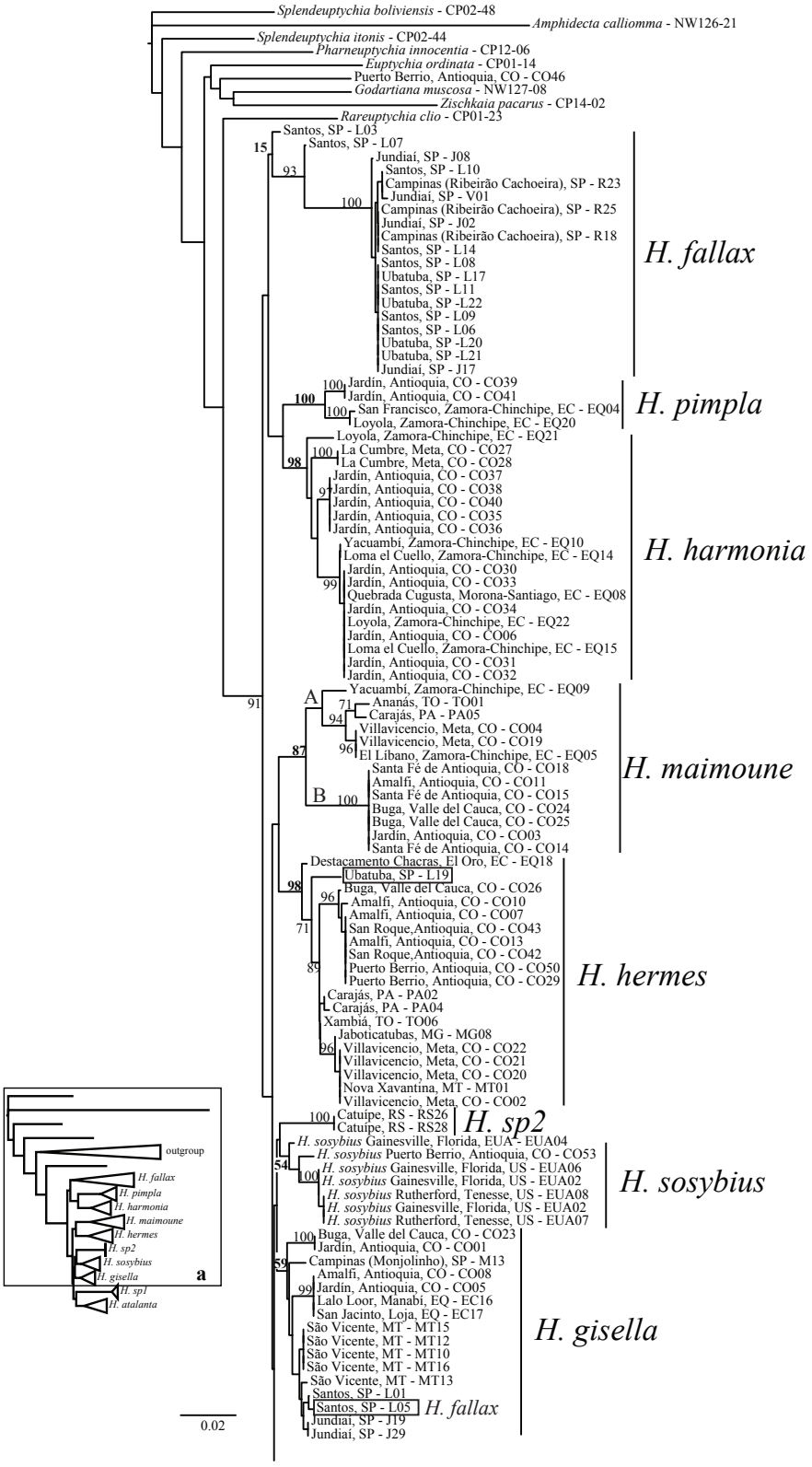

Figure 2. Neighbor-Joining distance tree based on 'barcode' data (*cox1* 5'), estimated under the Kimura two parameter model of nucleotide substitution. Numbers before nodes are values of 2,000 Bootstrap replicates. Values of species nodes are represented in bold. Individuals shown into squares are incongruencies between trees or genitalia. Letters show internal differentiation of *H. maimoune*.(continues)

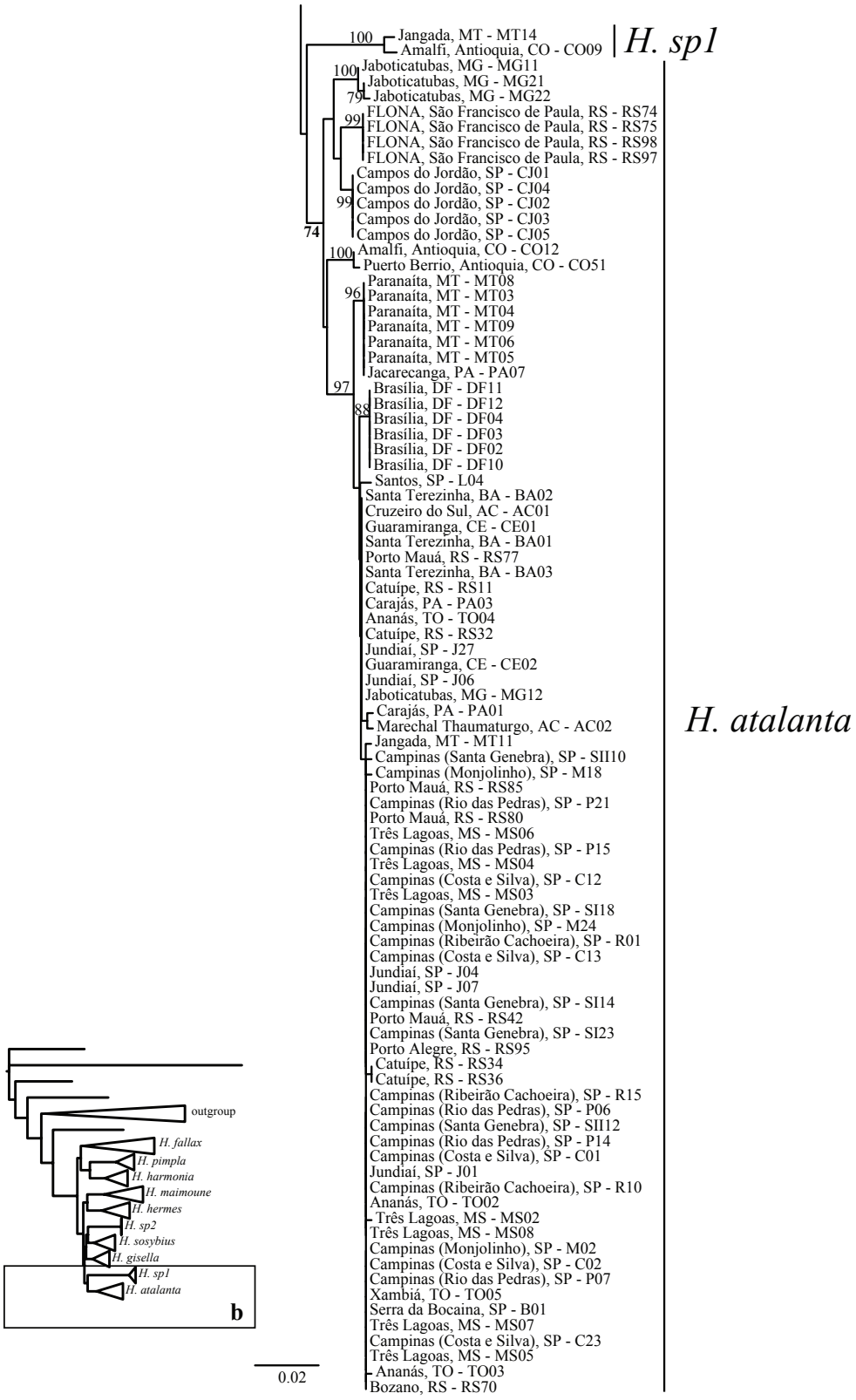

Figure 2 (continuation). Neighbor-Joining distance tree based on 'barcode' data (*cox1* 5'), estimated under the Kimura two parameter model of nucleotide substitution. Numbers before nodes are values of 2,000 Bootstrap replicates. Values of species nodes are represented in bold. Individuals shown into squares are incongruencies between trees or genitalia.

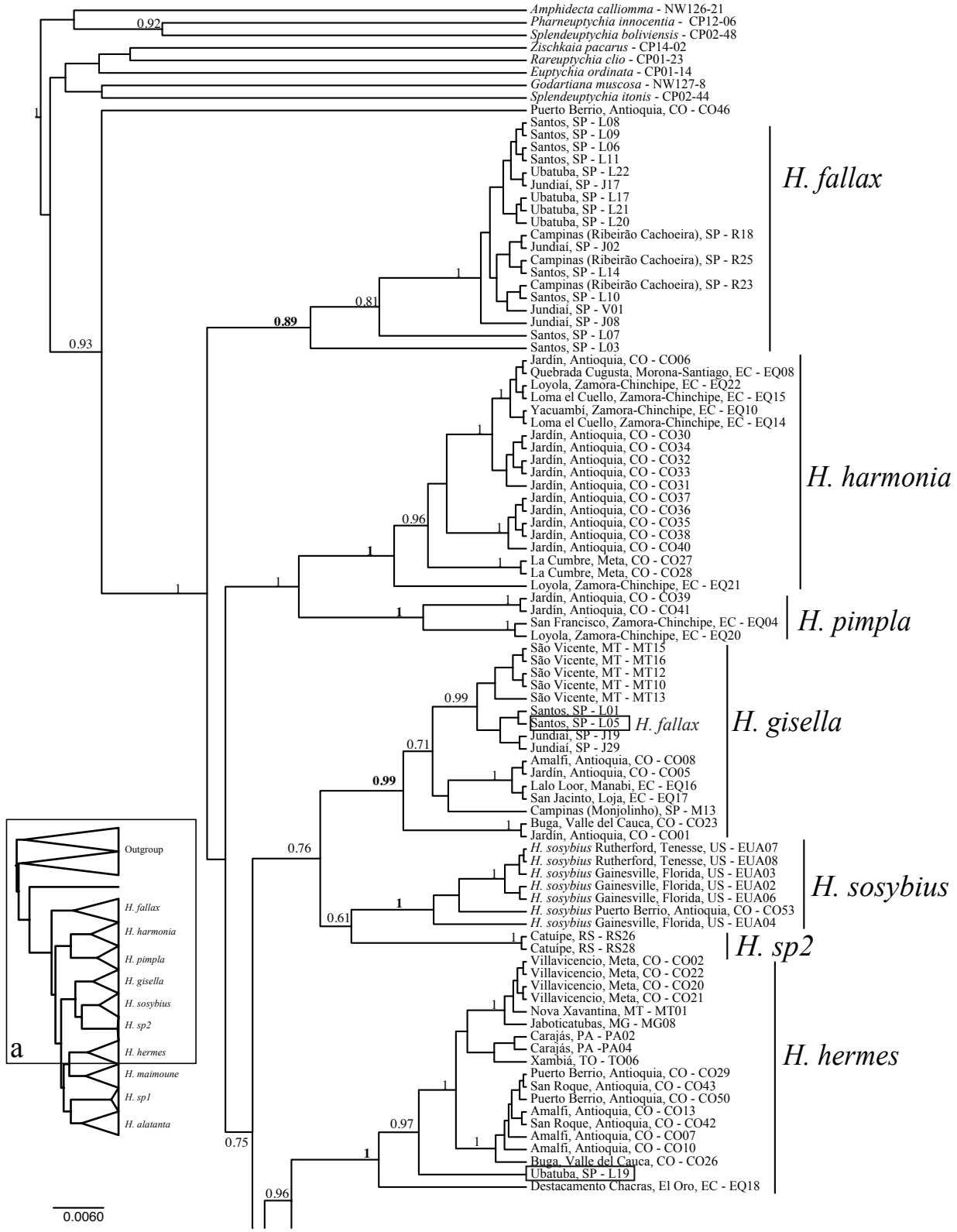

Figure 3. Bayesian Inference tree based on 'barcode' data (*cox1* 5') of *Hermeuptychia*, and obtained with the GTR + G model of nucleotide substitutions. Numbers before nodes are posterior probability values. Values of posterior probability for species nodes are represented in bold. Individuals shown into squares are incongruencies between trees or genitalia (continues).

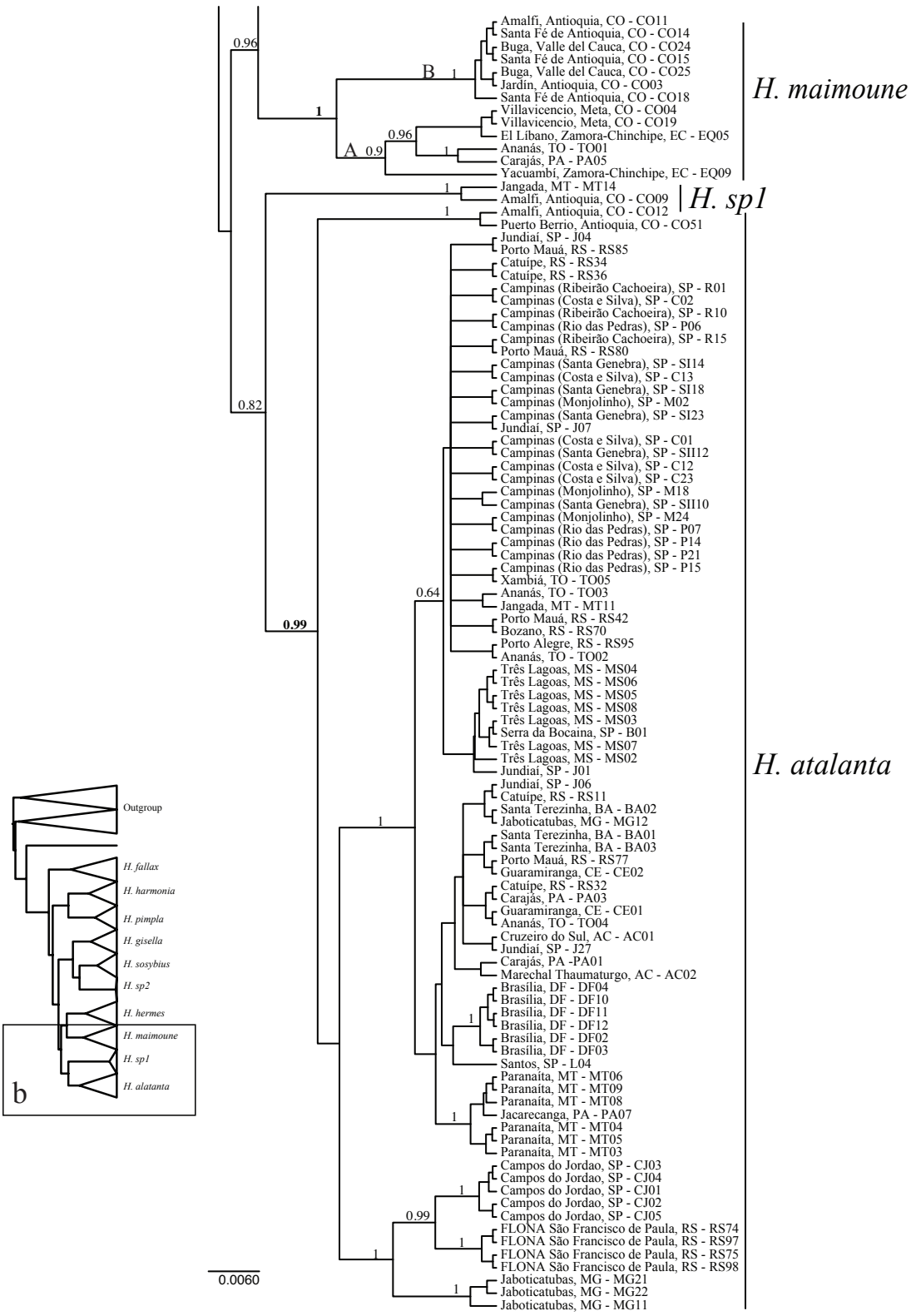

Figure 3 (continuation). Bayesian Inference tree based on 'barcode' data (*cox1* 5')of *Hermeuptychia*, and obtained with the GTR + G model of nucleotide substitutions. Numbers before nodes are posterior probability values. Values of posterior probability for species nodes are represented in bold. Letters show internal differentiation of *H. maimoune*.

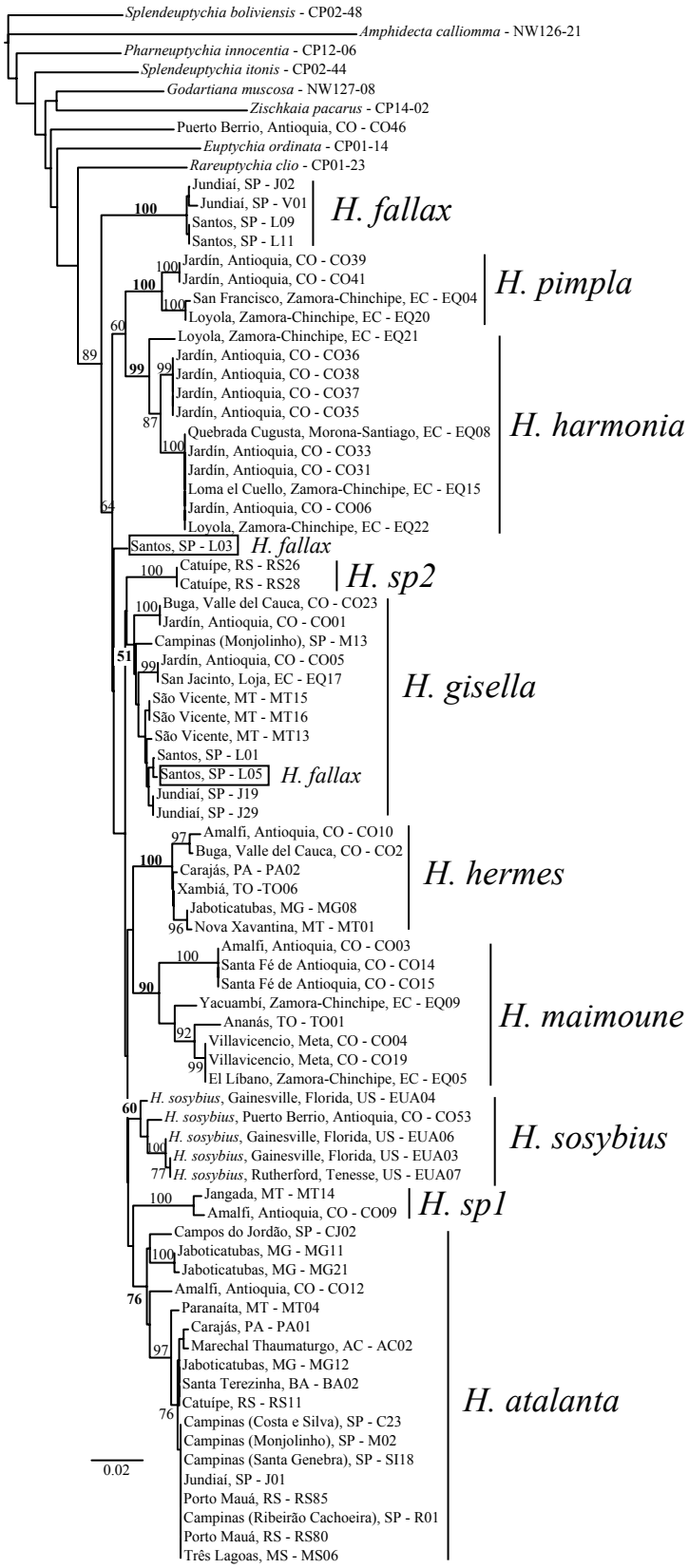

Figure 4. Neighbor-Joining distance tree, based on data of *cox1* 5', estimated under Jukes-Cantor model of nucleotide substitution. Numbers before nodes are values of 2,000 Bootstrap replicates. Values of species nodes are represented in bold. Individuals shown into squares are incongruencies between trees or genitalia

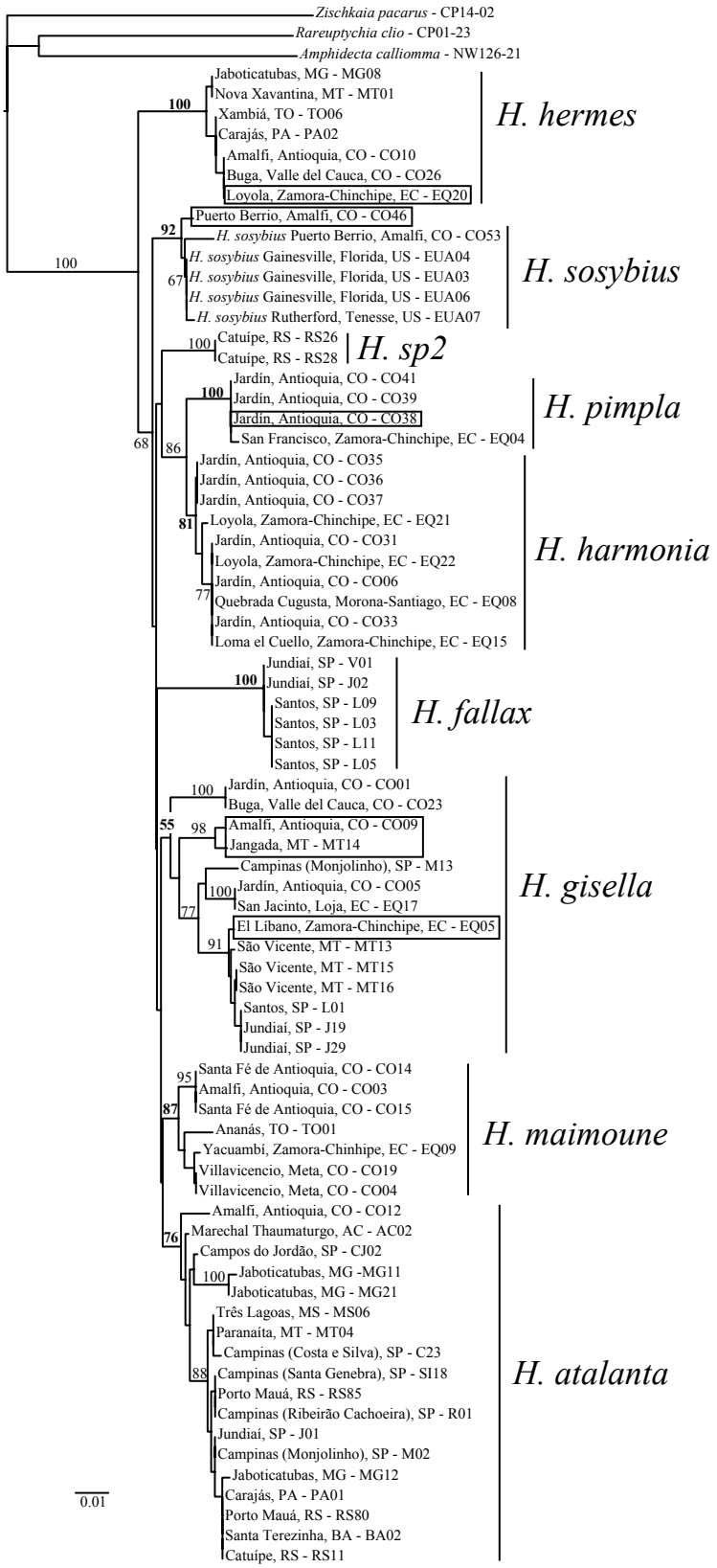

Figure 5. Neighbor-Joining distance tree, based on data of *nad6*, estimated under Jukes-Cantor model of nucleotide substitution. Numbers before nodes are values of 2,000 Bootstrap replicates. Values of species nodes are represented in bold. Individuals shown into squares are incongruencies between trees or genitalia

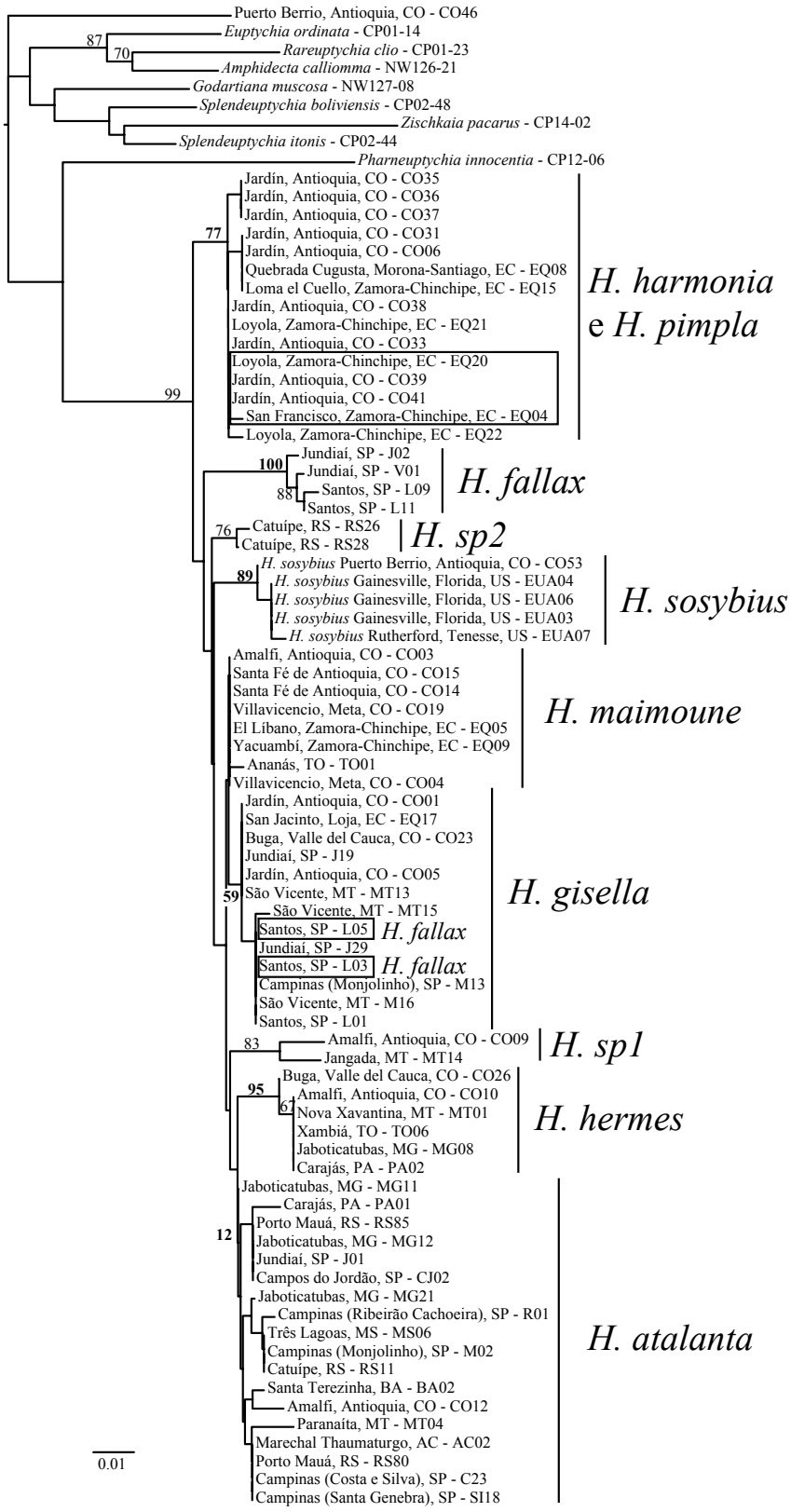

Figure 6. Neighbor-Joining distance tree, based on data of RpS5, estimated under Jukes-Cantor model of nucleotide substitution. Numbers before nodes are values of 2,000 Bootstrap replicates. Values of species nodes are represented in bold. *H. pimpla* (inside square) is seen as part of *H. harmonia*.

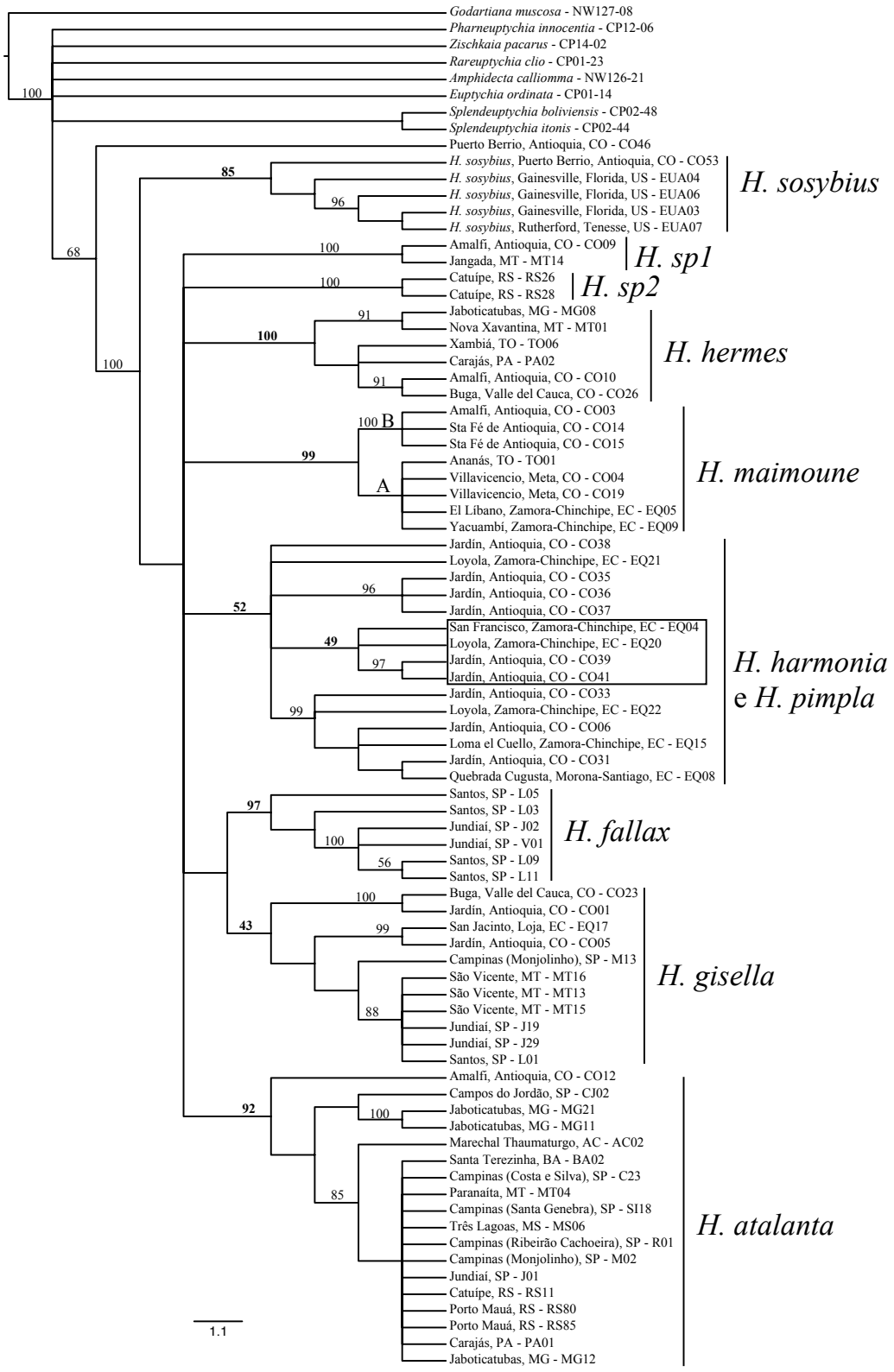

Figure 7. Consensus tree of 20,000 most parsimonious trees, based on concatenated data of *cox1* 5', *nad6*, and RpS5. Numbers before nodes are values of Bootstrap of 1,000 replicates. Values of species nodes are represented in bold. Individuals shown into squares are incongruencies between trees or genitalia. *H. pimpla* (inside square) is seen as part of *H. harmonia*.

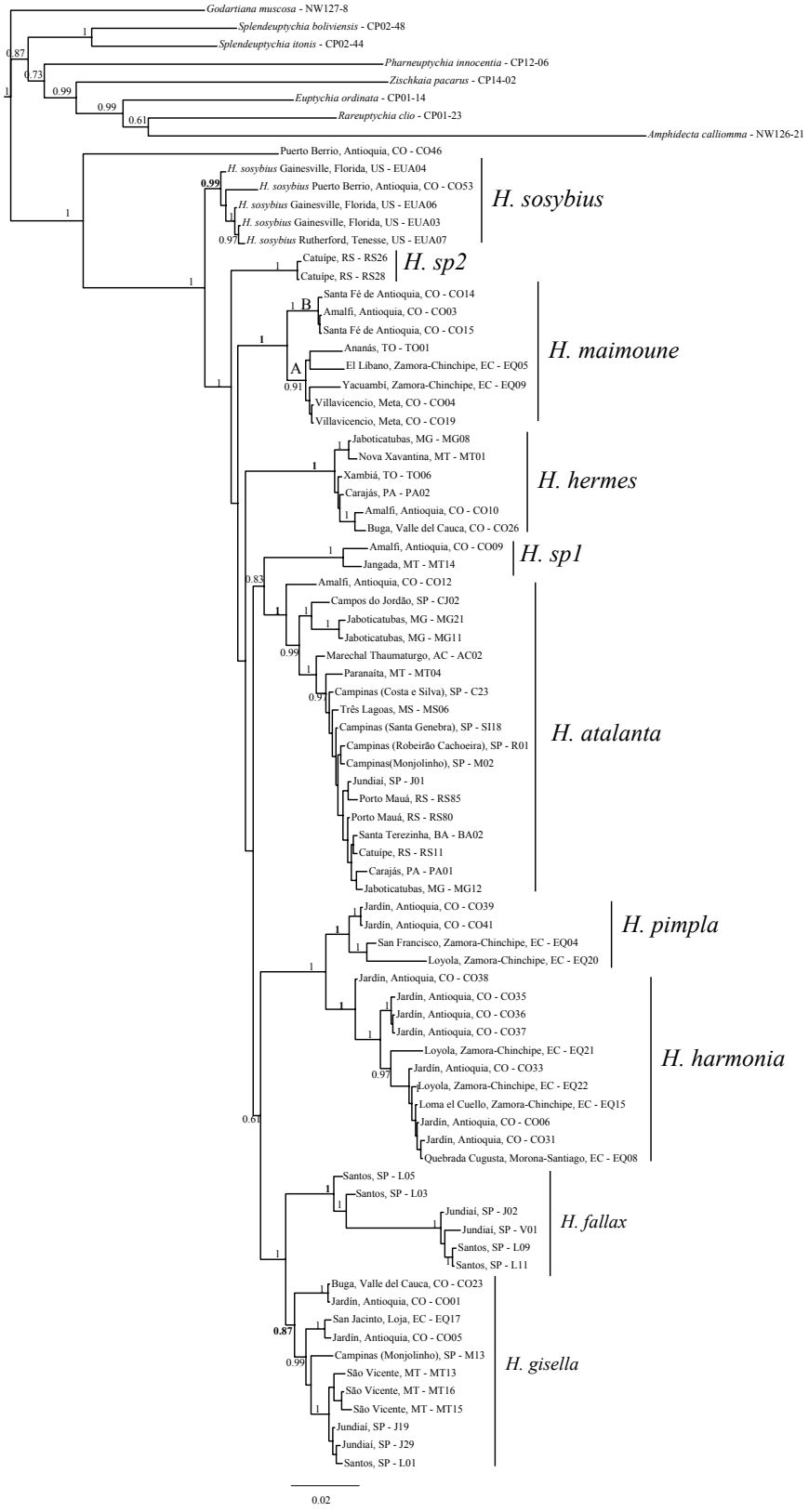

Figure 8. Bayesian Inference tree based on concatenated data of *Hermeuptychia* species, and obtained with the GTR + G model of nucleotide substitutions. Numbers before nodes are posterior probability values. Values of posterior probability for species nodes are represented in bold.

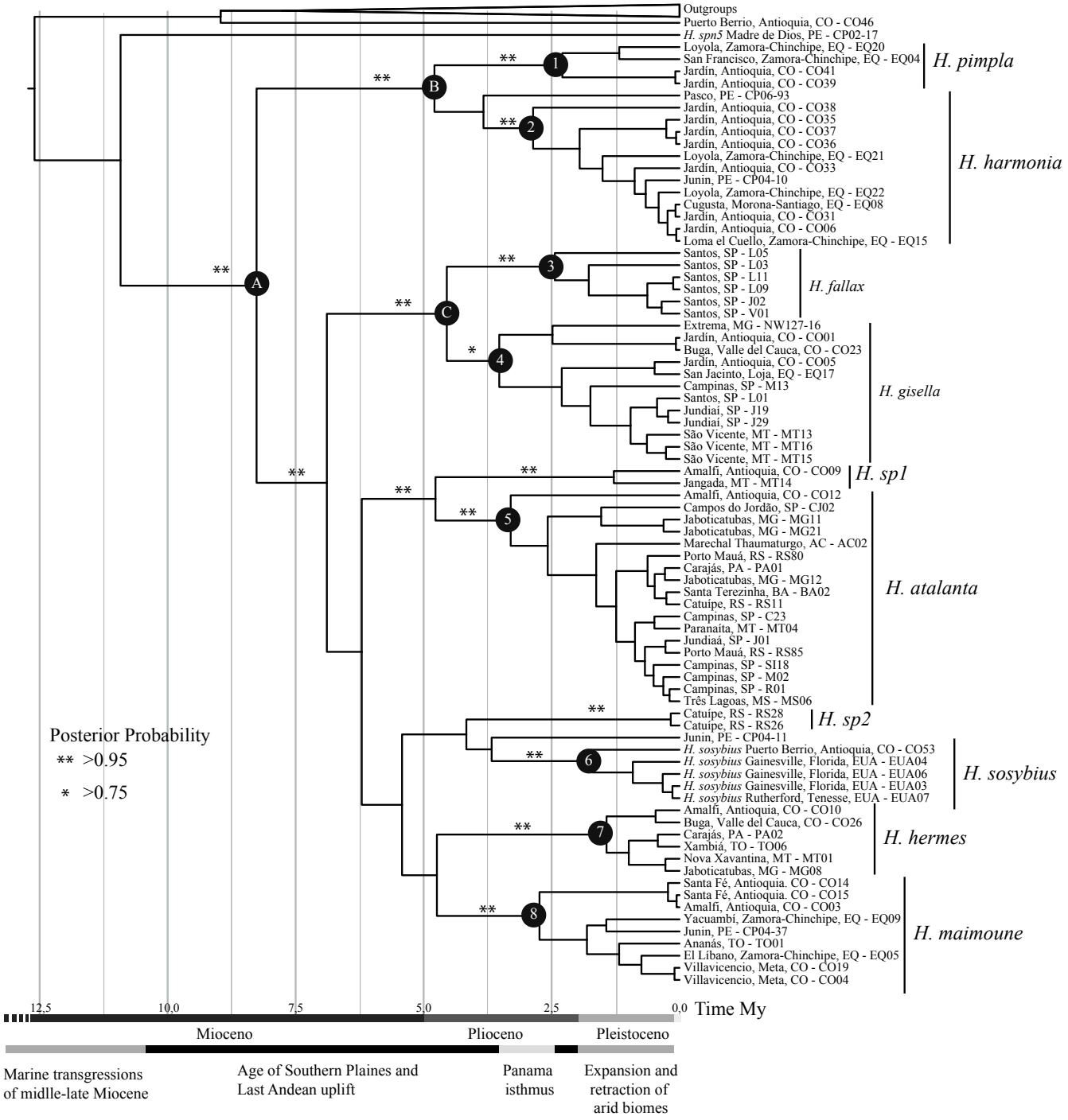

Figure 9. Estimated divergence time for species of *Hermeuptychia*. A. Beginning of diversification of the *Hermeuptychia* genus (8.2 ± 3.6 My). B Beginning of diversification of *H. harmonia* and *H. pimpla* (4.8 ± 2.0 My). C Beginning of diversification of *H. fallax* and *H. gisella* (4.5 ± 3.0 My). Speciation of: 1. *H. pimpla* (2.3 ± 2.6 My); 2. *H. harmonia* (2.8 ± 2.4 My); 3. *H. fallax* (2.4 ± 2.1 My); 4. *H. gisella* (3.5 ± 2.6 My); 5. *H. atalanta* (3.3 ± 2.4 My); 6. *H. sosybius* (1.7 ± 2.0 My); 7. *H. hermes* (1.4 ± 1.5 My); 8. *H. maimoune* (2.7 ± 2.2 My), group A (around 1.81 my) , group B (around 0.23 my). Geological information after Ortiz-Jaureguizar & Cladera 2006.

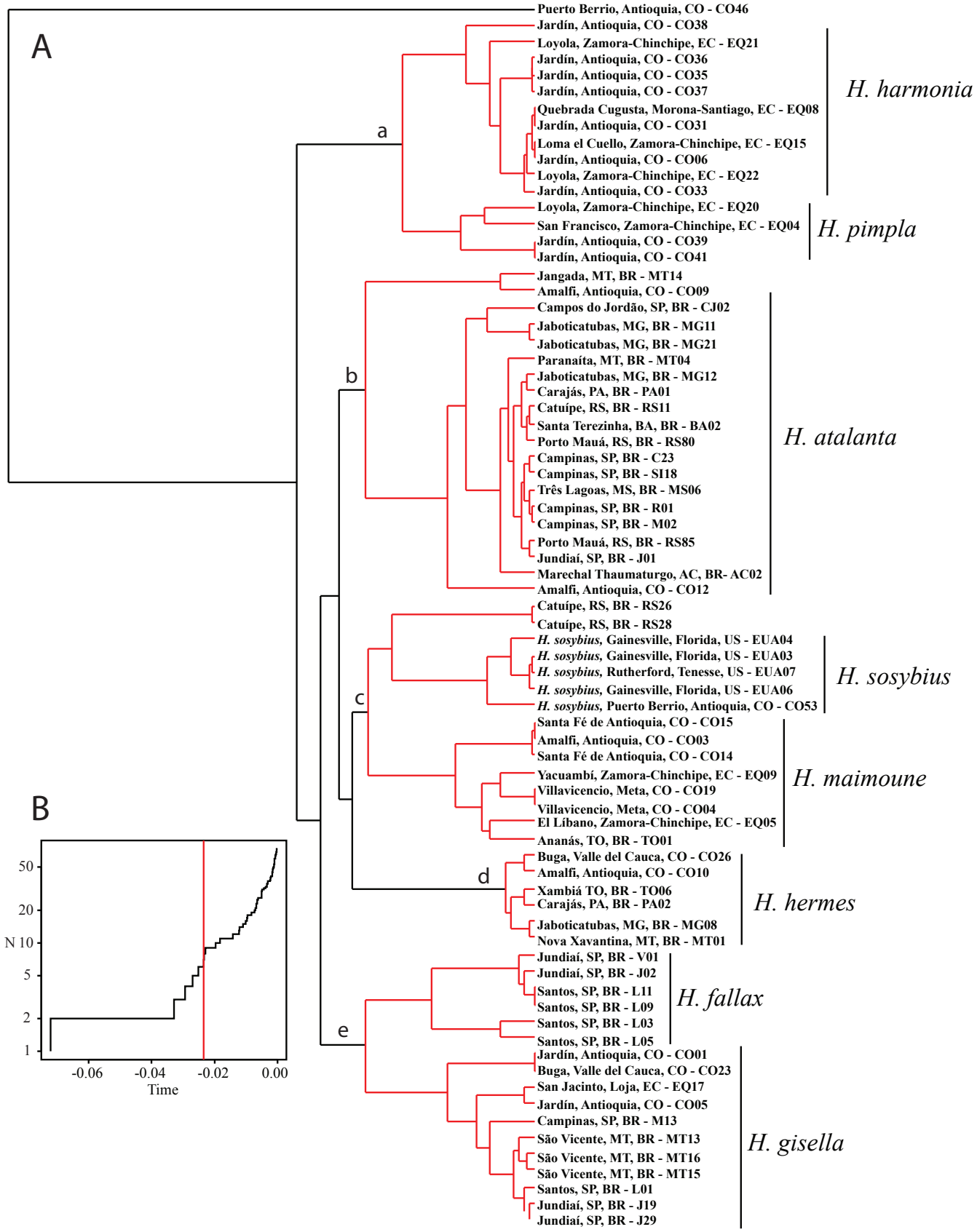

Figure 10. Molecular species delimitation. **A.** Five recovered "species" coded with letters, delimited by red branches. **B.** Single threshold model (= -0.0234).

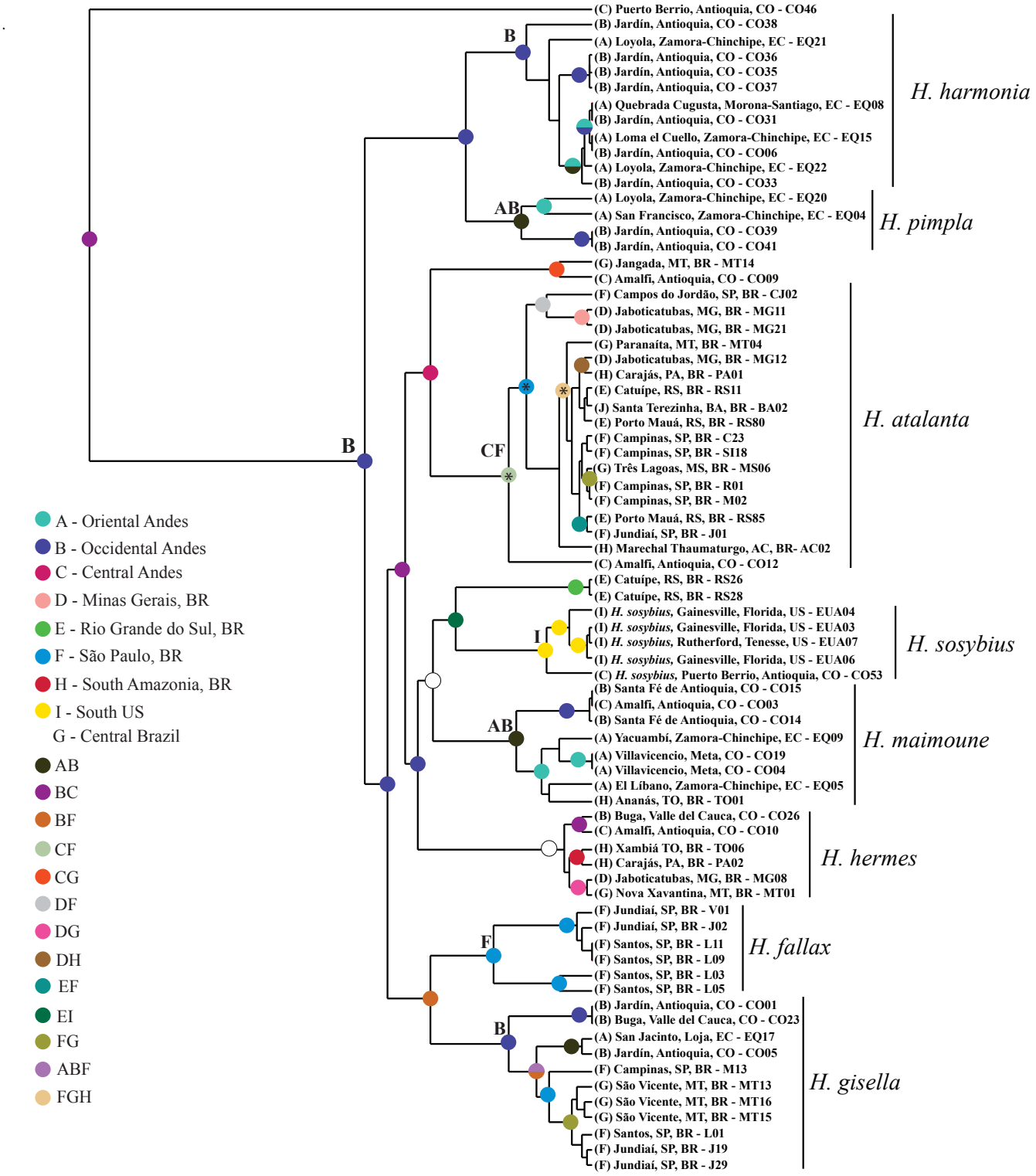

Figure 11. Ancestral distribution analysis. Current distributions are coded with letters at terminal nodes. Distributions are coded in color, and only shown for moderate do high supported nodes. White circles correspond to nodes were all possible combinations of current distributions are equally probable as ancestral distribution. Species ancestral distributions are coded with letters, as is the Hermeuptychia ancestral distribution. Nodes with asterisks are the most probable ancestral distribution

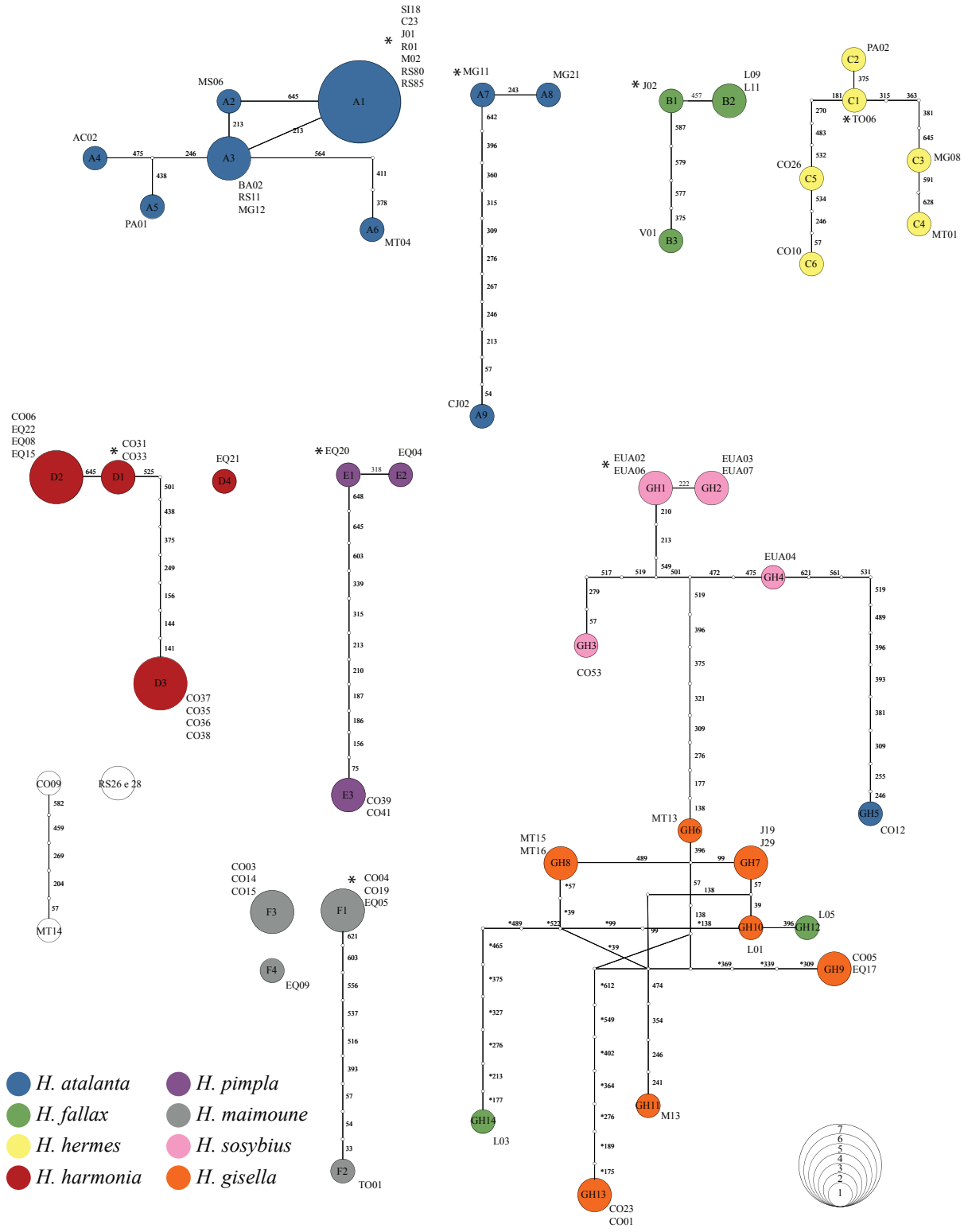

Figure 12. Minimum spanning network analysis for the *cox 1* 5' for the concatenated data. Color code represents species. White circles are not assigned to any known species to the moment. Circles size are directly proportional to the number of individuals. Small white circles indicates missing haplotypes. Each branch is equivalent to one base pair change. Individual codes are represented outside haplotype circles. Ancestral haplotypes are identified with an asterisk.

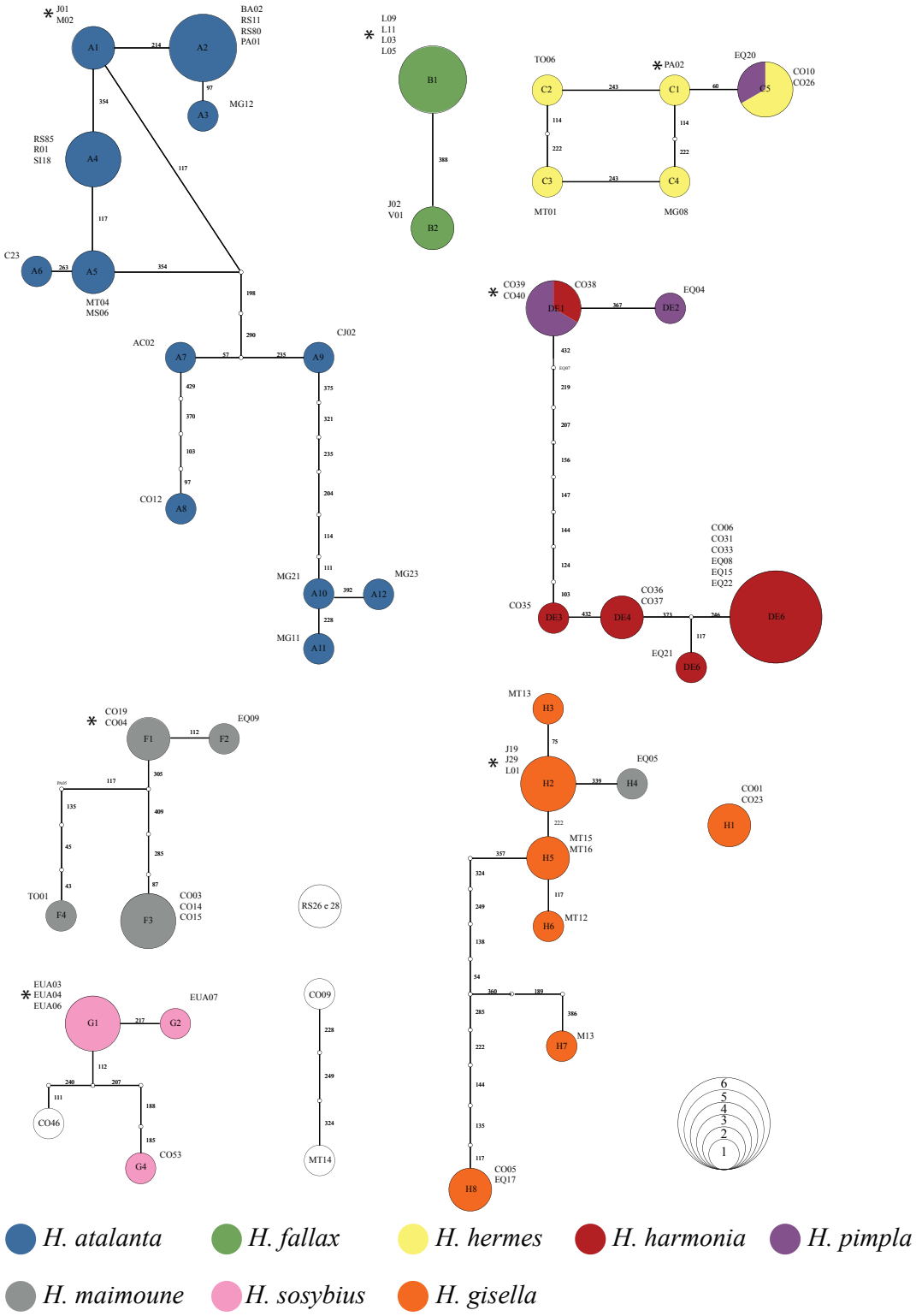

Figure 13. Minimum spanning network analysis for the *nad6* sequences. Color code represents species. White circles are not assigned to any known species to the moment. Circles size are directly proportional to the number of individuals. Small white circles indicates missing haplotypes. Each branch is equivalent to one base pair change. Individual codes are represented outside haplotype circles. Ancestral haplotypes are identified with an asterisk.

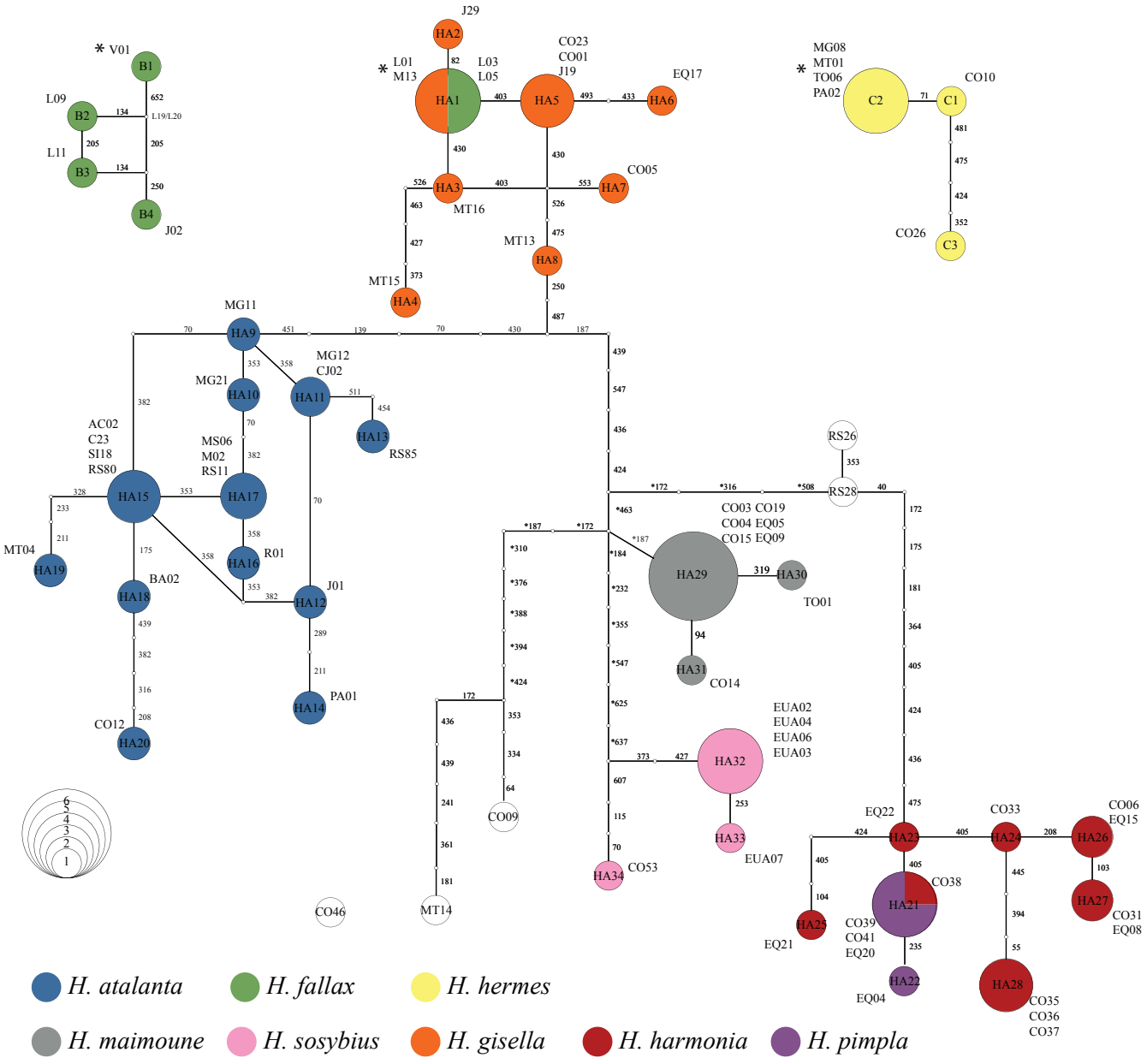

Figure 14. Minimum spanning network analysis for the RpS5 sequences. Color code represents species. White circles are not assigned to any known species to the moment. Circles size are directly proportional to the number of individuals. Small white circles indicates missing haplotypes. Each branch is equivalent to one base pair change. Individual codes are represented outside haplotype circles. Ancestral haplotypes are identified with an asterisk

3.2 Morphological study

Wing pattern

Hermeuptychia species have very variable wing patterns (see Att 6), and identifications based only in these patterns are prone to error. In spite of that, these species were originally described based

on wing patterns variations, mostly relied on presence, shape and color of the *ocelli*. In Brazil there are basically two forms of described *ocelli* pattern, one with all small black dots *ocelli*, a patten found in *H. atalanta*; and one with medium sized *ocelli* in a combination of one beige, one dark with white pupil, two beige and two black with white pupils. Although there is intraspecific variation, a few features can be used for identification of the three most common *Hermeuptychia* species in Brazil. *H. atalanta* commonly presents the pattern of all *ocelli* as small black dots, additionally, the dorsal face of the hindwing presents two more pale lines, and the eyes are lined with brown scales. *H. gisella* also presents lines in the dorsal hindwing, but these lines are darker in contrast with the lighter scales of *H. atalanta.* Furthermore, *H. gisella* has two white lines in its eyes margin, a feature present in many Satyrini, but not in other *Hermeuptychia* species. *H. fallax* is smaller and darker than *H. gisella* and does not have dorsal hindwings lines as the previous two species. All above observations can be found in the original descriptions (except the size pattern).

Ocelli pattern for individuals collected in Colombia and USA were more variable. Specimens of *H. sosybius,* described for South US, followed the pattern described in the original species description, allowing the identification of them by wing pattern once a trained eye is acquired. The species has more ferruginous scales in the lines and outside the ocelli than the other *Hermeuptychia* species. The lines are crocked, second and the fifth *ocelli* are bigger, the first, third and fourth are small and blind, the sixth is small but has the three areas well defined, a white pupil, a black ring and the yellow iris surrounded by ferruginous scales.

The original descriptions of *H. calixta* (synonymized by Lamas 2004 to *H. harmonia*)*, H. harmonia*, and *H. pimpla* are almost identical. The group formed by *H. harmonia* and *H. pimpla* can be identified by its ocelli pattern, the second is the largest and the third is residual, being positioned right bellow the second. Within this group there are two differences in wing pattern that usually lead to species discrimination; the first pattern presents second and fifth ocelli larger and more oval shaped, and is usually identified as *H. calixta*; and the second presents second and fifth ocelli generally smaller and round-shaped, and is usually identified as *H. harmonia*. However, these differences were not present in original descriptions, and identification merely followed an arbitrary agreement among taxonomists (Freitas pers. comm.). Neither molecular data from all three gene regions nor male genitalia supported the *ocelli* shape differentiation, thus confirming Lamas (2004) decision to synonymize *H. calixta* with *H. harmonia*. Among these individuals identified as either *H. harmonia* or *H. calixta*, we found representatives of *H. pimpla* based on both male genitalia and molecular data,

showing that no consistent wing pattern characters exists to tell these taxa apart. *H. pimpla* type could not be found neither by our inquiry nor by Lamas (2004), and the individual observed in his checklist is an individual from Felder collection with dubious identification, thus Forster's (1964) is the only register left for *H. pimpla*.

H. maimoune was the only clade identified by comparison with the type species and original description only. The individuals collected match the type photographs, the original description, but did not agree with any of Forster's (1964) male genitalia pictures. The differences from other species are rather subtle and hard to identify. The second and fifth ocelli of the hindwing are the biggest, but not as big as in *H. harmonia* or *H. pimpla*; first, second, fifth and sixth ocelli have white pupils, third and forth are blind, and all them have yellow irises. The internal lines are straight and external lines are also straighter than in the other species. The dorsal wings are dark to light brown, with one external line darker, one line lighter and another internal line darker in the hindwing. As the type is a male with intact abdomen, identification could still be confirmed.

Male genitalia

Sample data for extracted male genitalia is attached (Att.5), showing original sample location, collection, specific genitalia codes and assigned species, Fig. 15 shows observed genitalia parts following Klots (1956) nomenclature. *H. fallax* (Fig. 16) presented the most distinguished morphology, with *valvae* shape clearly distinct, more oval than elongated. It is the only species presenting a broad *valvae* end, with an acute medial point, as opposed to the thinner end of other *Hermeuptychia valvae.* The *H. cucullina* (Fig. 18) genitalia is the smallest of all, with short *aedeagus* with approximately the same diameter as the other species, and thus appearing to be short and large as opposed to Forster's (1964) description of the genus genitalia (elongated with a long and thin *aedeagus*). The male genitalia of *H. atalanta, H. maimoune* and *H. gisella* are very similar: *H. gisella* (Fig. 17) presents an enlarged and conspicuous *carina* in lateral view, and *H. atalanta* (Fig. 16) has slightly smaller processes in the apical end of the *valvae,* a more straight *aedeagus* and a smaller *uncus* when compared to *H. maimoune* (Fig. 18). *H. pimpla* (Fig. 18) and *H. harmonia* (Fig. 16), have also similar genitalia morphology, being distinguished from *H. pimpla* by the presence of a dorsally curved process at the *cucculus*, and by the internal margin of *valvae* bearing several small teeth. *H. hermes* (Fig. 16) can be distinguished from the other species as it has the longest *saccus.* This species also presents thinner *valvae* with the major body of the *valvae* and the *cucculus* process forming two parallel axis. *H. sosybius* (Fig. 17) is more easily

distinguished by its four prominent teeth at the end of *valvae*.With these diagnostic traits, a species identification key is presented below.

Differences in genitalia morphology between *H. maimoune* groups A and B are scarce. The Amazonian group (group A) has a hardly larger entrance to the *ductus ejaculatorius* in the *aedeagus* compared to the Andine group (group B). This difference, however, is comparatively small in relation to other well established *Hermeuptychia* species.

Forster (1964) did present *H. harmonia* and *H. calixta* as different taxonomic units, and the main difference according to the pictures is the lobe in the *sacculus* region of the *valvae* present in *H. harmonia* and absent in *H. calixta.* The only individual that did not present this lobe is CO06 an individual from Jardin, Colombia. However individuals from the same clade, and the same location presented the *saccular* lobe, as did all other inspected *H. harmonia* samples. These findings further support Lamas (2004) decision to synonymize these taxa.

From the group of individuals that presented a mixed pattern of *H. gisella* RpS5, and *H. fallax nad6*, with somewhat dubious *cox 1* (L03 and L05, but also L07 - only with *cox1*), only L03 is a male, and its genitalia follows perfectly the *H. fallax* pattern. The individuals RS26 and RS28, belonging to the unidentified taxa *H. sp2*, are males with *H. hermes* genitalia pattern. The individual CO46 and also MT14 and CO09 (the *H. sp1* group) are females, and could not be identified based on male genitalia pattern

Species identification key based on male genitalia morphology

1. a. Elongated *valvae*, lobe in *sacculus* region may or not be present…….……...…….2

1. b. *Valvae* with roughly oval shape, the *costal* region presents an appendix rising to articulate with the *tegumen*. No lobe in the *sacculus* region, *cucculus* with a small prolonged appendix with broad end in dorsal view, presenting an acute medial projection. *Valvae* folds in the posterior part of the *cucculus* with medium and central parts of *valvae* composed of only one tegumen layer and tubular *cucculus*. Dorsal view of uncus shows a narrow base and a broad medium region with a large pointed end. *Saccus* short and straight …..... ……..……..…….. *Hermeuptychia fallax*

2. a. Lobe in *sacculus* region absent or reduced. *Cucculus* region narrow and elongate, generally 0.45-0.50 of total *valvae* size. Opening of posterior region of *valvae* not conspicuous. *Uncus* curved in a semi-circular shape, with no process, dorsal view showing a prolonged base with posterior part rounded ending in a small pointed projection. *Saccus* short and straight……..…….…….…3

2. b. Conspicuous lobe in the *sacculus* region. *Cucculus* region narrow and short to medium sized, with less than 0.40 of total *valvae* size……..……..…….4

3. a. Internal margin of *cucculus* presenting numerous small teeth, *cucculus* curved dorsally…… ……..…….…… *Hermeuptychia pimpla*
3. b. Internal margin of *cucculus* with no teeth……...…….…… *Hermeuptychia harmonia*

4. a. *Saccus* short and straight.……....……5
4. b. Elongated and curved *saccus* at least as big as 0.7 of total *valvae* size. *Uncus* curved, leaf shaped in dorsal view..……...…….....…….....……. *Hermeuptychia hermes*

5. a. Teeth present in the extremity of *cucculus* region……...……...……6
5. b. Teeth absent in the extremity of *cucculus* region……...……...…….7

6. a. Four conspicuous teeth present in the end of *cucculus* region of the *valvae,* no marginal teeth. Medium sized *cucculus*. Long posterior opening of *valvae* folding. Curved *uncus* with dorsal appendix present or absent, slightly leaf shaped in dorsal view. *Saccus* short and straight. *Aedeagus* long and slightly curved …….……..…… *Hermeuptychia sosybius*
6. b. *Cucculus* region with small teeth located in the internal margin culminating with two small teeth in the apex. *Gnathos* presenting small teeth (generally four) in dorsal view. *Uncus* presenting a conspicuous process in lateral view, and leaf shaped in dorsal view. *Aedeagus* short, smaller than total *valvae* size, giving appearance of being short and large. *Valvae* generally more ornated, with teeth observed even in ventral *valvae* margin…….....…….…....*Hermeuptychia cucullina*

7. a. *Uncus* presenting conspicuous process in lateral view, dorsal view leaf shaped. Extremity of *cucculus* region of *valvae* slightly rounded…….....……... *Hermeuptychia gisella*
7. b. *Uncus* presenting no process, narrow shaped in dorsal view…….....……9

8. a *Uncus* straight, and long. *Cucculus* generally short. *Aedeagus* with proximal end straight and distal end curved dorsally…….……..…….....……...*Hermeuptychia atalanta*
8. b. *Uncus* slightly curved. *Aedeagus* with proximal end curved and presenting a thicker ring structure near insertion point of the *ductus ejaculatorius*……..…..... *Hermeuptychia maimoune*

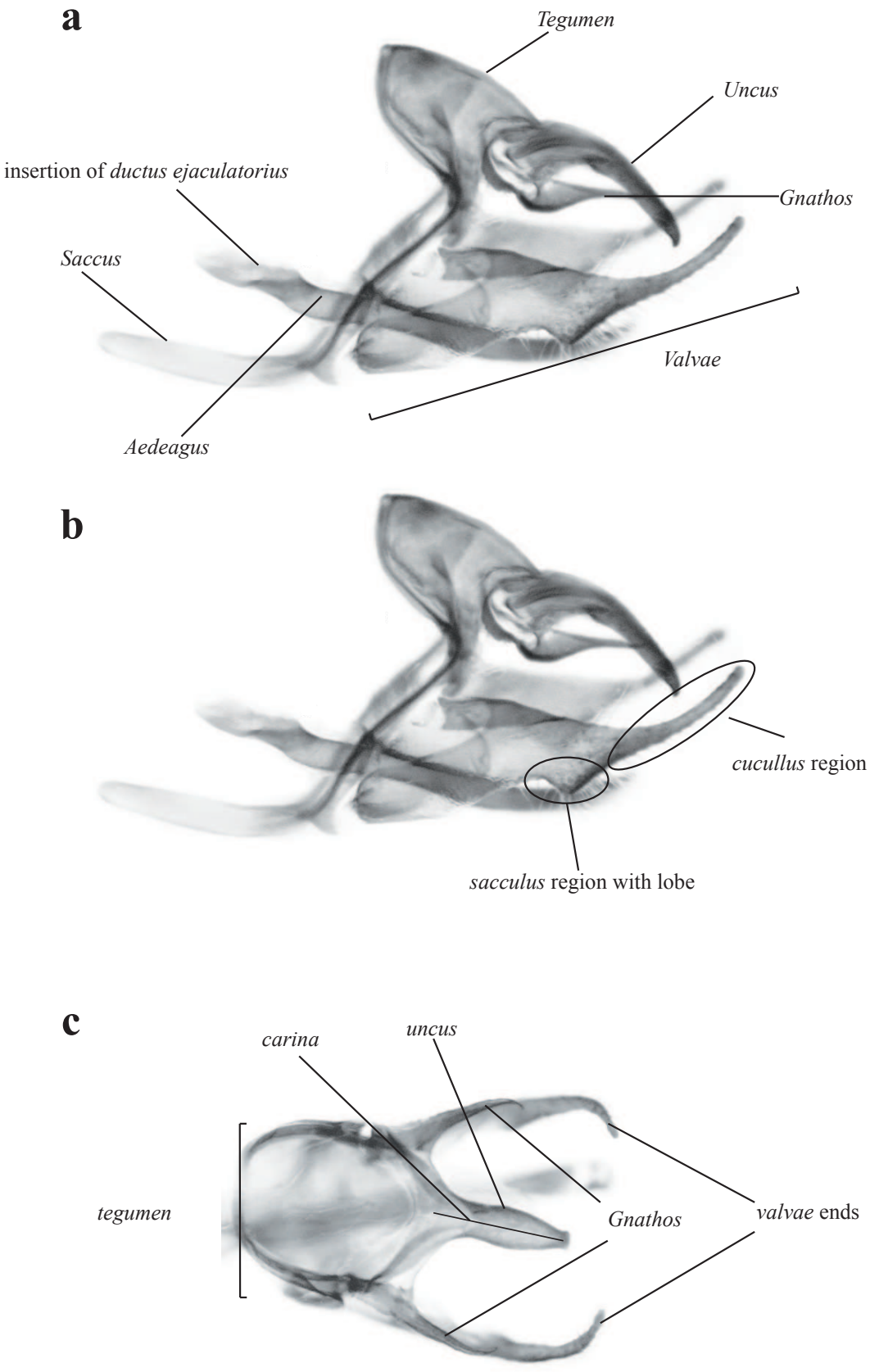

Figure 15. Male genitalia parts, nomenclature follows Klots (1956): **a.** General genitalia parts **b.** *valvae* regions **c.** dorsal view

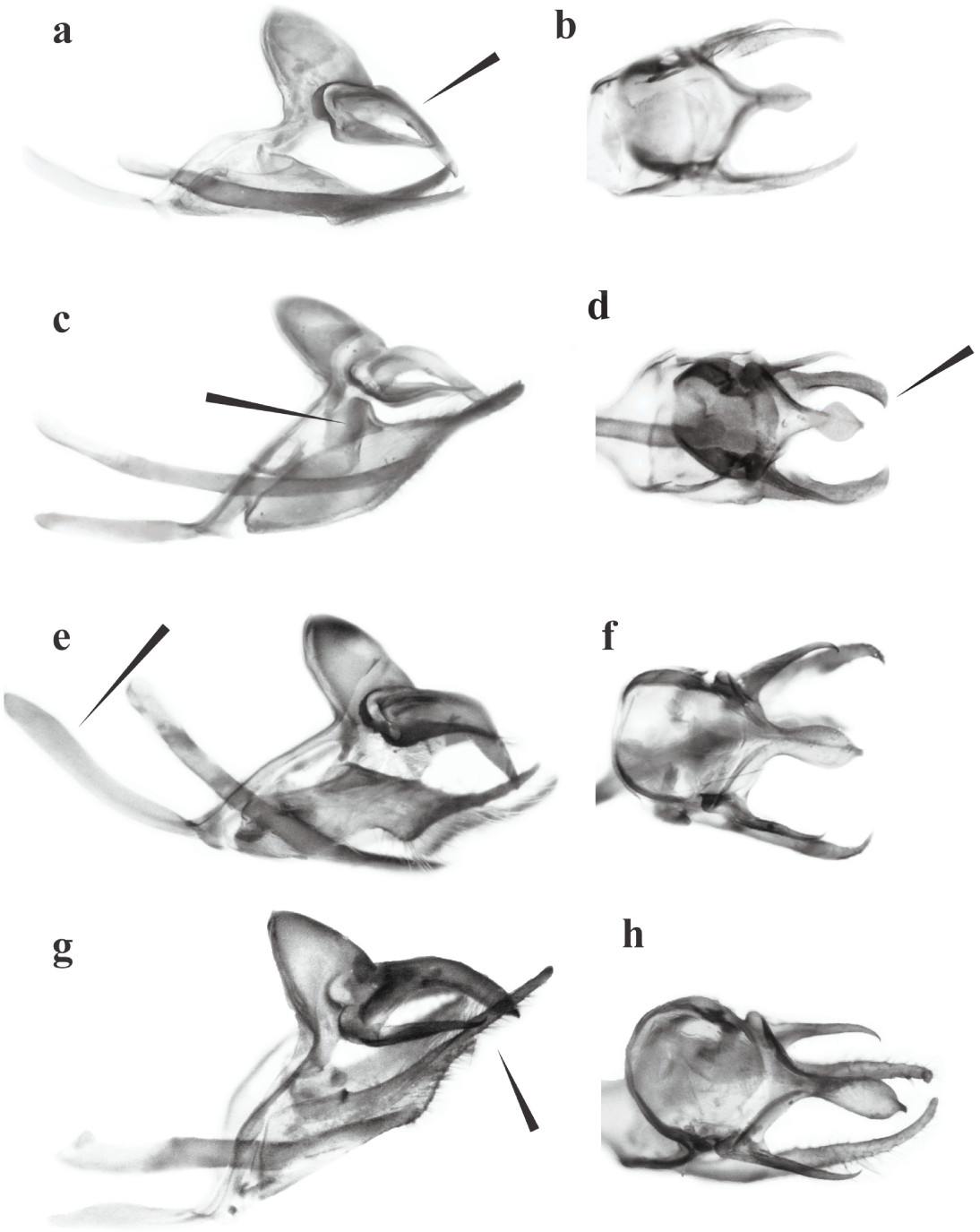

Figure 16. *H. atalanta* male genitalia: **a** lateral view, showing straight *uncus*; **b** dorsal view. *H. fallax* male genitalia: **c** lateral view, showing *valvae* roughly oval-shaped with appendix in *costal* region; **d** dorsal view showing broad *valvae* end. *H. hermes* male genitalia: **e** lateral view showing long *saccus* **f** dorsal view. *H. harmonia* male genitalia: **g** lateral view, showing long process in the *cucculus* region. **h** dorsal view.

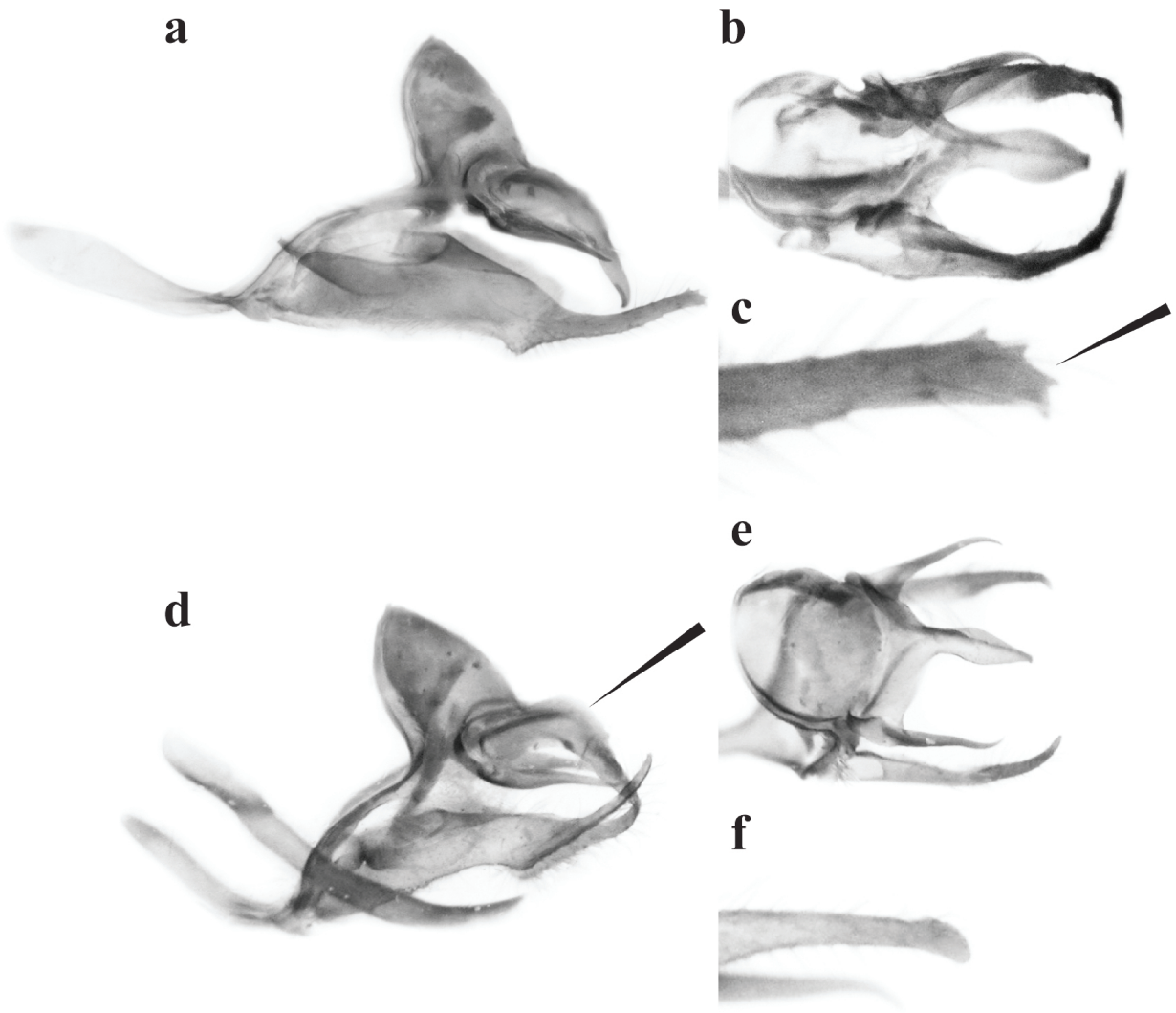

Figure 17. *H. sosybius* male genitalia: **a** lateral view; **b** dorsal view; **c** detail of end of *valvae* teeth. *H. gisella* male genitalia: **d** lateral view, showing conspicuous *carina*; **e** dorsal view; **f** detail of round *valvae* end.

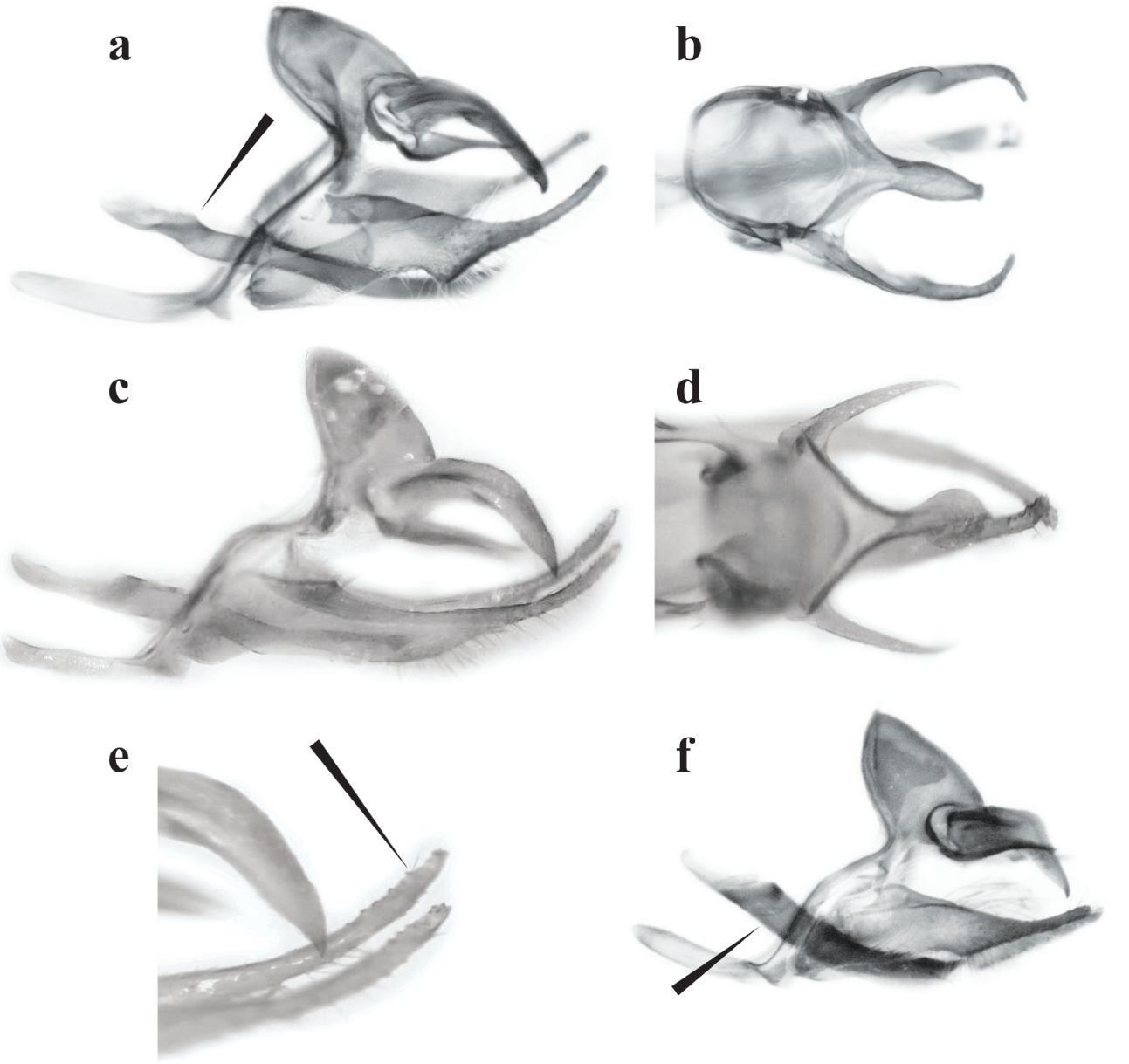

Figure 18. *H. maimoune* male genitalia: **a** lateral view, showing ring structure in the *ductus ejaculatorius* end of *aedeagus*; **b** dorsal view. *H. pimpla* male genitalia: **c** lateral view, showing elongated process in *cucculus* region of *valvae* curved dorsally*;* **d** dorsal view; **e** detail of teeth in the interior margin of *valvae*. *H. cucullina* male genitalia: **f** lateral view showing short *aedeagus.*

3.3 Geographical Patterns

Species geographical distribution (Fig 19. A-I) was plotted for all species studied. Additionally, *H. cucullina* was described for Bolivia, registered in Peru, and recently found in La Estella, Antioquia, Colombia; and Pirenópolis, Goiás, Brazil. Species altitudinal range were low to midland for the majority of *Hermeuptychia*: *H. atalanta* (range = 0m - 1800m, mean = 674m, standard deviation = 420); *H. gisella* (r = 0m - 1700m, m = 800m, sd =462); *H. fallax* (r = 0m - 900m, m = 258m, sd = 342); *H. maimoune* group B (r = 50m - 1030m, m = 802m, sd = 266); and *H. hermes* (r = 45m - 1030m, m = 677m, sd = 344). *H. maimoune* group A was midland dweller (r = 570m - 1600m, m = 933m, sd = 468). *H. harmonia* (r = 1210m - 2443m, m = 1945m, sd = 380) and *H. pimpla* (r = 1500m - 2443m, m = 1977m, sd = 359) were highland dwellers. Finally, *H. sosybius* was found only in lowland (r = 35m - 209m, m = 96m, sd = 82).

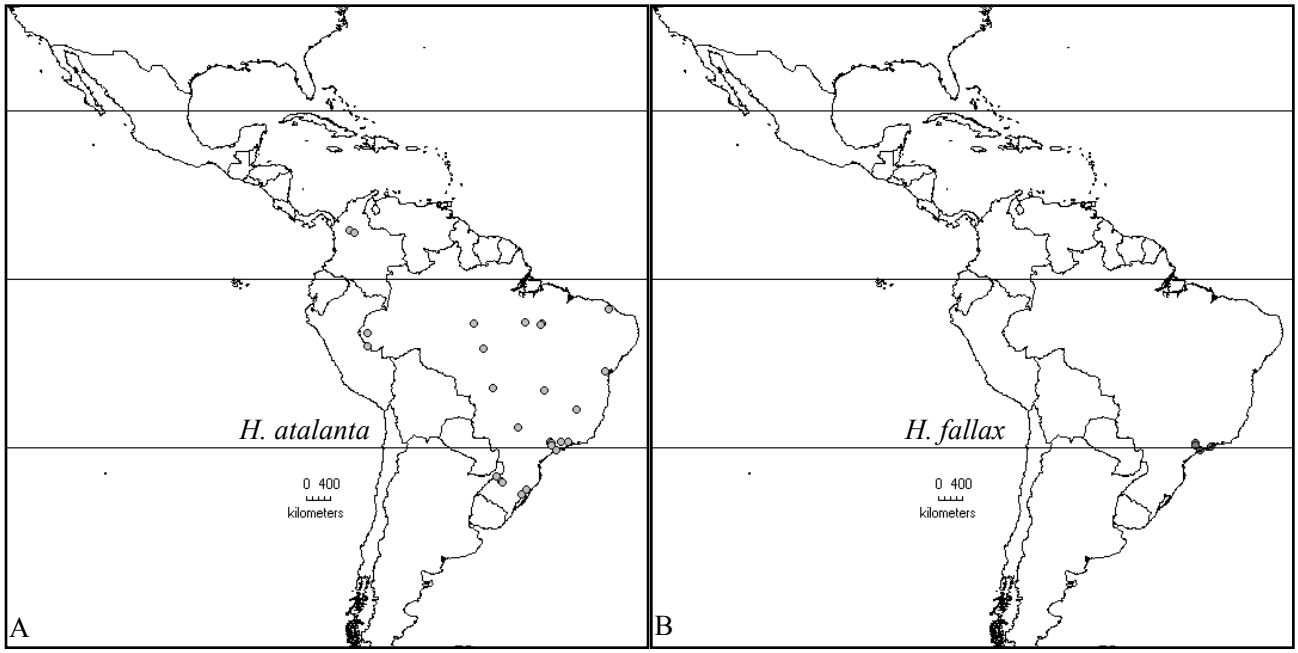

Figure 19. Geographical distribution of the sampled *Hermeuptychia,* Equator and Tropic lines are shown*:* **A** *H. atalanta*; **B** *H. fallax* (continues).

Figure 19 (cont). Geographical distribution of the sampled *Hermeuptychia*: **C** *H. hermes;* **D** *H. harmonia;* **E** *H. pimpla*; **F** *H. maimoune* (continues).

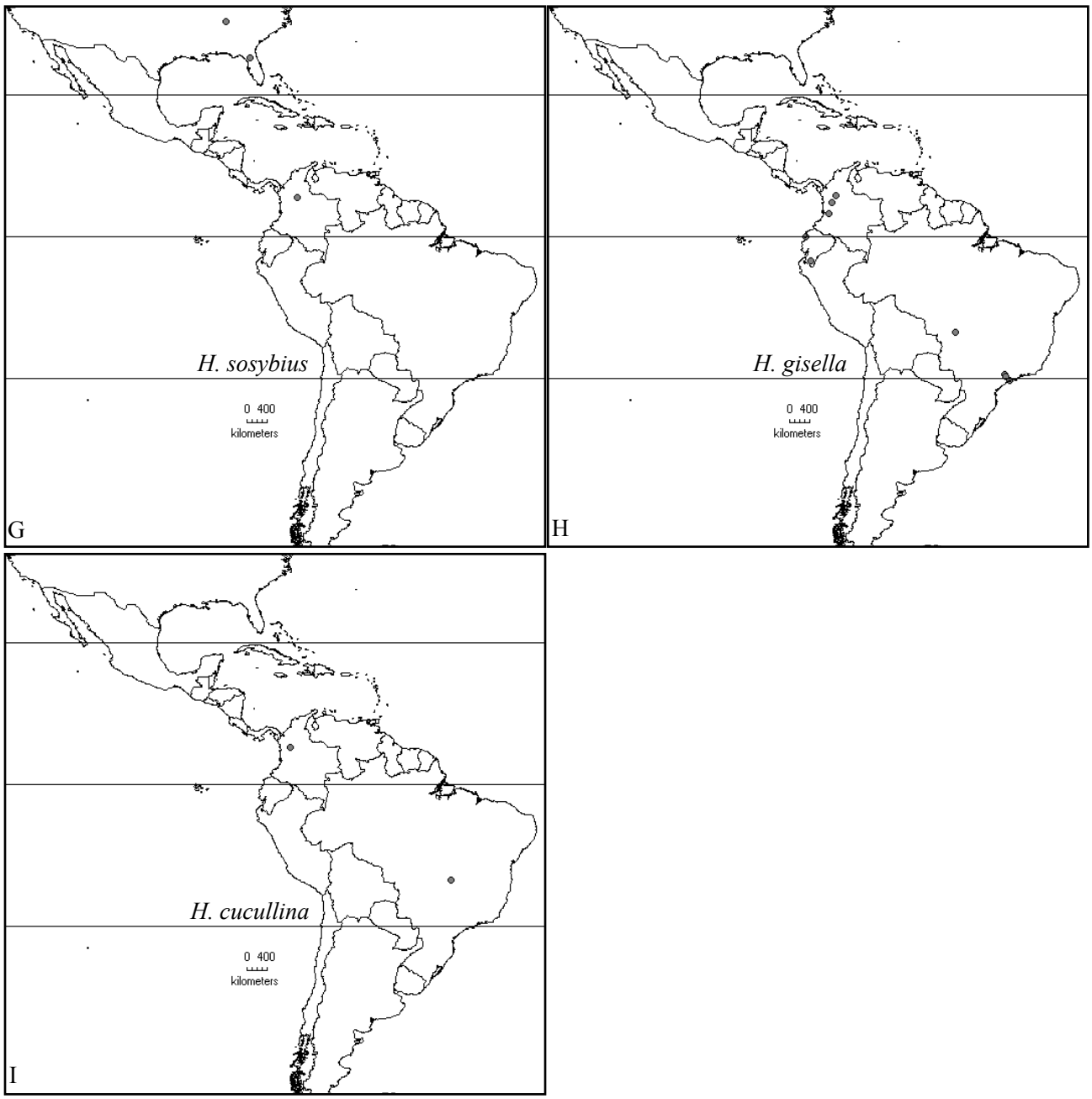

Figure 19. (cont.) Geographical distribution of sampled *Hermeuptychia*: **G** *H. sosybius*; **H** *H. gisella;* **I** *H. cucullina*

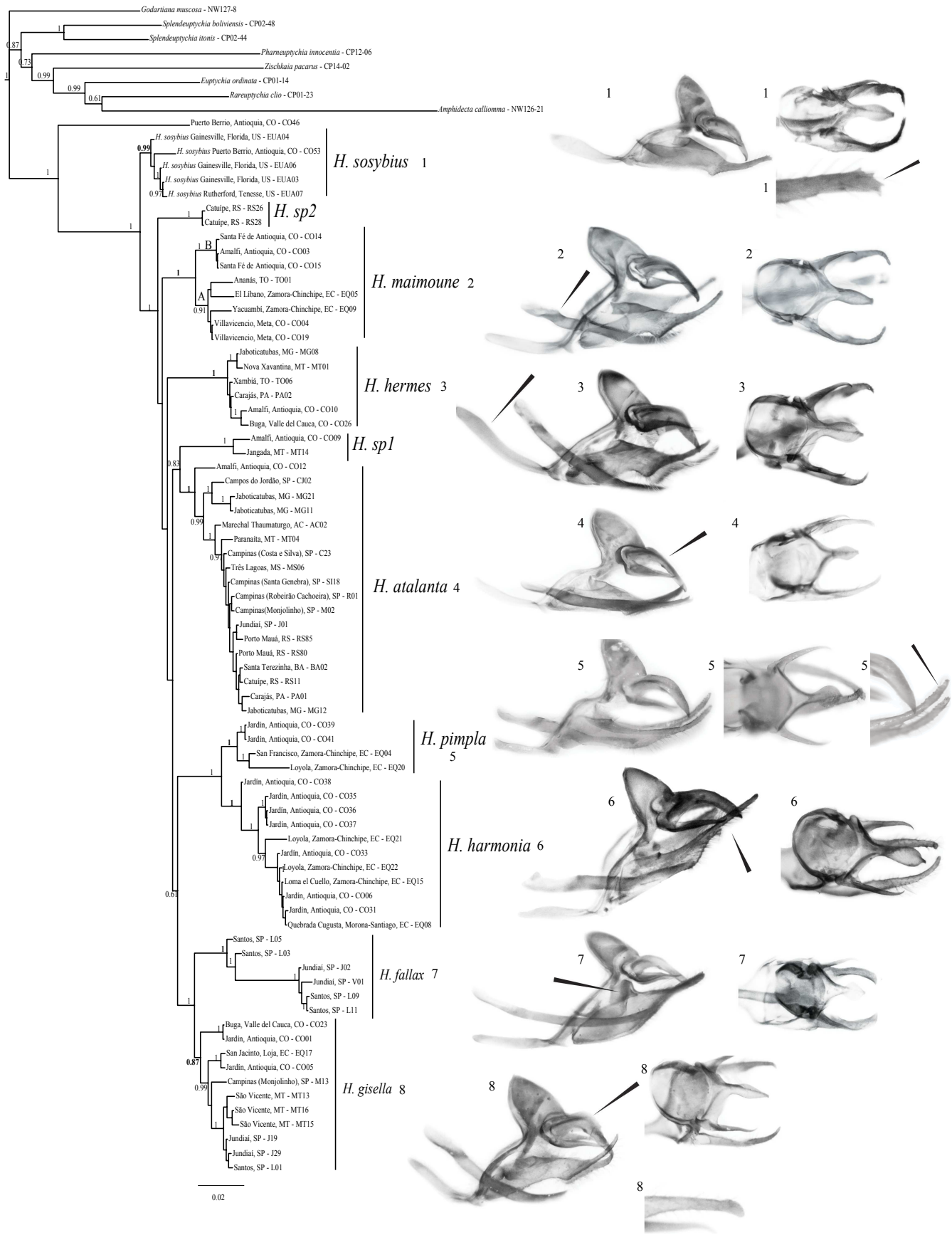

Figure 20. Synthesized main results, based on BI for concatenated data, and male genitalia photographs.

4. DISCUSSION

Phylogenetic hypotheses

The present work recovered *Hermeuptychia* as a monophyletic group (with CO46 as part of the outgroup) as did Peña *et al* (2010; 2011), a condition frequently not observed among several Euptychiina genera (Peña *et al.* 2010). Moreover, the present study helped to clarify the identity, delimitation and distribution of *Hermeuptychia*, a genus that was previously defined as an unresolved species complex needing taxonomic and phylogenetic revision (Marin *et al.* 2009; Marin *et al.* 2011). Main synthesized results for *Hermeuptychia* is shown in Fig. 19.

The relationship among most of the species within *Hermeuptychia* is not resolved, and the results found here are not comparable to the phylogenetic hypothesis among *Hermeuptychia* species found in the literature (Marin *et al.* 2009; Peña *et al.* 2010), since species identification in these previous studies were based only in wing pattern and clearly needs to be revised. Lack of resolution on species relationships is consistent with a rapid speciation event leading to an evolutionary radiation proposed for other Satyrini (Peña *et al.* 2011). Alternatively, other nuclear regions with low mutation levels, as EF-1α and wingless, could be applied to resolve internal relationships within the genus.

The hypothesis of rapid radiation found some support from the ancient history of *Hermeuptychia* inferred by our biogeographical analysis. S-DIVA analysis suggested that the ancestral distribution of most *Hermeuptychia* species, and of the genus itself, is the Andean mountain chain. Additionally, the initial estimated diversification of the genus (8.2 My) coincides with the last uplift of the Andes Mountain chains (late Miocene early Pliocene) (Gregory-Wodzicki 2000), as does Euptychiina diversification (Peña *et al.* 2010). These evidences are consistent to the diversification of the *Hermeuptychia* as a consequence of andean uplift, with posterior colonization of South America for most species, and Central and North Americas for *H. sosybius*. This pattern was already described for groups of butterflies (Elias *et al* 2009). This is somewhat intriguing for the genus *Hermeuptychia*, since at present there are only two species exclusive (*H. harmonia, H. pimpla*) from high altitudes (Marin *et al.* 2011), as well as for Euptychiina, since the majority of its species are lowland dwellers (Peña *et al.* 2010), however, Elias *et al* (2009) also arguments that the uplift of Andes could provide many opportunities for ecological diversification even for lowland dwellers, through influence on the river network of Amazonia lowlands, providing forest refugia during Pleistocene, or simply by increasing environmental complexity.

Sister species within Hermeuptychia

Although relationships among the species within *Hermeuptychia* remain uncertain, at least two clades, *H. harmonia* + *H. pimpla* and *H. fallax* + *H. gisella,* were recovered with all phylogenetic analyses applied on concatenated datasets (though with low support in MP phylogenetic hypothesis). These two clades have also similar divergence time. Possible hybrids between *H. harmonia* and *H. pimpla* were not identified in the present study, but these two species have very similar male genitalia and share an ancestral *nad6* haplotype (D1, Fig 15).

Hybrids between *H. fallax* and *H. gisella* were found in the present study even if these two species were not recovered as sister species in the distance trees (Fig 4-7). The pattern found for samples L03 and L05 is consistent with hybridization between these two species. The RpS5 haplotypes (HA1 Fig 16) are shared with L01 and M13 (both *H. gisella*), the genitalia morphology of L03 corresponds to *H. fallax* (L05 is a female) and the *nad6* haplotype (B1 Fig 15) is shared with L11 and L09 (both *H. fallax*). Further analysis of the original RpS5 chromatograms showed an heterozygous pattern (see Att 7), with *H. gisella* peaks higher than *H. fallax.* These traces are present in L03 and L05 RpS5 chromatograms, as expected for hybrids. Additionally, the chromatogram pattern for these individuals in the *nad6* region did not present the double peaks pattern, as expected of a maternal-inherited mitochondrial region. The *cox1 5'* region showed L05 as part of *H. gisella* (haplotype GH12 Fig 14, only one mutational step from L01), and L03 distant from the other *H. gisella* haplotypes, but still in the same network. The chromatograms of this region are not completely clean of double peaks, which could be explained by heteroplasmy (see Solignac *et al.* 1983; Kvist *et al.* 2003) with paternal leakage (Gyllensten et al. 1991; Schwartz & Vissing 2002; Kvist et al. 2003), nuclear paralogs (Calvignac *et al.* 2011) or contamination. An alternative to elucidate this question is to amplify and sequence L03 and L05 *cox1* region again following a dilution protocol to avoid mtDNA contamination, as described by Calvignac *et al* (2011). This protocol is intended to amplify only the most common *cox 1* sequence, preventing nuclear paralogs, and less frequent paternal mitochondria, to be amplified.

Depending on the species concept used, the sister species could be put together as a single taxonomic unit. *H. pimpla* and *H. harmonia* can be considered a single taxa due to molecular and morphological similarities, and *H. gisella* and *H. fallax* due to hybridization. Furthermore, both sister species co-occur, both are closely related in phylogenetic analysis and both have similar and almost indistinguishable wing patterns.

However, the absence of hybrids between *H. harmonia* and *H. pimpla,* consistent male genitalia morphological differences and molecular divergence support the differentiation of these two species. In the case of *H. gisella* and *H. fallax,* although hybrids can be found, there are major differences in male genitalia, and also differences in mating behavior (Peixoto & Benson 2011). It has been previously described that one of *Hermeuptychia* species presents territorial mating behavior (Peixoto 2009), since the authors sent samples for identification, after the present study, it was possible to identify that the territorial species is *H. fallax*.

The pattern found for individuals RS26 and RS28 could also be indicative of hybridization, but in this case, morphology corresponds to *H. hermes* and DNA could not match consistently any clade. Another possibility is DNA contamination, though we would still assume that this pattern should be found in other samples (as in the case of the hybridization hypothesis).

Species delimitation

All species within *Hermeuptychia* appeared separated in well defined clades using either the 'barcode' data alone or the concatenated data of mitochondrial and nuclear genes, and eight out of ten clusters corresponded to a clearly distinguished pattern of male genitalia, and could be easily assigned to previously described species. There are two clades, however, that remain unidentified. The *H. sp1* clade, composed by two females, and the *H. sp 2* clade, which have genitalia similar to that of individuals assigned as *H. hermes*. Because *H. cucullina* is still not present in the molecular analysis, it could still be possible that *H. sp1* corresponds to this species. Otherwise, it can be a new species, needing more study and further sampling, to be properly described.

The molecular species delimitation analysis (GMYC) failed to recover most of the morphological species. Only in the case of *H. hermes* and possibly *H. atalanta* the analysis with a single threshold recovered morphological identified species. This analysis also grouped the sister species *H. harmonia +H. pimpla* and *H. fallax+H.gisella* as taxonomic units, and grouped the remaining species *H. sosybius* and *H. maimoune.* Although interesting because it grouped sister species together, this analysis was not adequate for the *Hermeuptychia* and the threshold delimited was not significant.

In the specific case of *H. maimoune*, there is a high intraspecific genetic distance, consistent with phenetic and phylogenetic structure found in clusters of N-J analyses and phylogenetic inferences, respectively. This distance is specially high when only the 'barcode' region is analyzed, which suggest different species status for the separate clades. This differentiation represents geographical distribution,

with specimens grouped in two genetic clusters, one from oriental Andes + Brazilian Amazon (group A in Figs. 2 and 3), and the other from the occidental Andes (group B in Figs. 2 and 3). Nevertheless, both groups have identical wing patterns, and present hardly differentiation in genitalia morphology, especially when compared to the differences among other closely related species.

Subspecies are common in butterfly taxonomy, an in fact many of them can be describe as geographical races (Brown 1975), with some clear and easily identified variation in morphology. Furthermore, the divergence found here between *H. maimoune* subgroups is higher then the divergence found between subspecies of *Parides* (Silva-Brandão *et al* 2008a) and *Heliconius erato* (Brower 1994). Usually, there are no differences between subspecies genitalia (Freitas, pers. comm.). In view of all above evidence it is more parsimonious to assume that the two molecular groups found within *H. maimoune* correspond to recent diverged cryptic species still ongoing speciation process.

Use of proposed 'barcode' region in Hermeuptychia species identification

One of the most discussed problems with DNA 'barcodes' is inadequate sample size. Without proper sample size and representativeness of species distribution the 'barcode' could be unsuccessful to reveal species diversity thus failing to establish connection between some of species haplotypes and its 'barcode' signature (Vogler & Monaghan 2006). Meyer and Paulay (2005) found that 'barcode' performs poorly in incompletely sampled groups, and actually, in any DNA taxonomy effort, inadequate sample size could be hazardous. In this study sample size and representativeness was adequate for most species (with the probable exception of *H. pimpla,* a rare species, and *H. sosybius*, sampled only in the extremes of its distribution) thus, here, species could be properly recovered in the 'barcode' analyses, which was supported by the other molecular and morphological data.

Hermeuptychia species were successfully separated based on the 'barcode' region alone, with little incongruence between morphological and 'barcode' cluster identification (only 2% of the complete dataset for the 'barcode' region). This is only possible because of comprehensive sampling and good morphological taxonomy, as expected by Brower *et al.* (1996), and Vogler and Monaghan (2006).

Another problem of DNA 'barcodes' is the supposed lack of a barcoding gap (Meyer & Paulay 2005), that is, a clear threshold limit between intraspecific and interspecific genetic distance. In this study, 'barcode' genetic distances found between and within species did not overlapped (Fig.1a), provided that *H. maimoune* groups are computed separately. When *H. maimoune* cryptic species were

analyzed separately, the maximum intraspecific distance was 1.7 % for *H. fallax*, while the minimum interspecific distance was found between *H. gisella* and *H. sosybius* (2.4%). These values are within the 2% margin suggested by Hebert *et al.* (2004), and it is also proximate to the results found for *Parides* by Silva-Brandão *et al.* (2008a), with intraspecific genetic distances in *Hermeuptychia* more homogeneous than within *Parides*. The interspecific distance for congeneric taxa was similar to that found by Hajibabaei (2006), but the intraspecific distance was higher here, 0.8% for *Hermeuptychia* species, compared to 0.17%, 0.43% and 0.46% for three different moth families. When we analyze *H. maimoune* as a single unit, the minimum interspecific distance remains the same (2.4% between *H. gisella* and *H. sosybius*), but the maximum intraspecific distance is now 2.1% for *H. maimoune*, and thus the threshold disappears.

It is also possible for 'barcodes' to fail to individualize some groups due to the lack of enough genetic diversification between closely related species, mainly because of ongoing continuous process of speciation, as in the case of *Actinote* species (Silva-Brandão *et al.* 2008b). This phenomenon is called "gray zone" and represents the process of speciation itself, a period of time in which the two or more diverging species are not completely divided, and yet not a single taxonomic unit anymore (Wahlberg *et al.* 2009b). It is probably the case for *Hermeuptychia* species: although this time has already passed, and they are clearly "good" species, they were recently separated, which is corroborated by the divergence time estimative (1.4-3.5 My minimum and maximum species estimated divergence time, respectively). Even in the case of *H. maimoune* cryptic species, the 'barcode' region has already accumulated enough mutations to clearly separate these two clades.

Comparison among different gene regions

All different molecular methods recovered the same groups, using both the concatenated data or the barcode data. NJ distance trees with separated gene regions presented more incongruence when compared to morphological data than trees generated with concatenated datasets. NJ distance tree of RpS5 nuclear region presented less differentiation among groups, with lower support values, and with *H. harmonia* and *H. pimpla* grouped together, reflecting lower mutation rate of nDNA compared to the mtDNA regions. Furthermore, the RpS5 network was the most continuous showing most of *Hermeuptychia* species grouped in the same network.

The *nad6* NJ distance tree presented more spurious results than the other separated gene regions, with more discordances with morphological identification. However, this is also the only region that

recovered *H. fallax* as a monophyletic group, with small internal branches, and the region that better recovered species boundaries in the network analysis. This pattern is also present in the PBS analysis, in which the incongruencies due to *nad6* were the major responsible for low Bremer support. On the other hand, *nad6* was the main responsible for good support of *H. fallax* clade. These results are consistent with the *nad6* variation pattern. It is the most variable molecular region studied here, presenting 21% of variable nucleotide sites and 21% of variable amino acid sites. This pattern is not exclusive of the present study, but was also observed in other published mitochondrial genomes (Cameron & Whiting 2008; Yin *et al.* 2010). This mitochondrial region appears to be a highly variable gene region, with only a small portion of conservative amino acid sites in the middle of the sequence. Because of this pattern, *nad6* has been successfully used in population genetic studies (Albernaz 2011; Silva-Brandão *et al.* 2011 in press) showing genetic differentiation among populations even for widespread invasive species.

The *cox1* 5' ('barcode' region) presents an intermediate variability showing the best results for a single gene distance tree. The complete dataset presented the same bootstrap support to the species clusters, showing a better support only for *H. gisella* (bootstrap support = 59) than the NJ distance tree containing only the individuals chosen for the concatenated matrix (bootstrap support = 51). This is an indicative that sample selection did indeed represent all species groups and additional sampling would not significantly improve the final concatenated matrix.

Identification parameters, geographical distribution and taxonomical implications

Although male genitalia morphology can be hard to access in rapid species survey, with little training and the proposed species key it is possible to successfully identify males of all species of *Hermeuptychia*. Because it has not been done in the past, most published surveys lumped several species under *H. hermes* or *H. harmonia*, overestimating their geographical ranges and consequently ignoring all other species (Cardoso 1949; Brown & Mielke 1967, 1968; Brown 1992; Motta 2002; Iserhard & Romanowski 2004; Emery *et al.* 2006; Silva *et al.* 2007; Paz *et al.* 2008; Pinheiro *et al.* 2008; Sackis & Morais 2008; Vasconcelos *et al.* 2009; Iserhard *et al.* 2010; Silva *et al.* 2010). Therefore, the distributions presented in the present study, although species were not exhaustively sampled, are an important first attempt to clarify the actual species distributions in the Americas. The present study identified that the most common species found in Brazil is not *H. hermes,* but *H. atalanta,* not previously present in Brazilian listings. The challenge now is to find consistent and

objective differences in external morphology that could help in species identification on field or in collections without the need of dissecting genitalia or extracting DNA. If this could be attained, all specimens would be identified to species level, especially females, both in museum collections and field inventories, allowing a wider understanding about the geographical distribution of known species of *Hermeuptychia*.

Although not present in the molecular analysis, *H. cucullina* is present in the morphological analysis, and showed a distinct pattern from that of *H. gisella,* although the two taxa have been previously synonymized (Lamas 2004). Because of the differences found here and in Forster (1964), we suggest that *H. gisella* and *H. cucullina* are in fact valid taxonomic units with species status. Comparatively, as there were no differences between the wing patterns initially identified as *H. harmonia* and *H. calixta,* and because the differences found in Forster (1964) genitalia analyses are minimal and do not correspond to any clade in our work, our results support the decision to synonymize these taxa (Lamas 2004). Furthermore, *H. maimoune* can now be considered two cryptic species following a geographical allopatric pattern, and in need of taxonomic revision.

5. CONCLUSIONS

DeSalle *et al.* (2005) proposes an integrative approach to DNA taxonomy in which morphology, geography, DNA, ecology, and reproduction (meaning reproductive isolation) are part of a process to delimitate species. In this process, evidence from one source is used to generate a taxonomic hypothesis, and then evidence from the other sources can be used to test this hypothesis. For them, evidence from a single source is not enough to delimitate species, and it is crucial to sum evidence from different datasets. In this work we focused mainly on DNA and male genitalia morphology, but additional evidences from geography, in the case of *H. maimoune* cryptic species and ecology, in the case of the territorial *H. fallax*, were used to corroborate the proposed species delimitation. The great advantage of such a method is its testability, which ultimately gives taxonomy robustness and credibility, diminishing possibilities of errors.

The genus *Hermeuptychia* is monophyletic, presenting a radiation-like pattern of unresolved internal relationships. There are two pairs of sister species, the first composed by *H. harmonia* and *H. pimpla* supported by morphological and molecular similarities, and the second formed by *H. gisella* and *H. fallax* supported by phylogenetic proximity and incomplete reproductive isolation with hybrid formation. Both sister species pairs are sympatric, and have undistinguishable wing patterns.

Identification parameters for *Hermeuptychia* species remain, to the moment, restricted to male genitalia morphology, and a few wing characters that can separate species groups.

The proposed 'barcode' region (*cox1* 5') (Hebert *et al.* 2003b) can correctly distinguish *Hermeuptychia* species, and besides of being high congruent with male genitalia morphology, this is the only possible way for identifying females. The only possible method for 'barcode' identification of the genus is through tree analysis, and either NJ distance or BI methods recovered similar species delimitation. Comparison of genetic distances among and within species could lead to identification problems due to absence of a clear threshold limit between interspecific and intraspecific genetic distance.

Although mainly resolved, there are still some problems with *Hermeuptychia* species taxonomy. Firstly, *H. cucullina* is still absent in molecular analysis. Secondly, not all species relationships are resolved, and perhaps even further DNA sampling will not solve this, as the pattern found for Satyrini is consistent with rapid divergence and radiation (Peña *et al.* 2011). And, finally, there is still need for more study across the distribution range for some species (as *H. sosybius*), and further sampling could also reveal a broader species distribution range for all *Hermeuptychia* species. Further study of species ecology could also clarify specific differences, and help to explain evolutionary mechanisms and divergence patterns, as selection for ongoing diversification of *H. maimoune* cryptic species or identification mechanisms in mating systems of *H. gisella* and *H. fallax*.

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CONCLUSÃO GERAL

DeSalle *et al.* (2005) propõem uma abordagem integrada para a taxonomia, na qual evidências da morfologia, geografia, análises moleculares e de isolamento reprodutivo são parte de um processo para delimitar espécies. Neste processo, evidência de uma dessas fontes é utilizada para gerar uma hipótese taxonômica, e evidências de outras fontes são utilizadas para corroborar ou rejeitar essa hipótese. Para eles, evidências de uma única fonte não são suficientes para delimitar espécies e a soma de diferentes fontes de informação é crucial. Neste trabalho nós focamos principalmente em análises moleculares e de morfologia de genitália masculina, mas evidências adicionais de biogeografia, ecologia e isolamento reprodutivo foram usadas para corroborar a delimitação de espécies proposta aqui. A grande vantagem de tal abordagem é a sua capacidade de ser testada, o que contribui para fornecer à taxonomia maior robustez e credibilidade, diminuindo a possibilidade de erros.

O gênero *Hermeuptychia* é monofilético, apresentando um padrão similar ao de uma radiação evolutiva, com relações internas não resolvidas. Existem dois pares de espécies-irmãs, o primeiro composto por *H. harmonia* e *H. pimpla* que encontra suporte em similaridades morfológicas e moleculares, e o segundo formado por *H. gisella* e *H. fallax*, que encontra suporte na proximidade filogenética, e no isolamento reprodutivo incompleto com formação de híbridos. Ambos os pares de espécies-irmãs são simpátricos e tem padrões alares indistinguíveis. Os parâmetros de identificação para as espécies de *Hermeuptychia,* até o presente, restringem-se à morfologia de genitália masculina e a alguns poucos caracteres de asa, que podem separar grupos de espécies.

A região 'barcode', proposta por Hebert *et al.* (2003a, 2003b), é capaz de separar as espécies de *Hermeuptychia* com alta congruência com a identificação baseada em morfologia de genitália masculina, constituindo-se na única maneira possível de se identificar fêmeas. O único método possível para a identificação por 'barcode' é através de análises de árvores, e ambos os métodos de Neighbor-Joining, baseado em distância genética, e de Inferência Bayesiana recuperaram árvores semelhantes, com mesma delimitação de espécies. A comparação de distâncias intra e interespecíficas não seria adequada para identificação de novos indivíduos pois, poderia levar a conclusões errôneas devido à falta de um limite claro entre distâncias genéticas intra e interespecíficas.

Embora quase completamente resolvida, existem ainda alguns problemas com a taxonomia de *Hermeuptychia*. Em primeiro lugar, *H. cucullina* ainda está ausente nas análises moleculares. Além disso, nem todas as relações entre as espécies estão resolvidas, e talvez nem o aumento da amostragem

molecular poderia resolver esse problema, porque o padrão encontrado para Satyrini é o de uma rápida diversificação e radiação (Peña *et al.* 2011). Por fim, ainda existe a necessidade de ampliar a amostragem para englobar melhor a distribuição geográfica de algumas espécies (como *H. sosybius*). O aumento da amostragem ainda pode revelar um padrão de distribuição mais amplo para todas as espécies de *Hermeuptychia.*

A delimitação das espécies de *Hermeuptychia* foi possível somente através da observação de padrões morfológicos e moleculares em conjunto. É a continuidade desses padrões dentro das espécies e a sua descontinuidade entre espécies que nos faz percebê-las como unidades taxonômicas. Portanto, ao invés de debater qual o melhor conceito de espécie baseado nos processos que levam a sua diversificação seria mais interessante para o estudo da taxonomia e da evolução que se estabelecesse um conceito simples, de reconhecimento dos padrões que representam espécies, para que uma vez delimitadas os padrões de diversificação, estruturação e especiação pudessem ser estudados, sem que um argumento circular precise ser invocado

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ANEXOS

Att. 1. Sample data

DNA VOUCHER	COUNTRY	STATE	LOCALITY	LAT	LONG	ALT	COLLECTION	*COX 1*	*NAD 6*	RPS 5
H. atalanta					low to midland					
AC01	Brazil	Acre	Cruzeiro do Sul	-7.5130	-72.6802		NSP	x		
AC02	Brazil	Acre	Marechal Taumaturgo	-9.4000	-72.7000	200	AVLF	x	x	x
BA01	Brazil	Bahia	Santa Terezinha	-12.8500	-39.4667	600-800	AVLF	x		
BA02	Brazil	Bahia	Santa Terezinha	-12.8500	-39.4667	600-800	AVLF	x	x	x
BA03	Brazil	Bahia	Santa Terezinha	-12.8500	-39.4667	600-800	AVLF	x		
CE01	Brazil	Ceará	Guaramiranga; Serra do Baturité	-4.2500	-38.9333	866	AVLF	x		
CE02	Brazil	Ceará	Guaramiranga; Serra do Baturité	-4.2500	-38.9333	866	AVLF	x		
DF02	Brazil	Distrito Federal	Parque Nacional de Brasilia	-15.5992	-47.9592	1180	AVLF	x		
DF03	Brazil	Distrito Federal	Parque Nacional de Brasilia	-15.5992	-47.9592	1180	AVLF	x		
DF04	Brazil	Distrito Federal	Parque Nacional de Brasilia	-15.5992	-47.9592	1180	AVLF	x		
DF10	Brazil	Distrito Federal	Parque Nacional de Brasilia	-15.5992	-47.9592	1180	AVLF	x		
DF11	Brazil	Distrito Federal	Parque Nacional de Brasilia	-15.5992	-47.9592	1180	AVLF	x		
DF12	Brazil	Distrito Federal	Parque Nacional de Brasilia	-15.5992	-47.9592	1180	AVLF	x		
MT03	Brazil	Mato Grosso	Paranaíta	-9.6728	-56.4806		AVLF	x		
MT04	Brazil	Mato Grosso	Paranaíta	-9.6728	-56.4806		AVLF	x	x	x
MT05	Brazil	Mato Grosso	Paranaíta	-9.6728	-56.4806		AVLF	x		
MT06	Brazil	Mato Grosso	Paranaíta	-9.6728	-56.4806		AVLF	x		
MT08	Brazil	Mato Grosso	Paranaíta	-9.6728	-56.4806		AVLF	x		
MT09	Brazil	Mato Grosso	Paranaíta	-9.6728	-56.4806		AVLF	x		
MT11	Brazil	Mato Grosso	Jangada	-5.2667	-55.2333	560	NSP	x		
MS02	Brazil	Mato Grosso do Sul	Três Lagoas	-20.8000	-51.7167	300	NSP	x		
MS03	Brazil	Mato Grosso do Sul	Três Lagoas	-20.8000	-51.7167	300	NSP	x		
MS04	Brazil	Mato Grosso do Sul	Três Lagoas	-20.8000	-51.7167	300	NSP	x		
MS05	Brazil	Mato Grosso do Sul	Três Lagoas	-20.8000	-51.7167	300	NSP	x		
MS06	Brazil	Mato Grosso do Sul	Três Lagoas	-20.8000	-51.7167	300	NSP	x	x	x
MS07	Brazil	Mato Grosso do Sul	Três Lagoas	-20.8000	-51.7167	300	NSP	x		
MS08	Brazil	Mato Grosso do Sul	Três Lagoas	-20.8000	-51.7167	300	NSP	x		
MG11	Brazil	Minas Gerais	Jaboticatubas; Serra do Cipo	-18.2000	-43.5000	900	NSP	x	x	x
MG12	Brazil	Minas Gerais	Jaboticatubas; Serra do Cipo	-18.2000	-43.5000	900	NSP	x	x	x
MG21	Brazil	Minas Gerais	Jaboticatubas; Serra do Cipo	-18.2000	-43.5000	900	NSP	x	x	x
MG22	Brazil	Minas Gerais	Jaboticatubas; Serra do Cipo	-18.2000	-43.5000	900	NSP	x		
PA01	Brazil	Pará	Carajás	-6.0322	-50.5747	670	NSP	x	x	x
PA03	Brazil	Pará	Carajás	-6.0322	-50.5747	670	NSP	x		
PA07	Brazil	Pará	Jacareacanga	-6.1950	-57.8269	80	AVLF	x		
RS11	Brazil	Rio Grande do Sul	Catuípe	-28.2367	-54.0078	280	NSP	x	x	x

DNA VOUCHER	COUNTRY	STATE	LOCALITY	LAT	LONG	ALT	COLLECTION	*COX 1*	*NAD 6*	RPS 5
RS32	Brazil	Rio Grande do Sul	Catuípe	-28.2367	-54.0078	280	NSP	x		
RS34	Brazil	Rio Grande do Sul	Catuípe	-28.2367	-54.0078	280	NSP	x		
RS36	Brazil	Rio Grande do Sul	Catuípe	-28.2367	-54.0078	280	NSP	x		
RS42	Brazil	Rio Grande do Sul	Porto Mauá	-27.5772	-54.6744	120	NSP	x		
RS70	Brazil	Rio Grande do Sul	Bozano	-28.3728	-53.7792	355	ZUEC	x		
RS74	Brazil	Rio Grande do Sul	São Francisco de Paula; FLONA	-29.4333	-50.5667	630	ZUEC	x		
RS75	Brazil	Rio Grande do Sul	São Francisco de Paula; FLONA	-29.4333	-50.5667	630	ZUEC	x		
RS77	Brazil	Rio Grande do Sul	Porto Mauá	-27.5772	-54.6744	120	NSP	x		
RS80	Brazil	Rio Grande do Sul	Porto Mauá	-27.5772	-54.6744	120	NSP	x	x	x
RS85	Brazil	Rio Grande do Sul	Porto Mauá	-27.5772	-54.6744	120	NSP	x	x	x
RS95	Brazil	Rio Grande do Sul	Porto Alegre	-30.0581	-51.1994	54	ZUEC	x		
RS97	Brazil	Rio Grande do Sul	São Francisco de Paula; FLONA	-29.4333	-50.5667	630	ZUEC	x		
RS98	Brazil	Rio Grande do Sul	São Francisco de Paula; FLONA	-29.4333	-50.5667	630	ZUEC	x		
TO02	Brazil	Tocantins	Ananás	-6.1447	-48.3090		AVLF	x		
TO03	Brazil	Tocantins	Ananás	-6.1447	-48.3090		AVLF	x		
TO04	Brazil	Tocantins	Ananás	-6.1447	-48.3090		AVLF	x		
TO05	Brazil	Tocantins	Xambiá	-6.4428	-48.5632		AVLF	x		
B01	Brazil	São Paulo	Serra da Bocaina	-22.7139	-44.5944		ZUEC	x		
R01	Brazil	São Paulo	Campinas; Ribeirão Cachoeira	-22.8294	-46.9264	600-650	NSP	x	x	x
R10	Brazil	São Paulo	Campinas; Ribeirão Cachoeira	-22.8294	-46.9264	600-650	NSP	x		
R15	Brazil	São Paulo	Campinas; Ribeirão Cachoeira	-22.8294	-46.9264	600-650	NSP	x		
M02	Brazil	São Paulo	Campinas; Monjolinho	-22.8294	-46.9264	600-650	NSP	x	x	x
M18	Brazil	São Paulo	Campinas; Monjolinho	-22.8294	-46.9264	600-650	NSP	x		
M24	Brazil	São Paulo	Campinas; Monjolinho	-22.8294	-46.9264	600-650	NSP	x		
SI14	Brazil	São Paulo	Campinas; Santa Genebra	-22.8294	-46.9264	600-650	NSP	x		
SI18	Brazil	São Paulo	Campinas; Santa Genebra	-22.8294	-46.9264	600-650	NSP	x	x	x
SI23	Brazil	São Paulo	Campinas; Santa Genebra	-22.8294	-46.9264	600-650	NSP	x		
SII10	Brazil	São Paulo	Campinas; Santa Genebra	-22.8294	-46.9264	600-650	NSP	x		
SII12	Brazil	São Paulo	Campinas; Santa Genebra	-22.8294	-46.9264	600-650	NSP	x		
C01	Brazil	São Paulo	Campinas; Costa e Silva	-22.8294	-46.9264	600-650	NSP	x		
C02	Brazil	São Paulo	Campinas; Costa e Silva	-22.8294	-46.9264	600-650	NSP	x		
C12	Brazil	São Paulo	Campinas; Costa e Silva	-22.8294	-46.9264	600-650	NSP	x		
C13	Brazil	São Paulo	Campinas; Costa e Silva	-22.8294	-46.9264	600-650	NSP	x		
C23	Brazil	São Paulo	Campinas; Costa e Silva	-22.8294	-46.9264	600-650	NSP	x	x	x
P06	Brazil	São Paulo	Campinas; Rio das Pedras	-22.8294	-46.9264	600-650	NSP	x		
P07	Brazil	São Paulo	Campinas; Rio das Pedras	-22.8294	-46.9264	600-650	NSP	x		

DNA VOUCHER	COUNTRY	STATE	LOCALITY	LAT	LONG	ALT	COLLECTION	*COX 1*	*NAD 6*	RPS 5
P14	Brazil	São Paulo	Campinas; Rio das Pedras	-22.8294	-46.9264	600-650	NSP	x		
P15	Brazil	São Paulo	Campinas; Rio das Pedras	-22.8294	-46.9264	600-650	NSP	x		
P21	Brazil	São Paulo	Campinas; Rio das Pedras	-22.8294	-46.9264	600-650	NSP	x		
L04	Brazil	São Paulo	Santos; Rio Quilombo	-23.9042	-46.3908	0-50	NSP	x		
CJ01	Brazil	São Paulo	Campos do Jordão	-22.7000	-45.5833	1800	AVLF	x		
CJ02	Brazil	São Paulo	Campos do Jordão	-22.7000	-45.5833	1800	AVLF	x	x	x
CJ03	Brazil	São Paulo	Campos do Jordão	-22.7000	-45.5833	1800	AVLF	x		
CJ04	Brazil	São Paulo	Campos do Jordão	-22.7000	-45.5833	1800	AVLF	x		
CJ05	Brazil	São Paulo	Campos do Jordão	-22.7000	-45.5833	1800	AVLF	x		
J01	Brazil	São Paulo	Jundiaí; Serra do Japi	-23.2167	-46.9167	700-900	NSP	x	x	x
J04	Brazil	São Paulo	Jundiaí; Serra do Japi	-23.2167	-46.9167	700-900	NSP	x		
J06	Brazil	São Paulo	Jundiaí; Serra do Japi	-23.2167	-46.9167	700-900	NSP	x		
J07	Brazil	São Paulo	Jundiaí; Serra do Japi	-23.2167	-46.9167	700-900	NSP	x		
J27	Brazil	São Paulo	Jundiaí; Serra do Japi	-23.2167	-46.9167	700-900	NSP	x		
CO12	Colombia	Antioquia	Amalfi	6.7833	-75.1000	1000	MEFLG	x	x	x
CO51	Colombia	Antioquia	Puerto Berrio	6.5167	-74.4414	115	MEFLG	x		
H. fallax					low to midland					
R18	Brazil	São Paulo	Campinas; Ribeirão Cachoeira	-22.8294	-46.9264	600-650	NSP	x		
R23	Brazil	São Paulo	Campinas; Ribeirão Cachoeira	-22.8294	-46.9264	600-650	NSP	x		
R25	Brazil	São Paulo	Campinas; Ribeirão Cachoeira	-22.8294	-46.9264	600-650	NSP	x		
J02	Brazil	São Paulo	Jundiaí; Serra do Japi	-23.2167	-46.9167	700-900	NSP	x	x	x
J08	Brazil	São Paulo	Jundiaí; Serra do Japi	-23.2167	-46.9167	700-900	NSP	x		
J17	Brazil	São Paulo	Jundiaí; Serra do Japi	-23.2167	-46.9167	700-900	NSP	x		
V01	Brazil	São Paulo	Jundiaí; Serra do Japi	-23.2167	-46.9167	700-900	NSP	x	x	x
L03	Brazil	São Paulo	Santos; Rio Quilombo	-23.9042	-46.3908	0-50	NSP	x	x	x
L05	Brazil	São Paulo	Santos; Rio Quilombo	-23.9042	-46.3908	0-50	NSP	x	x	x
L06	Brazil	São Paulo	Santos; Rio Quilombo	-23.9042	-46.3908	0-50	NSP	x		
L07	Brazil	São Paulo	Santos; Rio Quilombo	-23.9042	-46.3908	0-50	NSP	x		
L08	Brazil	São Paulo	Santos; Rio Quilombo	-23.9042	-46.3908	0-50	NSP	x		
L09	Brazil	São Paulo	Santos; Rio Quilombo	-23.9042	-46.3908	0-50	NSP	x	x	x
L10	Brazil	São Paulo	Santos; Rio Quilombo	-23.9042	-46.3908	0-50	NSP	x		
L11	Brazil	São Paulo	Santos; Rio Quilombo	-23.9042	-46.3908	0-50	NSP	x	x	x
L14	Brazil	São Paulo	Santos; Rio Quilombo	-23.9042	-46.3908	0-50	NSP	x		
L17	Brazil	São Paulo	Ubatuba; Picinguaba	-23.3602	-44.8486	0-50	NSP	x		
L19	Brazil	São Paulo	Ubatuba; Picinguaba	-23.3602	-44.8486	0-50	NSP	x		
L20	Brazil	São Paulo	Ubatuba; Picinguaba	-23.3602	-44.8486	0-50	NSP	x		
L21	Brazil	São Paulo	Ubatuba; Picinguaba	-23.3602	-44.8486	0-50	NSP	x		
L22	Brazil	São Paulo	Ubatuba; Picinguaba	-23.3602	-44.8486	0-50	NSP	x		
H. gisella					low to midland					
MT10	Brazil	Mato Grosso	São Vicente	-15.8167	-55.4000	610	NSP	x		
MT12	Brazil	Mato Grosso	São Vicente	-15.8167	-55.4000	610	NSP	x		
MT13	Brazil	Mato Grosso	São Vicente	-15.8167	-55.4000	610	NSP	x	x	x
MT15	Brazil	Mato Grosso	São Vicente	-15.8167	-55.4000	610	NSP	x	x	x
MT16	Brazil	Mato Grosso	São Vicente	-15.8167	-55.4000	610	NSP	x	x	x
M13	Brazil	São Paulo	Campinas; Monjolinho	-22.8294	-46.9264	600-650	NSP	x	x	x
J19	Brazil	São Paulo	Jundiaí; Serra do Japi	-23.2167	-46.9167	700-900	NSP	x	x	x
J29	Brazil	São Paulo	Jundiaí; Serra do Japi	-23.2167	-46.9167	700-900	NSP	x	x	x

DNA VOUCHER	COUNTRY	STATE	LOCALITY	LAT	LONG	ALT	COLLECTION	*COX 1*	*NAD 6*	RPS 5
L01	Brazil	São Paulo	Santos; Rio Quilombo	-23.9042	-46.3908	0-50	NSP	x	x	x
CO01	Colombia	Antioquia	Jardín; Verda La Linda	5.5833	-75.8167	1600-1700	MEFLG	x	x	x
CO05	Colombia	Antioquia	Jardín; Verda La Linda	5.5833	-75.8167	1600-1700	MEFLG	x	x	x
CO08	Colombia	Antioquia	Amalfi	6.7833	-75.1000	1000	MEFLG	x		
CO23	Colombia	Valle del Cauca	Buga	3.8633	-76.2861	1030	MEFLG	x	x	x
EQ16	Equator	Manabí	Reserva Lalo Loor	-0.0914	-80.1489	90-180	FLMNH	x		
EQ17	Equator	Loja	San Jacinto	-4.1003	-79.3453	1240	FLMNH	x	x	x
H. harmonia					highland					
CO06	Colombia	Antioquia	Jardín; Verda La Linda	5.5833	-75.8167	1600-1700	MEFLG	x	x	x
CO27	Colombia	Valle del Cauca	La Cumbre	3.6847	-76.5300	1925	MEFLG	x		
CO28	Colombia	Valle del Cauca	La Cumbre	3.6847	-76.5300	1925	MEFLG	x		
CO30	Colombia	Antioquia	Jardín; Quebrada Bonita	5.5869	-75.8044	2065-2443	MEFLG	x		
CO31	Colombia	Antioquia	Jardín; Quebrada Bonita	5.5869	-75.8044	2065-2443	MEFLG	x	x	x
CO32	Colombia	Antioquia	Jardín; Quebrada Bonita	5.5869	-75.8044	2065-2443	MEFLG	x		
CO33	Colombia	Antioquia	Jardín; Quebrada Bonita	5.5869	-75.8044	2065-2443	MEFLG	x	x	x
CO34	Colombia	Antioquia	Jardín; Quebrada Bonita	5.5869	-75.8044	2065-2443	MEFLG	x		
CO35	Colombia	Antioquia	Jardín; Quebrada Bonita	5.5869	-75.8044	2065-2443	MEFLG	x	x	x
CO36	Colombia	Antioquia	Jardín; Quebrada Bonita	5.5869	-75.8044	2065-2443	MEFLG	x	x	x
CO37	Colombia	Antioquia	Jardín; Quebrada Bonita	5.5869	-75.8044	2065-2443	MEFLG	x	x	x
CO38	Colombia	Antioquia	Jardín; Quebrada Bonita	5.5869	-75.8044	2065-2443	MEFLG	x	x	x
CO40	Colombia	Antioquia	Jardín; Quebrada Bonita	5.5869	-75.8044	2065-2443	MEFLG	x		
EQ08	Equator	Morona-Santiago	Quebrada Cugusta	-2.2253	-78.2906	1750	FLMNH	x	x	x
EQ10	Equator	Zamora-Chinchipe	Yacuambí	-3.9267		1750	FLMNH	x		
EQ14	Equator	Zamora-Chinchipe	Loma El Cuello	-4.0681	-78.9397	1210	FLMNH	x		
EQ15	Equator	Zamora-Chinchipe	Loma El Cuello	-4.0681	-78.9397	1210	FLMNH	x	x	x
EQ21	Equator	Zamora-Chinchipe	Loyola	-4.5203	-79.0114	1500	FLMNH	x	x	x
EQ22	Equator	Zamora-Chinchipe	Loyola	-4.5203	-79.0114	1500	FLMNH	x	x	x
H. hermes					low to midland					
MT01	Brazil	Mato Grosso	Nova Xavantina	-14.6533	-52.3425		NSP	x	x	x
MG08	Brazil	Minas Gerais	Jaboticatubas; Serra do Cipo	-18.2000	-43.5000	900	NSP	x	x	x
PA02	Brazil	Pará	Carajás	-6.0322	-50.5747	676	NSP	x	x	x
PA04	Brazil	Pará	Carajás	-6.0322	-50.5747	676	NSP	x		
TO06	Brazil	Tocantins	Xambiá	-6.4428	-48.5632		AVLF	x	x	x

DNA VOUCHER	COUNTRY	STATE	LOCALITY	LAT	LONG	ALT	COLLECTION	*COX 1*	*NAD 6*	RPS 5
CO02	Colombia	Meta	Villavicencio; Bosque Bavaria	4.1789	-73.6494	570	MEFLG	x		
CO07	Colombia	Antioquia	Amalfi	6.7833	-75.1000	1000	MEFLG	x		
CO10	Colombia	Antioquia	Amalfi	6.7833	-75.1000	1000	MEFLG	x	x	x
CO13	Colombia	Antioquia	Amalfi	6.7833	-75.1000	1000	MEFLG	x		
CO20	Colombia	Meta	Villavicencio; Bosque Bavaria	4.1789	-73.6494	570	MEFLG	x		
CO21	Colombia	Meta	Villavicencio; Bosque Bavaria	4.1789	-73.6494	570	MEFLG	x		
CO22	Colombia	Meta	Villavicencio; Bosque Bavaria	4.1789	-73.6494	570	MEFLG	x		
CO26	Colombia	Valle del Cauca	Buga	3.8633	-76.2861	1030	MEFLG	x	x	x
CO29	Colombia	Antioquia	Puerto Berrio	6.5167	-74.4414	115	MEFLG	x		
CO42	Colombia	Antioquia	San Roque	6.4783	-74.8525	1000	MEFLG	x		
CO43	Colombia	Antioquia	San Roque	6.4783	-74.8525	1000	MEFLG	x		
CO50	Colombia	Antioquia	Puerto Berrio	6.5167	-74.4414	115	MEFLG	x		
EQ18	Equator	El Oro	Destacamento Chacras	-3.5500	-80.2000	45	FLMNH	x		
H. maimoune A					midland					
PA05	Brazil	Pará	Carajás	-50.5747	-50.5747	676	NSP	x		
TO01	Brazil	Tocantins	Ananás	-48.3090	-48.3090		AVLF	x	x	x
CO04	Colombia	Meta	Villavicencio; Bosque Bavaria	4.1789	-73.6494	570	MEFLG	x	x	x
CO19	Colombia	Meta	Villavicencio; Bosque Bavaria	4.1789	-73.6494	570	MEFLG	x	x	x
EQ05	Equator	Zamora-Chinchipe	El Líbano	-4.0803	-78.9694	1600	FLMNH	x	x	x
EQ09	Equator	Zamora-Chinchipe	Yacuambí	-2.2253	-78.2906	1250	FLMNH	x	x	x
H. maimoune B					low to midland					
CO03	Colombia	Antioquia	Amalfi	6.7833	-75.1000	1000	MEFLG	x	x	x
CO11	Colombia	Antioquia	Amalfi	6.7833	-75.1000	1000	MEFLG	x		
CO14	Colombia	Antioquia	Santa Fé de Antioquia	6.5356	-75.8281	517	MEFLG	x	x	x
CO15	Colombia	Antioquia	Santa Fé de Antioquia	6.5356	-75.8281	517	MEFLG	x	x	x
CO18	Colombia	Antioquia	Santa Fé de Antioquia	6.5356	-75.8281	517	MEFLG	x		
CO24	Colombia	Valle del Cauca	Buga	3.8633	-76.2861	1030	MEFLG	x		
CO25	Colombia	Valle del Cauca	Buga	3.8633	-76.2861	1030	MEFLG	x		
H. pimpla					highland					
CO39	Colombia	Antioquia	Jardín; Quebrada Bonita	5.5869	-75.8044	2065-2443	MEFLG	x	x	x
CO41	Colombia	Antioquia	Jardín; Quebrada Bonita	5.5869	-75.8044	2065-2443	MEFLG	x	x	x
EQ04	Equator	Zamora-Chinchipe	San Francisco	-3.9789	-79.0833	1900	FLMNH	x	x	x
EQ20	Equator	Zamora-Chinchipe	Loyola	-4.5203	-79.0114	1500	FLMNH	x	x	x
H. sosybius					lowland					
EUA02	USA	Florida	Gainesville	29.6517	-82.3247	35	AVLF	x		
EUA03	USA	Florida	Gainesville	29.6517	-82.3247	35	AVLF	x	x	x
EUA04	USA	Florida	Gainesville	29.6517	-82.3247	35	AVLF	x	x	x
EUA06	USA	Florida	Gainesville	29.6517	-82.3247	35	AVLF	x	x	x
EUA07	USA	Tenesse	Rutherford	35.7047	-86.3347	209	AVLF	x	x	x

DNA VOUCHER	COUNTRY	STATE	LOCALITY	LAT	LONG	ALT	COLLECTION	*COX 1*	*NAD 6*	RPS 5
EUA08	USA	Tenesse	Rutherford	35.7047	-86.3347	209	AVLF	x		
CO53	Colombia	Antioquia	Puerto Berrio	6.5167	-74.4414	115	MEFLG	x	x	x
H. sp1					midland					
MT14	Brazil	Mato Grosso	Jangada	-5.2667	-55.2333	562	NSP	x	x	x
CO09	Colombia	Antioquia	Amalfi	6.7833	-75.1000	1000	MEFLG	x	x	x
H. sp2					lowland					
RS26	Brazil	Rio Grande do Sul	Catuípe	-28.2367	-54.0078	280	NSP	x	x	x
RS28	Brazil	Rio Grande do Sul	Catuípe	-28.2367	-54.0078	280	NSP	x	x	x
					lowland					
CO46	Colombia	Antioquia	Puerto Berrio	6.5167	-74.4414	115	MEFLG	x	x	x

Att. 2. Data for sequences obtained from Peña *et al.* 20101.

SPECIES	VOUCHER	*COX1* ACCESSION NUMBER	RPS5 ACCESSION NUMBER	SAMPLE DATA
Godartiana muscosa	NW127-08	DQ338582	GQ865443	Serra do Japi, São Paulo, Brazil
Pharneuptychia inocentia	CP12-06	DQ338808	GU206035	Serra do Cipó, Minas Gerais, Brazil
Zisckaya pacarus	CP14-02	GQ864819	GQ865512	
Splendeuptychia boliviensis	CP02-48	GU205866	GU206041	Madre de Dios, Peru
Splendeuptychia itonis	CP02-44	DG338811	GQ357576	Madre de Dios, Peru
Rareuptychia clio	CP01-23	DQ338810	GQ865492	Madre de Dios, Peru
Amphidecta callioma	NW126-21	DQ338879	GQ357552	Mato Grosso, Brazil
Euptychia ordinata	CP01-14	GU205835	GU206008	Madre de Dios, Peru
Hermeuptychia sp.	CP02-17	GU205844	GU206016	Madre de Dios, Peru
Hermeuptychia sp.	CP04-11	GU205840	GU206012	Quebrada Siete Jeringas, Peru
Hermeuptychia sp.	CP06-93	GU205843	GU206014	Oxapamapa, Peru
H. maimoune	CP04-37	GU205841	GU206013	Río Colorado, Quebrada Perla, Peru
H. gisella	NW127-16	DQ338583	GQ357566	Extrema, Minas Gerais, Brazil
H. harmonia	CP04-10	GU205843	GU206015	Quebrada Siete Jeringas, Peru

Att 3. Mean genetic distances among groups based on K2P model of nucleotide substitution for 'barcode' region**.** Values above diagonal are standard error (obtained by bootstrap with 1,000 repetitions). Last column shows within groups mean genetic distance ± standard error. Values in bold are differences with J-C model, all differences are of +0.001.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**WITHIN GROUPS**
1 *H. atalanta*	-	0.010	0.006	**0.008**	0.009	0.008	0.009	0.009	0.007	0.009 ± 0.002
2 *H. fallax*	0.063	-	**0.009**	0.011	0.011	0.010	**0.010**	0.010	0.009	0.009 ± 0.002
3 *H. gisella*	**0.033**	**0.052**	-	0.008	0.009	0.006	**0.008**	0.008	0.005	0.010 ± 0.002
4 *H. maimoune A(amazonica*)	0.052	**0.073**	**0.046**	-	0.007	0.008	0.009	0.009	0.008	0.012 ± 0.003
5 *H. maimoune B(andina*)	0.055	**0.071**	**0.051**	0.035	-	0.009	0.011	0.010	0.009	0
6 *H. hermes*	0.044	0.069	**0.035**	0.048	0.054	-	0.009	0.009	0.007	0.010 ± 0.003
7 *H. pimpla*	**0.056**	0.060	0.043	0.055	**0.071**	0.054	-	0.007	0.008	0.011 ± **0.004**
8 *H. harmonia*	**0.051**	0.069	0.045	0.058	**0.065**	0.053	0.043	-	0.008	0.010 ± 0.003
9 *H. sosybius*	0.035	0.053	0.024	0.043	**0.048**	0.039	0.042	0.045	-	0.007 ± 0.002

Att. 4. Observed type specimen data.

TYPE SPECIMENS	COLLECTION	CONDITION	STATUS	TAXA STATUS (LAMAS)	SOURCE	LOCATION	SEX
H. atalanta	BMNH	without abdomen, wings intact	Syntype	Species	Photo from Lamas [A]	Venezuela	♂
H. cucullina	BMNH	intact	Lectotype	Species	Photo from Lamas [A]	Chaco (La Paz) Bolivia, 1893	♂
H. cucullina	BMNH	intact	Syntype	Species	Photo from Lamas [A]	Chaco (La Paz) Bolivia.	-
H. gisella	Buenos Aires	without abdomen, wings intact	Holotype	Synonym to *H. cucullina*	Photo from Lamas [A]	Yungas del Palmar, Bolivia	♂
H. fallax	BMNH	without abdomen, wings intact	Type	Species	Photo from Lamas [A]	-	-
H. harmonia	BMNH	loss of scales in wings	Type	Species	Photograph acquired by direct inquiry to BMNH	Quito, Ecuator	♀
H. calixta	ZMB	intact	Type	Synonym to *H. harmonia*	Photo from Lamas [A]	Bogota	-
H. hermes	BMNH	Without abdomen, right hindwing and part of left hindwing	Syntype	Species	Photograph acquired by direct inquiry to BMNH	-	♂
H. maimoune	BMNH	intact	Type	Species	Photograph acquired by direct inquiry to BMNH	Pebas, Peru	♂
H. pimpla ?	BMNH	without abdomen, wings intact	unknown	Species	Photo from Lamas [A]	Rio Negro	-

[A] Photos form Lamas, available at http://www.butterfliesofamerica.com/L/t/Hermeuptychia_a.htm

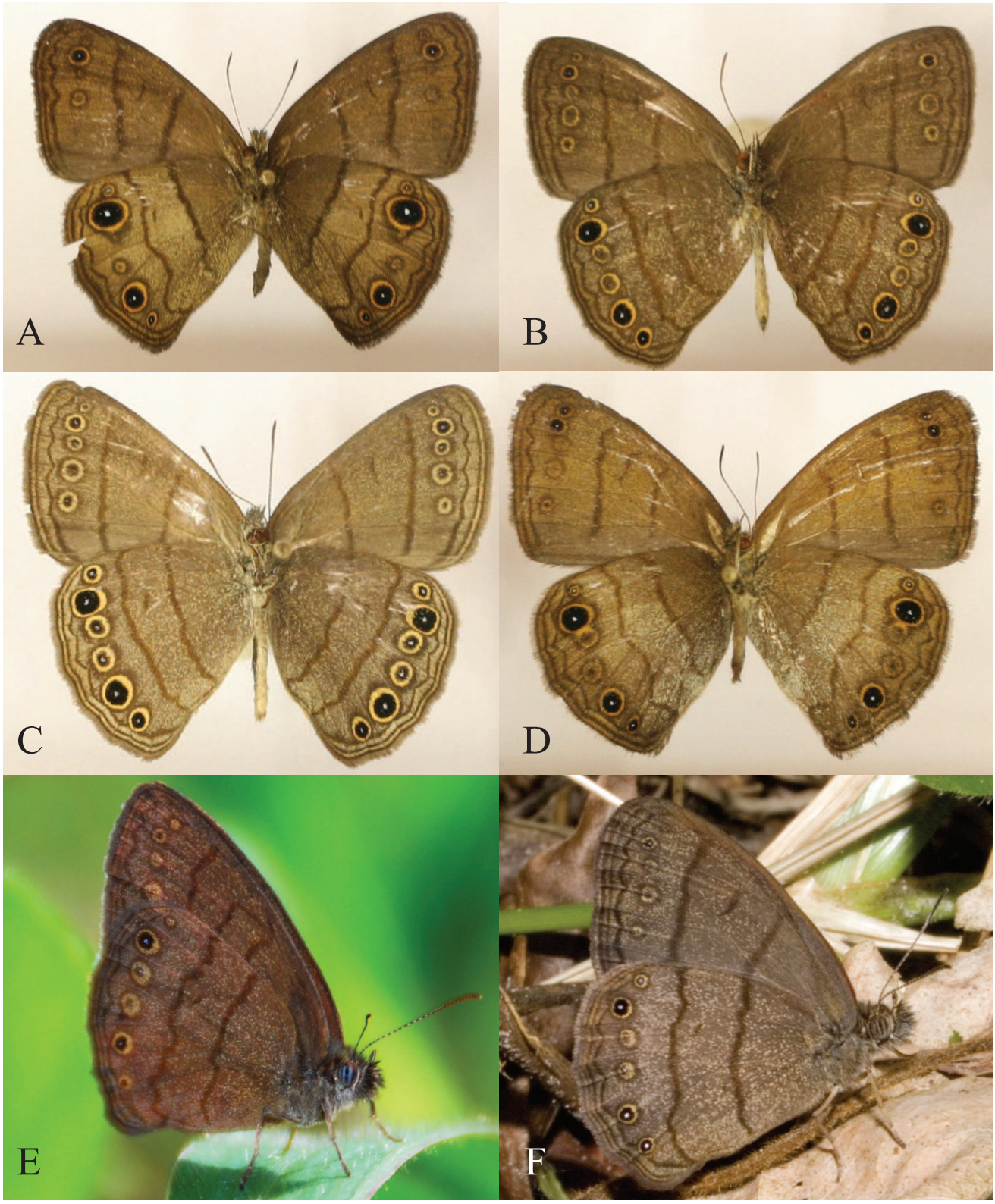
Att. 5. Sample date of extracted male genitalia.

DNA VOUCHER	SAMPLE LOCATION	COLLECTION	GENITALIA CODE	ASSIGNED SPECIES
C23	Campinas, São Paulo, BR	NSP	NSP0003	*H. atalanta*
CJ04	Campos do Jordão, São Paulo, BR	NSP	NSP0045	*H. atalanta*
RS80	Porto Mauá, Rio Grande do Sul, BR	NSP	NSP0005	*H. atalanta*
RS85	Porto Mauá, Rio Grande do Sul, BR	NSP	NSP0001	*H. atalanta*
MG08	Jaboticatubas, Minas Gerais, BR	NSP	NSP0013	*H. hermes*
MG11	Jaboticatubas, Minas Gerais, BR	NSP	NSP0017	*H. atalanta*
MG12	Jaboticatubas, Minas Gerais, BR	NSP	NSP0006	*H. atalanta*
MG21	Jaboticatubas, Minas Gerais, BR	NSP	NSP0014	*H. atalanta*
MG22	Jaboticatubas, Minas Gerais, BR	NSP	NSP0016	*H. atalanta*
PA01	Carajás, Pará, BR	NSP	NSP0035	*H. atalanta*
PA02	Carajás, Pará, BR	NSP	NSP0011	*H. hermes*
PA03	Carajás, Pará, BR	NSP	NSP0025	*H. atalanta*
L01	Santos, São Paulo, BR	NSP	NSP0021	*H. gisella*
L03	Santos, São Paulo, BR	NSP	NSP0019	*H. fallax*
L10	Santos, São Paulo, BR	NSP	NSP0034	*H. fallax*
L14	Santos, São Paulo, BR	NSP	NSP0022	*H. fallax*
L17	Santos, São Paulo, BR	NSP	NSP0024	*H. fallax*
L19	Ubatuba, São Paulo, BR	NSP	NSP0015	*H. fallax*
L20	Ubatuba, São Paulo, BR	NSP	NSP0010	*H. fallax*
L21	Ubatuba, São Paulo, BR	NSP	NSP0033	*H. fallax*
V01	Jundiaí, São Paulo, BR	NSP	NSP0018	*H. fallax*
RS26	Catuípe, Rio Grande do Sul, BR	NSP	NSP0030	*H. hermes*
RS28	Catuípe, Rio Grande do Sul, BR	NSP	NSP0031	*H. hermes*
RS32	Catuípe, Rio Grande do Sul, BR	NSP	NSP0032	*H. atalanta*
MT10	São Vicente, Mato Grosso, BR	NSP	NSP0040	*H. gisella*
MT11	Jangada, Mato Grosso, BR	NSP	NSP0039	*H. atalanta*

DNA VOUCHER	SAMPLE LOCATION	COLLECTION	GENITALIA CODE	ASSIGNED SPECIES
MT12	São Vicente, Mato Grosso, BR	NSP	NSP0037	*H. gisella*
MT13	São Vicente, Mato Grosso, BR	NSP	NSP0041	*H. gisella*
MT15	São Vicente, Mato Grosso, BR	NSP	NSP0036	*H. gisella*
MT16	São Vicente, Mato Grosso, BR	NSP	NSP0038	*H. gisella*
GO01	Pirenópolis, Goiás, BR	NSP	NSP0047	*H. cucullina*
TO02	Ananás, Tocantins, BR	NSP	NSP0046	*H. atalanta*
EUA02	Gainesville, Florida US	NSP	NSP0044	*H. sosybius*
EUA04	Gainesville, Florida US	NSP	NSP0012	*H. sosybius*
EUA08	Rutherford, Tennessee, US	NSP	NSP043	*H. sosybius*
CO02	Villavicencio, Meta, CO	NSP	NSP0023	*H. hermes*
CO05	Jardín, Antioquia CO	NSP	NSP0020	*H. gisella*
CO06	Jardín, Antioquia CO	NSP	NSP0007	*H. harmonia*
CO07	Amalfi, Antioquia CO	MEFLG	HGSM01	*H. hermes*
CO10	Jardín, Antioquia CO	MEFLG	HGSM05	*H. hermes*
CO11	Amalfi, Antioquia CO	MEFLG	HGSM06	*H. maimoune*
CO12	Jardín, Antioquia CO	MEFLG	HGSM07	*H. atalanta*
CO18	Santa Fé de Antioquia, CO	MEFLG	HGSM13	*H. maimoune*
CO21	Villavicencio, Meta, CO	MEFLG	HGSM16	*H. hermes*
CO22	Villavicencio, Meta, CO	MEFLG	HGSM17	*H. hermes*
CO26	Jardín, Antioquia CO	MEFLG	HGSM26	*H. hermes*
CO30	Jardín, Antioquia CO	MEFLG	GSM455	*H. harmonia*
CO31	Jardín, Antioquia CO	MEFLG	GSM452	*H. harmonia*
CO33	Jardín, Antioquia CO	MEFLG	GSM458	*H. harmonia*
CO38	Jardín, Antioquia CO	MEFLG	GSM485	*H. harmonia*
CO39	Jardín, Antioquia CO	MEFLG	GSM487	*H. pimpla*
CO51	Puerto Berrio, Antioquia, CO	MEFLG	GSM297	*H. atalanta*

DNA VOUCHER	SAMPLE LOCATION	COLLECTION	GENITALIA CODE	ASSIGNED SPECIES
CO53	Puerto Berrio, Antioquia, CO	MEFLG	GSM299	*H.sosybius*
EQ05	El Líbano, Zamora-Chinchipe, EQ	FLMNH	KW11-39	*H. maimoune*
EQ09	Yacuambí, Zamora-Chinchipe, EQ	FLMNH	KW11-40	*H. maimoune*
EQ20	Loyola, Zamora-Chinchipe, EQ	FLMNH	KW11 38	*H.pimpla*
EQ21	Loyola, Zamora-Chinchipe, EQ	FLMNH	KW11-54	*H. harmonia*
EQ22	Loyola, Zamora-Chinchipe, EQ	FLMNH	KW11-53	*H. harmonia*

Att. 7 *Hermeuptychia* wing pattern photographs. **A.** *H. pimpla* (CO40) from MEFLG; **B.** *H. atalanta* (CO51) from MEFLG. **C.** *H. sosybius* (CO53) from MEFLG; **D.** *H. pimpla* (CO41) from MEFLG, showing variation in the *H. harmonia* and *H. pimpla* wing pattern **E.** *H. gisella,* from Serra do Japi, SP-BR; **F.** *Hermeuptychia sp.* from Picinguaba, SP-BR.



Att. 6 Alignment of RpS5 sequences, showing heterozygous chromatogram for L03, with small *H. fallax* peaks and generally bigger *H. gisella* peaks. The pattern continues throughout the whole RpS5 sequence. In chromatograms (Finch TV), A is green, T is red, G is black and C is blue. In alignment (Se-Al), A is red and T is green, G and C colors remain the same. S is gray and means G or C; Y is dark green and stands for C or T; and R is pink and means A or G.