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**Dissertação de Mestrado**

**BASES MORFO-ECOLÓGICAS NA AVALIAÇÃO DO REFORÇO  
ADAPTATIVO E DIMORFISMO SEXUAL EM UM ANEL MIMÉTICO DE  
HELICONÍNEOS NEOTROPICAIS.**

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Porto Alegre, Fevereiro de 2014

**Bases morfo-ecológicas na avaliação do reforço adaptativo e dimorfismo sexual em um anel mimético de heliconíneos neotropicais.**

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*“O que for a profundidade do teu ser, assim será o teu desejo.  
O que for o teu desejo, assim será a tua vontade.  
O que for a tua vontade, assim serão os teus atos.  
O que forem teus atos, assim será o teu destino.”*

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## Resumo

O mimetismo é um tipo de interação interespecífica importante para a dinâmica de muitas espécies. Este mecanismo de defesa contra predadores se caracteriza por espécies distantes evolutivamente apresentarem similaridade morfológica. Associado à coloração aposemática, o mimetismo é uma forma de mostrar para o predador que a espécie mimética é impalatável, seja através de um sinal honesto ou desonesto. No mimetismo Mulleriano, ambas espécies são impalatáveis e portanto são chamadas de espécies co-miméticas. A coloração característica de espécies miméticas também pode sofrer influência da seleção sexual; ou seja, ser avaliada indiretamente quanto a outros atributos fenotípicos através de diferença quanto ao tamanho e a forma das estruturas envolvidas no mimetismo. Neste sentido, *Heliconius* é um gênero modelo em biologia evolutiva, devido a grande variação intra- e interespecífica no padrão de coloração e principalmente por apresentar diversas associações miméticas, tanto em nível específico quanto subespecífico. O anel mimético que apresenta ocorrência no Brasil se caracteriza por apresentar asas enegrecidas, com uma mancha vermelha na anterior e, uma amarela, na posterior, tendo sido moldado supostamente por pressão de predação associada a aves. Mesmo com alta similaridade entre as espécies identificamos variações morfológicas intra- e interespecífica. Estaria a variação intraespecífica variando de acordo com a outra espécie mimética? Ou seja, seria esperado maior convergência adaptativa ao mimetismo Mulleriano em áreas de simpatria. Essa foi a questão que norteou esta dissertação, onde avaliamos a variação morfológica através de uma escala sensível a pequenas variações (morfometria geométrica), pois tratam-se de espécies bastante similares. Com base em espécimes provenientes de museus, com ampla distribuição geográfica, determinamos a variação no tamanho e na forma da asa e da mancha vermelha, de espécies pertencentes ao anel mimético formado por: *H. erato phyllis* (com ampla distribuição na região central, leste e sul do Brasil), *H. besckei* (ocorrência em áreas de altitude no sudeste do Brasil), *H. melpomene burchelli* (ocorrência na região central e norte do Brasil) e *H. m. nanna* (distribuída ao longo do litoral nordeste do Brasil). Em paralelo, com vista à associação da forma à variação genotípica, foram obtidas e comparadas sequências de DNA em relação aos marcadores *COI* (mitocondrial) e *Optix* (nuclear, associado supostamente à codificação da mancha vermelha).

Os resultados mostraram que não há reforço adaptativo ao mimetismo Mulleriano, visto que as espécies e subespécies diferem significativamente entre si em áreas de simpatria. A variação na forma não esteve relacionada à variabilidade genética, a qual ocorreu de forma dissociada da distância geográfica. Assim, inferimos que outros mecanismos, além da seleção natural, estão envolvidos na evolução correspondente. Os resultados demonstraram que há dimorfismo sexual no tamanho e na forma da mancha vermelha, o que pode ser importante não somente para diferenciação entre as espécies/subespécies, mas também no contexto da seleção de parceiros. Assim, inferimos que a seleção sexual poderá estar envolvida na seleção dessas variações fenotípicas, o que resta ser elucidado. Neste sentido, os resultados desta dissertação questionam, de forma inédita, a seleção natural associada à predação como o único mecanismo envolvido na evolução de anéis miméticos de heliconíneos neotropicais.

**Palavras-chave:** mimetismo Mulleriano, seleção sexual, morfometria geométrica, fluxo gênico.



## Abstract

Mimicry is an important type of interspecific interaction to the dynamics of many species. This defense mechanism against predators is characterized when evolutionarily distant species present morphological similarity. Associated with aposematic coloration, mimicry is a way to show the predator that mimetic species are unpalatable, through either honest or dishonest signals. In Müllerian mimicry, both species are unpalatable and therefore are called co-mimetic species. The characteristic color of mimetic species may also be influenced by sexual selection; that is, to be evaluated indirectly regarding other phenotypic attributes through difference as to the size and shape of the structures involved in mimicry. In this sense, the genus *Heliconius* is model in evolutionary biology, due to the large intra- and interspecific variation in color pattern and especially for presenting various mimetic associations, at both specific and subspecific levels. The main mimetic ring that occurs in Brazil is characterized by having blackened wings, with a red spot on the front and a yellow on the back, allegedly having been shaped by predation pressure associated with birds. Even with high similarity between species, they can still be identified intra- and interspecific by morphological variation. Intraspecific variation would be varying according to other mimetic species? In the other words, one would expect greater adaptive convergence in Müllerian mimicry within areas of sympatry. That was the question that guided this dissertation, which evaluated the morphological variation through methodological tools sensitive to small variations (geometric morphometric), because these are very similar species. Based on specimens from museums with broad geographic distribution, we determined the variation in the size and shape of the wing and red spot for the species belonging to the mimetic ring formed by: *H. erato phyllis* (with wide distribution in Central, Eastern and Southern Brazil), *H. besckei* (occurrence in areas of elevation in Southeastern Brazil), *H. melpomene burchelli* (occurring in Central and Northern Brazil) and *H. m. nanna* (distributed along the Northeast coast of Brazil). In parallel, in order to test for the existence of association to genotypic variation, DNA sequences were obtained and compared with respect to the COI (mitochondrial) and Optix (nuclear, presumably associated with the encoding of the red spot) markers.

The results showed no adaptive enhancement to Mullerian mimicry, given that species and subspecies differ significantly in areas of sympatry. The variation in shape was not related to genetic variability, which occurred dissociated from geographical distance. Thus, we infer that other mechanisms, in addition to natural selection, are involved in the corresponding evolution. The results showed that there is sexual dimorphism in the size and shape of the red band, which can be important not only for the differentiation between species/subspecies but also in the context of the selection of partners. Thus, we infer that sexual selection may be involved in the selection of these phenotypic variations, which remains to be elucidated. In this sense, the results of this dissertation question, in an unprecedented way, natural selection associated with predation as the only mechanism involved in the evolution of mimetic neotropical heliconine rings.

**Key-words:** Müllerian mimicry, sexual selection, geometric morphometrics, gene flow.

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## Introdução geral

### Tribo Heliconiini (Lepidoptera: Nymphalidae)

As borboletas do maracujá, como são popularmente conhecidas, constituem um dos grupos mais bem estudados de borboletas. Isto deve-se em parte à coloração aposemática quando adultos, a participação em anéis miméticos e a variabilidade geográfica. Trabalhos envolvendo Heliconiini vão desde taxonômicos (Michener 1942, Emsley 1963, Turner 1976, Brown 1981, Brower 1994, Penz 1999), coevolutivos entre insetos e plantas (Ehrlich e Raven 1965, Benson et al 1976, Rodrigues e Moreira 2004, Jorge et al 2011), genéticos (Turner 1971, Sheppard et al 1985), de biologia de populações (Benson 1978, Mallet 1986) e de conservação (Brown 1981, Brown e Freitas 2000, Boggs et al 2003), especiação e hibridação (Mavárez et al 2006, Mallet et al 2007, Mallet 2008).

A tribo possui distribuição por toda região Neotropical e os gêneros mais diversos são *Heliconius* e *Eueides*, apresentando aproximadamente 50 espécies e 1500 raças geográficas (Lamas 2004). A alta diversidade fenotípica encontrada em *Heliconius* tem sido enfoque de diversos estudos (McMillan et al 1997, Naisbit et al 2003) e a fácil adaptação as condições de insetário e laboratório tem facilitado a utilização de indivíduos deste gênero como organismos modelo em estudos de ecologia comportamental e evolução (Gilbert 1991).

Biologistas evolucionistas tem estudado essas borboletas por mais de 150 anos, gerando resultados interessantes para a ecologia, sistemática e evolução de *Heliconius*, mas algumas lacunas ainda não foram preenchidas. É sabido que as borboletas do maracujá apresentam uma rápida especiação e divergência; além disso, em algumas espécies, mostram elevada convergência no padrão da asa devido ao mimetismo, mecanismo importante para evitar a predação.

## Ecologia Evolutiva: Mimetismo como forma de evitar a predação

A predação é um tipo de interação interespecífica muito importante na natureza. Além da predação influenciar a densidade e a sobrevivência do predador, essa interação também regula a densidade da presa, amenizando a dominância que a competição poderia originar e afeta fortemente a composição específica de um dado local. Em muitos casos, a predação previne a monopolização de um habitat por uma espécie competidora potencialmente mais apta. Esse processo é conhecido como competição aparente (Holt 1977), onde as interações entre as espécies competidoras são mediadas pelo consumidor.

Da mesma forma que os predadores desenvolveram evolutivamente atitudes comportamentais e estruturas destinadas à predação, presas também desenvolveram mecanismos para evitá-la. Quando as presas não podem se esconder ou escapar, geralmente adotam defesas protetoras, como produção de secreções químicas de cheiro forte ou dolorosas, além de substâncias químicas que as tornam impalatáveis (Ricklefs 2010). Características visuais associadas a mecanismos de defesa químicos, por exemplo, são bastante utilizadas como forma de evitar a predação. Na camuflagem, os indivíduos tendem a serem crípticos com o meio onde ocorrem. Dessa forma, passam despercebidos pelo predador, enquanto que no mimetismo as espécies frequentemente apresentam colorações aposemática. Assim, se destacam no ambiente, sinalizando advertência aos predadores devido a associação com a sua impalatabilidade (Joron 2009). O sinal emitido pela presa é considerado honesto quando espécies pertencentes ao anel mimético são impalatáveis e desonesto quando uma espécie palatável mimetiza uma impalatável.

Caracteriza-se como mimetismo a adaptação onde duas ou mais espécies distantes evolutivamente assemelham-se no padrão geral de coloração e/ou forma. O mimetismo envolve três protagonistas: ou seja, dois emissores do sinal de advertência e um receptor (Joron 2009). Muitos emissores irão convergir a um mesmo sinal formando um anel mimético (Joron 2009). Existem dois diferentes tipos de mimetismo: o Batesiano e o Mulleraiano. No mimetismo Batesiano clássico uma espécie aposemática modelo (impalatável) é mimetizada por outra (palatável). Enquanto que no mimetismo Mulleriano, que é o foco desta dissertação, todas as espécies participantes da semelhança são impalatáveis e são chamadas de espécies co-miméticas. Em 1879, Fritz Müller desenvolveu uma demonstração matemática em que duas presas impalatáveis podem se beneficiar a partir de semelhança mútua. A alta similaridade entre espécies impalatáveis resultam em um reforço no sinal de aviso de advertência a fim de evitar a predação e conseqüentemente aumentar a taxa de sobrevivência (Franks e Noble 2002, Endler 1988).

O mimetismo Mulleriano é benéfico tanto para a presa quanto para o predador, uma vez que o predador que melhor associa a impalatabilidade da presa com o seu sinal de advertência apresenta uma maior taxa de sobrevivência. Por outro lado, indivíduos raros ou com novas variantes dentro da população de presas não são reconhecidos como impalatáveis e por isso devem sofrer maior predação (Joron 2009). Por isso, os pássaros são reconhecidos como importantes agentes na seleção do mimetismo e na manutenção do polimorfismo espacial da cor em borboletas. Segundo Muller (1879), a aprendizagem dos pássaros implica em custos para as borboletas, que sendo miméticas acabam compartilhando os custos em educar o predador. A aprendizagem dos predadores pode estar

relacionada a diversos sinais visuais de advertência da asa como a coloração (Svádová et al 2009), o padrão (Ihalainen 2008), o tamanho (Marples 1993), a simetria (Forsman e Merilaita 1999); além disso, quanto aos níveis de impalatabilidade (Ihalainen et al 2006), à frequência relativa do co-mimético (Rowland et al 2010) e à forma e padrão do voo (Chai 1996).

### Mimetismo em *Heliconius*

O gênero *Heliconius* (Lepidoptera; Nymphalidae) compreende borboletas neotropicais altamente diversas em termos morfológicos, sendo um importante modelo em biologia evolutiva. Apresenta variação intra- e interespecífica no padrão de coloração (Brown 1979, Sheppard 1985), envolvidas em associações miméticas (Mallet 1989, Kapan 2001) e possui uma ecologia evolutiva peculiar (Brown 1981).

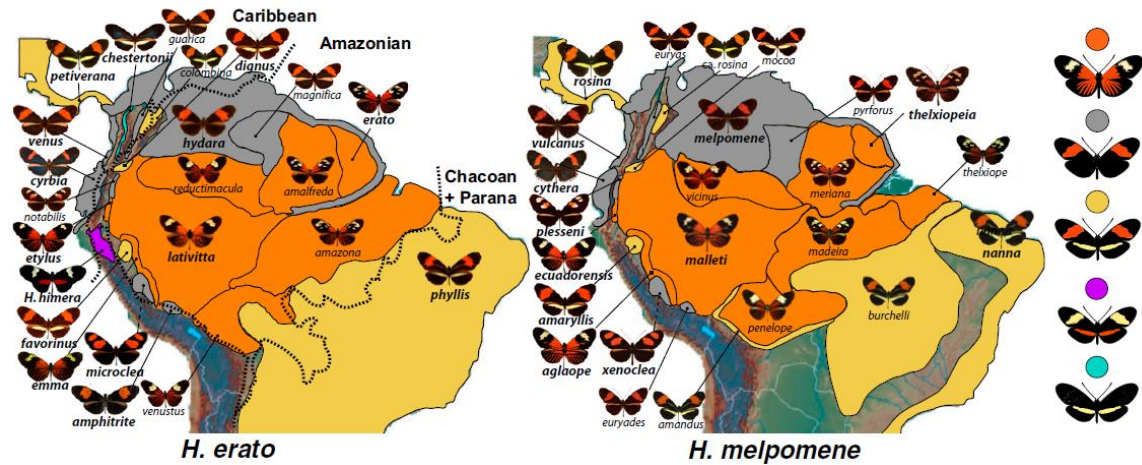
Devido a radiação adaptativa recente deste grupo, podemos observar casos de convergência fenotípica nos padrões de coloração das asas entre espécies distantes e, também, de divergência evolutiva entre táxons próximos (Turner 1981). Portanto, além dos padrões alares exercerem importante papel para o voo, rituais de corte (Joron 2005) e termorregulação (Berwaerts et al 2001), também são estruturas sujeitas a seleção natural. A variação fenotípica apresentada pelas borboletas deste gênero é utilizada para a classificação taxonômica intraespecífica em termos de subespécies (Sheppard et al 1985) [e.g., *H. erato* possui pelo menos 29 formas geográficas distintas intituladas subespécies (Brown 1979, Baigts 1992)].

A convergência evolutiva frequentemente ocorre espacialmente, onde a coloração aposemática dos adultos de espécies distantes evolutivamente

apresentam uma alta similaridade morfológica formando os anéis miméticos. Um exemplo clássico de anel mimético ocorre no mosaico geográfico entre *H. melpomene* e *H. erato*, que apresentam mais de 20 pares de formas geográficas distintas, mas compartilhadas espacialmente (Fig. 1; Hines et al 2011). Essas espécies são distantes filogeneticamente (Brown 1981, Beltran et al 2007), impalatáveis e possuem repetidos padrões mimético de coloração de advertência aos predadores (Benson 1972, Chai 1986, Pinheiro 2003). Sugere-se que *H. melpomene* tenha se originado na região litorânea do Brasil, enquanto *H. erato* colonizou posteriormente esta região (Quek et al 2010). Acredita-se que a variação do padrão de cor entre *H. erato* e *H. melpomene* seja resultado das mudanças paralelas ocorridas em refúgios do Pleistoceno (Turner e Mallet 1996; Brown et al 1974). Além disso, inferências recentes de história demográfica com base em dados de genética de populações sugerem uma hipótese alternativa ao 'mimetismo de advertência' (Eltringham 1916), onde *H. erato* por ser mais antiga, teria expandido primeiro, estabilizando posteriormente a diversidade dos padrões de cores em *H. melpomene* (Flanagan et al 2004; Quek et al 2010).

Além deste sistema amplamente conhecido, verifica-se um anel mimético mais complexo e único, envolvendo convergência fenotípica em três espécies de *Heliconius* na região Central, Sul e Nordeste do Brasil. Especificamente, *H. erato phyllis*, *H. besckei*, *H. melpomene burchelli* e *H. m. nanna* exibem um fenótipo quase idêntico incluindo um padrão de fundo escuro da asa (preto) com bandas vermelhas e manchas amarelas.





**Fig 1.** Convergência evolutiva com co-ocorrência espacial entre *H. erato* e *H. melpomene*. **Fonte:** Hines et al (2011).

Nesse contexto, como premissa básica para que seja corroborada evolução de animais miméticos por pressão de predação, é esperado haver uma maior convergência na forma dentre os taxa envolvidos quando em simpatria. Das espécies e subespécies pertencentes a esse anel mimético, *H. erato phyllis* possui a distribuição mais ampla em relação as demais, bem como maior variação na coloração das asas. Porém, estudo recentemente conduzido em nosso laboratório sugere que tal variação está associada em parte ao uso diferenciado das plantas hospedeiras (Passifloraceae) no estágio larval (Jorge et al 2011). Por outro lado, *H. besckei* é a espécie que possui a menor variação morfológica (sendo reconhecido um único padrão) e área de ocorrência mais restrita, particularmente nas regiões de altitude de Mata Atlântica (Brown 1981). Já *H. melpomene burchelli* possui distribuição no Brasil Central enquanto *H. m. nanna* está restrita a área de Mata Atlântica na região litorânea, e sugere-se que existam pequenas variações no padrão de coloração ao longo da área de ocorrência (Hines et al 2011). A distribuição de *H. besckei*, *H. melpomene burchelli* e *H. m. nanna* ocorre em

simpatria com *H. erato phyllis* em determinadas regiões. Assim, neste caso, é esperado que a variação espacial existente na forma da asa seja relacionada àquela do par mimético, quando em simpatria. Considerando que a distribuição das demais espécies é alopátrica, torna-se exequível testar tal hipótese em relação a variação na forma existente em *H. erato phyllis*, o que é objeto de estudo desta dissertação.

O conhecimento em escala fina acerca de variações morfológicas em relação à forma e padrão de coloração das asas nestes táxons são escassos. Entretanto, observações prévias em espécimes de coleção sugerem um cenário de variabilidade na morfologia ao longo da área de distribuição, pelo menos para *H. e. phyllis* e *H. m. nana* (GRP Moreira, dados não publicados).

Além disso, a variação do padrão de coloração das asas pode estar associado a um gene, à variação genética das populações ou a uma estruturação genética. Por isso estudos que abordem a morfologia associado à análise molecular geralmente desvendam cenários mais claros a respeito de quais fatores desempenham maior influência sobre as características miméticas correspondentes, o que também é contemplado no presente estudo.

### A genética do padrão de coloração mimética em *Heliconius*

Trabalhos extensivos tem mostrado que genes de tamanho considerável controlam a diferença no padrão de coloração entre raças geográficas dentro das espécies (Turner e Crane 1963, Sheppard et al 1985, Mallet 1989). Mallet (1989) sugeriu que tais diferenças são estabelecidas em apenas dois ou três loci que apresentam maior efeito fenotípico sobre o padrão de coloração das asas em heliconíneos. Entretanto, nem sempre essas variações correspondem aos

padrões tradicionais de distribuição, i.e., as vezes eles podem ocorrer dentro da mesma raça e em níveis ainda menores dentro de uma população, como também, entre os sexos (Stillwell e Fox 2009).

Existe uma forte ligação entre o loci do padrão de coloração e loci envolvidos em atributos comportamentais pode também facilitar o processo de rápida especiação (Kronforst et al 2006, Merrill et al 2011), característica deste gênero.

Estudos recentes tem mostrado que regiões homólogas entre espécies co-miméticos são responsáveis por controlar a variação do padrões de coloração vermelha nas asas de *Heliconius* (Counterman et al 2010, Baxter et al 2010, Joron et al 2006). Trabalhos comparativos de expressão de gene nesta região identificou o gene *Optix*, como sendo a sequência codificadora responsável por controlar a variação da cor vermelha em *H. erato* e *H. melpomene* (Reed et al 2011), e dessa forma, o uso de tal gene foi contemplado no presente estudo.

### Seleção sexual: Pressão sob o padrão de coloração

Em *Heliconius* as preferências masculinas coevoluiram com as mudanças miméticas no padrão da cor; ou seja, o isolamento reprodutivo pode ser reforçado pela seleção sexual, resultando em rápida especiação (McMillan et al 1997, Jiggins et al 2001, Mavárez et al 2006, Merrill et al 2011). Algumas diferenças morfológicas podem estar relacionadas a proteção contra predadores ou podem estar ligadas a diferença entre os sexos, sendo menos relevante para evitar a predação, mas importante para seleção sexual. Muitos trabalhos tem abordado o dicromatismo sexual (Silberglied e Taylor 1978, Kemp 2007) e o dimorfismo sexual do tamanho do corpo em borboletas (Singer 1982, Wiklund e Kaitala 1995).

Até o momento, apenas Klein e Araujo (2013) estudaram o dimorfismo sexual, ao avaliar o tamanho das bandas de cor através de medidas lineares em *H. erato phyllis* e *H. besckei*. Entretanto nenhum trabalho considerando a avaliação da mancha e da asa foi realizado utilizando uma ferramenta mais sensível a variação morfológica como a morfometria geométrica. Ou seja, essa ferramenta permite uma melhor avaliação da variação da forma e tamanho, visto que trata esses parâmetros de forma independente.

### Morfometria geométrica

A morfometria geométrica é uma descrição quantitativa, análise e interpretação da forma e da sua variação e covariação com outras variáveis (Rohlf 1990, Bookstein 1991). Essa ferramenta permite a captação da geometria da estrutura, preservando-a e removendo os efeitos, permitindo assim avaliar de forma consistente as mudanças na forma de estruturas biológicas (Bookstein 1991). Pode ser aplicada na descrição e comparação de estruturas em estudos de cunho sistemático, ecológicos, ontogenéticos e genéticos, entre outros.

Em geral, o método é baseado em marcos anatômicos e as análises são fundamentadas nas distâncias entre estes pontos (resíduos). Estes marcos representam coordenadas em um plano cartesiano (x,y) e devem passar por uma análise que remove efeitos que não são relativos a forma, como a inclinação, a escala e a direção (sobreposição de Procrustes). O centróide (centro geométrico de todos os marcos anatômicos da estrutura) é translado até a origem (0,0). Posteriormente, as configurações são escalonadas a um tamanho comum e rotadas até minimizar a diferença entre as comparações (Rohlf e Slice 1990). Dessa forma, pode-se realizar as devidas análises, pois os marcos estão

corrigidos a fim de manter apenas a forma das estruturas. Ou seja, restando apenas as diferenças dos marcos, os quais são utilizados para comparações multivariadas sobre a variação da forma (Bookstein 1991).

*Landmarks* são marcos anatômicos homólogos entre as espécies. O conceito de homologia operacional utilizado pela morfometria (correspondência biológica da posição de marcos de forma para forma, segundo Sneath e Sokal, 1973), diferem do conceito evolutivo ou taxonômico (similaridade devida a ancestralidade comum). Os tipos de dados mais ricos em informação utilizados pelos métodos morfométricos são provenientes de marcos anatômicos. Neste caso, a homologia é considerada um mapeamento de pontos, sendo importante a correspondência relacionada ponto a ponto, e não parte a parte, afinal o ponto é uma estrutura mais facilmente definida que a parte estrutural (Bookstein 1990). Os marcos anatômicos se dividem em três grupos, segundo classificação de Bookstein (1991): Tipo I – Justaposição de tecidos. Inclui pontos no espaço onde três estruturas se encontram, como suturas ósseas, ramificações de nervuras em folhas de árvores ou em estruturas dos sistemas nervosos ou arterial; Tipo II – Pontos de máxima curvatura ou outros processos morfogenéticos locais. Incluem extremidades de processo e vales de invaginações. São marcos menos confiáveis, mas podem ser interessantes para explicação biológicas, como o deslocamento de pontos de inserção de músculos; Tipo III – Pontos extremos. Estão relacionados a maior distância que podem ser medidas em uma estrutura. São considerados deficientes quanto a homologia e já foram considerado como não sendo marco.

Quando os landmark não são evidentes, em estruturas curvilíneas sem marcos homólogos, os estudos se baseiam em uma sequência de coordenadas

(*semi-landmarks*), na qual pelo menos o primeiro marco deve ser homólogo. Cada semi-landmark vai deslizar ao longo de uma linha até encontrar a posição do ponto correspondente no contorno da espécime referência (que contém as sequências dos pontos). Após esse tratamento, os marcos tipo *semi-landmark* recebem o mesmo tratamento dos landmarks (Brookstein 1997).

Posteriormente, os marcos são submetidos a técnica *thin-plate spline*, a qual pode ser utilizada para mapear as deformações na forma entre as configurações desejadas. As deformações (*partial warp scores*) podem ser utilizados como variáveis da forma a serem comparadas através de uma Análise de Componentes Principais (*relative warps*). Após gerar esses resultados, podemos realizar: 1) análise de variância para comparar o tamanho do centróide que é uma medida univariada; 2) análise de variância multivariada, para detectar se há diferença na forma entre tratamentos e interação entre eles; 3) análise canônica e discriminante para detectar grupos originados por formas distintas; 4) cálculo das distâncias de Mahalanobis para mostrar a proximidade das formas obtidas, dentre outras (Adams et al 2004).

O tamanho do centroide quando utilizado como medida de tamanho pode servir para comparar diferentes estruturas num mesmo indivíduo. A comparação do crescimento diferencial entre as estruturas chama-se alometria.

### Importância da alometria em sistemas biológicos

A alometria, termo cunhado por Huxley e Tessier (1936), estuda o padrão de crescimento diferenciado entre as partes do corpo e o impacto dessas mudanças sobre a ecologia e a evolução dos organismos. Huxley queria entender como o carangueijo *Uca pugnax* crescia para produzir uma quela exageradamente

grande. Então, estudou o tamanho da quela do macho em relação ao tamanho do corpo, considerando os diferentes estágios de vida. Como resultado, encontrou uma regressão de 1,57, valor maior do que 1 (isometria) demonstrando que o crescimento entre a quela e o corpo possui alometria positiva; ou seja, para qualquer unidade de aumento no tamanho do corpo no tempo, havia um aumento proporcionalmente maior no tamanho da quela. Portanto, a quela crescia em uma taxa maior do que o resto do corpo. Nesta comparação, a relação curvilínea foi linealizada através de uma escala logarítmica. Além de Huxley, vários outros pesquisadores tem estudado o crescimento diferenciado em outros órgãos e espécies (Gayon 2000).

Atualmente o termo alometria descreve como os atributos ou processos se escalonam entre si. De forma geral, se relaciona as relações biológicas de escala em geral, podendo incluir atributos morfológicos (ex. tamanho do cérebro e do corpo entre adultos), fisiológicos (ex. relação entre taxa metabólica e o tamanho do corpo em mamíferos) ou ecológicos (ex. relação entre o tamanho da asa e a performance de voo em aves).

A relação entre o crescimento diferenciado do corpo e das partes do corpo pode ser descrita usando uma equação linear ( $\log y = \beta \log x + \log \alpha$ ), onde  $x$  é o tamanho do corpo,  $y$  é o tamanho do órgão,  $\log \alpha$  é o intercepto da linha no eixo  $y$  e  $\beta$  é a inclinação da linha. Regressões lineares simples são usadas para descrever essa relação e estimar os expoentes da inclinação (coeficiente alométrico) e o intercepto da reta.

É chamado de alometria positiva ou hiperalometria quando o órgão tem uma taxa de crescimento maior do que o corpo ( $\beta > 1$ ). Já na alometria negativa ou hipoalometria a taxa de crescimento do órgão é mais baixa do que o

crescimento do corpo ( $\beta < 1$ ). É considerado crescimento isométrico quando o órgão cresce a uma taxa igual ao do corpo ( $\beta = 1$ ). O crescimento do coração humano é um exemplo de crescimento isométrico e, da cabeça, de hipoalometria.

Existem diferentes tipos de alometria, tais como ontogenética, filogenética e estática. Na alometria ontogenética, analisa-se o crescimento relativo nos indivíduos, onde  $x$  e  $y$  são atributos medidos no mesmo indivíduo ao longo da sua ontogenia e a inclinação da reta reflete a diferença na taxa de crescimento entre o órgão e o corpo. Já na alometria filogenética, analisa-se a taxa constante de crescimento diferencial nas linhagens. Enquanto que na alometria estática os atributos são medidos em diferentes indivíduos no mesmo estágio de desenvolvimento dentro de uma população ou espécies e a inclinação reflete como a variação no tamanho do atributo medido é acompanhada pela variação no tamanho do corpo dentro de uma espécie. Tanto a inclinação quanto o intercepto possuem um significado biológico. Por exemplo, considerando a alometria onde se compara diferentes indivíduos no mesmo estágio de desenvolvimento dentro de duas populações, a diferença no intercepto indica uma diferença proporcional do tamanho da asa por exemplo em relação ao tamanho do corpo entre as espécies. Já a diferença na inclinação indica como o tamanho relativo da asa muda com o tamanho do corpo dentro da espécie. Em 1924, no livro intitulado *Sexualidade e Hormônios*, Christian Champy propôs a expressão crescimento desarmônico, onde um crescimento contínuo de características secundárias em relação ao tamanho do corpo (Champy 1924). Nesse livro, Champy explora o fenômeno causado por hormônio sexual onde ocorre um aumento da taxa mitoses de divisões celulares em certas partes do corpo.



Padrões alométricos de características secundárias de adultos podem algumas vezes ser ligados a padrões de seleção sexual (Bonduriansky 2007).

## Avaliação do mimetismo, da seleção sexual e da alometria em um anel Mimético de *Heliconius* no Brasil

Nesta dissertação de mestrado, avaliamos o reforço adaptativo ao Mimetismo Mulleriano considerando a asa e a mancha vermelha da asa anterior em um anel mimético com distribuição no Brasil. As espécies analisadas foram: *Heliconius erato phyllis*, *H. besckei*, *H. melpomene burchelli* e *H. m. nanna*, as quais possuem distribuição restrita as áreas de alta altitude no sul do Brasil, região Central-Norte e litoral do Nordeste do Brasil. A variação morfológica intra e interespecífica encontrada empiricamente pode estar relacionada ao reforço adaptativo ao mimetismo Mulleriano. Esperávamos que a morfologia das demais espécies variasse de acordo com áreas de simpatria com *H. erato phyllis*. Já em áreas de alopatria encontraríamos um relaxamento dessa convergência evolutiva. Dessa forma, a variação morfológica tenderia a ser mais similar a *H. erato phyllis* em áreas de simpatria. E fora da área de simpatria encontraríamos um relaxamento ao padrão.

A variação do tamanho e forma da asa anterior e da mancha vermelha nesta asa foi avaliada através da morfometria geométrica, ferramenta bastante sensível a variação e com melhor precisão principalmente quanto a variação da forma, pois as coordenadas são digitalizadas em um sistema cartesiano o qual mantém as dimensões da área analisada, ao contrário de modelos lineares onde diferentes formas podem resultar em iguais valores de comprimento e largura.

Além disso, avaliamos a estruturação populacional dessas espécies. Neste sentido, conhecer a estrutura genética em *Heliconius* é importante para esclarecer a evolução dinâmica do mimetismo (Moura et al 2011). Espécies com alta capacidade de dispersão devem possuir ampla distribuição e por isso possuem maior fluxo gênico sobre áreas, resultando em populações mais homogêneas (Haag et al 1993; Krauss et al 2004, Kronforst e Fleming 2001; Vandewoestijne et al 1999). De maneira a remover o efeito de diferenças entre espécies com base em características ecológicas particulares, como estrutura populacional, dispersão diferencial e isolamento pela distância, avaliamos a ocorrência de fluxo gênico e estruturação genética em *H. e. phyllis*, *H. besckei*, *H. m. burchelli* e *H. m. nanna* ao longo do gradiente de distribuição.

1 **Convergent evolution of warningly wing color pattern in**  
2 ***Heliconius* butterflies: not only Müllerian mimicry.**

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31 \_\_\_\_\_  
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33 **Abstract.** *Heliconius* butterflies have been used as models in evolutionary biology,  
34 due in part to their wide intra- and interspecific variation in wing color pattern and  
35 for belonging to mimetic associations. Mullerian mimicry phenotypic patterns have  
36 supposedly converged in this case by predation pressure associated mainly with  
37 birds. Although from a gross morphology perspective similarities are notable,  
38 subtle differences on morphological traits are known to exist among mimetic  
39 members. This suggests other factors, such as phylogenetic constraints and/or  
40 sexual selection may also play a role in molding such mimetic patterns. In this  
41 study, we explored such questions in the “post man” mimetic ring existing in Brazil  
42 that includes: *Heliconius erato phyllis*, *H. besckei*, *H. melpomene burchelli* and *H.*  
43 *melpomene nanna*. By applying geometric morphometric tools, we evaluated  
44 spatial variation in size and shape of their forewing and corresponding red band,  
45 in association to DNA sequencing (*COI* and *Optix* genes). For *H. erato phyllis*, the  
46 most variable in wing shape and widest spread mimic ring member, results showed  
47 that differences on shape were randomly distributed in space. There was no  
48 convergence in shape towards a given mimetic pair even when in sympatry. Also,  
49 there was no correlation between spatial variation in shape and genetic variability,  
50 which was high but not geographic structured, neither associated with geographic  
51 distance. Also, there was no correlation between phylogenetic and Mahalanobis  
52 distances for the forewing and red band shape either, thus suggesting that  
53 phylogenetic constraints are not responsible for such differences. However, sexual  
54 dimorphism on the forewing red band shape was found for all lineages. A static  
55 allometric pattern for the centroid size occurred, but there was no sexual  
56 dimorphism on forewing and redband regarding size. Thus, there exist diagnosable  
57 and stable shape differences on such mimetic ring traits that contrary to what

58 theory on their evolution postulates have not converged by natural selection.  
59 Alternatively, our data suggest that sexual selection may have played a role in  
60 evolution of such divergences, keeping these subtle phenotypic differences in the  
61 wild.

62 **Key-words:** natural selection, sexual selection, phenotypic variation, mimetic  
63 rings, heliconian butterflies

64

## 65 INTRODUCTION

66 Phylogenetic distantly related species that show high morphological  
67 similarities are considered mimetics. Two types of mimicry are known: 1) Batesian  
68 (Bates 1862), where an edible species mimics an unpalatable one, and 2)  
69 Mullerian (Muller 1879), where both species are unpalatable, also known as co-  
70 mimics. A classical example of the latter is found within *Heliconius* butterflies  
71 (Lepidoptera: Nymphalidae), which have been used as models in evolutionary  
72 biology, due in part to their wide intra- and interspecific variation in wing color  
73 pattern and for belonging to such mimetic associations. In this case, convergence  
74 occurs at the spatial level, where species such as *H. erato* and *H. melpomene*  
75 show the same wing color pattern within a given area. But, they vary substantially  
76 among areas, forming several phenotypically distinct co-mimic pairs, called races  
77 or subspecies, that occurs in allopatry throughout the broad distribution ranges of  
78 each species (Turner 1981, Hines et al 2011).

79 That warningly wing color pattern in *Heliconius* butterflies are adaptive has  
80 been demonstrated experimentally in the wild by Benson (1972) and Mallet and  
81 Barton (1989). According to Joron (2009), mimetism is advantageous to mimetic

82 butterflies first for being under lower predation risk by birds, which associate their  
83 warningly color pattern to inpalatability. Second, because those variant individuals  
84 existing in a given population may not be recognized as unpalatable, and thus are  
85 likely under greater predation risk. It is also advantageous to the birds, since those  
86 individuals that visually better associate the signal existing on the butterfly wing  
87 indicating unpalatability have greater survivorship (Mallet and Barton 1989). As  
88 pointed out originally by Muller (1879), birds learn about how to differentiate such  
89 signals, and thus share initially with the butterflies the corresponding costs in terms  
90 of mortality. Learning in this case may be associated to several phenotypic,  
91 behavioral and ecological factors, associated with the butterflies, as for example:  
92 1) color (Svádová et al 2009) and pattern (Ihlainen et al. 2008) of the wings, 2)  
93 level of unpalatability (Ihlainen 2006) and 3) relative frequency of the comimics  
94 (Rowland et al 2010). As far as we are concerned, variation in the former factor  
95 has not been quantitatively examined in the wild for any *Heliconius* mimetic ring  
96 yet. Unless predators cue such signal in a broad scale, it is expected that within  
97 the scenario described above, natural selection alone would eventually lead to  
98 identical patterns regarding wing shape for each mimetic pair in *Heliconius*  
99 butterflies. In other words, as pointed out by Mallet et al (1996), “the ultimate  
100 prediction of Müllerian mimicry is that butterflies of a similar size should all  
101 ultimately converge on the same color pattern”. However, as demonstrated by  
102 Estrada and Jiggins (2008), phenotypic resemblances could impose costs for  
103 mimicry due to possible mistaken in species identity during courtship. Thus, there  
104 could be a conflict between the outcomes of natural and sexual selection in mimetic  
105 species.

106 We explored herein such questions for a striking *Heliconius* mimetic ring  
107 (called “post man”) existing in Brazil, including three species (*H. erato*, *H. besckei*  
108 and *H. melpomene*) that are not closely related phylogenetically (Figure 1). The  
109 latter is represented by two subspecies (*H. m. burchelli* and *H. m. nanna*) that  
110 similarly to *H. besckei* have smaller distribution ranges and are allopatric. The  
111 former is represented by the subspecies *H. erato phyllis* and has the widest  
112 distribution, occurring in sympatry with the other three on different areas located in  
113 Southern, Central, and Northern Brazil (Figure 2). This scenario allows one not  
114 only to test for the existence of expected phenotypic convergence in the mimetic  
115 signals in areas of sympatry but also to look for the existence of phylogenetic  
116 constraints on such traits, if any, since more than one taxonomic level is involved.

117 *H. erato phyllis* is known to vary substantially in space in terms of wing size  
118 and shape, due in part to use of several species of host plants (passion vines) that  
119 also vary in distribution (Rodrigues and Moreira 2002, Jorge et al 2011). However,  
120 as far as we have been concerned, there is no data, for example, about whether  
121 such a variation is spatially structured. There is also a lack of information about  
122 variation in size on phenotypic signal predators may cue in such mimetic rings,  
123 with the exception of those provided recently by Klein and Araújo (2013), for  
124 putative populations of *H. erato phyllis* and *H. besckei*. By applying geometric  
125 morphometric tools, including counter analysis, we evaluated in this study spatial  
126 variation in forewing size and shape and corresponding red band, base on field-  
127 collected specimens from taxa belonging to such mimetic ring. We covered most  
128 of the distribution ranges of such taxa, but giving emphasis to *H. erato phyllis* areas  
129 of sympatry with other species. We tested the null hypothesis that in such areas  
130 for this subspecies convergence in shape would be equal compared to others. To

131 test for existence of phylogenetic constraints a phylogenetic analysis based on  
132 unlinked (COI) and linked (*Optix*) color pattern genes was conducted for these  
133 taxa. For *H. erato phyllis*, genetic variation at population level was also evaluated.

134

## 135 MATERIAL AND METHODOS

### 136 **Insect samples**

137 From the total of 322 specimens used in this study, 229 dried-museum  
138 specimens were included in the geometric morphometric analyses, as follows (n =  
139 males, females): *H. erato phyllis* (31, 29), *H. besckei* (31, 26), *H. melpomene*  
140 *burchelli* (34, 24) e *H. melpomene nanna* (32, 22), for males and females,  
141 respectively. Most of them came from the Lepidoptera collection of Departamento  
142 de Zoologia, Universidade Federal do Paraná, Curitiba, PR (Appendix S1). They  
143 were randomly chosen according to their availability in the insect collection drawers,  
144 until completing at least 20 specimens for each species/subspecies. In the case of  
145 *H. erato phyllis*, those that were distributed outside the geographical boundaries  
146 established by Rosser et al (2012) for the other members in the mimetic ring under  
147 study were excluded for the samples. Thus, for this taxon, distribution of samples  
148 resulted in three different regions, forming areas of sympatry with *H. melpomene*  
149 *burchelli*, *H. melpomene nanna* and *H. besckei*, in Central, Northern and Southern  
150 Brazil, respectively (Figure 2).

151 Additional field-collected specimens that were kept under -20°C in the tissue  
152 collection of the Laboratório de Morfologia e Comportamento de Insetos (LMCI),  
153 from Federal University of Rio Grande do Sul state (UFRGS), Porto Alegre, RS,  
154 Brazil, were used in the molecular analyses, as follows: *H. erato phyllis* (n = 73),



155 *H. besckei* (n = 37), *H. melpomene burchelli* (n = 17), and *H. melpomene nanna*  
156 (n = 16).

157

### 158 **Geometric Morphometrics**

159 Each individual was photographed in the dorsal view by the same person,  
160 with a digital camera Sony Cybershot H20, 5 mega pixels of resolution, Iso200,  
161 one-shot, flash off and macro function activated. The choice of dorsal view was  
162 associated to the fact that is supposedly the side under greater exposure to natural  
163 and sexual selection. In addition to the whole wing, we analyzed also the  
164 corresponding red band (Figure 3A). We used a total of 19 landmarks (Jorge et al  
165 2011) for the whole wing (Figure 3B), and 8 landmarks added of 35 semi-  
166 landmarks for the red band (Figure 3C) (for a complete description of the  
167 landmarks, see Table S2).

168 Landmarks and semi-landmarks were digitized also by the same person  
169 using TPSDig, version 2.17 (Rohlf 2013). Images were scaled by software IMP –  
170 CoordGen6f (Sheets 2001) to comparisons of size among the mimic ring  
171 members. Size was estimated as the log transformed centroid size, which  
172 represents the squared root of the sum of squared distances of each landmark  
173 from the centroid of the configuration (Bookstein 1991).

174 Coordinates from the whole forewing and red band were superimposed  
175 using a generalized Procrustes analysis (GPA) (Dryden and Mardia 1998) using  
176 respectively the softwares R Studio version 0.97.551 (R Core Team 2013) and  
177 Matlab version 7.10.0.499 (MATLAB and Statistics Toolbox Release 2012). GPA  
178 removes differences unrelated to shape such as scale, position and orientation  
179 (Rohlf and Slice 1990, Adams et al 2004). To analysis of red band by using semi-

180 landmarks, we created a slider file using TPSUtil version 1.46 (Rohlf 2010); points  
181 were adjusted sliding along the outline curve until they match as much as possible  
182 the positions of the corresponding point in a reference specimen (Bookstein 1997).

183 The consensus configuration (mean shape) was calculated and the  
184 difference among mean landmarks and individual landmarks resulted in a residual  
185 matrix (Jolliffe 1986). This matrix was used in Principal Component Analysis (PCA),  
186 as the new shape variables. This procedure allowed us to reduce the  
187 dimensionality of the data set and to work on independent variables (Cordeiro-  
188 Estrela et al 2006).

189 For the analysis of shape and size variation of the forewing and red band  
190 we used R Studio and the libraries: MASS (Venables and Ripley 2002), ape  
191 version 1.8-2 (Paradis et al 2006) and ade4 (Dray and Dufour 2007). Geometric  
192 morphometrics procedures were carried out with RMORPH software: a geometric  
193 and multivariate morphometrics library for R (Baylac 2008).

194 We performed an one-way Analysis of Variance (ANOVA) followed by  
195 Tukey's pairwise comparison tests to verify whether size of the whole forewing and  
196 red band differs among the mimic ring members. Also, we performed Student's *t*-  
197 tests to verify differences between gender within these groups Regression  
198 analyses were performed between log-centroid size of the whole forewing and red  
199 band to test for existence of allometry, in relation to each member regarding  
200 isometry, and between gender within each mimic ring member. Slope lines and  
201 intercepts were compared using one-way Analysis of Covariance (ANCOVA).  
202 Allometric analysis and graphs were made by GraphPad Prism, version 5.00  
203 (Motulsky and Christopoulos 2003) and edited on CorelDraw.

204 The shape variation of the whole forewing and red band were scatterplotted  
205 through an exploratory analysis (type PCA). For this analysis, *H. erato phyllis*  
206 individuals were clustered in three groups as mentioned before. Such clusters  
207 totalized 28, 19 and 13 individuals, that occurs in sympatry with *H. besckei*, *H.*  
208 *melpomene burchelli* and *H. melpomene nanna*, respectively (Figure 2E). The  
209 shape variation analysis for the first two PCs of wing and red band were made by  
210 TPSRelw version 1.49 (Rohlf 2010), and edited on CorelDraw software. The  
211 consensus configuration for the whole forewing and red band was obtained for  
212 each mimic ring member. After, we carried out a Multivariate Analysis of Variance  
213 (MANOVA) of shape variables with the factors: clusters (species / subspecies) and  
214 gender. Also, we calculated Mahalanobis distance for morphometric data from the  
215 wholewing and red band. With the corresponding Mahalanobis distances we  
216 generated Neighbor joining trees. In addition, we tested the positive association ( $r$   
217  $> 0$ ) between the whole forewing and red band distances to genetic distances  
218 through a Mantel test using Pearson's correlation coefficient in XLStat software.

219

## 220 **Molecular Analyses**

### 221 **DNA data collection**

222 We surveyed a total of 109 samples belonging to *H. erato phyllis*, *H.*  
223 *besckei*, *H. melpomene nanna* and *H. m. burchelli*; the latter were pooled together  
224 in most analysis, and thus otherwise mentioned, treated as *H. melpomene*  
225 (Appendix S1). High quality DNA was purified from larvae tissue using the organic  
226 method of Cetyl Trimethyl Ammonium Bromide (CTAB). Specimens were amplified  
227 through polymerase chain reaction (PCR) for unlinked and linked loci to color  
228 pattern: (i) mitochondrial gene *cytochrome c oxidase subunit I (COI)* segment of

229 821 base pairs (bp), and (ii) the *Optix* gene region of 782 bp, respectively. Primers  
230 and conditions followed description by Beltrán et al (2007) and Hines et al (2011)  
231 for *COI* and *Optix*, respectively. Aliquots of PCR products were treated with  
232 Exonuclease I and FastAP Thermosensitive Alkaline Phosphatase (Thermo  
233 Scientific), sequenced using the BigDye chemistry, and analyzed on an  
234 ABI3730XL (Applied Biosystems Inc.) at Macrogen (Seoul, Republic of Korea).  
235 Sequences were aligned and visually inspected using the algorithm Clustal X in  
236 MEGA version 5 (Tamura et al 2011) running in full mode with no manual  
237 adjustment. Due to the high proportion of substitution in the third position of the  
238 *COI* codon, particularly in *H. erato phyllis*, we tested for substitution saturation in  
239 the three species with the program DAMBE version 5.2.61 (Xia and Xie 2001)  
240 using General Time-Reversible (GTR) model of sequence evolution (Rodríguez et  
241 al 1990) (Figure S1). All sequences generated herein have been submitted to  
242 GenBank (Tables S2-6).

243

#### 244 **Reconstructing phylogenetic trees**

245 In order to showed the convergence color pattern explored in this study  
246 under a phylogenetic tree, we reconstructed the relationship of *H. erato phyllis*, *H.*  
247 *besckei* and *H. melpomene* using some additional pair of sister species of each of  
248 these taxa based on the topology of Beltrán et al (2007). We incorporated  
249 sequences from ~2 Kb of two markers (*COI* and *Elongation factor 1-  $\alpha$*  [EF1- $\alpha$ ];  
250 Beltrán et al 2007) from 12 species, and performed a Bayesian inference (BI),  
251 implemented in BEAST 2.0 (Drummond et al 2012). Time of divergences and  
252 topology of lineages were estimated simultaneously. The tree prior was set to the  
253 Yule calibrated process, using the GTR model. The branch lengths were allowed

254 to vary under a relaxed clock model with an uncorrelated lognormal distribution  
255 (Drummond et al 2006). The analyses were run for 40 million generations with  
256 every 4000th generation sampled. One prior was specified in the form of a  
257 calibration point as the time of the most recent common ancestor (tMRCA) for  
258 *Heliconius* (18 mya; Wahlberg et al 2009). Convergence, effective sample sizes,  
259 and divergence times with upper and lower 95% highest posterior density (HPD)  
260 bounds were assessed in Tracer 1.4.8 (Rambaut and Drummond 2007).  
261 TreeAnnotator was used for all analyses, with 20% of the samples removed for  
262 burn-in to generate a tree that was then visualized and edited in FigTree v1.3.1  
263 (Rambaut 2009). In addition, we reconstructed two trees using only one sample of  
264 *H. erato phyllis*, *H. besckei* and *H. melpomene* to compare topologies of *COI* and  
265 *Optix* in order to investigate whether linked or unlinked loci results in different  
266 scenarios. We also performed a Bayesian analysis with the same parameter  
267 described previously, but with a Yule prior on branching rates. Finally, the genetic  
268 divergence between pairs of taxa was used to compare the whole forewing and  
269 red band shape distances with calculated using p-distance, using 1000 of  
270 bootstrap replication.

271

### 272 **Intraspecific genetic diversity**

273 Haplotypes of *COI* gene were directly inferred from polymorphic sequences  
274 using DNAsp version 5 (Librado and Rozas 2009) software. For *Optix* gene we  
275 used PHASE version 2.1 (Stephens et al 2001) to infer haplotypes. Phased  
276 haplotypes were used to construct relationships in the software NETWORK  
277 version 6.0 (Baldelt et al 1999). We used the median joining algorithm with default  
278 settings for constructing the networks. Genetic variation was estimated using

279 haplotype diversity (H) (Nei 1987)  $\pm$  standard deviation (SD) and nucleotide  
280 diversity ( $\pi$ ) (Nei and Jin 1989)  $\pm$  SD, with the software ARLEQUIN version 3.5  
281 (Excoffier et al 1992). We also calculated the total number of variable sites (S)  
282 using MEGA 5. Also, we tested spatial or demographic expansion estimating  
283 Tajima's  $D$  (Tajima 1989) and Fu's  $D$ ,  $F_s$  and  $F$  (Fu 1997), using DNAsp 5.0. We  
284 assessed significance with 1000 permutations.

285

### 286 **Population genetic structure**

287 We evaluated the existence of reduced gene flow (i.e., population genetic  
288 structure), as a proxy of genetic drift, which could induce preferential mates (due  
289 to small population size) and ultimately reflect wing shape and/or red-banded  
290 regional patterns. Thus, population structure for each gene and species was  
291 inferred based on admixture analysis of individuals across Southern, Central and  
292 Northern regions using Bayesian population assignment method implemented in  
293 BAPS version 4.1 (Corander et al 2008). We conducted admixture analysis of  
294 individuals without the geographic origin of the samples used as prior information.  
295 BAPS was run with the maximal number of groups (K) set to 1–11 for *H. erato*  
296 *phyllis*, K = 1-4 for *H. besckei*, and K =1-5 to *H. melpomene*. Each run was  
297 replicated six times. Results were according to the highest likelihood scores.  
298 Finally, we also implemented isolation by distance (IBD) analysis in order to  
299 evaluate the contribution of this variable to genetic differentiation, which might  
300 generate regional morphological patterns due to small population size. Mantel  
301 tests, also implemented in ARLEQUIN, were used to test each species for IBD;  
302 i.e., a correlation between pairwise genetic distance (based on  $F_{ST}$ ) and  
303 geographical distances (log-transformed, linear, kilometer distances).

## 304 RESULTS

305 **Forewing and red band shape and size**

306 The ANOVA for the whole forewing centroid size showed significant  
 307 differences ( $F_{3,225}=8.758$ ,  $P<0.001$ ). Forewing size of *H. besckei* was smaller than  
 308 *H. melpomene burchelli* and *H. melpomene nanna*, and *H. melpomene nanna* was  
 309 smaller when compared to *H. erato phyllis* ( $P < 0.001$ ). There was no difference  
 310 when the forewing size between *H. erato phyllis* and *H. melpomene burchelli* ( $P =$   
 311  $0.055$ ), and with *H. besckei* ( $P = 0.598$ ) were compared, neither between the two  
 312 subspecies of *H. melpomene* ( $P = 0.878$ ) (Tukey's multiple comparison tests;  
 313 Figure 4A). The ANOVA for the red band centroid size for clusters showed also  
 314 significant differences ( $F_{3,225} = 34.61$ ,  $P<0.001$ ). Corresponding Tukey's multiple  
 315 comparison tests were all significant ( $P < 0.001$ ), except for *H. melpomene nana*  
 316 when compared to *H. melpomene burchelli* ( $P > 0.05$ ), and for *H. erato phyllis* in  
 317 relation to *H. besckei* ( $P > 0.05$ ) (Figure 4B).

318 There was no significant difference between males and females to forewing  
 319 size to all species / subspecies (Table 1). To the contrary, regarding the red band  
 320 size there was significant difference between sexes for all species / subspecies,  
 321 except to *H. erato phyllis* (Table 2).

322 The relation of size between wing and red band showed negative allometry  
 323 only for *H. erato phyllis* ( $y=0.64x + 1.03$ ,  $r^2=0.45$ ,  $P<0.0001$ ). For the other mimic  
 324 ring member such a relationship was isometric, the corresponding linear regression  
 325 equations being as follows: *H. besckei* ( $y=0.94x + 0.99$ ,  $r^2=0.71$ ,  $P=0.48$ ), *H.*  
 326 *melpomene burchelli* ( $y=0.98x + 0.01$ ,  $r^2=0.75$ ,  $P=0.75$ ) *H. melpomene nanna*  
 327 ( $0.98x + 0.006$ ,  $r^2=0.781$ ,  $P=0.80$ ).

328 For all mimic ring members, males and females were not different from each  
329 regarding the allometric coefficient. But, when compared them to the isometric line,  
330 males and females of *H. e. phyllis* and males of *H. besckei* showed negative  
331 allometry. For the *H. melpomene* subspecies, males and females did not differ  
332 from isometry (Table 2; Figure 5).

333 Results from MANOVA on shape of the forewing and red band showed  
334 significant differences among all mimic ring members, between sexes, and also for  
335 the interaction between these two factors (Table 3).

336 The first ten PCs for whole wing shape explained 85.12% of variation. There  
337 was not a clear separation among mimic ring members in this case (Figure 6A).  
338 The consensus of forewing shape to each species/subspecies are given in Figure  
339 7. Thus, in spite of the MANOVA testing for positive significance, the forewing  
340 differences in shape were very small.

341 The first ten PCs for the red band explained 81.44% of the shape variation.  
342 To the contrary, in this case, there was a clear separation for all mimic ring  
343 members (Figure 6B). In the first PC, *H. besckei* was separated from the other  
344 species, while the second PC separated *H. erato phyllis* from *H. melpomene*  
345 *nanna*, and *H. melpomene burchelli* from *H. besckei* with partial overlap in this  
346 axes. The greater variation for the red band within a given mimic ring when  
347 compared to the whole forewing is clear seen on the corresponding consensus  
348 shape (Figure 7).

349 The MANOVAs on the whole forewing and red band shape for males and  
350 females showed significant differences for all mimic ring members (Table 4). In



351 general, the first PC showed the existence of difference between sexes, and for  
352 this reason we showed just the shape variation for the PC1 (Figure 8).

353

### 354 **Molecular Analyses**

355 We sequenced 1.6 kb region of unlinked (*COI*) and linked (*Optix*) color  
356 pattern loci to infer the population structure of *H. erato phyllis* and examine genetic  
357 diversity and phylogenetic relationships among this and the co-mimics (Figure 9).

358 There were marked differences within species in standing levels of genetic  
359 variation. *Heliconius erato phyllis* presented over two-fold variable sites and higher  
360 number of haplotypes than *H. besckei* and *H. melpomene* across *COI* and the  
361 *Optix* gene (Table 1). Also, Tajima's *D* and Fu's *D*, *F* and *F<sub>s</sub>* tests were positive  
362 and no significant for both genes and all co-mimics (Table 1). Gene trees  
363 reconstructed based on *COI* and *Optix* retrieved similar topologies, but with small  
364 differences in branch lengths (Figure 9A). *Heliconius erato phyllis* was the sister  
365 lineage to the group formed by *H. besckei* + *H. melpomene*.

366 Admixture analysis of population structure, in particular of *H. erato phyllis*,  
367 showed most of individuals with admixed ancestry, indicating that Southern,  
368 Central and Northern regions are not genetically structured (Figure 9B), neither in  
369 the co-mimics (Figure S2). For the *COI* gene a total of 36 haplotypes (Table S3)  
370 were found in *H. erato phyllis* (Figure 9C), 22 in Southern, 6 in Central and 13 in  
371 Northern region. A total of 4 haplotypes (H2, H8, H10, H16) are shared among  
372 regions. One haplotype in particular, the H16, is shared among three regions. In  
373 relation to the *Optix* gene we observed 21 haplotypes (Table S4) in *H. erato phyllis*,  
374 16 in Southern, 5 in Central and 9 in Northern region (Figure 9C). A total of 8  
375 haplotypes were shared among regions (H1, H2, H12, H4, H5, H8, H13, H17).

376 Haplotype 4 and H13 are shared among all three regions. Haplotypes found in the  
377 co-mimics are presented in Supplementary material (Tables S5, 6; Figure S2).

378 Mantel tests did not detect a pattern of IBD for populations (regions) of *H.*  
379 *erato phyllis* (Figure 9D), neither for the co-mimics (Figure S2), revealing a lack of  
380 positive association between pairwise genetic and geographical distances across  
381 the studied area (*COI*,  $r = 0.36$ ,  $P > 0.05$ ; *Optix*,  $r = 0.10$ ,  $P > 0.05$ ).

382 Neighbor-joining (NJ) trees of Mahalanobis distance based on wholewing  
383 and red band indicated that *H. erato phyllis* is more close related to *H. melpomene*  
384 subspecies (Figure 10A, B). Contrary to expected, in the NJ construct based on  
385 DNA loci (i.e. phylogenetic distances), we observed this species distantly related  
386 to *H. melpomene* subspecies, and close to *H. besckei* (Figure 10C). Mantel tests  
387 indicated that phylogenetic distance is not positive correlated with the Mahalanobis  
388 distance for both the whole fore wing ( $r = 0.90$ ;  $P = 0.20$ ) and red band ( $r = -0.99$ ;  
389  $P = 0.92$ ) shapes.

390

## 391 DISCUSSION

### 392 **Natural selection**

393 Results showed clearly that there exist differences among members of such  
394 a mimetic ring regarding shape on the forewing red band that are diagnosable and  
395 stable. For *H. erato phyllis*, the most variable in wing size and shape and widest  
396 distributed member, the corresponding variation was spatially random, not  
397 converging to any other comimic in the three areas of sympatry. Thus, natural  
398 selection due predation by birds should not be responsible for maintenance of such

399 forewing band shapes, and should not be reconsidered as the only factor driven  
400 evolution of visual cues (shape of warning color traits) in this mimetic ring.

401         It is possible that predatory birds cue the red band in a broader scale in this  
402 case, not recognizing such phenotypic variants in the wild as different entities while  
403 preying upon these butterflies. By being unperfect mimics, they could also benefit  
404 by showing a more generalized signal, where subtle differences existing among  
405 comimics would be neglected by the predator (Rowe et al 2004). According to  
406 Evans et al (1987), for example, *Coturnix coturnix* when preying upon three  
407 hemipteran species with significantly different body shapes avoids them equally  
408 because they all have bodies with the same warning color (black and red). It should  
409 be also mentioned that in this area of Brazil there are other members of such  
410 mimetic rings, as for example *Eresia lansdorfi* (Nymphalidae), where the forewing  
411 redband resemble those treated herein. Although occurring at low densities these  
412 species may influence evolution of visual cue involved in such a mimetic ring, by  
413 working as an enhancement mechanism, leading birds to wide selection towards  
414 a broader range of variation regarding such visual cues. Comimic species may in  
415 fact benefit from the presence of other comimics even when these are a little  
416 dissimilar (Rowe et al 2004, Ihalainen et al 2007, Rowland et al 2007).

417         At least initially during the phase of learning, chemical differences existing  
418 among comimics may be more important than visual similarities (Lindstrom et al  
419 1997, Darst and Cumming 2006). Heliconian butterflies have conspicuous exocrine  
420 glands on last abdominal segments that were originally presumed to be associated  
421 with defense in both sexes (Muller 1912, Ross et al 2001), but surprisingly have  
422 not been detailed explored in this regard within the context of Mullerian mimicry.  
423 Lately, they have been related to the production (males) and storage and dispersal

424 (females) of antiaphrosidiacs (e.g. Gilbert 1976, Shultz et al 2008). However, such  
425 functions may be not mutually exclusive. The possibility that chemicals produced  
426 by these structures have a role on species recognition remains also to be explored.

427

### 428 **Genetic variability and phylogenetic constraints**

429 The *Optix* gene that was used in this study is the most likely candidate to  
430 encode the genome region associated with the forewing red band in *Heliconius*  
431 butterflies (Reed et al 2011). Similarly to results obtained from sequences  
432 generated by the use of *COI*, this loci distinguished all mimetic ring members  
433 herein studied, leading to the same tree topologies in the phylogenetic analyses.  
434 However, even for *H. erato phyllis*, the widest distributed member and that showed  
435 the greatest genetic diversity there was no indication that such a variation is  
436 spatially structured. Thus, we inferred that it is unlike to associate differences found  
437 for shape to evolutionary effects, such as genetic drift, which is more intense in  
438 small population size resulted from genetic structure (or isolation).

439 Data showed that within a given species / subspecies in such mimetic ring,  
440 differences in whole forewing shape are smaller than on the red band.  
441 Furthermore, for all taxa variation within is smaller for the forewing red band than  
442 the forewing overall shape. Thus, results strongly suggested that selection on the  
443 forewing red band is greater than on the wing overall shape, under both specific  
444 and subspecific levels. In addition, the Mantel tests showed no correlation between  
445 phylogenetic distances obtained in the molecular analysis and Mahalanobis ones  
446 coming from the geometric morphometrics, in relation to both the forewing overall  
447 shape and forewing red band shape. Thus, there is no indication phenotypic  
448 evolution in such traits is phylogenetically constrained.

#### 449 **Species identity and sexual selection**

450           Since differences on shape found herein are stable, it is possible that they  
451 used in courtship first to identify their co-specifics. As sexual dimorphism was  
452 found regarding shape of the forewing red band, for all lineages explored in this  
453 study, results also suggest that sexual selection may be involved on evolution of  
454 this trait. In these cases, there could be a divergence in direction between natural  
455 and sexual selection (Estrada and Jiggins 2008). That wing color and pattern is  
456 used as visual stimuli during courtship in both sexes of *H. erato* was first  
457 demonstrated by Crane (1955). According to Emsley (1970), the red color is in fact  
458 used as a visual cue in *H. erato phyllis* courtship, and is likely also to be important  
459 for *H. melpomene melpomene*. However, preliminar tests conducted by him  
460 suggested the yellow color, which is present in the hindwings of the mimic ring  
461 members, and that was not herein taken into account, is important for *H. besckei*.  
462 It is questionable that such subtle differences on shape would be used as visual  
463 cues in this scenario. However, heliconian butterfly are known to have developed  
464 a good sense of vision and memory, including associative learning to refined hues  
465 of some colors, especially in the wave length interval from orange to red (Swihart  
466 1971, Barp et al 2011). Thus, such a possibility should not be discharged a priori  
467 without been experimentally tested.

468

#### 469 **Variation in size and allometry**

470           Similarly to what was obtained by Ramos and Freitas (1999), Jorge et al  
471 (2011) and Klein and Araujo (2013), we found no sexual dimorphism on overall  
472 forewing size in *H. erato phyllis*. Similar results were obtained for *H. besckei* and  
473 the two subspecies of *H. melpomene*. However, when the size red band was taken

474 into account, all member showed sexual dimorphism, except *H. erato phyllis*. Klein  
475 and Araujo (2013) found similar results for the latter species and also for *H.*  
476 *besckei*, suggesting in these mimetic members such trait might be under sexual  
477 selection pressure.

478 We interpreted the negative allometry found for the forewing red band in *H.*  
479 *erato phyllis* as evidence for the existence of a limiar in size as an effective visual  
480 cue used either in the context of predation and/or courtship. In other words, as  
481 being variable in the wild up to 50% in size (Rodrigues and Moreira 2002), those  
482 small individuals regarding overall forewing size would compensate for that by  
483 having a proportionally greater red band. This would not be the case of those  
484 belonging to *H. melpomene nanna*, as in average they vary less and are naturally  
485 bigger when compared to those of *H. erato phyllis*.

486

## 487 CONCLUSION

488 We demonstrated in this study that there are subtle differences in the  
489 forewing red band for members of the *Heliconius* postman mimetic ring existing in  
490 Brazil, which are diagnosable and stable. For *H. erato phyllis*, the most variable in  
491 wing shape and widest distributed member results demonstrated that such  
492 differences are randomly distributed in space, including three areas of sympatry  
493 with other comimics. Thus, we inferred that natural selection due predation by  
494 birds, which in theory would lead instead to convergence, is not the only  
495 responsible mechanism for the evolution of such a pattern. Also, there was no  
496 indication for the existence of phylogenetic constraints either, as divergence in  
497 shape occurred at a greater rate in the closest related phylogenetic lineages. On  
498 the other hand, we found conspicuous evidence for the existence of sex

499 dimorphism in relation to the forewing red band in particular, suggesting that sexual  
500 selection may play a role on phenotypic evolution in this case. Thus, we found  
501 support for the hypothesis that there is a conflict between natural and sexual  
502 selection, as already suggested for other mimetic species (Estrada and Jiggins  
503 2008). Whether the mimetic ring members herein tested use such subtle  
504 differences as visual cues to identify, compete and/or choose their partners  
505 remains to be tested. As postulated by Mallet et al (1996), mimicry theory is still  
506 open to discussion, and “poses more questions than it answers”.

507

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- 692 \* The references were formatted according to PLOS ONDE but with adaptations

## FIGURE LEGENDS

### **Figure 1. Bayesian phylogeny reconstructed using two concatenate genes [cytochrome oxidase c subunit I (COI) and Elongation factor-1a (EF1- $\alpha$ )] for three major clades within *Heliconius*.**

Species focus on the present study are marked in red: *H. erato* (“erato” clade), *H. melpomene* (“melpomene/cydnó” clade) and *H. besckei* (“silvaniform” clade). *Philaethria dido* was used to root the tree. Values below branches show Bayesian posterior probabilities for the equivalent node. Numbers below branches represent mean node age and the gray bars indicate confidence intervals of the estimates.

### **Figure 2. Putative individuals of the *Heliconius* mimic ring members and corresponding distributions in Brazil.**

From (A) to (D), respectively, *H. erato phyllis*, *H. besckei*, *H. melpomene burchelli*, and *H. melpomene nanna* (modified from Holzinger and Holzinger 1994). Maps represent the geographical locations of samples used in this study, as follows: (E) *H. erato phyllis*, with samples located in Central, Northern and Southern regions, represented by red, blue and green circles, respectively; (F) *H. besckei* (green squares); (G) *H. melpomene burchelli* and *H. melpomene nanna* (red and blue triangles, respectively). Gray areas show the overall distribution of each mimetic ring member, according to Rosser et al (2012). Biogeographical subregions are showed in brown (Amazon forest), pale yellow (Chacoan) and puple green (Atlantic Rain Forest), following Morrone (2006).

### **Figure 3. Location of landmarks on the forewing of *Heliconius melpomene burchelli*.**

(A) Photographed wing on millimeter paper. (B) Type I landmarks on whole wing, indicated by blue circles. (C) Type II landmarks (green circles) and semi-landmarks (yellow circles) on red band.

### **Figure 4. Variation in size on forewings of *Heliconius* mimic ring members.**

(A) Whole wing . (B) Red band. Box plots followed by the same letter do not differ statistically (Student’s *t*-tests,  $\alpha = 0.05$ )



**Figure 5. Variation in size of the red band in relation to forewing size among *Heliconius* mimic ring members.** (A) *H. erato phyllis*. (B) *H. besckei*. (C) *H. melpomene burchelli*. (D) *H. melpomene nanna*. Males and females are represented by blue and red circles, respectively. Isometry is indicated by dashed line.

**Figure 6. Two first axes of the principal component analysis (PCA) on shape residuals for the whole forewing (A) and red band (B) of *Heliconius* mimic ring members.** Percentage of shape variation explained by each PCA is given between parentheses. The shape deformations are showed near each axis, where the dashed line represents the shape at minimum values and the solid line represents the shape at maximum values. Circles represent *H. erato phyllis* individuals in sympatry with (●) *H. besckei*, (●) *H. melpomene burchelli* and (●) *H. melpomene nanna*; (■) *H. besckei*, (▲) *H. melpomene burchelli*, (▲) *H. melpomene nanna*.

**Figure 7. Consensus configuration (mean shape) for the whole forewing (left column) and red band (right column) of *Heliconius* mimic ring members..** (A) *H. erato phyllis*. (B) *H. besckei*. (C) *H. melpomene burchelli*. (D) *H. melpomene nanna*. Landmark types I and II, and semi-landmarks are indicated by blue, green and yellow circles, respectively.

**Figure 8. Two first axes of the principal component analysis (PCA) on shape residuals for the whole forewing (left column) and red band (right column) of *Heliconius* mimic ring members, in comparison to males (blue circles) and females (red circles).** (A) *H. erato phyllis*. (B) *H. besckei*. (C) *H. melpomene burchelli*. (D) *H. melpomene nanna*. Percentage of shape variation explained by each PCA is given between parentheses. The shape deformations are showed next to each graph, where the dashed line represents the shape at minimum values and the solid line represents the shape at maximum values.

**Figure 9. Molecular analysis of *Heliconius erato phyllis* based on cytochrome oxidase c subunit I [COI] (left column) and *Optix* (right column) genes.** (A) Bayesian phylogeny reconstructed using *H. erato phyllis*, *H. besckei* and *H. melpomene*. Numbers above branches indicate Bayesian posterior probability support. Scale bars on each side are equal to 0.005 and 0.003 for *COI* and *Optix*, respectively. (B) Bayesian estimated admixture coefficients (i.e., population genetic structure). Each column (vertical line) corresponds to one individual. Ancestral populations are represented by different colors. Each column is colored with different colors in proportions; that is, the estimated admixture coefficients of the corresponding individuals. The sampling region (Southern, Central, and Northern) was separated by horizontal lines above the colored area. (C) Median-joining network describing the genealogical relationships between haplotypes among the corresponding regions. Nucleotide substitutions are shown on the branches as small transverse bars. Circle sizes are proportional to haplotype frequency. Small white circles represent median vectors. (D) Isolation by distance, inferred by comparison between pairwise geographical and genetic distances. Data for all individuals were plotted, and association between these two variables was verified by using a Mantel test, to which the corresponding *p*-values are assigned.

**Figure 10. Neighbour-joining trees based on distances in *Heliconius mimic ring* members.** (A) and (B). Mahalanobis distances for shape on the whole forewing and red band, respectively. (C) Genetic distances based on *p*-distance using ~2 kb of three concatenate loci (*cytochrome oxidase c* subunit I + *Optix* + *wingless*).

## TABLES

**Table 1.** Effect of sex on forewing whole size and on red band in *Heliconius* mimic ring members.

| Species                | Whole wing |       |          | Red band |       |          |
|------------------------|------------|-------|----------|----------|-------|----------|
|                        | <i>t</i>   | df    | <i>P</i> | <i>t</i> | df    | <i>P</i> |
| <i>H. e. phyllis</i>   | 0.46       | 57.18 | 0.65     | 0.30     | 52.82 | 0.77     |
| <i>H. besckei</i>      | 0.76       | 52.34 | 0.45     | -4.10    | 47.80 | 0.0002*  |
| <i>H. m. burchelli</i> | 0.39       | 32.86 | 0.70     | -2.00    | 40.40 | 0.05*    |
| <i>H. m. nanna</i>     | 0.39       | 48.31 | 0.70     | -3.22    | 50.28 | 0.002*   |

\* Significant value for Student's *t*-tests, alpha = 0.05.

**Table 2.** Comparison of allometric coefficients (regression analysis) within and between sexes in *Heliconius* mimic ring members.

| Species                | Sex                       | n  | Slope   |       |        | Intercept |        |          |
|------------------------|---------------------------|----|---------|-------|--------|-----------|--------|----------|
|                        |                           |    | $\beta$ | F     | P      | $\alpha$  | F      | P        |
| <i>H. e. phyllis</i>   | male vs. female<br>$\psi$ |    |         | 0.14  | 0.71   |           | 0.24   | 0.63     |
|                        | male $\emptyset$          | 31 | 0.60    | 7.06  | 0.010* | 1.13      | $\phi$ | $\phi$   |
|                        | female $\emptyset$        | 29 | 0.68    | 10.02 | 0.002* | 0.91      | $\phi$ | $\phi$   |
| <i>H. besckei</i>      | male vs. female           |    |         | 0.80  | 0.37   |           | 51.80  | <0.0001* |
|                        | male                      | 32 | 0.82    | 7.39  | 0.01*  | 0.47      | $\phi$ | $\phi$   |
|                        | female                    | 25 | 0.94    | 0.27  | 0.61   | 0.09      | 918.55 | <0.0001* |
| <i>H. m. burchelli</i> | male vs. female           |    |         | 0.97  | 0.33   |           | 9.40   | 0.003*   |
|                        | male                      | 34 | 0.95    | 0.12  | 0.73   | 0.11      | 164.81 | <0.0001* |
|                        | female                    | 24 | 1.09    | 1.03  | 0.31   | -0.33     | 378.01 | <0.0001* |
| <i>H. m. nanna</i>     | male vs. female           |    |         | 0.35  | 0.56   |           | 48.69  | <0.0001* |
|                        | male                      | 32 | 0.94    | 0.99  | 0.32   | 0.14      | 470.41 | <0.0001* |
|                        | female                    | 22 | 0.94    | 0.86  | 0.36   | 0.12      | 724.32 | <0.0001* |

( $\psi$ ) Comparison of allometric coefficients between males and females; ( $\emptyset$ ) Comparison of allometric coefficients in relation to their isometric line; ( $\phi$ ) Slope differ so much, that is not possible to test whether the intercept differs significantly; \* Significante  $p$  value, alpha = 0.05.

**Table 3.** MANOVA results for shape variation on the forewing in *Heliconius* mimic ring members, taking into account as separate groups the two subspecies of *H. melpomene* and the three geographical areas for *H. erato phyllis* (total = six groups, 229 individuals).

|            | Category     | $\lambda$ Wilks | F     | P       |
|------------|--------------|-----------------|-------|---------|
| Whole wing | Sex          | 0.408           | 7.11  | <0.001* |
|            | Groups       | 0.008           | 7.98  | <0.001* |
|            | Sex * Groups | 0.284           | 1.41  | 0.001*  |
| Red band   | Sex          | 0.506           | 4.78  | <0.001* |
|            | Groups       | <0.001          | 22.29 | <0.001* |
|            | Sex * Groups | 0.116           | 2.65  | <0.001* |

\* Significant  $p$ -value with Bonferroni's correction, alpha = 0.008.

**Table 4.** MANOVA results for sex effect on forewing shape in *Heliconius* mimic ring members.

|            | Species/subspecies     | $\lambda$ Wilks | F     | P       |
|------------|------------------------|-----------------|-------|---------|
| Whole wing |                        |                 |       |         |
|            | <i>H. besckei</i>      | 0.093           | 4.98  | <0.001* |
|            | <i>H. e. phyllis</i>   | 0.158           | 3.17  | 0.003*  |
|            | <i>H. m. burchelli</i> | 0.213           | 2.19  | 0.027*  |
|            | <i>H. m. nanna</i>     | 0.139           | 2.35  | 0.044*  |
| Red band   |                        |                 |       |         |
|            | <i>H. besckei</i>      | 0.134           | 4.18  | <0.001* |
|            | <i>H. e. phyllis</i>   | 0.153           | 2.63  | 0.013*  |
|            | <i>H. m. burchelli</i> | 0.227           | 2.50  | 0.010*  |
|            | <i>H. m. nanna</i>     | 0.877           | 9.399 | <0.001* |

\* Significant p-value, alpha = 0.05.

**Table 5.** Genetic variability in *Heliconius* mimic members based on 821 bp of *cytochrome oxidase c* subunit I (COI) gene and 782 bp of the *Optix* gene. The number of individual surveyed (N), and standard indices, such as number of variable sites (S), number of haplotypes (H), haplotype diversity (Hd), nucleotide diversity ( $\pi$ ), and neutrality tests (all non-significant  $\alpha = 0.05$ ) are shown.

| Loci         | Species   | N         | S (% total)    | H         | Hd               | $\pi$               | Neutrality test   |                    |                    |
|--------------|---|-----------|----------------|-----------|------------------|---------------------|-------------------|--------------------|--------------------|
|              |   |           |                |           |                  |                     | Tajima's <i>D</i> | Fu & Li's <i>D</i> | Fu & Li's <i>F</i> |
| <i>COI</i>   |   |           |                |           |                  |                     |                   |                    |                    |
|              | <i>H. besckei</i>   | 32        | 10 (1%)        | 9         | 0.77±0.05        | 0.002±0.0004        | -0.386            | -0.787             | -0.776             |
|              | <b><i>H. erato phyllis</i></b>                                | <b>52</b> | <b>56 (7%)</b> | <b>36</b> | <b>0.97±0.01</b> | <b>0.010±0.0010</b> | <b>-0.868</b>     | <b>-2.086</b>      | <b>-1.954</b>      |
|              | <i>H. melpomene</i><br>( <i>nanna</i> +<br><i>burchelli</i> ) | 25        | 24 (3%)        | 6         | 0.72±0.05        | 0.010±0.0010        | 1.626             | 1.417              | 1.736              |
| <i>Optix</i> |   |           |                |           |                  |                     |                   |                    |                    |
|              | <i>H. besckei</i>   | 5         | 3 (<1%)        | 5         | 0.75±0.01        | 0.001±0.0003        | 0.699             | 0.174              | 0.337              |
|              | <b><i>H. erato phyllis</i></b>                                | <b>20</b> | <b>9 (1%)</b>  | <b>21</b> | <b>0.95±0.01</b> | <b>0.003±0.0002</b> | <b>0.794</b>      | <b>0.733</b>       | <b>0.883</b>       |
|              | <i>H. melpomene</i><br>( <i>nanna</i> +<br><i>burchelli</i> ) | 6         | 18 (2%)        | 6         | 0.81±0.06        | 0.008±0.0010        | 0.839             | 1.010              | 1.095              |

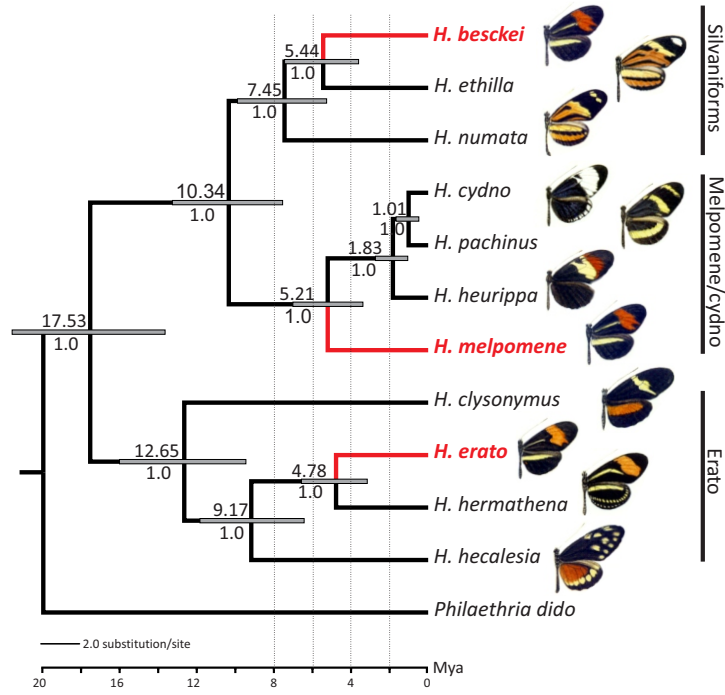


Fig 1- Rossato et al



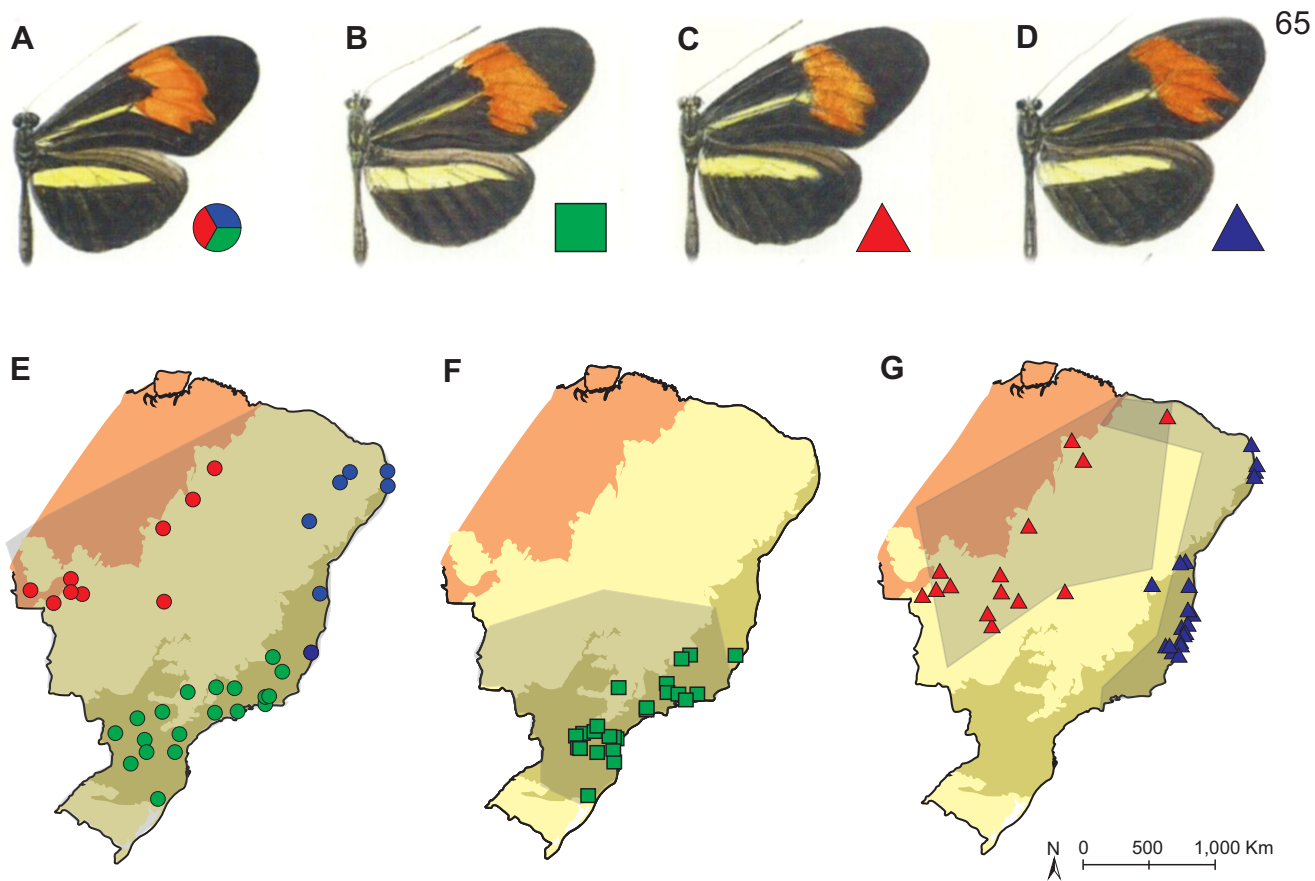


Fig 2- Rossato et al

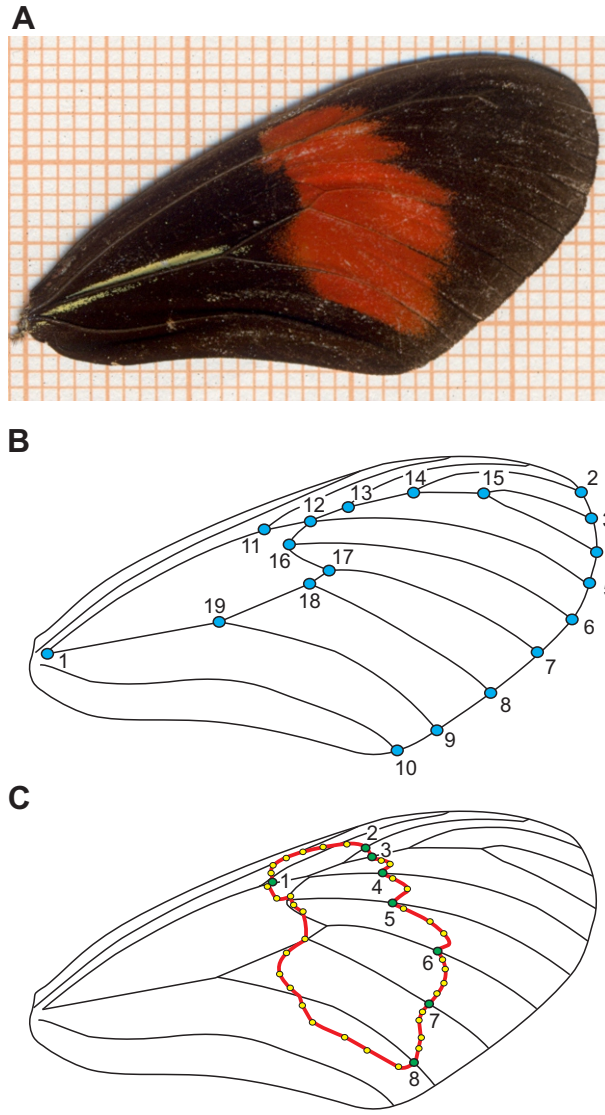


Fig 3- Rossato et al

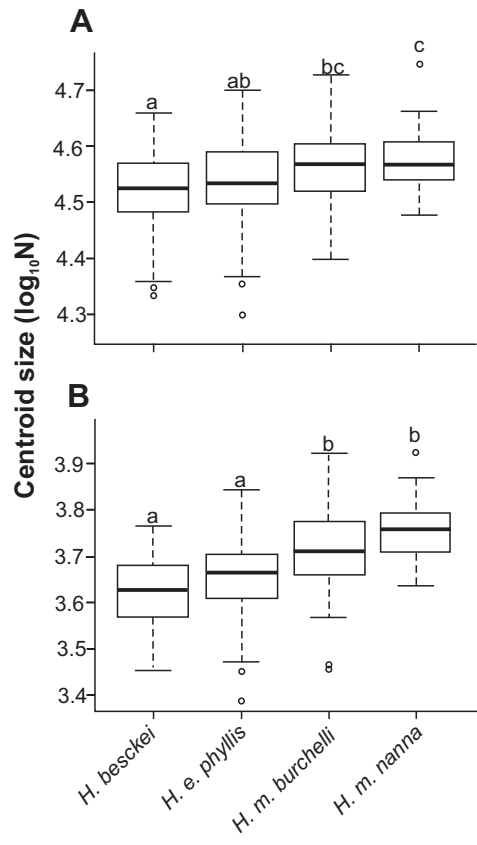


Fig 4- Rossato et al

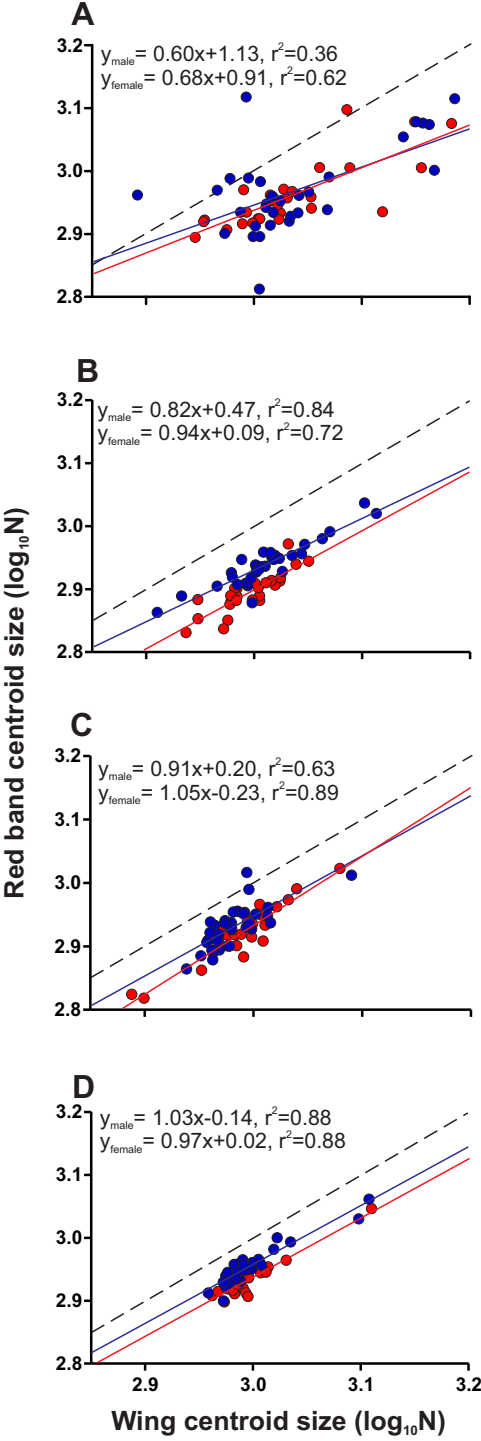


Fig 5- Rossato et al

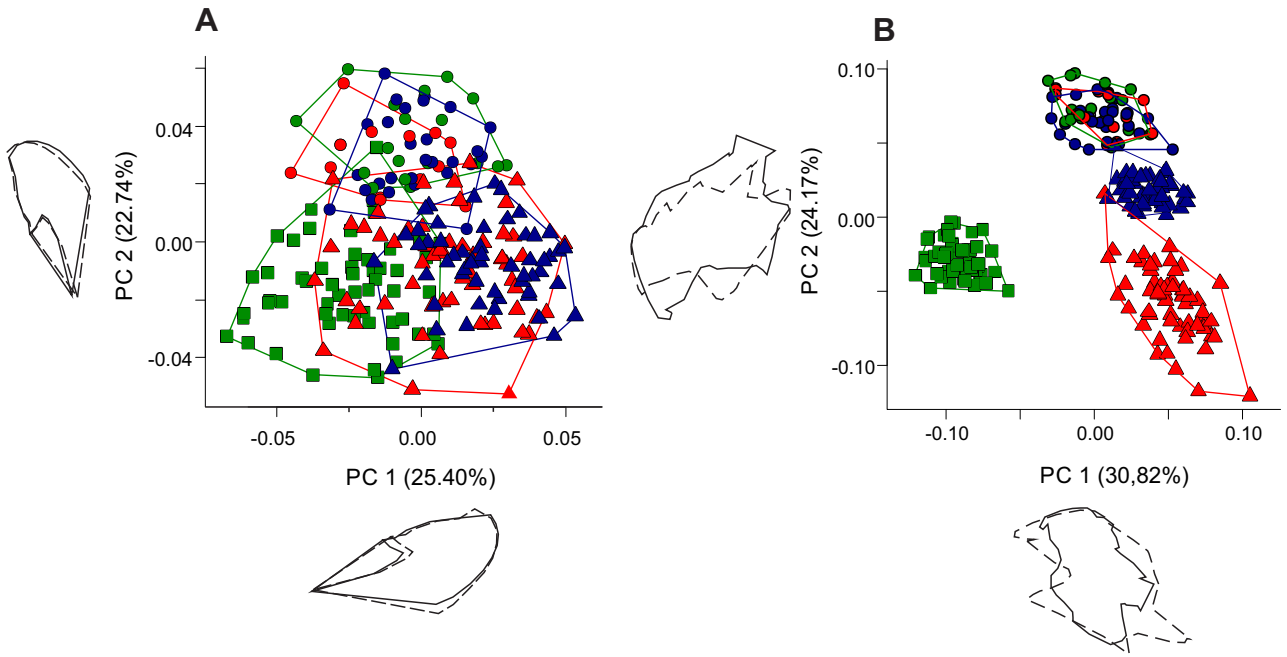


Fig 6- Rossato et al

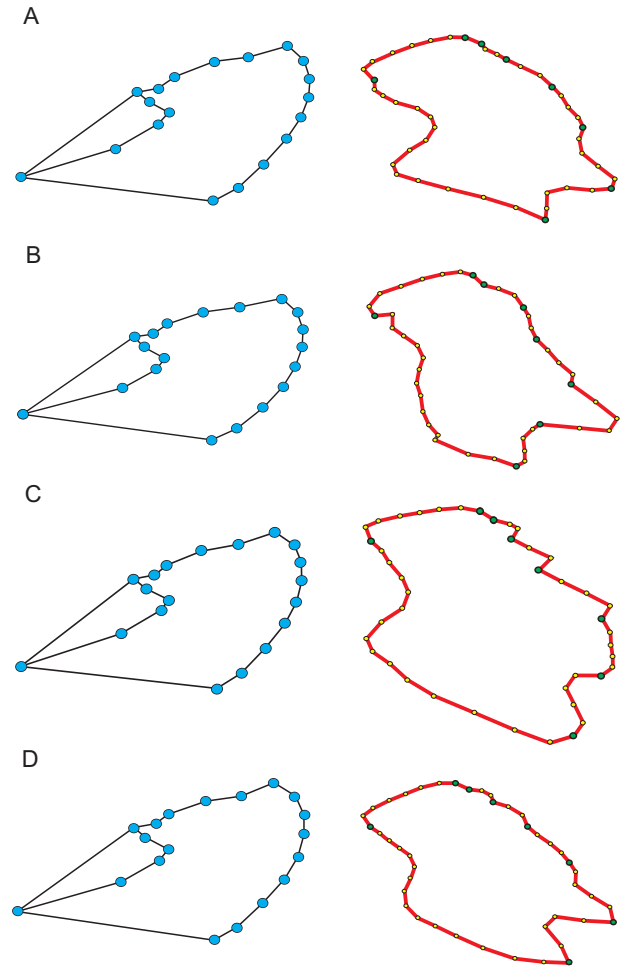


Fig 7- Rossato et al

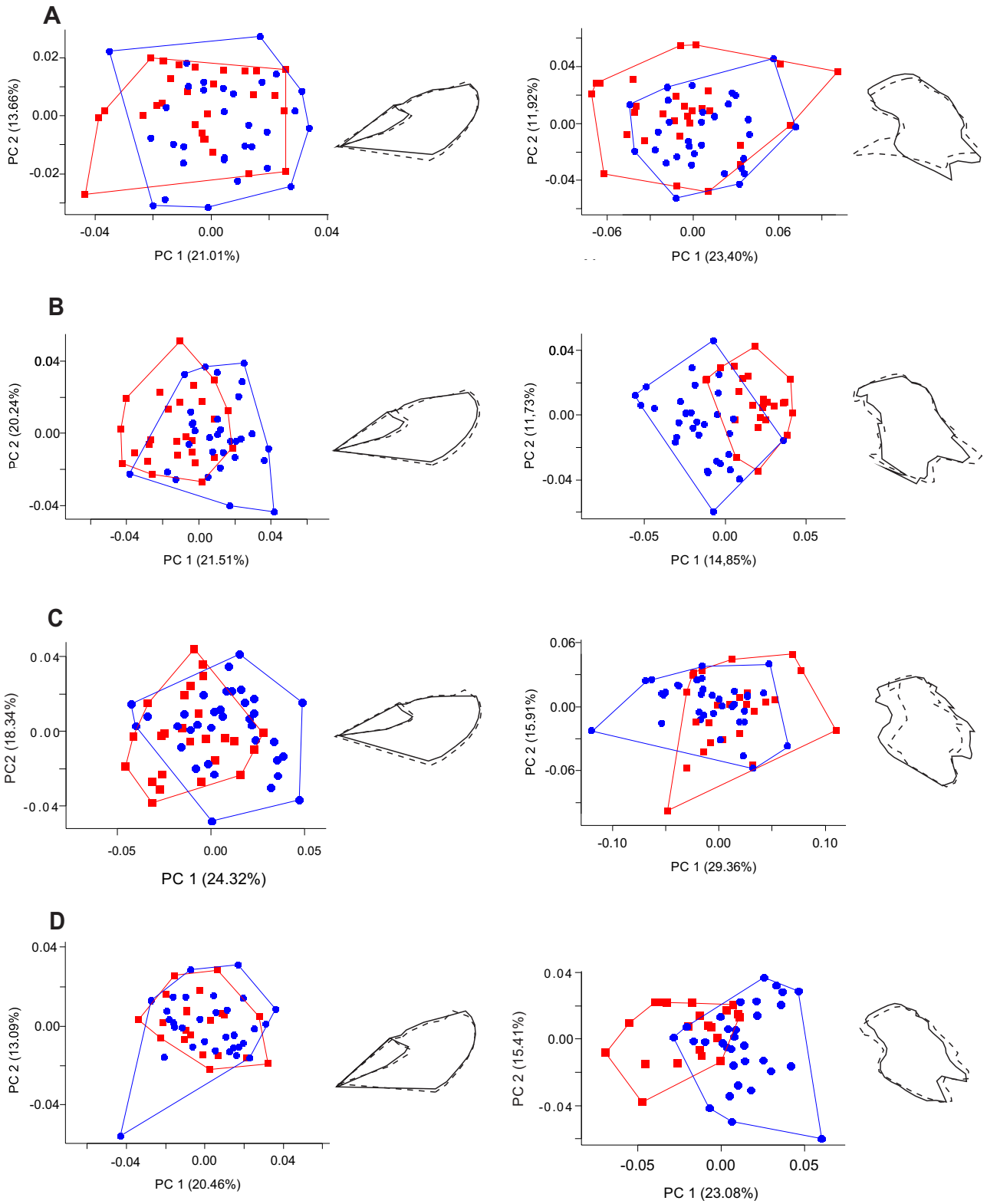
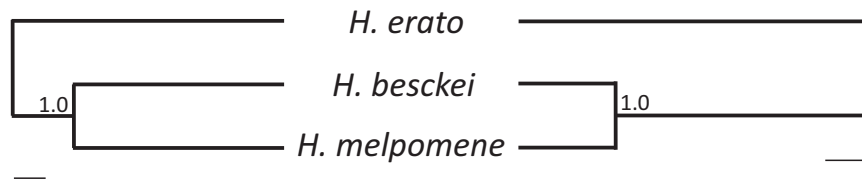
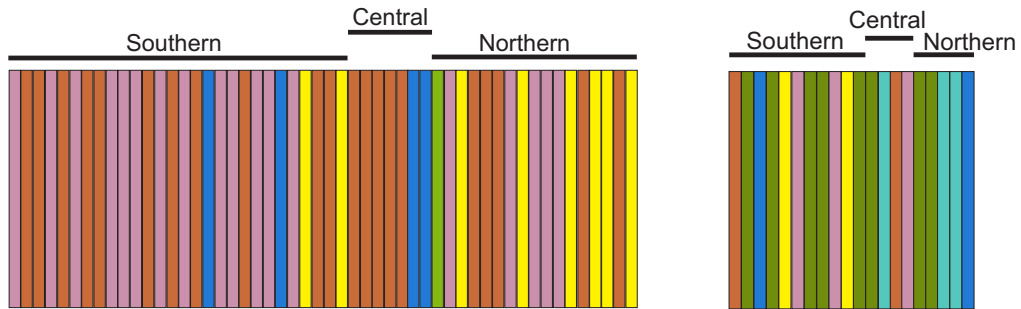


Fig 8- Rossato et al

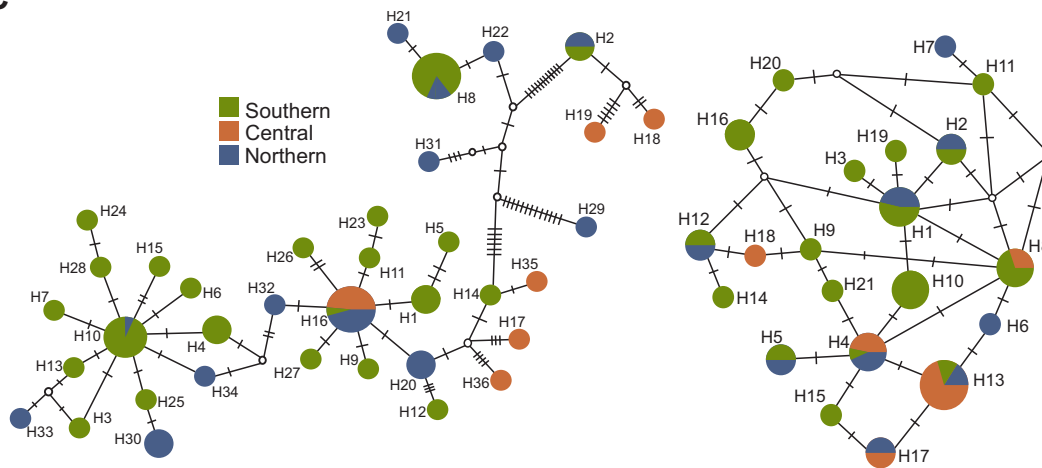
A



B



C



D

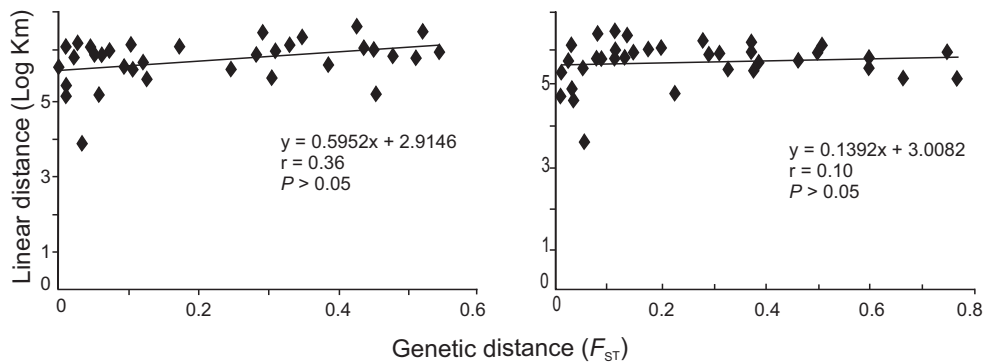


Fig 9- Rossato et al



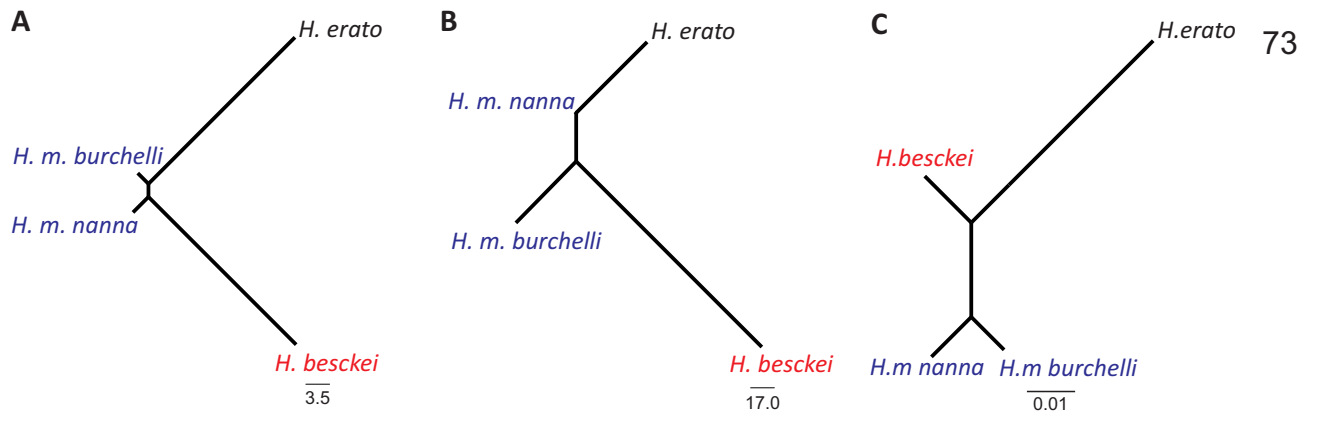


Fig 10- Rossato et al

## SUPPLEMENTARY MATERIALS

**Appendix S1.** *Heliconius* specimens used in morphometric and genetic analyses, listed per local and institution.

Specimens were obtained from the following collections: DZUP, Coleção Entomológica Pe. Jesus Moure, Universidade Federal do Paraná, Paraná, Brazil; LMCI, Coleção de Tecidos, Laboratório de Morfologia e Comportamento de Insetos, Universidade Federal do Rio Grande do Sul, Rio Grande do Sul, Brazil.  $\phi$  photographed geometric morphometric,  $\Psi$  used molecular analysis ( $\Psi^1 = \text{COI}$ ,  $\Psi^2 = \text{optix}$ ).

### a) *Heliconius erato phyllis*:

#### Bahia:

Camacan (Serra Bonita), 17-23.II.2013, Moreira GRM leg., 1♂ (LMCI 209-27  $\phi$   $\Psi^1$   $\Psi^2$ );

Senhor do Bonfim, 21.VI.1974, Laroca S leg., 1♂ (DZUP  $\phi$ ).

#### Ceará:

Ubajara, 23.X.2011, Kerpel & Ferreira leg., 2♂ (LMCI 183-02  $\Psi^1$ , LMCI 183-05  $\Psi^1$ ), 4♀ (LMCI 183-01  $\Psi^1$ , LMCI 183-03  $\Psi^1$   $\Psi^2$ , LMCI 183-04  $\Psi^1$ , LMCI 183-07  $\Psi^1$ ).

#### Espirito Santo:

Linhares, V.1978, Elias C leg., 1♀ (DZUP  $\phi$ );

Sooretama (ReBio), 28.II.-2.III.2010, Moreira GRP et al leg., 6♂ (LMCI 105-35  $\Psi^2$ , LMCI 105-36  $\Psi^1$ , LMCI 105-37  $\Psi^1$ , LMCI 105-38  $\Psi^1$ , LMCI 105-40  $\Psi^2$ , LMCI 105-41  $\Psi^2$ , LMCI 105-66  $\Psi^1$ , LMCI 105-67  $\Psi^1$ , LMCI 105-68  $\Psi^1$ ), 3♀ (LMCI 105-39  $\Psi^1$ , LMCI 105-40  $\Psi^1$ , LMCI 105-41  $\Psi^1$ ).

#### Goiás:

Goiás, VII.1976, XI.1976, Gifford D leg., 1♀, 1♂ (DZUP, Coll. Gifford,  $\phi$ );

Ilha do Bananal, 23.IX.1980, Gifford leg., 1♀, 1♂ (DZUP, Coll. Gifford,  $\phi$ );

Planaltina, 19-22.III.2013, Camargo AJA leg. 2♂ (LMCI 224-06  $\Psi^2$ , LMCI 224-10  $\Psi^1$ ), 1♀ (LMCI 224-01  $\Psi^1 \Psi^2$ ).

### **Maranhão:**

Feira Nova do Maranhão (Faz. Forquilha dos Brejos), 14-21.VIII.2011, Mielke O leg., 1♀ (DZUP  $\phi$ ).

### **Mato Grosso:**

Alto Xingu, 18.VII.1978, Gifford leg., 2♀♀ (DZUP  $\phi$ );

Alto Xingu, 18.VII.1978, Gifford leg., 1♂ (DZUP, Coll. Gifford,  $\phi$ );

Cáceres, 16.XI.1984, 19.XI.1984, Buzzi, Mielke, Elias & Casagrande leg., 2♀ (DZUP 'Proj. Polonoroeste'  $\phi$ );

Chapada dos Guimarães (Buriti), 700m, 23.XII.1968, 8.VIII.1973, 2♂♂ (DZUP, Coll. H Ebert 509 e 658,  $\phi$ );

Diamantino (Faz. S. João), 21.XI.1984, Mielke & Casagrande leg, 1♀ (DZUP  $\phi$ );

Diamantino (Faz. S. João, Rio Arinos, 300-400m), 1-8.VIII.1974, H & HD Ebert leg., 2♂♂ (DZUP, Coll. H Ebert,  $\phi$ ), (Hep116);

Jangada, 03.VI.2013, Rossato DO leg., 1♀ (LMCI 226-04  $\phi \Psi^1$ ), 3♂ (LMCI 226-01  $\Psi^1$ , LMCI 226-02  $\Psi^1$ , LMCI 226-03  $\phi \Psi^1$ );

Poconé, 04.VI.2013, Rossato DO leg., 1♀ (LMCI 227-01  $\Psi^1 \Psi^2$ );

Pontes e Lacerda (Fazenda Ouro Preto). 26.VII.2008, Queiroz & Silva leg, 1♂ (DZUP  $\phi$ ).

### **Minas Gerais:**

Caeté (Congo Soco), 20.XI.2009, Moreira GRP leg., 7♂♂ (LMCI 43-10  $\Psi^1$ , LMCI 43-12  $\Psi^1$ , LMCI 43-13  $\Psi^1$ , LMCI 43-14  $\Psi^2$ , LMCI 43-15  $\Psi^1$ , LMCI 43-16  $\Psi^1$ , LMCI 43-17  $\Psi^1$ , LMCI 43-19  $\Psi^1$ ), 1♀ (LMCI 43-14  $\Psi^1$ );

Cambuquira (900m), 19.IX.1969, Ebert H leg., 1♀ (DZUP, Coll. H Ebert 508,  $\phi$ );

Carangola (Fervedouro, 650m), 18.II.1972, Ebert leg., 1♀ (DZUP, Coll. H Ebert 954,  $\phi$ );

Marliéria (Parque Estadual do Rio Doce), 20.IX.1974, Gifford leg., 1♂ (DZUP  $\phi$ );

Poços de Caldas (100m), 22.IV.1967, 1♂ (DZUP, Coll. H Ebert 322,  $\phi$ ).

**Paraíba:**

João Pessoa, 14.XII.1952, Kesselring J leg., 1♂ (DZUP, Coll. Ebert 324, φ);

Patos, 27.VIII.2011, Kerpel S & Ferreira A leg., 1♀ (LMCI 181-02 φ), 1♂ (LMCI 181.01 φ);

Santa Teresinha, 05.VIII.2011, Kerpel S leg., 1♀ (LMCI 5180-02 φ), 1♂ (LMCI 180-06 φ).

**Paraná:**

Cascavel, 8.IX.1985, Mielke & Casagrande leg., 1♂ (DZUP φ);

Curitiba (Centro Politécnico), 31.IV.2009, Barão K leg., 2♀♀ (LMCI 54-16 φ Ψ<sup>1</sup>, LMCI 54-25 Ψ<sup>1</sup>), 5♂♂ (LMCI 54-18 φ, LMCI 54-21 Ψ<sup>1</sup>, LMCI 54-23 Ψ<sup>1</sup> Ψ<sup>2</sup>, LMCI 54-24 Ψ<sup>1</sup>, LMCI 54-25 Ψ<sup>2</sup>);

Fenix (300m), 23.XI.1986, Mielke & Casagrande leg., 1♂ (DZUP 'Profaupar' φ);

Guarapuava (Santa Clara, 650m), 21.XI.1986, Mielke & Casagrande leg., 1♂ (DZUP 'Profaupar' φ);

Jundiaí do Sul, 24.XI.1986, Mielke & Casagrande leg., 1♀ (DZUP 'Profaupar' φ);

Tunas do Paraná, 01.IV.2009, Moreira GRP leg., 1♀ (LMCI 52-60 φ Ψ<sup>1</sup> Ψ<sup>2</sup>), 1♂ (LMCI 52-59 φ Ψ<sup>1</sup> Ψ<sup>2</sup>).

**Pernambuco:**

Recife, 7.VII.1958, 23.VIII.1958, Ebert leg., 2♀♀ (DZUP, Coll. Ebert 327, 321 φ);

Recife (Camaragibe), 27.IV.1958, 1.IV.1961, H Ebert, 2♂♂ (DZUP, Coll. H Ebert '?', 328, φ).

**Rio de Janeiro:**

Petrópolis, 6.XII.1961, Gagarin leg., 1♂ (DZUP φ);

Petrópolis (Independência), 21.II.1934, Gagarin leg., 1♂ (DZUP, Coll. Gagarin, φ);

Rio de Janeiro (Lagoinha, 250m), 9.II.1952, Ebert H leg., 1♀ (DZUP, Coll. H Ebert 86, φ);

Rio de Janeiro, 15.V.1941, 1♀ (DZUP, Coll. Gagarin, φ), (Hep14);

Duque de Caxias (Imbariê, 25m), 20.XII.1955, Ebert H leg., 1♂ (DZUP, Coll. H Ebert 87, φ).

### **Rio Grande do Sul:**

São Francisco de Paula (FLONA), 20.IV.2006, Moreira GRP leg., 7♂♂ (LMCI 24-17 Ψ<sup>1</sup>, LMCI 24-18 Ψ<sup>1</sup>, LMCI 24-19 Ψ<sup>1</sup> Ψ<sup>2</sup>, LMCI 27-01 Ψ<sup>1</sup>, LMCI 27-02 φ Ψ<sup>1</sup>, LMCI 27-03 φ, LMCI 27-04 Ψ<sup>1</sup> Ψ<sup>2</sup>, LMCI 27-05 Ψ<sup>1</sup>).

### **Santa Catarina:**

Nova Teutônia (300-500m), II.1981, V.1981, Plaumann F leg., 2♀♀, 2♂♂ (DZUP φ);

São Bento do Sul, II.2010, Moreira GRP leg., 3♀♀ (LMCI 110-154 φ Ψ<sup>1</sup> Ψ<sup>2</sup>, LMCI 110-155 Ψ<sup>1</sup>, LMCI 110-156 Ψ<sup>1</sup> Ψ<sup>2</sup>), 4♂♂ (LMCI 110-157 φ Ψ<sup>1</sup> Ψ<sup>2</sup>, LMCI 110-158 Ψ<sup>1</sup> Ψ<sup>2</sup>, LMCI 110-159 Ψ<sup>1</sup>, LMCI 110-161 Ψ<sup>1</sup>).

### **São Paulo:**

Bocaina, 5.III.1966, Ebert H leg., 1♂ (DZUP, Coll. H Ebert 311, φ);

São Paulo, 29.IV.1971, Mielke leg., 1♀ (DZUP φ);

Ubatuba (80 m), 27.10.1962, '?' leg., 1♀ (DZUP, Coll. Ebert 329, φ).

### **Tocantins:**

Pedro Afonso, IX.1962, '?' leg., 1♀ (DZUP, Coll H. Ebert, φ).

**b) *Heliconius besckei***

**Espirito Santo:**

Santa Teresa, 1.IX.1966, 3.VII.1967, C & CT Elias leg., 2♀♀ (DZUP φ);

Santa Teresa (R. Lombardia), 1.VIII.1966, Mielke, Elias & Brown leg., 1♂ (DZUP φ).

**Minas Gerais:**

Brumadinho, 19.XI.2009, Moreira GRP leg., 1♀ (LMCI 90-01 φ Ψ<sup>1</sup>), 1♂ (LMCI 90-02 φ Ψ<sup>1</sup>);

Caeté (Gongo Soco), 20.XI.2009, Moreira GRP leg., 9♂♂ (LMCI 88-01 φ Ψ<sup>1</sup>, LMCI 88-02 φ Ψ<sup>1</sup>, LMCI 88-03 Ψ<sup>1</sup>, LMCI 88-04 Ψ<sup>1</sup>, LMCI 88-05 Ψ<sup>1</sup> Ψ<sup>2</sup>, LMCI 88-06 Ψ<sup>1</sup>, LMCI 88-07 Ψ<sup>1</sup>, LMCI 89-01 Ψ<sup>1</sup>, LMCI 89-02 Ψ<sup>1</sup>);

Cambuquira, 7.II.1985, Mielke & Casagrande leg., 1♀ (DZUP φ);

Carmo do Rio Claro, VIII.1948, Candido & '?' leg., 1♂ (DZUP, Coll. D'Almeida 6645, φ).

**Paraná:**

Alexandra (Paranagua), 3.IV.1970, Lauterjung M leg., 1♀ (DZUP φ);

Alexandra (Paranagua), 6.IX.1968, Mielke O leg., 2♂♂ (DZUP φ);

Castro, 15.XI.1971, Mielke & '?' leg., 1♀ (DZUP φ);

Curitiba (Cascatinha), 8.V.1966, 19.V.1966, 9.X.1966, 30.XI.1966, 29.I.1967, 1.XII.1967, Mielke O leg., 4♀♀, 7♂♂ (DZUP φ);

Curitiba (900m), 2.III.1968, Mielke O leg., 1♂ (DZUP φ);

Curitiba (Centro Politécnico), 31.III.2009, Mielke O leg., 1♀ (LMCI 53-14 Ψ<sup>1</sup>);

Guarapuava (1000m), 28.I.1972, Mielke & Schneider leg., 1♀ (DZUP φ);

Guarapuava (1200m), 3.II.1976, Mielke & Buzzi leg., 1♂ (DZUP φ);

Morretes (Marumbi), 13.V.1967, Mielke & Laroca leg., 1♀ (DZUP φ);

Morretes (Marumbi), 19.V.1966, Bianeli leg., 1♂ (DZUP φ);

Ponta Grossa (Lageado), III.1958, '?' leg., 1♀ (DZUP, Coll F Justus, φ);

Ponta Grossa, VI.1941, III.1958, '?' leg., 1♂, 2♀♀ (DZUP, Coll F Justus, φ);

Ponta Grossa (Vila Velha, 1000m), 5.II.1967, Mielke leg., 1♀ (DZUP φ);

Ponta Grossa (Piriquitos, 1000m), 21.IV.1967, Moura & Mielke leg. 1♂ (DZUP φ);

Prudentópolis (1200 m), 2.II.1976, Mielke & Buzzi leg., 1♀ (DZUP φ);

Tunas do Paraná, 15.IV. 2008, Moreira GRP leg., 6♂♂ (LMCI 22-05 Ψ<sup>1</sup>, LMCI 52-20 Ψ<sup>1</sup>, LMCI 52-21 Ψ<sup>1</sup>, LMCI 52-24 Ψ<sup>1</sup> Ψ<sup>2</sup>, LMCI 52-25 Ψ<sup>1</sup> Ψ<sup>2</sup>, LMCI 52-26 Ψ<sup>1</sup>).

### **Rio de Janeiro:**

Itatiaia (900m), 4.II.1936, '?' leg., 1♀ (DZUP, Coll. Gagarin, φ);

Itatiaia (800m), 26.II.1964, H Ebert leg., 1♀ (DZUP, Coll. H Ebert 434, φ);

Itatiaia (1400m), 20.XII.1957, '?' leg., 1♀ (DZUP, Coll. H Ebert 828, φ);

Mangaratiba (Sahy), 4.V.1932, '?' leg., 1♂ (DZUP, Coll. Ferr. D'Almeida 6651, φ);

Petrópolis (Independência, 900m), 18.X.1939, '?' leg., 1♀ (DZUP, Coll. Gagarin, φ).

### **Rio Grande do Sul:**

São Francisco de Paula, FLONA, 03.IV.2010, Millan C leg., 11♂♂ (LMCI 123-01 φ, LMCI 123-02 φ Ψ<sup>1</sup>, LMCI 24-11 Ψ<sup>1</sup>, LMCI 24-12 Ψ<sup>1</sup>, LMCI 24-13 Ψ<sup>1</sup>, LMCI 24-14 Ψ<sup>1</sup>, LMCI 27-08 Ψ<sup>1</sup>, LMCI 27-09 Ψ<sup>1</sup>, LMCI 27-10 Ψ<sup>1</sup>, LMCI 27-11 Ψ<sup>1</sup>, LMCI 123-3 Ψ<sup>1</sup>).

### **Santa Catarina:**

Brusque, 27-30.III.1970, Becker CF leg., 1♂ (DZUP φ);

Iraputã, 2.III.1937, '?' leg., 1♂ (DZUP, Coll. D'Almeida 6644, φ);

Joinville, 9.X.1971, 25.III.1978, Mielke O leg., 2♂♂ (DZUP φ);

Porto União, '?' leg., 1♀ (DZUP, Coll. D'Almeida 6647, φ);

São Bento do Sul, II.2010, Moreira GRP leg., 6♂♂ (LMCI 110-82 Ψ<sup>1</sup> Ψ<sup>2</sup>, LMCI 110-83 Ψ<sup>1</sup>, LMCI 110-84 Ψ<sup>1</sup>, LMCI 110-85 Ψ<sup>1</sup> Ψ<sup>2</sup>, LMCI 110-87 Ψ<sup>1</sup>), 1♀ (LMCI 110-86 Ψ<sup>1</sup>).

**São Paulo:**

Bocaina (Bananal), 8.I.1937, Travassos col., 1♂ (DZUP, Coll. D'Almeida 6650, φ);

São Paulo (Eldorado Represa Nova), 27.IV.1941, '?' leg., 1♀ (DZUP, Coll. D'Almeida 6643, φ);

São Paulo (capital), 3.VIII.1940, '?' leg., 1♀ (DZUP, Coll. D'Almeida 6646, φ);

São Paulo (Túnel da Mata Fria, 800m), 8.II.1985, Mielke & Casagrande leg., 1♀ (DZUP φ);

São Paulo (Ypiranga), VII.1941, '?' leg., 1♀ (DZUP, Coll. D'Almeida 6642, φ);

São Paulo (Cantareira), III.1934, L Trav F leg., 1♂ (DZUP, Coll. D'Almeida 6653, φ);

São Paulo (Ipiranga), 19.VIII.1943, '?' leg., 1♂ (DZUP, Coll. D'Almeida 6652, φ).



**c) *Heliconius melpomene burchelli***

**Ceará:**

Ubajara, 23.X.2011, Kerpel & Ferreira leg., 2♀ (LMCI 183-18 ♂, LMCI 183-13 ♀<sup>1</sup>), 7♂♂ (LMCI 183-10 ♂ ♀<sup>1</sup> ♀<sup>2</sup>, LMCI 183-11 ♀<sup>1</sup> ♀<sup>2</sup>, LMCI 183-12 ♀<sup>1</sup>, LMCI 183-14 ♀<sup>1</sup> ♀<sup>2</sup>, LMCI 183-15 ♀<sup>1</sup>, LMCI 183-16 ♀<sup>1</sup>).

**Distrito Federal:**

Brasilia (Esc. Fazendaria), 25.V.1977, Gifford D leg., 1♂ (DZUP, Coll. Gifford, ♂).

**Goiás:**

Ilha do Bananal, 23.IX.1980, Gifford leg., 3♀♀ (DZUP, Coll. Gifford, ♂);

Iporá (420m), 21.VI.1972, Mielke & Brown leg., 1♂ (DZUP ♂);

Mineiros (Parque das Emas), XI.1980, Gifford leg., 1♀ (DZUP, Coll. Gifford, ♂);

Planaltina, 19-22.III.2013, Camargo AJA leg., 2♂♂ (LMCI 224-22 ♀<sup>1</sup>, LMCI 224-23 ♀<sup>1</sup>).

**Maranhão:**

Feira Nova do Maranhão, 13-18.IV.2011, Mielke & Casagrande, 1♀ (LMCI 184-02 ♂ ♀<sup>1</sup>), 4♂♂ (LMCI 184-01 ♂ ♀<sup>1</sup>, LMCI 184-03 ♀<sup>1</sup>, LMCI 184-04 ♀<sup>1</sup>, LMCI 184-05 ♀<sup>1</sup>);

Imperatriz (Exc. Depto. Zool.), 13, 15, 22.XII.1972, 19.XII.1973, 4, 5, 6, 7, 12, VII.1974; 11♂♂, 3♀♀ (DZUP ♂).

**Mato Grosso:**

Alto Araguaia, 11.VIII.1924, Ebert leg., 1♀ (DZUP, Coll. H Ebert 566, ♂);

Alto Xingu (Porto Jacaré), VIII.1948, Candido J leg., 7♂♂, 2♀♀ (DZUP, Coll. D'Almeida 6654-6662, ♂);

Barra do Garça (São Felix), 25.VII.1968, Claudionor Elias leg., 1♀, 8 ♂♂ (DZUP ♂);

Barra do Garça (Vale dos Sonhos, 400m), 22.VI.1972, Mielke & Brown leg., 3♂♂ (DZUP ♂);

Barra do Garça (520m), Mielke & Brown leg., 1♂ (DZUP φ);

Cáceres, 19.XI.1984, Buzzi, Mielke, Elias & Casagrande leg., 1♀ (DZUP Proj. 'Polonoroeste' φ);

Chapada dos Guimarães (Buriti, 600m), 25.VI.1972, 26.VI.1972, Mielke & Brown leg., 3♀♀, 1♂ (DZUP φ);

Chapada dos Guimarães (Buriti, 700m), 22.XII.1968, 1♀ (DZUP, Coll. H. Ebert 494, φ);

Chapada dos Guimarães, 23.XI.1983, Buzzi, '?', Yamamoto & Hoffmann leg., 1♀ (DZUP φ);

Chapada dos Guimarães, 28.III-8.IV.1972, Elias C leg., 2♀♀ (DZUP Proj. 'Polonoroeste' φ);

Diamantino (Rio Arinos, Faz. S. João, 300-400m), 23.VII.1975, H & HD Ebert leg., 1♀ (DZUP, Coll. H Ebert 647, φ);

Nova Xavantina, 09.I.1977, 14.I.1977, Gifford leg., 2♀♀ (DZUP, Coll. Gifford, φ);

Nova Xavantina (Bacaba, UNIMAT), 17-19.VIII.1997, Mielke leg, 1♀ (DZUP φ).

**d) *Heliconius melpomene nanna***

**Bahia:**

Camacan (Serra Bonita), 17-23.II.2013, Moreira GRM leg., 1♀ (LMCI 209-26 φ Ψ<sup>1</sup> Ψ<sup>2</sup>), 1♂ (LMCI 209-25 φ Ψ<sup>12</sup>);

Itamaraju (100m), 20.VII.1977, Mielke, Moura & Elias leg., 1♂ (DZUP φ);

Itamari, 5.II.1971, Elias leg., 1♂ (DZUP φ);

Jitaúna (Rio das Contas, 150m), 26.III.1961, Ebert leg., 1♀ (DZUP φ);

Mucuri, 1.III.1971, Elias leg., 1♂ (DZUP φ);

Prado, 5.III.1971, Elias leg., 1♂ (DZUP φ);

São João do Paraíso, 22.VII.1977, Mielke, Moura & Elias leg., 1♀ (DZUP φ).

**Espirito Santo:**

Baixo Guandu, 11.II.1970, C & CT Elias leg., 1♀ (DZUP φ);

Colatina (Itapina), 23.XII.1967, Elias T leg., 1♂ (DZUP φ);

Conceição da Barra, 20.VI.1968, 04, 10.X.1968, 04.VII.1969, 04.X.1969, 17.XI.1969, I.1986, C & CT Elias leg., 5♂♂, 2♀♀ (DZUP φ);

Conceição da Barra, '?'.1982, Elias leg., 1♂ (DZUP φ);

Itapina, 23.XII.1967, Elias T leg., 1♀ (DZUP φ);

Jacaraípe, 5.II.1967, Elias leg., 1♀ (DZUP φ);

Linhares (R. Sooretama), 28, 30.VII.1966, Mielke & Brown leg., 1♀, 2♂♂ (DZUP φ);

Linhares, XI-XII.1965, Ma?er A leg., 1♀ (DZUP φ);

Linhares, V.1981, Elias C leg., 1♂ (DZUP φ);

Pedro Canário, 19.VII.1977, Mielke, Moura & Elias leg., 1♂ (DZUP φ);

Santa Teresa (750m), 25-29.III.1970, Ebert K leg., 1♀ (DZUP, Coll. H Ebert 951, φ);

Santa Teresa, 5.II.1967, 15.III.1967, 26.IX.1968, 11.VIII.1969, III.1971, C & CT Elias leg., 1♀, 9♂♂ (DZUP φ);

Santa Teresa, 10.IV.1966, Elias leg., 1♂ (DZUP φ);

São Mateus, X.1985, Elias leg., 2♀♀, 1♂ (DZUP φ);

Sooretama (ReBio), 28.II. -2.III.2010, GRPM et al leg., 8♂ (LMCI 105-13 Ψ<sup>1</sup> Ψ<sup>2</sup>, LMCI 105-14 Ψ<sup>1</sup>, LMCI 105-15 Ψ<sup>1</sup>, LMCI 105-16 Ψ<sup>1</sup>, LMCI 105-61 Ψ<sup>1</sup>, LMCI 105-62 Ψ<sup>1</sup> Ψ<sup>2</sup>, LMCI 105-63 Ψ<sup>1</sup> Ψ<sup>2</sup>, LMCI 105-64 Ψ<sup>1</sup>) 1♀ (LMCI 105-65 Ψ<sup>1</sup>).

### **Rio Grande do Norte:**

Natal (Parnamirin), 15.IX.1949, Alvarenga leg., 1♀ (DZUP, Coll. D'Almeida 6664, φ).

### **Minas Gerais:**

Aimorés, 4, 17.IV.1970, Elias C leg., 2♂♂ (DZUP φ).

### **Paraíba:**

João Pessoa, Clerot leg., 1♀ (DZUP, Coll. D'Almeida 12405, φ);

João Pessoa, 5.VII.1952, Diniz leg., 1♂ (DZUP, Coll. D'Almeida 11.346, φ);

Municipality '?', VI.1953, 1♀ (DZUP, Coll. Gagarin, φ);

Municipality '?', 24.VI.1953, J. Kesselring leg., 1♀ (DZUP, Coll. Gagarin, φ).

### **Pernambuco:**

Goiana, 7.XII.1954, H. Ebert leg, 1♀ (DZUP, Coll. H. Ebert 71, φ);

Recife (Camaragibe, 20-80m), 27.VII.1962, H Ebert leg., 1♀ (DZUP, Coll. H Ebert 428, φ);

São Lourenço da Mata (Tiuma, 100m), 9.VIII.1958, H Ebert leg, 1♀ (DZUP, Coll. H. Ebert 429, φ);

São Lourenço da Mata (Tiuma), 15.VII.1973, Mielke & Kesselring leg., ♂ (DZUP φ).

**Table S2.** Morphological definition of forewing landmark and semilandmark depicted in Figure 3.

|          | Mark     | Anatomic | Type                     | Description                                |
|----------|----------|----------|--------------------------|--|
| Forewing | 1        |          | Land I                   | Humeral vein (hv) basis                    |
|          | 2        |          | Land I                   | Radial 3 (R3) distal point                 |
|          | 3        |          | Land I                   | Radial 4 (R4) distal point                 |
|          | 4        |          | Land I                   | Radial 5 (R5) distal point                 |
|          | 5        |          | Land I                   | Media 1 (M1) distal point                  |
|          | 6        |          | Land I                   | Media 2 (M2) distal point                  |
|          | 7        |          | Land I                   | Media 3 (M3) distal point                  |
|          | 8        |          | Land I                   | Cubital anterior 1 (CuA1) distal point     |
|          | 9        |          | Land I                   | Cubital anterior 2 (CuA2) distal point     |
|          | 10       |          | Land I                   | Anal 1 (1A) distal point                   |
|          | 11       |          | Land I                   | Radial 1 (R1) proximal point               |
|          | 12       |          | Land I                   | Media 1 (M1) proximal point                |
|          | 13       |          | Land I                   | Radial 2 (R2) proximal point               |
|          | 14       |          | Land I                   | Radial 3 (R3) proximal point               |
|          | 15       |          | Land I                   | Radial 4-5 (R4 and R5) proximal point      |
|          | 16       |          | Land I                   | Media 2 (M2) proximal point                |
|          | 17       |          | Land I                   | Media 3 (M3) proximal point                |
|          | 18       |          | Land I                   | Cubital anterior 1 (CuA1) proximal point   |
|          | 19       |          | Land I                   | Cubital anterior 2 A (CuA2) proximal point |
| Red band | 1        |          | Land II                  | Radial 1 (R1)                              |
|          | 2        |          | Land II                  | Radial 2 (R2)                              |
|          | 3        |          | Land II                  | Radial 3 (R3)                              |
|          | 4        |          | Land II                  | Media 1 (M1)                               |
|          | 5        |          | Land II                  | Media 2 (M2)                               |
|          | 6        |          | Land II                  | Média 3 (M3)                               |
|          | 7        |          | Land II                  | Cubital anterior 1 (CuA1)                  |
|          | 8        |          | Land II                  | Cubital anterior 2 (CuA2)                  |
|          | 9 to 14  |          | Semi                     | between landmark 1 and 2                   |
|          | 15 to 16 |          | Semi                     | between landmark 3 and 4                   |
|          | 17 to 18 |          | Semi                     | between landmark 4 and 5                   |
|          | 19 to 21 |          | Semi                     | between landmark 5 and 6                   |
|          | 22 to 25 |          | Semi                     | between landmark 6 and 7                   |
|          | 26 to 29 |          | Semi                     | between landmark 7 and 8                   |
| 30 to 43 |          | Semi     | between landmark 8 and 1 |  |

Land I = Landmark type I, Land II = Landmark type II, Semi = Semi landmark.



**Table S4.** *Optix* gene haplotypes found in *Heliconius erato phyllis*.

| #   | Genbank<br>Accession<br>Number | Nucleotide site |   |   |   |   |   |   |   |   |   |
|-----|--------------------------------|-----------------|---|---|---|---|---|---|---|---|---|
|     |                                | 2               | 3 | 4 | 4 | 5 | 5 | 5 | 6 | 6 | 6 |
|     |                                | 7               | 1 | 1 | 2 | 0 | 1 | 9 | 4 | 8 |   |
|     |                                | 0               | 4 | 7 | 0 | 1 | 0 | 1 | 2 | 1 |   |
| H1  |                                | C               | A | G | C | C | C | C | G | G |   |
| H2  |                                | .               | . | . | . | T | . | . | . | . |   |
| H3  |                                | .               | C | . | . | . | . | . | . | . |   |
| H4  |                                | .               | . | . | . | . | . | T | A | . |   |
| H5  |                                | T               | . | . | . | . | . | T | A | . |   |
| H6  |                                | .               | . | . | . | . | . | . | A | C |   |
| H7  |                                | T               | . | . | . | T | . | . | A | C |   |
| H8  |                                | .               | . | . | . | . | . | . | A | . |   |
| H9  |                                | .               | . | C | . | . | . | . | A | . |   |
| H10 |                                | .               | . | . | . | . | . | T | . | . |   |
| H11 |                                | T               | . | . | . | T | . | . | A | . |   |
| H12 |                                | .               | . | C | . | . | T | . | . | . |   |
| H13 |                                | .               | . | . | . | . | . | T | A | C |   |
| H14 |                                | .               | . | C | . | . | T | . | . | C |   |
| H15 |                                | .               | . | . | A | . | . | T | A | . |   |
| H16 |                                | T               | . | C | . | . | . | . | . | . |   |
| H17 |                                | .               | . | . | A | . | . | T | A | C |   |
| H18 |                                | .               | . | C | . | . | T | . | A | . |   |
| H19 |                                | .               | . | . | A | . | . | . | . | . |   |
| H20 |                                | T               | . | C | . | T | . | . | . | . |   |
| H21 |                                | .               | . | C | . | . | . | T | A | . |   |

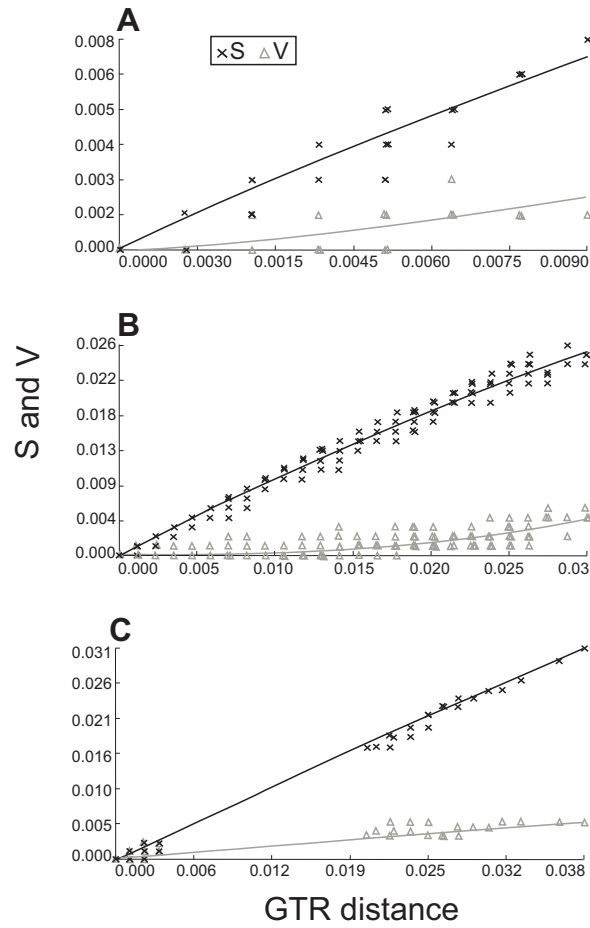
**Table S5.** *Cytochrome oxidase c subunit I* (COI) gene haplotypes found in *Heliconius besckei*.

|    | Genbank<br>Accession<br># Number | Nucleotide site |   |   |   |   |   |   |   |   |   |
|----|----------------------------------|-----------------|---|---|---|---|---|---|---|---|---|
|    |                                  | 2               | 2 | 5 | 5 | 5 | 6 | 7 | 7 | 7 |   |
|    |                                  | 6               | 2 | 8 | 4 | 8 | 9 | 0 | 4 | 5 | 6 |
|    |                                  | 3               | 8 | 8 | 3 | 8 | 7 | 6 | 2 | 9 | 2 |
| H1 |                                  | T               | A | A | T | C | T | C | C | A | C |
| H2 |                                  | C               | . | T | C | . | C | T | . | . | . |
| H3 |                                  | C               | G | T | C | . | C | T | . | . | . |
| H4 |                                  | C               | . | . | C | . | . | . | . | . | . |
| H5 |                                  | C               | . | . | C | . | . | . | . | G | . |
| H6 |                                  | C               | . | . | C | A | . | . | . | . | . |
| H7 |                                  | C               | . | . | C | . | . | . | . | . | T |
| H8 |                                  | C               | . | . | . | . | . | . | . | . | . |
| H9 |                                  | .               | . | . | . | . | . | . | T | . | . |

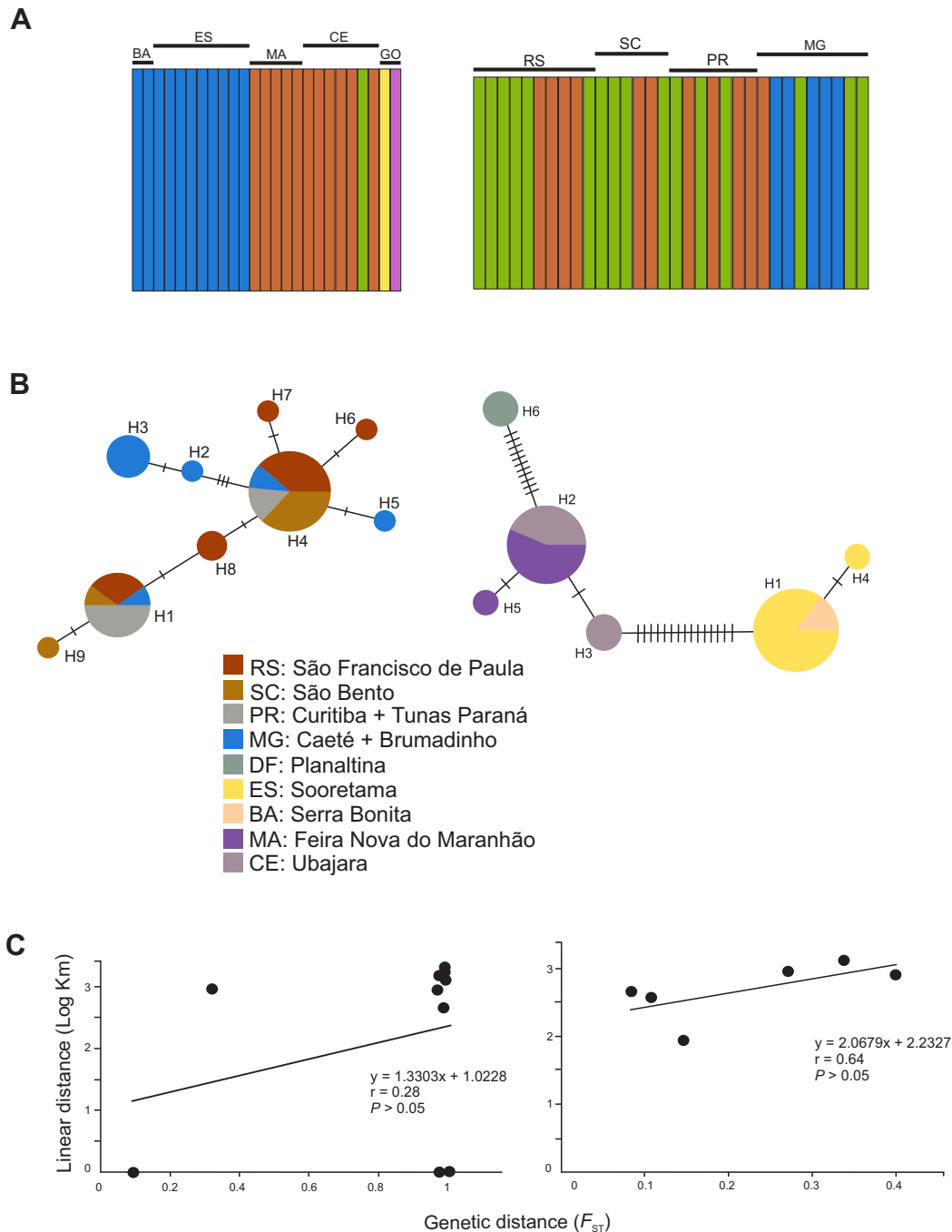


**Table S6.** *Cytochrome oxidase c subunit I (COI) gene haplotypes found in *Heliconius melpomene*.*

| #  | Genbank<br>Accession<br>Number | Nucleotide site |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|----|--------------------------------|-----------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|    |                                | 1               | 1 | 1 | 1 | 1 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 5 | 5 | 5 | 5 | 5 | 5 | 6 | 6 | 6 | 6 | 7 | 7 | 7 |
|    |                                | 5               | 2 | 2 | 3 | 5 | 9 | 1 | 5 | 0 | 2 | 3 | 6 | 8 | 9 | 2 | 3 | 8 | 9 | 2 | 5 | 5 | 7 | 8 | 0 | 0 | 9 | 9 | 0 | 1 | 4 | 6 |
|    |                                | 4               | 3 | 9 | 5 | 6 | 8 | 9 | 5 | 6 | 1 | 0 | 0 | 1 | 0 | 6 | 8 | 0 | 8 | 5 | 5 | 8 | 3 | 8 | 6 | 9 | 3 | 9 | 2 | 1 | 2 | 2 |
| H1 |                                | C               | A | A | T | C | A | C | C | G | C | G | T | T | C | G | T | T | C | C | C | T | G | A | C | T | T | A | A | T | T | T |
| H2 |                                | T               | G | G | C | . | . | T | T | A | T | A | C | C | T | A | C | C | . | . | T | . | A | T | T | A | . | . | T | . | C | . |
| H3 |                                | T               | G | G | C | . | . | T | T | A | T | A | C | C | T | A | C | C | . | . | T | . | . | T | T | A | . | . | T | . | C | . |
| H4 |                                | .               | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | . | . | . | . | . | . | . | . |
| H5 |                                | T               | G | G | C | . | . | T | T | A | T | A | C | C | T | A | C | C | . | . | T | . | A | T | T | A | . | . | T | . | C | . |
| H6 |                                | ?               | ? | . | . | T | T | . | T | . | T | A | C | . | T | A | C | C | T | A | T | C | A | C | T | A | C | G | . | C | C | C |



**Figure S1.** Saturation plots for GTR distances in relation to transitions (S) and transversions (V). Data correspond to 821 bp of the CO-I gene regarding to *H. besckei* (A), *H. erato phyllis* (B) and *H. melpomene* (C).



**Figure S2.** Molecular analysis of *Heliconius besckei* and *H. melpomene* based on cytochrome oxidase c subunit I [COI] (left column) and Optix (right column) genes. (A) Bayesian estimated admixture coefficients (i.e., population genetic structure). Each column (vertical lines) corresponds to one individual. Ancestral populations are represented by different colors. Each column is colored with different colors in proportions corresponding to estimated admixture coefficients of the corresponding individual. The sampling populations were separated by horizontal lines above the colored area. (B) Median-joining network describing the genealogical relationship between haplotypes of populations sampled. Nucleotide substitutions are shown on the branches as small transverse bars. Circle sizes are proportional to haplotype frequency. Small white circles represent median vectors. (C) Isolation by distance, inferred by comparison of pairwise geographical and genetic distances. Data for all individuals were plotted, and the association between these two variables was verified by using a Mantel test, to which the corresponding p-values are assigned.

## Considerações Finais

A seleção do padrão de coloração no anel mimético formado por *Heliconius erato phyllis*, *H. besckei*, *H. melpomene burchelli* e *H. melpomene nanna* não ocorre em uma escala refinada, como aquela da morfometria geométrica, ferramenta bastante sensível a variação na forma. Podemos observar que as espécies mantiveram a sua forma padrão diferenciada, principalmente quanto à mancha vermelha da asa anterior, mesmo quando em simpatria. Assim, na escala de abordagem deste estudo, podemos afirmar que não somente a seleção natural está envolvida na evolução do padrão de coloração envolvido no anel mimético avaliado.

Para o tamanho da asa, encontramos valores significativamente diferentes, mas com ampla sobreposição tanto em nível específico quanto subespecífico. Quanto à alometria, foram obtidas relações isométricas entre asa e mancha vermelha para ambas subespécies de *H. melpomene* e, hipoalometria, para as demais, sendo maior em *H. erato phyllis*. Assim, nestas últimas, inferimos que o investimento na mancha é proporcionalmente maior em indivíduos menores, indicando existir um limiar de tamanho quanto ao reconhecimento dessa estrutura pelos predadores.

A forma da mancha vermelha, e não da asa em si, foi a característica mais importante quanto ao dimorfismo sexual obtido, indicando fortemente a existência de seleção sexual a respeito.

Estudos considerando anéis miméticos em especial, a cerca de como esse padrão morfológico e de coloração se mantem e evolui em cada espécie tem instigado diversas pesquisas por séculos. Os resultados aqui gerados em nível de detalhamento refinado quanto à variação morfológica da asa e da mancha vermelha em espécies miméticas são inéditos. Eles refutam a seleção natural por predação como hipótese única para a evolução correspondente, com base na ausência de reforço adaptativo ao mimetismo Mulleriano em áreas de simpatria neste anel mimético. Além disso, a análise molecular demonstrou a ocorrência de fluxo gênico; ou seja, de que há troca de material genético entre as diferentes populações de *H. erato phyllis*, as quais não são estruturadas geograficamente.

Assim, esta dissertação traz a discussão sobre o tema no sentido de questionar o real papel do mimetismo Mulleriano quando a similaridade morfológica e a influência de outros fatores sob a manutenção das características mantidas. Sugerimos que a seleção sexual possa estar atuando não somente em nível interespecífico, mas também na seleção de um padrão diferenciado para machos e

fêmeas em cada espécie, o que dever ser testado experimentalmente no futuro.

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