



*Biological Journal of the Linnean Society*, 2012, **106**, 540–560. With 7 figures

## Ecologically relevant cryptic species in the highly polymorphic Amazonian butterfly *Mechanitis mazaeus* s.l. (Lepidoptera: Nymphalidae; Ithomiini)

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Received 1 September 2011; revised 1 December 2011; accepted for publication 1 December 2011

The understanding of mimicry has relied on a strong biosystematic framework ever since early naturalists first recognized this textbook example of natural selection. We follow in this tradition, applying new biosystematics information to resolve problems in an especially difficult genus of tropical butterflies. *Mechanitis* species are important components of Neotropical mimetic communities. However, their colour pattern variability has presented challenges for systematists, and has made it difficult to study the very mimicry they so nicely illustrate. The South American *Mechanitis mazaeus* and relatives have remained particularly intractable. Recent systematists have recognized one highly polytypic species, whereas earlier work recognized the melanic Andean foothill races as a distinct species: *Mechanitis messenoides*. Recent molecular evidence suggests *M. mazaeus* and *M. messenoides* are genetically well differentiated, but evidence of morphological and ecological differences indicative of separate species was still lacking. Thus, it remains to be conclusively demonstrated whether this is an extreme case of a polymorphic mimetic species, or whether distinct co-mimetic lineages are involved. Here we provide evidence that *M. mazaeus* and *M. messenoides* are ecologically distinct and identify consistent morphological differences in both adult and immature stages. These ecological and morphological differences are correlated with mitochondrial sequence data. In spite of some overlap in almost all traits, wing shape, adult colour pattern, and larval colour pattern differ between the two species, in addition to clutch size and larval host use in local sympatry. Although three well-differentiated mitochondrial DNA (mtDNA) haplogroups were identified within these two species, one for *M. mazaeus* and two within *M. messenoides*, no morphological or ecological differences were found between two mtDNA haplogroups, both of which appear to belong to *M. messenoides*. We conclude that *M. mazaeus* and *M. messenoides* are distinct although highly polymorphic species, each with multiple sympatric co-mimetic forms, and suggest that further work is needed to clarify the identity of other phenotypes and subspecies of *Mechanitis*. © 2012 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2012, **106**, 540–560.

ADDITIONAL KEYWORDS: Amazonia – biodiversity – DNA barcoding – host plant – integrative taxonomy – Müllerian mimicry – mitochondrial DNA – Neotropics – *Solanum*.

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## INTRODUCTION

Forbes (1948: 16) wrote about the *Mechanitis mazaeus* complex: ‘... while variation is chiefly racial, there is always among specimens with the normal coloring of any race, a proportion far from their proper area, especially in the case of the more striking types, so that the distinction of race and dimorphic form becomes nearly meaningless’.

Since its inception, investigations into mimicry have relied strongly on a sound systematic foundation for diagnosing convergent wing pattern evolution. Bates (1862) recognized mimicry between pierid and ithomiine butterflies because of differences in morphology between butterfly families: pierids walk on six legs whereas ithomiines, like all nymphalids, use only four. Müller (1879) also recognized convergent wing patterns between different lineages of danaine butterflies, diagnosed by wing venation and androconial structures. In addition to these iconic examples, Eltringham (1916) combined data from morphology, behaviour, and immature stages, to clarify relationships among *Heliconius* species (especially the co-mimics *Heliconius erato* and *Heliconius melpomene*). Contemporary studies integrating multiple lines of evidence, including molecular, morphological, life-history, and ecological data (Will, Mishler & Wheeler, 2005; Schlick-Steiner *et al.*, 2010), provide the best approaches for resolving species identity in cases involving strong convergent evolution (such as mimicry).

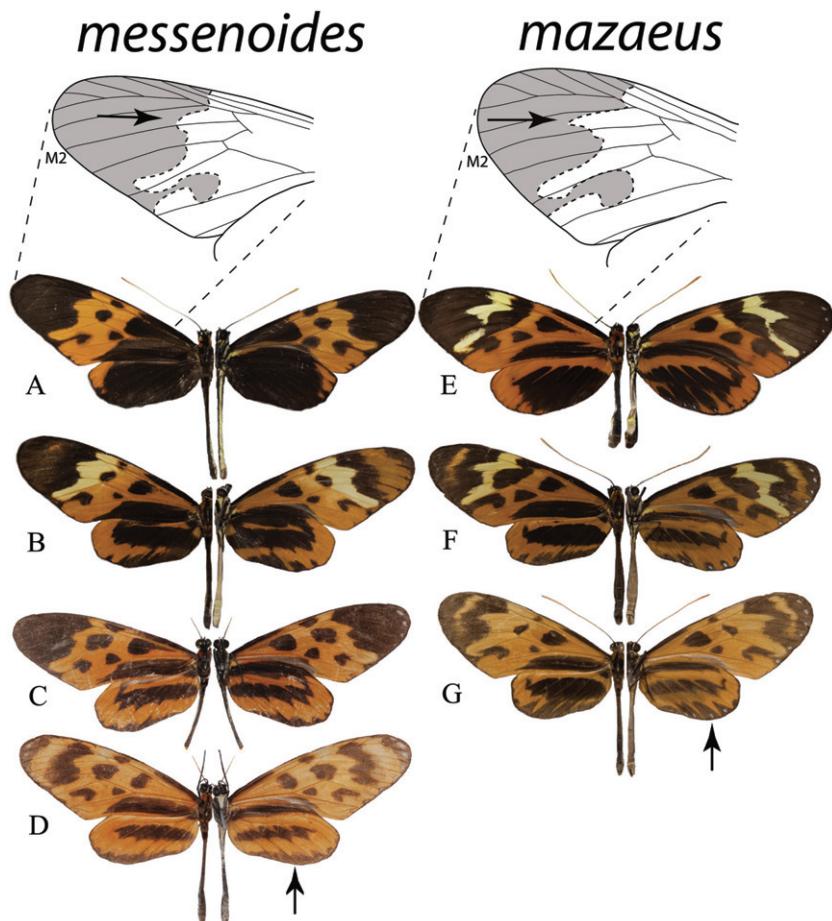
Butterflies in the genus *Mechanitis* are some of the most abundant and widespread Neotropical butterflies (Brown, 1977, 1979). *Mechanitis* species are unpalatable (Brown, 1984, 1985), and bear warning black, orange, and yellow ('tiger') wing patterns. Their extraordinary abundance and ubiquity suggests that they are important members of mimetic communities, probably serving as mimics of larger ithomiines, such as *Melinaea*, as well as models for less abundant ithomiines, and mimetic species in other groups of Lepidoptera (e.g. Heliconiinae, Nymphalinae, and Pericopinae; Bates, 1862; Brown & Benson, 1974; Beccaloni, 1997). In addition, they have been implicated in spatiotemporal dynamics in mimetic communities, and in maintaining local and regional mimetic polymorphism in taxa such as *Heliconius numata* (Brown & Benson, 1974). Given the likely significance of *Mechanitis* species in mimetic communities, a strong systematic foundation for the genus is important for further studies of the ecology and evolution of mimicry (e.g. Elias *et al.*, 2008; Hill, 2010).

The genus *Mechanitis* has a reputation for being taxonomically 'difficult' (Fox, 1967), and the *M. mazaeus* species group has been particularly challenging. *Mechanitis mazaeus* occurs in hyper-variable populations, including between five and seven sympatric forms in parts of Ecuador and

Peru (Fig. 1). Two opposing views are apparent in later revisions of *M. mazaeus*. Several authors [D'Almeida, (1951, 1978), Fox (1967), D'Abrera (1984), Beccaloni (1997), and Neild (2008)] concluded that at least some sympatric forms of Forbes' *M. mazaeus* represent separate species, in particular several Andean and west Amazonian phenotypes, referred to as *Mechanitis messenoides*. On the other hand, although Brown (1977: 188) agreed that '*mazaeus*-related phenotypes occur in highly polymorphic populations', his conclusions were similar to those applying to *H. numata* (Brown & Benson, 1974; Joron *et al.*, 1999; Joron & Iwasa, 2005). Brown (1977) regarded local polymorphisms in *M. mazaeus* to be a result of dispersal from nearby Pleistocene refuge areas, coupled with predator selection for matching different models.

These taxonomic arrangements represent two opposing hypotheses for the *M. mazaeus* group that relate to mimicry, speciation, and evolution. Has selection for mimicry facilitated speciation, resulting in multiple sympatric species? Or is *M. mazaeus* a hypervariable species with sympatric morphs maintained by gene flow and selection to mimic multiple model species? Fox, D'Almeida, and Brown all admitted the potential shortcomings of using colour pattern to delimit species in *Mechanitis* – mimetic similarity makes species boundaries difficult to determine in a group where structural morphological characters (e.g. genitalia) are not useful (Forbes, 1924; D'Almeida, 1951; Fox, 1967; K. Willmott, pers. observ.). Thus, additional types of data are needed to answer these questions.

In a paper related to the present study, Dasmahapatra *et al.* (2010a) identified three divergent mitochondrial haplogroups among *M. mazaeus* (*sensu* Lamas, 2004): one group with the *M. mazaeus* phenotype and two separate groups with the *M. messenoides* phenotype. Genome-wide amplified fragment length polymorphism (AFLP) markers indicated significant differentiation between sympatric *M. mazaeus* and *M. messenoides* lineages, but not among the two *M. messenoides* haplogroups (Dasmahapatra *et al.*, 2010a). This indicates *M. messenoides* is likely to be a separate species, supporting the conclusions of several earlier authors. Yet morphological and ecological differences between *M. mazaeus* and *M. messenoides*, the usual hallmarks of butterfly species, have not been documented. Recent systematic work on ithomiines has made use of characters from both adult and juvenile stages, as well as DNA sequences, to resolve higher relationships (Brower *et al.*, 2006; Willmott & Freitas, 2006). However, no studies have integrated life history, morphology, and DNA sequence data to clarify species boundaries in ithomiines.



**Figure 1.** Sympatric colour pattern forms of *Mechanitis mazaeus* and *Mechanitis messenoides* found in eastern Ecuador and Peru. The wing colour pattern traits useful for distinguishing the species are indicated with black arrows: (1) forewing postmedial band yellow/orange colour produced in cell M1 in *M. mazaeus*; (2) white spots in ventral hindwing in *M. mazaeus*. Seven individuals are illustrated with dorsal views on the left and ventral views on the right: A, typical male *Mechanitis messenoides deceptus*; B, male *Mechanitis messenoides messenoides* with reduced dark area in hindwing, most individuals of this morph have hindwing dark, similar to A; C, male *M. messenoides* individual resembling *M. mazaeus mazaeus* ('nigroapicalis' phenotype) with reduced dark in hindwing and paler orange; D, male *M. messenoides* individual resembling *M. mazaeus fallax* ('phasianita' phenotype) with extensive coloration in the forewing apex, individuals of this form show variation in the extent of forewing apex coloration and the extent of black in the hindwing; E, female *M. mazaeus fallax* with extensive dark in hindwing ('fallax' phenotype); F, male *M. mazaeus fallax* ('elevata' phenotype); G, male *M. mazaeus mazaeus*, lacking yellow in the forewing.

Here, we synthesize multiple lines of evidence from genetics, ecology, and morphology of both adults and immature stages to shed light on the nature of mimetic polymorphism within *M. mazaeus*. We use mitochondrial haplogroups to help discover consistent morphological and ecological traits that firmly establish species limits in the *M. mazaeus* complex. The data reject Brown's hypothesis that *M. mazaeus* exists as a single polymorphic interbreeding population. Instead, our data suggest the existence of two species that are often sympatric, both of which exhibit mimetic polymorphism.

## MATERIAL AND METHODS

### TAXON SAMPLING AND SEQUENCE ACQUISITION

In total, we analysed 658 bp of the mitochondrial gene cytochrome c oxidase subunit I (*COI*) for 238 *Mechanitis* individuals (Table 1). We used previously published sequences for 70 individuals, representing a number of phenotypes, from across the range of *M. mazaeus* s.l. (Elias *et al.*, 2007; Dasmahapatra *et al.*, 2010a), with more intensive sampling in Peru and Ecuador, including *M. mazaeus fallax* and other synonymized phenotypes of *M. mazaeus* [*M. maz.*

**Table 1.** Summary of sample size, country of origin, and mtDNA haplogroup for the *Mechanitis* taxa analysed in this study

<i>Mechanitis</i> taxon (based on adult colour pattern)	Ecuador	Peru	Panama	Venezuela	Brazil	mtDNA haplogroup
<i>lysimnia macrinus</i>			4			H
<i>lysimnia solaria</i>				2		H
<i>lysimnia utemai</i>			2	1		H
<i>lysimnia roqueensis</i>	6	5				E
<i>lysimnia</i> ssp.		1				E
sp.				1		H
<i>mazaeus fallax</i>	36	16				H
<i>mazaeus mazaeus</i>		1				H
<i>mazaeus pannifera</i>				2		H
<i>mazaeus pothetoides</i>					1	H
<i>mazaeus</i> ssp. (no adult)	12					H
<i>mazaeus</i> ssp.		1				H
<i>messenoides deceptus</i>	27(A); 6(F)	9(A); 9(F)				A, F
<i>messenoides messenoides</i>	26(A); 6(F)					A, F
<i>mazaeus fallax</i> ('phasianita' phenotype)		8(A)				A
<i>mazaeus mazaeus</i> ('nigroapicalis' phenotype)		3(F)				F
<i>messenoides messenoides</i> × <i>deceptus</i>	3(F)					F
<i>mazaeus</i> ssp.		1(F)				F
<i>mazaeus</i> ssp. (no adult)	7(A); 3(F)					A, F
<i>menapis mantineus</i>	3					G
<i>menapis saturata</i>			2			G
<i>polymnia bolivarensis</i>				1		C
<i>polymnia casabranca</i>					1	C
<i>polymnia</i> cf. <i>dorissides</i>		1				B
<i>polymnia isthmia</i>			5			I
<i>polymnia proceriformis</i>	10					B, C, D
<i>polymnia</i> cf. <i>proceriformis</i>		5				B, D
<i>polymnia veritabilis</i>			1			I
<i>polymnia</i> ssp. nov.	10					D, I

For haplogroups A and F the number of individuals from Ecuador and Peru is indicated.

*mazaeus* ('nigroapicalis' phenotype), *M. maz. fallax* ('elevata' phenotype), *M. maz. fallax* ('phasianita' phenotype) (Fig. 1). We obtained new sequence data from 107 *M. mazaeus* individuals, primarily from eastern Ecuador, where an intensive study of larval morphology and ecology was conducted (details below). We also added sequence data for 61 individuals (26 of which are new) of the other three *Mechanitis* species *Mechanitis polymnia* (*M. p. bolivarensis*, *M. p. casabranca*, *M. p. isthmia*, *M. p. proceriformis*, *M. p. dorissides*, *M. p. veritabilis*, and *M. p.* ssp. nov. from eastern Ecuador), *Mechanitis lysisimnia* (*M. l. roqueensis*, *M. l. macrinus*, *M. l. solaris*, and *M. l. utemai*), and *Mechanitis menapis* (*M. m. mantineus* and *M. m. saturata*) from a wide range of localities for comparison. Previously published sequence data for a single *Forbestra olivencia*, a member of the sister genus to *Mechanitis*, was also included for comparison. GenBank accession numbers for the *F. olivencia* and *Mechanitis* individuals from previous work are

indicated in Table S1. Locality data and accession numbers for new individuals published here are given in Tables 1, S1, and S2 (GenBank accession numbers JF450926–JF451058).

For sequences generated in this study, DNA was generally extracted from reared or field-collected adults. However, in cases where rearing did not produce adults, DNA was extracted from early stages (i.e. eggs and larvae) in order to associate host-plant data or larval morphology with mitochondrial DNA (mtDNA) haplogroups. When available, tissues preserved in ethanol or dimethyl sulfoxide (DMSO) salt solution (20% DMSO, 0.25 M EDTA, saturated with NaCl) were used, otherwise DNA was extracted from the legs of dried adult specimens. Whole genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen). The manufacturer's protocol was followed with some exceptions. For some individuals, and all eggs and larvae, the lysis step was performed with an overnight incubation and we used an initial elution

volume of 150 µL for adult tissue and 100 µL for eggs and larvae, with a second elution volume of 100 µL. DNA was amplified and sequenced for *COI* using primers and polymerase chain reaction (PCR) protocols described in Dasmahapatra *et al.*, (2010a). Sequence contigs were assembled and edited using SEQMAN (DNASTAR) and CODONCODE ALIGNER (CodonCode Corporation), and then finally checked against amino acid translations for stop codons in MESQUITE (Maddison & Maddison, 2011). Alignments were performed with the MUSCLE algorithm (Edgar, 2004) and checked manually. MEGA 5.05 (Tamura *et al.*, 2011) was used to generate a neighbour-joining tree using raw sequence differences, with bootstrap values generated from 1000 iterations.

#### ECOLOGY AND MORPHOLOGY

Larval ecology and morphological data were gathered in eastern Ecuador along the Río Napo, between 2000 and 2007. Sampling was conducted on the north side of the river in the communities of Pilche and Sani Isla, in the forests surrounding Garzacocha, Provincia Sucumbíos ( $00^{\circ}29.87'S$ ,  $76^{\circ}22.45'W$ ), and on the south side of the river (Provincia Orellana) in the communities of Añangu ( $00^{\circ}31.41'S$ ,  $76^{\circ}23.73'W$ ) and Sani Isla ( $00^{\circ}30.69'S$ ,  $76^{\circ}21.07'W$ ). *Mechanitis* butterflies were collected as adults, eggs, and larvae. Eggs and larvae were reared in plastic containers and bags under ambient conditions. Host plants were photographed and mounted for identification. Notes and photographs were taken on larval morphology, behaviour, and development time. Reared and field-collected adults (from across the geographic range) were scored for colour pattern and androconial scale characters using a dissecting microscope. Adult and larval specimens will be deposited in the Essig Museum of Entomology at UC Berkeley. Host-plant vouchers are deposited in the MECN Herbarium in Quito, the University and Jepson Herbaria at UC Berkeley, the New York Botanical Garden, and The Natural History Museum, London.

In addition to basic observations on eggs and larvae, we conducted a host-switch experiment between two of the commonly used hosts of *M. mazaeus* and *M. messenoides* at these sites, *Solanum pedemontanum* and *Solanum leucopogon*, respectively. Egg clutches laid on one host were divided approximately in half, with one half given the novel host and the other the original host. Larvae were reared in plastic tubs. Larval mortality for each individual in a clutch was recorded. For each clutch, we calculated the average percentage mortality in the first instar, and from first instar to pupal formation. Instar duration was recorded either individually for each clutch member (small clutches) or for the individuals as a group (i.e. start and end time

of a particular instar). For each clutch, we calculated the average duration of first instars, and the average duration of larvae to reach the fifth instar. Differences between host plants were evaluated for each clutch with paired one-tailed Student's *t*-tests. Paired tests were used because individuals within a clutch received one of two treatments. One-tailed tests were used to test the specific hypothesis that performance decreased when feeding on a novel host.

A post-hoc analysis of wing shape between *M. mazaeus* and *M. messenoides* was carried out on a subset of individuals to test if the species differ in wing shape, as suggested by Beccaloni (1997). Individuals in the wing shape analysis were classified as belonging either to *M. mazaeus* or to *M. messenoides* based on DNA sequence, wing colour pattern, or larval morphology, all of which we found can be used to separate the species (see below). The wing shape of field-caught individuals was quantified with camera images taken in the same plane as the wings. For each individual, the fore- and hindwings were arranged in a manner similar to that attained during flight (Betts & Wootton, 1988). Aspect ratio and wing centroid were calculated from the images using vertical wing chords following the equations presented in Ellington (1984). The aspect ratio is the ratio of wing length to mean wing chord, and is higher in species with long, narrow wings. The wing centroid refers to the non-dimensional position of the centre of the wing area (i.e. the first radial moment of the wing area), and tends to be higher in butterfly species with the wing area shifted distally. Differences in wing shape were evaluated with Student's *t*-tests and sexes were analysed separately.

## RESULTS

### GENOTYPIC CLUSTERING

Genotypic clusters, or haplogroups, within the mitochondrial tree were defined using a threshold of 1.5% between-cluster raw sequence divergence (Dasmahapatra *et al.*, 2010a). This revealed three well-supported *COI* haplogroups (bootstrap values  $> 89\%$ ) among *M. mazaeus* s.l.: one *M. mazaeus* and two *M. messenoides* (Fig. 2). The *COI* haplogroups identified here correspond to those in Dasmahapatra *et al.* (2010a): *M. mazaeus* (haplogroup H), *M. messenoides* (haplogroup A), and *M. messenoides* (haplogroup F). The three haplogroups have high bootstrap support and appear more genetically distinct at this locus than other morphologically well-characterized species of *Mechanitis*. In addition, there is moderate support for monophyletic mtDNA lineages within the three *M. mazaeus*/*M. messenoides* haplogroups (Fig. 2). The use of a 1.5% threshold is somewhat arbitrary, but pairwise distances between any two of the three *M. mazaeus*/*M. messenoides* haplogroups is  $> 3.4\%$ .

(Table 2), indicating that these three haplogroups are robust to substantial increases in the threshold.

Although the two *M. messenoides* haplogroups were monophyletic, one divergent branch comprising three subspecies of *M. lysimnia* (*M. l. macrinus*, *M. l. solaris*, and *M. l. utemai*), indicated as haplogroup Hb in Fig. 2), made the *M. mazaeus* haplogroup paraphyletic. This divergent lineage arising within *M. mazaeus* was also identified in Dasmahapatra *et al.* (2010a). For purposes of comparing adult colour pattern, larval morphology, and ecology here, *M. mazaeus* (haplogroup Ha) is considered monophyletic and the *M. lysimnia* (haplogroup Hb) are ignored. The average raw percentage pairwise distance between *M. messenoides* haplogroup A and *M. messenoides* haplogroup F is 3.4%, between *M. mazaeus* haplogroup H and *M. messenoides* haplogroup F is 3.6%, and between *M. mazaeus* haplogroup H and *M. messenoides* haplogroup A is 4.0%. If we remove *M. lysimnia* individuals nested within *M. mazaeus* haplogroup H and calculate distances between just *M. mazaeus* haplogroup Ha and *M. messenoides* haplogroup F, the value is 3.6%, whereas between just *M. mazaeus* haplogroup Ha and *M. messenoides* haplogroup A the distance is 3.9%. Other pairwise divergence comparisons are given in Table 2.

As in *M. mazaeus/M. messenoides*, the mitochondrial diversity found within *M. polynnia* clustered into multiple haplogroups. *Mechanitis polynnia* had four different haplogroups with high bootstrap values, three of which were identified in Dasmahapatra *et al.* (2010a) (Fig. 2, *M. polynnia* haplogroups B, C, and D). The new *M. polynnia* haplogroup identified here (haplogroup I) is composed of *M. polynnia* samples from Panama and a single individual from Ecuador. *Mechanitis lysimnia* mostly clustered into one well-supported species-specific haplogroup (E). However, several *M. lysimnia* subspecies (haplogroup Hb) clustered with *M. mazaeus* (haplogroup Ha). An additional individual (4–279; Fig. 2) was not similar enough to cluster with other *M. lysimnia*, here with >50% bootstrap support, but this individual did cluster with the *M. lysimnia* haplogroup E in Dasmahapatra *et al.* (2010a) when other *M. lysimnia* individuals were included. *Mechanitis menapis* were clustered into a single species-specific haplogroup (G), although the bootstrap value is relatively low. Genetic distances based on the COI barcode region showed a marked gap in the distribution of average raw percentage pairwise mtDNA distances within and between the various haplogroups (Fig. 3; Table 2).

#### ADULT MORPHOLOGY

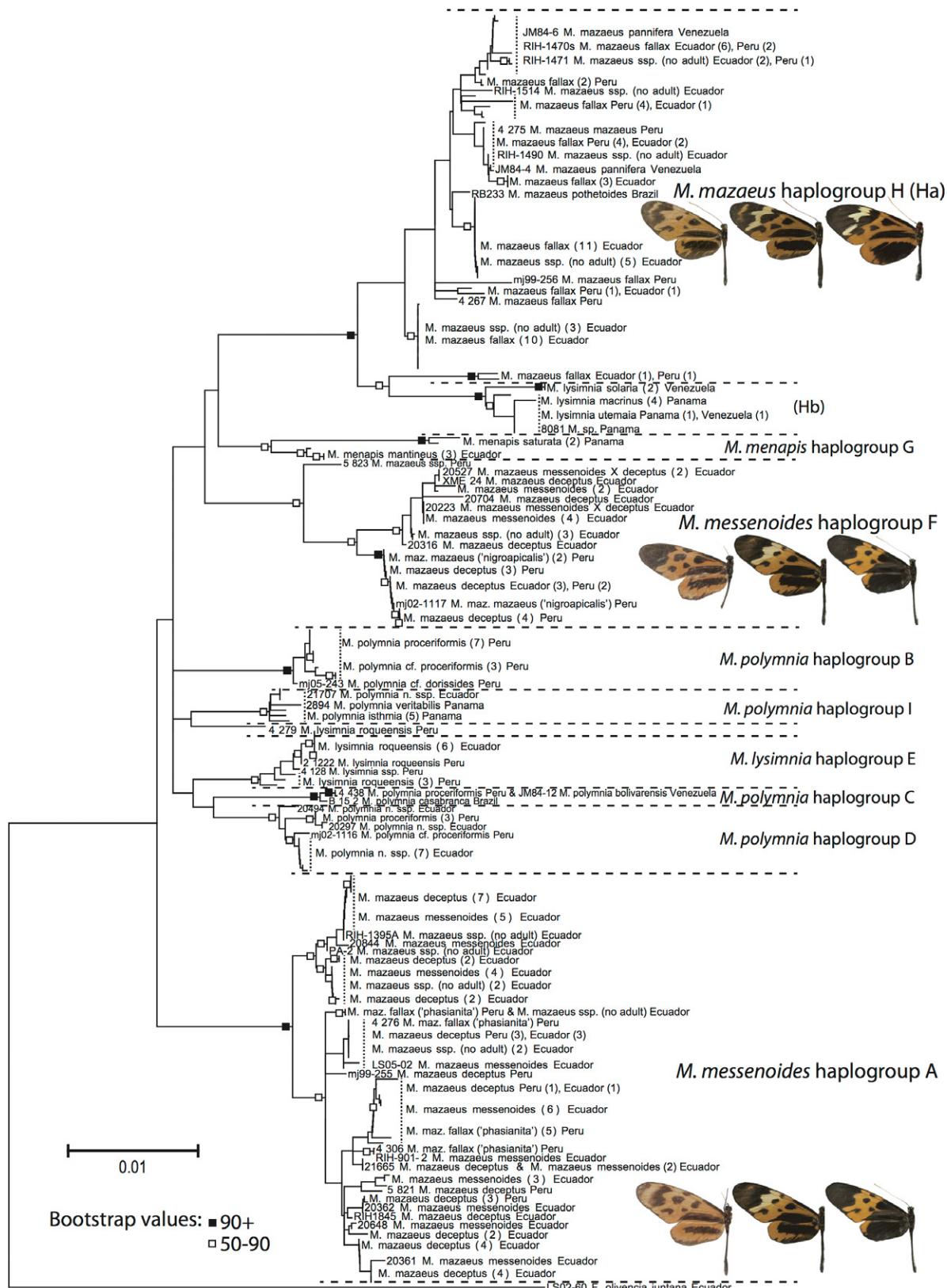
##### Colour pattern

A total of 153 adult specimens from across the ranges of *M. mazaeus* and *M. messenoides* were examined for

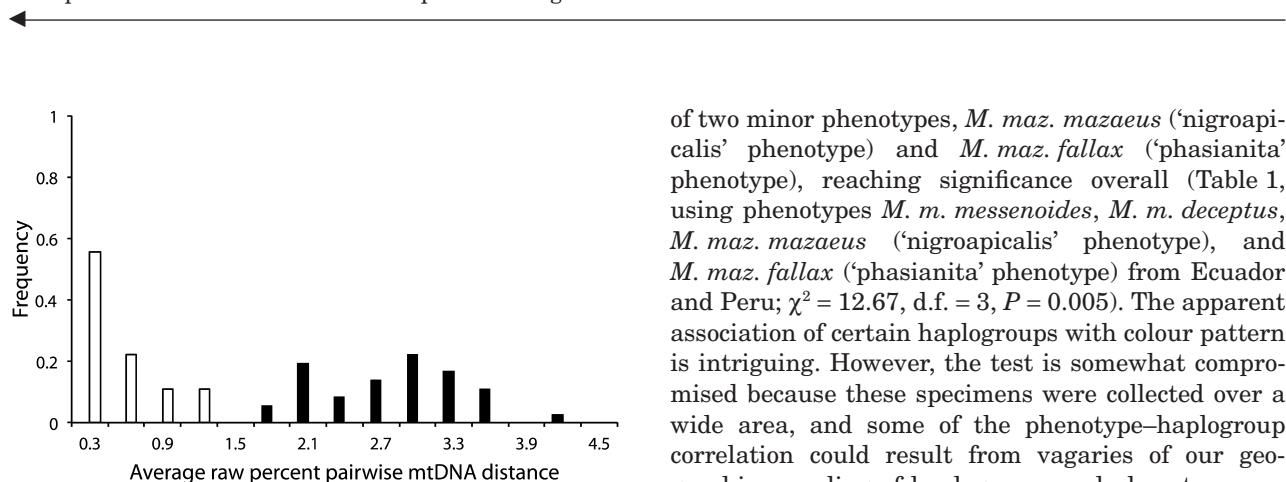
**Table 2.** Average raw percentage pairwise mtDNA distances, within (along diagonal) and between haplogroups

Haplogroup	<i>menapis</i> (G)	<i>messenoides</i> (A)	<i>polynnia</i> (B)	<i>messenoides</i> (F)	<i>polynnia</i> (I)	<i>lysimnia</i> (E)	<i>polynnia</i> (C)	<i>polynnia</i> (D)	<i>mazaeus +</i> <i>'lysimnia-H'</i> (H)	<i>mazaeus</i> + <i>'lysimnia'</i> (H)	<i>lysimnia</i> (H)	<i>mazaeus</i> + <i>'lysimnia-H'</i> (Hb)	<i>Only mazaeus</i> (Ha)	<i>Only mazaeus</i> (Hb)	<i>Only mazaeus</i> (Ha)
<i>menapis</i> (G)		<b>1.1</b>	<b>0.6</b>												
<i>messenoides</i> (A)	3.2		<b>0.1</b>												
<i>polynnia</i> (B)	2.8	2.7		<b>0.5</b>											
<i>messenoides</i> (F)	2.6	3.4	2.7		<b>0.5</b>										
<i>polynnia</i> (I)*	2.2	2.3	1.9	2.8		<b>0.2</b>									
<i>lysimnia</i> (E)	2.7	2.6	2.1	2.8	1.9		<b>0.2</b>								
<i>polynnia</i> (C)	2.8	2.6	2.3	3.3	1.8	2.0		<b>0.1</b>							
<i>polynnia</i> (D)	2.5	2.5	2.1	2.8	1.9	1.7	1.6		<b>0.2</b>						
<i>menapis +</i> <i>'lysimnia-H'</i> (H)	3.1	4.0	3.3	3.6	3.1	3.1	3.5	3.3		<b>0.9</b>					
Only <i>'lysimnia-H'</i> (Hb)	3.5	4.4	4.0	3.6	3.2	3.6	3.1	2.9	3.4	3.7	NA	<b>0.3</b>			
Only <i>mazaeus</i> (Ha)	3.1	3.9	3.2							3.2	NA	<b>2.1</b>		<b>0.5</b>	

\*The *polynnia* haplogroup I is an additional haplogroup found here as a result of the addition of samples not analysed in Dasmahapatra *et al.* (2010a). Below the line are values when the *Mechanitis lysimnia* samples nested within *mazaeus* haplogroup H are treated as a separate haplogroup.



**Figure 2.** Mitochondrial *COI* neighbour-joining tree indicating haplogroup clusters for 238 *Mechanitis* individuals. Nodes with bootstrap values of 90 and above are indicated with filled squares; empty squares indicate values ranging from 50 to 90. Species, subspecies/phenotype, and country are given for each individual, with numbers in parentheses indicating multiple individuals. Scale bar: 1% sequence divergence.



**Figure 3.** Average raw percentage pairwise mtDNA distances within (unshaded) and between (shaded) haplogroups. The haplogroups compared are indicated in Table 3, and only comparisons involving the entire haplogroup H are plotted (i.e. no comparisons involving haplogroups Ha and Hb are included).

correlations between wing colour pattern morph (subspecies) and mtDNA genotype (Table 1); 149 of these specimens were scored for particular colour pattern characters (Fig. 4; four were unavailable to score). Colour pattern races and subspecies of *M. mazaeus* s.l. were very strongly associated with either *M. mazaeus* haplogroup H or *M. messenoides* haplogroups A and F. *Mechanitis mazaeus* haplogroup H included phenotypes similar to the types of *M. maz. fallax*, *M. maz. mazaeus*, *M. maz. pannifera*, *M. maz. potheoides*, as well as a phenotype considered a synonym of *M. maz. fallax* (the ‘elevata’ phenotype). The two *M. messenoides* haplogroups included the subspecies *M. mes. deceptus* and *M. mes. messenoides*, as well as phenotypes resembling synonyms of *M. maz. mazaeus* (the ‘nigroapicalis’ phenotype) and *M. maz. fallax* (the ‘phasianita’ phenotype). Morphological and genetic differences between *M. messenoides* haplogroup A and haplogroup F, and between *M. mazaeus* haplogroup H and *M. messenoides* haplogroups A and F, are described in the following sections.

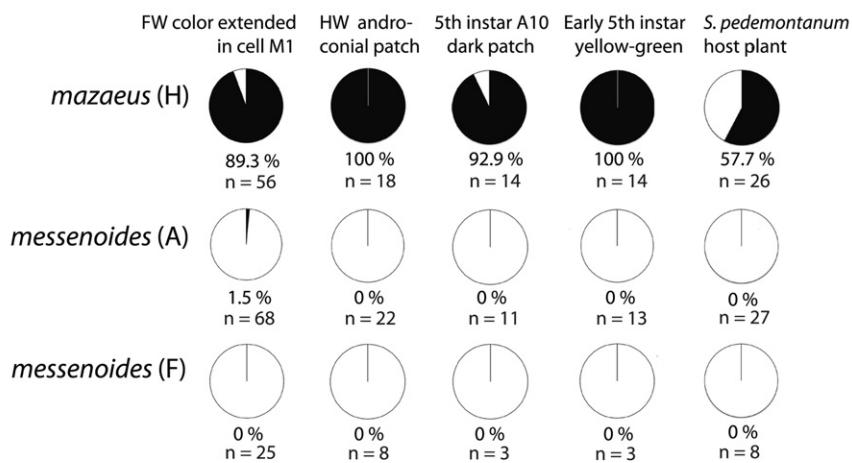
*Mechanitis messenoides* haplogroups A versus F: *Mechanitis messenoides* haplogroups A and F did not differ from one another with respect to characters distinguishing them from the *M. mazaeus* haplogroup H. Haplogroups A and F differed little in the frequency of the most common phenotypes, but appeared to differ more strongly in the frequency

of two minor phenotypes, *M. maz. mazaeus* (‘nigroapicalis’ phenotype) and *M. maz. fallax* (‘phasianita’ phenotype), reaching significance overall (Table 1, using phenotypes *M. m. messenoides*, *M. m. deceptus*, *M. maz. mazaeus* (‘nigroapicalis’ phenotype), and *M. maz. fallax* (‘phasianita’ phenotype) from Ecuador and Peru;  $\chi^2 = 12.67$ , d.f. = 3,  $P = 0.005$ ). The apparent association of certain haplogroups with colour pattern is intriguing. However, the test is somewhat compromised because these specimens were collected over a wide area, and some of the phenotype–haplogroup correlation could result from vagaries of our geographic sampling of haplogroups and phenotypes.

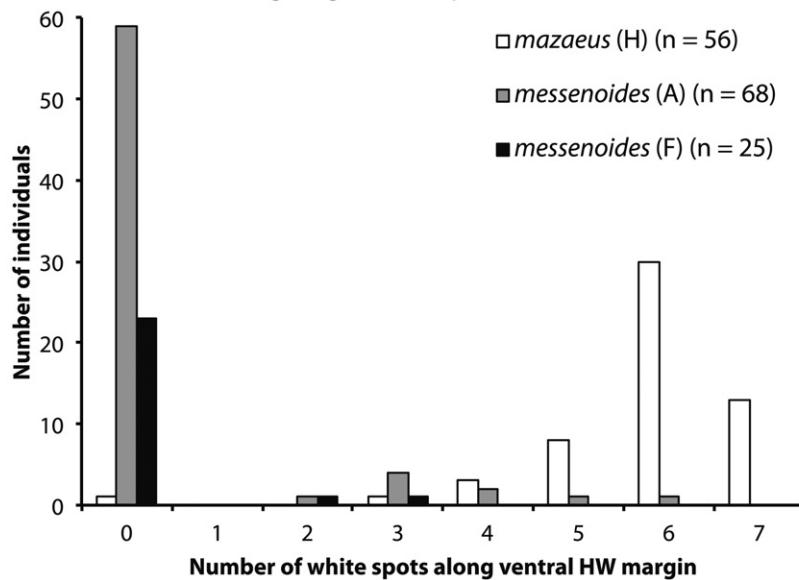
*Mechanitis mazaeus* haplogroup H versus *M. messenoides* haplogroups A and F: Three wing characters are strongly correlated with *M. mazaeus* haplogroup H, and in combination allowed *M. mazaeus* to be distinguished from *M. messenoides* in all but one of the examined specimens (Fig. 4). First, in our samples from Ecuador and Peru, where *M. mazaeus* co-occurs with *M. messenoides*, the dorsal forewing postmedial band was markedly extended in cell M1 in 94.3% (50 of 53) of the *M. mazaeus* haplogroup individuals scored (Fig. 4A), including two additional *M. mazaeus* individuals from Venezuela and one from Brazil, representing different subspecies that did not exhibit a markedly extended postmedial band in M1 (samples from geographic areas lacking *M. messenoides*), reduced this to 89.3% (50 of 56). In contrast, only 1.5% (1 of 68) of *M. messenoides* haplogroup A, and 0% (0 of 25) of *M. messenoides* haplogroup F had this phenotype (1.1% if *M. messenoides* haplogroups are combined).

Second, the ventral hindwing margin contained three or more white spots in 98.2% of *M. mazaeus* haplogroup H individuals scored (mean no. spots = 5.82, mode no. spots = 6,  $N = 56$ ; Fig. 4B). This included the individuals from Venezuela and Brazil that did not appear to have the *M. mazaeus* haplogroup H phenotype for the postmedial band character, and each of these individuals had seven ventral hindwing spots. The vast majority of individuals in the two *M. messenoides* haplogroups had no ventral hindwing white spots (*M. messenoides* haplogroup A, 13.2% with spots, mean no. spots = 0.49, mode no. spots = 0,  $N = 68$ ; *M. messenoides* haplogroup F, 8.0% with spots, mean no. spots = 0.20, mode no. spots = 0,  $N = 25$ ; *M. messenoides* haplogroups A and F, 11.8%

**A.** Characters of adult and immature stages that differ between *M. mazaeus* and *M. messenoides*.



**B.** Number of ventral hindwing marginal white spots

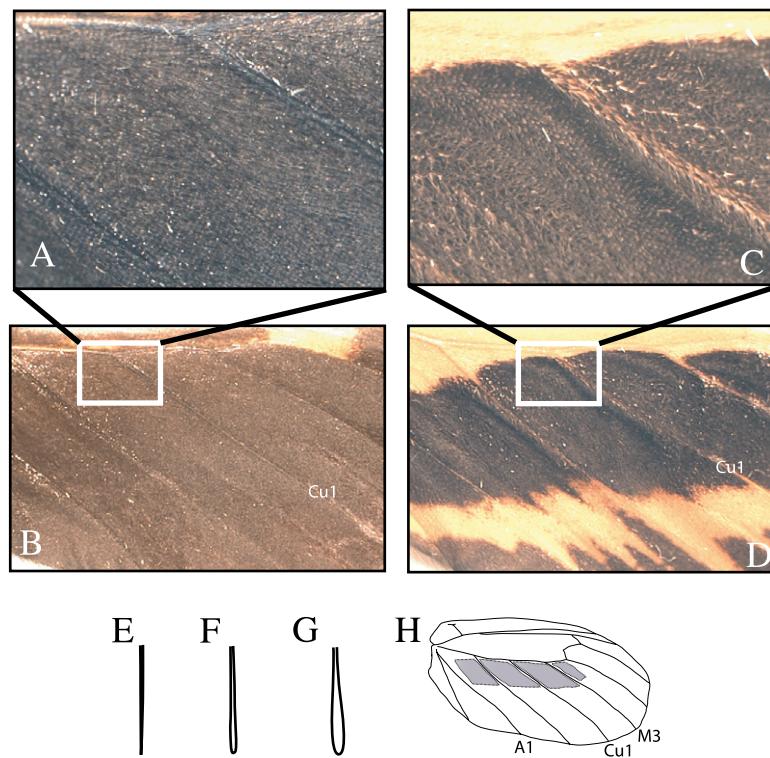


**Figure 4.** Summary of morphology and host use differences between *Mechanitis mazaeus* and *Mechanitis messenoides* mtDNA haplogroups. A, morphology of adults, immature stages, and use of the host plant *Solanum pedemontanum*. Dark shading indicates the presence of a trait. Note that where *Mechanitis mazaeus* and *Mechanitis messenoides* are sympatric, the forewing M1 cell character identified 50 of 53 individuals (94.3%). B, distribution of the number of white spots on the ventral hindwing margin.

with spots, mean no. spots = 0.41, mode no. spots = 0,  $N = 93$ ). The number of ventral hindwing marginal white spots was significantly higher in *M. mazaeus* haplogroup H compared with both *M. messenoides* haplogroup A (Wilcoxon rank-sum test:  $z = 9.82$ ,  $P < 10^{-4}$ ) and *M. messenoides* haplogroup F ( $z = 7.32$ ,  $P < 10^{-4}$ ; Fig. 4B). The *M. messenoides* haplogroups A and F did not differ in number of ventral hindwing spots ( $z = 0.77$ ,  $P = 0.44$ ).

Third, *M. mazaeus* males have androconial patches in the dorsal subdiscal hindwing black band (Figs 1,

4, 5). All of the genotyped male *M. mazaeus* haplogroup H individuals ( $N = 18$ ) had roughened androconial patches in Cu1 and M3. The androconial patches were also present in an additional 19 male *M. mazaeus*, determined by a combination of wing pattern and/or larval characters. These patches can occur in cells A1, Cu2, Cu1, M3, and M2, but they are particularly pronounced and were always present in cells Cu1 and M3 (Fig. 5C, D, H). The patches are confined to the black band area near the discal cell, even in individuals with extensive dark areas that merge



**Figure 5.** Androconial scale differences in the dorsal hindwing of *Mechanitis mazaeus* and *Mechanitis messenoides*. The left two panels illustrate *M. messenoides*, which lacks the androconial patches (A, B). The right two panels illustrate *M. mazaeus* with androconial patches present (C, D). Note the roughened texture in (C) and (D), as well as the more vertically oriented scales in patches in (C). The images in (A) and (C) show magnified views of the wing in cell Cu1, with vein Cu1 running diagonally from the centre at the top down to the right. A normal cover scale found in the dorsal hindwing subdiscal band of both species is shown in (E). Androconial scale types found in both *M. mazaeus* and *M. messenoides* are shown in (F) and (G). Androconial scale type (G) is not common in *M. messenoides* but is common in the androconial patches of *M. mazaeus*. Areas of the dorsal hindwing in *M. mazaeus* that typically contain extensive androconial patches are indicated with shading in (H). Wing veins are labelled on the outer wing margin for reference.

from the wing margin. Under a stereomicroscope these patches look rough in texture and are composed of dense, robust, elongate elliptical ground scales that are elevated instead of lying flat. The ground scales in these patches are very different from the ground scales in dark areas of the wing margin. The cover scales in these patches differ from regular cover scales (shown in Fig. 5E) in being wider and parallel sided or spatulate (Fig. 5F, G). These cover scales can obscure flat-lying ground scales in small elongate patches on some individuals, but are predominantly found mixed with the elevated ground scales giving the patches a densely scaled rough appearance.

Among the genotyped male *M. messenoides*, the androconial patches were always absent (*M. messenoides* haplogroup A:  $N=9$  *M. mes. messenoides*;  $N=9$  *M. mes. deceptus*;  $N=4$  *M. maz. fallax* ('phasianita' phenotype); *M. messenoides* haplogroup F:  $N=5$  *M. mes. messenoides*;  $N=1$  *M. mes. deceptus*;  $N=2$  *M. maz. mazaeus* ('nigroapicalis' phenotype)).

The androconial patches were also absent in an additional 22 male *M. messenoides* identified only by wing colour pattern and larval characters ( $N=9$  *M. mes. deceptus*;  $N=10$  *M. mes. messenoides*;  $N=2$  *M. mes. holmgreni*;  $N=1$  *M. maz. fallax* ('phasianita' phenotype)). These specimens are from across the range of *M. messenoides* (from Colombia to Bolivia), and include two 'ballucatus' paratypes (a junior synonym of *M. mes. holmgreni*; Lamas, 2004) and a male identified by R. Fox as *M. egaensis phasianita* in the Harvard Museum of Comparative Zoology (R. Hill, pers. observ.). Further comments on scale morphology in *M. messenoides* are given in the Appendix S1.

#### Adult wing shape

Using the above wing pattern characters to classify wild-caught adults from Garzacocha, Ecuador, as either *M. mazaeus* or *M. messenoides* indicated that these taxa also differ in wing shape. Within species,

males and females differed in aspect ratio (*M. messenoides*,  $t = 11.25$ ,  $p_{\text{two-tailed}} < 0.0001$ ; *M. mazaeus*,  $t = 8.33$ ,  $p_{\text{two-tailed}} < 0.0001$ ), but not wing centroid (*M. messenoides*,  $t = 0.24$ ,  $p_{\text{two-tailed}} = 0.81$ ; *M. mazaeus*,  $t = 0.27$ ,  $p_{\text{two-tailed}} = 0.79$ ), and so sexes were analysed separately when comparing groups. Aspect ratio was significantly higher ( $t = 4.81$ ,  $p_{\text{two-tailed}} < 0.0001$ ) for male *M. messenoides* (mean = 5.32, SD = 0.16,  $N = 11$ ) compared with male *M. mazaeus* (mean = 5.01, SD = 0.17,  $N = 19$ ), confirming that *M. messenoides* males have a relatively narrower wing. Wing centroid did not significantly differ ( $t = 0.30$ ,  $p_{\text{two-tailed}} = 0.77$ ) between *M. messenoides* males (mean = 0.46, SD = 0.011,  $N = 11$ ) and *M. mazaeus* males (mean = 0.47, SD = 0.009,  $N = 19$ ). Females did not significantly differ in either wing shape measure ( $t < 1.4$  and  $p_{\text{two-tailed}} > 0.18$  for both variables).

#### IMMATURE MORPHOLOGY AND ECOLOGY

A total of 84 clutches of immature *M. mazaeus* and *M. messenoides* were collected. Sequences were generated for 61 of these clutches, providing data on larval morphology and ecology for the three *M. mazaeus*/*M. messenoides* haplogroups.

#### Larval morphology

Larval colour pattern differed strongly between the *M. mazaeus* and *M. messenoides* haplogroups, but no differences were observed between *M. messenoides* haplogroups (Fig. 4). No differences were observed in the pupa.

*Mechanitis mazaeus* and *M. messenoides* larvae can be clearly distinguished by two characters. First, in the early fifth instar (also detectable in the third and fourth instars) *M. messenoides* is whiter in colour (pale grey), and has bright yellow at the base of the lateral projections, whereas *M. mazaeus* is dingy yellow-green (olivaceous), and the colour at the base of the lateral projections is not markedly different from the nearby dorsolateral tissue (Figs 6D–G, 7D–F). Second, late-stage fifth instars become very similar but can be distinguished by the presence of a sclerotized patch on A10 above the anus (present in *M. mazaeus*, but absent in *M. messenoides*; Figs 6H, 7H).

#### Host plant use

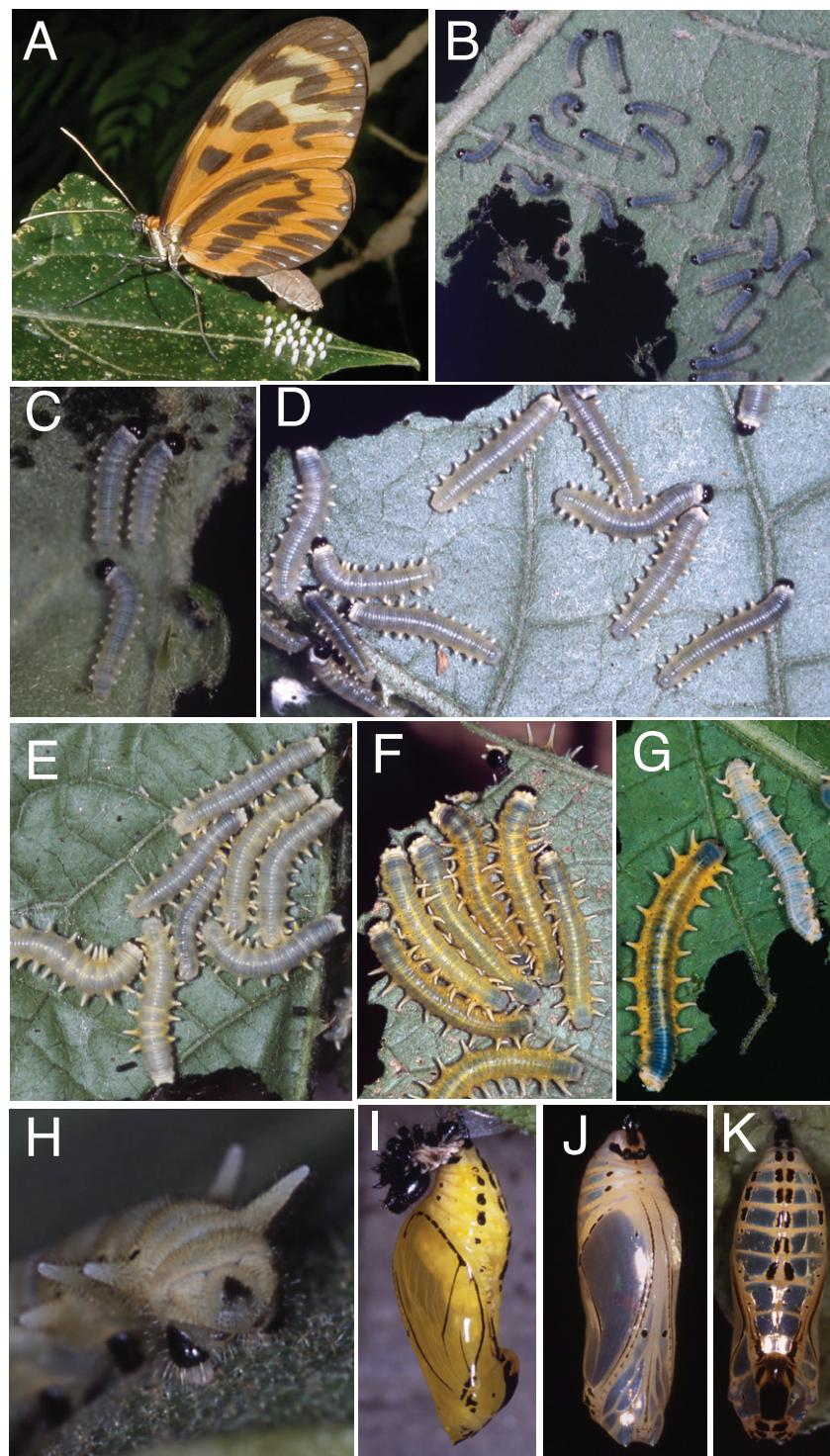
The *M. messenoides* haplogroups A and F overlapped broadly in host use (Tables 3 and S1), and frequency of host use did not differ between them (Pearson's  $\chi^2 = 7.0$ , d.f. = 6,  $P = 0.32$ ). Immatures of both *M. messenoides* haplogroup A and *M. messenoides* haplogroup F were found on *Solanum leucopogon*, *Solanum monarchostemon*, *Solanum morellifolium* and *Solanum cf. sessiliflorum*. In addition, *M. messenoides* haplogroup A and *M. messenoides* haplogroup F used

other plants resembling 'naranjilla' (*Solanum quitoense*, grown for its edible fruits) in the *Solanum* subgenus *Leptostemonum* clade (Bohs, 2005; Levin, Myers & Bohs, 2006: 1), a small tree species, hereafter called 'tree naranjilla', and seedlings of naranjilla-like *Solanum*, hereafter collectively called 'seedling naranjilla'. These unidentified subgenus *Leptostemonum* clade species are probably *Solanum candum*, *Solanum sessiliflorum*, or *Solanum quitoense* (Jørgensen *et al.*, 1999), but details of morphology were not available to make an exact determination. One host was not shared between *M. messenoides* haplogroups: *M. messenoides* haplogroup A was found on *Solanum cf. cacosmum* ( $N = 2$ , only 7%), whereas *M. messenoides* haplogroup F was not (Table 3). Calculating Schoener's percentage overlap index (Schoener, 1970) for the two *M. messenoides* haplogroups gives a value of 56.0% host overlap.

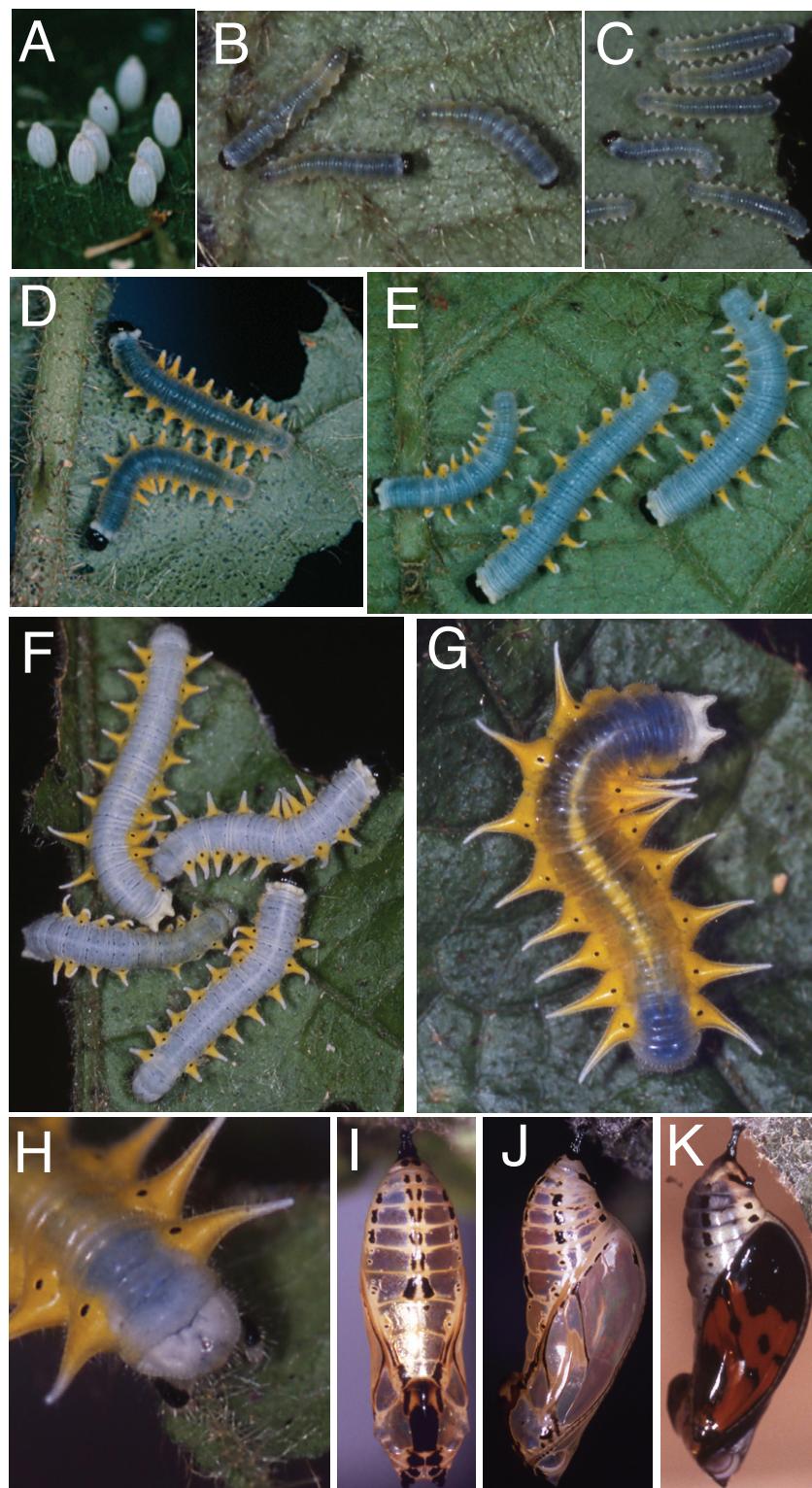
Comparison of *M. mazaeus* and *M. messenoides* indicated divergent host use, and the frequency of host use differed significantly between them ( $\chi^2 = 54.5$ , d.f. = 7,  $P < 0.001$ ). In total, host data for 23 additional clutches that lacked sequence data were incorporated after species determination was made using the adult colour pattern, larval morphology, and clutch size results reported here. This raised the total number of clutches with host data to 84 (34 *M. mazaeus* and 50 *M. messenoides*), and host use is summarized in Table 3. Briefly, the most common host of *M. mazaeus*, *S. pedemontanum*, was never used by *M. messenoides* (Fig. 4A; Table 3), although the most common host of *M. messenoides*, *S. leucopogon*, was used by *M. mazaeus*. Furthermore, five of the *Solanum* species used by *M. messenoides* were never used by *M. mazaeus*, and *M. mazaeus* was never found on 'seedling naranjilla'. Schoener's percentage overlap index indicates that the overlap in host use between *M. mazaeus* and *M. messenoides* is 25.6%. Although abundance was not quantified, the host species *S. pedemontanum* and *S. leucopogon* were both commonly found growing in the same forest light gaps.

#### Novel host performance

Larval performance on both *S. pedemontanum* and *S. leucopogon* was evaluated for 11 clutches of *M. mazaeus* laid on *S. pedemontanum*, four clutches of *M. mazaeus* laid on *S. leucopogon*, and five clutches of *M. messenoides* (all haplogroup A) laid on *S. leucopogon*. Clutches were split approximately in half to compare performance on the novel and original host, resulting in the same number of clutches being compared for each host. Results are summarized in Tables 4 and 5. Overall, the results do not indicate a strong performance disadvantage when reared on the novel host, and adults of both species were suc-



**Figure 6.** Immature stages of *Mechanitis mazaeus*. A, female laying a large clutch of eggs on *Solanum pedemontanum*. B, first instars. C, second instars. D, third instars, exhibiting pale-yellow lateral protuberances. E, fourth instars, showing only subtle differences in colour between dorsum and lateral protuberances. F, two-day-old fifth instars, illustrating dingy yellow-green dorsum and dark sclerotized patch on segment A10. G, first-day (right) and fourth-day fifth instars, indicating the change observed in this instar. H, detail of fifth instar segment A10, with dark sclerotized patch. I, first-day pupa. J, ventrolateral view of mature pupa. K, dorsal view of mature pupa.



**Figure 7.** Immature stages of *Mechanitis messenoides*. A, eggs, note small clutch size. B, first instars. C, second instars. D, third instars, with bright-yellow lateral projections, contrasting with the dorsum coloration (present from this instar to early in the fifth instar). E, fourth instars. F, one- and two-day-old fifth instars, illustrating the pale-grey dorsum. G, mature five-day-old fifth instar. H, detail of five-day-old fifth instar segment A10, showing a lack of the dark heavily sclerotized patch. I, dorsal view of pupa. J, lateral view of pupa. K, pupa showing *Mechanitis messenoides deceptus* wing coloration.

**Table 3.** *Solanum* host use for *Mechanitis mazaeus* and *Mechanitis messenoides* in eastern Ecuador (Prov. Orellana and Sucumbíos)

	Records by mtDNA haplogroup			Records by species	
	<i>mazaeus</i> (H)	<i>messenoides</i> (A)	<i>messenoides</i> (F)	<i>mazaeus</i>	<i>messenoides</i>
<i>S. pedemontanum</i>	15 (58%)			20 (59%)	
<i>S. leucopogon</i>	5 (19%)	8 (30%)	1 (12.5%)	6 (18%)	17 (34%)
<i>S. monarchostemon</i>		1 (4%)	1 (12.5%)		2 (4%)
<i>S. morellifolium</i>		2 (7%)	1 (12.5%)		3 (6%)
<i>S. 'tree naranjilla'</i>	6 (23%)	1 (4%)	2 (25%)	8 (23%)	4 (8%)
<i>S. cf. sessiliflorum</i>		1 (4%)	1 (12.5%)		5 (10%)
<i>S. 'seedling naranjilla'</i>		12 (44%)	2 (25%)		17 (34%)
<i>S. cf. cacosmum</i>		2 (7%)			2 (4%)
Total records=	26	27	8	34	50

The three left-hand columns are records for separate mtDNA haplogroups. The two right-hand columns are records for species determined using all available data, including the adult and immature traits found in this study.

cessfully reared on the novel hosts. The results for both larval development time and mortality showed little evidence of the increased performance of *M. mazaeus* on *S. pedemontanum* and *M. messenoides* on *S. leucopogon*.

The average percentage mortality of *M. mazaeus* clutches laid on *S. leucopogon* was lower when reared on *S. pedemontanum*, but this was not significant for first instars ( $P = 0.19$ ) or when considering all stages from first-instar to pupa ( $P = 0.057$ ) (Table 4). The average percentage mortality for *M. messenoides* clutches was higher on its novel host *S. pedemontanum*. This increase was marginally significant for first instars ( $P = 0.050$ ), but not significant when considering all stages from first-instar to pupa ( $P = 0.16$ ) (Table 4).

Larval development times were longer for *M. mazaeus* when reared on *S. leucopogon*, whether laid on *S. leucopogon* or *S. pedemontanum*, and were significantly longer for immatures laid on *S. pedemontanum* (0.5 day longer in first instar,  $P = 0.01$ , and 1.0 day longer from first to fifth instar,  $P = 0.008$ ) (Table 5). *Mechanitis messenoides* clutches also showed suggestive evidence of longer development times on *S. leucopogon*, despite the decreased mortality on this host, but this was not significant for first instars (0.1 day longer,  $P = 0.30$ ) or from first to fifth instars (0.9 day longer,  $P = 0.17$ ) (Table 5).

#### Clutch size

No significant difference in clutch size was observed between the two *M. messenoides* haplogroups ( $t = 0.45$ ,  $P = 0.66$ ; *M. messenoides* haplogroup A, mean no. eggs = 8.85, SD = 4.81,  $N = 13$ ; *M. messenoides* haplogroup F, mean no. eggs = 10, SD = 2.58,  $N = 4$ ). Clutch size was significantly larger for the *M. mazaeus* haplogroup ( $t = 5.19$ ,  $P < 0.0001$ ;

*M. mazaeus* clutches ranged from six to 57 eggs, mean = 28.6, SD = 15.0,  $N = 23$ ) compared with the pooled *M. messenoides* haplogroups (ranged from one to 16 eggs, mean = 9.1, SD = 4.3,  $N = 17$ ). This pattern was maintained across hosts within the *M. mazaeus* haplogroup H and *M. messenoides* haplogroup A, where sample sizes were sufficient to allow comparison. *Mechanitis mazaeus* did not differ in the number of eggs per clutch among three *Solanum* hosts (*S. pedemontanum*, *S. leucopogon* and *S. 'tree naranjilla'*;  $F = 0.60$ ,  $P = 0.56$ ), nor did the *M. messenoides* haplogroup A differ between two hosts (*S. leucopogon* and *S. 'seedling naranjilla'*;  $t = 0.10$ ,  $P = 0.92$ ).

## DISCUSSION

### DIFFERENTIATION OF *M. MAZAEUS* AND *M. MESSENOIDES*

Our results clearly indicate that *M. mazaeus* s.l. (Lamas, 2004) is composed of two species, *M. mazaeus* and *M. messenoides*. Analysis of mtDNA revealed that *M. mazaeus* (haplogroup H) is well differentiated from *M. messenoides* (haplogroups A and F; Fig. 2, Table 2; see also Dasmahapatra *et al.*, 2010a). Strong differences are also evident in the nuclear genome, as seen in the AFLP analysis of Dasmahapatra *et al.* (2010a). We have demonstrated that several morphological traits of adults and immature stages are associated with the observed genetic differentiation (Figs 1, 4–7). Furthermore, each species is ecologically distinct with respect to mimetic relationships and other ecological traits. Interestingly, both species are polymorphic, with some co-mimetic forms between the two species (Fig. 1). Given the polymorphic and mimetic nature of these species, additional study of named mimetic forms, including subspecies, is required to assign them to the correct species (see Appendix S1 and Table S3).

**Table 4.** Summary of larval mortality on *Solanum pedemontanum* and *Solanum leucopogon*

Species	Oviposition host	Reared host	Mortality of first instar		Paired one-tailed Student's <i>t</i> -test		Mortality from first instar to pupa		Paired one-tailed Student's <i>t</i> -test	
			N	Mean %	t	P	N	Mean %	t	P
<i>Mechanitis mazaerae</i>	<i>S. pedemontanum</i>	<i>S. pedemontanum</i>	11	0.0	1.33	0.10	11	74.4	0.02	0.49
	<i>S. pedemontanum</i>	<i>S. leucopogon</i>	11	2.2			11	74.1		
<i>Mechanitis mazaerae</i>	<i>S. leucopogon</i>	<i>S. leucopogon</i>	4	26.5	0.93	0.19	4	88.2	1.85	0.057
	<i>S. leucopogon</i>	<i>S. pedemontanum</i>	4	12.1			4	47.0		
<i>Mechanitis messenoides</i>	<i>S. leucopogon</i>	<i>S. leucopogon</i>	5	0.0	1.86	0.050*	5	18.3	1.08	0.16
	<i>S. leucopogon</i>	<i>S. pedemontanum</i>	5	20.0			5	55.0		

Eggs and larvae of *Mechanitis messenoides* were never found on *S. pedemontanum* in the wild. Sample size N indicates number of clutches. The percentage mortality was calculated for each clutch, and the average was calculated among clutches on each host. \* $P \leq 0.05$ .

**Table 5.** Summary of larval development times on *Solanum pedemontanum* and *Solanum leucopogon*

Species	Oviposition host	Reared host	Duration of first instar (days)		Paired one-tailed Student's <i>t</i> -test		Time to fifth instar (days)		Paired one-tailed Student's <i>t</i> -test	
			N	Mean # d	t	P	N	Mean # d	t	P
<i>Mechanitis mazaerae</i>	<i>S. pedemontanum</i>	<i>S. pedemontanum</i>	11	3.1	2.42	0.01*	6	12.3	2.82	0.008*
	<i>S. pedemontanum</i>	<i>S. leucopogon</i>	11	3.6			6	13.3		
<i>Mechanitis mazaerae</i>	<i>S. leucopogon</i>	<i>S. leucopogon</i>	3	3.3	1.21	0.15	2	12.3	1.85	0.10
	<i>S. leucopogon</i>	<i>S. pedemontanum</i>	3	3.0			2	10.8		
<i>Mechanitis messenoides</i>	<i>S. leucopogon</i>	<i>S. leucopogon</i>	5	3.1	0.55	0.30	5	12.2	1.01	0.17
	<i>S. leucopogon</i>	<i>S. pedemontanum</i>	5	3.0			5	11.3		

Eggs and larvae of *M. messenoides* were never found on *S. pedemontanum* in the wild. Sample size N indicates number of clutches. In some cases here the sample size of clutches compared differs from Table 4, and between first-instar duration and time to fifth instar, as a result of complete mortality on one host in the paired comparison. \* $P \leq 0.05$ .

### MORPHOLOGICAL DIFFERENTIATION OF *M. MAZAEUS* AND *M. MESSENOIDES*

#### *Adults*

Using molecular markers as a guide allowed us to evaluate colour pattern variation and identify characters useful for field identification of these polytypic species. The mimetic polymorphism and variation in adult colour pattern characters observed in this study highlights the difficulty encountered by previous workers in arranging *Mechanitis* solely based on colour pattern. In fact, we encountered several individuals that were difficult to identify based on colour pattern alone (see Appendix S1). However, in contrast to Brown (1977), who found no clear separation based on wing pattern, we identified two adult colour pattern characters that correlate very strongly with observed genetic differentiation (Figs 1, 4). In addition, males can be distinguished by whether they possess dorsal hindwing androconial patches (Fig. 5) and by wing shape.

It appears that the two wing colour pattern characters will serve to differentiate *M. mazaeus* from *M. messenoides* where they co-occur across their ranges. The differences observed in our samples from Ecuador and Peru also appear to discriminate the species at a site in Colombia (see Brown, 1977: fig. 83), indicating that these characters may be useful in Colombia. Based on a relatively small sample of *M. mazaeus* individuals outside of Ecuador and Peru, and images in Fox (1967), Brown (1977), and Neild (2008), it appears that the postmedial band character is less extended in cell M1 in some subspecies farther east (e.g. *M. mazaeus pannifera* in Venezuela and *M. mazaeus fallax* ('elevata' phenotype) in Brazil). However, the number of ventral hindwing spots appears more stable. Introgression from these more easterly subspecies into *M. mazaeus* in western Amazonia may explain the few individuals lacking the extended postmedial band phenotype in our sample (Fig. 4A).

#### *Immatures*

Our study highlights the great utility of using immature stages to help resolve species boundaries. Immature stages have proven useful in helping resolve relationships of nymphalid butterflies at various taxonomic levels (Eltringham, 1916; Brown & Freitas, 1994; Penz, 1999; Penz & Peggie, 2003; Freitas & Brown, 2004; Willmott & Freitas, 2006), and clearly warrant attention in difficult groups. Studying immature morphology is particularly relevant in cases that involve the strong convergent evolution of adult phenotypes, as in the mimetic species described here.

An examination of larval morphology revealed striking differences between *M. mazaeus* and *M. mes-*

*senoides* (Figs 4A, 6, 7). In later instars the overall body coloration differs, with *M. mazaeus* larvae being yellow-green, showing little contrast between the dorsolateral coloration and colour at the base of the lateral tubercles, and increasingly developing dorsal yellow hues (Fig. 6D–F). *Mechanitis mazaeus* is also differentiated by the presence of a dark sclerotized patch above the anus on A10 in the 5th instar, which is lacking in *M. messenoides* (compare Figs 6H, 7H). Some variation was present in the A10 patch precluding identification based on this trait alone (see Appendix S1).

### ECOLOGICAL DIFFERENTIATION OF *M. MAZAEUS* AND *M. MESSENOIDES*

The host plant ecology data reported here supports the close relationship between *M. mazaeus* and *M. messenoides*, and sheds light on the ecological interaction between these two species, which differed in the frequency with which they used different hosts (Table 3). They show less overlap in host use than that found between *M. messenoides* haplogroups, having only two hosts in common, and each species was found to use unique hosts (Table 3; see also Appendix S1). Thus, sympatric *M. mazaeus* and *M. messenoides* appear segregated on local *Solanum* hosts, similar to other *Mechanitis* species (Vasconcellos-Neto, 1986). Clutch size was also significantly different between the species.

Observations on host use and larval performance reported here indicate that divergent host use may facilitate the coexistence of *M. mazaeus* and *M. messenoides*. Although competition may play a role, particularly for prime oviposition sites on young leaves, it is not likely to be the only factor contributing to divergent host use. The *Solanum* hosts of *M. mazaeus* and *M. messenoides* do not seem to be a very limiting resource as they are common at Garzacocha and Añangu. However, *Mechanitis* clutches are likely to be capable of defoliating seedlings or the smallest plants of these otherwise large-leaved trees/shrubs and leafy vines.

There does not appear to be much evidence of physiological adaptation to common hosts. Both *M. mazaeus* and *M. messenoides* were reared to adulthood on the other species' most commonly used host (*S. leucopogon* and *S. pedemontanum*, respectively), and the host-switch experiment did not indicate strong increased mortality on novel hosts (Table 4). This is particularly striking as *M. messenoides* was never found using *S. pedemontanum* in the forest. However, *M. mazaeus* broods laid on *S. pedemontanum* took an extra half-day in the first instar, and an extra day to reach the fifth instar, when reared on *S. leucopogon* (Table 5). This may be partly the result

of the physical defenses that *S. leucopogon* presents for early instars. In contrast to *S. pedemontanum*, the younger leaves and petioles of *S. leucopogon* are covered in dense hairs (trichomes) that could make it difficult for young larvae to move about and feed. However, the gregarious behaviour of *Mechanitis* larvae may mitigate the effect of trichome defenses (Rathcke & Poole, 1975; Young & Moffett, 1979).

Extra time in the larval stage on a particular host probably represents a significant increase in the chance of predation/parasitism, and is therefore a potentially important variable determining host use among populations. Observations in Costa Rica indicate wasps removed entire groups of *M. polymnia isthmia* larvae in the first and second instars, but not once the larvae reached the third instar (Young & Moffett, 1979). The difference in clutch size between *M. mazaeus* and *M. messenoides* may also be the result of predation or parasitism pressures on different hosts. Young & Moffett (1979) indicated that although predation rates on eggs and young larvae of *M. p. isthmia* are high (> 80%), plants on which attacks occurred were patchily distributed. They also reported (Young & Moffett, 1979: 314) that predaceous orthopterans sometimes do not eat entire clutches of *M. p. isthmia* eggs, and it is typical for this to occur with smaller clutch sizes (i.e. fewer than ten eggs). The small clutch size in *M. messenoides* and large clutch size in *M. mazaeus* may represent two different strategies for dealing with predators and parasites: one a bet-hedging strategy that lays many clutches of small size; the other a satiation strategy. Overall, it seems likely that predators and parasitoids play an important role in shaping host use and ecology in these species.

In addition, elevational differences have long been recognized among taxa in the *M. mazaeus* species group, and our results indicate that this is an important factor in *M. mazaeus* and *M. messenoides*. Although sympatric over a large area of the upper Amazon, *M. messenoides* and *M. mazaeus* actually have distinct elevational preferences in the Andean foothills. In eastern Ecuador *M. messenoides* occurs commonly above 1000 m a.s.l. up to as high as 1600 m a.s.l., whereas *M. mazaeus* is rarely found above 700 m a.s.l., and not above 950 m a.s.l. *Mechanitis messenoides* is thus typically a member of mimicry complexes that are essentially Andean, whereas *M. mazaeus* belongs to complexes that are typically lowland. Whether initial shifts in wing pattern driven by changing communities of co-mimics across the Andean elevational gradient were responsible for the initial divergence between *M. messenoides* and *M. mazaeus* remains to be investigated.

#### DIFFERENTIATION BETWEEN *M. MESSENOIDES*

##### HAPLOGROUPS A AND F

Despite the large sequence differences existing between *M. messenoides* mtDNA haplogroups A and F (Table 2), no strong morphological differences were found (Fig. 4). The tantalizing possibility that the two abundant and relatively distinct *M. messenoides* subspecies, *M. mes. messenoides* and *M. mes. deceptus*, are actually species that exhibit morphological/ecological differences correlated with haplogroups A and F was not supported. The only difference found between haplogroups A and F was in the frequency of the relatively rare 'phasianita' and 'nigroapicalis' phenotypes, although more data are needed to confirm this. Our analysis included small sample sizes for these morphs, raising the possibility that the association will not be maintained with further and more appropriate local geographic sampling. In addition, whether or not these morphs are distinct or represent extremes of variation also requires further study.

Overall, the data indicate a lack of ecological differences between the two *M. messenoides* haplogroups that is consistent with the lack of morphological difference. The two haplogroups did not differ in clutch size, and broadly overlapped in host use (Table 3). Any host-use differences noted are very likely the result of only having a few observations for *M. messenoides* haplogroup F. The lack of morphological and ecological differences between *M. messenoides* haplogroups A and F is consistent with the genome-wide AFLP analysis and nuclear gene sequencing of Dasmahapatra *et al.* (2010a), which found no support for genotypic clustering within *M. messenoides*. Given the questions surrounding the forms 'phasianita'/ 'nigroapicalis', and the lack of other strong differences between haplogroups A and F, it seems the mtDNA polymorphism within *M. messenoides* represents ancestral polymorphism rather than differentiation associated with adult or immature-stage morphology.

#### POLYMORPHISM AND MIMICRY IN *M. MAZAEUS* AND *M. MESSENOIDES*

In combination, our ecological, morphological, and genetic data from eastern Ecuador and Peru provide strong evidence that both *M. messenoides* and *M. mazaeus* are separate species, with each being polymorphic with multiple sympatric forms (Fig. 1). The sympatric forms of both species participate in mimicry complexes with sympatric species of *Melinaea*, *Forbestra*, *Hypothisis*, *Heliconius*, and other butterflies and moths. Thus it appears that D'Almeida (1951, 1978), Fox (1967), and Brown (1977) were all partly correct: *M. mazaeus* does, as Brown

(1977: 188) saw it, 'occur in highly polymorphic populations'. However, there are in actuality at least two ecologically and genetically distinct separate species existing in sympatry, as hypothesized by D'Almeida (1951, 1978), Fox (1967), D'Abrera (1984), and Beccaloni (1997): we here identify these as *M. mazaeus* and *M. messenoides*.

Although both *M. mazaeus* and *M. messenoides* are often highly polymorphic, the frequency of morphs in each species varies across our sampling area. For example, in eastern Ecuador, the melanic forms *M. messenoides messenoides* and *M. messenoides deceptus* are both found in high frequency. In the vicinity of Añangu and Garzacocha *M. messenoides messenoides* is most common, comprising 57.6% of *M. messenoides* observed in the forest (91 *M. mes. messenoides*, 65 *M. mes. deceptus*, and two other forms; M. Elias, R. Hill, K. Willmott, unpubl. data). Near Villavicencio, Colombia, however, the *M. messenoides* population is nearly fixed for the *M. messenoides messenoides* form (J. Mallet, pers. observ.). To the south, the *M. messenoides deceptus* morph dominates, and this form also increases to very high frequency at higher elevations (~1000 m a.s.l.) in eastern Ecuador and northern Peru (K. Willmott, pers. observ.). Although we have not quantified it here, similar variation in frequency of morphs from different localities occurs in *M. mazaeus* (K. Willmott, pers. observ.).

The frequency of sympatric forms of *M. mazaeus* and *M. messenoides* appears correlated with the abundance of other mimetic species. *Mechanitis messenoides messenoides* and *M. messenoides deceptus* are both abundant in eastern Ecuador, where they fly with other members of the well-represented 'yellow-bar tiger' and 'orange and black tiger' mimicry complexes, respectively (Elias *et al.*, 2008; Hill, 2010; see also Beccaloni, 1997). The third *M. messenoides* form, the 'nigroapicalis' phenotype (Fig. 1C), lacks yellow markings and is present in this area in very low frequency, but flies there with the relatively rare and strikingly similar *M. mazaeus mazaeus*, also lacking yellow. To the south, the *M. messenoides* form resembling the 'phasianita' phenotype (Fig. 1D) becomes more abundant, tracking the increased presence of a mimicry complex more common in eastern Peru. Thus it appears that the change in abundance of unpalatable co-mimetic species at larger geographic scales explains the local frequency of morphs in *M. mazaeus* and *M. messenoides*. However, smaller scale spatial and temporal fluctuation in co-mimics may also play a role (Brown & Benson, 1974; Kapan, 2001). The existence and maintenance of polymorphism in local populations of *M. mazaeus* and *M. messenoides* is thus a result of the dynamics of selection for fitting into local mimicry complexes.

It is also worth noting that *M. mazaeus* and *M. messenoides* differ in their mimetic interactions. The two species differ in which mimicry complexes they participate, indicating another dimension by which they differ ecologically. In eastern Ecuador, *M. mazaeus fallax* is abundant and variable, with individuals that match either the 'yellow-bar tiger' or, more commonly, the 'tiger' mimicry complexes (see Beccaloni, 1997). Thus, both *M. mazaeus* and *M. messenoides* participate in the 'yellow-bar' and 'tiger' complexes, but only *M. messenoides* fits well with the 'orange and black tiger' complex. In sum, our analysis indicates that these two species exist in stable polymorphic populations that are probably maintained because of a combination of selection to match other coexisting aposematic species, and immigration of forms that are more abundant in adjacent geographic areas (Brown & Benson, 1974; Joron *et al.*, 1999; Joron & Iwasa, 2005).

#### GENETIC DIFFERENTIATION IN *MECHANITIS* SPECIES

Despite the usefulness of genetic tools for helping determine species boundaries among *Mechanitis* butterflies, obvious difficulties are indicated in our mtDNA results, as in the nuclear results of Dasmahapatra *et al.* (2010a), and much work remains to be done. Although *Mechanitis* belongs to a lineage that diverged early in ithomiine evolution (Brower *et al.*, 2006; Willmott & Freitas, 2006), there appears to have been rampant recent radiation, given the obvious paraphyly in mtDNA lineages. *Mechanitis* and *Forbestra* diverged just 12.85 Mya (Wahlberg *et al.*, 2009), indicating that they are among the youngest ithomiine genera. The recent origin of *Mechanitis* coupled with high effective population sizes and extensive ancestral polymorphism has probably resulted in the widespread mtDNA lineage paraphyly. This is particularly striking in *M. lysimnia* and *M. polymnia*, two species thought to be well differentiated on morphological grounds, which appear paraphyletic with multiple mtDNA haplogroups (Fig. 2). This pattern is potentially exacerbated by continued hybridization and introgression. Vasconcellos-Neto & Brown (1982) described hybridization occurring relatively frequently in dense dry-season populations between *M. polymnia* and *M. lysimnia*. In this situation, pheromone signals from males of one abundant species caused conspecific females to become receptive, thereby facilitating pairing to heterospecific males. Interestingly, some evidence for hybridization between these species is suggested by an earlier STRUCTURE analysis of AFLP data (Dasmahapatra *et al.*, 2010a).

Although several *Mechanitis* species now appear to contain multiple mtDNA haplogroups, it is rare for

two species to belong to the same haplogroup. One exception is the splinter lineage, composed of *M. lysimnia macrinus*, *M. lysimnia utemai*, and *M. lysimnia solaris*, which is strongly supported as belonging to the *M. mazaeus* haplogroup (Fig. 2). No other *M. lysimnia* or *M. polymnia* individuals were found with mtDNA lineages of other species. This exception may represent an extreme case of ancestral polymorphism, or it could indicate something else. Perhaps there has been introgression of mtDNA between *M. lysimnia solaris* and *M. mazaeus* in Venezuela, with further introgression among *M. lysimnia* subspecies in Colombia, Panama, and Ecuador. Alternatively, it could be that this is a previously unrecognized *M. mazaeus* lineage. The paraphyletic nature of *Mechanitis* species and status of north-west South American *Mechanitis* species will be investigated in further detailed phylogenetic studies using greater subspecies coverage.

In conclusion, by integrating evidence from adult and larval morphology with ecology and genetic markers, we were able to clarify the identity of mimetic species in a region harbouring some of the world's most diverse forests. In retrospect, it becomes clear that the taxonomic difficulty of the *M. mazaeus* species complex stems from the difficulty of using wing colour pattern characters in this genus. These are remarkably variable within species, and because of mimicry, rather similar between species. Genetic data, host plant information, and larval morphology all help, but also show some variability and a tendency to partially overlap among species. Some of these difficulties may be the result of shared ancestral polymorphism, whereas other polymorphisms may be the result of occasional hybridization. The difficulty of inferring species boundaries may be a common problem for other insect taxa with large, relatively stable population sizes across this vast region.

A biogeographic pattern of elevationally distinct sister taxa is common in the east Andean foothills, not only among co-mimics of *M. mazaeus* and *M. messenoides* [e.g. the ithomiine genera *Melinaea* and *Hypothenemis*, and the heliconiine genera *Heliconius* and *Eueides*; Dasmahapatra *et al.*, 2010b], but also in many other butterfly groups (e.g. *Adelpha*; Willmott, 2003). Further intensive studies incorporating multiple lines of evidence, such as we describe here, are needed to test species limits among all of these taxa. If our results are broadly applicable, the world's biologically richest region is likely to be even more diverse than previously thought.

#### ACKNOWLEDGEMENTS

We thank Ecuador's Museo Ecuatoriano de Ciencias Naturales and Ministerio del Ambiente for permits,

La Selva Jungle Lodge and the Napo Wildlife Center for logistical support, L. Tipan, J. Vaca, A. Toporov, and R. Aldaz for field assistance, S. Knapp and M. Nee for help with host-plant determinations, and A. Wu, L. Go, J. Sachs, E. Simms, M. Brunell, and S. Luthy for help and support with lab work. M. Joron kindly provided specimens from Peru. We would also like to thank M. Kronforst, R.H.'s undergraduate research students at the University of the Pacific, and five anonymous reviewers for their comments on the article. R.H. was supported by the Margaret C. Walker Fund for teaching and research in systematic entomology, the Department of Integrative Biology at UC Berkeley, Center for Systems Biology at Harvard University, and Department of Biological Sciences at University of the Pacific. M.E., K.W., and C.J. were funded by the Leverhulme Trust (UK). K.W. acknowledges the support of the National Science Foundation (DEB 0639861). K.D. and J.M. were supported by funding from NERC and BBSRC.

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

**Appendix S1.** Supplementary discussion.

**Table S1.** Host-plant records, voucher information, and accession numbers for *Mechanitis mazaeus* and *Mechanitis messenoides* from eastern Ecuador. GenBank accession numbers for the *Forbestra olivencia* and *Mechanitis* individuals from previous work are: FJ445857–FJ445860, FJ445863, FJ445865–FJ445869, FJ445870, FJ445872–FJ445879, FJ445881–FJ445891, FJ445899–FJ445904, FJ445906, FJ445908, FJ445910–FJ445920, FJ445923, FJ445924, FJ445926, FJ445928–FJ445935, FJ445941–FJ445954, EU068843–EU068856, EU068966, EU068981, EU068993–EU068900, EU069070–EU069075, and DQ157513–DQ157515 (Brower *et al.*, 2006; Elias *et al.*, 2007; Dasmahapatra *et al.*, 2010a).

**Table S2.** Additional new *Mechanitis* individuals included in analysis of *COI* in this study.

**Table S3.** Taxonomic changes involving *Mechanitis mazaeus* and *Mechanitis messenoides*.

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