A CRYPTIC SPECIES OF *HELICONIUS* BUTTERFLY PROVIDES EVIDENCE OF VICARIANT SPECIATION IN THE AMAZON VIA A HOST PLANT SHIFT

Neil Rosser[1,2*], André V. L. Freitas[3], Blanca Huertas[4], Mathieu Joron[5], Gerardo Lamas[6], Claire Mérot[7], Fraser Simpson[8], Keith Willmott[9], James Mallet[2], Kanchon K. Dasmahapatra[1].

1. Department of Biology, University of York, Wentworth Way, York YO10 5DD, UK

2. Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, Massachusetts 02138, USA

3. Departamento de Biologia Animal and Museu de Zoologia, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, São Paulo, Brazil.

4. Department of Entomology, Natural History Museum, Cromwell Road, London SW7 5BD, UK.

5. Centre d'Ecologie Fonctionnelle et Evolutive, UMR 5175 CNRS - Université de Montpellier - Université Paul Valéry Montpellier - EPHE, 1919 route de Mende, 34293 Montpellier, France

6. Museo de Historia Natural, Universidad Nacional Mayor de San Marcos, Apartado 14-0434, Lima-14, Peru.

7. IBIS, Université Laval, 1030 Avenue de la Médecine, Québec, Canada.

8. Department of Genetics, Evolution and Environment, University College London, London WC1E 6BT, United Kingdom;

9. McGuire Center for Lepidoptera and Biodiversity, Florida Museum of Natural History, University of Florida, Gainesville, FL, USA

*Author for correspondence; neil_rosser@fas.harvard.edu

ABSTRACT

The evolution of reproductive isolation via a switch in mimetic wing coloration has become the paradigm for speciation in aposematic *Heliconius* butterflies. Here, we provide an interesting counterexample to this, by demonstrating the presence of a cryptic species within *Heliconius demeter* Staudinger, 1897. Amplified fragment length polymorphisms identify two sympatric genotypic clusters in northern Peru, corresponding to subspecies *Heliconius demeter ucayalensis* H. Holzinger & R. Holzinger, 1975 and *Heliconius demeter joroni* **ssp. nov.** These subspecies are reciprocally monophyletic for the mitochondrial genes *CoI* and *CoII* and the nuclear gene *Ef1a*, and exhibit marked differences in adult/larval morphology and host plant use. *CoI* sequences from 13 of the 15 currently recognized subspecies show that mtDNA differences are reflected across the range of *H. demeter*, with a deep phylogenetic split between the southern and northern Amazonian races. As such, our data suggest vicariant speciation driven by disruptive selection for larval performance on different host plants. We raise *Heliconius demeter eratosignis* (Joicey & Talbot, 1925) to *Heliconius eratosignis* based on nomenclatural priority, a species comprising *H. demeter ucayalensis* and two other southern Amazonian races. *Heliconius demeter joroni* **spp. nov.** remains within *H. demeter sensu stricto*, along with northern Amazonian and Guianese subspecies. This study documents the discovery of a cryptic species in one of the best-studied groups of insects, and implies that numbers of species estimated using current taxonomy may be underestimates.

Keywords: Butterflies, cryptic species, DNA taxonomy, genotypic species concept, mimicry, vicariant speciation, host plant shift.

INTRODUCTION

In recent years, speciation studies have increasingly focused on the drivers and genetic basis of reproductive isolation (Via 2001; Butlin et al. 2008). In particular, ecological speciation has become the focus of an extensive and productive field of research (Nosil 2012). Ecological speciation is said to occur when ecologically-based selection between populations pleiotropically creates reproductive isolation (Schluter 1996). For example, Bahamas mosquito fish (*Gambusia hubbsi* Breder, 1934) living in habitats with different predation intensities have evolved different body shapes as a response. Because the fish also use body shape as a mate choice cue, this has created reproductive isolation that is twice as strong between populations under different predation regimes than between populations under the same predation regimes (Langerhans et al. 2007). One of the main alternatives to ecological speciation is mutation order speciation, in which reproductive isolation is created through the fixation of different, potentially epistatic incompatibilities in separate populations subject to similar selective pressures (Clarke et al. 1988; Mani and Clarke 1990; Orr 1995; Turelli and Orr 2000). For example, reproductive isolation may evolve as a by-product of intragenomic conflict resolution, such as meiotic drive or cytoplasmic male sterility (Hurst and Pomiankowski 1991; Schluter 2009). Consequently, species formed by mutation order speciation may be less likely to exhibit phenotypes obviously divergent to a human observer than those formed by ecological speciation, i.e. purely mutation order effects are liable to produce “cryptic” species. In addition, because mutation order speciation is thought likely to occur only in allopatry (Gavrilets 2004; Nosil and Flaxman 2011), it does not predict the ecological divergence that ecological speciation with gene flow requires

(Coyne and Orr 2004). However, mutation order speciation via sexual selection may lead to clearly observable divergence in sex-related traits. For example, under Fisherian runaway selection, population divergence is driven by arbitrary female preference for a male trait, and could lead to marked divergence among populations (Lande 1981; Clarke et al. 1988).

Heliconius butterflies are chemically defended and aposematic, i.e. they advertise their defence to would-be predators using bright colours on their wings. To minimise the per capita cost incurred while predators learn the association between the warning signal and prey unprofitability, many *Heliconius* species mimic one another. This mutualistic interaction is known as Müllerian mimicry (Müller 1879). Within *Heliconius*, a number of distinct mimetic phenotypes exist (e.g. blue and yellow, red and black). Groups of sympatric species exhibiting the same phenotype are said to be co-mimics in a "mimicry ring". It has been convincingly shown that when a population switches to a different mimicry ring this can create reproductive isolation with the ancestral population. The reasons for this are twofold. Firstly, hybrids with intermediate colour patterns are selected against by predators that do not recognise them as aposematic (Merrill et al. 2012). Secondly, *Heliconius* males of several species have been shown to preferentially court females with the same colour pattern as themselves (Jiggins et al. 2001b; Kronforst et al. 2006; Mavárez et al. 2006; Chamberlain et al. 2009; Merrill et al. 2011). Because divergence in an ecologically relevant adaptive trait also creates reproductive isolation, *Heliconius* have become known as a prime examples of ecological speciation (Nosil 2012). Furthermore, most *Heliconius* sister species pairs have different mimetic phenotypes (Turner

1976; Rosser et al. 2015), leading reproductive isolation by mimicry shift to become a paradigm for speciation in the genus (Jiggins 2008; Mérot et al. 2017).

Nonetheless, there are instances of *Heliconius* sister species that do not appear to have diverged in wing colour pattern. For example, *Heliconius sara* (Fabricius, 1793) and *H. leucadia* Bates, 1862 are sympatric sister species with almost identical blue and yellow phenotypes. *Heliconius numata* (Cramer, 1780) and *H. ismenius* Latreille, 1817 are parapatric sister species with similar "tiger" colour patterns. In addition, modern taxonomy and DNA sequencing have revealed a number of cryptic races belonging to the *H. cydno*/*timareta* superspecies from the tropical eastern Andes (Brower 1996; Lamas 1997; Giraldo et al. 2008; Mallet 2009; Mérot et al. 2013; Arias et al. 2017). These taxa were hitherto unrecognised as members of the *H. cydno-timareta* clade because they exhibit colour patterns extremely similar to those of races of *H. melpomene* (Linnaeus, 1758), itself the sister to the *H. cydno-timareta* lineage. It has been shown that in some cases, this striking phenotypic similarity is likely due to adaptive introgression of colour patterns between *H. melpomene* and *H. timareta* Hewitson, 1867 (*Heliconius* Genome Consortium 2012). These examples suggest that speciation in *Heliconius* may sometimes occur without a mimicry shift, and at the very least demonstrate that closely related species can maintain their identities with similar mimetic phenotypes and despite occasional hybridisation (Mérot et al. 2017).

Based on previous systematic research, *Heliconius demeter* Staudinger, 1897 was held to comprise 15 described subspecies with red, yellow and black phenotypes (Figure 1) that participate in the "dennis-rayed" *Heliconius* mimicry ring (Brown and Benson 1975; Lamas

2004). The taxon is widely distributed throughout most of Amazonia and the Guiana shield, but is usually scarce when compared to closely related co-mimics, such as *H. erato* (Linnaeus, 1758). Interestingly, several northern Amazonian and Guianese races of *H. demeter* are sexually dimorphic, with hindwing rays in males fused at their base to form a bar. Sexual dimorphism in colour pattern is very rare in *Heliconius*, and known in only one other species; *Heliconius nattereri* C. Felder & R. Felder, 1865, from south-eastern Brazil.

In the easternmost cordillera of the Andes in northern Peru, we discovered what we at first took to be two *H. demeter* races, *H. demeter* cf. *demeter* and *H. demeter* cf. *ucayalensis* H. Holzinger & R. Holzinger, 1975*,* flying together in sympatry near the city of Tarapoto. *H. demeter* cf. *demeter* is sexually dimorphic, but *H. demeter* cf. *ucayalensis* is not. At first we viewed these taxa as somewhat divergent subspecies, since we had evidence for contacts of multiple butterfly subspecific taxa in a 'suture zone' in this area (Dasmahapatra et al. 2010). In the present study, detailed examination of inconspicuous morphological characteristics, coupled with reciprocal monophyly at single locus DNA markers and the presence of distinct multilocus genotypic clusters, reveal that these races of *H. demeter* in fact comprise two distinct species. Furthermore, morphological, behavioural and molecular phylogenetic evidence show that this split is apparent across Amazonia, with *H. demeter sensu lato* comprising two clades; *H. demeter* cf. *demeter* and the northern races, and *H. demeter ucayalensis* and the southern races. In accordance with nomenclatural priority, the southern clade is recognized as *H. eratosignis* (Joicey & Talbot, 1925), a species comprising four subspecies (Lamas and Jiggins 2017; Table S1*),* and this nomenclature is adopted from hereon. Additionally, it was noted that *H. demeter* cf. *demeter*

from Tarapoto were divergent from those in the western Amazonian lowlands, with Tarapoto specimens exhibiting a strongly narrowed forewing yellow band, similar to that of *H. melpomene aglaope* C. Felder & R. Felder, 1862 and *H. erato emma* Riffarth, 1901. Accordingly, this Andean population is here described as a **new subspecies**: *Heliconius demeter joroni*.

MATERIALS AND METHODS

Morphological and behavioural analysis

To identify species-specific diagnostic characters in the 15 currently recognised subspecies of *H. demeter* and *H. eratosignis*, all type series and specimens held in the Natural History Museum London (NHMUK) were examined in detail. In addition, we examined holotypes, allotypes, syntypes and other material held at the Florida Museum of Natural History (FLMNH), Museum für Naturkunde, Berlin (MNB), the Natural History Museum at the San Marcos National University, Lima, Peru (MUSM), the Naturhistorisches Museum, Wien (NHMW), the National Museum of Brazil, Rio de Janeiro (MNRJ), the Museum of Zoology "Adão José Cardoso" at the University of Campinas, Brazil (ZUEC), and the Museum of Zoology at the University of São Paulo, São Paulo, Brazil (MZUSP) (Table S2).

For morphometric analyses of wing shape, images of the ventral and dorsal surfaces of dissected forewings and hindwings of 75 *H. eratosignis ucayalensis*, 31 *H. demeter joroni* **ssp. nov.**, 16 *H. demeter bouqueti* specimen from Tarapoto and French Guiana were captured using a high-resolution flatbed scanner or a Nikon D90 digital camera with a Nikon micro 105/2.8GEDVR lens. In addition, we conducted a global geometric morphometric analysis using 31 photographs

of museum specimens representing eight other subspecies. All specimens used in morphometric analysis are shown in Table S3. Specimens used in morphometric analysis

Wing shape was described on the forewing and the hindwing, using respectively 20 and 18 landmarks, placed at vein intersections and vein termini on the ventral side, as visible on Figure S1. Standard tests of repeatability were carried out by taking the landmarks five times per wing on subsamples of five butterflies from a single subspecies and sex. Landmark coordinates were digitalized using TpsDig2 (Rohlf 2010) and superimposed using a general Procrustes analysis (Bookstein 1991; Zelditch et al. 2004). Wing size was measured using log-transformed centroid size (Bookstein 1991). Differences in size between *H. d. joroni* **ssp. nov.** and *H. e. ucayalensis* were investigated with a one-way ANOVA with size as a dependent variable and species/sex as factors. Because of multiple comparisons, p-values were corrected following Benjamini and Hochberg (1995).

To study shape, dimensionality reduction was employed to correct for the effect of using a large number of variables relative to the number of specimens. We used the minimum subset of principal components (PCs) that minimized the total cross-validated misclassification percentages between groups defined *a priori* (Baylac and Friess 2005). To explore shape differences between *H. d. joroni* **ssp. nov.** and *H. e. ucayalensis*, a MANOVA was applied to those subsets of PCs, with shape as dependent variable and sex/species as factor. Given the high sexual dimorphism, species discrimination based on shape was investigated for each sex separately through a Canonical Variate Analysis (CVA) with a leave-one-out cross validation procedure (CV). All statistics and

morphometrics were performed in R. 2.13.1 (R Development Core Team, 2011) with ade4 (Chessel et al. 2004) and Rmorph libraries (Baylac 2007).

Genitalia of three male *H. demeter joroni* **ssp. nov.** and seven male *H. eratosignis ucayalensis* collected from Tarapoto were prepared from material preserved in salt-saturated DMSO. Tips of the abdomens were removed and soaked in 10 % KOH for 10 minutes at 70 °C, and then transferred to distilled water. Scales were removed with a fine brush and the valves extruded. Genitalia were removed and further cleaned. Temporary slides were prepared in 25 % ethanol, and the interior surface of each left valva photographed.

Observations on host plant use and larval morphology were made around Tarapoto. To supplement field observations of host plant use, wild caught adult females were placed in a cage with 22 locally common *Passiflora* species (Table S4. List of *Passiflora* species used in captive host plant oviposition tests.) and allowed to oviposit. Geographic localities for *H. demeter* and *H. eratosignis* were obtained from those published in Rosser et al. (2012) and supplemented with subsequent collections in Bolivia, Brazil, French Guiana, Peru and Suriname between 2011 and 2017, as well as collections by Keith Brown from 1970 to 1999, by AVLF in Mato Grosso and Acre from 1994 to 2016, and from Eurides Furtado from 1978 to 1998.

Molecular analysis

Details of specimens used for molecular work are shown in Table S5. Wings were removed from samples collected in French Guiana and around Tarapoto, in northern Peru, and the bodies preserved at -20°C in salt-saturated DMSO. Both wings and tissue of the French Guiana

specimens are held at the Muséum National d'Histoire Naturelle, Paris (MNHN), while the Peruvian specimens are held at the University of York, UK. In addition to these, single legs were obtained from dried museum specimens of 11 other subspecies from the Florida Museum of Natural History (FMNH; identification numbers beginning with "KW" in Table S5). DNA was extracted from single legs using the QIAamp DNA Micro Kit (QIAGEN), and from one-third of the thorax of the remaining specimens using the DNeasy Blood and Tissue Kit (QIAGEN).

Approximately 2200 bp of mtDNA comprising the *CoI* and the 5' end of the *CoII* genes was amplified by PCR in three sections in seven *H. demeter joroni* **ssp. nov.** and 12 *H. eratosignis ucayalensis* collected around Tarapoto, as well as in 12 *H. demeter bouqueti* Nöldner, 1901 from French Guiana. Four autosomal nuclear genes *Elongation factor 1-α* (*Ef1α*), *Tektin*, *Ribosomal protein L5* (*Rpl5*), *Mannose-phosphate isomerase* (*Mpi*) and the sex-linked *Triose phosphate isomerase* (*Tpi*) were also successfully sequenced for varying numbers of these three taxa. Only small amounts of degraded DNA could be obtained from the museum specimens. Therefore, for these samples the first ~760 bp of the mitochondrial *CoI* was amplified in two shorter sections. All PCR products were cleaned and cycle sequenced with the PCR primers using the BIG DYE TERMINATOR v. 3.1 Cycle Sequencing Kit (Applied Biosystems), and sequences obtained using an ABI3730xl DNA Analyzer (Applied Biosystems). Table S6 contains details of the primers used and PCR conditions. A representative sequence from each *sara-sapho* group *Heliconius* species was downloaded from Genbank to act as outgroups. GenBank accession numbers for all sequences used are provided in Table S7 and Table S8. *S*equences were aligned using ClustalW, and the alignments then checked by eye.

Phylogenetic analysis of sequence data was carried out using MEGA7 (Kumar et al. 2016). For each gene, we found the nucleotide substitution model that best described the substitution pattern using all sites, a Neighbour-joining tree and Bayesian Inference Criterion (BIC). We then found the maximum likelihood (ML) tree for each gene assuming the selected model of sequence evolution, and estimated tree reliability using 1000 bootstrap replicates. To obtain higher resolution nuclear data, eight specimens each of *H. eratosignis ucayalensis*, *H. demeter joroni* **ssp. nov.** and *H. demeter bouqueti* from French Guiana were genotyped using four AFLP primer combinations: *TaqI*-CAG with *EcoRI*-ATG, *TaqI*-CGA with *EcoRI*-AGC, *TaqI*-CAG with *EcoRI*-AGC and *TaqI*-CCA with *EcoRI*-ACA. The AFLP protocol used is similar to that described in (Vos *et al.* 1995), and the primer sequences and reaction conditions are described in Madden *et al.* (2004). The AFLP products were resolved by electrophoresis through 6% acrylamide gels, visualised by autoradiography, and scored by eye. A total of 81 loci were polymorphic and could be scored unambiguously in the remaining specimens.

The Bayesian clustering program STRUCTURE 2.2 (Pritchard et al. 2000) was used to evaluate the number of genetic clusters indicated by these AFLP genotypes using standardized inference criteria (Evanno et al. 2005). Following a 100,000 step burn-in period, data were collected over 100,000 Markov chain Monte Carlo repetitions. STRUCTURE analysis was carried out on the data set, increasing *K* from 1 to 10. At each value of *K*, the analysis was repeated three times to check between-run consistency. The AFLP data was also used to calculate pairwise Nei-Li genetic distances (Nei and Li 1979) between all genotyped individuals. These distances were then used to calculate average genetic distances between each of the three taxa.

RESULTS

Adult morphology

Examination of *Heliconius demeter joroni* **ssp. nov.** and *H. eratosignis ucayalensis* wings from Peru revealed two diagnostic morphological characters strictly concordant with genetic classification of these two species. 1) In the proximal region of the narrow costa-subcosta space on the underside of the forewing, *H. demeter joroni* **ssp. nov.** exhibits a strong yellow 3-5mm long streak placed in the anterior half of the space along the costal vein, often associated with black scales posteriorly (Figure 2a). In *H. eratosignis ucayalensis* this region is uniformly orange (Figure 2b). Brown and Benson (1975) also noticed this character difference between Southern and Northern "yellow rather than red basocostal line on the ventral forewing", but concluded it was "variable." They likely did not appreciate the distinctness of this trait, due to a failure to observe long series of these species in sympatry, which we here document for the Tarapoto region. 2) In males of *H. demeter joroni* **ssp. nov.** the red rays on the dorsal hindwing fuse to form a hindwing bar (Figure 2c), while in males of *H. e. ucayalensis* they do not (Figure 2d). This character is inapplicable to females, all of which have unfused red rays, and to geographic forms of *H. demeter* from the Guianas and nearby that lack rays. One clear difference in genital morphology was observed between the males of the *H. d. joroni* **ssp. nov.** (n=3) and *H. e. ucayalensis* (n=7): the posterior tip of the valva present a rounded profile in *H. d. joroni* while in *H. e. ucayalensis* this region had a characteristic convex depression (Figure S5). However, the utility of this trait is unclear given the small sample sizes.

Using the presence/absence of the yellow costal streak on the underside forewing, the existing 15 named subspecies of *H. demeter* could be unambiguously classified as either belonging to *H. demeter* or *H. eratosignis CoI* haplogroups (see below). With the exception of *Heliconius demeter titan* Neukirchen, 1995, in all male specimens the presence of the yellow costal streak was also perfectly concordant with fused or reduced hind wing rays (Table S9). In *H. d. titan* the hindwing rays in male appeared intermediate between those of *H. demeter* and *H. eratosignis*, and *H. d. titan* was also intermediate for other less clear cut characters (see subspecies description for *H. d. joroni* **ssp. nov.**).

Description of the new subspecies from Tarapoto

Heliconius demeter joroni Lamas and Rosser, **new subspecies** (Figure S6)

Heliconius demeter [n. ssp.] Lamas, MS: Lamas, 2004: 268.

Diagnosis: *Heliconius demeter joroni* **ssp. nov.** is similar to *H. demeter demeter*, but differs from Staudinger's syntypes of. *H. demeter* from Iquitos, Loreto, Peru (now in the MNB), in having a much narrower yellow postmedian band on the dorsal forewing. It is known only from the Cordillera Escalera, near Tarapoto, Peru (Figure 1), where its co-mimics include *Heliconius eratosignis ucayalensis*, *Heliconius elevatus pseudocupidineus* Neustetter, 1931, *Neruda aoede cupidineus* (Stichel, 1906) and *Eueides tales michaeli* Zikán, 1937 among others. Males are easily distinguishable from all sympatric taxa through the fused rays on the hindwing dorsum and the yellow costal streak on the forewing underside. Females may be distinguished from co-mimics through the configuration of the rays (which radiate from the cell), small size, length of

the antennae (longer than the forewing discal cell) and the presence of the forewing underside yellow costal streak. Both sexes usually exhibit a single row of white submarginal dots along the anal margin of the ventral hindwing, which can be used to help separate the females from *H. erato emma* and *E. tales michaeli*. However, this character can be faint or even missing in *H. demeter joroni* **ssp. nov.**, and occasionally present in *H. erato emma*. In addition, the latter is confined to the Amazonian lowlands adjacent to the Cordillera Escalera, and at present there is no evidence to suggest that they regularly co-occur, barring occasional migrants.

Male. Forewing length: (35.5–40 mm, mean = 38.25 mm, n = 10). Forewing dorsum with a yellow postmedian band from R_1 to Cu_1, with maximum width of 8mm. The band usually more or less straight or bowed slightly outwards distally (indented distally in *H. e. ucayalensis*). At the edges of the band a slight overlap of yellow scales on the black background, producing a greenish tinge both discally and distally, but this character less pronounced than in *H. demeter demeter* or *H. eratosignis ucayalensis*. Some specimens exhibit a faint greenish spot in the middle of cell Cu_1-Cu_2. Dennis brick red, reaching roughly two-thirds the length of the discal cell. Anal bar of dennis shorter than other dennis elements, and tends to become separated from the anal margin (longer and tends to fill nearly to the anal margin in *H. e. ucayalensis*). Forewing more elongate and pointed than in *H. e. ucayalensis*, usually with a bulge in the margin near end of Cu_1 (absent in *H. e. ucayalensis*). Ventral surface similar to dorsum, but with ~~the~~ dennis and postmedian band less bright and reduced relative to dorsum. Base of the narrow costa-subcosta space with a strong yellow 3-5mm long streak placed adjacent to costa, often

associated with black scales posteriorly. Anal cell space (aft of 2A) tends to be narrower than in *H. e. ucayalensis*, fitting with the narrower friction patch

Hindwing: On dorsum the grey friction patch is narrow, and the ray in cell Rs-M_1 is strongly present, forming the anterior tip of the bar of fused rays (in *H. e. ucayalensis* rays are unfused and the friction patch is broad leading to almost complete loss, or reduction to a smudge, of the ray). On the ventral side a yellow costal streak, a single row of white submarginal dots along the anal margin, and some diffuse red spots at the bases of Cu_2, Sc+R_1 and the discal cell. Rays reduced relative to the dorsal side, and unfused.

Female. Forewing length: 35–39.5 mm, mean = 36.8 mm , n = 5). As the male, except no friction patch or greenish tinge to forewing postmedian band on dorsum, and no greenish spot in the middle of cell Cu_1-Cu_2. Hindwing: the subcostal ray on cell Sc+R_1-Rs is expressed on dorsum in full orange-red (expressed in pale whitish scales in *H. e. ucayalensis*). Also distinguishable from males by the five-segmented prothoracic tarsus (fused in male) and external genitalia.

Type material: Holotype ♂, PERU, San Martín, Tarapoto, San Roque, 500m, 06º22'S, 76º26'W, 28.iii.2016 (N. Rosser leg.). Deposited in the Natural History Museum at the San Marcos National University, Lima, Peru (MUSM). Paratypes (all from PERU, San Martín): 2♂, same data as holotype; 1♂, 5♀, km 17 Tarapoto-Yurimaguas, 1000m, 06º27'S, 76º17'W, 20.xi.1999 (G. Valencia leg.); 1♂, km 17 Tarapoto-Yurimaguas, 1000m, 06º27'S, 76º17'W, 11.xii.1999 (M. Joron leg.); 1♂, km 19 Tarapoto-Yurimaguas, 1300m, 06º27'S, 76º17'W, 26.viii.2002 (C. Jiggins

leg.); 1♂, km 22 Tarapoto-Yurimaguas, 940m, 06º27'S, 76º17'W, 16.xi.2005 (M. Joron leg.); 2♂, km 19 Tarapoto-Yurimaguas, La Antena, 1300m, 06º27'S, 76º18'W, 22.vii.2007 (M. Joron leg.); 1♂, Fundo Biodiversidad, 950m, 06º28'S, 76º17'W, 21.xi.2007 (G. Lamas leg.). All deposited in MUSM.

Etymology: The subspecies is named in recognition of the French biologist Dr. Mathieu Joron, for his contribution to the knowledge of the mimetic butterfly fauna of San Martín, Peru. Dr. Joron is presently a Senior Scientist at the Centre d'Ecologie Fonctionnelle et Evolutive in Montpellier. He began studying the butterflies of San Martín during his PhD and has continued to do so throughout his career, with a particular focus on *Heliconius numata*. A masculine noun in the genitive case.

Wing shape morphometrics

Morphometric analyses found no significant difference in wing centroid size between *H. e. ucayalensis* and *H. d. joroni* **ssp. nov.** (FW: $F_{1,103}$=1.62, p=0.20, HW: $F_{1,103}$=0.52, p=0.47), although in both species females were larger than males (Fig. S2; FW: F1,104=16.9 p<0.001, HW F1,104=28.9 p<0.001). However, forewing and hindwing shape differ significantly between *H. e. ucayalensis* and *H. d. joroni* **ssp. nov.** (FW: $F_{20,84}$=12.3, pillai=0.74 p<0.0001, HW: $F_{20,84}$= 16.0, pillai=0.79 p<0.0001). *Heliconius d. joroni* **ssp. nov.** has proportionally more elongated forewings than *H. e. ucayalensis,* characterized by a reduction around the Cu_1 veins while *H. e. ucayalensis* has more rounded wings (Figure 3 & Fig. S3), confirming the perception of human observers (see description of *H. d. joroni* **ssp. nov.**). Hindwings are also more elongated in *H. d. joroni* **ssp. nov.**,

with a smaller discal cell, and more rounded in *H. e. ucayalensis.* Hindwing shape can be used as criteria to distinguish between *H. e. ucayalensis* and *H. d. joroni* ssp. nov, with about 92-93% of accurate reassignment. Forewing shape differences between *H. e. ucayalensis* and *H. d. joroni* ssp. nov is much stronger in males (allowing proper reassignment of over 93% of the samples), than in females (for which reassignment is not better than random). Wing shape differences (with more elongated wings in *H. demeter* and more rounded wings in *H. eratosignis*) were also consistently observed in other subspecies, as shown by the analysis including *H. d. bouqueti* samples (Figure 3) and the museum specimens of the other races (Figure 3).

Host plant ecology and immature morphology

In the wild near Tarapoto, confirmed host plant records for *H. e. ucayalensis* comprised clusters of 12-20 yellow ovoid eggs (n = 3), or groups of 1-4 gregarious larvae (n = 2) encountered on new leaves of *Passiflora skiantha* Huber (Passifloraceae) at Urahuasha (-6.466309°, -76.334911°) and San Roque de Cumbaza (-6.363100°, -76.440460°) (Figure 4e, g). Both male and female *H. e. ucayalensis* were also often caught investigating *P. skiantha* plants in these and other nearby localities. When placed in an insectary with 22 local species of *Passiflora*, wild caught females (n = 6) laid 78 eggs on *P. skiantha*, in clusters of 12-33 eggs (n = 4) usually on new leaves, and once on the meristem (Figure 4a). One other female laid a single egg on *Dilkea retusa* Mast. (Passifloraceae). This latter female did also show considerable interest in *P. skiantha* prior to ovipositing on *D. retusa*, but the *P. skiantha* plant had no new growth at the time. Final instar larvae are characterised by a black head, thoracic and prolegs, spines and anal shield (Figure 4b). Aside from the spiracles and a black band comprising a pair of elongated

black spots running laterally on the dorsal side of the prothorax, only faint black spotting is observed on the thorax and abdomen, which are yellow. The larvae are notable for having annular black stripes running laterally and dorsally, starting around the midpoints of each abdominal segment, approximately through the spiracles and through the base of the spines. In between these black stripes, there are also fainter bands of darker coloration running between the abdominal segments. The pupae are fairly typical for *Heliconius* in the *H. erato* clade, with long head horns (Figure 4d). The base colouration is predominantly brown but with some paler bands/ patches, and with distinct narrow white bands running horizontally and diagonally in the abdominal segments. There are three pairs of silver spots on the dorsal side of first abdominal segments, and an additional pair on the head. The horns are more darkly coloured, and the spines are black. The horns are similar in length to those of *H. erato* and *H. charithonia* (Linnaeus, 1767), but are more elongate, and taper to a point. Spines on the abdominal segments are somewhat longer than in *H. erato* and *H. charithonia*, and similar in length to those of *H. sara*.

Around Tarapoto we noted an association between presence and abundance of *D. retusa* and *H. d. joroni* **ssp. nov.** On several occasions *H. d. joroni* **ssp. nov.** females were caught in the vicinity of *D. retusa* plants (on two of which solitary eggs were found) at Biodiversidad (-6.460556°, -76.289928°), San Roque de Cumbaza and Pucayaquillo (-6.588195°, -76.222451°) and the Antena (-6.45716°, -76.29858°). On two occasions, two eggs were found on separate plants at Biodiversidad and La Antena. However, in general finding and rearing eggs and larvae proved difficult. This is probably because it is difficult to find *D. retusa* plants with new growth suitable for immature stages of *Heliconius*, at least in the plants accessible to human observers.

Nonetheless, on 28th March 2016 a single first instar larva and a yellow ovoid egg were found on a *D. retusa* plant above San Roque de Cumbaza (Figure 4f). The larva was reared to final instar, but failed to pupate. Its identity was confirmed as *H. d. joroni* **ssp. nov.** using *CoI* DNA barcoding. This final instar larva appeared morphologically broadly similar to *H. e. ucayalensis* (Figure 4c). However, black annular stripes running between the spines were absent, and instead the larvae was characterised by regular black spotting between the spines. The base colour also appeared more greenish yellow than in *H. e. ucayalensis*; however, on the basis of a single individual it is unclear whether this is a reliable diagnostic character.

While we provide data on larval morphology and host plant use only from northern Peru, previously published data suggest that the specific differences we found in sympatry are widely applicable across the ranges of *H. demeter* and *H. eratosignis*. *Heliconius demeter terrasanta* Brown & Benson, 1975 has solitary, spotted final instar larvae and uses *Dilkea* sp. in the Brazilian state of Pará, while *H. e. eratosignis* has been recorded using *Passiflora* ca. *citrifolia* Salisb. in Rondônia and has gregarious, striped final instar larvae (Brown and Benson 1975).

Molecular Data

The selected model of sequence evolution for each gene, along with associated parameter values and Bayesian Inference Criterion score are shown in Table S10. Analysis of mtDNA sequences (*CoI* + *CoII*) revealed a deep divergence between two haplogroups corresponding to *H. d. joroni* **ssp. nov.** + *H. d. bouqueti* and *H. e. ucayalensis* (Figure 5). The net proportional distance between these haplogroups is 5.2 %, and reciprocal monophyly of was well supported (bootstrap

percentages of 97% and 99%, respectively). *H. d. bouqueti* and *H. d. joroni* **ssp. nov.** also formed two well supported reciprocally monophyletic groups (bootstraps of 88% and 97%, respectively).

In addition, we were able to obtain ~760bp of *CoI* sequence for 13 of the 15 previously recognized subspecies of *H. demeter sensu lato* (*H. demeter* + *H. eratosignis*) (Figure 5). The resulting phylogeny indicated 2 reciprocally monophyletic groups, corresponding to the northern *H. demeter* and southern *H. eratosignis*. The southern clade was well supported (100% bootstrap), and comprised *H. eratosignis ucayalensis*, along with *H. eratosignis eratosignis*, *H. eratosignis tambopata* Lamas, 1985 and *H. eratosignis ulysses* Brown & Benson, 1975. The northern clade comprised *H. d. demeter* and *H. d. bouqueti*, along with *H. d. angeli* Neukirchen, 1997, *H. d. karinae* Neukirchen, 1990, *H. d. neildi* Neukirchen, 1997, *H. d. terrasanta*, *H. d. titan* and *H. d. turneri* Brown & Benson, 1975. The bootstrap support for this northern clade was only moderate (64%), however this is due to the uncertain placement of *H. d. titan*, which appears as sister to a well-supported (100%) monophyletic clade containing the other races.

Of the five nuclear loci examined, only *ef1a* showed *Heliconius d. demeter* + *H. d. bouqueti* and *Heliconius e. ucayalensis* to form reciprocally monophyletic groups (Figure 6). Bootstrap support for these two groupings was only moderate (65% and 62%, respectively), and the two only exhibited two fixed nucleotide differences across 798bp of *ef1a* sequence. *Tpi* showed *H. e. ucayalensis* to be monophyletic (76%), but with the paraphyly or monophyly of *H. demeter* uncertain; the ML tree indicates the former, but with bootstrap support of only 28%. *Mpi* also recovered *H. d. demeter* as a well-supported monophyletic group (97%), but found *H. e.*

ucayalensis and *H. d. bouqueti* to be polyphyletic. *Rpl5* and *Tektin* revealed polyphyly in all three taxa.

Between-run consistency was high in the STRUCTURE analysis of AFLP genotypes: replicate runs at each K-value yielded virtually identical likelihoods. The optimal number of genotypic clusters was three, corresponding cleanly to each of the three taxa (Figure 7). Significantly, what this shows is that the two sympatric Peruvian taxa, *H. d. joroni* **ssp. nov.** and *H. e. ucayalensis*, form separate genotypic clusters. The average Nei-Li pairwise genetic distances between *H. d. joroni* **ssp. nov.**, *H. d. bouqueti* and *H. e. ucayalensis* calculated using AFLP genotypes are: *H. d. joroni* **ssp. nov.**-*H. d. bouqueti* 0.46, *H. d. joroni* **ssp. nov.**-*H. e. ucayalensis* 0.72 and *H. d. bouqueti*-*H. e. ucayalensis* 0.70. Therefore, the sympatric Peruvian taxa (*H. d. demeter* and *H. e. ucayalensis*) are genetically more divergent to one another compared to the allopatric *H. d. demeter*-*H. d. bouqueti.*

Geographic distribution

Races of *H. demeter* and *H. eratosignis* are mapped in Figure 1, with photos of the type of each race. Races of *H. demeter* occupy the Guianas and much of the Amazon basin. *H. eratosignis* races occur in the west and south of the Amazon basin. In Tarapoto, the two species fly together at a number of sites in the Cordillera Escalera. However, only *H. eratosignis* has been recorded from the adjacent Amazonian lowlands, despite considerable sampling in the area. Museum data and observations by Keith Brown suggest that the two overlap (at least broadly) in the extreme south of Pará and northern Mato Grosso, in Brazil. There may well also be a contact zone on the

Juruá river, between Porto Walter and Eirunepé, as both *H. demeter demeter* and *H. eratosignis tambopata* are known to occur there. However, the exact position of contact in this very large area is unclear. In data published by Brown (1979) two additional contact zones are indicated, at Pucallpa, Peru and near Cobija on the Brazilian/Bolivian border. We were unable to locate the relevant specimens in museum collections, however, we consider these points unreliable. The first is probably a generalised locality, with the specimens potentially coming from a large area of northern Peru. The second is likely explained through the co-occurrence of both *H. eratosignis ulysses* and *H. eratosignis tambopata*, as the latter was not described until 1985.

DISCUSSION

Gene genealogies can be used in concert with morphological differences to diagnose species within single populations, because reciprocal monophyly within an interbreeding population becomes highly improbable when multiple individuals are sequenced. Similarly, the existence of clusters of multilocus genotypes within a sympatric population comprises strong evidence for distinct species, because linkage disequilibria between alleles at unlinked loci are highly unlikely to arise without barriers to reproduction. We have shown that in northern Peru, *H. d. joroni* **ssp. nov.** and *H. e. ucayalensis* sampled from a small geographic area comprise two monophyletic groups for mtDNA markers *CoI* + *CoII*, and form distinct genotypic clusters using AFLP data. Furthermore, the 5.2% net mtDNA divergence between *H. d. joroni* **ssp. nov.** + *H. d. bouqueti* and *H. e. ucayalensis* is equivalent to interspecific genetic distances between other *sara-sapho* group species, and is greater than distances between many other pairs of *Heliconius* species, such as those within the *cydno-melpomene* species group (Beltrán et al. 2002; Giraldo et al. 2008;

Kozak et al. 2015). Thus, together with the observed differences in larval and adult morphology, wing shape, behaviour and host plant use, our data strongly imply the existence of two species. Additionally, the *CoI* phylogeny from 13 of the 15 races of *H. demeter* and *H. eratosignis* resolved two reciprocally monophyletic groups, comprising *H. d. joroni* **ssp. nov.** and the northern Amazonian races, and *H. e. ucayalensis* and the southern Amazonian races. These groups are consistent with morphological criteria (e.g. the forewing costal streak), and are also apparent in the morphometric analysis of wing shape (Figure 3 & Fig. S3). Both clades were well supported, except regarding the position of *H. d. titan*, whose assignment to *H. demeter* rather than *H. eratosignis* was only marginally favoured by molecular and morphological data. *Heliconius demeter titan* is also notable for discordant morphological characters, and for its long mtDNA branch lengths and reciprocal monophyly with *H. demeter*. Because *H. d. titan* appears to be broadly sympatric with several other *H. demeter* races, it may even present a further cryptic species within this clade.

In contrast to the mtDNA and AFLP data, only one of the five nuclear markers sequenced (*ef1a*) showed reciprocal monophyly between *H. d. bouqueti* + *H. d. joroni* **ssp. nov.** and *H. e. ucayalensis*. However, two other nuclear genes (*Tpi* and *Mpi*) did show monophyletic groups corresponding to subspecies or species. Gene genealogies that fail to resolve relationships between closely related species are not unusual in *Heliconius* and may reflect either the retention of ancestral polymorphisms or introgression following speciation (Maddison 1997; Beltrán et al. 2002; Bull et al. 2006), or simply uninformative genetic data. Because effective population sizes are lower for maternally inherited mtDNA and the sex-linked *Tpi* than for autosomal loci, they

are expected to coalesce more recently (Palumbi et al. 2001), and so monophyly at these loci is consistent with ancestral polymorphisms. In addition, if ongoing introgression was producing the observed patterns, we might expect polyphyly between the sympatric taxa *H. e. ucayalensis* and *H. d. joroni* **ssp. nov.**, but with *H. d. bouqueti* phylogenetically distinct, due to its geographic isolation. Nonetheless, females are the heterogametic sex in butterflies, and, in accordance with Haldane's rule, female sterility is the first manifestation of intrinsic postzygotic reproductive isolation (Jiggins et al. 2001a; Naisbit et al. 2002). Introgression should therefore be inhibited at maternally inherited *CoI* and sex-linked *Tpi* (Sperling 1994), and so monophyly at these loci is consistent with introgression, as well as ancestral polymorphisms. Therefore, we cannot rule this out as the cause of nuclear genealogies failing to reflect species boundaries, especially given the abundant evidence for gene flow between closely related *Heliconius* (Dasmahapatra et al. 2007; Mallet et al. 2007; *Heliconius* Genome Consortium 2012; Pardo-Díaz et al. 2012; Martin et al. 2013). On-going introgression between *H. demeter* and *H. eratosignis* would seem remarkable given that they diverged ~4.5 million years ago (assuming mtDNA evolution of 1.1-1.2% per million years (Brower 1994)), but not implausible given the known importance of colour pattern as a prezygotic reproductive isolating barrier in *Heliconius* (Merrill et al. 2011, 2012). Previous studies of cryptic *Heliconius* have suggested that hybridisation between closely related co-mimics may be higher than between non-mimics, but quantitative comparisons are difficult (Giraldo et al. 2008; Mérot et al. 2013, 2017). It would be interesting to investigate whether the other similarly divergent co-mimetic sister pair *H. leucadia* Bates, 1862 and *H. sara* also exhibit similar phylogenetic discordance.

It is striking that three of the recently described cryptic species pairs of *Heliconius* are distinguishable using a streak in the costa-subcosta space on the forewing underside (the present study; Giraldo et al. 2008; Mérot et al. 2013). Many other co-mimetic *Heliconius* are distinguishable using seemingly inconsequential red dots and streaks at the base of the ventral hindwing (Emsley 1965; Holzinger and Holzinger 1994). While this variation might be attributable to relaxed selection from predators on the underside of the hindwing, their repeated utility in distinguishing species leads one to speculate whether they are important for the butterflies themselves in terms of conspecific recognition. Indeed, these areas of the wing are perhaps the most visible to the female while they are being courted by males. This hypothesis could conceivably be tested using colour pattern manipulations and assortative mating experiments.

Given that *H. demeter* and *H. eratosignis* do not differ greatly in mimetic pattern it is interesting to speculate about the drivers of divergence between them. In other recently described cryptic species, phenotypic similarity is most parsimoniously explained by convergence through introgression of colour pattern alleles, rather than divergence without mimicry shift (Mallet 2009; *Heliconius* Genome Consortium 2012; Pardo-Díaz et al. 2012). However, in the case of *H. demeter* and *H. eratosignis*, the available data suggest that speciation occurred from start to finish without a significant mimicry shift. The present geographic distributions of the species are suggestive of vicariance between the north and south Amazon basis, which seems consistent with the species' phenotypic similarity. For example, 7-10 million years ago a huge wetland (the "Acre system") bisected Amazonia from east to west (Hoorn et al. 2010), and this could have

initiated allopatric divergence through stochastic processes (e.g. mutation order speciation). This might also explain the poly- and paraphyly at nuclear loci, because monophyly would be slow to develop in the resulting large populations (Maddison 1997). However, *H. demeter* and *H. eratosignis* do differ in ecological traits which may have played a part or driven their speciation. Sexual dimorphism in colour pattern is very unusual in *Heliconius*, and finding that closely related species differ markedly in mating signals is often considered indicative of speciation via sexual selection (Panhuis et al. 2001). The greenish scales exhibited by males produce a seemingly non-mimetic phenotype that could be the product of sexual selection, but seem unlikely to be involved in speciation because they are present in both species. In contrast, fused rays are exhibited only by *H. demeter*. In some regions, such as near the Andes, this leads to males being rather poor mimics of other similar *Heliconius* species, and could therefore be interpreted as the product of female choice for a male trait. However, in other regions, such as in French Guiana, the dimorphism seems to be a mixed strategy, with males mimicking species such as *Heliconius egeria* (Cramer, 1775) and females mimicking species such as *H. erato*. This latter explanation for *H. demeter*'s fused rays therefore seems to fit the scenario of speciation in allopatry, followed by more recent contact in the Amazon headwaters.

Heliconius demeter and *H. eratosignis* are also unusual in their apparent host plant specificity because most *Heliconius* sister species use overlapping suites of *Passiflora* spp. (Rosser et al. 2015). Host plant shifts are frequently associated with speciation in phytophagous insects (Bush 1969; Drès and Mallet 2002), and there is some evidence for their importance in *Heliconius* (Jorge et al. 2011; Merrill et al. 2013; Rosser et al. 2015). This could be either because the

butterflies tend to mate in the vicinity of their host plants (Deinert et al. 1994), or due to disruptive selection for hybrid larvae performance on alternative hosts (Funk 1998). In the Cordillera Escalera, the host plants of *H. eratosignis* and *H. demeter*, *P. skiantha* and *D. retusa* respectively, are notable for tending to co-occur. Thus, at least on the basis of this population the former model does not seem likely to generate strong reproductive isolation. In contrast, it seems plausible that the clear phenotypic and phylogenetic differences between *P. skiantha* and *D. retusa* and could produce disruptive selection on larval performance. Furthermore, *H. demeter* and *H. eratosignis* are the only sister species pair within *Heliconius* known to comprise a species with gregarious larvae and one with solitary larvae (Beltrán et al. 2007). While the Dobzhansky-Muller incompatibilities underlying such a model of speciation are typically thought to require geographically induced reduction in gene flow (Bank et al. 2012), this concords once again with the hypothesis of Amazonian vicariance. Whatever the drivers of divergence in *H. demeter* and *H. eratosignis*, their limited geographic overlap, co-mimicry, sexual dimorphism, and marked differences in host plant use and oviposition behaviour highlight them as an interesting counter-example to more typical *Heliconius* sister species.

While the discovery of yet another cryptic species in a group as intensively studied as *Heliconius* may seem extraordinary, our finding joins a long series of similar recent discoveries in other butterflies (e.g. Willmott et al. 2001; Hebert et al. 2004; McBride et al. 2009; Dincă et al. 2011; Hill et al. 2012; Barbosa et al. 2015). In particular, *H. demeter* and *H. eratosignis* seem to parallel the discovery of cryptic species in Afrotropical *Cymothoe* butterflies (Nymphalidae). Strong host plant and ecological differences have evolved between *Cymothoe egesta* (Cramer,

[1775]) and *Cymothoe confusa* Aurivillius, 1887, formerly considered races of a single widely distributed species. These differences are apparently insufficient to allow sympatry, bar a narrow region of overlap between allopatric ranges (McBride et al. 2009). It is important to distinguish cases such as these, involving the elevation of previously known taxa to species level, from the discovery of entirely unrecognised species. Nonetheless, the rate at which cryptic species are being discovered in groups as well-studied as butterflies suggests that numbers of species estimated on the basis of current taxonomy have been even more woefully underestimated than the most careful recent analyses suggest (Purvis and Hector 2000; Stork 2018). Despite its limitations (Elias et al. 2007), DNA barcoding may hold the greatest potential to identify putative cryptic species for further study.

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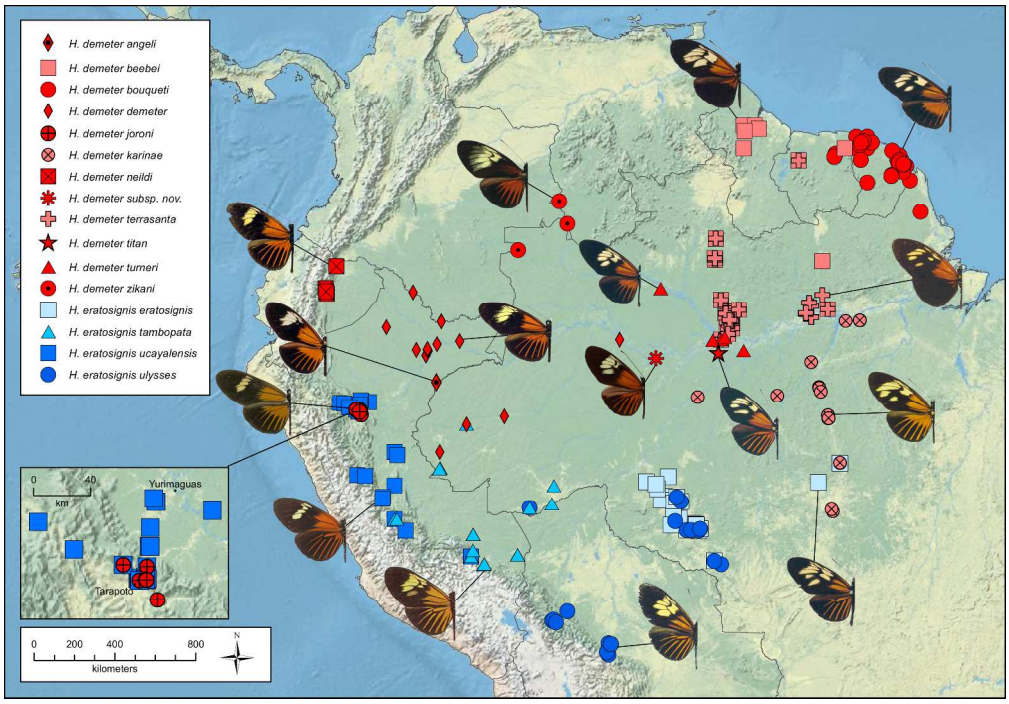

Figure 1. Distribution of races of H. demeter and H. eratosignis. Photos of type specimens are all males, except for H. e. ucayalensis. The inset shows fine scale sympatry between H. d. joroni ssp. nov. and H. e. ucayalensis in the Tarapoto area of Peru. A second probable contact zone is apparent in northern Mato Grosso. While the map also shows sympatry between H. demeter demeter and H. eratosignis tambopata on the Juruá river in Acre, we only have evidence for broad-scale co-occurrence, and whether the two are actually in contact is unclear. Heliconius demeter beebei Turner, 1966 and H. demeter terrasanta appear to conform to the type specimens only around the type localities (in Terrasanta, Pará, and in Guyana). Between these, most populations appear to be either polymorphic or exhibit intermediate phenotypes (mixed square and cross symbols in the map). Heliconius demeter subsp. nov. refers to three males in the FLMNH recognised by W. Neurkichen as distinct from other described subspecies. These individuals may prove to have affinities to H. demeter titan.

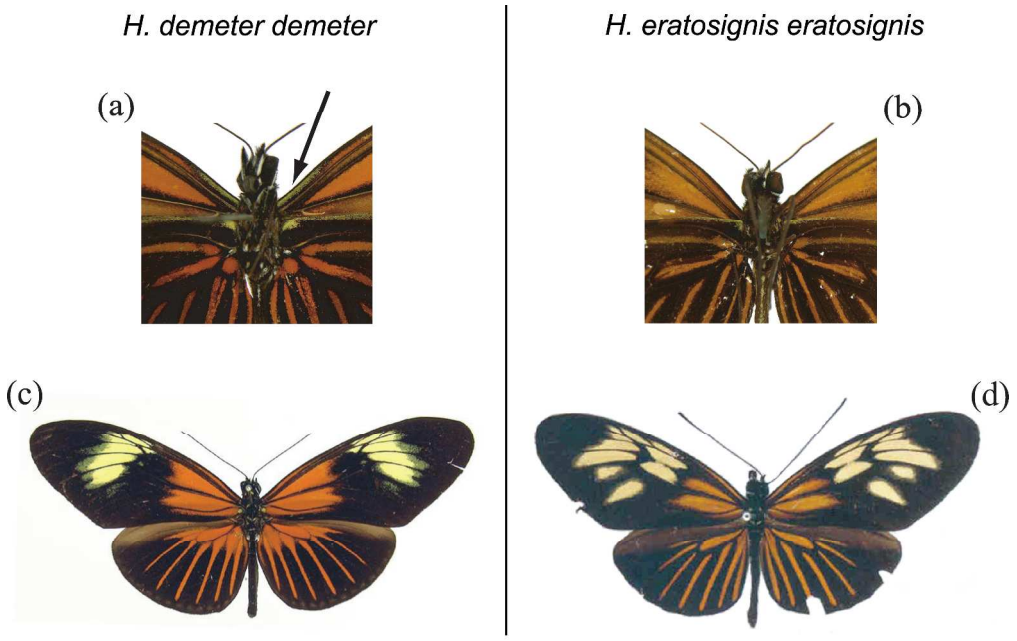

Figure 2. Diagnostic features for H. demeter and H. eratosignis. All H. demeter races are characterised by a yellow streak in the proximal region of the costal-subcostal space on the underside of the forewing (a), and by the fusion of the hindwing rays to form a bar in males (except in H. d. titan which has an intermediate phenotype, and H. d. beebei and H. d. terrasanta, which have reduced rays) (c). These characters are absent in H. eratosignis (b, d). Pictured races are H. d. demeter from "Iquitos, Mich[ael]." (NMB), and H. e. eratosignis "River System, Cuyaba-Corumba, Mato Grosso, Brazil" (NHMUK).

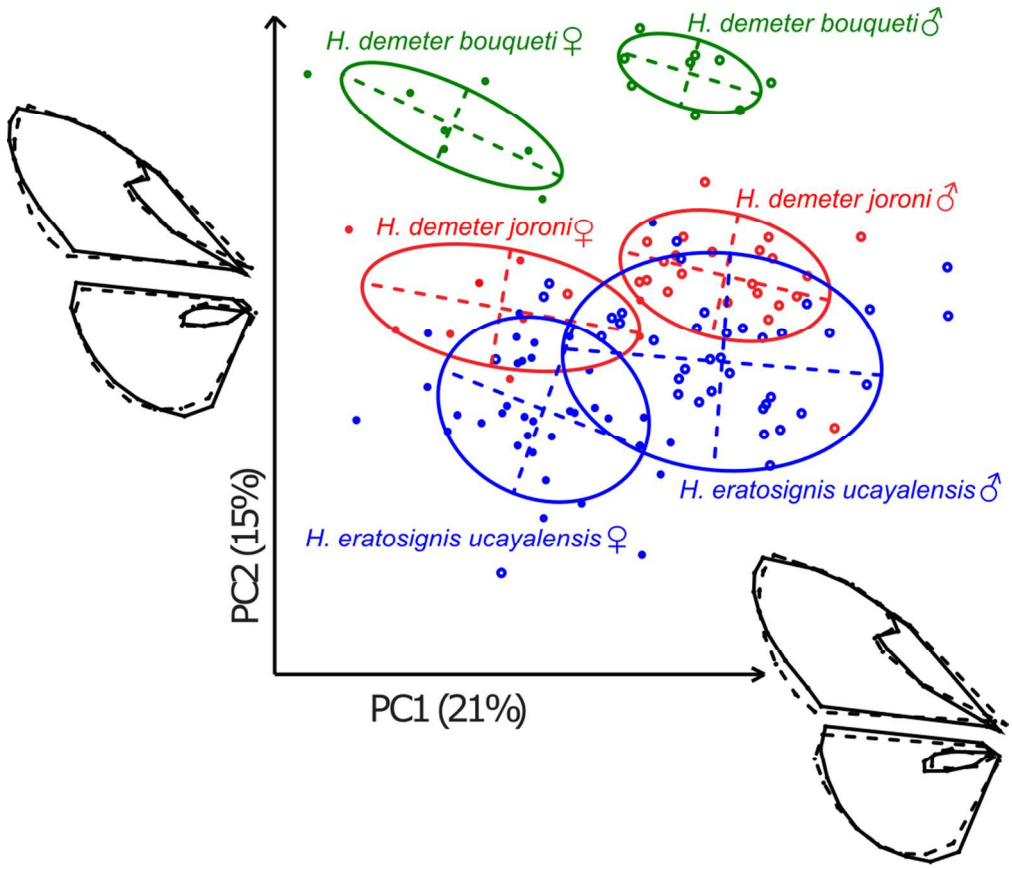

Figure 3 Principal component (PC) analysis of wing shape variation between H. eratosignis and H. demeter. Males are represented with open circles and females by filled circles. Ellipses represent a graphical summary of the distribution. H. e. ucayalensis is shown in blue, F. d. joroni in red and H. d. bouquet in brown. Shape variation captured by each of PC1 and PC2 are illustrated next to each axis, where dotted lines represent minimum values of the axis, and full lines represent maximum values. PC1 captures shape differences between males and females across both species. PC2 captures variation between species as well as between H. demeter subspecies. Museum specimen (types, syntypes, etc) are represented with a pink letter for H. demeter corresponding to the subspecies (a, angeli, b, bouqueti, d, demeter, k, karinae, n, neildi, t, titan, z, zikani) and with a letter in light blue for H. eratosignis (e, eratosignis, u, ucayalensis, y, ulysses).

Figure 4. Clockwise from top left. a) Heliconius eratosignis ucayalensis ovipositing on P. skiantha in our insectary in Tarapoto. b) Heliconius eratosignis ucayalensis final instar larva, found wild as a 2nd instar larva on P. skiantha at Urahuasha (Peru) on 24/3/16. c) Heliconius demeter joroni ssp. nov. final instar larva, found wild as first instar larva on D. retusa at San Roque de Cumbaza (Peru) on 28/3/16. d) Heliconius eratosignis ucayalensis pupa. e) Passiflora skiantha in flower at El Túnel, near Tarapoto (Peru). f) Dilkea retusa flowering at San Roque de Cumbaza. g) A clutch of wild H. eratosignis ucayalensis eggs on P. skiantha from San Roque de Cumbaza. All photos by NR.

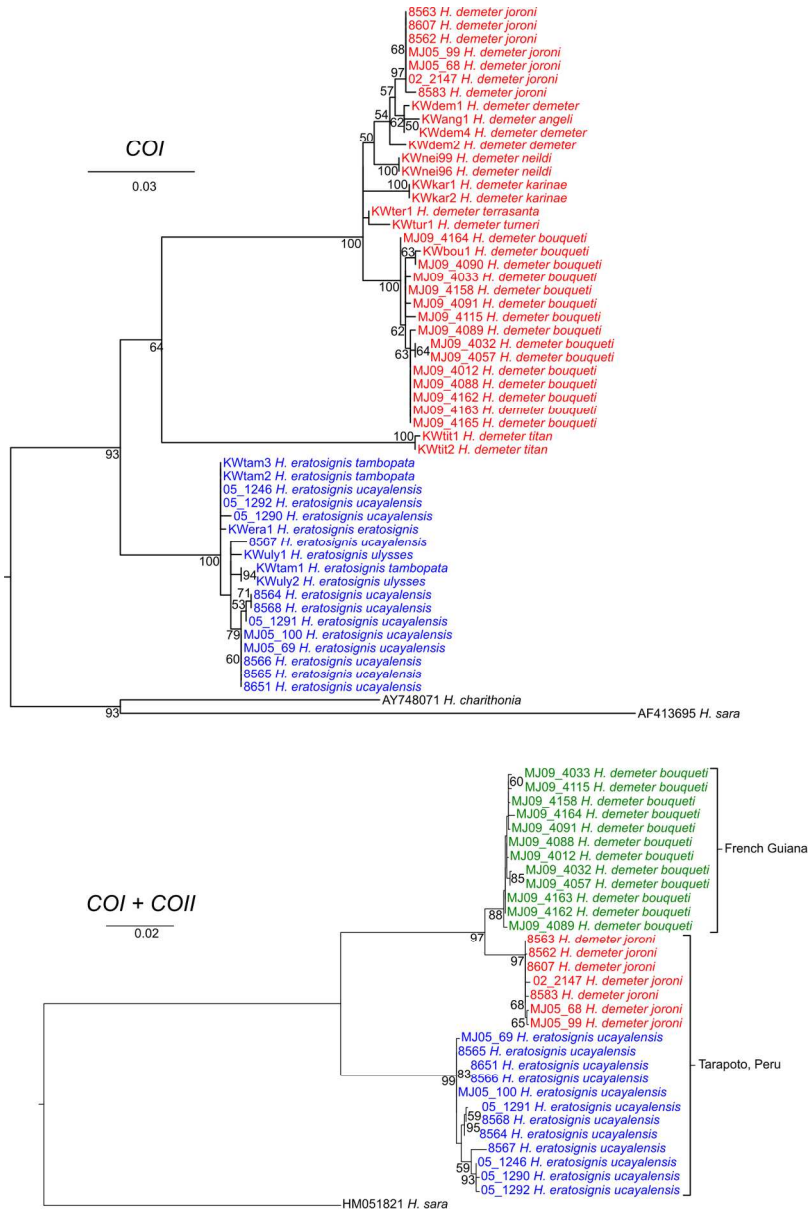

Figure 5. Maximum likelihood phylogeny of A) 13 of the 15 currently recognised subspecies of H. demeter and H. eratosignis based on ~760 bp of mitochondrial CoI sequence; B) H. demeter and H. eratosignis specimens from Tarapoto (Peru) and H. demeter from French Guiana based on ~2200bp of mitochondrial CoI + CoII sequence. Bootstrap values greater than 50% are shown.

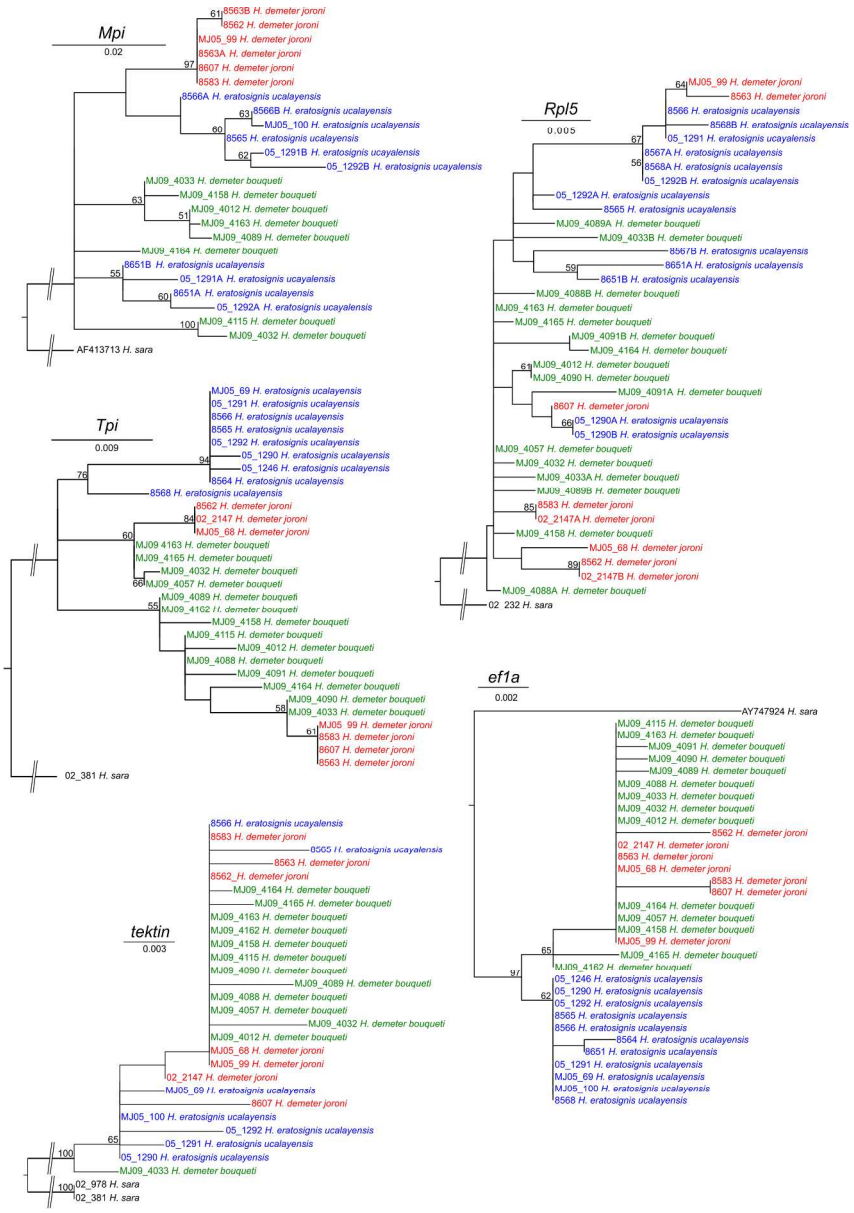

Figure 6. Maximum likelihood trees of H. demeter and H. eratosignis specimens from Tarapoto (Peru) and H. demeter from French Guiana based on sequences of five nuclear loci Mpi, Tpi, Tektin, Rpl5 and Ef1α. Bootstrap values greater than 50% are shown. Otherwise identical voucher numbers terminating in A or B refer to alleles from heterozygous individuals.

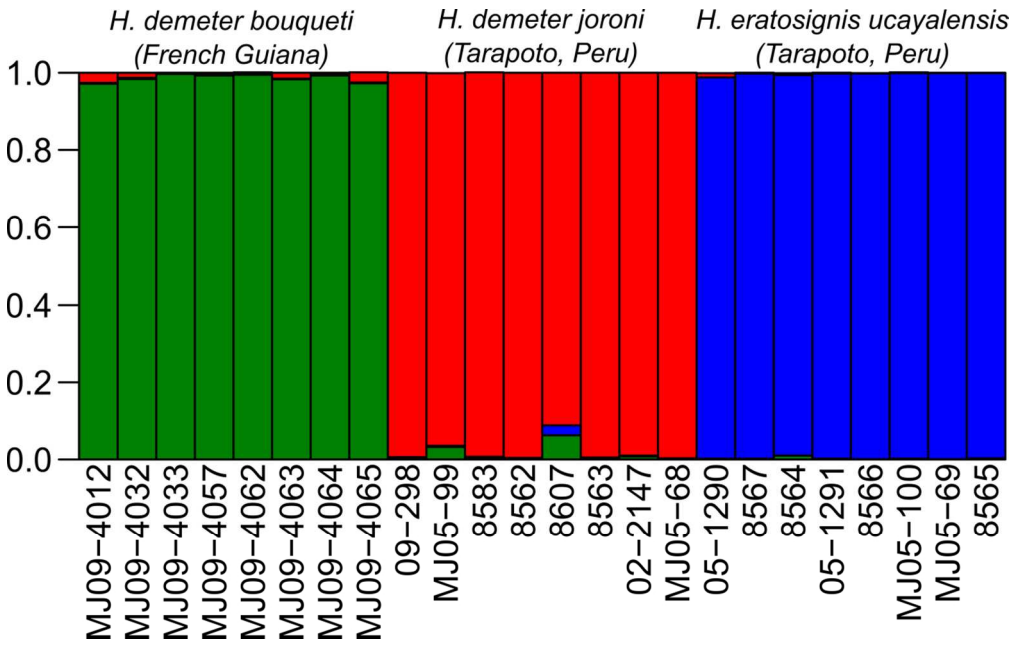

Figure 7. STRUCTURE analysis of AFLP genotypes from H. demeter and H. eratosignis specimens from Tarapoto (Peru) and H. demeter from French Guiana using the optimal number of clusters (K=3). Each of the 24 individuals is represented by a vertical bar broken into three segments. The proportion of each colour in the bar indicates the posterior mean probability of ancestry from each genetic cluster.