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Cryptic speciation associated with geographic and ecological divergence in two Amazonian *Heliconius* butterflies

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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 a counterexample to this, by documenting two cryptic species within the taxon formerly considered *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 *Ef1α*, and exhibit marked differences in 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 also comprising ***H. eratosignis ucayalensis* comb. nov.** and three other southern Amazonian races. ***Heliconius demeter joroni* spp. nov.** remains within *H. demeter s.s.*, along with northern Amazonian and Guianan subspecies.

ADDITIONAL KEYWORDS: butterflies – cryptic species – genotypic clusters – host plant shift – integrative taxonomy – mimicry – vicariant speciation.

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INTRODUCTION

Cryptic species can be defined as species that are, or have been, erroneously classified as a single nominal

species due to their superficial morphological similarity (Bickford *et al.*, 2007). In recent years, the integration of DNA sequences into taxonomy has led to the recognition of increasing numbers of such species (Hebert *et al.*, 2004). Whether the origins of cryptic species can be consistently ascribed to any particular evolutionary processes is unclear (Bickford *et al.*, 2007). On the face of it, this might seem unlikely, given that they are defined principally through humans' visual perception of morphology. For example, while divergent selection may often lead to sister species with markedly different body shapes or colours (Jiggins *et al.*, 2001b; Langerhans *et al.*, 2007), in other cases it may affect traits causing reproductive isolation that have no clear morphological basis, such as behaviours (Janzen *et al.*, 2009). Nonetheless, some theories of speciation might be more predisposed to the creation of cryptic species, such as when reproductive isolation results from the chance fixation of different, epistatic incompatibilities in separate populations subject to similar selective pressures (Clarke *et al.*, 1988; Mani & Clarke, 1990; Orr, 1995; Turelli & Orr, 2000). It also has been shown that, at least for butterflies in the western Mediterranean, cryptic species are rarely sympatric (Vodă *et al.*, 2015). While this may reflect to some extent the ability of taxonomists to diagnose species with limited range overlap, it is also consistent with phenotypic similarity constraining coexistence (Pigot & Tobias, 2013).

Heliconius butterflies are chemically defended and aposematic, i.e. they advertise their defence to would-be predators using bright colours on their wings. To minimize 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 patterns). 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 contribute to reproductive isolation. The reasons for this are twofold. First, hybrids with intermediate colour patterns are selected against by predators that do not recognize them as aposematic (Merrill *et al.*, 2012). Second, *Heliconius* males have been shown to preferentially court females with similar colour patterns to their own (Jiggins *et al.*, 2001b; Jiggins *et al.*, 2004; Kronforst *et al.*, 2006; Mavárez *et al.*, 2006; Chamberlain *et al.*, 2009; Merrill *et al.*, 2011), even when those females belong to a different species (Estrada & Jiggins, 2008). Because divergence in an ecologically relevant adaptive trait also creates reproductive isolation, *Heliconius* have become a prime example of so-called 'ecological speciation' (Nosil, 2012). Furthermore, most *Heliconius* sister-species pairs differ in mimetic phenotype (Turner, 1976; Rosser *et al.*, 2015). Consequently, reproductive isolation by mimicry shift has 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 unrecognized as members of the *H. cydno–timareta* clade because they exhibit colour patterns extremely similar to those of sympatric subspecies of *H. melpomene* (Linnaeus, 1758), itself the sister to the *H. cydno–timareta* lineage. 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 demonstrate that closely related, co-mimics can maintain their identities in sympatry, despite occasional hybridization (Mérot *et al.*, 2017).

Based on previous systematic research, *H. demeter* Staudinger, 1897 was held to comprise 15 described subspecies with red, yellow and black phenotypes (Fig. 1) that participate in the 'dennis-rayed' *Heliconius* mimicry ring (Brown & 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 north 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 rare in *Heliconius*, and only one other species exhibits it prominently: *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 near the city of Tarapoto. *Heliconius demeter* cf. *demeter* is sexually dimorphic, but *H. demeter* cf. *ucayalensis* is not. At first we viewed these taxa as somewhat divergent subspecies, since there are contact zones between many butterfly subspecies in this area (Dasmahapatra *et al.*, 2010). In the

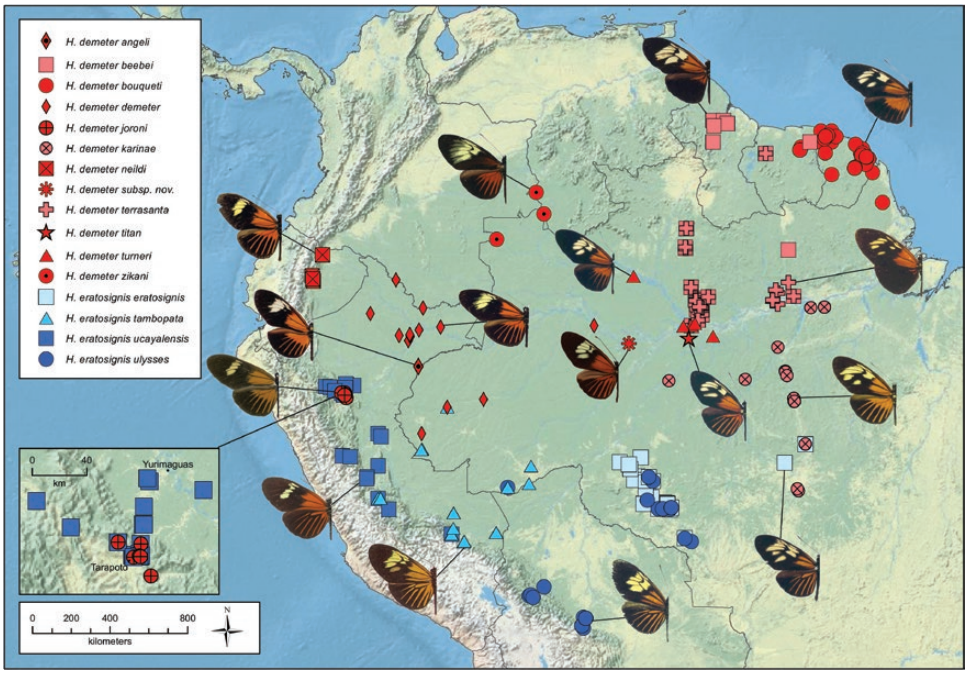

Figure 1. Distribution of races of *H. demeter* and *H. eratosignis*. Photos of type specimens are all males, except for *H. e. ucayalensis*. The inset shows fine-scale sympatry between ***H. d. joroni* ssp. nov.** and *H. e. ucayalensis* in the Tarapoto area of Peru. *Heliconius demeter beebei* Turner, 1966 and *H. d. 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* ssp. nov.** refers to three males in the FLMNH recognized by W. Neukirchen as distinct from other described subspecies. These individuals may prove to have affinities to *H. demeter titan*.

present study, we show that these sympatric subspecies of '*H. demeter*' in fact comprise two distinct species, corresponding to *H. demeter* cf. *demeter* and other northern and central Amazonian subspecies, and *H. demeter* cf. *ucayalensis* and the south Amazonian races. In accordance with nomenclatural priority, the southern clade is recognized as *H. eratosignis* (Joicey & Talbot, 1925), a species comprising four subspecies (Lamas & Jiggins, 2017; Supporting Information, Table S1), and this nomenclature is adopted in this paper from here on. Additionally, it was noted that *H. demeter* cf. *demeter* specimens from Tarapoto are divergent from those in the *H. demeter* type locality near Iquitos, and accordingly this population is here described as a new subspecies: *Heliconius demeter joroni* ssp. nov.

MATERIAL AND METHODS

Morphological and behavioural analysis

To identify species-specific diagnostic characters in the 15 currently recognized subspecies of *H. demeter* and *H. eratosignis*, all type series and specimens held in the Natural History Museum London (NHMUK) were examined. In addition, we examined holotypes, allotypes, syntypes and other material held at the Florida Museum of Natural History (FLMNH), the 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) (Supporting Information, 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. and 16 *H. demeter bouqueti* Nöldner, 1901 specimens from Tarapoto and French Guiana were captured using either 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 Supporting Information, Table S3.

Forewing and hindwing shape were described using 20 and 18 landmarks, respectively, which were placed at vein intersections and vein termini on the ventral side (Supporting Information, Fig. 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 digitized using TpsDig2 (Rohlf, 2010) and superimposed using a general Procrustes analysis (Bookstein, 1991; Zelditch *et al.*, 2004). Wing size was measured using the 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 the response, and species and sex as predictive factors. *P*-values were corrected for multiple comparisons following Benjamini & 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 & Friess, 2005). To explore shape differences between *H. d. joroni* ssp. nov. and *H. e. ucayalensis*, a MANOVA was applied to the PC subsets, with shape as the response and sex and species as predictive factors. 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. d. joroni* ssp. nov. and seven male *H. e. ucayalensis* collected from Tarapoto were prepared from material preserved in salt-saturated DMSO. The tips of the abdomens were removed and soaked in 10% KOH for 10 min at 70 °C, and then transferred to distilled water. The scales were first removed with a fine brush and the valves extruded. The genitalia were then removed and further cleaned. Temporary slides were prepared in 25% ethanol, and the interior surfaces of each left valva were photographed.

Observations on host plant use and larval morphology were made near Tarapoto, Peru. To supplement field observations of host plant use, wild caught adult females were placed in a cage with 22 locally common *Passiflora* species (Supporting Information, Table S4) 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 by NR in Bolivia, Brazil, French Guiana, Peru and Suriname between 2011 and 2017, by AVLF in Mato Grosso and Acre from 1994 to 2016, and by Keith Brown (from 1970 to 1999) and Eurides Furtado (from 1978 to 1998) in Brazil.

Molecular analysis

Details of the specimens used for molecular work are shown in Supporting Information, Table S5. Wings were removed from samples collected in French Guiana and around Tarapoto, 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, single legs representing 11 other subspecies were obtained from dried museum specimens in the FMNH (identification numbers beginning with 'KW' in Supporting Information, Table S5). DNA was extracted from these 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 *cytochrome oxidase I* (*COI*), *tRNA-leu* and the 5' end of *cytochrome oxidase II* (*COII*) was amplified by PCR in three sections, for seven *H. demeter joroni* ssp. nov., 12 *H. eratosignis ucayalensis* and 12 *H. demeter bouqueti*. 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 *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). Supporting Information, Table S6 contains details of the primers used and PCR conditions. Sequences from *Heliconius* species in the *sara–sapho* clade were downloaded from Genbank to act as outgroups. GenBank accession numbers for all sequences used are provided in Supporting Information, Table S7 and Table S8. Sequences 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 node

support 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* 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, visualized by autoradiography, and scored by eye. A total of 81 loci were polymorphic and could be scored unambiguously.

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 dataset, 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 were also used to calculate pairwise Nei-Li genetic distances (Nei & 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 *H. demeter joroni* ssp. nov. and *H. eratosignis ucayalensis* wings from Peru revealed two diagnostic morphological characters strictly concordant with the mitochondrial DNA 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 (Fig. 2A). In *H. eratosignis ucayalensis* this region is uniformly orange (Fig. 2B). Brown & Benson (1975) also noticed this character difference between northern and southern Amazonian populations, but did not recognize its significance, probably because they lacked a long series of these taxa from a sympatric population. (2) In males of *H. demeter joroni* ssp. nov., the red rays on the dorsal hindwing fuse to form a hindwing bar (Fig. 2C), while in males of *H. e. ucayalensis* they do not (Fig. 2D). This character is inapplicable to females, all of which have unfused red rays, and to the geographic forms of *H. demeter* from north-eastern South America 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 presents a rounded profile in *H. d. joroni* ssp. nov., while in *H. e. ucayalensis*

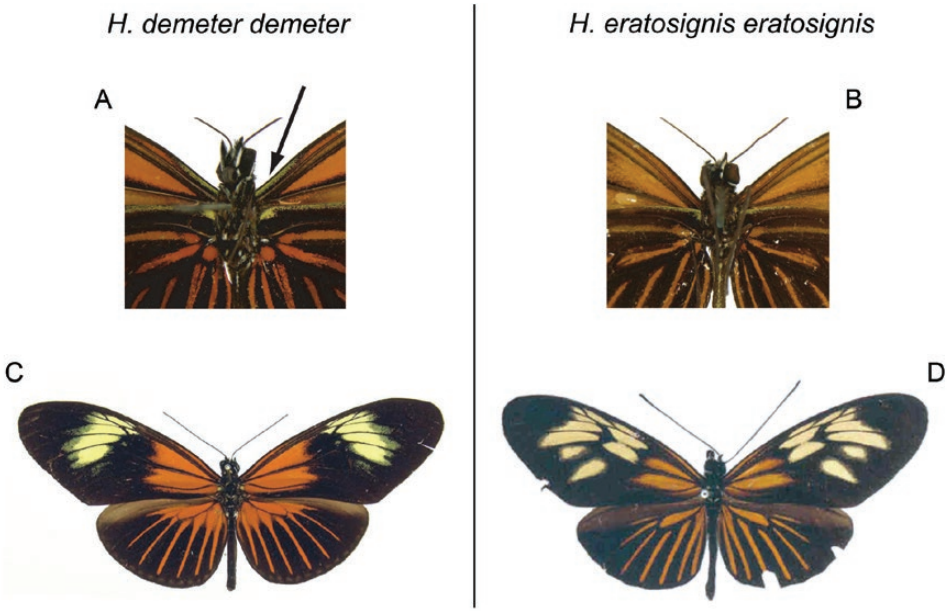

Figure 2. Diagnostic features for *H. demeter* and *H. eratosignis*. All *H. demeter* races are characterized by a yellow streak in the proximal region of the costal-subcostal space on the underside of the forewing, A, and by the fusion of the hindwing rays to form a bar in males, C, except in *H. d.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

this region has a characteristic convex depression Supporting Information, Fig. S2). However, the utility of this trait is unclear given the small sample sizes.

Using the presence/absence of the yellow costal streak on the ventral 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 (Supporting Information, Table S9). In *H. d. titan* there is a clear yellow costal streak, but the hindwing rays in the male are only partially fused, and *H. d. titan* was also intermediate for other less clear-cut characters (see subspecies description for *H. d. joroni* ssp. nov.). Its *COI* sequence is also divergent from the other *H. demeter* (see below).

DESCRIPTION OF THE NEW SUBSPECIES FROM TARAPOTO

HELICONIUS DEMETER JORONI LAMAS & ROSSER **SSP. NOV.**

(FIG. 3)

Heliconius demeter [ssp. nov.] Lamas, MS: Lamas, 2004: 268. Lamas & Jiggins, 2017: 224.

Figure 3. Holotype ♂ of ***Heliconius demeter joroni*** Lamas and Rosser **ssp. nov.** Upper photo: dorsal, lower photo: ventral. Scale bar = 10 mm.

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 (Fig. 1), where its co-mimics include *Heliconius eratosignis ucayalensis*, *Heliconius elevatus pseudocupidineus* Neustetter, 1931, *Heliconius 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*. This character can be faint or even missing in *H. demeter joroni* ssp. nov. and occasionally present in *H. erato emma*. However, 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 8 mm. The forewing 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. d. demeter* or *H. e. ucayalensis*. Some specimens exhibit a faint greenish spot in the middle of cell Cu_1-Cu_2. Dennis (i.e. the basal patch on the forewing) 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 dennis and postmedian band less bright and reduced relative to dorsum. Base of the narrow costa–subcosta space with a strong yellow 3–5 mm long streak placed adjacent to the 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 the 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 ♂ (Fig. 3), PERU, San Martín, Tarapoto, San Roque, 500m, 06°22'S, 76°26'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, 06°27'S, 76°17'W, 20.xi.1999 (G. Valencia leg.); 1♂, km 17 Tarapoto-Yurimaguas, 1000m, 06°27'S, 76°17'W, 11.xii.1999 (M. Joron leg.); 1♂, km 19 Tarapoto-Yurimaguas, 1300m, 06°27'S, 76°17'W, 26.viii.2002 (C. Jiggins leg.); 1♂, km 22 Tarapoto-Yurimaguas, 940m, 06°27'S, 76°17'W, 16.xi.2005 (M. Joron leg.); 2♂, km 19 Tarapoto-Yurimaguas, La Antena, 1300m, 06°27'S, 76°18'W, 22.vii.2007 (M. Joron leg.); 1♂, Fundo Biodiversidad, 950m, 06°28'S, 76°17'W, 21.xi.2007 (G. Lamas leg.). All deposited in MUSM.

Etymology

The subspecies name (a masculine noun in the genitive case) recognizes the contribution of the French evolutionary biologist Dr Mathieu Joron 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*.

Wing shape morphometrics

Morphometric analyses found no significant difference between the wing centroid sizes of *H. e. ucayalensis* and *H. d. joroni* ssp. nov. (FW: $F_{1,103} = 1.62$, $P = 0.20$; HW: $F_{1,103} = 0.52$, $P = 0.47$). 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 demeter joroni* ssp. nov. has proportionally more elongated forewings than *H. e. ucayalensis*, characterized by a reduction around the Cu_1 vein, while *H. e. ucayalensis* has more rounded wings (Fig. 4; Supporting Information, Fig. S4), confirming the perception of human observers (see description of *H. d. joroni* ssp. nov.). The 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 a criterion to distinguish between *H. e. ucayalensis* and *H. d. joroni* ssp. nov, with 92% of females and 93% of males accurately reassigned. Forewing shape differences between *H. e. ucayalensis* and *H. d. joroni* ssp. nov. are much stronger in males (allowing accurate reassignment of 93% of

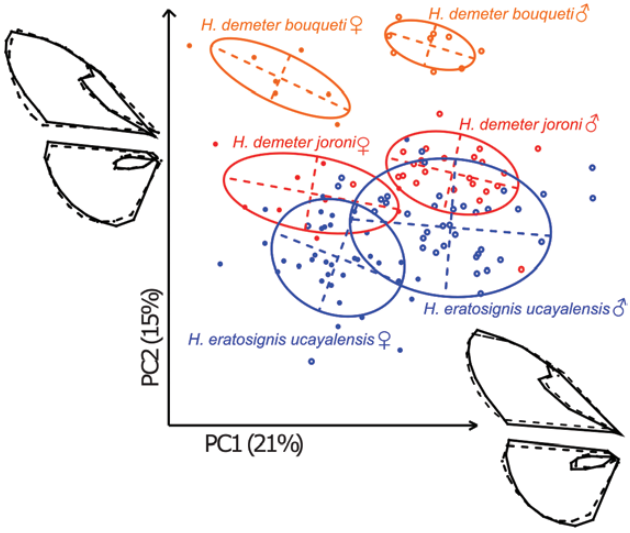

Figure 4. Principal component (PC) analysis of wing shape variation between *H. eratosignis* and *H. demeter*. Males are represented with open circles and females by filled circles. Ellipses represent a graphical summary of the distribution. *Heliconius e. ucayalensis* is shown in blue, ***H. d. joroni* ssp. nov.** in red and *H. d. bouqueti* in orange. Shape variation captured by PC1 and PC2 are illustrated next to each axis, where dotted lines represent minimum values of the axis, and solid lines represent maximum values. PC1 captures shape differences between the sexes across both species. PC2 captures variation between species as well as between *H. demeter* subspecies.

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 (Fig. 4) and the museum specimens of the other races (Supporting Information, Fig. S5). In both species, size and shape exhibit sexual dimorphism with females having larger, wider wings than males (Fig. 4; Supporting Information, Fig. S3) (size FW: $F_{1,104} = 16.9$ $P < 0.001$, HW $F_{1,104} = 28.9$ $P < 0.001$; shape FW: $F_{20,84} = 15.2$, pillai = 0.78 $P < 0.0001$, HW: $F_{20,84} = 19.4$, pillai = 0.82 $P < 0.0001$).

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: subgenus *Astrophea*) at Urahuasha (–6.466°, –76.335°) and San Roque de Cumbaza (–6.363°, –76.441°) (Fig. 5E, 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

Figure 5. Immature stages and host plants of *Heliconius eratosignis* and *Heliconius demeter* near Tarapoto. A, *Heliconius eratosignis ucayalensis* ovipositing on *P. skiantha* in our insectary. B, *Heliconius eratosignis ucayalensis* final instar larva, found wild as a 2nd instar larva on *P. skiantha* at Urahuasha. on 24/3/16. C, ***Heliconius demeter joroni*** Lamas and Rosser **ssp. nov.** final instar larva, found wild as first instar larva on *D. retusa* at San Roque de Cumbaza on 28/3/16. D, *Heliconius eratosignis ucayalensis* pupa. E, *Passiflora skiantha* in flower at El Túnel. 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.

expanding young shoot (Fig. 5A). 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 characterized by a black head, legs and prolegs, spines and anal shield (Fig. 5B). 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. However, the larvae are notable for having black, annular stripes that start around the midpoints of each abdominal segment and run laterally and dorsally, approximately through the spiracles and 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 typical for *Heliconius* in the *H. erato* clade, with long head horns (Fig. 5D). The base coloration 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 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 at Biodiversidad (–6.460556°, –76.289928°), San Roque de Cumbaza, Pucayaquillo (–6.5882°, –76.2224°) and at La Antena (–6.45716°, –76.29858°). On two occasions, pairs of eggs were found on plants at Biodiversidad and La Antena; however, in general, finding eggs and larvae proved difficult. This is probably because it is difficult to find *D. retusa* with new growth suitable for the immature stages of *Heliconius*, at least in those plants accessible to human observers. Nonetheless, on 28 March 2016 a single first instar larva and a yellow ovoid egg were found on a *D. retusa* plant above San Roque de Cumbaza (Fig. 5F). The larva was reared to final instar using *D. retusa* (it refused *P. skiantha*), but failed to pupate. Its identity was confirmed as *H. d. joroni* ssp. nov. using *COI* DNA barcoding. This final instar larva was broadly similar to *H. e. ucayalensis* morphologically (Fig. 5C). However, the black annular stripes running between the spines were absent, and instead the larva was characterized by regular black spotting between the spines. The base colour also appeared a 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 only provide data on larval morphology and host plant use 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. *Heliconius eratosignis eratosignis* has been recorded using *Passiflora* ca. *citrifolia* Salisb. (subgenus *Astrophea*) in Rondônia, and has gregarious, striped final instar larvae (Brown & Benson, 1975).

Molecular data

The models of sequence evolution selected for each gene, along with associated parameter values and Bayesian Inference Criterion score, are shown in Supporting Information, Table S10. Analysis of mtDNA sequences (*COI* + *COII*) revealed a deep divergence between two haplogroups corresponding to *H. d. joroni* ssp. nov. and *H. d. bouqueti* + *H. e. ucayalensis* (Fig. 6). The net proportional distance between these haplogroups is 5.2%, and reciprocal monophyly was well supported (bootstrap percentages of 97% and 99%, respectively). Within the *H. demeter* cluster, *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 in the clade formed by *H. demeter* + *H. eratosignis* (Fig. 6). The resulting phylogeny indicated two reciprocally monophyletic groups, comprising the northern (*H. demeter*) and southern (*H. eratosignis*) races. The southern clade was well supported (100% bootstrap), and comprised *H. e. ucayalensis*, along with *H. e. eratosignis*, *H. e. tambopata* Lamas, 1985 and *H. e. 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 *H. demeter*.

Of the five nuclear loci examined, only *Ef1α* showed *H. d. demeter* + *H. d. bouqueti* and *H. e. ucayalensis* to form reciprocally monophyletic groups (Supporting Information, Fig. S6). Bootstrap support for these two groupings was only moderate (65% and 62%, respectively), and the two exhibited only two fixed nucleotide

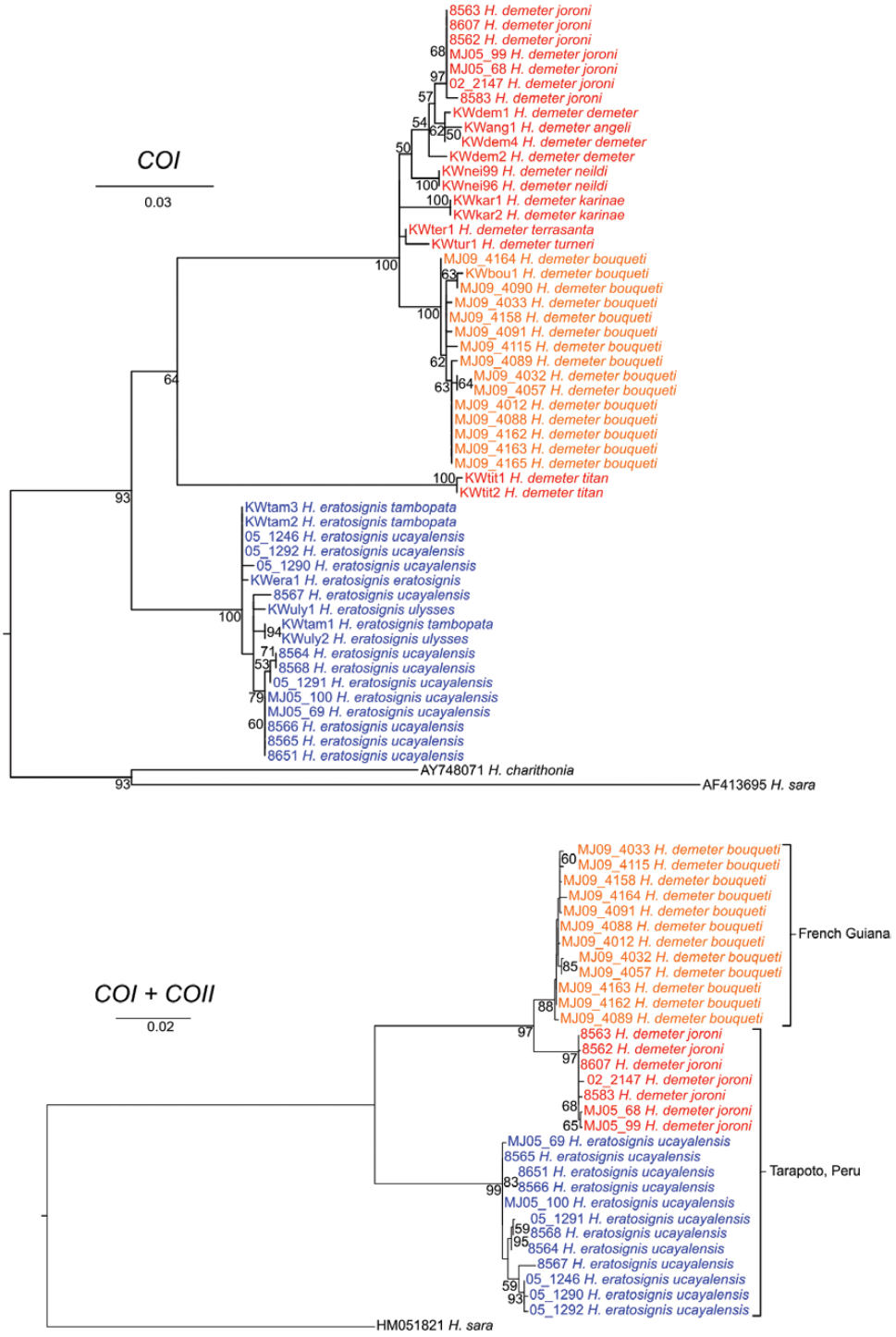

Figure 6. Maximum likelihood phylogenies for, A, 13 of the 15 currently recognized subspecies of *H. demeter* (red) and *H. eratosignis* (blue), based on ~760 bp of mitochondrial CoI sequence, and, B, ***Heliconius demeter joroni*** Lamas and Rosser **ssp. nov.** (red), *H. eratosignis ucayalensis* (blue) and *H. demeter bouqueti* (orange), based on ~2200 bp of mitochondrial *COI* + *COII* sequence. Bootstrap values greater than 50% are shown.

differences across 798 bp of *Ef1α* 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. joroni* ssp. nov. as a well-supported monophyletic group

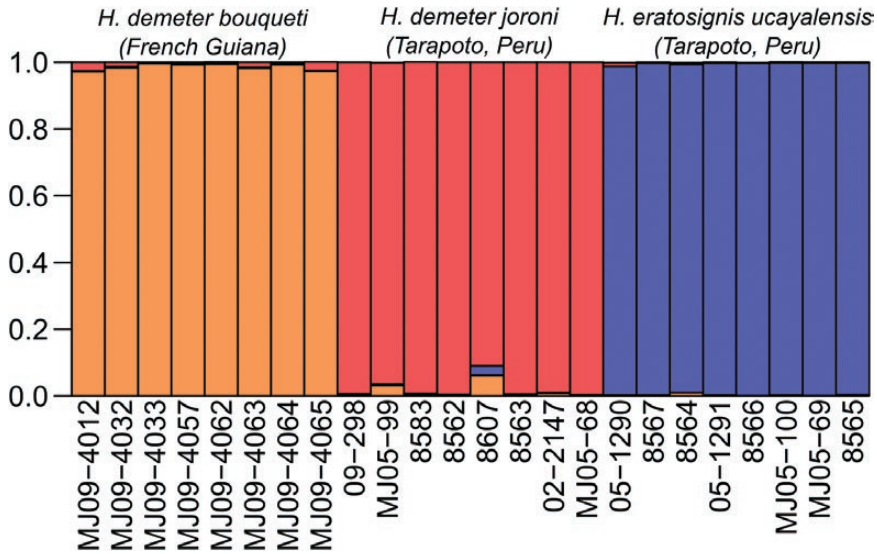

Figure 7. Structure analysis of AFLP genotypes from *H. demeter* and *H. eratosignis* specimens from Tarapoto (Peru) and *H. demeter* from French Guiana using the optimal number of clusters (K = 3). Each of the 24 individuals is represented by a vertical bar broken into three segments. The proportion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

(97%), but found *H. e. ucayalensis* and *H. d. bouqueti* to be polyphyletic. *Rpl5* and *Tektin* showed 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 (Fig. 7). The two sympatric Peruvian taxa, *H. d. joroni* ssp. nov. and *H. e. ucayalensis*, form clearly 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 than in the allopatric *H. d. demeter*–*H. d. bouqueti* comparison.

Geographic distribution

Subspecies of *H. demeter* and *H. eratosignis* are mapped in Fig. 1, with photos of a type specimen 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. Only *H. eratosignis* has been recorded from the adjacent Amazonian lowlands, despite considerable sampling in the area. Museum data and observations by Keith Brown (1979) 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 and excluded them from the distribution map in Fig. 1. The first is probably a generalized 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 at the time (Lamas, 1985).

DISCUSSION

Gene genealogies can be used in concert with morphological differences to diagnose species within single populations, because reciprocal monophyly within a freely 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 recombination.

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 the 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 sister pairs of *Heliconius* species, such as those within the *cydno–melpomene* species group (Beltrán *et al.*, 2002; Giraldo *et al.*, 2008). 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 that are sympatric in at least one area. Additionally, the *COI* phylogeny of 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 evident in the morphometric analysis of wing shape (Fig. 4; Supporting Information, Fig. S5). Both clades were well supported, excepting the uncertain position of *H. d. titan*, whose assignment to *H. demeter* rather than to *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 broadly sympatric with other *H. demeter* races, it may even represent a further cryptic species within this clade.

In contrast to the mtDNA, only one of the five nuclear markers sequenced (*Ef1α*) 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, introgression following speciation, or simply uninformative genetic data (Maddison, 1997; Beltrán *et al.*, 2002; Bull *et al.*, 2006). Because effective population sizes are lower for the maternally inherited *COI* + *COII* and sex-linked *Tpi* than for the autosomal loci, they are expected to coalesce more recently (Palumbi *et al.*, 2001), and so finding monophyly at these loci remains consistent with the hypothesis of ancestral polymorphisms. In addition, if 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. However, females are the heterogametic sex in butterflies, and, in accordance with Haldane's rule, female sterility is an early manifestation of intrinsic postzygotic reproductive isolation (Jiggins *et al.*, 2001a; Naisbit *et al.*, 2002). Introgression should, therefore, be more inhibited at *COI* + *COII* and *Tpi* (Sperling, 1994), thus their monophyly could still be consistent with autosomal introgression between the species. As such, we cannot rule out introgression as a possible cause of incongruence between nuclear genealogies and 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), and 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 hybridization between closely related co-mimics may be higher than between non-mimics, although 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* exhibit similar phylogenetic discordance.

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

In other recently described cryptic *Heliconius*, phenotypic similarity is most parsimoniously explained by convergence through introgression of colour pattern alleles (Mallet, 2009; *Heliconius* Genome Consortium, 2012; Pardo-Díaz *et al.*, 2012). 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 basin. This seems consistent with the species mimetic

similarity, because allopatric speciation does not require ecological divergence (Coyne & Orr, 2004). It might also explain the poly- and paraphyly at nuclear loci, because monophyly would be slow to develop in the large vicariant populations (Maddison, 1997). Nonetheless, *H. demeter* and *H. eratosignis* do differ in other ecologically relevant traits that may have played a part in 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 (in reality, interspersed black and yellow 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 somewhat poorer mimics of other *Heliconius* species than are females 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*. A mimetic explanation for the fused rays of *H. demeter* may, therefore, be more likely than sexual selection, and furthermore fits the hypothesis of vicariance, 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 & 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 (Bush, 1969), or due to disruptive selection for larval performance on alternative hosts (Funk, 1998). *Heliconius demeter* and *H. eratosignis* belong to a clade of *Heliconius* known to exhibit 'pupal mating', in which mating sometimes occurs on the host plant before the females have fully emerged from their pupae (Deinert *et al.*, 1994), thus the former model seems possible. It also seems plausible that the evolutionary and phenotypic divergence between *P. skiantha* and *D. retusa* could produce disruptive selection on larval performance. For example, *P. skiantha* contains cyanogenic glycosides (secondary defence compounds) not found in *D. retusa* (Érika de Castro & Neil Rosser, unpublished). 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; Kozak *et al.*, 2015). Their larvae may also be involved in mimicry with other *Heliconius* species (Brown & Benson, 1975): *Heliconius eratosignis* larvae are nearly identical to the gregarious larvae of *H. doris* (Linnaeus, 1771) and *H. xanthocles* Bates, 1862 (Brown & Benson, 1975; Mallet & Jackson, 1980), whereas, *H. demeter* larvae are more similar to those of *H. ricini* (Linnaeus, 1758). Whatever the drivers of divergence in *H. demeter* and *H. eratosignis*, their limited geographic overlap, comimicry, sexual dimorphism, and marked differences in host plant use and oviposition behaviour, highlight them as an interesting counter-example to other *Heliconius* sister species. In particular, *H. demeter* and *H. eratosignis* exhibit striking parallels to cryptic species in the Afrotropical butterfly genus *Cymothoe* (Nymphalidae). Strong host plant and ecological differences have evolved between *C. egesta* (Cramer, 1775) and *C. confusa* Aurivillius, 1887, formerly considered subspecies of a single widely distributed species. These differences are apparently insufficient to allow sympatry, bar a narrow region of overlap between their otherwise allopatric ranges (McBride *et al.*, 2009).

Our use of integrative taxonomy (Dayrat, 2005; Pante *et al.*, 2015) to diagnose a cryptic species of *Heliconius* joins a series of similar, recent discoveries in other butterflies (Willmott *et al.*, 2001; Hebert *et al.*, 2004; McBride *et al.*, 2009; Dincă *et al.*, 2011; Hill *et al.*, 2012; Barbosa *et al.*, 2015). Frequently, cryptic taxa are initially flagged by molecular markers, after which subtle differences in morphology or behaviour are recognized as species-specific (Janzen *et al.*, 2009). Thus, despite its limitations (Elias *et al.*, 2007; Silva-Brandão *et al.*, 2009), DNA barcoding still holds great potential to screen putative cryptic species for further study. While the net contribution of cryptic species to biodiversity remains to be established (Stork, 2018), the continual discovery of hidden species in a group as intensively studied as butterflies suggests that predictions of global species richness based on current knowledge may be gross underestimates (Adis, 1990; Bickford *et al.*, 2007).

DATA ACCESSIBILITY

DNA sequences have been submitted to GenBank; accession numbers are given in the Supplementary Information.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Landmarks used for the analysis of wing shape and size with geometric morphometry (displayed on *H. eratosignis ucayalensis* male).
Figure S2. Morphology of genitalia. Arrow indicates possible fixed difference. Voucher IDs in brackets.
Figure S3. Forewing (left) and hindwing (right) centroid size for *H. demeter joroni* ssp. nov. and *H. eratosignis ucayalensis.* Groups labelled with the same letter do not show significant centroid size difference.
Figure S4. Principal component analysis displaying wing shape variation between *H. demeter joroni* ssp. nov. (red) and *H. eratosignis ucayalensis* (blue). Males are represented with open circles and females by filled circles. Ellipses represent a graphical summary of the distribution. Top: forewing, bottom: hindwing. Shape variation is illustrated next to each axis, where broken shapes represent minimum negative values of the axis, and full lines represent maximum values.
Figure S5. Principal component (PC) analysis of wing shape variation between *H. eratosignis* and *H. demeter* with the inclusion of additional subspecies from museum collections (types, syntypes, etc.). As in Fig. 4 in the main text, the analysis included *H. e. ucayalensis* (in blue), *H. d. joroni* ssp. nov. (in red) and *H. d. bouqueti* (in orange); males are represented with open circles and females by filled circles. The additional museum specimens are represented with red letters for *H. demeter* (orange for *H. d. bouqueti*), corresponding to the subspecies: a, *H. d. angeli*; b, *H. d. bouqueti*; d, *H. d. demeter*; k, *H. d. karinae*; n, *H. d. neildi*; t, *H. d. titan*; z, *H. d. zikani;* and with a blue letters for *H. eratosignis*: e, *H. e. eratosignis*; u, *ucayalensis*; y, *H. e. ulysses*. Ellipses represent a graphical summary of the distribution. Shape variation captured PC1 and PC2 are illustrated next to each axis, with dotted lines represent minimum values of the axis, and full lines representing the 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.
Figure S6. Maximum likelihood trees of *H. demeter* and *H. eratosignis* specimens (red and blue respectively) from Tarapoto (Peru) and *H. demeter* from French Guiana (orange) 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.
Table S1. Revised synonymy of taxa formerly considered part of *H. demeter.* Letters a–m are valid subspecies names according to our revision and that of Lamas (2004).
Table S2. Type specimens of *H. demeter* and *H. eratosignis* examined. [1] Figured in: Holzinger, H. & Holzinger, R. 1975. *Heliconius demeter ucayalensis*, eine neue Subspezies aus Peru (Lepidoptera: Nymphalidae). Z. ArbGem. öst. Ent. 26:29–152. [2] Figured in: Lamas, G. 1985. Los Papilionoidea (Lepidoptera) de la Zona Reservada de Tambopata, Madre de Dios, Perú.- I : Papilionidae, Pieridae y Nymphalidae (en parte). Revista Peruana de Entomología 27: 59–73.
Table S3. Specimens used in morphometric analysis.
Table S4. List of *Passiflora* species used in captive host plant oviposition tests.
Table S5. Details of samples used for molecular work.
Table S6. PCR conditions for the amplicons used in this study. All reactions were carried out in 10-μL volumes using 10×buffer (Sigma), 0.5 μM each of forward and reverse primers and 0.25U *Taq* polymerase (Sigma). PCR cycling conditions were: initial denaturing at 94 ºC for 2 min; followed by 35 cycles of 94 ºC for 45 s, annealing temperature (T_a) for 45 s, 72 ºC for 60 s; final extension at 72 ºC for 5 min.
Table S7. GenBank Accession numbers for sequences used in nuclear phylogenies.
Table S8. GenBank accession numbers for sequences used in mtDNA phylogenies.
Table S9. Subspecies classified by presence or absence of strong costal yellow streak on the ventral forewing. Descriptions based on examination of holotype, syntype, allotype detailed in Table S2; fw = forewing; hw = hindwing.
Table S10. Models of sequence evolution and estimated parameters, selected using Bayesian Inference Criterion.