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Genetic differentiation without mimicry shift in a pair of hybridizing *Heliconius* species (Lepidoptera: Nymphalidae)

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Butterflies in the genus *Heliconius* have undergone rapid adaptive radiation for warning patterns and mimicry, and are excellent models to study the mechanisms underlying diversification. In *Heliconius*, mimicry rings typically involve distantly related species, whereas closely related species often join different mimicry rings. Genetic and behavioural studies have shown how reproductive isolation in many pairs of *Heliconius* taxa is largely mediated by natural and sexual selection on wing colour patterns. However, recent studies have uncovered new cases in which pairs of closely related species are near-perfect mimics of each other. Here, we provide morphometric and genetic evidence for the coexistence of two closely related, hybridizing co-mimetic species on the eastern slopes of the Andes, *H. melpomene amaryllis* and ***H. timareta* ssp. nov.**, which is described here as ***H. timareta thelxinoe***. A joint analysis of multilocus genotyping and geometric morphometrics of wing shape shows a high level of differentiation between the two species, with only limited gene flow and mixing. Some degree of genetic mixing can be detected, but putative hybrids were rare, only one of 175 specimens being a clear hybrid. In contrast, we found phenotypic differentiation between populations of ***H. timareta thelxinoe***, possibly indicative of strong selection for local mimicry in different communities. In this pair of species, the absence of breakdown of genetic isolation despite near-identical wing patterns implies that factors other than wing patterns keep the two taxa apart, such as chemical or behavioural signals, or ecological adaptation along a strong altitudinal gradient. © 2013 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2013, ••, ••–••.

ADDITIONAL KEYWORDS: geometric morphometrics – hybridization – multilocus genotype – reproductive isolation – speciation – wing shape.

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INTRODUCTION

Heliconius butterflies have become important models in evolutionary biology because of their remarkable intra- and interspecific variation in warning colour patterns (Brown, 1979; Sheppard *et al*., 1985), mimicry associations (Mallet, 1989; Kapan, 2001; Langham, 2004) and other aspects of their evolutionary ecology (Brown, 1981). More recently, these butterflies have become models for ecological speciation research, especially to understand genetic changes and phylogenetic patterns associated with speciation (Jiggins *et al*., 2001; Beltran *et al*., 2002; Naisbit *et al*., 2002; Salazar *et al*., 2005; Bull *et al*., 2006; Mavarez *et al*., 2006; Kronforst, 2008; Heliconius Genome Consortium, 2012; Rosser *et al*., 2012).

Recent research supports a major role for wing pattern divergence in the promotion of genetic divergence and in keeping species isolated despite occasional hybridization. Indeed, two of the most important known processes reducing gene flow between races or closely related species in *Heliconius* involve colour patterns: assortative mating in part mediated by colour patterns (McMillan, Jiggins & Mallet, 1997; Jiggins *et al*., 2001; Jiggins, Estrada & Rodrigues, 2004; Mavarez *et al*., 2006; Merrill *et al*., 2010) and selection against nonmimetic hybrid forms (Mallet, 1989; Kapan, 2001; Naisbit, Jiggins & Mallet, 2001). Wing patterns thus combine premating isolation via assortative mating and postmating isolation as a result of increased predation on nonmimetic hybrids or geographically distant forms (Mallet & Barton, 1989; Pinheiro, 2003), making them so-called 'magic traits' for ecological speciation (Mallet, Jiggins & McMillan, 1998; Merrill *et al*., 2010). In *Heliconius*, closely related species tend to differ in wing colour pattern (Beltran, 2004; Rosser, 2012), perhaps corroborating the extensive experimental evidence that shifts in colour patterns favour speciation (Mallet *et al*., 1998; Jiggins *et al*., 2001; Chamberlain *et al*., 2009).

Strong assortative mating has been shown in several pairs of hybridizing species in *Heliconius* (Jiggins *et al*., 1996, 2001; McMillan *et al*., 1997). For instance, partially interfertile *H. melpomene* Linnaeus and *H. cydno* Doubleday, which diverged in the last million years (Beltran *et al*., 2002), coexist in sympatry over much of Central America (Brown, 1979). Assortative mating between *H. melpomene* and *H. cydno* forms a strong overall barrier to hybridization mediated, in part, by strong male preference based on visual cues, the probability of courting a female of a different colour pattern being reduced by 75% (Jiggins *et al*., 2001). Other sources of reproductive isolation are documented, such as the lower mating success of hybrids (Naisbit *et al*., 2001) (itself possibly colour mediated), hybrid sterility (Naisbit *et al*., 2002; Salazar *et al*., 2005) and habitat preference (Estrada & Jiggins, 2002). Nonetheless, one of the main teachings of the recent studies is the functional association between a divergence in colour pattern and genetic isolation in pairs of closely related *Heliconius* taxa living in contact (sympatry/parapatry) (Brower & Egan, 1997; Mallet *et al*., 1998; Jiggins *et al*., 2001; Merrill *et al*., 2011).

However, a few pairs of closely related *Heliconius* species have been found recently which are perfect co-mimics of each other (Brower, 1996; Giraldo *et al*., 2008; Mallet, 2009) and therefore do not conform well to a model of wing pattern-mediated speciation. Three pairs of hybridizing co-mimics have been identified, in which one species of the *H. cydno* clade is a close mimic of the local coexisting race of *H. melpomene* (sister to the *H. cydno* clade), and recent studies have documented hybrid exchange of adaptive genes between these co-mimics (Heliconius Genome Consortium, 2012; Pardo-Diaz *et al*., 2012). The *cydno* clade consists of several young species: *H. cydno* Doubleday, represented by several subspecies differing in wing pattern at low elevations in Central America and west of the Andes; *H. pachinus* Salvin, in northern Panama; and a series of taxa found at mid-elevation on the eastern foothills of the Andes, represented in Colombia by *H. heurippa* Hewitson, *H. timareta tristero* Brower, *H. timareta florencia* Giraldo and *H. timareta* ssp. nov. (Giraldo *et al.*, 2008), in Ecuador by the polymorphic *H. timareta timareta* Hewitson and in Peru by *H. timareta timoratus* Lamas (Fig. 1).

On the eastern side of the north-central Peruvian Andes, long-term population and molecular ecological studies have now revealed the existence of another taxon, described here in the Appendix as *H. timareta thelxinoe*, also belonging to the *H. cydno* clade, and with near-perfect resemblance to the local wing pattern form of *H. melpomene amaryllis* (Heliconius Genome Consortium, 2012; Pardo-Diaz *et al*., 2012). *Heliconius timareta thelxinoe* occurs at altitudes between 1000 and 1700 m in the eastern Andes of San Martín, Peru. Although it predominates at higher elevations, *H. timareta thelxinoe* is also found in sympatry with *H. melpomene amaryllis* in the lower part of its altitudinal range, in an extended zone within the Río Mayo valley between 1000 m and 1400 m (Fig. 1). Considering that *H. melpomene* and *H. cydno* are maintained in isolation, partly as a result of divergence in wing patterns (Jiggins *et al*., 2001; Merrill *et al*., 2011), a high level of hybridization between the co-mimics *H. melpomene amaryllis* and *H. timareta thelxinoe* would confirm the predominant role of colour pattern shift in the promotion of isolation. However,

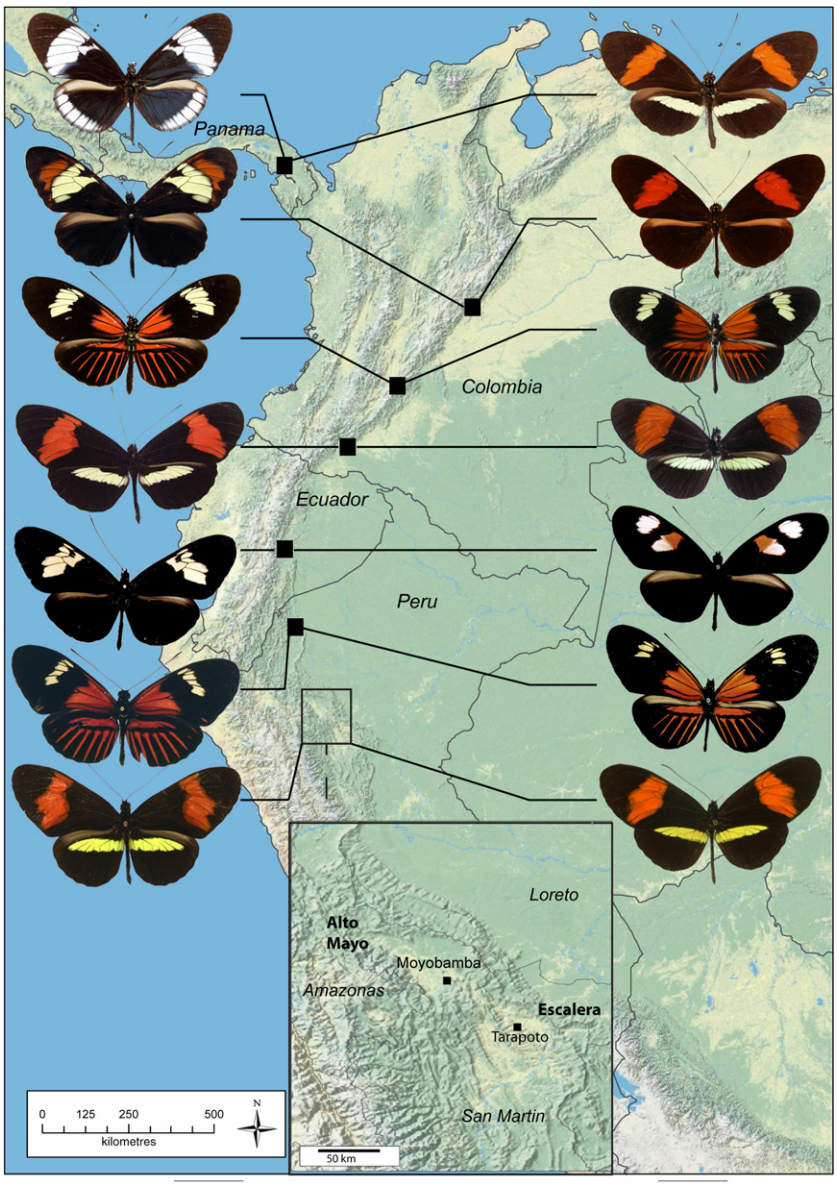

Figure 1. Map of the *Heliconius melpomene/H. cydno* clade where they overlap in Panama and along the eastern slopes of the Andes. From top to bottom: left: *H. cydno chioneus*, *H. heurippa*, *H. timareta florencia*, *H. timareta tristero*, *H. timareta timareta*, *H. timareta timoratus*, *H. timareta thelxinoe*; right: *H. melpomene rosina*, *H. melpomene melpomene*, *H. melpomene malleti*, *H. melpomene bellula*, *H. melpomene plesseni*, *H. melpomene ecuadorensis*, *H. melpomene amaryllis*. The inset shows a map of the study area, with the Escalera and Alto Mayo populations separated by approximately 200 km.

strong differentiation between *H. melpomene* and *H. timareta thelxinoe* would suggest an important role of factors other than colour pattern in assortative mating and selection against hybrids. Here, we analyse genetic and morphometric variation to investigate genomic vs. colour pattern differences between *H. timareta thelxinoe* and its co-mimic *H. melpomene*, and its relationship with other subspecies of the *H. timareta* lineage. Then, we combine multilocus genotyping with morphometric analysis of wing pattern and wing shape on a larger population sample to evaluate the importance of phenotypic similarity in the differentiation and hybridization in this pair of co-mimics, and to suggest new avenues for speciation research in this group of butterflies.

MATERIAL AND METHODS

SPECIMENS

Figure 1 and Supporting Information Table S1 show the geographical distribution and sampling information for all species and races analysed. A total of 223 individuals (133 *H. melpomene amaryllis*, 89 *H. timareta thelxinoe* and one specimen later identified as a possible hybrid) were sampled in the Escalera and Alto Mayo areas near Tarapoto, Departamento de San Martín, Peru. Sampling localities were chosen along an altitudinal continuum from Tarapoto (300 m) to montane forests (1800 m). Bodies were preserved in salt-saturated 20% dimethylsulphoxide (DMSO) with ethylenediaminetetraacetic acid (EDTA) and wings were stored in envelopes. Specimens of other *cydno* group species and the Amazonian race *H. melpomene aglaope* were included in the phylogenetic and morphometric analysis. The description of *H. timareta thelxinoe* is appended to this study. The holotype (Fig. 2A) and 11 paratypes are deposited at the Museo de Historia Natural, Universidad Nacional Mayor de San Marcos, Lima, Peru.

DNA SEQUENCE ANALYSIS

Mitochondrial sequences for *cytochrome oxidase I* (*CoI*), *tRNA-leu* and the 5′ end of *cytochrome oxidase II* were obtained for 13 *H. timareta thelxinoe* and three *H. timareta tristero* specimens using the primers and PCR conditions described in Dasmahapatra *et al*. (2010) (Table S1). Mitochondrial sequences were obtained from GenBank for *H. melpomene* (Supporting Information Tables S1 and S2), for species in the *cydno* group (*H. timareta timareta*, *H. cydno*, *H. heurippa*, *H. timareta tristero*, *H. pachinus*) and for the outgroup species *H. numata* (accession numbers in Table S2). Nuclear sequences of the sex-linked gene *triosephosphate isomerase* (*Tpi*) were amplified and sequenced for three *H. timareta thelxinoe* [primers and PCR conditions as in Dasmahapatra *et al*. (2010)], and additional sequences for the other taxa were obtained from GenBank (Tables S1 and S2). Sequences generated for the present study are available in GenBank (Accession numbers KC435427–KC435446).

Phylogenetic relationships were inferred using maximum likelihood with RAxML Blackbox (Stamatakis, Hoover & Rougemont, 2008) and Bayesian inference in Beast 1.6.2 (Drummond & Rambaut, 2007). In maximum likelihood and Bayesian inference, each gene was analysed with a GTR + G model, as suggested by the model testing performed with jModel Test 1.1 (Posada, 2008), and parameters of this model were estimated independently for each gene. In Bayesian inference, the tree prior was set to the Yule speciation process. Branch lengths were modelled under a relaxed clock assumption with an uncorrelated log-normal distribution. Default

Figure 2. A, *Heliconius timareta thelxinoe* male, from the Alto Mayo, Peru. Holotype (Museo de Historia Natural, Universidad Nacional Mayor de San Marcos, Lima, Peru – MUSM); B, *H. melpomene amaryllis* male, from Tarapoto, Peru (MUSM). Dorsal view (left) and ventral view (right). C, Landmarks used for geometric morphometrics, shown on a ventral view of *H. timareta thelxinoe* wings.

parameters were used for all priors. We ran the analyses for 10 million generations, and sampled every 10 000 generations. Using the Tracer program, we confirmed that the run had converged to a stationary distribution after 250 samples (burn-in). The 750 samples remaining were used to describe the posterior distribution. The posterior distribution of the trees was summarized using the maximum credibility tree with average values for branch length using TreeAnotator 1.7.1 (Drummond & Rambaut, 2007).

Morphometric analyses

Images of ventral and dorsal forewings (FW) and hindwings (HW) were captured using a high-resolution flatbed scanner, to score phenotypic indices and for geometric morphometric analyses on the specimens. This procedure was performed on specimens with undamaged wings: 88 *H. timareta thelxinoe*, 113 *H. melpomene amaryllis* and one possible hybrid, and also on specimens from related *H. cydno* group species (*H. timareta timareta*, *H. timareta tristero*, *H. cydno chioneus*, *H. heurippa*) and *H. melpomene aglaope* (Table S1).

Phenotypic indices

We designed six phenotypic indices to integrate variation at six chosen wing characters that tend to differ between *H. melpomene amaryllis* and *H. timareta thelxinoe* and are easy to score in the field (Fig. 2A, B). Unlabelled photographs of all specimens were scored for these characters, ranging from '0' (typical of *H. melpomene amaryllis*) to '1' (typical of *H. timareta thelxinoe*). Specimens were scored in a random order by a single individual. These characters were (from '0' to '1') as follows: FW: size of the red line at the base of the underside costal vein (absent to well marked), hue of the underside red FW patch (red–orange to pinkish), position of the red patch relative to the discal cell on both ventral and dorsal sides (broad intersection vs. no intersection with cell); HW: size of the basal underside red spots (minute or absent dots to large smudgy spots), hue of the HW bar (yellow to whitish). The six character indices were analysed using a principal component analysis (PCA), and a mean index was calculated. Cross-validation (CV) percentages of discriminant analyses were used to quantify the classification accuracy of the characters separately and all together.

Geometric morphometrics

Phenotypic variation was quantified on both sides of the wing using the size and shape of the venation pattern, the wing outline and colour patches. The overall structure of the wing, irrespective of colour pattern, 'wing', was described using the two-dimensional coordinates of a subset of 15 and 14 landmarks on the FW and HW surfaces respectively, placed at vein intersections and vein termini (Fig. 2C: 1–15 on FW; 26–39 on HW). Wing landmarks were taken in *H. timareta thelxinoe*, *H. melpomene amaryllis* and in related species, on the ventral side on which the veins are more visible. The size and shape of the red and yellow colour patches, characteristic of the so-called 'postman' pattern of this pair of mimetic species, and hereafter called 'colour patch', were described by the two-dimensional coordinates of a subset of 10 landmarks placed at the intersection of the outline of the colour patches and veins (Fig. 2C: 16–25 and 41–49). Colour patch landmarks were taken on both dorsal and ventral sides. Wing and colour patch data were analysed jointly and separately. Landmark coordinates were scored by the same person using TpsDig2 (Rohlf, 2010). Landmark coordinates were superimposed using a general procrustes analysis (Rohlf & Slice, 1990; Bookstein, 1991, 1996; Goodall, 1995; Dryden & Mardia, 1998; Zelditch *et al.*, 2004). Standard tests of repeatability were performed by taking the landmarks five times per wing on subsamples of five butterflies from a single species, population and sex.

Overall size was measured using log-transformed centroid size [CS; see Bookstein (1991)] from the ventral measures of wing landmarks. We detected no size differences between males and females in either *H. melpomene amaryllis* ($N_{\text{males}} = 90$, $N_{\text{females}} = 23$; Student's t-test: $F_{1,111} = 0.0029$, $P = 0.96$) or *H. timareta thelxinoe* ($N_{\text{males}} = 75$, $N_{\text{females}} = 13$; Student's t-test: $F_{1,86} = 0.22$, $P = 0.64$). Overall differences in size between species were investigated with a one-way analysis of variance (ANOVA) with size as a dependent variable and species (or location of the population within each species) as factor, and visualized using boxplots (Fig. 3B). Because of multiple comparisons, P values were corrected following Benjamini & Hochberg (1995). HW and FW size variations between species were congruent, and so we defined a specimen's reference size as the sum of the logarithm of each wing's CS.

To study shape, we employed a dimensionality reduction 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 species, we used a one-way multivariate ANOVA (MANOVA) on those subsets of PCs with shape as dependent variable and species as factor. Species discrimination based on shape was investigated through a canonical variate

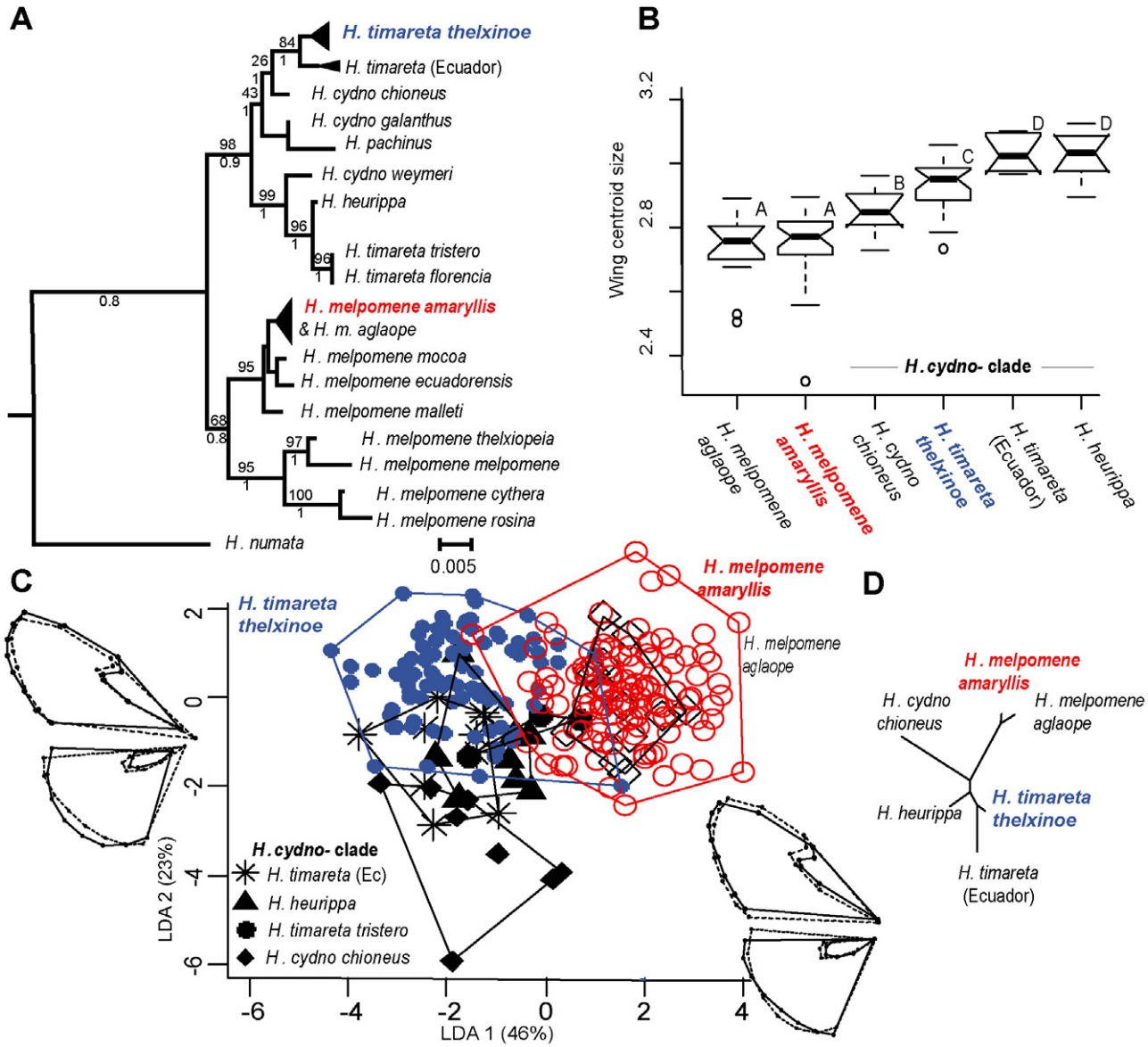

Figure 3. A, Maximum likelihood (ML) phylogenetic relationships between *Heliconius melpomene* and multiple *H. cydno* clade members, including the new and the previously described races of *H. timareta*, based on mitochondrial sequences of *cytochrome oxidase I* (*CoI*) and *cytochrome oxidase II* (*CoII*) and nuclear sequences of *triosephosphate isomerase* (*Tpi*). ML bootstrap supports are shown above the branches; Bayesian posterior probability values are shown below the branches. B, Wing centroid size variation. Confidence intervals of the median are visualized by notches. C, Canonical variant analyses (CVA) based on wing shape. Shape variation is illustrated next to each axis, where broken shapes represent minimum negative values of the axis, and full lines represent maximum values. *Heliconius melpomene amaryllis* is represented with open red circles, *H. melpomene aglaope* with open black diamonds and *H. timareta thelxinoe* with blue circles. Other *H. cydno* clade taxa are represented with black symbols. D, Unrooted neighbor-joining dendrogram of the Malahanobis distances between species based on forewing and hindwing shape.

analysis (CVA) with a leave-one-out CV procedure. Differences in shape can be visualized as deformations along the factorial axes calculated by multivariate regressions (Monteiro, 1999). We then explored shape differences between populations of different geographical origins within each species using the same methods. All subsets of wing shape and colour patch shape (FW and HW, ventral and dorsal sides) were analysed separately and then pooled. The overall phenotypic similarities between species were depicted using a neighbor-joining (NJ) tree computed from the matrix of Malahanobis' D^2 distances.

Species identity was first assessed by eye and confirmed by microsatellite genotype data for 160 of 202 samples. Differences between *H. timareta* and *H. melpomene* were first explored using only the 160 genotyped specimens and a linear discriminant analysis (LDA). This LDA was computed on a combined dataset of all the landmarks on both FW and HW (CV rate = 100%). A predictive LDA was then used to identify the nongenotyped specimens. The confidence with which a specimen is assigned to one of the two groups corresponds to the posterior probability (PP) of classification, which depends on the relative distance of the specimens to the group means (Table S1).

The potential effects of sexual dimorphism were explored before further analyses. In taxa for which we had a sufficient number of specimens to test for sexual dimorphism, males and females did not differ in size, but did differ in shape. Nevertheless, we found no significant interactions between sex and species for size [interaction terms of an analysis of covariance (ANCOVA): $F_{1,197} = 0.056$, $P = 0.81$] or shape [interaction terms of a multivariate ANCOVA (MANCOVA): FW: $F_{8,190} = 1.73$, $P = 0.09$; HW: $F_{8,190} = 1.5$, $P = 0.15$]. Therefore, sexual dimorphism is homogeneous across taxa, and sexes were pooled in the rest of our analyses.

We analysed allometry (the relationship between size and shape) by testing for homogeneity of within-species allometric patterns using MANCOVAs with shape as the dependent variable, log CS as a covariate and species as a factor.

All statistics and morphometrics were performed in R. 2.13.1 (R Development Core Team, 2011) with ade4 (Chessel, Dufour & Thioulouse, 2004), APE (Paradis, Claude & Strimmer, 2004) and Rmorph (Baylac, 2012) libraries.

Multilocus microsatellite analysis

Multilocus genotypes were derived by examining variation at 11 microsatellite loci developed for *Heliconius* (Supporting Information Table S3) using the primers and PCR conditions adapted from Flanagan *et al*. (2002) and Mavarez & Gonzalez (2006). A preliminary set of specimens of 59 *H. melpomene amaryllis* and 28 *H. timareta thelxinoe* was analysed using GeneMapper with the Genescan Rox-500 size standard for allele size determination (Applied Biosystems). A secondary set of specimens of 37 *H. melpomene amaryllis* and 59 *H. timareta thelxinoe*, including one putative hybrid and four reference individuals per species from the first set, was analysed using GeneMarker 2.2.0 with the Genescan-500Liz size standard. Linkage disequilibrium and departure from Hardy–Weinberg expectations within each population were tested using exact tests implemented in GENEPOP 4.1.4 (Rousset, 2008). We used FSTAT 2.9.3 (Goudet, 2001) to survey within-species genetic diversity in terms of expected heterozygosity (H_E), observed heterozygosity (H_O) and allelic richness (A), estimated on the smallest sample size per locus per population ($N = 74$ for the locus Hel4 in *H. timareta thelxinoe*). Allelic frequency and F statistics (Weir & Cockerham, 1984) were calculated using GENETIX 4.05 (Belkhir *et al*., 1996–2004).

We used two multilocus Bayesian clustering methods to assign individuals to species and to detect admixed individuals (e.g. admixed genotypes). STRUCTURE 2.2 (Pritchard, Stevens & Donnelly, 2000) was run with 500 000 updates of the Markov chain after an initial 'burn-in' of 50 000 updates, to achieve chain convergence. PPs of being a member of a cluster (q) were estimated for a set of models with different numbers of clusters ($K = 1$–4), ancestry type (with/without admixture) and allele frequency estimation (correlated vs. independent). Then, for the best model, the proportion of an individual's genotype belonging to each cluster (q values) was used as a genotypic introgression index. The detection of putative hybrids or admixed individuals was also performed using NEWHYBRIDS 1.1 (Anderson & Thompson, 2002). This method outputs the PP that an individual in the sample set belongs to each of the six possible genotypic classes, i.e. pure *H. melpomene amaryllis*, pure *H. timareta thelxinoe*, F1, *amaryllis* backcross, *thelxinoe* backcross and F2. This PP reflects directly the level of certainty that an individual truly belongs to the genotypic class. In order to test the assumption that variation at these microsatellite loci is sufficient to detect the introgressive proportions expected under a scenario of recurrent backcrossing, we used HYBRIDLAB 1.0 (Nielsen, Bach & Kotlicki, 2006) to simulate populations of F1, F2, three generations of backcrosses and two 'pure' populations. The genotypes used as parents for the simulations were selected in the populations as far as possible from the zone of altitudinal sympatry: 25 *H. melpomene amaryllis* from low altitude (Tarapoto, Moyobamba) and 25 *H. timareta thelxinoe* from the highest altitude (Alto Mayo), with a mean phenotypic index close to '0' or '1', respectively. The 10 simulated populations were analysed as above in STRUCTURE 2.2 and NEWHBRIDS 1.1.

RESULTS

Species relationships

Mitochondrial data and nuclear DNA

Bayesian and maximum likelihood analyses on a combination of mtDNA and nuclear DNA support the existence of two well-supported clades: an *H. melpomene* clade including *H. melpomene amaryl-*

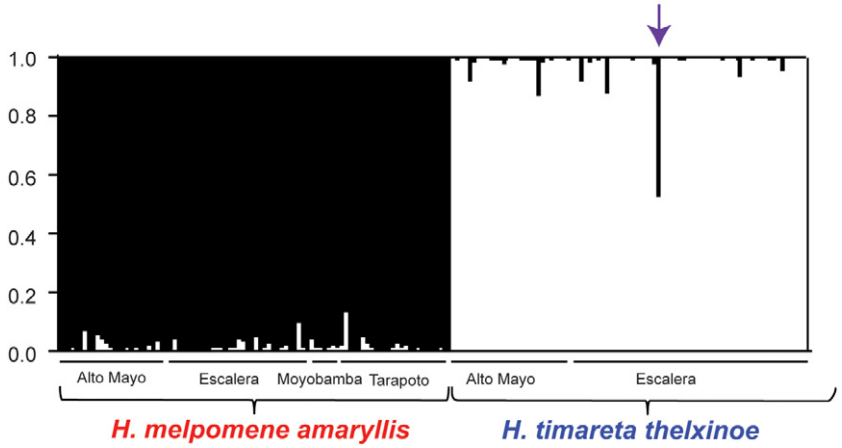

Figure 4. Genetic differentiation between *Heliconius timareta thelxinoe* and *H. melpomene amaryllis* based on a multilocus microsatellite analysis. Results shown are the assignment analysis performed in STRUCTURE 2.3.1 using $k = 2$. The relative genome contribution of the two clusters to each individual is shown in black and white. The putative hybrid specimen is indicated by an arrow.

lis and *H. melpomene aglaope*; and a clade containing *H. cydno* and related species *H. heurippa*, *H. timareta* and *H. timareta tristero*, and including our *H. timareta thelxinoe* specimens (Fig. 3A). This is consistent with the phylogeny based on genome-wide restriction site-associated DNA (RAD) markers presented in Heliconius Genome Consortium (2012), and confirms the identity of this population as a member of the *cydno* clade, closely related to *H. timareta timareta* from Ecuador, which is also the geographically nearest *cydno* cognate in our dataset.

Morphometric proximity

Species differ notably in size (ANOVA: $F_{6,237} = 44$, $P < 0.0001$), *H. melpomene* races being smaller than *cydno* group taxa, including *H. timareta thelxinoe* (Fig. 3B), and in shape (Pillai = 2.2, $F_{6,237} = 52$, $P < 0.0001$). The first two axes of the CVA (Fig. 3C) separate three groups with distinct wing shapes. Axis 1 (46%) separates *H. melpomene* from all *H. cydno* group taxa, *H. melpomene* having slightly shorter, rounder wings with a proportionally longer discal cell. Axis 2 (26%) separates *H. cydno chioneus*, with very rounded HW, from all other species. According to the NJ network (Fig. 3D), *H. timareta thelxinoe* and *H. timareta timareta* from Ecuador cluster together. Nevertheless, these two subspecies differ significantly in wing shape (Pillai = 0.51, $F_{10,88} = 9.2$, $P < 0.01$). In contrast, the two *H. melpomene* races cluster together in the NJ network and do not differ in wing shape (Pillai = 0.03, $F_{10,115} = 0.35$, $P = 0.96$).

Genetic differentiation

Genetic variation within each species is shown in Table S3. A total of 256 alleles was found, *H. melpomene* showing more diversity than *H. timareta thelxinoe* (for a population of 74 individuals, the estimated allelic richness $A = 19$ vs. $A = 10$, respectively). No significant linkage disequilibrium was found between loci. Some loci did show significant deviations from Hardy–Weinberg expectations in each species, presumably because of the presence of null alleles, as reported in previous studies (Flanagan *et al.*, 2002; Mavarez & Gonzalez, 2006). Genetic differentiation between the two species was strong and significant ($F_{ST} = 0.14$, $P < 0.001$). This differentiation is confirmed here by Bayesian clustering using STRUCTURE and NEWHYBRIDS (Fig. 4). Initial runs without prior species information identified two clusters with high q values (average $q > 0.98$), corresponding to our identifications based on phenotypic indices; only two of 92 specimens (05-1079 and MJ11-3040), identified as *H. timareta* using phenotypic indices, were unambiguously assigned to *H. melpomene* using microsatellites, and subsequently classified as *H. melpomene*. These results were robust to parameter variations (ancestry with vs. without admixture; correlated vs. independent allelic frequencies). One specimen (MJ11-3025) could not be assigned an ID because of intermediate PPs ($q = 0.48$), and was therefore given a putative hybrid status. Using admixture analyses with STRUCTURE, a few specimens were identified to a species with a PP $q < 0.95$, indicating possible admixed ancestry: four in 83 *H. timareta* ($q = 0.84$ to $q = 0.94$) and six in 92 *H. melpomene* ($q = 0.87$ to $q = 0.94$) (Fig. 4). Using NEWHYBRIDS, the same specimens and two additional *H. melpomene* specimens were identified as intermediate (PP < 0.70); most were assigned to a first-generation backcross, albeit with moderate probability (0.30–0.70). The putative hybrid was assigned

to F1 or a backcross hybrid. Analyses on simulated hybrids and backcrosses (Supporting Information Fig. S1) suggest that our set of markers can distinguish pure individuals from individuals with a certain amount of introgression (F1, F2 and backcross 1), although second and third generations of backcrosses are harder to identify. The putative hybrid is a male butterfly from the Escalera population and has an *H. timareta* phenotype (wing shape, red costal line, large red dots).

Morphological differences between *H. melpomene amaryllis* and *H. timareta thelxinoe*

Phenotypic indices

Heliconius melpomene amaryllis and *H. timareta thelxinoe* differ significantly based on the PCA on the six phenotypic indices (Pillai = 0.81, $F_{6,195} = 147$, $P < 0.0001$), and are clearly separated by the first axis (PC1: 72%, Fig. 5A). Moreover, within *H. timareta thelxinoe*, these indices show geographical variation, specimens from the Escalera being more similar to *H. melpomene amaryllis* than to specimens from the Alto Mayo (Fig. 5A: mean indices: *H. melpomene amaryllis* = 0.23 ± 0.09, *H. timareta thelxinoe* Escalera = 0.67 ± 0.12, *H. timareta thelxinoe* Alto Mayo = 0.89 ± 0.07). Overall, a combination of the six characters gives a relatively good identification: using an LDA combining the six indices, the CV rate reaches 95% (vs. 78–89% for each criterion alone) (Fig. 5A).

Wing size and colour patch size

Heliconius timareta thelxinoe is larger than *H. melpomene amaryllis* for both HW ($F_{1,200} = 137$, $P < 0.0001$) and FW ($F_{1,200} = 165$, $P < 0.0001$) (Fig. 3B). No size differences were found among the Alto Mayo and Escalera populations within each species, either for *H. timareta thelxinoe* ($P = 0.54$) or *H. melpomene amaryllis* ($P = 0.85$). The wing size of the putative hybrid is smaller than 75% of *H. melpomene amaryllis* and at the minimum of the *H. timareta thelxinoe* range size. The FW red patch does not differ in absolute size between the two species (*t*-test on CS: $P = 0.08$); however, because *H. melpomene amaryllis* has smaller wings, the proportion of the FW covered by the red patch is significantly larger for this species than for *H. timareta thelxinoe* (*t*-test on CS ratios: $F_{1,200} = 116$, $P < 0.0001$). *Heliconius timareta thelxinoe* in the Escalera has a significantly larger red patch than in the Alto Mayo ($F_{1,87} = 14$, $P < 0.0001$). On the HW, *H. melpomene amaryllis* has a smaller yellow band and a smaller proportion covered by yellow than *H. timareta thelxinoe* (*t*-test on CS: $F_{1,200} = 117$, $P < 0.0001$; on CS ratios: $F_{1,200} = 12.6$, $P = 0.0004$), but no differences were observed between populations ($P = 0.75$).

Wing shape and colour patch shape

Heliconius timareta thelxinoe and *H. melpomene amaryllis* differ in wing shape ($F_{8,193} = 64$, Pillai = 0.72, $P < 0.0001$; Fig. 5B) and colour patch shape ($F_{10,191} = 87$, Pillai = 0.85, $P < 0.0001$; Fig. 5C). *Heliconius timareta thelxinoe* has proportionally slightly elongated FWs, characterized by a shorter discal cell and a longer distal part of the wing, than *H. melpomene amaryllis*. The red FW patch in *H. timareta thelxinoe* is proportionally not as rounded as in *H. melpomene amaryllis*, tending towards a broad 'Z' shape, and the yellow HW bar is more slender and a little more curved (Figs 2A, B, 5C). Allometry was not significant for wing shape or HW yellow patch shape within each species; a slight allometric effect was observed only for the FW red patch shape of *H. melpomene amaryllis* ($R^2 = 0.15$, $P = 0.03$). Further analyses considering allometry-free residuals do not change the level of discrimination between species. Therefore, although the wing and the yellow patch differ in both shape and size between species, no allometric component seems to be involved in such a difference. In contrast, allometry is observed for red patch shape; however, the same level of between-species discrimination was found (CV rate = 90%) using raw data vs. allometric-free residuals.

An LDA, first run on a subset of the samples identified by genetic data (143 of 202), and then applied to the remaining specimens (59), confirmed the a priori phenotypic identification (CV rate = 99%). One specimen initially assigned to *H. melpomene amaryllis* on the basis of phenotypic indices, and missing genetic data, was unambiguously identified as *H. timareta thelxinoe* based on wing shape (FW and HW venation, PP of identification > 0.95). Wing shape or colour patch shape alone also gave good discrimination (97% and 96%, respectively).

Geographical variations (Escalera vs. Alto Mayo populations) were observed in both species. However, only a slight effect of location was found in *H. melpomene* for wing shape and colour patch shape (MANOVA: $P = 0.05$ and $P = 0.01$, respectively), most of their variation being shared and LDA not separating the two populations (CV rate = 60–70%). In contrast, significant shape differences were found between populations of *H. timareta thelxinoe* (wing shape: $F_{14,74} = 5.5$, $P < 0.001$; colour patch shape: $F_{15,73} = 13$, $P < 0.001$; Fig. 5B, C) and LDA allowed population assignation with high CV rates (86% and 93%, respectively). The shape of the red FW patch showed the strongest differences, being noticeably proportionally more rounded and larger in Escalera

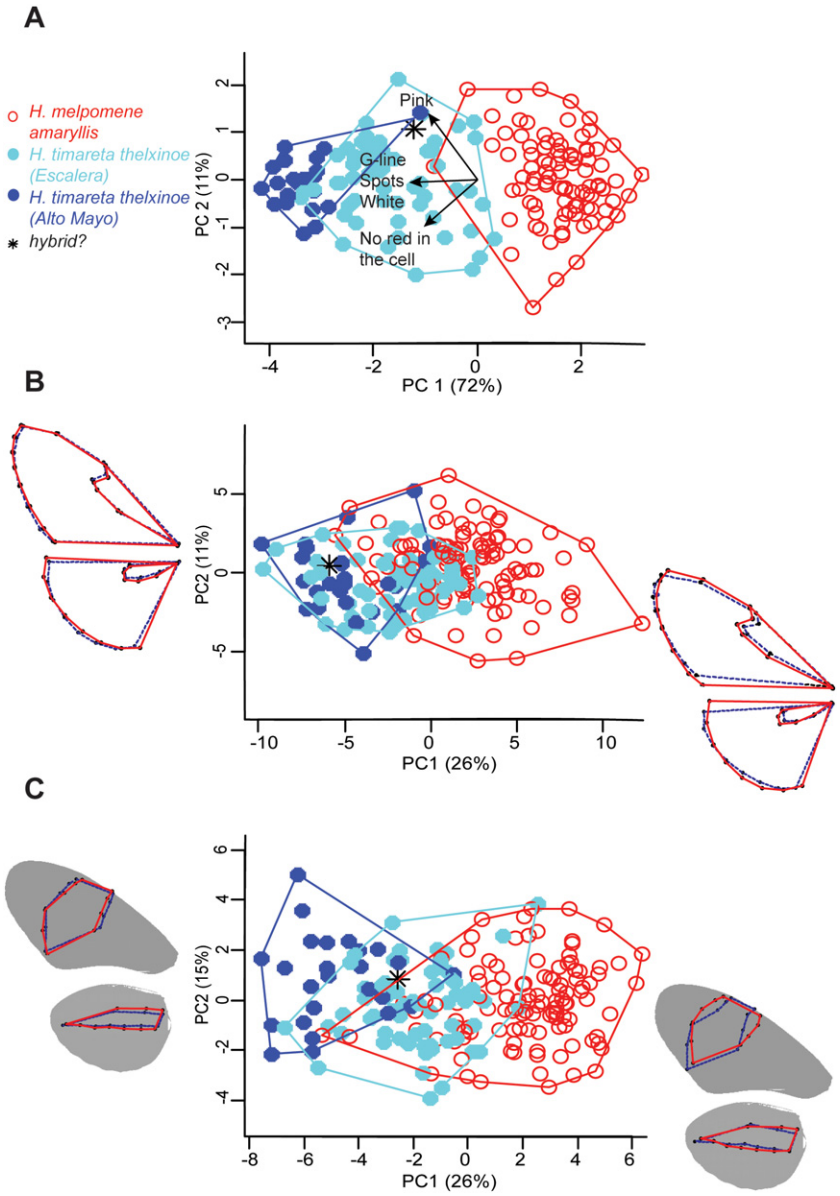

Figure 5. Morphological differentiation between *Heliconius timareta thelxinoe* and *H. melpomene amaryllis*. Two populations of *H. timareta thelxinoe* are shown, collected in the Alto Mayo (dark blue) and Escalera (light blue). A, Principal component analysis (PCA) of variation in phenotypic indices. The directions of variation at the six characters scored by six indices are represented by arrows on the plot. B, PCA of variation in wing shape. C, PCA of variation in colour patch shape (dorsal side). Shape variation is indicated next to each axis, with broken blue shapes representing minimum values and full red lines representing maximum values along each axis. The putative hybrid specimen is indicated by an asterisk.

specimens, making them more similar to *H. melpomene amaryllis* (Fig. 5C).

DISCUSSION

Heliconius timareta thelxinoe from northern Peru was previously recognized as an altitudinal form of *H. melpomene amaryllis* (J. Mallet & G. Lamas, unpubl. data). However, strong differentiation at nuclear and mitochondrial loci is mirrored by subtle, but significant, morphological differentiation, allowing the recognition of two species co-occurring in a zone of sympatry between 1000 and 1300 m above sea level. Morphological differentiation has provided the characters used to identify specimens in each taxon, whose genome-wide phylogenetic position was recently elucidated on the basis of RAD markers (Heliconius Genome Consortium, 2012; Nadeau *et al.*, 2013).

Analyses of wing shape across several species allowed the evaluation of the relative position of this new taxon with respect to the morphological distribution of closely related taxa: here, shape coordinates show the highest phenotypic proximity of *H. timareta thelxinoe* with *H. timareta timareta* from Ecuador, and give a clear nesting of the new taxon within a large and diverse *H. cydno* clade well separated from *H. melpomene* populations. The genetic and phenotypic proximity of the altitudinal form with *H. timareta timareta* from Ecuador allow us to assign this taxon to a new subspecies of *H. timareta*, and therefore a new eastern Andean taxon.

In a clade in which different genes may often have very distinct genealogies and may suggest varied evolutionary histories, geometric morphometric data can provide additional support for taxonomic assignation. Here, analyses of wing shape suggest the existence of two major groups: *H. cydno* and an eastern Andean group containing the several subspecies of *H. timareta*, but also *H. heurippa* and *H. timareta tristero*. This is highly consistent with a close relationship between all members of the eastern Andean clade shown recently using molecular markers (Nadeau *et al*., 2013). However, the ranking of these eastern Andean taxa may remain subject to debate as no contact zone between their populations has been documented. Nevertheless, the geographical position of *H. tristero* nested within the broader range of *H. timareta* logically leads us to consider it as a subspecies of *H. timareta*, with a similar phenotype to that of *H. timareta thelxinoe* (Appendix).

A limited amount of gene flow is often found between closely related species of *Heliconius*, even 3 million years after speciation (Mallet, 2007; Kronforst, 2008; Heliconius Genome Consortium, 2012; Nadeau *et al*., 2013), but the frequency of F1 hybrids varies depending on the pair of species considered. It can reach 5% in the parapatric contact zone of *H. erato* and *H. himera*, despite their divergence in colour pattern (Mallet *et al*., 1998; Mallet, 2007). Rates tend to be lower between sympatric pairs of *H. melpomene* and *H. cydno* clade taxa, but recent studies based on RAD loci have shown that gene flow increases with geographical proximity between pairs of *H. melpomene* and *H. cydno*/*timareta* clade taxa (Nadeau *et al*., 2013). In the case of *H. cydno* and *H. melpomene*, with strong colour pattern differences, hybrid frequency was estimated to be around 0.05% in their zone of sympatry (Mallet, 2007). However, this figure is derived from a composite estimate of the frequency of phenotypic hybrids across many collecting studies, and does not take into account the relative frequencies of parental species in the study areas. One F1 hybrid museum specimen is known from the zone of sympatry between *H. heurippa* and *H. melpomene*. No F1 hybrids were identified in a sample of 84 individuals of these species by Mavarez *et al*. (2006), although microsatellite genotypes suggested some admixed ancestry for several specimens (three of 84 specimens). In the case of *H. timareta florencia* and *H. melpomene malleti*, showing only slight wing pattern differences, a 2% frequency of F1 hybrids was found (three of 142 specimens) (Giraldo *et al*., 2008). In our study, one putative F1 hybrid was identified in 175 genotyped specimens, based on the microsatellite data. Eight *H. melpomene amaryllis* and three *H. timareta thelxinoe* probably have admixed genotypes, possibly involving some level of backcrossing. Therefore, although a comparison of the occurrence of rare events across multiple studies is inherently difficult, the frequency of hybrids appears to be generally higher for mimetic pairs of taxa than for pairs of taxa with different colour patterns. This would confirm the role of wing pattern cues for species recognition in the wild, although with a relatively limited effect overall. It is unclear to what extent this could alternatively be explained by a different hybridizing propensity of *H. melpomene* with *H. timareta* than with *H. cydno* irrespective of pattern, and/or to differences in ecological differentiation. Hybrid frequency estimates should therefore be obtained for areas in which *H. timareta* and *H. melpomene* differ strongly in wing pattern.

At the population level, shape and genotype show a good concordance. Over 99% of the specimens identified by genotyping are assigned to the same species with a predictive LDA on wing shape and colour patch shape. However, a few specimens show morphological elements atypical of their species assignation (Supporting Information Notes S1). Cases of conflicting assignations, such as when wing shape and colour patch shape do not correspond to the same taxon, might indicate limited cross-species sharing of alleles controlling phenotypic elements, as might be expected given recent evidence of past gene exchange at the *B*/*D* and *N*/*Yb* alleles controlling red elements in this pair of species (Heliconius Genome Consortium, 2012; Pardo-Diaz *et al*., 2012). More conflicting cases were found when the assignment procedure was based on colour patch shape than on wing shape (23 individuals vs. 12, respectively; see Notes S1). In this case, wing venation may appear to be a more stable character for species assignation, whereas wing pattern elements might flow more freely between hybridizing mimetic species.

Our measurements show significant variations in wing shape and colour patch shape between Escalera and Alto Mayo populations of *H. timareta thelxinoe*: specimens from the Escalera are, on average, more

similar to *H. melpomene amaryllis* than to specimens from the Alto Mayo, most noticeably when considering the shape of the FW red patch. Phenotypic indices are in good accordance with this observation, with Escalera specimens having lower average values, indicating a phenotype more similar to *H. melpomene*. Specifically, Escalera specimens of *H. timareta thelxinoe* have, on average, a more orange tinge of the FW patch, a fainter FW red costal line, reduced ventral HW dots and a brighter yellow HW bar.

The Escalera populations sit at the lower altitudinal limit (1000–1300 m) and at the eastern edge of *H. timareta*'s distribution in this region, where it is rare relative to *H. melpomene*. In this locality, the postman mimicry ring is thought to be driven by the highly abundant *H. erato favorinus* bearing a wing pattern nearly identical to local *H. melpomene*, with very round and red–orange FW patch. In contrast, Alto Mayo populations sit at higher elevations (1300–1600 m), an altitude at which *H. melpomene* and *H. erato* become markedly less frequent, and where *H. telesiphe telesiphe* Doubleday becomes abundant (C. Mérot, pers. observ.). *Heliconius telesiphe* bears a double crimson FW patch and a narrow white HW bar, reminiscent of the sinuous red FW patch and the whiter HW band of *H. timareta* samples from the Alto Mayo. The shift in local butterfly communities and in the most abundant postman patterns could therefore influence local selection on 'postman' mimetic patterns. The phenotypic differences in the wing patterns of *H. timareta* between the Escalera and Alto Mayo populations could therefore stem either from higher rates of hybridization and introgression of wing patterning alleles from *H. melpomene*, or from stronger selection for resemblance to *H. melpomene* and *H. erato favorinus* in the Escalera, or from both phenomena.

Higher hybridization rates with *H. melpomene* would be expected to lead to greater similarities between species for all traits and markers, including those not involved in mimicry. From the microsatellite data, there is no evidence for a higher frequency of admixed individuals in the Escalera, although the overall low hybridization frequency may not provide sufficient power to evaluate subtle differences in hybridization rates. Conversely, if the mimicry optimum is different in the two areas, we would expect traits under strong selection, such as hue and shape of the colour patch, to show stronger differences between *H. timareta thelxinoe* populations. These are indeed the characters showing stronger differences between Escalera and Alto Mayo populations relative to wing shape, providing some support for the hypothesis of adaptive mimicry variation between populations. Recent findings have indicated that the postman mimicry of *H. timareta thelxinoe* and *H. melpomene amaryllis* itself involves the introgression of entire genomic segments containing the wing patterning alleles (Heliconius Genome Consortium, 2012; Nadeau *et al*., 2013), suggesting that variations in mimicry selection in different communities could build on an initial resemblance involving past or ongoing hybridization in certain populations.

The differentiation of *H. timareta thelxinoe* and *H. melpomene amaryllis* demonstrates yet another new mimetic relationship among two sister species complexes coexisting in the eastern Andes. The role of wing pattern divergence in the determination of assortative mating has been well established between these two clades [e.g. *H. melpomene* vs. *H. cydno* (Jiggins *et al*., 2001; Merrill *et al*., 2010), *H. melpomene* vs. *H. heurippa* (Mavarez *et al*., 2006)], and even within each clade [*H. cydno* vs. *H. pachinus* (Kronforst *et al*., 2006), *H. melpomene* races (Jiggins *et al*., 2004; Merrill *et al*., 2011), and *H. cydno* polymorphic forms (Chamberlain *et al*., 2009)]. A model of speciation driven partly by mimicry shifts (Jiggins *et al*., 2001) was apparently supported by the observations of strong assortative mating based on wing pattern, thought to act as a major premating mechanism. A direct prediction from this model is that hybridization rates should be correlated with wing pattern similarity along the speciation continuum of this clade; mimetic pairs of these two groups should show higher levels of hybridization. Our results, together with other recent studies (Giraldo *et al*., 2008), can be used to evaluate this prediction, and question the predominant role of mimicry shifts in driving speciation in *Heliconius*.

Four *H. timareta* races distributed along the eastern Andes share wing patterns with local *H. melpomene* populations: *H. timareta tristero* (Colombia; Brower, 1996), *H. timareta florencia* (Colombia; Giraldo *et al*., 2008), *H. timareta timoratus* (Ecuador/Peru border; Lamas, 1997) and *H. timareta thelxinoe* (northern Peru; this study); a fifth taxon, *H. timareta timareta* from central Ecuador, shows polymorphism, but no resemblance, to co-occurring *H. melpomene* (Fig. 1; for range details, see Rosser *et al*., 2012). Given that races and recently diverged species within the *H. cydno* clade still share much ancestral genetic diversity (Brower & Egan, 1997; Mallet *et al*., 1998; Beltran *et al*., 2007; Kronforst, 2008), the biogeographical history of speciation in this clade is still unclear. Consequently, we cannot rule out a role of wing pattern divergence in the ancient split between the two clades, followed by a secondary mimicry of *H. melpomene* by eastern Andean *H. timareta* populations. Our data, together with other recently published studies, suggest that these taxa hybridize to varying degrees, but do not suggest very high rates of hybridization (< 2%). In addition, mate choice

experiments between *H. timareta florencia* and *H. melpomene malleti* showed strong assortative mating despite near-identical wing patterns (Giraldo *et al*., 2008). This strongly suggests that mechanisms other than wing pattern differences predominate in maintaining genetic isolation between sympatric eastern Andean pairs of species.

Colour pattern similarity is known to promote elevated rates of approach between *H. melpomene* and *H. erato*, and should similarly lead to frequent approaches between *H. melpomene* and *H. timareta* (Estrada & Jiggins, 2008). Therefore, as for other butterfly groups (Andersson *et al*., 2007; Nieberding *et al*., 2008), short-range cues, such as pheromones, are likely to mediate mating and hybridization propensity in *Heliconius*. Wing shape variation, however subtle to the human eye, may also be found even within species (Jones *et al*., 2013), and may represent other dimensions involved in recognition, for instance through their effect on flight behaviour (Srygley, 1999). Ventral characters, such as the hue of the FW red patch here, are visible for the female when courted, and might also represent a male character affecting female mate choice (Papke, Darrell & Rutowski, 2007). Ecologically, the species differ markedly in altitudinal range, *H. melpomene amaryllis* being found mostly below 1200 m, whereas *H. timareta thelxinoe* occurs between 1000 and 1800 m. Both species overlap in microhabitat preferences in the zone of sympatry, feeding on the same flowers (C. Mérot, pers. observ.); however, altitudinal shifts represent steep ecological gradients in thermal ecology and host plant communities (Hodkinson, 2005), suggesting that ecological divergence and specialization along the altitudinal gradient could play an important role in the isolation of these species (Willmott & Freitas, 2006; Elias *et al*., 2009; Schoville, Roderick & Kavanaugh, 2012).

Under wing pattern-mediated speciation, genomic regions involved in wing patterning differences are predicted to show restricted gene flow and elevated rates of nucleotide divergence between colour differentiated taxa, relative to regions of the genome with no involvement in species isolation (Nosil, Funk & Ortiz-Barrientos, 2009; Nosil & Feder, 2012). This prediction fits recent observations of elevated divergence in the genomic regions containing the colour patterning genes *B*/*D* and *Yb* in comparison between colour differentiated races of *H. melpomene* and *H. timareta* (Nadeau *et al*., 2012). In contrast, if wing patterns do not play an important role in genetic isolation when species are co-mimetic, as seems to be the case here, the prediction is for wing patterning regions encoding similar phenotypes in both taxa to flow more freely across the species boundary, and for regions showing suppressed gene flow to be situated elsewhere in the genome. This is perhaps reflected in the observation of a stronger resemblance of *H. timareta thelxinoe* and *H. melpomene amaryllis* in their zone of altitudinal overlap, and the fact that more individuals show colour pattern coordinates in conflict with morphometric and genotypic species assignation (Notes S1). The combination of morphometric measurements and genotyping may allow the identification of individuals to be used to test these predictions employing genomic sequencing.

CONCLUSION

Overall, our study reveals a new pair of closely related co-mimetic species in the eastern Andes, which provides an interesting challenge to the model of speciation mediated by wing colour pattern variation in *Heliconius*. Our results highlight a good association of multilocus genotypes and morphometric variation, and show that near-perfect wing pattern mimicry does not dramatically weaken species differentiation. This implies that other dimensions of the ecological niche play a major role in the maintenance of genetic isolation in this group of butterflies. The determination of which traits diverge first during ecological speciation and how they affect the divergence at other traits provides an exciting prospect for speciation research in butterflies. Phenotypic variation observed between populations, such as here between two areas, which may result from a response to differences in mimetic communities, could be a first clue to untangle how mimicry and ecological factors trigger isolation or hybridization.

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APPENDIX: DESCRIPTION OF THE NEW SUBSPECIES

HELICONIUS TIMARETA THELXINOE LAMAS & MÉROT, NEW SUBSPECIES (FIG. 2A)

Diagnosis

This taxon belongs to the *Heliconius cydno* clade [part of the '*numata* group' of Holzinger & Holzinger (1994) and of the '*melpomene–cydno* group' of Brower & Egan (1997)] (Heliconius Genome Consortium, 2012; Nadeau *et al*., 2013). It is superficially most similar to partly sympatric *Heliconius melpomene amaryllis* C. Felder & R. Felder, 1862, but the latter is easily distinguished by the FW red post-median band broadly entering the distal fifth or more of the discal cell, the absence of a ventral red dash at the base of the FW costal cell, and the strongly reduced red basal spots on the HW ventral surface. It is also quite similar to allopatric *Heliconius timareta tristero* Brower, 1996, but is distinguished from the latter by the different shape of the FW post-median band, particularly the much shorter element in cell Cu2-2A in *tristero*. It does not resemble in colour pattern any other known (described or undescribed) subspecies of *Heliconius timareta* Hewitson, 1867, but shares with all of them the ventral FW long, red costal dash and the ventral HW large, red basal spots (Giraldo *et al*., 2008).

Male

FW length 41–44 mm (mean, 42.1 mm; $N = 9$). *Dorsal* wing colour deep dark brown, FW traversed by an irregularly shaped, vermillion red post-median band, extending from the subcostal vein to the anterior half of cell Cu2-2A and sometimes barely entering the distal end of the discal cell, HW with a broad yellow discal bar extending from cell Rs-M1 to the base of the anal margin. *Ventral* wing colour paler brown, FW with long (> 4 mm) vermillion red dash at the base of the costal cell and crossed by an irregularly shaped, pink to brick red post-median band, less developed than on the dorsal surface, HW with a conspicuous yellow line, extending from the wing base for about one-half to two-thirds the length of the costa, a series of five large (> 2 mm in length), vermillion red basal spots from cell Sc+R1-Rs to the anal margin, and a yellowish to whitish discal bar from cell Rs-M1 to the anal margin.

Female
FW length 41.5–44 mm (mean, 43.2 mm; $N = 3$). Very similar to male, but easily distinguished by the dull dark brown costal area of the dorsal HW (gleaming mealy yellowish in male), the five-segmented prothoracic tarsus (fused together in male) and the external genitalia.

Type material
Holotype ♂, PERU, *San Martín*, entre El Afluente y Nuevo Edén, 1550 m, 05°39′S, 77°42′W, 7.ix.2007 (C. Ramírez) (MJ07-478), in the Museo de Historia Natural, Universidad Nacional Mayor de San Marcos, Lima, Peru (MUSM) (Fig. 2A). Paratypes (8♂, 3♀, all from PERU, deposited in MUSM): *Amazonas*: 1♂, Aramango, Numparque, *c*. 1500 m, *c*. 05°26′S, 78°21′W, vi.2007 (M. Büche *leg*.). *San Martín*: 2♂, km 19 Tarapoto–Yurimaguas, La Antena, 1300 m, 06°27′S, 76°18′W, 22.vii.2007 (M. Joron); 1♂, same locality, but viii.2002 (M. Joron); 1♂, same locality, but 1260 m, 22.x.2011 (C. Mérot); 1♂, same data as holotype (MJ07-479); 1♂, same data as holotype, but (J. W. Chung) (MJ07-476); 1♂, same data as holotype, but 1600 m (J. W. Chung) (MJ07-477); 1♀, same data as holotype, but 1600 m (M. Joron) (MJ07-480); 1♀, same data as holotype, but 1650 m (M. Joron) (MJ-0481); 1♀, Puente Serranoyacu, 1200 m, 05°40′S, 77°40′W, 23.x.2002 (M. Joron).

Etymology
Following a long tradition in Heliconiina nomenclature, we name this subspecies after Thelxinoe, one of the Greek Muses, goddesses presiding over the various kinds of poetry, arts and sciences. A noun in apposition.

Taxonomy and variation
All available evidence (morphological, genetic, biogeographical, behavioural, etc.) strongly supports the hypothesis that this new taxon is conspecific with *H. timareta*, a member of the *Heliconius cydno* clade (Heliconius Genome Consortium, 2012; Nadeau *et al*., 2013; this study). Individual variation is not pronounced, and is expressed mainly in the size and shape of the FW red post-median band. The male from Amazonas shows significant reduction of the FW post-median band elements in cells Sc-R1 to M1-M2. One female (MJ07-481) from San Martín shows an additional dorsal, post-median, vermillion red line, in the anal cell. Morphometric analyses showed slight geographical differences between the populations of Alto Mayo and Escalera (this study).

Known (described and undescribed) *Heliconius timareta* subspecies are as follows (in a latitudinal distribution, north to south): (1) an undescribed '*cydno* cognate' from the Río Pato, Caquetá, eastern Colombia (Giraldo *et al*., 2008); (2) *florencia* Giraldo *et al*., 2008, also from Caquetá, eastern Colombia, south of the Río Pato '*cydno* cognate'; (3) *timareta* Hewitson, 1874, a polymorphic subspecies from eastern Ecuador; (4) an undescribed subspecies from southeastern Ecuador (Holzinger & Holzinger, 1994); (5) *timoratus* Lamas, 1997, from northern Amazonas, Peru, close to the border with Ecuador [the geographical location of this subspecies was placed erroneously in fig. 6 of Giraldo *et al*. (2008)]; and (6) *thelxinoe* (this study) (Fig. 1).

The known geographical distribution of *Heliconius tristero* Brower, 1996 (Putumayo, southeastern Colombia) lies approximately in the middle of the distribution area of *timareta*. Given such biogeographical pattern, and because *tristero* shows no significant morphological or genetic differences from *timareta*, we propose herein to downgrade it to subspecies rank, as follows: *Heliconius timareta tristero*, **new status**.

Distribution
Currently known only from the departments of Amazonas and San Martín in northern Peru, along the eastern slopes of the Andes, at elevations between 1000 and 1700 m. It is the southernmost member of *H. timareta*.

Habitat and behaviour
Heliconius timareta thelxinoe is found in humid montane forest, usually foraging as adults on orange Cucurbitaceae flowers, such as *Psiguria* or *Gurania*, in small sunny gaps or at forest edges. This habitat is also visited by other species of *Heliconius*, such as *H. melpomene*, *H. erato* and *H. telesiphe* within their altitudinal range.

Males are more frequently seen than females, flying fast in sunny patches and chasing females or other males. Females can mate several times, with up to three spermatophores found in a single female after dissection. Females lay solitary eggs, usually on young stems of the host plant. Recorded host plants include *Passiflora oerstedii* Mast, *Passiflora riparia* Mast and *Passiflora (Astrophea)* sp. The larva is very similar to that of *H. melpomene*, but that of *H. melpomene* has a paler cephalic capsule in the fourth and fifth instars than that of *H. timareta*.

SUPPORTING INFORMATION

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

Figure S1. Results of the analysis in STRUCTURE for simulated populations. From left to right: wild populations of *Heliconius melpomene amaryllis* and *H. timareta thelxinoe*, F1 hybrids, first generation of backcross (Bx1) with *H. timareta thelxinoe* (Ti.) and then *H. melpomene amaryllis* (Melp.), F2 hybrids, second and third generation of backcross (Bx2, Bx3) and simulated pure populations of *H. timareta thelxinoe* and *H. melpomene amaryllis*.

Table S1. List of specimens used in this study. P_q, genotypic posterior probability of belonging to *Heliconius timareta thelxinoe* (STRUCTURE). P_m, morphometric probability of having *timareta* shape from the discriminant analysis. I, mean phenotypic index.

Table S2. Samples and sequences used for the phylogenetic tree with accession numbers in GenBank.

Table S3. Genetic polymorphism of the studied sample. Allelic richness (A) is estimated for the smallest population ($N = 74$). H_O represents the observed heterozygosity and H_E the expected heterozygosity. Significant deviations from Hardy–Weinberg expectations are indicated by asterisks ($^*P < 0.05$; $^{**}P < 0.01$). Loci come from Flanagan *et al*. (2002) [1], and Mavarez *et al*. (2006) [2].

Notes S1. Assignment procedure based on repeated discriminant analysis.