A new subspecies in a *Heliconius* butterfly adaptive radiation (Lepidoptera: Nymphalidae)

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A fundamental goal in evolutionary biology is to understand how evolutionary patterns and processes shape natural diversity. This, however, requires a complete characterization of the phenotypic and genetic variation between and within species. Here, we used molecular, morphological and behavioural data to describe a new and stable subspecies of *Heliconius timareta*, named *Heliconius timareta linaresi* subsp. nov. This race differs phenotypically from other red-coloured *H. timareta* and instead exhibits a black and yellow wing pattern more similar to *Heliconius cydno*. However, mtDNA, microsatellite and amplified-fragment length polymorphism data indicate a closer relationship with *H. timareta* than *H. cydno* and *Heliconius melpomene*. Larval morphology and host plant preferences are similar to other *H. timareta* and *H. cydno* races. Thus, our combined data indicate that this taxon is a novel subspecies of *H. timareta*, clearly differentiated from *H. cydno* and *H. melpomene*.


INTRODUCTION

The *Heliconius* butterflies have undergone an adaptive radiation in warning coloration and are an excellent system in which to study how phenotypic variation originates in nature (Emsley, 1965; Turner, Johnson & Eanes, 1979; Turner, 1981; Brower, 1996b; Supple et al., 2014; Merrill et al., 2015). Typically, *Heliconius* butterflies are involved in Müllerian mimicry rings where distantly related species converge onto a few common colour patterns to advertise their toxicity to predators (Müller, 1879). In contrast, closely related taxa normally display divergent colour patterns and are part of different mimicry rings (Mallet & Gilbert, 1995). This phenomenon has resulted in a complex and colourful mosaic of patterns with more than 40 recognized species and more than 400 colour pattern forms found in forest across tropical Central and South America (Brown, 1979, 1981; Mallet & Gilbert, 1995; Mallet, McMillan & Jiggins, 1998; Lamas et al., 2004).

Despite considerable taxonomic, field and molecular studies, we are still discovering new taxa (Brower, 1996a; Giraldo et al., 2008; Mallet, 2009; Moreira & Mielke, 2010; Mérot et al., 2013; Nadeau et al., 2014). The recently diverged species *Heliconius melpomene* Linnaeus, *Heliconius cydno* Doubleday and *Heliconius timareta* Hewitson form a closely related species complex with partially overlapping distributions in the North Andes of South America and in Central America (Brown, 1979; Brower, 1996a; Giraldo et al., 2008; Mallet, 2009; Mérot et al., 2013). *Heliconius melpomene* is largely sympatric with both *H. cydno* and *H. timareta*, while *H. cydno* and *H. timareta* are parapatric with respect to each other. *Heliconius cydno* and *H. melpomene* are well-studied species that show strong
assortative mating, differ in habitat use, host plant preference (Mallet et al., 1998; Jiggins et al., 2001; Naisbit, Jiggins & Mallet, 2001; Kronforst, Young & Gilbert, 2007; Merrill et al., 2011, 2012, 2013), and they mimic different and unrelated species of Heliconius. Heliconius cydno has typically yellow or white elements and most often mimics H. sapho Drury and H. eleuchia Hewitson, while H. melpomene has red and yellow wing patterns and mimics Heliconius erato Linnaeus (Flanagan et al., 2004). Heliconius timareta, the third member of this radiation, was previously thought to be limited to an isolated polymorphic population in Ecuador. However, recent studies have revealed the existence of several distinctive populations along the eastern slopes of the Andes from southern Colombia to Peru (Brower, 1996a; Giraldo et al., 2008; Mallet, 2009; Mérot et al., 2013; Nadeau et al., 2014). These new forms generally exhibit red-coloured pattern elements, which are acquired through adaptive introgression from H. melpomene (The Heliconius Genome Consortium, 2012; Pardo-Díaz et al., 2012; Wallbank et al., 2016) (but see Brower, 2011, 2013) and are almost indistinguishable from local H. melpomene races. Nonetheless, the two are reproductively isolated both with respect to mate choice and hybrid viability (Sánchez et al., 2015). In this case, assortative mating is almost certainly related to differences in pheromones (Mérot et al., 2015; Sánchez et al., 2015). More extensive geographic sampling has revealed additional populations of H. timareta, including a recent study that discovered a new form in eastern Ecuador (Nadeau et al., 2013, 2014). With a widespread sampling and new genetic data, we are beginning to resolve the evolutionary relationships between these three species and understand how these species varies across the genome.

Here, we used an integrative framework (similar to Braby, Eastwood & Murray, 2012) that uses morphological (adult morphology, wing pattern and larval head capsule coloration), genetic (mtDNA, Tpi, microsatellites) and behavioural data (host plant choice) to test the distinctiveness of a new race of H. timareta, named here as Heliconius timareta linaresi Arias & Lamas subsp. nov. This new race is endemic to the eastern cordillera of the Southern Colombian Andes. Genetically it clusters within the H. timareta clade, but, unlike the most of the more southern H. timareta, linaresi has a black-yellow H. cydno wing colour pattern. Interestingly, linaresi does not seem to entirely mimic any other taxa in its distribution, although we cannot discard that other black-yellow Heliconius occurring in the same general area (Heliconius congener Weymer, Heliconius sara Fabricius and Heliconius wallacei Reakirt) could be comimetics. However, none of them present a complete yellow forewing band. The only form that perfectly match H. t. linaresi colour pattern is H. c. cordula Neustetter (Figs 1, 2), a species found further north on the eastern slopes of the Andes. In addition to describing morphological, ecological and genetic attributes of the H. t. linaresi, we discuss the implications of this new form for our understanding of speciation in this group of butterflies.

**METHODS**

**Sampling and stock populations**

Adult individuals of H. t. linaresi subsp. nov. were collected between 2005 and 2009 in Las Morras (02°41′04″N, 79°53′17″W) and Puerto Rico (1°56′44″N, 75°13′16″W) near the Pato River, in Caquetá (Colombia). A subset of these individuals was kept alive in separate insectaries (2 × 3 × 2 m²) in La Vega (Cundinamarca) and was used to establish stock populations. These were used to carry out host plant choice experiments and larval morphology description (see below). Adults were provided with ample nectar and pollen (Lantana and Psiguria), as well as artificial nectar solution (10%...
sugar solution). In addition, several *Passiflora* spp. host plants for immature stages were provided. Wings were removed from wild specimens and bodies preserved in dimethyl sulfoxide for phenotypic and molecular analyses. DNA extractions were performed from one-third of the thorax of each individual by using a DNeasy tissue kit (QIAGEN) following manufacturer’s protocol.

**DNA Sequence Analyses**

We sequenced the mitochondrial region spanning the Col and CoI genes (1525 bp) for 12 *H. t. linaresi* individuals and the nuclear Z (X)-linked gene *Tpi* (648 bp) for 11 *H. t. linaresi* specimens. Primer sequences and conditions were used as outlined by Beltrán et al. (2002). The fragments obtained were sequenced in an ABI Prism 3100 sequencer (PE Applied Biosystems). Sequences for the Col-CoI and *Tpi* gene regions were downloaded from GenBank for *H. melpomene*, for other closely related species in the cydno complex (*H. cydno* races, *H. timareta* races, *H. heurippa* Hewitson and *H. pachinus* Salvin), and for the outgroup species *H. numata* Cramer (GenBank accession numbers; Table S1). All sequences were aligned and checked by eye using MacClade 4.08a (Maddison & Maddison, 2001).

The sequences generated in this study are available in GenBank (accession numbers KU877714–KU877725 [*Tpi*], KU877726–KU877737 [Col-CoI]; Table S1).

Phylogenetic analyses were conducted using maximum likelihood (ML) with RAxML Blackbox (Stamatakis, Hoover & Rougemont, 2008) and Bayesian inference (BI) in Beast Xsede 1.7.5 (Drummond & Rambaut, 2007) from the Cipres cluster web service (Miller, Pfeiffer & Schwartz, 2010). Both genes were analysed using GTR + G nucleotide substitution model, which was preferred by the Akaike information criterion (AIC) using JModel test 1.1 (Darriba et al., 2012). For ML analyses, branch stability was estimated after 5000 bootstrap replicates. BI analyses were modelled under a Yule speciation process and branch lengths under the assumption of relaxed clock with an uncorrelated log-normal distribution. The analysis was run for 40 million generations and sampled every 5000 bootstrap replicates. BI analyses were modelled under a Yule speciation process and branch lengths under the assumption of relaxed clock with an uncorrelated log-normal distribution. The analysis was run for 40 million generations and sampled every 4000 generations. Mixing properties and convergence of the Markov Chain Monte Carlo (MCMC) were evaluated by visual inspection of the parameter trend plots and by examining that the effective sample size (ESS) was higher than 200 after a burn in of 2500 samples in the Tracer program (Drummond & Rambaut, 2007). Finally, 7500 trees from the posterior distribution

**Figure 2.** Colour pattern similarities and differences among *H. c. cordula*, *H. t. linaresi* subsp. nov., *H. t. timareta* f. timareta. From left to right *H. c. cordula*, *H. t. linaresi* subsp. nov., and *H. t. timareta* f. timareta are presented: (a) Complete dorsal view of the three taxa. Note the black-blue iridescent coloration of *H. c. cordula*, but the opaque black coloration of *H. t. linaresi* subsp. nov. and *H. t. timareta* f. timareta; (b) dorsal view of the Forewing (FW) – the FW has three principal pattern elements in a black background consisting by an irregular yellow postmedial band extending from distal end of discal cell to R$_1$–R$_3$ fork and laterally from subcostal to CU$_{1b}$, a yellow bowtie element in the discal cell and an oval element below CU$_{1b}$. Both *H. t. linaresi* subsp. nov. and *H. c. cordula* show a similar FW yellow band, but irregular in *H. t. timareta* f. timareta; (c) ventral view of FW – the ventral FW has two principal pattern elements in a black background consisting by a yellow band similar between the three taxa and a ‘red line’ present in *H. t. linaresi* subsp. nov. and *H. t. timareta* f. timareta, but absent in *H. c. cordula*. (d) Ventral view of Hindwing (HW) – the ventral HW has two colour pattern elements in a black background, the ‘forceps’ that is present in all *H. c. cordula*, absent in *H. t. timareta* f. timareta, but with a small remnant of this element in *H. t. linaresi* subsp. nov.; the ‘red spots’ element which is present in *H. t. linaresi* subsp. nov. and *H. t. timareta* f. timareta, but absent in *H. c. cordula*. Arrows point to important diagnostic traits between the three forms.
were evaluated and summarized with average branch length values using the maximum credibility tree in TreeAnnotator 1.7.1 (Drummond & Rambaut, 2007).

MULTILOCUS MICROSATellite ANALySES
Nuclear DNA variation was described at seven microsatellite loci for 203 individuals [51 H. cydno individuals (18 H. c. cydnides Staudinger and 33 H. c. cydno Doubleday), 83 H. m. malleti Lamas, 53 H. t. florencia Giraldo and 16 H. t. linaresi] using primers and conditions delineated in Arias et al. (2012). Reaction fragments were run in an ABI Prism 3100 sequencer (PE Applied Biosystems). Allele sizes were established using ABI GeneMapper v4.0 (PE Applied Biosystems) with Genescan LIZ-500 (Applied Biosystems) as size standard. Departure from Hardy–Weinberg equilibrium and linkage disequilibrium were tested using Arlequin 3.5 (Schneider, Roessli & Excoffier, 2000). Levels of differentiation (FST) (Weir & Cockerham, 1984) among populations were calculated with Arlequin 3.5 (Schneider et al., 2000). We used a Bayesian model-based clustering algorithm to assign individuals to species and to detect admixed individuals using the software Structure 2.3.4 (Pritchard, Stephens & Donnelly, 2000). The analysis was run under an admixture model with correlated allele frequencies between populations, which assumes that allele frequencies between populations are similar due to migration or ancestral polymorphism (Falush, Stephens & Pritchard, 2003). The number of ancestral clusters, K, were determined using an ad hoc statistic ΔK based on the rate of change in the log probability of data for K between 1 and 10 in 20 runs (Evanno, Regnaut & Goudet, 2005), where each run consisted of 10⁶ iterations, after a burn in period of 10⁵ iterations.

ADULT MORPHOLOGY AND LARVAL COLORATION
We assessed colour pattern qualitative diagnostic traits in adult forewing and hindwing between wild caught individuals of H. t. linaresi (4 females, 13 males), H. c. cordula (10 females, 10 males) and H. t. timareta f. timareta Hewitson (5 females, 5 males), which exhibit a similar phenotype (Figs 1, 2). We further look at H. t. linaresi head capsule larval coloration. Giraldo et al. (2008) found that H. t. florencia displays similar head capsule coloration to H. cydno races and different from H. melpomene. To test for differences between H. cydno, H. melpomene, H. t. Florencia and H. t. linaresi, we raised six larvae from six different wild-caught females from Las Morras (Caqueta) and compared them to 107 larvae head capsules from 54 H. melpomene (H. m. malleti, H. m. bellula and H. m. vulcanus), 29 H. cydno (H. c. cordula, H. c. cydnides and H. c. zelinde) and 24 H. t. florencia individuals. Pictures were taken with a Sony digital still camera DSC-S85 under similar light conditions with a colour standard and processed with the software Scion Image (Scion Corporation, Frederick, MD, USA). Four RGB indexes were calculated following Giraldo et al. (2008) protocol. Finally, we tested for significant differences between indexes with a one-way analysis of variance (ANOVA) and Tukey’s honest significant difference (HSD) post hoc test by using R statistical package (R Core Team, 2014). P < 0.01 was taken to be statistically significant.

HOST PLANT CHOICE
Individual insectaries were equipped with host plants of six Passiflora species known to be used by races of the H. cydno/H. melpomene complex [Passiflora edulis Sims, P. maliformis Linnaeus, P. ligularis Juss, P. arborea Spreng, P. quadrangularis Linnaeus, P. oerstedii Mast], and two host plant controls frequently used by H. erato races [P. suberosa Linnaeus and P. rubra Linnaeus] (Benson, Brown & Gilbert, 1975; Gilbert, 1982). All plants used were fresh and of the same age. We tested plant preference of seven females of H. t. linaresi, nine H. t. florencia and ten H. m. malleti that were kept separated in the individual insectaries. Eggs laid per plant by each female were collected and counted twice a week. A multinomial laying probability, PJx, that represents the probability of choice by a female type j to a plant type i, for each combination of j-type female and i-type plant was obtained using ML. The laying probability for each species/group is

\[ P_{jxt} = \frac{P_i}{\sum_{i=1}^{m} P_i} \]

where n is the total number of tested plants and P represents the laying proportion of eggs for each plant. This probability was maximized by the expression

\[ \sum_{j=1}^{m} \sum_{i=1}^{n} a \ln(P_{ji}) \]

where m represent the total number of species/groups tested and a represent the total number of counted eggs per plant. Different parameter models were compared using a reductionist strategy, starting with a model that assumes three parameters corresponding to different laying proportions across plants for the three groups (H. m. malleti, j; H. t. florencia, j and H. t. linaresi, j; \[ {\sum_{j=1}^{n} a \ln(P_{ji})} \]). These initial model was contrasted with a two (all possible two species/groups arrangements \[ {\sum_{j=1}^{n} a \ln(P_{ji})} \]) and one-parameter model (\[ {\sum_{j=1}^{n} a \ln(P_{ji})} \]) using a G = –2ΔlogeL

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RESULTS

MITOCHONDRIAL AND Tpi DATA

We assessed variation at the mtDNA gene Col-CoI (n = 150) and the Z(X) linked nuclear gene Tpi (n = 137) across the H. cydno, H. melpomene and H. timareta radiations. MtDNA analyses with ML and BI generated a phylogeny with three clades: (1) a poorly supported clade containing H. cydno races, the closely related species H. cydno, and H. pachinus, the six H. t. florenca individuals and one individual of H. pachinus and (3) a well-supported clade that contained the remaining H. pachinus specimens, all the H. t. timareta and H. t. thelxinoe individuals, a cluster formed by 12 H. t. linaresi individuals and one H. t. florenca (Fig. 3A, S1). In contrast, ML and BI analyses for the Tpi gene showed two well-supported clades: (1) a H. melpomene clade, mainly clustered by east and west of the Andes and (2) a clade containing H. cydno races, the closely related species H. heurippa and H. pachinus, all H. timareta races and our 12 H. t. linaresi specimens (Fig. 3B, S2).

MICROSATELLITE ANALYSES

We assessed variation at seven microsatellite loci for H. t. linaresi, H. t. florenca, H. m. malleti and two allopatric populations of H. cydno. In general, H. t. linaresi exhibited a mean observed heterozygosity of 0.45 ± 0.26, which was slightly lower than that of H. t. florenca and H. cydno (0.48 ± 0.21 and 0.48 ± 0.22, respectively). In contrast, H. m. malleti showed the highest heterozygosity (0.50 ± 0.14). Some loci displayed significant deviations from Hardy–Weinberg equilibrium within populations. These deviations were caused by heterozygote deficiencies and were most likely due to presence of null alleles, as has been observed in similar studies (Flanagan et al., 2002; Mavarez & Gonzalez, 2006; Arias et al., 2012). Genetic differentiation was measured with and without null alleles correction with similar results. Overall, genetic differentiation between H. t. linaresi and H. m. malleti was strong and significant (FST = 0.12, P < 0.001) and consistent with strong differentiation between H. timareta and H. melpomene species (Giraldo et al., 2008; Mérot et al., 2013). Congruently, H. cydno was also strongly and significantly differentiated from H. t. linaresi (FST,H. c. cydnos = 0.14, FST,H. c. cydnos = 0.11, P < 0.001). In contrast, genetic differentiation between H. t. linaresi and H. t. florenca was lower but still significant (FST = 0.04, P < 0.01). The Bayesian model-based clustering method implemented in Structure was consistent with the observed genetic differentiation. The best estimate of the number distinct clusters K was three, which corresponded to the three Heliconius species (Fig. 4A, S3). These results are consistent with a similar analysis (a population assignment test in structure) previously performed with a broader scan of the genome with Amplified-Fragment Length Polymorphism (AFLP) markers and a larger sample of H. cydno, H. timareta and H. melpomene races (Fig. 4B; Arias et al., 2014).

ADULT MORPHOLOGY AND LARVAL HEAD CAPSULE COLORATION

We investigated differences in colour pattern by comparing H. t. linaresi individuals with H. c. cordula and H. t. timareta f. timareta, populations that both display similar black-yellow wing pattern (Fig. 2). We detected six clear differences between H. t. linaresi, H. c. cordula and H. t. timareta f. timareta specimens: (a) in general, H. t. linaresi and H. t. f. timareta wings are opaque black, whereas H. c. cordula wings are blackish-blue iridescent (Fig. 2A); (b) the forewing (FW) yellow postmedian band with a smooth distal border in H. t. linaresi and H. c. cordula individuals, while H. t. timareta f. timareta individuals present a narrower and more irregular FW postmedian band (Fig. 2A, B); (c) the yellow FW postmedian spot (‘oval’ element), present below vein Cu1, that is shared between H. t. linaresi and H. c. cordula, but absent in H. t. timareta f. timareta; (d) a ‘red line’ at the base of the costal vein on the ventral side of FW, present in H. t. linaresi and H. t. timareta f. timareta, but rarely visible in H. c. cordula (less than 3% of the individuals present this line; Fig. 2C); (e) the ‘forceps’ element on the ventral side of the hindwing (HW) (Linares, 1989) that is present in all H. c. cordula and is absent in H. t. timareta f. timareta; however, there is a small remnant of this element at the base of the HW in H. t. linaresi and (f) the basal ‘red spots’ on the HW ventral side, present in H. t. linaresi and H. t. timareta f. timareta (Fig. 2D), but absent in H. c. cordula (a first dot is located between the A1 + 2 and the Cu1 veins, a second dot at the base of the discal cell and a third dot located between the Sc+R1 and the Rs veins).

We also found that H. t. linaresi has a dark yellow-orange larval head capsule tone and light narrow bands on the dorsal view behind the head, similar to other H. cydno/H. timareta races. This was very different from the coloration of H. melpomene races where the larval head capsule is pale yellow and there are two dark broad bands just behind the head. In fact, we found significant differences in larval head capsule coloration between species/groups (ANOVA, d.f. = 4, P = 2.2 × 10^-16). In particular, larval
Figure 3. CoI-CoII and Tpi Phylogenetic trees. Phylogenetic relationships of Heliconius timareta linaresi subsp. nov. (indicated with a star) and closely related cognates (H. cydno, H. melpomene and H. timareta subspecies). Posterior probability values (on the numerator) were estimated using Bayesian analysis and bootstrap support (on the denominator) derived from a maximum likelihood analysis are displayed over the branches.
head capsule coloration analyses showed that offspring of wild H. t. linaresi females share similar colour indexes (b’ and LM) with H. t. florencia and other H. cydno races (Tukey’s HSD test $P > 0.01$; Fig. S4), but are significantly different from H. m. malleti and other H. melpomene races (Tukey’s HSD test $P < 0.01$, Fig. S4).

**Host plant choice**

Female laying behaviour was compared between 7 H. t. linaresi (353 eggs), 10 H. m. malleti (471 eggs) and 9 H. t. florencia (729 eggs). Heliconius t. linaresi females displayed a similar laying behaviour to H. t. florencia, ovipositing on several Passiflora species (P. edulis 63%, P. ligularis 19%, P. oerstedii 17% and P. quadrangularis, P. arborea and P. maliformis 1%; Fig. S5). In contrast, H. melpomene females laid eggs mainly on two Passiflora species (P. oerstedii 70%, P. ligularis 29%, Fig. S5). The model that best explained the data was a two-parameter model, which distinguished the laying behaviour of H. timareta races from that of H. melpomene (LnL = -638.749; $P = 0.00001; j_1 \neq j_2 \neq j_3$). Moreover, previous studies found that H. c. cordula also oviposited on several Passiflora species, but in a different proportion to H. timareta races from that of H. melpomene (Salazar, unpublished data). These results suggest that H. t. linaresi shows different laying behaviour than H. melpomene, but behaves more similar to H. cydno and H. t. florencia.
A common practice is to define newly discovered local populations as subspecies based on their different appearance from known populations. However, information about the complete phenotypic variation, geographic range, connectivity and natural history is not well understood in many cases. Furthermore, many such studies do not use a clear criterion to delimit species and subspecies boundaries (Braby et al., 2012). In this study, we used an integrative approach by gathering multiple sources of evidence (morphology, behaviour, genetics, ecology, etc.) to diagnose distinctiveness in evolving populations. This approach has been used to delimit species and subspecies boundaries in Australian satyrine butterflies (Braby et al., 2012), Philaethria butterflies (Barão et al., 2014), European wood white butterflies (Dincă et al., 2011), killer whales (Hoelzel et al., 2007), Alaskan song sparrows (Prueitt & Winker, 2010) and other examples in Mallet (2008) and James (2010). All available evidence – molecular, morphological and ecological – is consistent with a new distinctive subspecies of *H. timereta* on the south-eastern slopes of the Colombian Andes, which we call *H. t. linaresi* (Appendix 1).

**Molecular evidence**

The molecular data strongly supported the hypothesis that *H. t. linaresi* is a new member of the *H. timareta* lineage. Mitochondrial DNA places *H. t. linaresi* as a discrete clade within the larger *H. timareta* radiation. This result is congruent with recent mtDNA analysis, which surveyed the ‘barcode’ region for a much larger number of races of *H. cydno*, *H. melpomene* and *H. timareta* (Arias et al., 2014). In both studies, *H. timareta linaresi* clustered monophyletically with races of *H. timareta*, to the exclusion of both *H. cydno* and *H. melpomene*. Likewise, our microsatellite loci assigned all *H. t. linaresi* individuals to the *H. timareta* cluster, clearly differentiating it from both *H. melpomene* and *H. cydno*. A similar pattern was observed in a previous AFLP analysis of this radiation, but using a larger sample of *H. cydno* races (Fig. 4; Arias et al., 2014). Congruently, *F_{ST}\text{m* estimates between *H. t. linaresi* and both *H. m. malleti* and *H. cydno* races were high and similar to comparable estimates from earlier studies of genetic differentiation among the *H. melpomene*, *H. cydno* and *H. timareta* radiations (Giraldo et al., 2008; Martin et al., 2013; Mérot et al., 2013; Nadeau et al., 2013; Arias et al., 2014).

The *Z(X)* linked gene *Tpi* similarly differentiated all *H. cydno* and *H. timareta* individuals from all *H. melpomene* individuals. As expected, *H. t. linaresi* fell within a distinctive lineage containing *H. cydno/H. timareta*. Within this lineage, there were two clear clusters of *Tpi* alleles, both containing multiple races of *H. cydno* and *H. timareta*. However, within both lineages, there are no *Tpi* alleles shared between the two species. In contrast, the *H. t. linaresi* individuals we analysed shared nearly identical alleles with both *H. t. timareta* and *H. t. florencia*. Perhaps, the strong differentiation observed between *H. melpomene* and *H. cydno/H. timareta* clades could be the result of rapid coalescent of the species alleles due to lower effective population size of loci on the Z chromosome and/or the accumulation of Z linked factors that contribute to postzygotic isolation. In fact, previous studies have found a statistical association between *Tpi* and linked loci with sterility in *F_{1}* females between *H. cydno* females and *H. melpomene* males (Naisbit et al., 2002; Salazar et al., 2005). There was a similar pattern between *H. t. linaresi* and *H. melpomene*, where *F_{1}* female hybrids between *H. t. linaresi* female and *H. m. malleti* male were sterile (Sánchez et al., 2015). The pattern observed in these studies suggests that *H. timareta* and *H. cydno* show a similar degree of Z effect in their postzygotic isolation with *H. melpomene*. Additionally, *H. t. linaresi* and *H. m. malleti* showed strong prezygotic reproductive isolation (Sánchez et al., 2015). In contrast, experimental crosses between *H. t. linaresi* and *H. c. cordula* are completely interfertile (Sánchez et al., 2015). Nonetheless, there is evidence for some premating isolation where females of *H. t. linaresi* mate at a low frequency with *H. c. cordula* males, while *H. t. linaresi* males easily mate with *H. c. cordula* females (Sánchez et al., 2015). This asymmetric mating preference, with an almost identical colour pattern form (e.g. *H. c. cordula*, see below), suggests that mechanisms other than colour pattern, such as pheromone signals and/or courtship behaviour, are likely to be involved.

Notably, genetic differentiation as measured by *F_{ST\text{m}}* analysis of microsatellite loci between *H. t. linaresi* and other *H. timareta* subspecies was approximately five times lower than that was observed between *H. timareta* subspecies and either *H. cydno* and *H. melpomene*. Nonetheless, these differences were significant, suggesting restricted gene flow among parapatrically distributed *H. timareta* races. As an example, genetic differences between *Heliconius t. linaresi* and *H. t. florencia* were slight, but significant. The two subspecies have a parapatric distribution and hybrids between them have been collected in areas of overlap. However, the strong differences in colour pattern between *H. t. linaresi* and *H. t. florencia* (black-yellow vs. dennis-ray, respectively, Fig. 1) likely play a role in some degree of premating reproductive isolation between the two (Sánchez et al., 2015).
MORPHOLOGICAL AND NATURAL HISTORY EVIDENCE

In the eastern foothills of the Andes, there are two other taxa that are phenotypically similar to H. t. linaresi: to the north H. c. cordula and further south, one of the forms of the polymorphic race of H. t. timareta from eastern Ecuador. When we compared FW and HW patterns, we found five main traits that differ between these taxa. Three of these, the lack of wing iridescence, ‘red line’ and ‘red spots’, were shared between H. t. linaresi and H. t. timareta; one trait (FW yellow band size and form) was shared between H. t. linaresi and H. c. cordula and one trait was different between the three taxa (‘forceps’; Fig. 2). We further compared head capsule larval coloration between H. t. linaresi and several H. melpomene, H. cydno and H. timareta races (Fig. S4). These results indicate that H. t. linaresi is more closely related to the H. timareta/H. cydno clade and is clearly divergent from H. melpomene (except for H. m. malleti; Fig. S4). Similar results were found previously in comparisons between H. t. florencia/H. cydno and H. melpomene races (Giraldo et al., 2008).

Ecological differentiation between H. melpomene and H. cydno has been documented in habitat preference and host-plant use (see Jiggins, 2008; Merril et al., 2013). Typically, H. timareta/H. cydno races are associated with high altitudinal ranges (between 500 and 2000 m) in the understorey forest of the north-eastern Andes, while H. melpomene races are usually correlated with tropical lowland habitats (0–1000 m) across Central and South America. Heliconius t. linaresi has been collected during the last 10 years (~120 individuals) at two different locations on the understorey forest of the north-eastern Andes slopes: (1) at Puerto Rico (Caquetá, Colombia) within an altitude of ~1100 m a.s.l. and (2) at Las Morras (Caquetá, Colombia) within an altitude of ~1300 m a.s.l. Interestingly, H. timareta and H. cydno races, including H. t. linaresi, are geographic replacements of each other along the north-eastern slopes of the Andes (Fig. 1), which suggest some kind of competitive exclusion between these two species. Additionally, H. cydno/H. timareta and H. melpomene show contrasting host-plant use. On the one hand, Heliconius timareta/H. cydno races are usually not host-specific, whereas H. melpomene races, in general, are more host-specific (Smiley, 1978; Giraldo et al., 2008; Jiggins, 2008; Merril et al., 2013). The host-plant choice experiments carried out here imply that H. t. linaresi is not host-specific, similar to H. t. florencia and other H. cydno races (Fig. S5). Therefore, habitat preference and host-plant use again imply some degree of ecological isolation between H. t. linaresi and H. melpomene. Overall, morphological and natural history data support the existence of a well-established population clearly differentiated from H. melpomene races and more closely related to the H. timareta/cydno clade. Moreover, despite the phenotypic similarity of the black-yellow pattern between H. t. linaresi and H. c. cordula, distinctive colour pattern traits suggest that this novel entity is more closely related to the H. timareta lineage.

IMPLICATIONS FOR THE HELICONIUS BUTTERFLY RADIATION

The rapid radiation of the Neotropical Heliconius butterflies has long interested biologists. In particular, the diversification of the closely related species H. melpomene, H. cydno and H. timareta has received great attention in the last few years (Jiggins, 2008; Kronforst & Papa, 2015; Merril et al., 2015). Recent phylogenetic and phylogeographic studies of the radiation support the separation of H. melpomene, H. cydno and H. timareta as three distinct species (Nadeau et al., 2013; Arias et al., 2014; Kozak et al., 2015). However, the history of H. cydno/H. timareta diversification is still not well understood. It has been suggested that a proto H. cydno/H. timareta evolved in Central America and spread down the north-eastern slopes of the Andes and into the Magdalena and Cauca valleys, across to the western slopes of the Andes (Arias et al., 2014). At some point, the H. timareta lineage diverged and acquired colour pattern alleles through introgression from H. melpomene and started mimicking races of this species in the north-eastern slopes of the Andes (Pardo-Díaz et al., 2012; The Heliconius Genome Consortium, 2012) (but see Brower, 2011, 2013), whereas the H. cydno lineage largely tracked the phenotypic variation of the distantly related H. sapho and H. eleuchia. In general, H. cydno races have a complete yellow or white FW band (Fig. 1), suggesting that the proto H. timareta/H. cydno form likely had a similar pattern. Perhaps, H. t. linaresi represents a remnant population of the proto H. cydno/H. timareta that spread into the slopes of the north-eastern Andes and that later has acquired red elements by introgression from H. melpomene races in different locations. Notably, H. t. linaresi is not obviously mimetic with other taxa in its distribution, which contrasts with other H. timareta races, which fall in the same mimicry ring as H. melpomene (Lamas, 1997; Giraldo et al., 2008; Nadeau et al., 2014).

In general, the genetic pattern observed together with morphological and natural history data supports the existence of a well-established race of H. timareta, clearly differentiated from H. cydno and H. melpomene species. This study highlights the importance of the use of a comprehensive approach to understand the phenotypic and genetic diversity within species, which is crucial to completely comprehend the mechanisms that promote adaptation and speciation in recent radiations.
DESCRIPTION OF THE NEW SUBSPECIES

**Heliconius timareta linaresi** Arias & Lamas, subsp. nov. (Fig. S5)

**Diagnosis**

As other recently described subspecies in the *Heliconius timareta* group (Giraldo et al., 2008; Mérot et al., 2013), this new taxon belongs to the *Heliconius cydno* clade [part of the ‘numata’ group of Holzinger & Holzinger (1994) and of the ‘melpomene–cydno lineage’ of Brower & Egan (1997)] (Arias et al., 2014). *Heliconius timareta linaresi* subsp. nov. is phenotypically similar to *H. timareta* timareta f. timareta Hewitson, 1867 and *H. c. cordula* Neustetter (1913) (Fig. 2 and S6). However, it is clearly distinguishable by the following six traits: (a) the opaque black background coloration typical of *H. timareta* subspecies that contrasts with the blackish-blue iridescent coloration of *H. c. cordula*; (b) the form of the forewing (FW) yellow postmedian band with a smooth distal border in *H. t. linaresi* subsp. nov. and *H. c. cordula*, but irregular in *H. t. timareta* f. timareta (Fig. 2); (c) the yellow FW postmedian spot present below vein Cu 2 present in *H. t. linaresi* subsp. nov. and *H. cordula* but absent in *H. t. timareta* f. timareta; (d) a ‘red line’ at the base of the costal vein on the ventral side of FW, present in *H. t. linaresi* subsp. nov. and *H. t. timareta* f. timareta, but absent in *H. c. cordula* (Fig. 2); (e) the ‘forceps’ element on the ventral side of the hindwing (HW), absent in *H. t. timareta* f. timareta, present in *H. c. cordula* and a small remnant in *H. t. linaresi* subsp. nov. and (f) the basal ‘red spots’ on the HW ventral side ventral side, present in *H. t. linaresi* subsp. nov. and *H. t. timareta* f. timareta, but absent in *H. c. cordula*. (Fig. 2, S6).

**Male**

FW length 38–44 mm (mean 41.8 mm; N = 9). Dorsal wing colour opaque black, FW traversed by a yellow postmedian band that extends from the distal end of the discal cell to the base of vein R 3, curving smoothly from subcosta to vein Cu 1, a yellow trapezoidal spot in the distal third of the discal cell and another yellow, oval spot in the distal fourth of cell Cu 2-2A; HW completely opaque black, except for the area above subcosta, which has a shiny light buff. Ventral wing colour paler brown, FW with a ‘red line’ element (2.43–5.10 mm in length; N = 12) located at the base of the costal cell and crossed by a pale yellow postmedian band mirroring that present on the dorsal side; HW with a series of three (>2 mm in length) basal ‘red spot’ elements from cell Sc + R1 – Rs to cell Cu 2-2A, a conspicuous yellow costal streak, extending from the base to one half to two-thirds the length of the costa, and a brown remnant of the ‘forceps’ element behind vein 2A to the anal margin.

**Female**

FW length 37–44 mm (mean 40.5 mm; N = 4). Phenotypically similar to the male, but distinguishable by the dull dark brown costal area of the dorsal HW, the five-segmented prothoracic tarsus (fused together in males) and the clearly different external genitalia.

**Type material**

Holotype ♂, Colombia, Caquetá, San Vicente del Caguán, Vereda Las Morras, close to the Pato river, 2°06′49.61″N 74°47′09.40″W, 1300 m, 12.i.2006 (M. Linares) (IAvH-E-163671), in the Colección de Mariposas del Instituto de Investigación de Recursos Biológicos Alexander von Humboldt, Villa de Leyva, Colombia (Fig. S5). Paratype ♂, same data as holotype, but 5.ix.2009 (M. Linares) (ANDES-E16852), deposited in the Museo de Historia Natural de la Universidad de los Andes, Bogotá, Colombia (Fig. S5); Paratype ♀, same data as holotype, 1300 m, but 14.i.2007 (M. Linares) (IAvH-E-163672), in the Colección de Mariposas del Instituto de Investigación de Recursos Biológicos Alexander von Humboldt, Villa de Leyva, Colombia.

**Etymology**

Here we propose to name this new *H. timareta* subspecies as *H. timareta linaresi* subsp. nov. after Mauricio Linares who has dedicated his scientific career to studying evolution and speciation of Colombian *Heliconius* butterflies. A noun in the genitive case.

**Taxonomy and variation**

All available evidence (morphological, genetic, biogeographic and behavioural) supports the existence of a well-established race of *H. timareta*, a member of the *H. cydno* clade (Arias et al., 2014; this study). Individual variation is not pronounced; however, there is some variation on the size of the ‘forceps’ element.

Known (described and undescribed) *H. timareta* subspecies are as follows (in a latitudinal distribution, north to south): (a) *linaresi* (this study); (b) *florencea* Giraldo et al. (2008), from Caquetá, eastern Colombia; (c) *tristero* Brower (1996a) sensu Mérot et al. (2013), from Putumayo, south-eastern Colombia; (d) *timareta* Hewitson 1867, a polymorphic subspecies from eastern Ecuador; (e) an as yet undescribed subspecies from eastern Ecuador (Nadeau et al., 2014); (f) an undescribed subspecies from south-eastern Ecuador (Holzinger & Holzinger, 1994); (g) *timoratus* Lamas.
(1997), from northern Amazonas, Peru, close to the border with Ecuador and (h) thelaxioina Mérot et al. (2013), from north-eastern Peru (Fig. 1).

**Distribution**

Currently known near the vicinity of San Vicente del Caguan (Caquetá), along the eastern slopes of the Colombian Andes, at elevations between 1100 and 1300 m.

**Habitat and behaviour**

*Heliconius timareta linaresi* is found in the forest understory, usually foraging as adults on orange Cucurbitaceae flowers, such as *Psiguria* or *Gurania*, in small sunny gaps or at forest edges. Males are more frequently seen than females, flying fast in sunny patches and chasing females or other males. Females lay solitary eggs, usually on young stems of the host plant. Host-plant choice experiments suggest that *H. t. linaresi* is not host specific. The larva has a dark yellow-orange head capsule tone and light narrow bands on the dorsal region behind the head similar to *H. t. florencia*.

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Figure S1. Coalescent reconstruction in BEAST for the COI-COII locus. Trees are visualized with DensiTree (Bouckaert & Heled, 2014) displaying all trees of the Markov chain Monte Carlo chain with a burn-in of 7500 trees. Higher levels of uncertainty are represented by lower densities in green. A root-canal tree is presented in blue to guide the eye.

Figure S2. Coalescent reconstruction in BEAST for the TPI locus. Trees are visualized with DensiTree (Bouckaert & Heled, 2014) displaying all trees of the Markov chain Monte Carlo chain with a burn-in of 7500 trees. Higher
levels of uncertainty are represented by lower densities in green. A root-canal tree is presented in blue to guide the eye.

**Figure S3.** Estimation of the number of Heliconius clusters ($K$). In (a) is showed the mean Ln probability of the data [Ln $P(K)$] (Pritchard, Stephens & Donnelly, 2000) and in (b) is displayed the second-order rate of change ($\Delta K$) (Evanno, Regnaut & Goudet, 2005). The highest point in (b) suggest that the best estimate is $K = 3$.

**Figure S4.** Larvae colour dispersion index $b'$ and index LM, based on intensity and brightness of the head capsule. Larvae of *H. melpomene* and *H. cydno/H. timareta* are distinguishable by cephalic colour, which is pale yellow in *H. melpomene* and bright orange in *H. cydno/H. timareta*. Moreover, *H. cydno/H. timareta* larvae has two light narrow bands on the dorsal view behind the head, while *H. melpomene* larvae present two dark broad bands just behind the head. Heliconius melpomene races are *H. m. malleti, H. m. bellula* and *H. m. vulcanus* and *H. cydno* races are *H. c. cordula, H. c. cydnides* and *H. c. zelinde*. In fact, we found significant differences in head capsule coloration between species/groups (ANOVA, d.f. = 4, $P = 2.2 \times 10^{-16}$). In particular, head capsule coloration analyses showed that offspring of wild *H. t. linaresi* females share a similar colour index with *H. t. florencia* and other *H. cydno* races (Tukey’s HSD test $P > 0.01$), but significantly different from *H. m. malleti* and other *H. melpomene* races (Tukey’s HSD test $P < 0.01$). Results from the Tukey’s test are presented next to colour code legend, where species/groups that present the same letter do not show significant differences in $b'$ and LM indexes.

**Figure S5.** Host plant preference. Egg laying percentage for each type of female: *H. m. malleti* ($j_1$), *H. t. florencia* ($j_2$) and *H. t. linaresi* ($j_3$). Labels, oerstedii: *Passiflora oerstedii*; Granadilla: *P. ligularis*; Passion fruit: *P. edulis*; other: *P. quadrangularis, P. arborea* and *P. maliformis*; suberosa, rubra: *P. suberosa* and *P. rubra*. Heliconius t. linaresi females displayed a similar laying behaviour to *H. t. florencia*, ovipositing on many species, but different from *H. m. malleti* (Ln $L = –638.749$ for a two parameter model, $j_1 \neq j_2 = j_3$).

**Figure S6.** *H. timareta* subspecies. Heliconius timareta linaresi (Holotype). Individual is lodged at the Colección de Mariposas del Instituto de Investigación de Recursos Biológicos Alexander von Humboldt, Villa de Leyva, Colombia (accession numbers: IAvH-E-163671). (a) Dorsal view and (b) ventral view.

**Table S1.** Individuals used in phylogenetic analyses. Gene accession number and locality of individuals included in the phylogenetic analyses.