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A New Subspecies of *Heliconius hermathena* (Nymphalidae: Heliconiinae) from Southern Amazonia

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Abstract

The present paper describes *Heliconius hermathena curua* Freitas & Ramos **ssp. nov.** This subspecies exhibits a non-mimetic phenotype typical of *H. hermathena*, but is characterized by the merging of the yellow streak over the forewing cubitus with the red postmedian band in the dorsal forewing. The subspecies is known from two localities in the south of Altamira, Pará State, Brazil, where it inhabits an isolated patch of "campina" vegetation more than 600 km from the nearest known *H. hermathena* populations. Geographic isolation of the population is supported by molecular data; based on the mitochondrial gene COI, all individuals of *H. hermathena curua* **ssp. nov.** form a monophyletic group and all haplotypes found in it are unique, suggesting that gene flow is not currently on-going. Given the fragile situation of Amazonian white sand forests and the proximity of the population to areas of intensive agriculture, this new subspecies and its habitat deserve attention.

Introduction

The genus *Heliconius* Kluk, 1780 is one of the best studied butterfly groups, with 48 described species and hundreds of named wing color forms (Lamas 2004, Lamas & Jiggins 2017). Although most species of *Heliconius* are lowland tropical forest organisms, there are some exceptions. For example, *Heliconius besckei* (Ménétriés, 1857) and *Heliconius clysonymus* Latreille, (1817) are montane species from southeastern Brazil and the northern Andes/southern Central America, respectively (Brown Jr 1979), and *Heliconius himera* (Hewitson, 1867) occurs in dry forests in Peru and Ecuador (Descimon & Mast De Maeght 1984). Another notable exception is *Heliconius hermathena* (Hewitson, 1854). *Heliconius hermathena* is associated with open Amazonian vegetal formations, including the white sand physiognomies known locally as "campina" or "campinarana," where the high light and low humidity produce harsh conditions that are restrictive for most *Heliconius* (Brown Jr. & Benson 1977; for detailed descriptions of these habitats, see Ducke & Black 1953, Anderson 1981 and Adeney *et al* 2016). The species has been studied in detail by Brown Jr. & Benson (1977), who described aspects of its natural history, behavior, geographic distribution, host plants, immature stages, and variation in color pattern, and by Seixas *et al* (2017), who described its population biology. Currently, six subspecies have been described. Five of these are characterized by a red, black, and yellow wing color phenotype and differ only in subtle variations in the shape and size of the color pattern elements. Since they are quite distinct from the other butterflies in the region, include all known *Heliconius*, they are thought to exhibit a non-mimetic pattern (Brown Jr. & Benson 1977, Brown Jr. & Fernández 1985, Neukirchen 1992). The exception is *Heliconius hermathena vereatta*

Stichel, 1912, which is characterized by the absence of yellow, making it seemingly mimetic with other sympatric species of *Heliconius*, such as *Heliconius erato* (Linnaeus, 1758) and *Heliconius melpomene* (Linnaeus, 1758) (Brown Jr. & Benson 1977).

About four decades ago, while exploring several areas in the still recently opened Cuiabá-Santarém highway, the last author of this paper collected a series of eight individuals of *H. hermathena* in south Pará, near the border of Mato Grosso state. The wing pattern was noted to be sufficiently distinct from previously described taxa to merit subspecies status (as stated by KSB in his field notes). The sites were revisited in October 2016 and April 2018, revealing that the population is still present. Based on material collected in both expeditions, a new subspecies of *Heliconius hermathena* is therefore described from the southern limits of Brazilian Amazonia, in a region transitional to the cerrado savannas.

Material and Methods

Butterflies were collected in the extreme south of the Brazilian State of Pará, at two localities adjacent to the Cuiabá–Santarém highway (BR-163), in Altamira municipality: (1) "Salto do Curuá" (Curuá falls) (8°43′52.46″S; 54°58′7.28″W) and (2) a site 6 km north of the Mato Grosso–Pará frontier (9°27′41.72″S; 54°51′30.27″W). The vegetation in this region comprises a mosaic of white sand ecosystems, including typical open campina vegetation and some patches of cerrado savannas, as well as evergreen Amazonian forest along the rivers.

Specimens of all subspecies of *H. hermathena* were studied and collected by AVLF, RRR, and KSB at several field locations in the Neotropics and were examined in major public and private collections in Europe and North and South America (including the type specimens of all *H. hermathena* valid taxa and names). In addition, we also examined the Lamas collection of neotropical butterfly type specimen photographs at the MUSM (also available online in Warren *et al* 2013), representing all relevant names of *H. hermathena* (see Lamas 2004). The distributions of all the subspecies were mapped, using data compiled from museums and literature.

Acronyms for examined collections are **NHMUK**—Natural History Museum, London, UK and **ZUEC**—Museu de Zoologia da Universidade Estadual de Campinas, Campinas, São Paulo, Brazil.

Subspecies relationships

In order to establish the evolutionary relationships of the new subspecies with other populations of *H. hermathena*, tissue samples for *H. hermathena curua* **ssp. nov**. and five other currently recognized subspecies were collected from nine localities in Amazonia (Table 1). Collecting efforts were concentrated in areas with white sand ecosystems, especially in regions with campina or campinarana habitat. The butterflies were sampled with entomological nets (i.e., actively searched). Each individual was geo-referenced, and adult butterflies were stored in entomological envelopes.

Total genomic DNA was extracted using a CTAB buffer (Doyle & Doyle 1990) or DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Each sample was run on agarose gel in 1× buffer 1.0% SB to determine the quality of extracted DNA. The final DNA concentration and 280/260 and 260/230 ratios (for estimates of the proportions of DNA-RNA and proteins in samples) were estimated using a NanoDrop spectrophotometer (Techno Scientific, Concord, Canada).

COI amplification

The 5′ end of the gene for cytochrome *c* oxidase I (COI) was amplified using primer pairs K698 (5′-TACAATTTATCGCCTAAACTTCAGCC-3′) and Wyman (5′-GYTGAGCTCAWACAATAAATCCTA-3′) (Wahlberg *et al* 2009). The reactions were performed in a final volume of 25 uL, using 1 uL total DNA (concentration = 20 ng/ul), 3.0 mM $MgCl_2$, 40 mM dNTPs, 0.2 mM of each primer, 1 U of DNA GoTaq polymerase (Promega, Madison, WI, USA), and 10% 10× Taq buffer. The PCR program for amplifying COI includes an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and polymerization at 72°C for 1.5 min, followed by an extension step at 72°C for 10 min. After amplifications, the amplicons were purified by the column method, using the kit QIAquick PCR Purification Kit (Qiagen), or using ExoSAP-IT (GE Healthcare, Bucks, UK). The resulting fragment was sequenced in an automatic sequencer ABI 3570 (Life Technologies, California, USA). Sequences were analyzed and aligned manually using the program Geneious 7.0 (Biomatters Ltd., Auckland, New Zealand).

Population genetics analyses

The program MEGA 7.0.16 (Kumar *et al* 2016) was used to estimate the genetic distance within and between *H. hermathena* subspecies under Kimura's 2-parameter (K2P) substitution model (Kimura 1980). MEGA was also used to infer a distance tree using the Neighbor-Joining (NJ) algorithm (Saitou & Nei 1987) under the same K2P model. Branch supports were inferred with 1000 bootstrap replicates. The program TCS 1.21 (Clement *et al* 2000) was used to estimate a minimum spanning network of genealogies of haplotypes and to calculate the number of haplotypes for each population. The relatedness of two haplotypes was limited by a probability of parsimony for DNA pairwise differences lower than 0.95.

Table 1 Locality data for all DNA samples of *H. hermathena* subspecies.

Subspecies	Code	Locality	N	Coordinates
H. h. sabinae	HHBA	Barcelos, AM	22	– 0°59′29.87″S; – 62°55′31.28″W
H. h. sheppardi	HHCA	Manaus, AM	9	– 2°35′29.23″S; – 60°1′48.87″W
	HHPFCO	Pres. Figueiredo, AM (Cachoeira das Orquídeas)	4	– 2°2′8.82″S; – 59°59′59.17″W
	HHPFCA	Pres. Figueiredo, AM (Cachoeira das Araras)	8	– 1°59′33.04″S; – 60°3′12.12″W
	HHPFI	Pres. Figueiredo, AM (Cachoeira Iracema)	7	– 1°59′7.5″S, – 60°3′43″W
H. h. vereatta	HHFA	Faro, PA	18	– 2°11.23′S; – 56°40.83′W
H. h. duckei	HHFA-AIBI	Faro, PA	7	– 1°58′48.60″S; – 56°42′32.46″W
H. h. hermathena	HHMU	Maués, AM	9	– 3°22′36.55″S; – 57°43′17.18″W
	HHSA	Santarém, PA	13	– 2°27′38″S; – 54°43′59″W
H. h. curua **spp. nov**	HHCU	Salto do Curuá, Altamira, PA	8	– 8°43′52.46″S; – 54°58′7.28″W

Results

Taxonomy

Heliconius hermathena curua Freitas & Ramos **ssp. nov.** (Figs 1, 2, 3, 4, and 5).

Diagnosis. Distinguished from *H. hermathena vereatta* by the presence of yellow bars, spots, and streaks in both fore- and hindwings. Distinguished from all other subspecies by the yellow streak over the forewing cubitus, which is strongly elongated and changes to red in the space Cu_I-Cu_2, and usually merges (in about 70% of the known individuals) with the transverse red postmedian band.

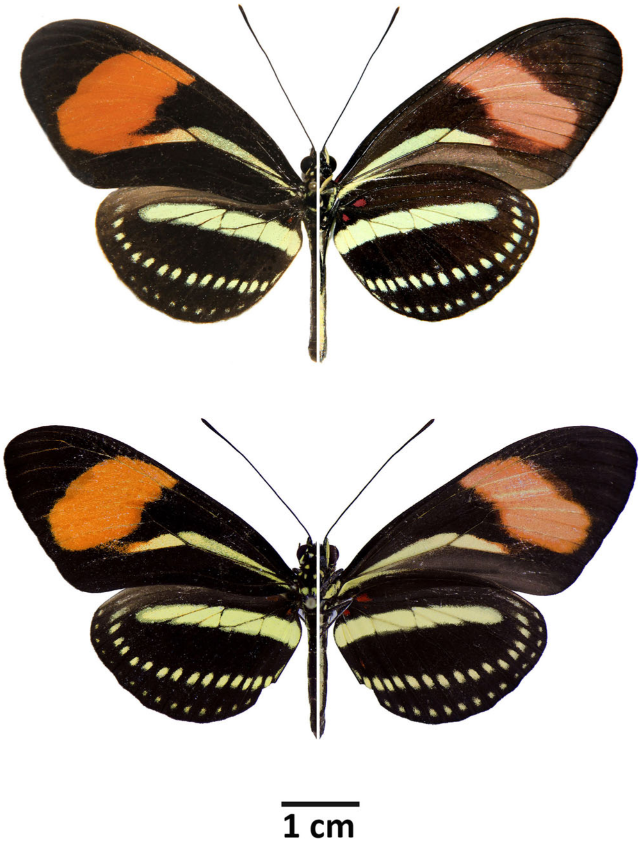

Fig 1 Holotype male (above) and allotype female (below) of *Heliconius hermathena curua* **ssp. nov.** (dorsal left, ventral right).

Description. Antennae entirely black, three-quarters the length of the forewing (19–21 mm in males, *n* = 4; 21–22 mm in females, *n* = 2), with 35–37 antennomeres; club with 10 antennomeres, not conspicuously developed. Thorax black with small yellow markings. Patagium black with a small yellow dot. Forewing length 31–40 mm in males (mean = 38.0 mm, SD = 2.79 mm, *n* = 11), 32–41 mm in females (mean = 36.0 mm, SD = 3.03 mm, *n* = 6); hindwing length 20–25 mm in males (*n* = 6), 21–26 mm in females (*n* = 4). Wings with a black background, patterned with yellow and red markings as follows: dorsal forewing with a broad postmedian red band; a yellow streak over the forewing cubitus elongated in space Cu_I-Cu_2, changing to red in the distal portion and usually merging with the red postmedian band; ventral forewing with similar patterns, the red band mixed with yellow scales resulting in a orangish tint; dorsal hindwing with a broad yellow cubital stripe (broader than in all other subspecies except *H. hermathena sabinae*, Fig 2); two series of doubled intervenal submarginal yellow spots, the internal series with spots larger than the external series; ventral hindwing with two red basal spots; a single small red raylet on the anal border is present on some individuals (absent in the holotype).

Type material. HOLOTYPE. Male (Fig 1, above). Deposited in the Museu de Zoologia da Universidade Estadual de Campinas (ZUEC), Campinas, São Paulo, Brazil. With the following labels separated by transverse bars: / Holotypus / Brazil, Pará, Altamira, Salto do Curuá, Km 102 - BR 163 (Rod [ovia]. Cuiabá-Santarém), 8°43′52.46″S; 54°58′7.28″W, 3.XI.2016, Ramos, RR & Beirão, MV / DNA voucher – HHCU1 / ZUEC LEP 10618 /.

ALLOTYPE. Female (Fig 1, below). Deposited in the Museu de Zoologia da Universidade Estadual de Campinas (ZUEC), Campinas, São Paulo, Brazil. With the following labels separated by transverse bars: / Allotypus / Brazil, Pará, Altamira, Salto do Curuá, Km 102 - BR 163 (Rod[ovia]. Cuiabá-

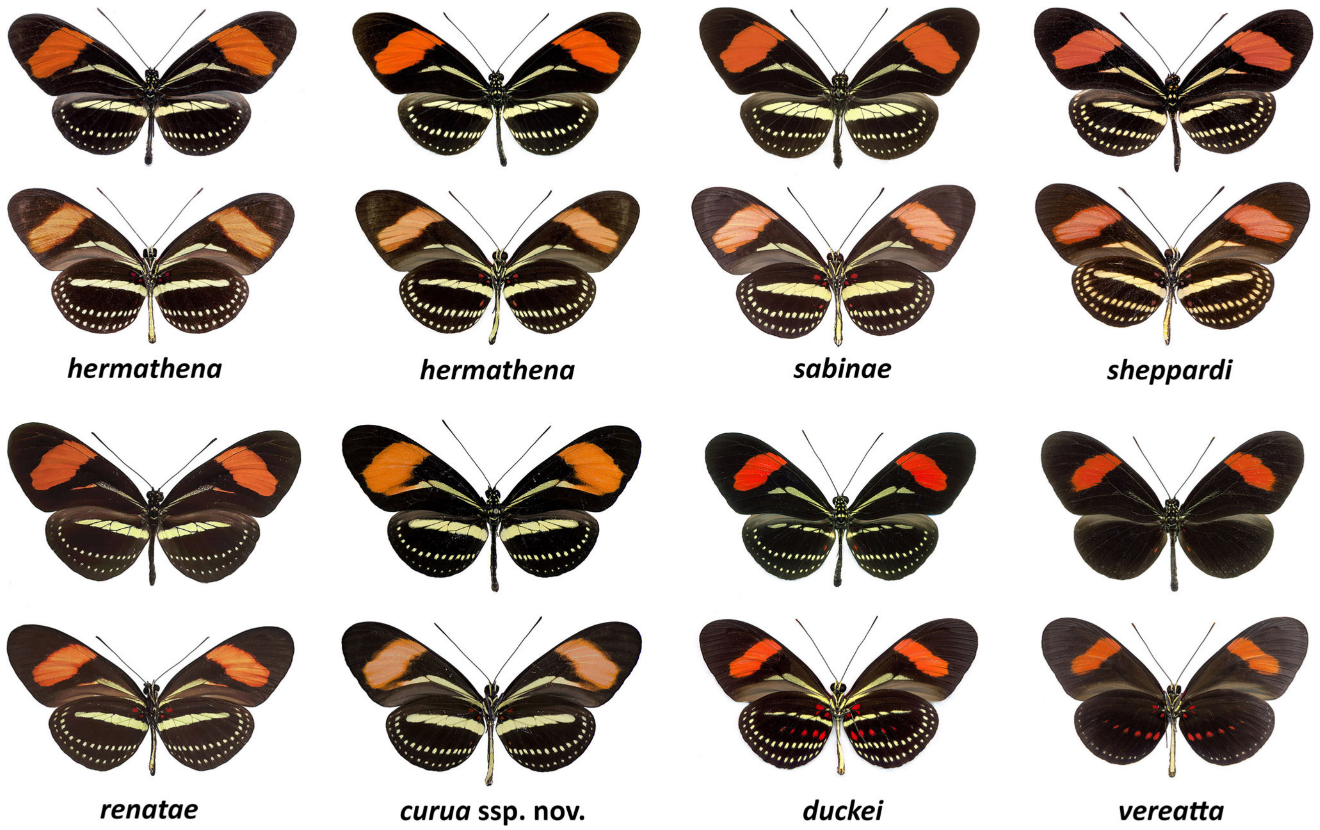

Fig 2 Comparative plate of the seven described subspecies of *Heliconius hermathena* (dorsal above, ventral below). Names for each subspecies are below each specimen. Localities for figured specimens are as follows (from left to right, top to bottom): *H. h. hermathena*, Santarém, Pará, Brazil; *H. h. hermathena*, Maués, Amazonas, Brazil; *H. h. sabinae*, Barcelos, Amazonas, Brazil; *H. h. sheppardi*, Presidente Figueiredo, Amazonas, Brazil; *H. h. renatae*, San Carlos de Rio Negro, Amazonas, Venezuela; *H. h. curua* **ssp. nov.** (paratype, ZUEC LEP 10619) Altamira, Pará, Brazil; *H. h. duckei*, Faro, Pará, Brazil; *H. h. vereatta*, Faro, Pará, Brazil.

Santarém), 8°43′52.46″S; 54°58′7.28″W, 3.XI.2016, Ramos, RR & Beirão, MV / DNA voucher – HHCU3 / ZUEC LEP 10623 /.

PARATYPES. BRAZIL, *Pará* - **Altamira** - Salto do Curuá, Km 102 - BR 163 (Rod[ovia]. Cuiabá-Santarém), 8°43′52.46″S; 54°58′7.28″W, 1 male, 18-19.X.1977, Brown, KS, *leg.* (ZUEC LEP 10624) **(ZUEC)**, 3 males, 19.X.1977, Brown, KS *leg.* (ZUEC LEP 10625, ZUEC LEP 10626, ZUEC LEP 10631) **(ZUEC)**, 3 females, 18.X.1977, Brown, KS *leg.* (ZUEC LEP 10627, ZUEC LEP 10629, ZUEC LEP 10630) **(ZUEC)**, 1 female, 18.X.1977, Brown, KS *leg.* **(NHMUK)**, 1 male, 3.XI.2016, (DNA voucher HHCU4), Ramos, RR & Beirão, MV *leg*. (ZUEC LEP 10621) **(ZUEC)**, 2 males, 1 female, 30.X.2016, (DNA vouchers HHCU2, HHCU6, HHCU5), Ramos, RR & Beirão, MV *leg.* (ZUEC LEP 10619 (Fig 2), (ZUEC LEP 10620, ZUEC LEP 10622)) **(ZUEC)**, 2 males, 4.IV.2018, Ramos, RR & Gusmão, P. *leg*. (ZUEC LEP 10633, ZUEC LEP 10634) **(ZUEC)**, 1 male, 4.IV.2018, Ramos, RR & Gusmão, P. *leg*. (ZUEC LEP 10632) **(ZUEC)**, Córrego XV de Novembro, 21-22.X.1977, 9°27′41.72″S; 54°51′30.27″W, 1 female, Brown, KS *leg.* (ZUEC LEP 10628) **(ZUEC).**

Etymology. The subspecific epithet, curua, refers to the place where most individuals have been observed and collected, the "Salto do Curuá" (Curua Waterfalls). A noun in apposition.

Variation. The general wing pattern of *H. hermathena curua* **ssp. nov.** belongs to the non-mimetic pattern of *H. hermathena*. On the basis of all known individuals, the main diagnostic differences in the wing pattern are observed in the yellow streak over the forewing cubitus, which in addition to being longer than in all other known non-mimetic subspecies, can merge (Figs 1 and 2) or not (Fig 1) with the red postmedian band in dorsal forewing. Other minor variation is the presence/absence of a single small red raylet on the anal border of the ventral hindwing.

Distribution. *Heliconius hermathena curua* **ssp. nov.** is known from only two localities 80 km apart along the Cuiabá-Santarém highway, in the south of Altamira municipality, Pará State, North Brazil, very near to the border of Mato Grosso State (Fig 3). Both collecting sites are inside a very isolated patch of "campina" vegetation in southern Amazonia (Fig 3), more than 600 km from the nearest *H. hermathena* populations known so far.

Habitat and natural history. The two sites where this new subspecies has been collected are covered by open campina

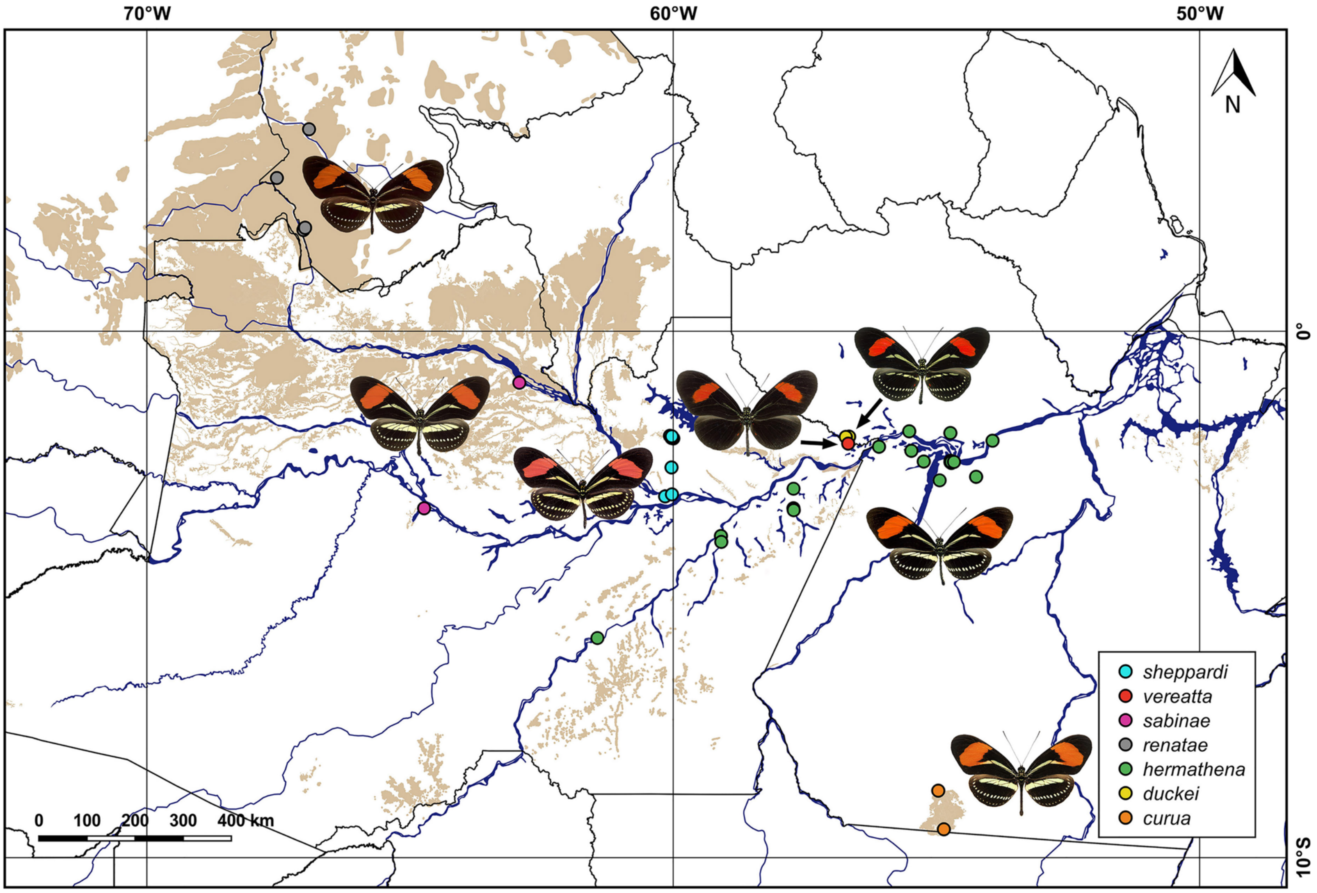

Fig 3 Map showing the known distribution of all described subspecies of *Heliconius hermathena*. Areas of white sand environments are in light brown (following Adeney *et al*. 2016).

vegetation (Fig 4a–c). The sandy soil is partially flooded during the rainy season, and several water bodies keep the ground humid even during the dry season. The general behavior of *H. hermathena curua* **ssp. nov.** is quite similar to that reported for other subspecies of *H. hermathena* (see Brown & Benson 1977, Seixas *et al* 2017). Adults fly in sunny areas partially shaded by low vegetation during early morning, but search for more shaded areas when temperatures are high around midday. Both sexes tend to fly low (0.5–2 m high) and exhibit a clear preference for flying in the more open vegetation along trails and paths. Nonetheless, they demonstrate high maneuverability when flying through the dense and tangled vegetation. Adults were observed visiting three species of flowers, including *Lantana camara* L. (Verbenaceae) and two species of *Palicourea* (Rubiaceae). A potential larval host plant, *Passiflora hexagonocarpa* Barb. Rodr. (Passifloraceae) was observed in the study site (Fig 4d), but no immatures or oviposition behavior were observed.

Evolutionary relationships and genetic distances

All individuals of *H. hermathena curua* **ssp. nov.** form a cohesive group closely related to specimens from Faro, Pará (*H. hermathena vereatta* and *H. hermathena duckei*) and from Manaus, Amazonas (*H. hermathena sheppardi*) (Fig 5a). Most subspecies were recovered as monophyletic groups, except *H. hermathena sheppardi*, that appeared in two clades corresponding to two sampled sites (with a relatively high pairwise mean distance between them, see Table 2). Within population mean genetic distances were usually lower than pairwise mean genetic distances except in two comparisons: (1) the mean genetic distance is lower between *H. h. sabinae* and *H. h. sheppardi* from Presidente Figueiredo than the mean distance within *H. h. sabinae* and (2) the same was observed between the two forms from Faro, Pará (*H. h. vereatta* and *H. h. duckei*) (Table 2). The shortest pairwise mean genetic distance was found between *H. hermathena curua* **ssp. nov.** and the individuals from Faro, Pará (Table 2). The eight individuals of *H. hermathena curua* **ssp. nov.** present three different haplotypes of COI, the most frequent of which is separated by only one mutational step from a haplotype present in Faro, Pará, and by three steps from a haplotype present in Barcelos, Amazonas (*H. hermathena sabinae*; H6) (Fig 5b). The most frequent haplotype within *H. hermathena curua* **ssp. nov.** differs by three mutational steps from a haplotype present in Maués,

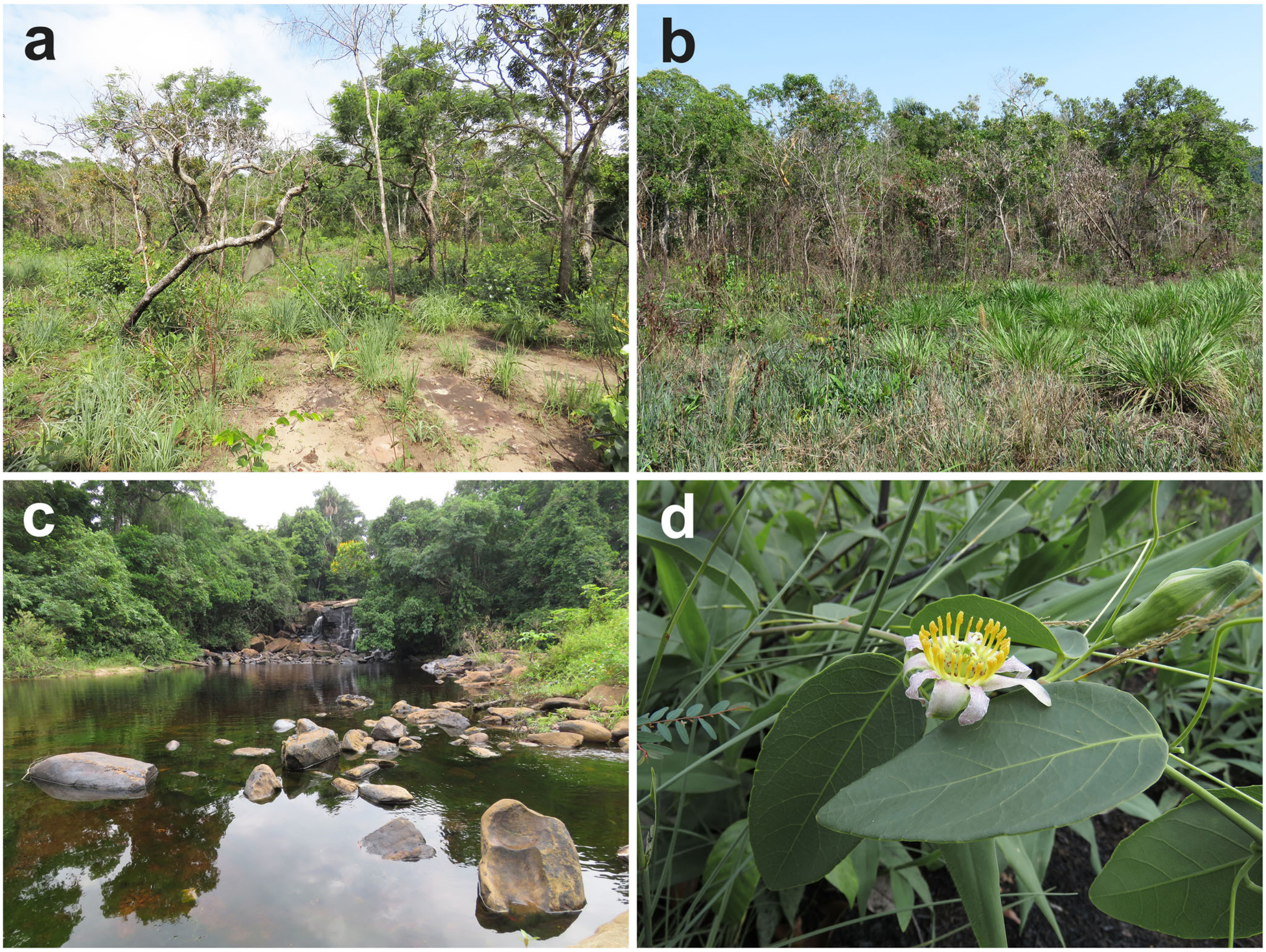

Fig 4 Habitat of *Heliconius hermathena curua* **ssp. nov.** **a**, **b** General view of the open habitats of campina–cerrado mosaic. **c** The evergreen forest near the Curuá Falls (in the background, a small waterfall with low water volume due to the dry season). **d** *Passiflora hexagonocarpa* Barb. Rodr. (Passifloraceae), the likely host plant for *H. hermathena curua* **ssp. nov.**

Amazonas (*H. hermathena hermathena*), the population most geographically close to the new subspecies. All sequences are available in GenBank under accession numbers MK035598 – MK035702.

Discussion

The description of a new subspecies in such a well-studied group of butterflies as *Heliconius* is surprising in itself. However, the importance of the present study goes beyond this. This is the most southerly known population of *H. hermathena*, and it inhabits the most isolated patch of campina vegetation in southern Amazonia, near the transition with the cerrado savannas of central South America. It is therefore probably the southern limit of the distribution of this species. Furthermore, *H. hermathena curua* **ssp. nov.** is separated by more than 600 km from the nearest known *H. hermathena* populations in the central Amazon (Fig 3). This isolation is likely to be real and not an artifact of collecting bias for two reasons. (1) The last author of the present study (KSB) collected intensively along the entire Cuiabá–Santarém highway in 1977 (very soon after its opening in 1976), and all his lists of species and collected material are available at the ZUEC and were carefully revised. (2) The region of the Curuá Falls is an island of campina vegetation, isolated by almost 600 km of rainforest from the other areas of white sand ecosystems (Fig 3, see also Adeney *et al* 2016). Furthermore, *H. hermathena curua* **ssp. nov.** is phenotypically well defined and distinct from all other known populations of non-mimetic *H. hermathena*; the long yellow streak over the forewing cubitus usually merging with the red postmedian band in the forewing is unique for this subspecies (but is similar to the pattern of the sympatric *Heliconius melpomene burchelli* Poulton, 1910).

Although we only analyzed a small number of individuals, the geographic isolation of *H. hermathena curua* **ssp. nov.** is also supported by our molecular data; the population is

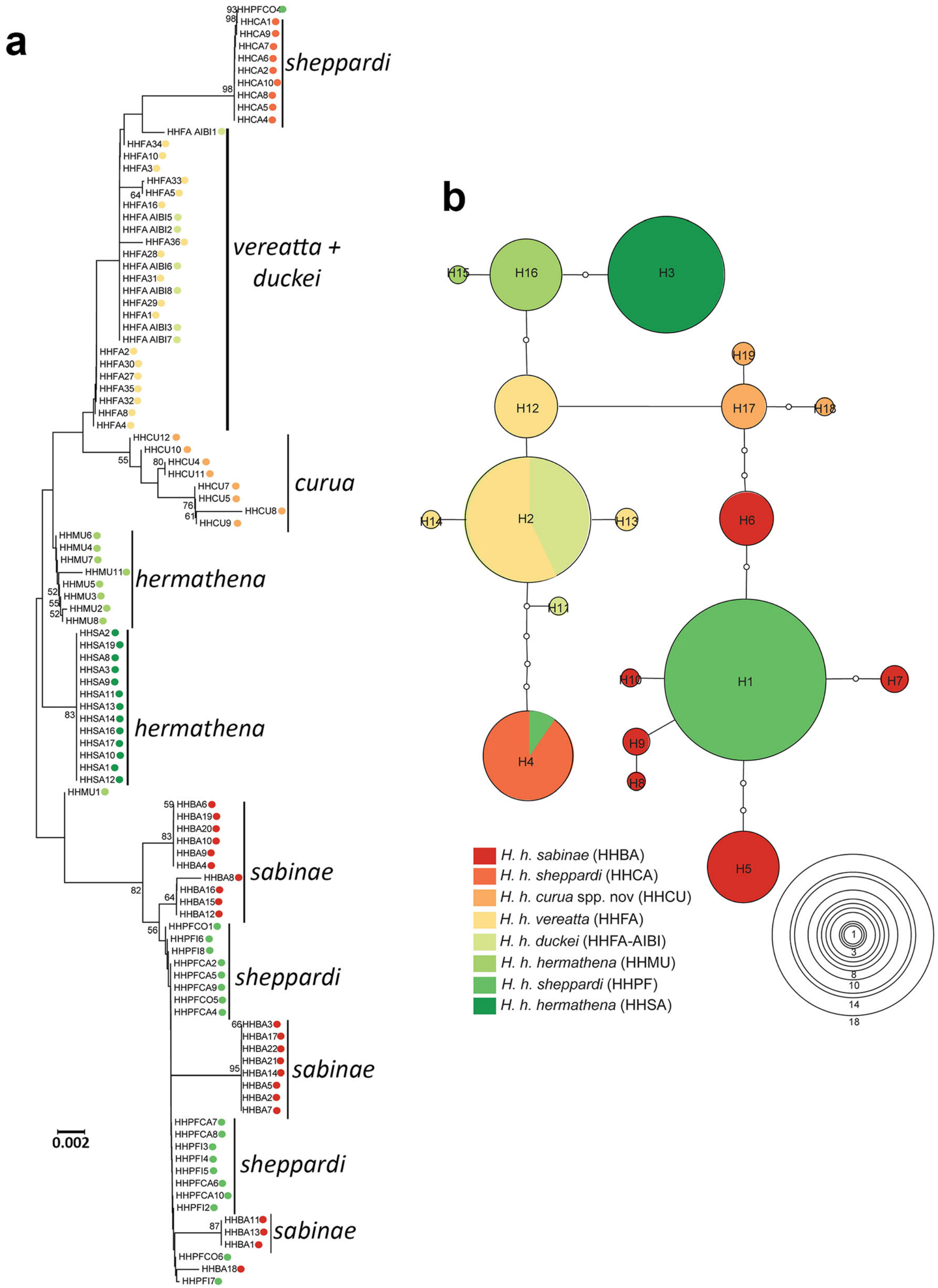

Fig 5 **a** Neighbor-joining distance tree of *H. hermathena* subspecies. **b** Haplotype network of *H. hermathena* subspecies. Different colors indicate localities where the individuals of each subspecies were sampled (further details in Table 1). Area of the circles is directly proportional to the number of specimens showing such haplotype; small white circles designate non-sampled haplotypes. Each branch is equivalent to one base pair change.

Table 2 Within population mean genetic distance (first diagonal, in italics) and pairwise mean genetic distance of COI sequences, estimated with Kimura's 2-parameter substitution model. All samples from Presidente Figueiredo region were combined under HHPF.

	HHBA	HHPF	HHCA	HHFA	HHFA-AIBI	HHSA	HHMU	HHCU
H. h. sabinae (HHBA)	*0.006*							
H. h. sheppardi (HHPF)	0.005	*0.002*						
H. h. sheppardi (HHCA)	0.024	0.020	*0*					
H. h.a vereatta (HHFA)	0.018	0.015	0.009	*0.002*				
H. h. duckei (HHFA-AIBI)	0.019	0.015	0.009	0.001	*0*			
H. h. hermathena (HHSA)	0.017	0.014	0.013	0.008	0.009	*0*		
H. h. hermathena (HHMU)	0.016	0.014	0.015	0.005	0.006	0.004	*0*	
H. h. curua **spp. nov.** (HHCU)	0.015	0.014	0.017	0.010	0.010	0.013	0.011	*0.002*

monophyletic and all haplotypes found in it are unique, suggesting that gene flow is not currently on-going. Nonetheless, *H. hermathena curua* **ssp. nov.** is only weakly differentiated from other known conspecific populations. This could be indicative of recent isolation of the subspecies, which may have originated from founders coming from the lower Amazon River (in the east of the Amazon and west of the Pará States).

The present population of *H. hermathena curua* **ssp. nov.** therefore represents important evidence of ancient connections between the open habitats of central and south Amazonia, that may have been mediated by natural or even anthropic causes. Recent data has shown that rainfall in Amazonia was highly variable over the last 45,000 years, ranging from 58 to 142% relative to modern levels (Wang *et al* 2017). These fluctuations may have caused reductions in rain forests, and periodic expansions of dry, open forests, thus allowing migration and gene flow between now isolated populations of *H. hermathena*. Human populations may also have influenced the prevalence and extension of open forests. For example, de Souza *et al* (2018) showed that the entire southern rim of Amazonia exhibited high population densities of earth-builder cultures between AD 1250 and 1500, who lived far from the floodplains of the main rivers. These large human settlements may also have promoted a recent connection between open ecosystems across southern Amazonia, allowing the exchange of species such as *H. hermathena*. Further phylogeographic analysis of *H. hermathena* incorporating genomic dating may shed light on these interesting questions and help reveal the processes underlying the observed biogeographical patterns.

Finally, given the fragile situation of Amazonian white sand forests in general (Adeney *et al* 2016), and the proximity of the isolated campina habitat at Curuá Falls to areas of intensive agriculture in northern Mato Grosso and southern Pará, the description of *H. hermathena curua* **ssp. nov.** highlights the importance of future conservation strategies across the entire region.

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Nomenclature

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