Supplementary Materials for:
Supergene formation is associated with a major shift in genome-wide patterns of diversity in a butterfly

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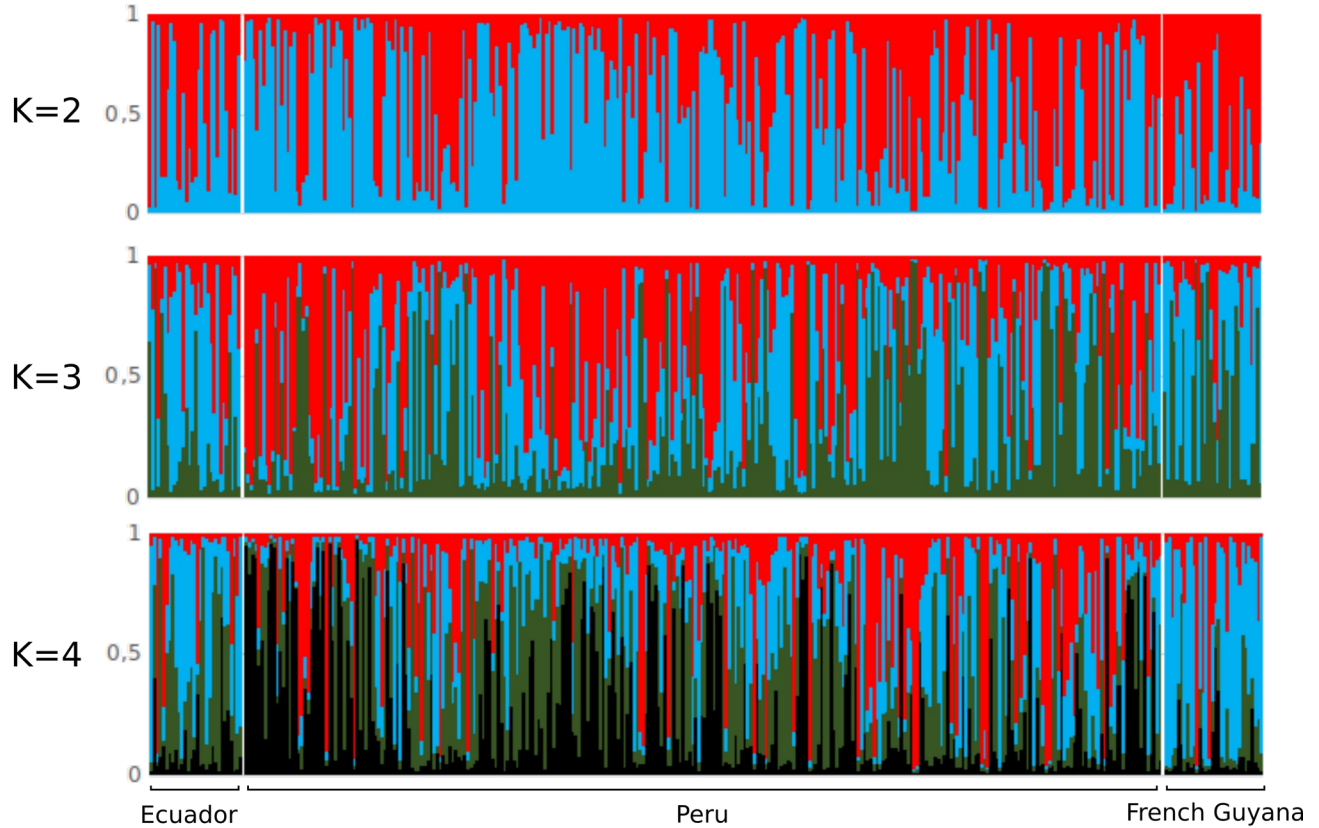

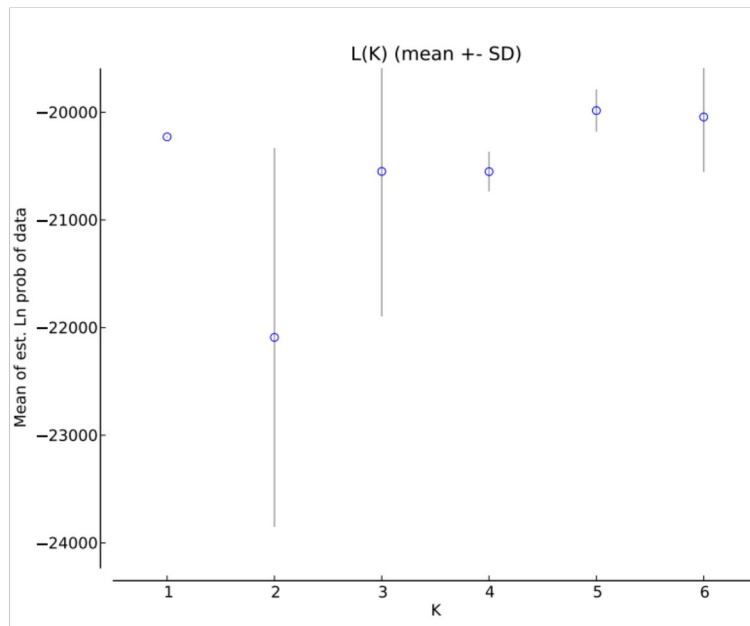* contributed equally
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This file contains:
Figure S1 to S5
Table S1-S8 (Table S3 provided as a separate file)
Text S1

A

B

Figure S1 | Genotype assignation plot using Structure showing general lack of population structure across the Amazon in *H. numata*.

Detection of the number of groups (K) in STRUCTURE analysis of 360 *H. numata* individual genotyped at 14 microsatelites loci[1]. **A,** CLUMPACK[2] results following 20 STRUCTURE[3] run for each K ranging from 2 to 4 and setting both the length of burning period and the number of MCMC reps after burning at 100000. **B,** Mean

value of log probability of the data L(K), as function of K ranging from 1 to 6 estimated by the program STRUCTURE HARVESTER[4] and following the method of Evano et al. (2005)[5].

References

1. Chouteau, M., Whibley, A., Angers, B. & Joron, M. Development of microsatellite loci from a reference genome for the Neotropical butterfly Heliconius numata and its close relatives. *Entomological Science* **18**, 283–287 (2015).

2. Kopelman, N. M., Mayzel, J., Jakobsson, M., Rosenberg, N. A. & Mayrose, I. Clumpak: a program for identifying clustering modes and packaging population structure inferences across K. *Molecular Ecology Resources* **15**, 1179–1191 (2015).

3. Pritchard, J. K., Stephens, M. & Donnelly, P. Inference of Population Structure Using Multilocus Genotype Data. *Genetics* **155**, 945–959 (2000).

4. Earl, D. A. & vonHoldt, B. M. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources* **4**, 359–361 (2012).

5. Evanno, G., Regnaut, S. & Goudet, J. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* **14**, 2611–2620 (2005).
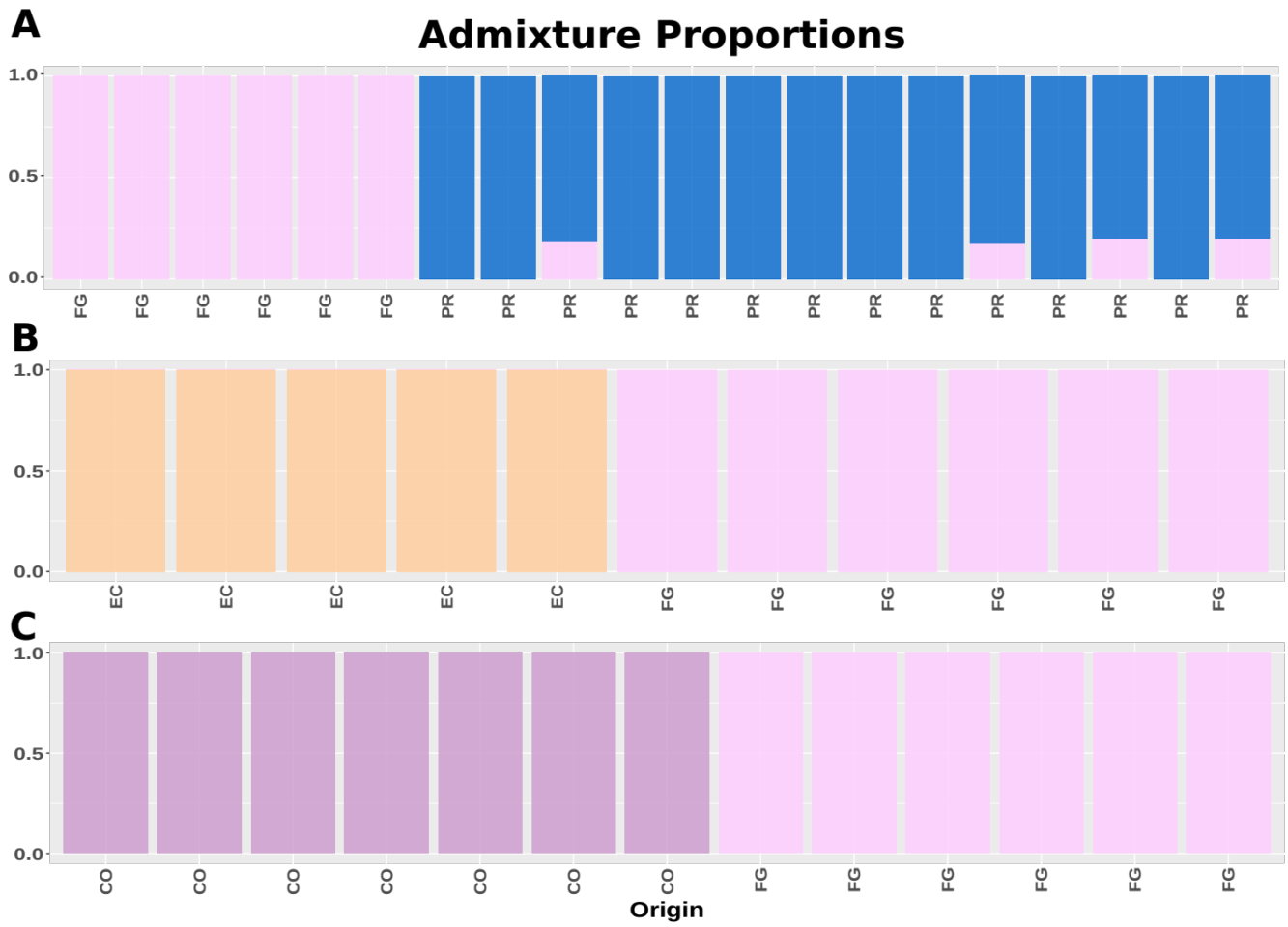
Fig S2| Admixture analyses revealed substructure between French Guiana and other subgroups.

Analysis was performed by sampling random individuals from Peru (PR) in panel A; or all individuals from other localities, namely Ecuador (EC) in panel B and Columbia (CO) in panel C.
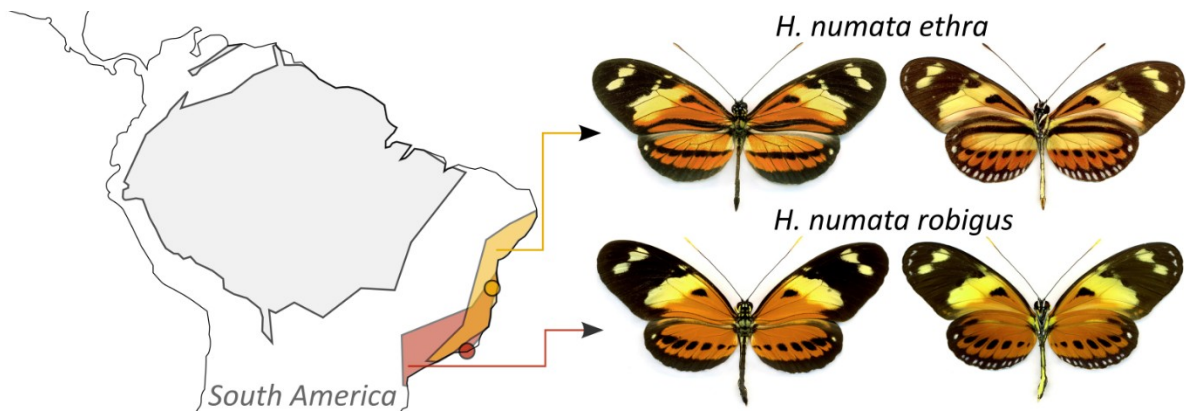
Figure S3 | Geographic distribution, sampling sites and phenotype of the forms *H. numata ethra* (yellow area) and *H. numata robigus* (red area).

Orange and red circles indicate sampling sites for *H. n. ethra* and *H. n. robigus*, respectively. The dorsal (left) and ventral (right) views of a specimen of each race are shown. The grey area on the map represents the range of distribution of the rest of the *H. numata* races. Geographic distribution of the races is according to Rosser *et al.* 2012.
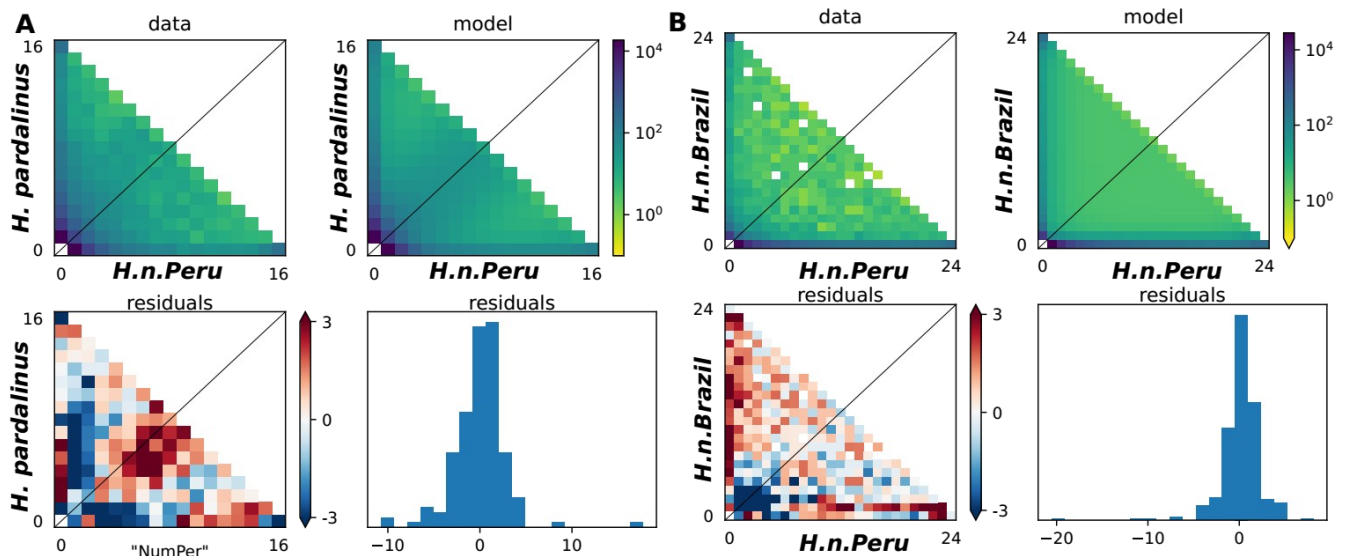
Figure S4 | Differences between observed and predicted jSFS along with model residuals from dadi for the best models. A) Comparison between H. *numata* from the Amazonian rain-forest in Peru and *H. pardalinus.* The best model is one of secondary contact with linked selection and population size change (SCA2NG). Migration was highly asymmetric. Site frequency spectrum for Peru in panel A) was down sampled to match the number of individuals in *H. pardalinus.*

B) Comparison between *H. numata* from Mata Atlântica in Brazil versus *H. numata* from the Amazonian forest in Peru. The best model is one of isolation with migration with linked selection and population size change (IMA2NG). B)

$W_{\text{Mann-Whitney}} = 41085.00$, $p = 9.09\text{e-}06$, $\hat{r}^{\text{rank}}_{\text{biserial}} = -0.20$, $\text{CI}_{95\%}$ [-0.29, -0.12], $n_{\text{obs}} = 642$
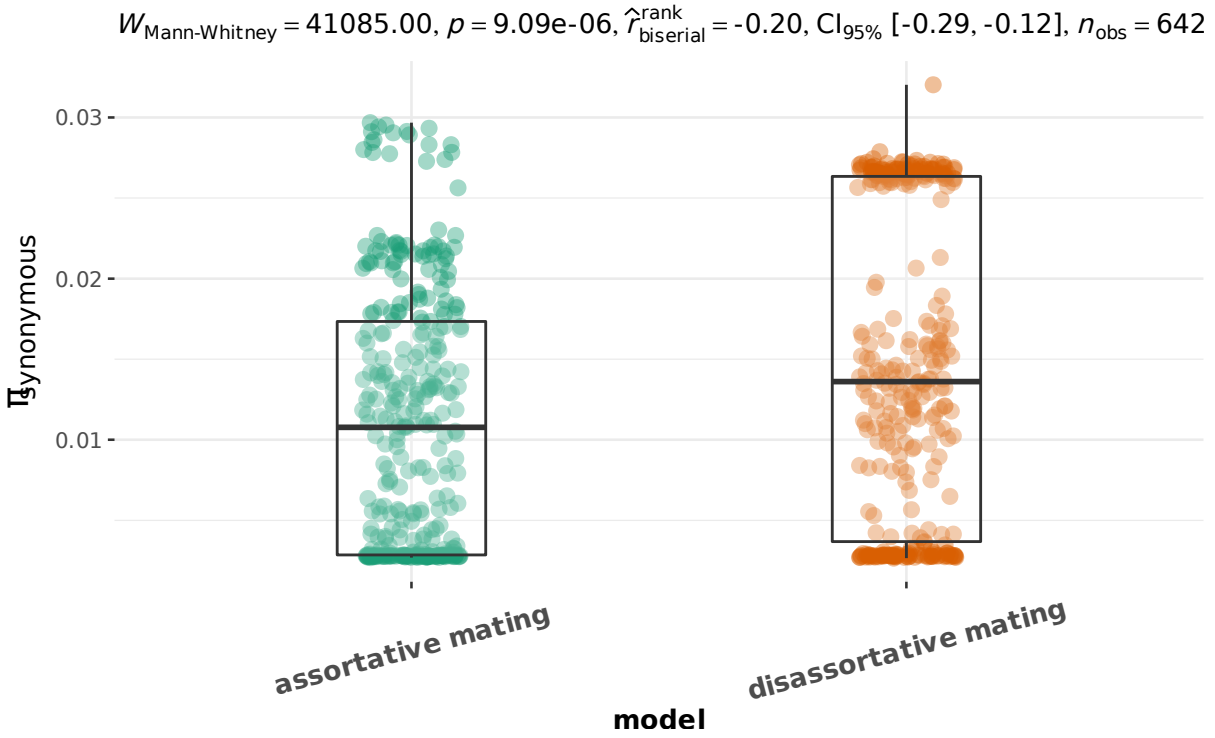
Figure S5 | Slightly higher synonymous diversity in models of disassortative mating compared to models of assortative mating (p <0.01). Most of the difference is due to models with m = 0.0001 as seen on Figure 4.

Table S1. Band size of the *cortex* amplicons corresponding to the different gene orders at the supergene *P* in Peruvian populations of *H. numata*

		Expected band size (bp)		
		Arrangement *Hn0:*	Arrangement *Hn1*	Arrangement *Hn123*
First set of *cortex* primers (amplicon 1)[1]	F: CGCAACGTTATCGCCTAGATAGGTTCG R: AANGCGAAASMACTGAYAACACGWG	~500	~1100	~800
Second set of *cortex* primers (amplicon 2)[2]	F: CGTAGCGACCCGAGATTCTT R: AATACATGGCCACAGTTGATTC	~380	~920	~650

[1]Jay et al. 2021 [2] Saenko et al., 2019

Table S2. Supergene alleles in the *numata* populations from the Brazilian Atlantic Forest

Form	Population	Specimen	*cortex* amplicon 1 (bp)	*cortex* amplicon 2 (bp)	Summary
H. numata ethra	Reserva Serra Bonita	BH16-0020	Null	Null	**Arrangement *Hn0*: 8/13** Null allelles: 5/13
H. numata ethra	Reserva Serra Bonita	BH16-0021	~500	~380	
H. numata ethra	Reserva Serra Bonita	BH16-0022	Null	Null	
H. numata ethra	Reserva Serra Bonita	BH16-0023	~500	~380	
H. numata ethra	Reserva Serra Bonita	BH16-0024	~500	~380	
H. numata ethra	Reserva Serra Bonita	BH16-0026	Null	Null	
H. numata ethra	Reserva Serra Bonita	BH16-0027	~500	~380	
H. numata ethra	Reserva Serra Bonita	BH16-0028	Null	Null	
H. numata ethra	Reserva Serra Bonita	BH16-0029	Null	Null	
H. numata ethra	Reserva Serra Bonita	BH16-0030	~500	~380	
H. numata ethra	Reserva Serra Bonita	BH16-0031	~500	~380	
H. numata ethra	Reserva Serra Bonita	BH16-0032	~500	~380	
H. numata ethra	Reserva Serra Bonita	BH16-0033	~500	~380	
H. numata robigus	Praia do Sono	BH16-0042	~500	~380	**Arrangement *Hn0*: 11/11**
H. numata robigus	Praia do Sono	BH16-0043	~500	~380	
H. numata robigus	Praia do Sono	BH16-0044	~500	~380	
H. numata robigus	Praia do Sono	BH16-0045	~500	~380	
H. numata robigus	Praia do Sono	BH16-0046	~500	~380	
H. numata robigus	Praia do Sono	BH16-0047	~500	~380	
H. numata robigus	Praia do Sono	BH16-0048	~500	~380	
H. numata robigus	Praia do Sono	BH16-0049	~500	~380	
H. numata robigus	Praia do Sono	BH16-0050	~500	~380	
H. numata robigus	Praia do Sono	BH16-0051	~500	~380	
H. numata robigus	Praia do Sono	BH16-0052	~500	~380	
H. numata silvana	Oriximiná	HEL069	~500	~380	**Arrangement *Hn0*: 2/2**
H. numata silvana	Oriximiná	HEL085	~500	~380	

Table S3 | Sample name and metadata. Includes sample id, species identification, morph, origin, sequencing depth, sample accession and type of analyses in which a given sample was used.

See attached xls sheet file

Table S4 | Fst and Dxy between morphs or populations of *Heliconius* species and distance in km. The first column represents the pair of populations compared. In some cases, where the subspecies ranges do not overlap (geographic races), the names of the subspecies studied are indicated instead of the populations. This is the case for *zuleika, melicerta, clearei* and "Peru" (geographic races/subspecies of *H. hecale*) for instance. Within *H. numata*, populations named Moyobamba, Tunel and Urahuasha are subsamples of the "Tarapoto" population indicated on Fig 1.

See next pages

population1	population2	Species	F_{ST}	Distance
Urahuasha	Moyobamba	H.numata (Amazon)	0,018154	83
Moyobamba	Michaela	H.numata (Amazon)	0,021686	84
Tunel	Moyobamba	H.numata (Amazon)	0,01771	87
Moyobamba	FG	H.numata (Amazon)	0,055996	2924
Moyobamba	Colombia	H.numata (Amazon)	0,02706	879
Moyobamba	Bresil	H.numata (Brazil) vs H.numata (Amazon)	0,57124	3954
Moyobamba	Ecuador	H.numata (Amazon)	0,023722	550
Moyobamba	Madre_de_Dios	H.numata (Amazon)	0,049239	1042
PuertoBermudez	Moyobamba	H.numata (Amazon)	0,022862	524
Moyobamba	La_Merced	H.numata (Amazon)	0,028	574
Urahuasha	Michaela	H.numata (Amazon)	0,010698	55
Urahuasha	Tunel	H.numata (Amazon)	0,0040785	22
Urahuasha	FG	H.numata (Amazon)	0,038642	2873
Urahuasha	Colombia	H.numata (Amazon)	0,017033	911
Urahuasha	Bresil	H.numata (Brazil) vs H.numata (Amazon)	0,50322	3872
Urahuasha	Ecuador	H.numata (Amazon)	0,014587	606
Urahuasha	Madre_de_Dios	H.numata (Amazon)	0,035378	962
Urahuasha	PuertoBermudez	H.numata (Amazon)	0,0063779	458
Urahuasha	La_Merced	H.numata (Amazon)	0,017939	516
Tunel	Michaela	H.numata (Amazon)	0,0090059	53
Michaela	FG	H.numata (Amazon)	0,037372	2842
Michaela	Colombia	H.numata (Amazon)	0,012697	857
Michaela	Bresil	H.numata (Brazil) vs H.numata (Amazon)	0,53097	3891
Michaela	Ecuador	H.numata (Amazon)	0,010968	558
Michaela	Madre_de_Dios	H.numata (Amazon)	0,035196	993
PuertoBermudez	Michaela	H.numata (Amazon)	0,0069017	506
Michaela	La_Merced	H.numata (Amazon)	0,01899	567
Tunel	FG	H.numata (Amazon)	0,040786	2868
Tunel	Colombia	H.numata (Amazon)	0,01503	910
Tunel	Bresil	H.numata (Brazil) vs H.numata (Amazon)	0,56533	3867
Tunel	Ecuador	H.numata (Amazon)	0,013215	607
Tunel	Madre_de_Dios	H.numata (Amazon)	0,03683	958
Tunel	PuertoBermudez	H.numata (Amazon)	0,0047838	457
Tunel	La_Merced	H.numata (Amazon)	0,017184	515
FG	Colombia	H.numata (Amazon)	0,038624	2563
FG	Bresil	H.numata (Brazil) vs H.numata (Amazon)	0,59106	3143
FG	Ecuador	H.numata (Amazon)	0,040995	2857
Madre_de_Dios	FG	H.numata (Amazon)	0,064761	2661
PuertoBermudez	FG	H.numata (Amazon)	0,046453	2940
La_Merced	FG	H.numata (Amazon)	0,050235	3019
Colombia	Bresil	H.numata (Brazil) vs H.numata (Amazon)	0,53668	4353
Ecuador	Colombia	H.numata (Amazon)	0,0058787	407
Madre_de_Dios	Colombia	H.numata (Amazon)	0,039283	1697
PuertoBermudez	Colombia	H.numata (Amazon)	0,012295	1343
La_Merced	Colombia	H.numata (Amazon)	0,024572	1414
Ecuador	Bresil	H.numata (Brazil) vs H.numata (Amazon)	0,53699	4339
Madre_de_Dios	Bresil	H.numata (Brazil) vs H.numata (Amazon)	0,57678	2942
PuertoBermudez	Bresil	H.numata (Brazil) vs H.numata (Amazon)	0,64886	3525
La_Merced	Bresil	H.numata (Brazil) vs H.numata (Amazon)	0,57264	3535
Madre_de_Dios	Ecuador	H.numata (Amazon)	0,038934	1521
PuertoBermudez	Ecuador	H.numata (Amazon)	0,012336	1064
La_Merced	Ecuador	H.numata (Amazon)	0,021665	1121

Table S4 (suite)

population1	population2	Species	F_{ST}	Distance
PuertoBermudez	Madre_de_Dios	H.numata (Amazon)	0,03854	585
Madre_de_Dios	La_Merced	H.numata (Amazon)	0,043319	603
PuertoBermudez	La_Merced	H.numata (Amazon)	0,015199	87
amaryllis	aglaope	H.melpomene	-4,84E-05	30
Ser	But	H.pardalinus	0,42796	30
zuleika	melicerta	H.hecale	0,031355	400
Peru	But	H.pardalinus	0,010075	400
Ser	Peru	H.pardalinus	0,42679	430
Clarescens	boulleti	H.ismenius	-0,015628	511
Peru	Ecuador	H.elevatus	0,012688	571
Peru	melicerta	H.hecale	0,29052	1776
melicerta	clearei	H.hecale	0,35583	1900
zuleika	Peru	H.hecale	0,31119	2100
Peru	clearei	H.hecale	0,39312	2230
zuleika	clearei	H.hecale	0,37628	2300
French_Guiana	Ecuador	H.elevatus	0,20174	2730
Peru	French_Guiana	H.elevatus	0,19544	2900
French_Guiana	amaryllis	H.melpomene	0,33315	2900
French_Guiana	aglaope	H.melpomene	0,30753	2930
Peru	Brazil	H.ethilla	0,43403	4150
Tunel	SE_Brazil	H.numata (SE Brazil) vs H.numata (Amazon)	0,48327	4107
Urahuasha	SE_Brazil	H.numata (SE Brazil) vs H.numata (Amazon)	0,43968	4112
SE_Brazil	Bresil	H.numata (Brazil) vs H.numata (SE Brazil)	0,012293	862
SE_Brazil	Colombia	H.numata (SE Brazil) vs H.numata (Amazon)	0,46257	4101
SE_Brazil	Ecuador	H.numata (SE Brazil) vs H.numata (Amazon)	0,46496	4307
SE_Brazil	FG	H.numata (SE Brazil) vs H.numata (Amazon)	0,51405	2074
SE_Brazil	La_Merced	H.numata (SE Brazil) vs H.numata (Amazon)	0,49424	3991
SE_Brazil	Madre_de_Dios	H.numata (SE Brazil) vs H.numata (Amazon)	0,49896	3397
SE_Brazil	Michaela	H.numata (SE Brazil) vs H.numata (Amazon)	0,45999	4103
SE_Brazil	Moyobamba	H.numata (SE Brazil) vs H.numata (Amazon)	0,49207	4185
SE_Brazil	PuertoBermudez	H.numata (SE Brazil) vs H.numata (Amazon)	0,5753	3946

Table S5 | Effective population sizes and divergence times in years as inferred by G-PhoCS.
Historical effective population size (Ne) and split time (T) are referred to using species initials; for instance, the population size of the ancestror of *H. pardalinus* and *H. elevatus* is referred to as Ne_PE. Mean, standard deviation and HPD interval (minimum and maximum) at 95% are presented. NumataA stands for *H. numata* from the Amazon and French Guiana, NumataB stands for *H. numata* from the Atlantic forests of Brazil.

Parameter	Mean	S.D.	HPDmin	HPDmax
Ne_Cydno	5311544	57966.764	5195578.9	5423236.8
Ne_NumataA	23089618	1089100.2	20680171	25441513
Ne_NumataB	1144291	15608.681	1114302.6	1175578.9
Ne_Pardalinus	15132304	972850.99	12661145	16301789
Ne_Elevatus	11833709	805558.7	10600079	13721697
Ne_Ismenius	1023584.4	11708.262	1000789.5	1046605.3
Ne_PE	6835145.4	498638.94	6224605.3	8051000
Ne_Numata	4963871.5	431830.73	4406328.9	5621407.9
Ne_IN	3492728.6	1567408.1	628144.74	6529223.7
Ne_PEIN	2441101.7	1818552.5	74986.842	5999723.7
Ne_CPEIN	3199022.4	39979.576	3121052.6	3278289.5
T_PE	1375611.6	84100.673	1171210.5	1452960.5
T_Numata	1429080.5	48163.774	1348973.7	1541921.1
T_IN	2412324.2	187762.79	1903421.1	2569671.1
T_PEIN	2543031.1	8980.2061	2525486.8	2560947.4
T_CPEIN	2543227.2	8977.8798	2525500	2560973.7

Table S6 | Migration bands analysed with G-PhoCS.
Mean values, standard deviation, minimum and maximum confidence interval at 95% showing the probability that the estimated total migration was greater than 0.

	Mean	S.D.	HPD.C.I..minimum	HPD.C.I..maximum	Probability
Numata -> Ismenius	0.80433	0.22333	0.49155	1.10236	1
Ismenius -> Numata	0.00017	0.00048	0	0.00095	0
Elevatus -> Pardalinus	1.77953	0.53181	0.65674	2.41723	1
Pardalinus -> Elevatus	0.0567	0.10494	0	0.32894	0.282
PE -> IN	0.00019	0.00057	0	0.00113	0
IN -> PE	0.00015	0.00047	0	0.00092	0
PEIN -> Cydno	0.00039	0.00161	0	0.00224	0
Cydno -> PEIN	8e-05	0.00036	0	0.00034	0
Numata -> PE	0.19306	0.04095	0.12593	0.25093	1
PE -> Numata	0.02067	0.02045	0	0.05907	0.342
Ismenius -> PE	0.00018	0.00044	0	0.00099	0
PE -> Ismenius	0.11323	0.02506	0.06787	0.16708	1
Pardalinus -> Ismenius	0.00033	0.00084	0	0.00189	0
Ismenius -> Pardalinus	0.00013	0.00025	0	0.00066	0
Elevatus -> Ismenius	0.00087	0.00202	0	0.00609	0
Ismenius -> Elevatus	9e-05	0.00016	0	0.00045	0

Table S7: Model choice from $\partial\mathbf{a}\partial\mathbf{i}$**.** Comparison between *H. numata* from peru and *H. numata* from Brazil and *H. numata* from Peru versus *H. pardalinus.* SI = Strict Isolation, IM = Isolation W. Migration, AM = Ancient Migration. G = suffix indicating Growth in the daughter populations. 2N = suffix indicating heterogeneity of effective population size. A = Suffix indicating growth in the Ancestral population.

H. numata Peru (Amazonian) – vs – *H. numata* Brasil (Mata Atlântica)		*H. Numata* Peru (Amazonian) – vs – *H. pardalinus*	
Model	**AIC**	**Model**	**AIC**
SI2NG	13878	SI2N	10297
SI2N	11405	SI2NG	9917
AM2N	9838	IM2N	6211
IM2N	9762	AM2N	6183
SC2N	8963	SC2N	5923
IM2NG	7587	SC2NG	4029
AMA2NG	6713	SCA2NG	4029
IMA2NG	6179	SIA2NG	3310
AM2NG	6089	IM2NG	3131
SC2NG	5542	AMA2NG	2932
SCA2NG	*2888*	*IMA2NG*	*2099*

Text S1 | Supplementary Methods

Analysis of the supergene occurrence Atlantic populations of Brazil of H. numata

Heliconius numata robigus and *H. n. ethra* are distributed along the South-Eastern coastal border of Brazil (Figure S2). They are both confined to the Atlantic forests of Brazil (*Mata Atlântica* biome) and are geographically isolated from the rest of the races of *H. numata*, which are predominantly distributed throughout the Amazon basin (grey area in Figure S2). Populations of *H. n. robigus* and *H. n. ethra* are not locally polymorphic (Brown and Mielke, 1972), something we confirmed by observing the collected specimens in two of the biggest Lepidoptera collections in Brazil, that of the Museo de Zoologia da Universidade de São Paulo (MZ/USP) and that of the Museo Nacional da Universidade Federal de Rio de Janeiro (MN/UFRJ). Instead, *H. n. robigus* and *H. n. ethra* share a quite large transition zone where any of these races or wing color intermediates between them can be found. Importantly, however, these races are distinguishable on the basis of rather few wing colour elements (Figure S2) when compared to the large phenotypic differences existing between polymorphic morphs of *H. numata*. We collected 11 and 13 specimens of *H. numata* forms *robigus* and *ethra* at Praia do Sono, Paraty, Rio de Janeiro State (see red circle in Figure S2) and at Reserva Serra Bonita, Camacã, Bahia State (see orange circle in Figure S2), respectively. Butterfly bodies were preserved in a NaCl-saturated EDTA-DMSO solution and wings were stored separately in glassine envelopes. We isolated gDNA from the preserved bodies using the DNeasy Blood and Tissue Kit (Qiagen). We genotyped gene *cortex* for the collected *H. n. robigus* and *H. n. ethra* specimens to determine whether or not the Brazilian populations harbour the ancestral *Hn0* gene order at the supergene *P*. *Cortex* controls whole-wing variation in black, yellow, white, and orange/red elements in *H. numata* color pattern (Nadeau *et al.* 2016). Allelic variants at gene *cortex* have been consistently associated with the different gene orders at the supergene *P* in Peruvian populations of this species (Joron et al., 2011). Primers that allow assessing this allelic variation are anchored on exons 2 and 3 of gene *cortex* (Table S1). Standard PCR master mixes and thermal cycling conditions were used. Thermal cycling conditions were 94°C for 3 min, 40 cycles of 94°C for 30 sec, 53°C annealing for 45 sec, 72°C for 90 sec, and a final extension at 72°C for 5 min. We scored amplicon length variation of gene *cortex* in agarose gels stained with BEt. Two specimens of *H. numata silvana* from an Amazonian population of Brazil were used as a control for the size of the *silvana*-like band (arrangement *Hn0*). Results (Table S2) show that *H. numata* populations from the Atlantic forest of Brazil are devoid of any rearrangements.

Analyses of the slope of Fst versus distance, as measured in kilometers

As stated in the main text, we tested for the existance and intensity of an isolation by distance (IBD) signal among species and between population of *H. numata* using a linear model. If IBD is stronger in species not polymorphic for the inversion we should observed significantly steeper slopes in these species. The slopes of F_{ST}/1-Fst versus log(distance) in km was calculated using the R package *lsmeans* (Lenth 2016); the slope difference among species or between populations within species was estimated with an ANOVA and its significance evaluated with function pairs of this package.

As shown in Fig. 2, *H. numata* has a much lower slope except for those Fst which involved the Brazilian samples. In order to quantify whether these slopes were significantly different, we performed the following analyses, using ANOVA and the package *lsmeans*. We analysed the slope of Fst/1-Fst vs. log10(distance) involving only *H. numata* and compared with the slope of Fst vs. distance within all other *Heliconius* (i.e., *H. elevatus, H. ethilla, H. ismenius, H. hecale, H. pardalinus* and *H. melpomene*) together, and tested whether these slopes were significantly different. Using the function lm of the package *lsmeans*, we analysed the interaction of distance and group (all other Heliconii or *H. numata* only) on Fst. The analysis of variance (ANOVA) showed that both distance and group have a significant effect on Fst, but not their interaction. Using the functions *lm* and pairs of *lsmeans*, we observed that the slope of Fst vs. distance for each group were significantly different (p=0.04) but with a greater slope for *H. numata*. We proceeded then to a similar analysis removing the Brazilian comparisons from *H. numata* set. In that case, the analysis of variance of Fst showed that distance, species and the interaction between distance and group had a significant effect. Using *lstrends*, the difference between the slope of Fst vs. distance for *H. numata* and other *Heliconius* was significant (p<0.0042).

Testing for an effect of the inversion on population differentiation

In order to test whether the difference in slopes in *H. numata* came from the absence of the inversion supergene in the Brazilian samples, we performed similar analyses to those above mentioned, with and without

chromosome 15, where the inversions are located. Firstly, we tested the significance of the slope of Fst/(1-Fst) vs. log(distance) using an analysis of variance of a linear model including as response variable distance, group (Amazonian or Atlantic) and their interaction. All these three components were significant. Using *lstrends* and the *pairs* function, the slope of Fst vs. distance for *H. numata* without the Atlantic locality was significantly different from the slope of Fst vs. distance for comparisons which included the Atlantic population (p<0.0001). Therefore the slope of Fst vs distance was significantly higher when including the Atlantic population.

We then performed the same analysis for Fst computed without chromosome 15. The analysis of variance resulted in significant effects on Fst from distance, group (Atlantic or Amazon) and the interaction of distance and group. The slope of Fst vs. distance for comparisons including only Amazonian populations and the slope including all comparisons were also significantly different (p<0.0001). Therefore the slope of Fst vs distance without chromosome 15 was significantly higher when including the Atlantic population. The result were the same with or without chromosome 15 and suggests that including or not the inversion in our computation did not have a major influence. Overall the slope of the signal of IBD was lower when comparing only Amazonian populations than when the Atlantic population was considered.