Supplementary Information for Martin *et al.*,

Diversification of complex butterfly wing patterns by repeated regulatory evolution of a *Wnt* ligand

SI Figures S1-5

SI Tables S1-5

SI Methods

SI References

Supplementary Figures

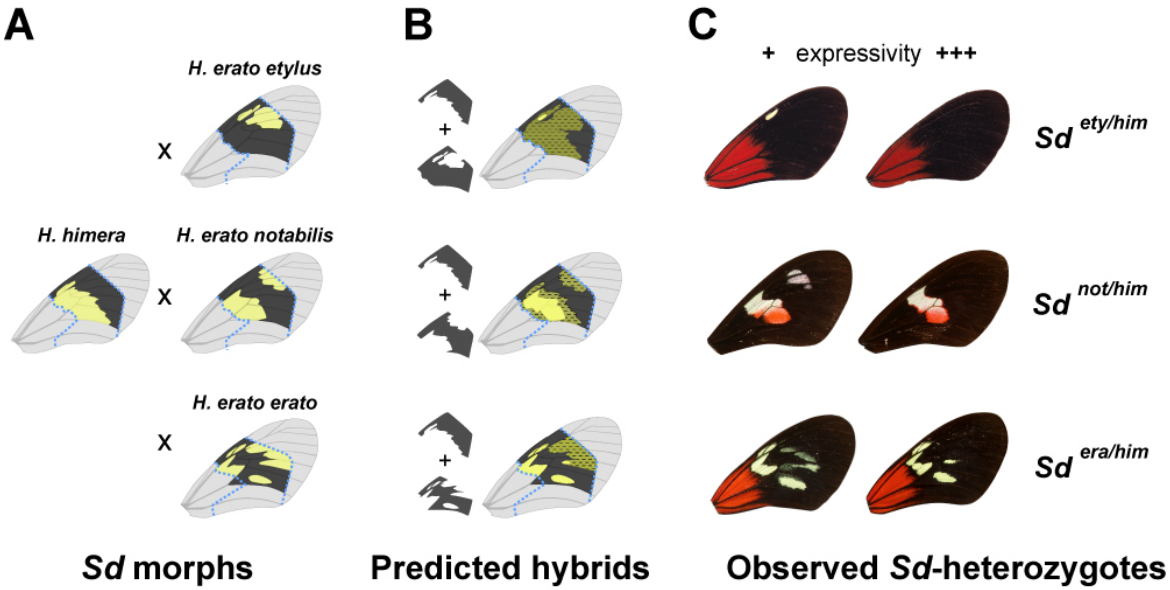

Fig. S1 Additive effects of *Sd* variation in central forewing black patterns. (*A*) *Heliconius erato* x *H. himera* crosses between various *Sd* phenotypes. Black patterns and the corresponding light-colored pattern shapes were drawn by superimposition of wing pictures. (*B*) Apposition of the parental black patterns (as drawn in *A*) results in the predicted heterozygous *Sd* phenotypes under the hypothesis of additive black pattern variation. Stippled areas denote zones of non-overlap between the parental black patterns, *i.e.* zones where black color is inherited from a single parent (hemizygous state). (*C*) Spectrum of phenotypic variation observed in *Sd*-heterozygous phenotypes validates the

model of additive central black pattern (in *B*). Presence-absence of the yellow spot along the anterior wing margin (the R-spot) in $Sd^{ety/him}$ may be due to modifier loci (1). Also, the *H. himera* black pattern shows variable expressivity in the *notabilis* and *erato* backgrounds, as seen by color varying from back-peppered color (+) to pure black (+++) in fields at the $Sd^{him/-}$ hemizygous state.

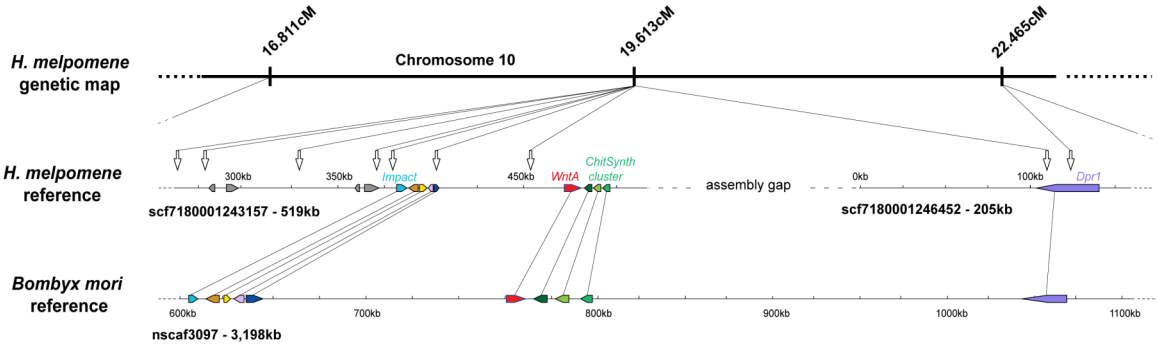

Fig. S2 Contiguity of the *WntA* and *Dpr1 Heliconius* genomic scaffolds and micro-synteny with the silkworm genome. The *Heliconius WntA* and *Dpr1* scaffolds are separated by an assembly gap but are directly contiguous on the H. melpomene genetic map, as their respective halves cover markers (white arrows) that co-localize at position 19.613cM of chromosome 10 (2). Colinearity of the *Impact-Dpr1* syntenic block with the silkworm genome assembly (3) provides independent evidence of contiguity between the two scaffolds, and suggests that *the ChitSynth – Dpr1* interval is devoid of genes. Genomic coordinates are provided in Table S4. Scaffolds *scf180001243157* and *scf7180001246452* can be accessed on Genbank (accession numbers HE668478 and HE669520) and via the Heliconius Genome Project Browser.

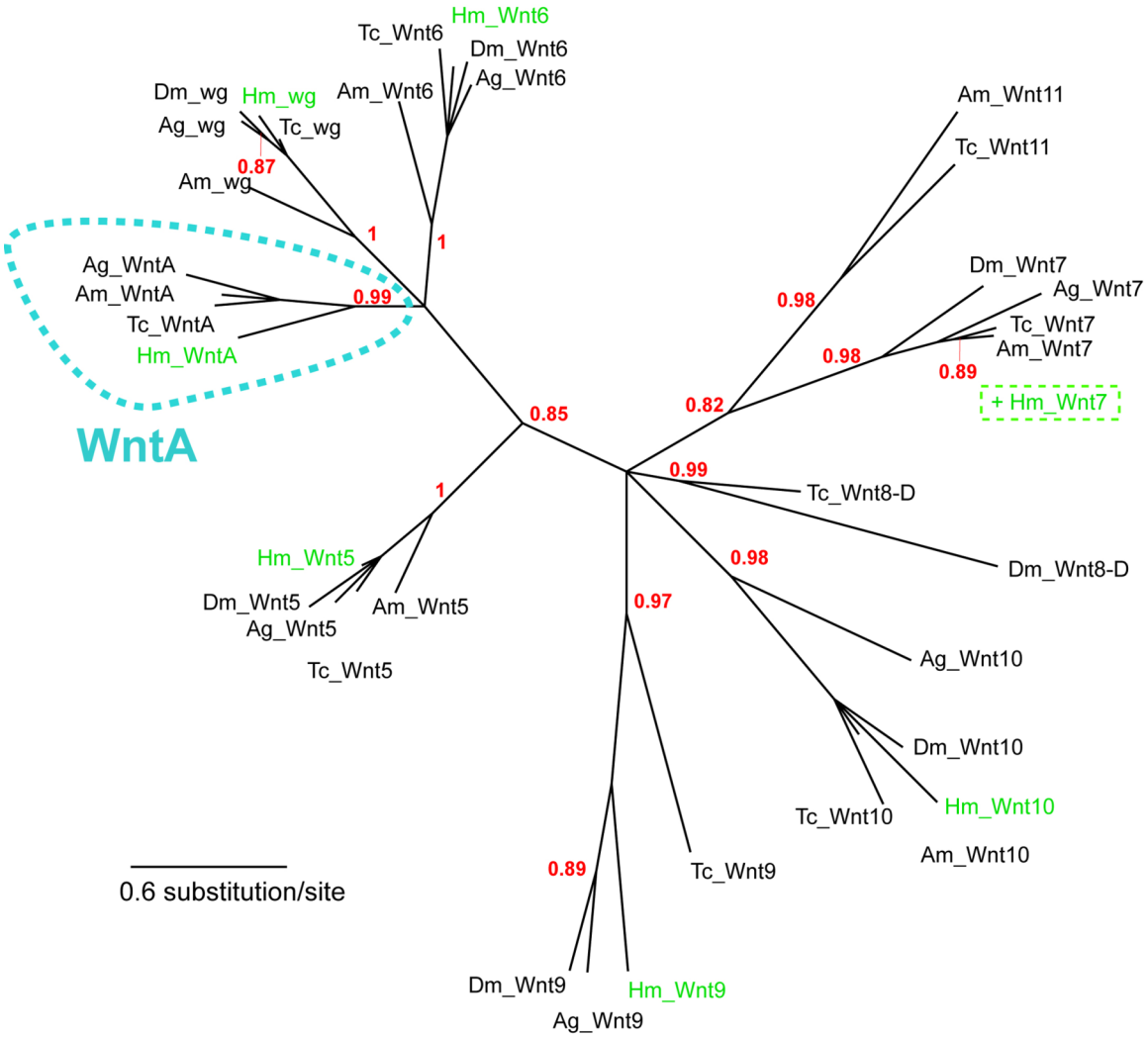

Fig. S3 Phylogenetic relationships of insect Wnt ligands and annotation of *H. melpomene* Wnt proteins. While a *Wnt7* orthologue is present in the *H. melpomene* genome, it was not included in the phylogenetic analysis because of its missing first exon (assembly gap). Note the loss of *WntA* in *Drosophila*. Bootstrap values superior to 0.80 are indicated at the corresponding nodes. Species and accession numbers are as follows. *Anopheles gambiae* : Ag_wg (XM_553580.3), Ag_Wnt5 (XM_319487.4), Ag_Wnt6 (XM_318816.4), Ag_Wnt7 (XM_001238161.2), Ag_Wnt9 (XM_318818.4), Ag_Wnt10 (XM_318815.4), Ag_WntA (XM_557821.3), *Apis mellifera* : Am_wg (XM_396946.3), Am_Wnt5 (XM_397473.2), Am_Wnt6 (XM_396945.3), Am_Wnt7 (XM_395388.4), Am_Wnt10 (XM_396944.3), Am_Wnt11 (XM_001121348.1), Am_WntA (XP_624751.3); *Drosophila melanogaster* : Dm_wg (NM_078778.3), Dm_Wnt5 (NM_057576.3), Dm_Wnt6 (NM_135264.2), Dm_Wnt7 (NM_057462.3), Dm_Wnt8-D

(NM_142015.2), Dm_Wnt9 (NM_057624.2), Dm_Wnt10 (NM_135265.2); *Heliconius melpomene* : Hm_wg (HMEL011440), Hm_Wnt5 (HMEL007382), Hm_Wnt6 (HMEL011436), Hm_Wnt7 (HMEL004238, partial), Hm_Wnt9 (HMEL011441), Hm_Wnt10 (HMEL011434), Hm_WntA (JN944582); *Tribolium castaneum* : Tc_wg (NM_001114350.1), Tc_Wnt5 (XM_969591.1), Tc_Wnt6 (XM_962962.2), Tc_Wnt7 (XM_968066.1), Tc_Wnt8-D (XM_966346.1), Tc_Wnt9 (XM_962805.1), Tc_Wnt10 (XM_963117.2), Tc_Wnt11 (XM_964168.1), Tc_WntA (EFA07513.1).

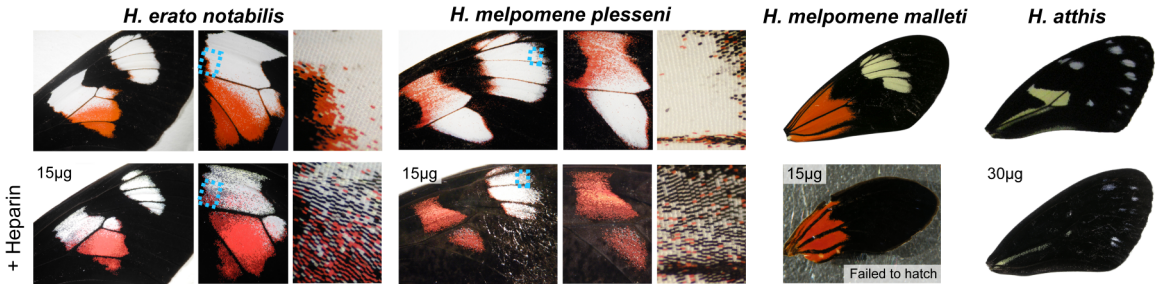

Fig. S4 Heparin injections result in black-pattern specific expansions (additional data). See Fig. 2C-D and Main Text for details.

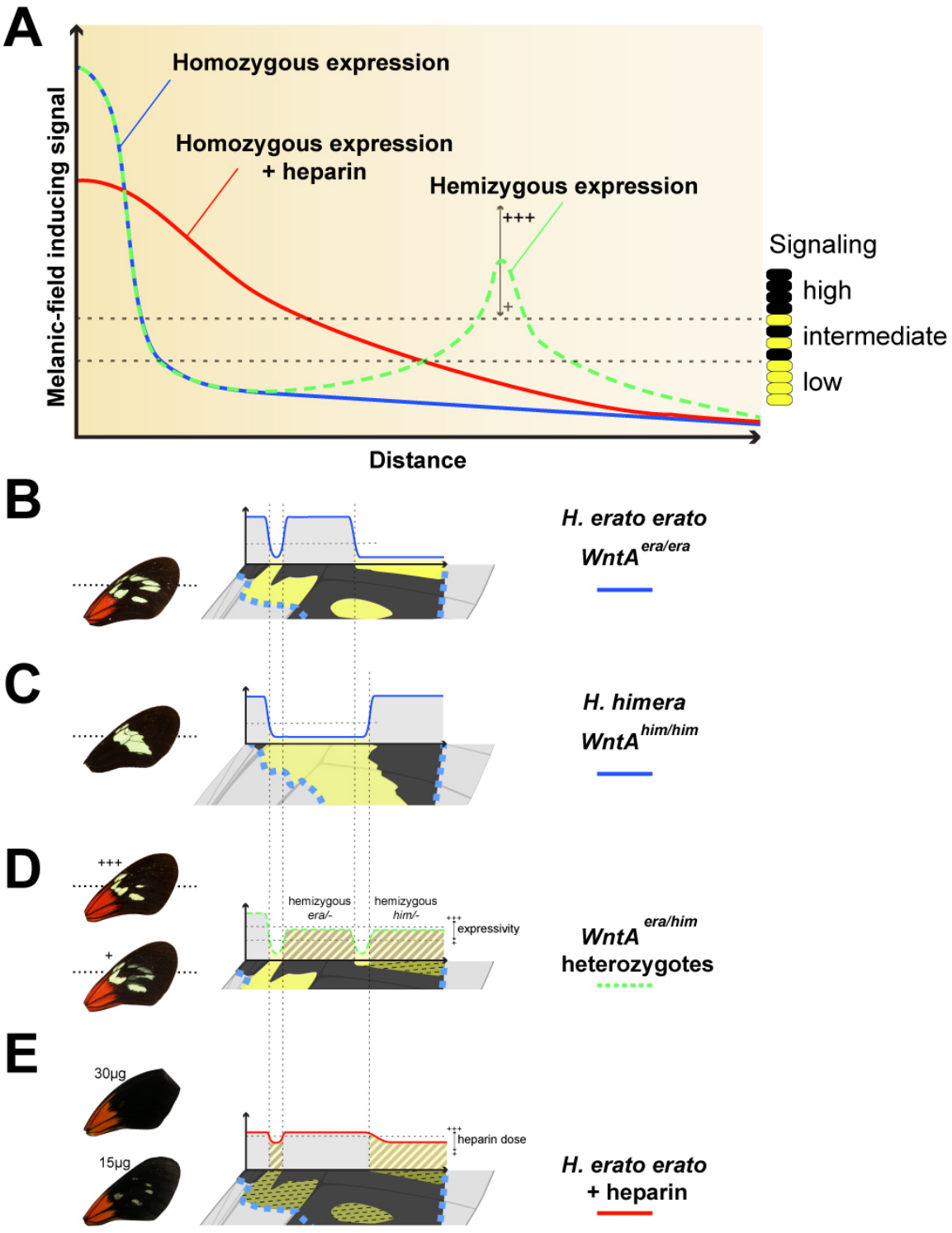

Fig. S5 A heparin-sensitive signal induces black patterns. (*A*) One-dimensional, schematic representation of the distribution gradient of a putative diffusible black pattern inducer or morphogen expressed as a focal source in a naturally-occurring butterfly (*blue*

line). *Green line*: hybrid (heterozygous) patterns are expected to result in local peaks of normal expression where the parental black patterns overlap, as well as in domains expressing hemizygous levels of the diffusible factor where black patterns are parent-specific. *Red line*: heparin injections are expected to increase ligand diffusivity and stability (see Main Text), therefore spreading the distribution gradient. Both hybrid crosses and heparin injections may result in increased signaling at the wing-wide level and reveal an intermediate signaling state by interfering with the spatial dynamics of the gradient. (*B*-*E*) Extension of the working model to one-dimension wing transects. (*B*) *H. erato erato* wild-type forewing; (*C*) *H. himera* wild-type forewing*;* (**d**) $WntA^{era/him}$ individuals generated from a F2 cross. All the genotyped $WntA^{era/him}$ individuals show zones of variable melanic pattern expressivity (peppered yellow to pure black ; Fig. S1) that correspond to pattern positions that are only present in *H. himera* (stippled yellow); (*E*) Heparin-injected *H. erato erato* with dose-dependent melanization of the yellow patterns (peppered yellow to pure black). *WntA*-heterozygosity and heparin dose-dependent effects can be explained by a spread of the melanic field inducer, and are consistent with a role of *WntA* in black pattern induction.

Supplementary Tables

Table S1 | Candidate *H. melpomene* genome scaffolds after RAD mapping

***H. melpomene* scaffold[a] - GenBank accession number**	***H.erato* SNP position(s) on scaffold**	**Piled-up *H.erato* SNP count (coverage)**	**[*Sd* $^{him/him}$]**		**[*Sd* $^{not/not}$]**	
			allele 1	**allele 2**	**allele 1**	**allele 2**
scf7180001243157[b] (*WntA*) - HE668478	11440	70	39	0	0	31
	46372	97	0	25	72	0
	208013-208019	53	0	22-23	30-31	0
	383321	55	0	23	32	0
	487893	82	45	0	0	37
scf7180001249804 - HE670851	296944	61	44	0	0	17
	531087-531118	62	43-45	0	0	19
scf7180001250214 - HE671162	45200-45209	55-56	29-30	0	0	26
	57584	59	35	0	0	24
scf7180001246279 - HE669460	236440	59	35	0	0	24
scf7180001249002 - HE670311	145575-145598	204-205	145-146	0	0	59
scf7180001249397 - HE670566	48427	78	53	0	0	25
scf7180001249723 - HE670792	146780	56	34	0	0	22
scf7180001249836 - HE670875	60158	196	108	0	0	88
scf7180001250713 - HE671542	223441	110	82	0	0	28

[a] SNPs with a perfect association to phenotype and coverage >50X (>1X per individual) are shown.

Nine scaffolds show SNPs with this level of stringency.

[b] The *WntA* scaffold shows the highest number of independently associated positions

and was retained for further fine-mapping.

Table S2 | *Sd* and *Ac* fine-mapping markers

***Sd*-linked markers in the *H. himera* x *H. erato* F2 crosses**

Locus	Primer orientation on transcript	Primer sequence (5'>3')	Position[a] on *H. melpomene scf7180001243157*	Genotyping method
Cpg24	forward	TCAAGGTCCACGTTGACAAG	296935 (+)	Sequencing with forward primer
	reverse	GGGTAGGGGACCTCTTTGAC	297454 (-)	
JHEBP-Mat1	forward	CAGTCTTGACAAAGTCGCACA	402431 (-)	Amplicon lenght polymorphism
	reverse	AGGTGGAGGTCTCCAGTTT	401029 (+)	
	forward	CGGGGACGTTTTAGACAGC	403101 (+)	Sequencing with forward primer
	reverse	TGCAAAATCCTCCTCCTTTTT	403621 (-)	
intergenic428kb	NA[b]	TGATCTAATTAGTATGGTTCGTTGC	427670 (+)	Sequencing with forward primer
	NA[b]	TCATCTGTTTTAAGTGTAATCTATCG	428043 (-)	
WntA	forward	TCAGCACAGCGAAGAGTATG	450192(+)	Sequencing with forward primer
	reverse	TTGTCATTTGAACGACTAATGTG	450788 (-)	
	forward	CGTAAACAACGCGAAGACAA	473545 (+)	Sequencing with forward primer
	reverse	GAACTGGCCGTACTGGTGTT	474310 (-)	
	forward	CAGGCGGTACGTGGTCGT	481158 (+)	Sequencing with forward primer
	reverse	CGGCTCACAATAGTCCGGG	482177 (-)	
Chitsynth1	forward	TGAGCGAATTCAACGATCAG	488191 (-)	Sequencing with forward primer
	reverse	GTCGTCATCAGGTGGGCTAT	487830 (+)	
Chitsynth3	forward	TGAGTTGGCCTACAACTTCG	497150 (-)	Sequencing with forward primer
	reverse	GCTCTGCTAGCTGCTGGACT	496604 (+)	
Dpr1	forward	AGATCTTCACATATTAACAGCCG	NA	Sequencing with reverse primer
	reverse	CCAACTGCCAACTGAAAATG	NA	

***Ac*-linked markers in the *H. melpomene malleti* x *H. melpomene melpomene* F2 cross**

Locus	Primer orientation on transcript	Primer sequence (5'>3')	Position[a] on *H. melpomene scf7180001243157*	Genotyping method (*enzyme*)	
Cpg24	forward	TCAAGGTCCACGTTGACAAG	296935 (+)	Sequencing with forward primer	
	reverse	GGGTAGGGGACCTCTTTGAC	297454 (-)		
Mat1	forward	TGTCCAGAATGTAATGTACCACTGCGA	403487 (+)	Restriction fragment length (*Sau3AI*)	
	reverse	TCCTTGGCATCCCCTTCAGCGA	404259 (-)		
Chitsynth3	forward	CCTTCGACGATGGTGTAAACGTGCAAA	497654 (-)	Restriction fragment length (*BstBI*)	***<<< WntA***
	reverse	AGGCACTGGGGCAAGGTGGC	497048 (+)		

***Ac*-linked markers in the *H. cydno galanthus* x *H. pachinus* F2 cross and *H. cydno alithea* male-informative backcrosses**

Locus	Primer orientation on transcript	Primer sequence (5'>3')	Position[a] on *H. melpomene scf7180001243157*	Genotyping method
Cpg24	forward	TCAAGGTCCACGTTGACAAG	296935 (+)	Sequencing with forward primer
	reverse	GGGTAGGGGACCTCTTTGAC	297454 (-)	
WntA	forward	CAGGCGGTACGTGGTCGT	481158 (+)	Sequencing with forward primer
	reverse	CGGCTCACAATAGTCCGGG	482177 (-)	
Chitsynth3	forward	TGAGTTGGCCTACAACTTCG	497150 (-)	Sequencing with forward primer
	reverse	GCTCTGCTAGCTGCTGGACT	496604 (+)	
Dpr1	forward	AGATCTTCACATATTAACAGCCG	NA	Sequencing with reverse primer
	reverse	CCAACTGCCAACTGAAAATG	NA	

[a] 5'-ends of primers are positioned on the *H. melpomene* reference scaffold scf7180001243157 for comparison between species

NA: not applicable

Table S3 | *Sd* and *Ac* fine-mapping results

***Sd* fine-mapping in the *H. himera* x *H. erato* F2 crosses**

Locus	Position[a] on *H. melpomene scf7180001243157*	**F2 recombinants** (informative individuals) **in *H. himera* x**			**Total - without inferred genotypes**	**Total - with inferred genotypes**
		H. erato etylus	***H. erato notabilis***	***H. erato erato***		
Cpg24	296935 (+) 297454 (-)	**2** (257)	**0** (91)	NT[c]	**2** (348)	**2** (348)
JHEBP-Mat1	402431 (-) 401029 (+) 403101 (+) 403621 (-)	**2** (257) non-informative	0[b] **0** (91)	NT[c] NT[c]	**2** (257) **0** (91)	**2** (348)
intergenic428kb	427670 (+) 428043 (-)	**1** (257)	**0** (91)	NT[c]	**1** (348)	**1** (348)
WntA	450192(+) 450188 (+) 473545 (+) 474310 (-) 481158 (+) 482177 (-)	**0** (257) **0** (257) non-informative	0[b] 0[b] **0** (91)	NT[c] NT[c] **0** (107)	**0 (458)**	**0 (458)**
Chitsynth1	488191 (-) 487830 (+)	**0** (257)	**0** (91)	NT[c]	**0** (348)	**0** (348)
Chitsynth3	497150 (-) 496604 (+)	**0** (257)	**1** (91)	NT[c]	**1** (348)	**1** (348)
Dpr1	NA NA	**7** (311)	NT[c]	NT[c]	**7** (311)	**7** (311)

***Ac* fine-mapping in the *H. melpomene malleti* x *H. melpomene melpomene* F2 cross**

Locus	Position[a] on *H. melpomene scf7180001243157*	**F2 recombinants** (informative individuals)	
Cpg24	296935 (+) 297454 (-)	**2** (51)	
Mat1	403487 (+) 404259 (-)	**0** (51)	
Chitsynth3	497654 (-) 497048 (+)	**0** (51)	**<<< *WntA***

***Ac* fine-mapping in the *H. cydno galanthus* x *H. pachinus* F2 cross**
and x *H. cydno alithea* male-informative backcrosses

Locus	Position[a] on *H. melpomene scf7180001243157*	**Recombinants** (informative individuals) **in *H. c. galanthus* x**		
		H. pachinus	***H. c. alithea***	**Total**
Cpg24	296935 (+) 297454 (-)	**0** (65)	**0** (64)	**0** (129)
WntA	481158 (+) 482177 (-)	**0** (65)	**0** (64)	**0** (129)
Chitsynth3	497150 (-) 496604 (+)	**0** (65)	**0** (64)	**0** (129)
Dpr1	NA NA	**1** (28)	**1** (55)	**2** (83)

[a] 5'-ends of primers are positioned on the *H. melpomene* reference scaffold scf7180001243157 for comparison between species

[b] Absence of recombination between two perfectly associated markers was inferred for three markers in the *himera* x *notabilis* brood

[c] NT : not tested

Table S4 | Gene annotations in the *Ac* and *Sd* intervals

Genetic interval	Gene ID	Range[a] (bp)	Strand[a]	*Bombyx* orthologue ; Range (strand) on *nscaf3097* (SilkDB)	NCBI BLASTP hit - accession [*species*]	E-value	Annotated molecular function
-	***Cpg24***	292749-297843	+	(BGIBMGA13899 ; *nscaf3099*)	Cuticular protein glycin-rich CPG24 - BAG30722 [*Papilio xuthus*]	5E-170	Unknown
[*Ac*]	***Conserved transcript FY037235***	362202-362832	-	no hit	no hit	-	Unknown
[*Ac*]	***Poxn***	365755-380944	+	(unannotated ; *nscaf3099*)	Pox neuro - NP_476686 [*Drosophila melanogaster*]	5E-69	Paired-box family transcription factor - various developmental processes
[*Ac*]	***Impact***	385746-390167	+	BGIBMGA013784 ; 605260-609383 (+)	Imprinted and ancient (Impact) protein - BAE23558 [*Mus musculus*]	7E-77	Unknown - discovered as a conserved imprinted locus in mammals
[*Ac*]	***Mus312***	391110-398225	-	BGIBMGA013759 ; 614135-620461 (-)	Mutagen-sensitive 312 - NP_648104 [*Drosophila melanogaster*]	2E-24	DNA repair and recombination
[*Ac*]	***Maltp1***	396876-400472	+	unannotated ; 621003-623852 (+)	Mucosa-associated lymphoid tissue lymphoma translocation protein 1 (Maltp1) NP_776216 [*Homo sapiens*]	3E-18	Caspase-like - regulation of proteolysis and apoptosis
[*Ac*]	***JHEBP***	400264-402628	-	unannotated ; 625729-628748 (-)	Juvenile hormone esterase binding protein - AAD38067 [*Manduca sexta*]	5E-141	Regulation of juvenile hormone activity
[*Ac*]	***Mat1***	4003080-405490	+	BGIBMGA13786 ; 635189-643724 (+)	Menage-a-trois 1 protein (Mat1) - NP_610605 [*Drosophila melanogaster*]	1E-85	Basal transcription factor - RNA pol II-dependent transcription initiation
[*Ac, Sd*]	***WntA***	450400-483854	+	BGIBMGA013787 ; 771657-781653 (+)	(Fig. S3)	-	Secreted ligand of the Wnt-family - morphogenesis
[*Ac, Sd*]	***ChitSynth1***	486270-489436	-	BGIBMGA013758 ; 789788-792617 (-)	Chitin deacetylase 5a - ADB43611 [*Helicoverpa armigera*]	4E-154	Polysaccharide deacetylase - chitin biosynthesis
[*Ac, Sd*]	***ChitSynth2***	490610-494251	-	BGIBMGA013757 ; 797560-804535 (-)	Chitin deacetylase 5a - ADB43611 [*Helicoverpa armigera*]	4E-157	Polysaccharide deacetylase - chitin biosynthesis
[*Ac*]	***ChitSynth3***	495897-498544	-	BGIBMGA013756 ; 810851-816660 (-)	Chitin deacetylase 5a - ADB43611 [*Helicoverpa armigera*]	0	Polysaccharide deacetylase - chitin biosynthesis
-	***Dpr1***	NA	-	BGIBMGA013755 ; 1042967-1066089 (-)	Dpr1 - NP_995908 [*Drosophila melanogaster*]	1E-63	Sensory perception of salty taste

[a] relative to the *H. melpomene scf7180001243157* reference scaffold

Table S5 | Pairwise comparisons of *Heliconius WntA* coding sequences

dN/dS[a]	*H. m. melpomene* [*ac/ac*]	*H. m. malleti* [*Ac/Ac*]	*H. c. galanthus* [*ac/ac*]	*H. c. alithea* [*Ac/Ac*]	*H. e. petiverana*	*H. e. lativitta*	*H. e. notabilis*	*H. himera*
***H. m. melpomene* [*ac/ac*]**	0							
***H. m. malleti* [*Ac/Ac*]**	0/14	0						
***H. c. galanthus* [*ac/ac*]**	0/8	0/18	0					
***H. c. alithea* [*Ac/Ac*]**	0/10	0/10	0/18	0				
H. e. petiverana	6/45	6/45	6/42	6/46	0			
H. e. lativitta	4/39	4/40	4/39	4/40	2/12[b]	0		
H. e. notabilis	4/41	4/42	4/41	4/42	2/14[b]	0/2	0	
H. himera	8/40	8/40	8/40	8/41	2/11	4/7	4/5	0

[a] The matrix features pairwise dN/dS ratios between *WntA* full-length coding sequences (885bp) of various species and morphs

[b] Both non-synonymous substitutions affect codon 233, resulting in a single Arg (*H. e. petiverana*) - Leu (*H. e. lativitta/notabilis*) amino-acid substitution

yellow : no amino-acid substitutions were observed between *H. melpomene* and *H. cydno* morphs of different *Ac* phenotypes

red : no amino-acid substitutions were observed between the parapatric *H. e. lativitta* and *H. e. notabilis* morphs (Eastern Ecuador) of different *Sd* phenotypes

SI Methods

RAD mapping

Preparation of a RAD sequencing library followed a previous protocol (4) with the following adaptations. Genomic DNA from 25 $Sd^{not/not}$ and 25 $Sd^{him/him}$ F2 individuals from a single *H. himera* x *H. erato notabilis* family were combined equimolarly (50ng/individual) into two separate $Sd^{not/not}$ and $Sd^{him/him}$ DNA pools. Each pool was digested with the restriction enzyme *NcoI*, heat inactivated, and directly ligated to custom barcoded, Illumina Paired-End sequencing forward adapters ($Sd^{him/him}$ PE1 :5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT[CAGTA]G-3; 3'-TTACTATGCCGCTGGTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA[GTCAT]CGTACp-5'; $Sd^{not/not}$ PE1 : 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT[CGTAG]G-3'; 3'-TTACTATGCCGCTGGTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA[GCATC]CGTACp-5' ; brackets denote five-nucleotide barcodes). The two barcoded samples were then mixed together, sheared to an average size of 400bp using a Covaris S220 shearer, size selected to 300-600bp by agarose gel extraction, and blunt ligated to a custom reverse adapter (PE2 : 5'-GTTCGTCTTCTGCCGTATGCTCTAGCCAGAGCCGTAAGGACGACTTGGCGAGAAGGCTAG*A-3' ; 3'-ATCGTCACTCGGCATTCCTGCTGAACCGCTCTTCCGATCp-5' ; asterisk denotes a phoshorothiate bond modification). The resulting library was amplified by Touchdown PCR (18 cycles with denaturation at 98ºC and extension at 72ºC ; annealing step : 5 cycles at 65ºC -1ºC/cycle and 13 cycles at 60ºC) using the high-fidelity Phusion Taq polymerase and custom amplification primers (PE1 : 5'-AATGATACGGCGACCACCG*A-3' ; PE2 : 5'-CAAGCAGAAGACGGCATACGA*G-3' ; asterisks denote phoshorothiate bond modifications), gel extracted between 300-700bp, and sequenced on a single lane of an Illumina Genome Analyser GAII generating 72bp Paired-End reads. Custom Perl scripts

were used to separate the resulting forward and reverse *.fastq* files into new *.fastq* files separated by barcode/pool. The resulting files were aligned to the reference assembly of the *H. melpomene* genome using MosaikAligner (version 1.0.1388) with the following relevant switches: *-hs 12 -mm 15 -a all -mhp 100 -act 30 -p 6 -bw 29 -m all*. We allowed for a large number of mismatches, as we were aligning to a reference genome from a related species, with an estimated divergence of time of 13-26 MY between the *erato/himera* and *melpomene* clades (5). We used the MosaikText command to generate an *.axt* file, followed by a custom Perl script to parse that file and generate a SNP table. To be considered valid a candidate SNP had to have a minimum pileup depth of 30, a minimum minor allele count of 5, and a minimum minor allele frequency of 5% , averaged over the two pools (6). Running the pipeline resulted in a SNP table containing 46,236 SNPs, each mapped to a *H. melpomene* scaffold, with minor and major allele counts in the two pools and a median coverage of 86X. The 2-plex barcoding design resulted in sequence bias, as we found a 3:2 ratio of $Sd^{him/him}$: $Sd^{not/not}$ sequences. We identified SNPs closely linked to the wing-patterning element as those with a minimum coverage of 50X that were differentially fixed in the two pools. After this stringent treatment, only the *WntA* scaffold (*scf7180001243157*) showed more than two associated SNPs, including three SNP positions that escaped from sequence bias ($Sd^{him/him} < Sd^{not/not}$; Table S1).

Cloning

Full-length *WntA* coding sequences from *H. erato, H. himera, H. cydno* and *H. melpomene* morphs were amplified from wing cDNA first-strands using semi-nested PCR (first round Forward primer 5'-GCCAGTTCAAACGTCAAACA-3'; second round Forward primer 5'-CCTCGTTGAGAGACAGAAACA-3'; Reverse primer 5'-CTAGTTGCAGACGTGGTGGT-3') and directly sequenced on both strands. MEGA 3.0 was used for calculating pairwise dN/dS ratios(7). A 565bp fragment of the *WntA* coding region was amplified from *H. erato* wing cDNA first strands by PCR (Forward primer 5'-GGCGGTTGAAGAGTGTCAAT-3'; Reverse primer 5'-GTTGCAGACGTGGTGGTC-3'). A 565bp *H. erato erato* amplicon and a 885bp *H. cydno alithea* amplicon were then cloned in the pCRII vector (Invitrogen Corporation, Carlsbad, California, United States),

and used for antisense riboprobe synthesis with the DIG RNA labeling kit (Roche Applied Science, Indianapolis, Indiana, United States).

In Situ Hybridizations

Fifth-instar larvae were anaesthetized in ice cold water and their wing disks dissected in Phosphate Buffer Solution (PBS), incubated in cold fixative (formaldehyde 9% in PBS containing 50 mM ethylene glycol tetraacetic acid) for 30-35 min, rinsed in PBS with 0.01% Tween20 (PBST), dehydrated with increasing concentrations of methanol and kept for long-term storage in methanol at -20°C. This sample preparation technique allowed transport from the place of tissue collection (Panama) to the molecular laboratory (USA), but is unsuitable for pupal wings during the 50hr that follow pupation, due to the fragility of the tissues. For *in situ* hybridization, wing disks preserved in methanol were rehydrated in increasing concentrations of cold PBST, washed in cold PBST, incubated 5 min with 25 µg/mL proteinase K in cold PBST, washed in cold PBST containing 2mg/mL glycine, washed in cold PBST and freed from their peripodial membrane using fine forceps. The tissues were then post-fixed 20min on ice in PBS containing 5.5% formaldehyde, washed in cold PBST, gradually transferred to a standard hybridization buffer (5X saline sodium citrate pH 4.5, 50% formamide, 0.01% Tween20, 100µg/mL denatured salmon sperm DNA, final pH 5-6 at 22°C), placed at 62°-65°C for an hour, incubated in hybridization buffer supplemented with 1 g/L glycine and 30-50 ng/mL riboprobe for 16-40h hr at 62°C, washed eight times 15-30 min in hybridization buffer, returned to room temperature, and gradually stepped back into, and washed in, PBST. For secondary detection of the riboprobe, the tissues were blocked for 30 min in Tris buffer saline, 0.01% Tween20 (TBST, pH=7.5) supplemented with 1 g/L bovine serum albumin, incubated with a 1:4000 dilution of anti-digoxigenin alkaline phosphatase F_{ab} fragments (Roche Applied Science, Indianapolis, Indiana, United States), washed ten times (10-120 min per wash) in cold TBST, incubated in an alkaline phosphatase buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.01% Tween20), and finally stained with BM Purple (Roche Applied Science) for 4-8hr at room temperature. Stained tissues were then washed in PBST 2 mM ethylene diamine tetraacetic acid and slide mounted in PBS

containing 60% glycerol. mRNA *in situ* hybridizations were photographed with a Nikon Coolpix P5100 digital camera (Nikon Inc. USA, Melville, New York, United States) mounted with a LNS- 30D/P51 adapter (Zarf Enterprises, Spokane, Washington, United States) on a Leica S4E microscope (Leica Microsystems, Buffalo Grove, Illinois, United States). All the presented results were replicated in at least three individuals per morph at informative stages.

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