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

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Although animals display a rich variety of shapes and patterns, the genetic changes that explain how complex forms arise are still unclear. Here we take advantage of the extensive diversity of *Heliconius* butterflies to identify a gene that causes adaptive variation of black wing patterns within and between species. Linkage mapping in two species groups, gene-expression analysis in seven species, and pharmacological treatments all indicate that *cis*-regulatory evolution of the *WntA* ligand underpins discrete changes in color pattern features across the *Heliconius* genus. These results illustrate how the direct modulation of morphogen sources can generate a wide array of unique morphologies, thus providing a link between natural genetic variation, pattern formation, and adaptation.

Müllerian mimicry | Wnt pathway | Mendelian genetics | evolutionary-developmental biology

Cell fate induction by signaling molecules is a central characteristic of developmental pattern formation (1, 2), thus the evolution of genes encoding signaling molecules is expected to drive pattern evolution and contribute to morphological diversity (3, 4). An empirical test of this hypothesis relies on linking molecular and phenotypic evolution using forward genetic approaches (5–7), and indeed a handful of signaling gene variants underlying morphological differences in natural populations have been identified (8–14). Here we show that genetic variation linked to a Wnt-family signaling molecule drives the diversification of complex wing patterns in *Heliconius erato* and *Heliconius melpomene* mimetic butterflies (15). These species are unpalatable and each has undergone parallel adaptive radiations, such that the geographic range of each species is composed of a nearly identical patchwork of distinct geographic races (16, 17). Local populations of coexisting butterflies share common wing patterns that provide mutualistic protection (Müllerian mimicry) from avian predators (18, 19). With their extensive within-species variation (16, 20) and numerous cases of mimetic convergence between species, *Heliconius* wing patterns form an ideal model to study the repeatability of complex trait evolution (7, 15, 21–24).

Results

Wing Pattern Shape Variation Repeatedly Maps to *WntA*. The great diversity in forewing band shape across the *H. erato* wing color pattern radiation is controlled by a single locus of large effect called *Short band* (*Sd*) (15, 22, 25). Pattern variation occurs through changes in the size and position of black pattern elements, which in turn define the shape of yellow and white areas of the forewing (Fig. 1*A* and *SI Appendix*, Fig. S1). We positionally cloned this locus using a combination of restriction-associated amplified DNA (RAD) markers (26) and traditional linkage mapping. Our RAD screen of a *H. erato notabilis* × *Heliconius himera* F2 brood pinpointed a single scaffold from the *H. melpomene* genome (27) that showed an enrichment of *Sd*-associated SNPs (*SI Appendix*, Table S1). Further fine-scale linkage mapping identified a 69-kb zero-recombinant interval centered on the gene encoding the signaling ligand WntA (Fig. 1*B* and *SI Appendix*, Tables S2–S4). Indeed, there was no recombination between black pattern phenotypes and *WntA* across 458 F2 offspring from crosses between *H. himera* and three different morphs of *H. erato*. Thus, natural *Sd* allelic variation controls at least four distinct pattern shape variants in *H. erato* (as shown in Fig. 1*A*). Furthermore, we found perfect linkage with a 1.1-cM resolution [~200 kb (28)] between *WntA* and the *Anterior cell spot* (*Ac*) locus that controls variation of forewing pattern shape in the *H. melpomene*/*Heliconius cydno* clade (15, 22, 29). Pattern variation thus has a monogenic basis in two independent radiations, re-emphasizing that repeated evolution of butterfly color pattern mimicry is driven by a relatively small toolkit of large-effect loci (15, 21, 22, 24).

***WntA* Expression Is Variable and Marks Presumptive Black Patterns.** WntA is a member of the Wnt-family of signaling ligands (*SI Appendix*, Fig. S3), of which Wingless has previously been identified as a morphogen involved in *Drosophila* and lepidopteran wing pattern formation (30, 31). Genetic support for *WntA* as the source of adaptive variation in *Heliconius* wing patterns was corroborated by expression studies. *WntA* mRNA expression prefigured black patterns in a total of seven *Heliconius* species examined (Fig. 2*A*). For example, *WntA* distribution varied between the *Sd*-informative *H. erato* and *H. himera* morphs, correlating with the position of black color in the central portion of the wing; *WntA* expression also showed convergence between the comimics *H. erato notabilis* and *H. melpomene plesseni* by prefiguring the black stripe dividing their forewing color patches. It is noteworthy that this median split is also visible in the

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Data deposition: The RAD-mapping raw reads were deposited in the Short Read Archive (accession no. SRA045716.2); cDNA sequences were deposited in the GenBank database (accession nos. JN944582–JN944589); and genomic sequences can be accessed on the GenBank database (accession nos. HE668478 and HE669520) and on the Heliconius Genome Database, www.butterflygenome.org (scf180001243157 and scf7180001246452).

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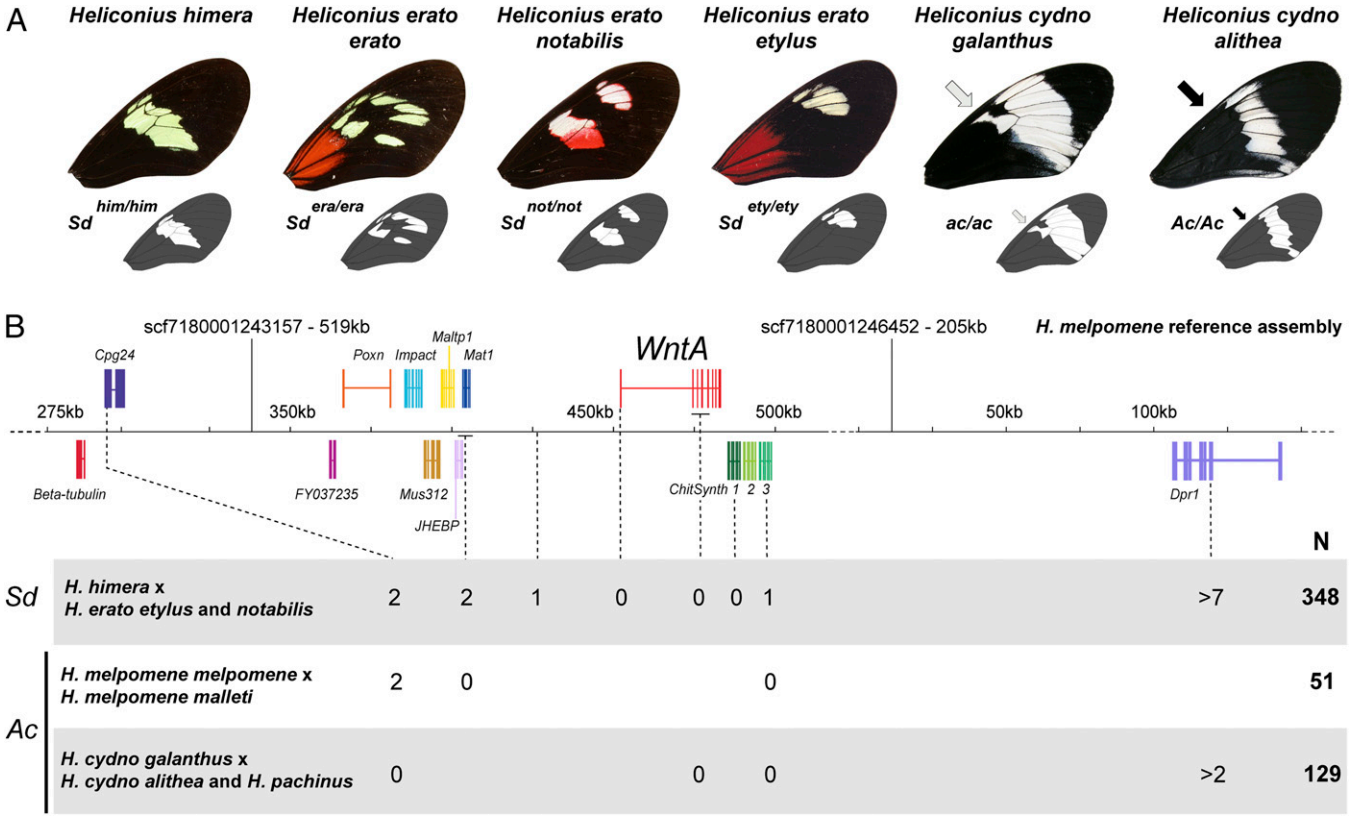

Fig. 1. Linkage mapping of forewing pattern shape variation to *WntA*. (*A*) Examples of forewing pattern shapes found in *Heliconius* shown with their associated *Sd* and *Ac* genotypes. Mapped *Ac* polymorphisms in the *H. melpomene*/*H. cydno* clade consist of presence or absence of a proximal melanic patch (arrows). (*B*) Linkage-mapping of *Sd* and *Ac*. Crossing-over events between genetic variation and individual phenotypes out of *N* individuals are featured at each marker. The depicted region spans the halves of two scaffolds separated by an assembly gap; however, the reference *H. melpomene* genetic map (27) and the colinearity of the *Impact-Dpr1* syntenic block with the silkworm genome assembly (46) provide independent evidence of contiguity between these two scaffolds (*SI Appendix*, Fig. S2).

complementary pattern of *optix* expression that marks the *H. melpomene plesseni* red/white patterns at later stages (24) (Fig. 2*B*). The spatial association of *WntA* expression with intraspecific color pattern variation, combined with the genetic mapping data, strongly argues that *WntA cis*-regulatory evolution is driving wing pattern variation (6). Consistent with this conclusion, we observed no coding variation within the *H. melpomene*/*H. cydno* clade or between parapatric races of *H. erato* with divergent forewing band shapes (*SI Appendix*, Table S5).

Heparin-Sensitive Signal Determines Pattern Boundaries. Our results imply that *WntA* controls pattern formation and variation, suggesting that modulating WntA protein distribution should result in pattern alterations. To test this prediction, we injected heparin into early pupae of a variety of *Heliconius* morphs. Heparin is an analog of heparan sulfate proteoglycans, a class of extracellular matrix compounds that play a key role in morphogen gradient formation (32). In particular, heparin-like chains bind Wnt-family ligands and promote their mobility in epithelial tissues, resulting in the expansion of their extracellular gradients (32–41). There is precedent for this effect in the butterfly *Junonia coenia*, where heparin injections resulted in specific expansions of the wing patterns associated with *wingless* expression (30, 42). We found that heparin injections had dose-dependent effects on *WntA*-associated *Heliconius* patterns (Fig. 2*C* and *SI Appendix*, Fig. S4). High dosages resulted in complete melanization of the central wing, and intermediate dosages caused partial expansion of *WntA*-associated black patterns with fields of peppered scales along expanding pattern boundaries. We thus conclude that an extracellular heparin-sensitive signal determines scale cell fate in a concentration-dependent manner. By themselves, these pharmacological results might be explained by possible heparin effects on a number of ligands (32); it is therefore important to note that heparin injections produced phenocopies of *WntA*-heterozygous phenotypes observed in *H. erato* and *H. cydno* hybrids (Fig. 2*D*). *WntA* heterozygosity is predicted to spread WntA distribution by driving its expression in adjacent domains, and similarly, heparin injections are expected to promote Wnt-ligand spread (32–41). The comparable outcomes of heparin injections and *WntA* heterozygosity thus suggest that heparin-induced pattern expansions are largely mediated by WntA (*SI Appendix*, Fig. S5).

Discussion

In this study we provide phylogenetically replicated genetic, expression, and pharmacological data that implicate *WntA* in pattern induction and adaptive evolution of black wing patterns in *Heliconius* butterflies (Fig. 3). It is worth noting the caveat, however, that the strongest support for the role of a specific gene in a developmental process is targeted loss- or gain-of-function work, which we lack because of technical limitations of our study system. This said, our heparin injection results were entirely consistent with the expectations of a targeted Wnt gain-of-function experiment (32–41). Heparin may interact with secreted molecules other than WntA, but it is too large to cross cell membranes and none of the *WntA*-neighboring genes encode extracellular products that could interact with heparin to explain its effects (*SI Appendix*, Table S4). Thus, although we are unable

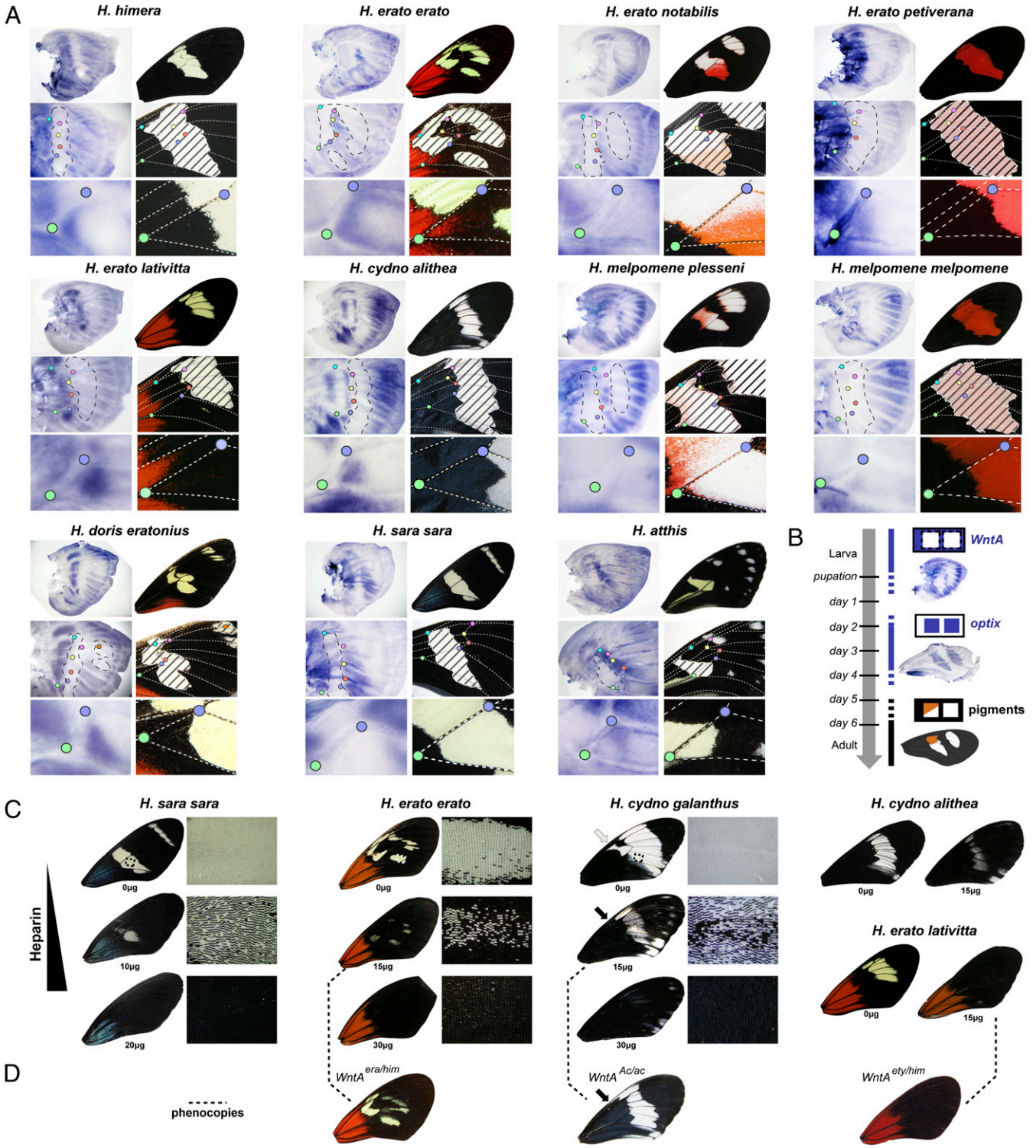

Fig. 2. Variable expression of *WntA* explains pattern shape diversity in *Heliconius*. (*A*) Larval wing disk in situ hybridizations showing *WntA* expression in presumptive black territories, and delineating pattern boundaries. Vein landmarks define homologous positions between larval and adult wings. In the intermediate panels, dashed lines corresponding to the presumptive position of the light-color patterns (hashed on adult wings) were added to facilitate the comparison of larval and adult wing topologies. (*B*) *WntA* marks presumptive *optix*-negative territories in *H. melpomene plesseni*. Expression of *optix* was reproduced from ref. 24. (*C*) Heparin injections result in dose-dependent expansions of black patterns that phenocopy the effects of *WntA* heterozygosity (*D*, dashed lines). The absence of effect on basal iridescent (*H. sara sara*) and red (*H. erato erato* and *H. erato lativitta*) patterns rules out a generic effect of heparin on wing scale phenotypes. *H. erato lativitta* resembles *H. erato etylus* (used in mapping crosses) and both originate from Eastern Ecuador low-lands.

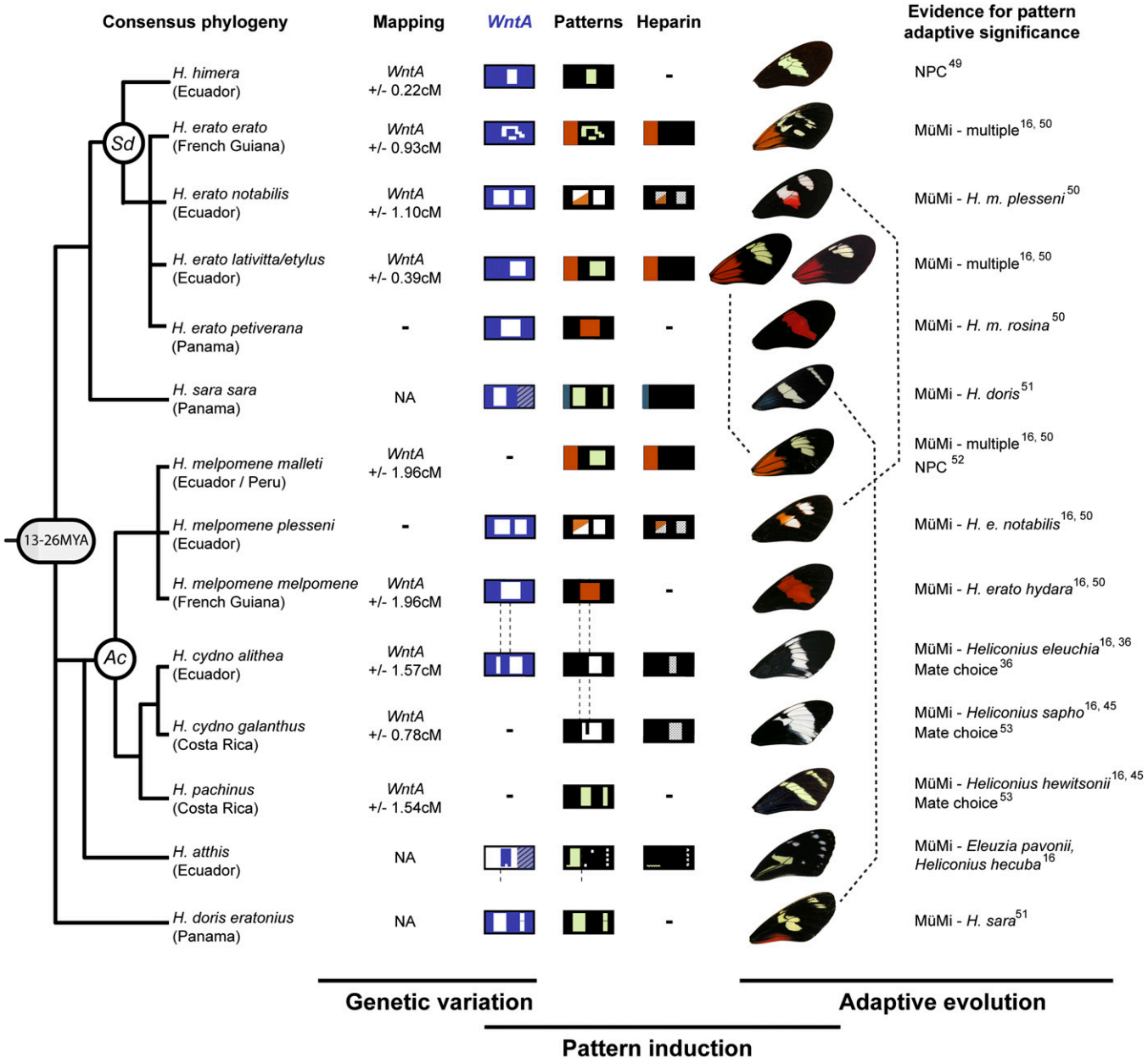

Fig. 3. Summary of phylogenetic replication for linkage mapping of forewing pattern variation (*Sd* and *Ac* loci), *WntA in situ* hybridizations, and heparin injections presented in this study. Multiple lines of evidence suggest that forewing pattern shapes are adaptive across the genus (16, 36, 45, 49–53). NA, not applicable; NPC, narrow phenotypic cline; MüMi: Müllerian mimicry; —, not assessed because of stock limitations or unavailability. Note that *H. erato lativitta/H. melpomene malleti*, and *H. erato notabilis/H. melpomene plesseni* are convergent comimics that occur in Eastern Ecuador low- and highlands, respectively (dashed lines). Phylogenetic relationships between species are derived from a recent molecular phylogeny (47) after retaining nodes with bootstrap support ≥0.98. Estimated age of *Heliconius* radiation is derived from a recent molecular clock study (48). Only a subset of *Heliconius* wing pattern diversity is represented here.

to formally rule out wing patterning functions for genes genetically linked to *WntA*, all results positively indicate that *cis*-regulatory variation at *WntA* is sufficient to explain the natural wing pattern variation considered in this study.

This first discovery of a Wnt-pathway gene driving variation in natural populations complements developmental evolution studies [e.g., the previous reports of wing color patterning by *wingless* (30, 31)] with one important difference: we show that genetic changes at a *Wnt* locus itself are responsible for pattern variation. *WntA* is deployed early during wing development and may determine pattern boundaries and identities rather than acting directly as a melanic activator, as suggested by its complementarity with pattern-specific *optix* expression at later stages (Fig. 2*B*). As such, linking a patterning molecule to the evolution of a protean, highly variable trait fills an empirical gap between the genetics of adaptive change (6, 43) and the developmental pathways that generate complex phenotypic diversity (3, 4). Because of its repeated association with mimetic phenotypes in two independent color pattern radiations, *cis*-regulatory changes of *WntA* expression also appear to represent a path of least resistance in evolution of novel wing patterns. Spatial shifts of morphogen sources may thus be a key mechanism for generating phenotypic novelty through quantum leaps across the landscape of possible morphologies.

Methods

Mapping Crosses. The *H. himera* × *H. erato* and *H. melpomene malleti* × *H. melpomene melpomene* families are described elsewhere (25, 28, 44). Segregation of the recessive *ac* allele in the *H. melpomene* cross is only visible if a yellow color of the forewing band is inherited from *H. melpomene malleti*, because of epistasis of *Ac* with the *N* locus (28, 29). Therefore, [*ac*/*ac*] vs. [*Ac*/–] segregating phenotypes were scored based on the presence vs. absence of a yellow "hourglass" pattern in the discal cell, respectively, and nonyellow banded individuals were not included in the analysis. The *H. cydno galanthus* (*ac*/*ac*) × *H. pachinus* (*Ac*/*Ac*) (one F2 cross) and *H. cydno galanthus* (*ac*/*ac*) × *H. cydno alithea* (*Ac*/*Ac*) (four male-informative backcross) families are described elsewhere (22, 45).

RAD Mapping. Preparation of a RAD sequencing library followed the canonical protocol (26) with adaptations to a bulked segregant analysis design. In brief, genomic DNA from 25 $Sd^{not/not}$ and 25 $Sd^{him/him}$ F2 individuals from a single *H. himera* × *H. erato notabilis* family were combined equimolarly (50 ng per individual) into two separate $Sd^{not/not}$ and $Sd^{him/him}$ DNA pools. Each pool was digested with the restriction enzyme NcoI, ligated to barcoded adapters, and sequenced on a single lane of an Illumina Genome Analyzer GAII generating 72-bp paired-end reads. The resulting reads were sorted by barcode and aligned to the reference assembly of the *H. melpomene* genome. A custom script was used to generate a table of 46,236 SNPs that each mapped to a *H. melpomene* scaffold with a median coverage of 86×. Library preparation and data analysis details are included in *SI Appendix, SI Methods*.

Individual Genotyping. The *H. melpomene* reference scaffold *scf7180001243157* was referenced for linkage mapping of the *Sd* and *Ac* traits. PCR primers and genotyping methods are detailed in *SI Appendix*, Table S2.

Animals, Gene Cloning, and in Situ Hybridizations. The butterflies used for expression analysis and sequence comparison originated from phenotypically pure stocks (i.e., homozygous for wing pattern alleles) maintained in the outdoor insectaries at the *Heliconius* Stock and Rearing Center at the Smithsonian Tropical Research Institute in Gamboa, Panama. Isolation of *WntA* cDNA and in situ hybridization was performed as previously described (24), and primer sequences and minor modifications are included in *SI Appendix, SI Methods*.

Heparin Injections. Heparin sodium salt (Sigma-Aldrich) was dissolved in sterile H_2O at a concentration of 5 μg/μL, aliquoted, and kept frozen. Pupae aged 12–16 h after pupation were injected with 5 μg/μL heparin or sterile H_2O using a pulled glass micropipette mounted on a 10-μL cut pipette tip and a 2- to 20-μL pipette. Pupae were surface-sterilized with ethanol and injected on the left side in an interstice that separates the baso-posterior parts of the developing forewing and hindwing. As in a previous report (42), H_2O controls showed no effects, heparin had systemic effects on both left and right wing patterns, and control and heparin injections did not produce local damage artifacts. All of the results presented in Fig. 2*C* were replicated at least three times per morph and dosage (including H_2O control), but, because of stock limitations, the results presented in *SI Appendix*, Fig. S4 were replicated in only two individuals per morph and without H_2O control.

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