Ancient homology underlies adaptive mimetic diversity across butterflies

Jason R. Gallant, Vance E. Imhoff, Arnaud Martin, Wesley K. Savage, Nicola L. Chamberlain, Ben L. Pote, Chelsea Peterson, Gabriella E. Smith, Benjamin Evans, Robert D. Reed, Marcus R. Kronforst & Sean P. Mullen

Convergent evolution provides a rare, natural experiment with which to test the predictability of adaptation at the molecular level. Little is known about the molecular basis of convergence over macro-evolutionary timescales. Here we use a combination of positional cloning, population genomic resequencing, association mapping and developmental data to demonstrate that positionally orthologous nucleotide variants in the upstream region of the same gene, WntA, are responsible for parallel mimetic variation in two butterfly lineages that diverged >65 million years ago. Furthermore, characterization of spatial patterns of WntA expression during development suggests that alternative regulatory mechanisms underlie wing pattern variation in each system. Taken together, our results reveal a strikingly predictable molecular basis for phenotypic convergence over deep evolutionary time.
As different organisms evolve similar phenotypes in response to the same selective pressure, is evolution constrained by genetic architecture or, as Mayr famously postulated, do many roads lead to Rome? Phenotypic convergence can arise from molecular convergence at one or more functional levels (that is, mutation, gene, pathway and so on) or by totally independent means. Given the opportunity for widespread functional diversity, a long-standing question in biology is whether evolution is predictable and, if so, under what circumstances? Our knowledge about the molecular basis of convergent evolution comes primarily from examples of convergence among closely related populations or species in response to a shared environment. This work suggests that re-use of genes and pathways is common on short evolutionary timescales, but there is an expectation that the constraints that promote molecular convergence should erode over evolutionary time, leading to a diversity of functional mechanisms in comparisons among distantly related organisms. We currently lack a detailed knowledge of the specific molecular mechanisms underlying convergence at these phylogenetic depths.

Butterfly wing patterns provide a unique opportunity to address the molecular basis of convergent evolution over deep evolutionary time because despite their incredible diversity, the wing patterns of this old evolutionary radiation are built from a conserved ground plan. This permits us to investigate whether similar shifts in wing pattern among distantly related butterflies are controlled by homologous genes or pathways, and whether the causative nucleotide variation is conserved over evolutionary time. Prior work suggests two very different predictions. On one hand, natural color pattern variation routinely maps back to a core set of melamin pathway genes; this is true in both invertebrates and vertebrates, although the specific genes and pathways differ markedly between these two clades. This suggests that the same pathway can be a recurrent target of selection for color pattern variation in a given clade. In contrast, recent work on butterfly wing patterning suggests that its genetic basis is highly labile over evolutionary time. For instance, comparative analyses of gene expression show that while a set of developmental genes are used routinely for eyespot patterning, the specific genes that are expressed in a given species and wing position are variable, as are the links between each gene and the adult phenotype it specifies. Furthermore, the gene optix has recently been shown to specify red and brown wing pattern elements in Heliconius butterflies, but not outside the genus, suggesting recent co-option in this one lineage. Overall, these contrasting observations yield two very different hypotheses for the genetic control of butterfly wing pattern variation across deep evolutionary time, one predicting ancient homology and the other recent innovation.

Results

Positional cloning of the color-patterning locus. To investigate the genetic basis of wing pattern diversity, we first compared the genetic architecture of pattern formation in two butterfly systems, Limenitis and Heliconius, that exhibit convergent variation in mimetic color patterns (Fig. 1). Natural selection for mimicry between Limenitis and its unpalatable model, Battus philenor, has produced a hybrid zone between wing pattern races of Limenitis arthemis, a non-mimetic, white-banded (ancestral) form and an unbanded, mimetic (derived) form. We used crosses between mimetic (L. a. astyanax) and non-mimetic (L. a. arthemis) individuals to map the position of the genomic region controlling mimetic wing pattern variation (Supplementary Fig. 1 and Supplementary Table 1). Our crosses revealed that white-band patterning in Limenitis segregates as a single Mendelian locus, and based on syntenic comparisons maps to a homologous

Figure 1 | Biogeographic distribution of study populations. (a) Parapatric distribution of hybridizing mimetic (L. a. astyanax) and non-mimetic (L. a. arthemis) admiral butterflies (distribution data from BAMONA). White dots indicate population samples; note that the toxic model, B. philenor, geographically overlaps with the distribution of the mimetic form of Limenitis. (b) Parapatric distribution of H. c. galanthus and H. pachinus in Costa Rica. (c) Sympatric distribution of H. c. alitheia in Ecuador. Black arrows illustrate medial pattern polymorphism on the forewing. Black arrows in each panel highlight variation in the melanic forewing pattern of each species. Scale bars in kilometres.
chromosomal position in *Heliconius* that is known to contain the colour-patterning locus, Ac (Supplementary Fig. 1). Martin et al.\(^\text{20}\) demonstrated that the Ac locus, which controls medial pattern shape in *Heliconius* forewings, maps to a genomic interval containing the diffusible signalling ligand WntA. WntA is a member of a larger family of Wnt signalling genes\(^\text{21}\) that encode secreted ligands involved in cell signalling across a wide range of developmental processes\(^\text{22}\) including examples of insect pigmentation\(^\text{23,24}\). By fine-mapping the colour-patterning chromosome in *Limenitis*, we reduced the zero-recombinant window to a 291-kb interval that contained just three genes, two chitin synthase genes and WntA (Supplementary Fig. 1 and Supplementary Table 2). Taken together, these results suggest that variation in the function or regulation of WntA likely mediates medial pattern formation in both *Heliconius* and *Limenitis*, two species that diverged 65 million years ago\(^\text{25}\).

**Developmental patterns of gene expression.** To test this hypothesis, we examined the developmental basis of wing pattern formation in *Limenitis* using a combination of heparin injections, *in situ* hybridization (Fig. 2) and RNA sequencing (RNAseq) experiments (Fig. 3). First, to investigate WntA signalling, we injected heparin into early *Limenitis* pupae of a white-banded progeny. Heparin binds Wnt family ligands in a wide range of organisms, promoting their transport through the extracellular matrix of developing tissues\(^\text{26–28}\), and, in this case, resulted in a fully melanized adult wing pattern lacking the white band, similar...
to the mimetic form (Fig. 2a). These results, reminiscent of previous heparin injections in other butterfly species20,29, suggest a role for heparin-sensitive signals such as Wnt molecules in patterning the medial region of the butterfly wing. Next, we examined WntA expression in 5th larval instar wing discs and found that WntA mRNA expression forms an elongated anteroposterior expression domain (Fig. 2b) outside of the white band delineating the contour of its proximal boundary. This spatial correlation, observed in both forewings and hindwings, as well as across all observed stages (Supplementary Fig. 2), suggests that WntA expression in the early developmental specification of the white band.

Despite this, we found identical spatial patterns of WntA across all observed stages (Supplementary Fig. 2), suggesting that WntA has a role in the early developmental specification of the white band and, again, found a perfect correspondence between genotype and absence of white band in mimetic and non-mimetic Limenitis respectively. Asterisk indicates significant differential expression (P<0.0001).

**Population genomics.** Next, to characterize patterns of nucleotide variation across the colour-patterning region in Limenitis, we generated a single, contiguous reference scaffold by sequencing bacterial artificial chromosome (BAC) clones spanning our zero-recombinant interval (Fig. 4a). Subsequent sequencing and analysis of 30 full Limenitis genomes (Supplementary Table 2), including both parents of the mapping brood, aligned to our BAC reference, identified a 30-kb segregating haplotype, consisting of 173 fixed single-nucleotide polymorphisms (SNPs) in complete linkage disequilibrium (LD), located 23 kb upstream of the 5′-UTR (E1) between mimetic (n=3) and non-mimetic (n=3) Limenitis only during the 5th instar stage. NS, not significant. Arrows indicate presence and absence of white band in mimetic and non-mimetic Limenitis respectively. Asterisk indicates significant differential expression (P<0.0001).
phenotype (Fig. 5; Supplementary Fig. 3 and Supplementary Table 2). Importantly, genome-wide patterns of molecular variation revealed no evidence of geographic population structure19 (Supplementary Figs 5 and 6; and Supplementary Tables 3 and 4), and no other portion of the genome showed an association with phenotype. In addition, because we found no overlap between associated SNPs and the WntA exons, these results rule out coding mutations as a possible molecular switch controlling the presence/absence of white bands in Limenitis. Finally, to investigate the mechanism maintaining extended LD upstream of WntA, we analysed patterns of structural variation, and found evidence for a single 9-kb-long interspersed element (LINE) retrotransposon, situated near the centre of the mimetic haplotype but absent in the non-mimetic allele, that occurs in the 60-kb-long first intron of WntA that was also perfectly associated with phenotype (Fig. 3b, vertical blue bar, and Supplementary Fig. 4). LINE elements can suppress recombination via the insertion of a non-homologous and non-collinear sequences, as well as by altering local DNA methylation patterns31, and, therefore, may be responsible for maintaining LD across the large (30 kb) haplotypes we found.

Taken together, our results suggest that differential expression of a WntA 5’-UTR sequence during late larval development underlies adaptive phenotypic divergence between mimetic and non-mimetic Limenitis. At the molecular level, differential expression of the 5’-UTR may have arisen either directly (by interfering with gene function) or indirectly (by facilitating the accumulation of cis-regulatory mutations) from a LINE insertion in the first intron of WntA. Alternatively, the extensive LD we observed may instead reflect strong natural selection on multiple cis-regulatory SNPs interspersed across the haplotype interval. Under either scenario, differential expression of the 5’-UTR is a reasonable proximate mechanism given that such sequences regulate many aspects of protein translation32, and, in this case,
the differentially expressed WntA 5'-UTR contains a predicted internal ribosome entry site (IRES) motif that could mediate such effects (Supplementary Fig. 7). This latter observation suggests that a post-transcriptional regulatory mechanism may act as the molecular switch in Limenitis, controlling the presence/absence of white bands.

Comparative genomics of colour pattern evolution. While these results provide unique insights into the molecular basis of adaptation, our primary goal was to identify and compare the proximate basis of melanin pattern formation in Limenitis and Heliconius. To do this, we first focused on two closely related species in Costa Rica, H. cydno galanthus (n = 10) and H. pachinus (n = 10), which vary markedly in their Müllerian mimicry phenotypes as a result of allelic variation at Ac\(^2^{10}\) (Fig. 4c); H. pachinus has a melanized patch of scales, which is lacking in H. c. galanthus. Examination of SNPs across the 581-kb Ac scaffold from the published Heliconius genome sequence\(^33\) identified 170 SNPs and a 1.8-kb indel fixed between these two species, all of which occur upstream of the WntA coding region. To refine the phenotypic association, we then analysed an additional 25 genomes from a single phenotypically variable species, all of which occur upstream of the WntA coding region.

Role of ascertainment bias in phylogenetic replication

Figure 6 | The empirical landscape of parallel genetic evolution. The graph features 118 genes involved in parallel genetic evolution, where de novo mutations have independently driven phenotypic evolution of a phenotypic trait in at least two lineages. The y axis marks the estimated divergence time between the two lineages (for genes with >2 entries, the most distant pair is shown). The literature is also partitioned across the x axis according to criteria thought to influence the discovery of parallel genetic evolution: (1) type of ascertainment bias on the genetic nature of the trait\(^38\); (2) cases of natural variation versus cases including domesticated variation\(^38\), and (3) regulatory versus terminal differentiation and effector genes\(^39\). See Supplementary Data 1–3 for data and references.
constraints, with certain genetic or developmental architectures limiting the functional mechanisms available in the production of novel morphologies. With the maturation of the genetic age comes the ability to examine the molecular basis of phenotypic evolution at multiple functional levels and in multiple biological systems. Such work promises to reveal the extent to which the evolutionary process is predictable over varying evolutionary timescales. While several studies have identified instances of genetic parallelism over large divergence times, biases on genetic function may favour the discovery of genetic parallelism in studies that repeatedly focus on the same small sets of candidate genes.

Here we have leveraged the power of association mapping in naturally hybridizing populations to demonstrate that a positionally orthologous region of the WntA locus has independently driven the evolution of mimetic wing patterns in two butterfly species. Although additional functional work is needed to evaluate the regulatory consequences of these mutations, the discovery of parallel genetic evolution of WntA is remarkable because it was identified in two independent mapping studies without initial bias on the genetic basis of the trait, owing to the exceptionally large divergence times between Heliconius and Limenitis, and because, unlike melanin pathway genes that have also been repeatedly linked to pigmentation variation, WntA is a regulatory gene involved in the early deployment of spatial information in undifferentiated tissues (for example, embryos and wing discs). Surprisingly, our results suggest that modulation of this conserved developmental gene has occurred in tandem between these two deeply divergent butterfly lineages, implying an unexpected and remarkable level of predictability in the evolutionary process.

Methods

Positional cloning. All Limenitis specimens utilized for genetic linkage mapping were collected from a single locality in Pennsylvania (Supplementary Table 2). Wild-captured, mated female specimens were captured and fed a mixture of honey and water twice daily, and were secured on Prunus serotina and/or Salix babylonica to encourage oviposition. Larvae were raised directly on host plants with one brood per enclosure. Pupae were collected and placed in labelled containers to prevent them from escaping. Newly emerged butterflies were dissected, and wing discs were transferred to envelopes numbered with their sibling group and according to their order of emergence. Adults were then photographed and crossed via hand-pairing. Mapping families were generated by backcrossing heterozygous mimetic males to fully banded, non-mimetic females (homozygous recessive at the major gene controlling mimicry); these linkage maps consisted of 90 banding patterns and were uninformative because there is no recombination during oogenesis. Following mating, the wings and tissues of male butterflies were immediately archived, and the wings and tissues of female butterflies were archived after oviposition was ceased. Pupae from mapping crosses were photographed upon emergence, and wing discs were archived.

Medial banding in Limenitis is controlled by two, incompletely dominant alleles at a single locus, and at least one dominant modifier that influences the penetrance of the white-banded allele in heterozygous individuals. All progeny from mapping crosses displayed either the mimetic phenotype (heterozygous with dominant modifier) or the white-banded (homozygous) phenotype. The spotted wing banding pattern consists of homogenous females that are uninformative because there is no recombination during oogenesis. Following mating, the wings and tissues of male butterflies were immediately archived, and the wings and tissues of female butterflies were archived after oviposition was ceased. Pupae from mapping crosses were photographed upon emergence, and wing discs were archived.

Developmental patterns of gene expression and functional tests. Mimetic and non-mimetic female Limenitis were wild captured from Baltimore county, Maryland, and White Mountain National Forest, New Hampshire, respectively. Lab-reared offspring from true-breeding individuals were mated to full-sibs, and the resulting progeny were utilized as reported for all following developmental and functional tests.

Individuals aged 7–16 h after pupation were injected with 10 or 20 μg of heparin or saline into the 2 μl of a 1:5 dilution of mWntA. The resulting progeny were utilized as reported for all following developmental and functional tests.

Individuals aged 1–5 h after pupation were injected with 10 or 20 μg of heparin or saline into the 2 μl of a 1:5 dilution of mWntA. The resulting progeny were utilized as reported for all following developmental and functional tests.

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The tissues were then post fixed 20 min on ice in PBS containing 5.5% formaldehyde, transferred to a standard hybridization buffer, incubated in supplemented hybridization buffer for 16–40 h at 62 °C. Tissues were washed eight times, and, for secondary detection of the riboprobe, the tissues were blocked and then incubated with a 1:4,000 dilution of anti-digoxigenin alkaline phosphatase Fab fragments (Roche Applied Science, Indianapolis, Indiana, USA). Tissues were again washed 10× in PBS for 10–120 min, and finally washed with BM Purple (Roche Applied Science) for 4–8 h at room temperature. Stained tissues were then washed in PBST 2 mM EDTA and slide mounted in PBS containing 60% glycerol. mRNA in situ hybridizations were photographed with a Nikon Coolpix P510 digital camera (Nikon Inc., Melville, New York, USA) mounted with a LNS-30D/FS1 adapter (Zeiss Enterprise, Spokane, Washington, USA) on a Leica S5E microscope (Leica Microsystems, Buffalo Grove, Illinois, USA).

Mimetic and non-mimetic individuals were captured in Massachusetts and New Hampshire, respectively, and allowed to oviposit in the laboratory. Mimetic and non-mimetic individuals were then crossed to siblings to ensure that they were true-breeding for each respective phenotype. Individuals from each cross were sampled at the 5th instar (n = 3 mimetic and n = 3 non-mimetic), prepupa (identified by stereotypic ‘j-curve’ hanging from leaves; n = 3 mimetic and n = 3 non-mimetic), early pupation (n = 30 pupa post hatching; n = 3 mimetic and n = 3 non-mimetic), and late pupation (≥ 48 h post pupation; n = 3 mimetic and n = 3 non-mimetic). Wing discs were dissected from each individual in cold PBS, wing discs were then stored in RNAlater (Ambion, Inc.) following manufacturer’s instructions. RNA was extracted using an RNAeasy-kit (Qiagen, Inc.), and individual RNAseq libraries were prepared using the TrueSeq RNA sample prep kit (Illumina, Inc.) at the Michigan State University Genomics core facility. Each library was pooled and sequenced across two lanes of an Illumina HiSeq 2500, using 2 × 150 bp reads.

Individual reads were quality filtered and trimmed using custom python scripts, then aligned to BAC sequences representing mimetic and non-mimetic haplotypes using the TopHat pipeline. TopHat identified genes and exons automatically and filtered those from each individual by filtering those sampled at the 5th instar (n = 3 mimetic and n = 3 non-mimetic), prepupa (n = 3 mimetic and n = 3 non-mimetic), early pupation (n = 30 pupa post hatching; n = 3 mimetic and n = 3 non-mimetic), late pupation (≥ 48 h post pupation; n = 3 mimetic and n = 3 non-mimetic). Wing discs were dissected from each individual in cold PBS, wing discs were then stored in RNAlater (Ambion, Inc.) following manufacturer’s instructions. RNA was extracted using an RNAeasy-kit (Qiagen, Inc.), and individual RNAseq libraries were prepared using the TrueSeq RNA sample prep kit (Illumina, Inc.) at the Michigan State University Genomics core facility. Each library was pooled and sequenced across two lanes of an Illumina HiSeq 2500, using 2 × 150 bp reads. Individual reads were quality filtered and trimmed using custom python scripts, then aligned to BAC sequences representing mimetic and non-mimetic haplotypes using the TopHat pipeline. TopHat identified genes and exons automatically and filtered those from each individual by filtering those sampled at the 5th instar (n = 3 mimetic and n = 3 non-mimetic), prepupa (n = 3 mimetic and n = 3 non-mimetic), early pupation (n = 30 pupa post hatching; n = 3 mimetic and n = 3 non-mimetic), late pupation (≥ 48 h post pupation; n = 3 mimetic and n = 3 non-mimetic). Wing discs were dissected from each individual in cold PBS, wing discs were then stored in RNAlater (Ambion, Inc.) following manufacturer’s instructions. RNA was extracted using an RNAeasy-kit (Qiagen, Inc.), and individual RNAseq libraries were prepared using the TrueSeq RNA sample prep kit (Illumina, Inc.) at the Michigan State University Genomics core facility. Each library was pooled and sequenced across two lanes of an Illumina HiSeq 2500, using 2 × 150 bp reads.

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Author contributions
S.P.M. conceived the project and led the study. M.R.K. contributed to experimental design and provided Heliconius samples. S.P.M. M.R.K., A.M. and J.R.G. wrote the manuscript. J.R.G. assembled the BAC clones, constructed the sequencing libraries for all taxa, mapped reads, called SNPs, performed the RNAseq experiments and performed all bioinformatics analyses. V.E.I. reared insects and led the genetic mapping study. A.M. and R.D.R. conducted the developmental analyses. W.K.S. collected insects, aided in genotyping the mapping progeny and contributed to figure production. N.L.C. screened the BAC libraries. B.L.P. performed TaqMan genotyping assays of the hybrid zone transect samples. B.E. assisted with transcriptome annotations related to the mapping work. C.P. and G.E.S. assisted with insect rearing and wing disc dissections. All authors contributed to editorial revisions of the manuscript.

Additional information
Accession codes: All Illumina short reads have been archived in the NCBI Short Read Archive with the following BioProject accession numbers: PRJNA226620 and PRJNA252628.

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