The Functional Basis of Wing Patterning in *Heliconius* Butterflies: The Molecules Behind Mimicry

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ABSTRACT Wing-pattern mimicry in butterflies has provided an important example of adaptation since Charles Darwin and Alfred Russell Wallace proposed evolution by natural selection >150 years ago. The neotropical butterfly genus *Heliconius* played a central role in the development of mimicry theory and has since been studied extensively in the context of ecology and population biology, behavior, and mimicry genetics. *Heliconius* species are notable for their diverse color patterns, and previous crossing experiments revealed that much of this variation is controlled by a small number of large-effect, Mendelian switch loci. Recent comparative analyses have shown that the same switch loci control wing-pattern diversity throughout the genus, and a number of these have now been positionally cloned. Using a combination of comparative genetic mapping, association tests, and gene expression analyses, variation in red wing patterning throughout *Heliconius* has been traced back to the action of the transcription factor *optix*. Similarly, the signaling ligand *WntA* has been shown to control variation in melanin patterning across *Heliconius* and other butterflies. Our understanding of the molecular basis of *Heliconius* mimicry is now providing important insights into a variety of additional evolutionary phenomena, including the origin of supergenes, the interplay between constraint and evolvability, the genetic basis of convergence, the potential for introgression to facilitate adaptation, the mechanisms of hybrid speciation in animals, and the process of ecological speciation.

KEYWORDS *Heliconius*; adaptation; mimicry; speciation

Background

> ...the study of butterflies—creatures selected as the types of airiness and frivolity—instead of being despised, will someday be valued as one of the most important branches of Biological science" (Bates 1864, p. 413)

Henry Walter Bates discovered mimicry—the evolutionary phenomenon in which natural selection by predators causes unrelated species to appear similar—by collecting and studying butterflies in South America (Bates 1862). It is hard to imagine that anyone in Bates' time actually "despised" the study of butterflies, and it is clear that this field has not yet risen to the esteemed position that Bates predicted. Yet butterflies, and their mimetic wing patterns in particular, have recently provided a wealth of insight into the genetic basis of adaptation (Joron *et al.* 2011; Reed *et al.* 2011; *Heliconius* Genome Consortium 2012; Martin *et al.* 2012; Kunte *et al.* 2014; Timmermans *et al.* 2014). Animal pigmentation, more generally, has been a staple of research in genetics and evolutionary biology for over a century (Cott 1940; Bennett and Lamoreux 2003; True 2003; Hoekstra 2006; Kronforst *et al.* 2012). External appearance is so intimately connected to both survival and reproduction that it is frequently the target of intense natural and sexual selection, and the combination of these two evolutionary forces routinely generates great diversity in pigment patterns, both within and between species (True 2003; Caro 2005; Hoekstra 2006; Joron *et al.* 2006a; Hubbard *et al.* 2010; Manceau *et al.* 2010; Kronforst *et al.* 2012). Perhaps no group of animals is more diverse in terms of pigmentation and patterning than butterflies (Nijhout 1986, 1991; McMillan *et al.* 2002). Butterflies, including skippers (family Hesperiidae) and butterfly moths (family Hedylidae), are a monophyletic clade within the insect order Lepidoptera (Heikkila *et al.* 2012). The clade consists of an

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estimated 18,000 species, and many of these can be identified on the basis of wing pattern alone (Nijhout 1991). Color pattern in butterflies is a morphologically simple phenotype, largely determined by the pigments deposited in tiny scales that line the wing surface like microscopic roofing tiles. In addition, colors like blue, some whites and greens, and features like iridescence are determined by scale structure, which shapes the way light reflects off the wing surface (Ghiradella 1991).

While wing pattern itself is structurally simple, the dizzying array of distinct patterns depicted on these miniature mosaics is exceptionally complex. The reasons for such extreme diversity are manifold because wing pattern plays a role in a variety of biological processes from thermoregulation to predator avoidance and mate attraction (Beldade and Brakefield 2002; McMillan *et al.* 2002). Evolutionary phenomena that have played particularly important roles in shaping butterfly wing patterns are aposematism and mimicry (Joron and Mallet 1998; Mallet and Joron 1999). Aposematism, or warning coloration, is widespread among butterflies as many species feed on chemically defended host plants and then sequester these compounds themselves to defend against predation. Like other toxic or defended organisms, chemically defended butterflies have subsequently evolved bold warning patterns that likely serve to enhance predator learning and/or allow predators to better distinguish toxic species from co-occurring palatable species. The existence of chemical defense and warning coloration has secondarily generated a permissive environment for the evolution of mimicry. Defensive mimicry is broadly divided into two categories: classic Batesian mimicry, in which an undefended mimic evolves to look like a toxic model (Bates 1862), and Müllerian mimicry, in which mutually defended species converge on a shared warning pattern as a means of enhancing predator learning (Müller 1879).

Wing-pattern mimicry in butterflies, which encompasses hundreds of examples of both Batesian and Müllerian mimicry, has served as an important model of adaptation since the earliest days of modern evolutionary theory. Darwin (1863) himself was amazed by Bates' discovery of butterfly mimicry, writing: "It is hardly an exaggeration to say, that whilst reading and reflecting on the various facts given in this Memoir, we feel to be as near witnesses, as we can ever hope to be, of the creation of a new species on this earth," p. 223. More recently, R. A. Fisher (1930) dedicated an entire chapter to mimicry in his classic book, *The Genetical Theory of Natural Selection*, calling mimicry theory "the greatest post-Darwinian application of natural selection," p. 146. Since then, butterfly wing patterning, and mimicry in particular, has been explored theoretically and empirically, in the context of Mendelian and population genetics, evolutionary genomics, and evo-devo (Nijhout 1986, 1991; Mallet and Joron 1999; Beldade and Brakefield 2002; McMillan *et al.* 2002; Papanicolaou *et al.* 2005; Joron *et al.* 2006a; Parchem *et al.* 2007; Beldade *et al.* 2008; Papa *et al.* 2008a; Kronforst *et al.* 2012; Supple *et al.* 2014).

Butterflies in the genus *Heliconius* are conspicuous members of neotropical butterfly communities and some of the more influential players in original mimicry theories. From those early days, *Heliconius* has transformed into something of an emerging model system for the study of ecology, evolution, and behavior, especially in the context of mimicry, leading Turner (1977a) to assert that they were the "best studied terrestrial invertebrates of no economic importance outside the Drosophilidae." While that is no longer true (*e.g.*, *Caenorhabditis elegans*), there has been a recent coordinated effort among an international team of researchers to characterize the genes underlying wing-pattern mimicry in *Heliconius* and then to use this information to address fundamental questions in evolutionary biology. Given the rapid progress that has been made in the past few years, now is an ideal time to review this research program, the history of work that facilitated it, and prospects for new insight in the near future. Here, we briefly introduce the biology of *Heliconius* and outline historical work on the Mendelian genetics of *Heliconius* wing patterning that primed it to become the emerging ecological model system that it is today. Then we highlight exciting recent discoveries in which the hunt for wing-patterning genes has come to fruition. Finally, we focus on perhaps the most important issue of all: the critical questions about basic evolutionary processes that can be addressed now that these wing-patterning genes are in hand.

The Biology of *Heliconius* and Related Genera

Host plants, chemical defense, and pollen feeding

A long history of research focused on *Heliconius* has revealed a detailed portrait of their fascinating biology (reviewed in Brown 1981). *Heliconius*, which consists of 43 species, is one of nine genera in the nymphalid tribe Heliconiini (Penz 1999; Beltran *et al.* 2007), commonly referred to as passion-vine butterflies because of their close affiliation with their *Passiflora* host plants (Gilbert 1971; Benson *et al.* 1975; Smiley 1978). *Passiflora* and *Heliconius* are cyanogenic, and it is this chemical defense that protects *Passiflora* from most herbivores and protects *Heliconius* from predation. Interestingly, a minority of *Heliconius* species are cyanogenic because they sequester cyanogens (simple monoglycoside cyclopentenyl cyanogens) from their host plants as larvae (Engler *et al.* 2000). Most *Heliconius* species actually synthesize aliphatic cyanogenic glycosides *de novo* from amino acid precursors (Nahrstedt and Davis 1981, 1983), and there is a trade-off in the ability to sequester *vs.* manufacture cyanogens across the genus (Engler-Chaouat and Gilbert 2007).

Heliconius also interact intimately with other plants, specifically those in the cucurbit genera *Gurania* and *Psiguira*, which provide a specialized resource to adult *Heliconius* in the form of pollen (Gilbert 1975). *Heliconius* are unique among butterflies in that they feed on pollen as adults, in addition to nectar (Gilbert 1972; Boggs *et al.* 1981). While *Heliconius* collect pollen from many flowers (Estrada and Jiggins 2002), they show a specialized, coevolved relationship with *Gurania* and *Psiguira*, plants that provide substantial pollen rewards to *Heliconius* and appear to be largely pollinated by *Heliconius*

(Gilbert 1975). The nutritional benefit provided by pollen feeding has significant impacts on many aspects of *Heliconius* biology, such as cyanogenesis, reproduction, and longevity (Dunlap-Pianka *et al.* 1977; Brown *et al.* 1991). Other notable aspects of *Heliconius* biology include home-range behavior (Mallet 1986 a,b; Gilbert 1991), male production of specialized "anti-aphrodisiac" compounds (Gilbert 1976; Schulz *et al.* 2007, 2008), the unique pupal-mating behavior of some *Heliconius* species (Deinert *et al.* 1994; Estrada and Gilbert 2010; Estrada *et al.* 2010), and their pronounced visual acuity (Zaccardi *et al.* 2006), which is in part a product of a *Heliconius*-specific UV opsin duplication (Briscoe *et al.* 2010; Yuan *et al.* 2010).

Müllerian mimicry

Wing-pattern mimicry is one of the most-studied aspects of *Heliconius* biology (Brown 1981; Mallet and Joron 1999; Joron *et al.* 2006a; Papa *et al.* 2008a; Kronforst *et al.* 2012). Many examples of mimicry in *Heliconius* involve convergence between species within the genus, and frequently between pairs of species. These co-mimetic pairs generally consist of distantly related species within the genus (Eltringham 1916; Turner 1976), with one member coming from each of the two major *Heliconius* subclades (Figure 1). For example, species and subspecies in the *H. cydno* group generally mimic species from the *H. sara/H. sapho* clade, while *H. melpomene* always mimics *H. erato*. There are exceptions to these patterns, such as species in the silvaniform clade, which are members of larger mimicry rings (groups of co-mimetic taxa) that include other *Heliconius* and more distantly related taxa (Brown and Benson 1974). In addition, mimicry in certain regions of the Amazon basin stands out as an extreme example of convergence, where *Heliconius* species from across the phylogeny, as well as a variety of other butterflies and even day-flying moths, have converged on the same red rayed wing pattern (Mallet 1999).

The dynamics of *Heliconius* mimicry appear to play out at a fine spatial scale, yielding both striking convergence among species as well as incredible diversity within species. Perhaps the most extreme example of this is the case of *H. melpomene* and *H. erato* (Brown *et al.* 1974; Sheppard *et al.* 1985; Brower 1994, 1996b; Turner and Mallet 1996; Flanagan *et al.* 2004; Quek *et al.* 2010; Hines *et al.* 2011; Nadeau *et al.* 2014). These two species are distributed throughout much of South and Central America, and they mimic one another wherever they co-occur. Like many other examples of pairwise mimicry in *Heliconius*, one of these species comes from each of the two major within-*Heliconius* subclades. Strikingly, the shared wing patterns of *H. melpomene* and *H. erato* switch geographically, in tandem, producing a patchwork of mimicry phenotypes across their range (Figure 2). This has resulted in >20 named wing-pattern races in each species, but there are common themes among phenotypes that cluster them into three general categories: red-banded, rayed, and "postman." The evolutionary history of this shared diversity between *H. melpomene* and *H. erato* remains a bit of a mystery. Historically, Sheppard, Turner, Brown, and others believed that this geographical patchwork emerged from a strict co-evolutionary process whereby *H. melpomene* and *H. erato* radiated in parallel, over time and space, and that this was driven by populations of each species becoming isolated in Pleistocene forest refugia (Brown *et al.* 1974; Brown 1981; Sheppard *et al.* 1985; Brower 1996b; Turner and Mallet 1996). However, recent phylogeographic work suggests that this may not be the case. For example, the radiation of *H. erato* seems to predate that of *H. melpomene* by almost 1 million years (3.1 *vs.* 2.1 MYA) (Quek *et al.* 2010; Hill *et al.* 2013; but see Cuthill and Charleston 2012). This time discrepancy may be reflective of a larger trend as divergence events throughout the clade that includes *H. erato*, *H. sara*, and *H. sapho* appear to be older than those in the clade that includes *H. melpomene*, *H. cydno*, and the silvaniforms (Kozak *et al.* 2015). The emerging picture is that *H. erato* likely radiated first, and it was this established diversity that served as a template for a subsequent *H. melpomene* radiation. If so, the ultimate source of *H. erato* diversification remains an open question although there is speculation that genetic drift may have played a role (Turner and Mallet 1996; Mallet 2010).

Measuring selection on wing patterns

Butterfly mimicry is an appealing evolutionary study system because, unlike many natural systems, we have a good understanding of the targets of selection (wing-pattern traits that match mimicry models) as well as the agents of selection (predators). Furthermore, for *Heliconius* specifically, there are abundant and varied empirical data documenting natural selection on wing pattern as well as quantitative estimates of the strength of selection. An important early experiment revealed the strength of purifying selection on *Heliconius* wing patterns. Benson (1972) altered the wing pattern of *H. erato* in Costa Rica by obscuring the red forewing band on a minority of individuals in the population. Subsequent recapture results revealed that the altered phenotypes disappeared from the population rapidly, and those that were re-caught, showed a higher frequency of bird beak marks on their wings. Mallet and Barton (1989) followed this with a reciprocal transplant experiment, moving *H. erato* butterflies across a racial hybrid zone. Subsequent recapture revealed rapid disappearance of the foreign morph, combined with elevated instances of bird attack, yielding a selection coefficient estimate of 0.51 overall, or $s \sim 0.17$ per mimicry locus. In this same hybrid zone, and the coincident hybrid zone between matching races of *H. melpomene*, Mallet *et al.* (1990) measured cline widths and linkage disequilibrium among mimicry loci to estimate selection on wing pattern, resulting in estimates of $s \sim 0.23$ per mimicry locus for *H. erato* and $s \sim 0.25$ per mimicry locus for *H. melpomene*. These are large selection coefficients, as predicted by the very narrow hybrid zones ($\sim$10 km).

While effectively documenting selection on wing patterns, this work focused on purifying selection within species rather than the convergence between species predicted by mimicry theory. Kapan (2001) filled this gap by experimenting with a *Heliconius* mimicry system in western Ecuador

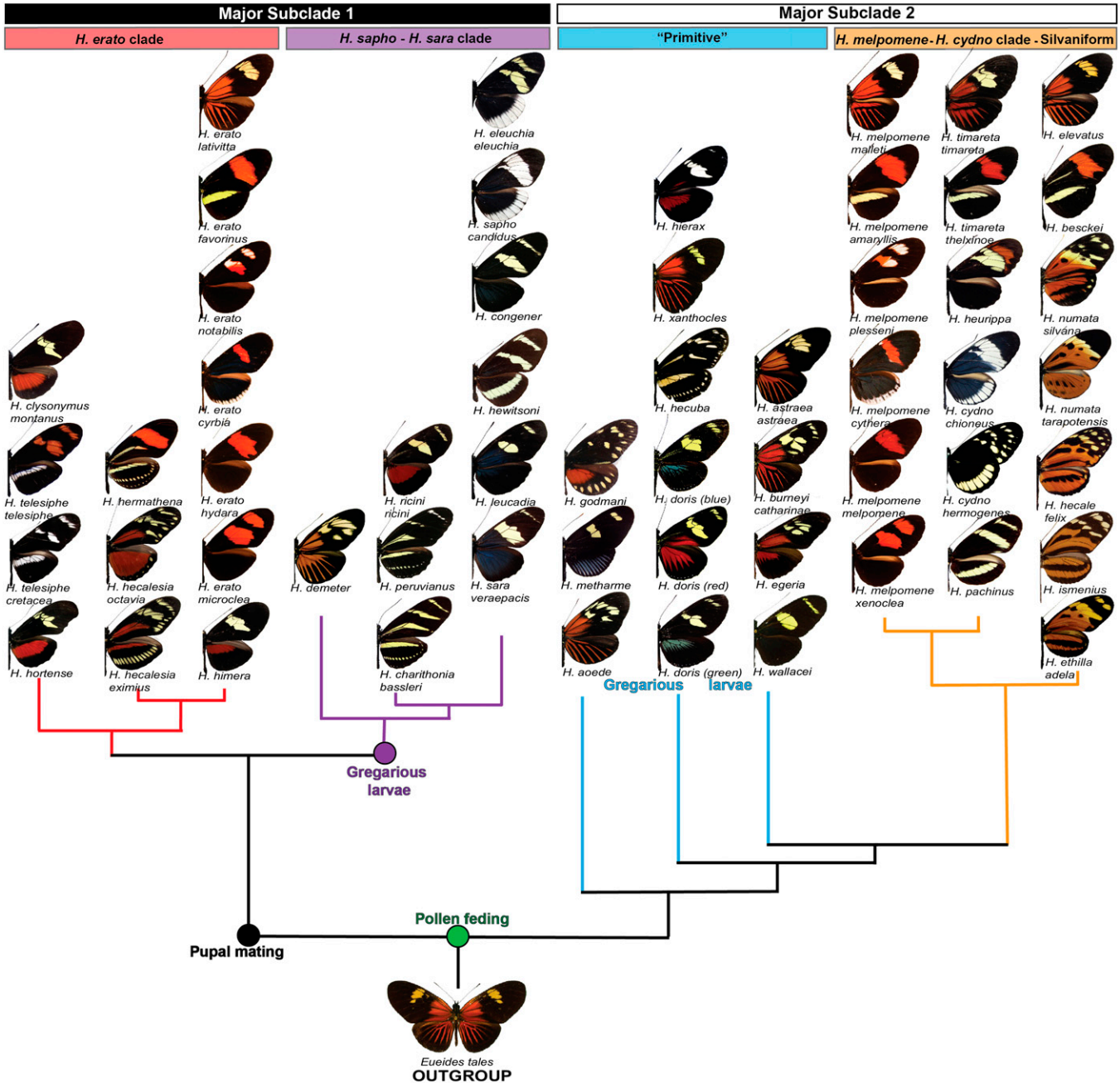

Figure 1 Phenotypic diversity across *Heliconius* butterflies. Phylogenetic relationships among all major *Heliconius* clades are shown (Kozak *et al.* 2015). Names and color patterns of representative species and subspecies are depicted, together with behavioral traits that characterize phylogenetic nodes across the genus.

that contained two divergent and monomorphic species, white-winged *H. sapho* and yellow-winged *H. eleuchia*, and a third polymorphic species, *H. cydno alithea*. *Heliconius cydno alithea* exists as two morphs, a white form that mimics *H. sapho* and a yellow form that mimics *H. eleuchia*. Kapan (2001) moved white and yellow *H. cydno alithea* morphs among sites in western Ecuador, where the abundances of *H. sapho* and *H. eleuchia* varied. Consistent with previous work, *H. cydno* individuals that differed from the locally abundant model disappeared from the population faster than those that matched the model. Furthermore, Kapan (2001) showed that increasing the density of experimentally released individuals reduced the effect, presumably as a result of predator learning. These results provided the first field test of Müllerian mimicry theory, showing natural selection for convergence between different toxic species.

Mimicry and speciation

The evolution of mimicry in *Heliconius* has a further, second-order effect on biodiversity because divergent natural selection

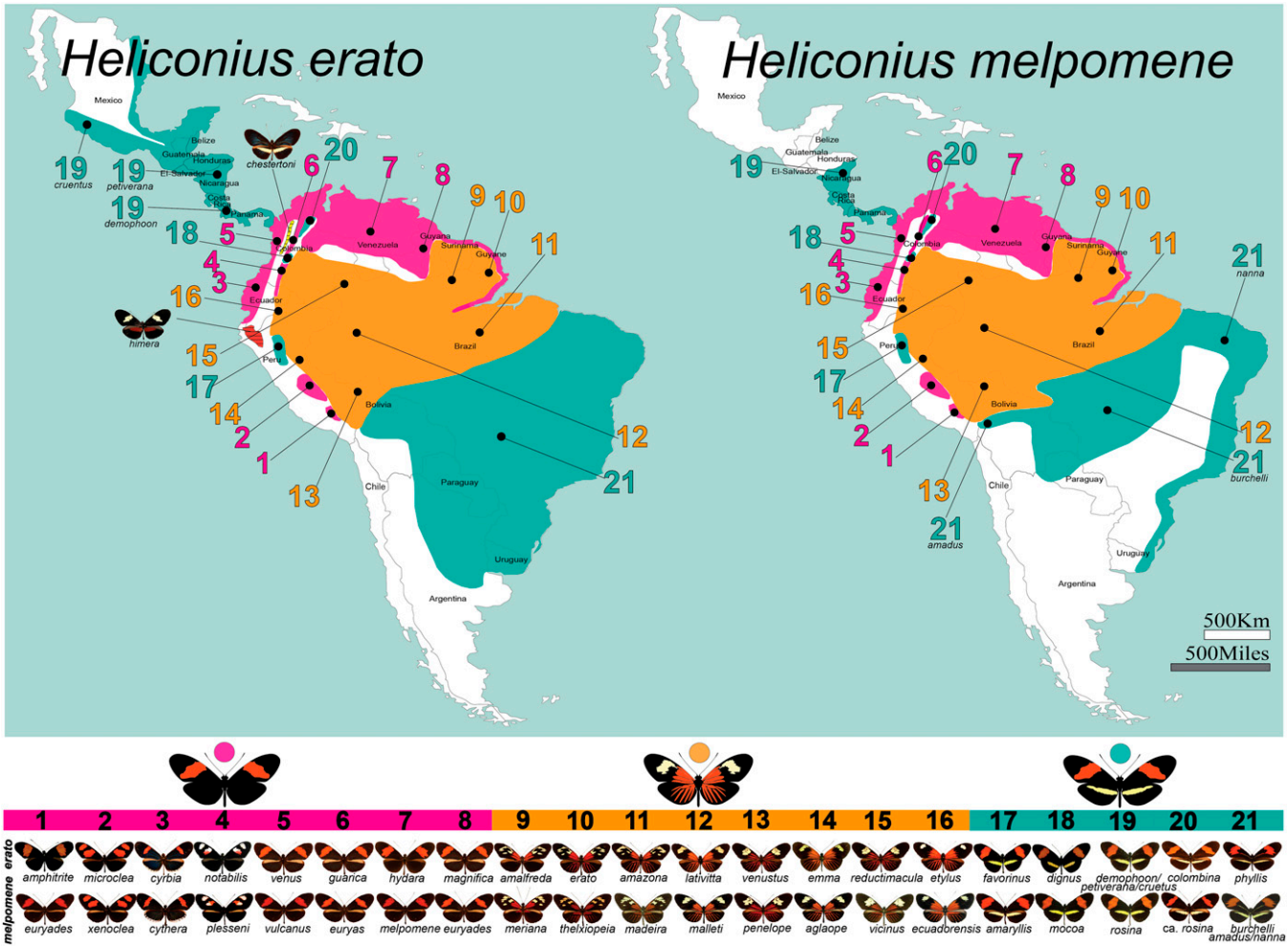

Figure 2 Geographic distributions of the *H. erato* and *H. melpomene* convergent radiations. Approximate distributions of the three major phenotypes and locations of many described subspecies, or "races," of the Müllerian co-mimics *H. erato* and *H. melpomene*. Matching wing-color-pattern variation and subspecies names are shown below the maps. Two incipient species that are part of the *H. erato* radiation, *H. himera* and *H. erato chestertoni*, are also presented.

for mimicry appears to play an important role in the early stages of speciation (Jiggins 2008). Given the strong selection on wing patterns documented in previous experiments, what happens when a subpopulation of one species leaps the adaptive valley to join a different mimicry ring? The prediction is that divergence in color pattern should generate reproductive isolation because recombinant wing patterns are nonmimetic and thus likely to be sampled by predators. This is a form of hybrid incompatibility, but in contrast to intrinsic incompatibilities such as hybrid sterility or inviability, this incompatibility is extrinsic because it is generated by the fit (or lack thereof) between a hybrid and the environment. Recently, Merrill *et al.* (2012) tested this hypothesis using two different approaches—behavioral assays of field-caught birds when presented with live butterflies and estimates of natural predation using clay butterfly models—and found that predators do indeed attack hybrids more often than the parental types. Other work has also shown that the parental species are not attracted to hybrids as potential mates (Naisbit *et al.* 2001). The result is that divergent natural selection to match different mimicry rings immediately generates, as a by-product, pronounced extrinsic, postzygotic isolation as well as disruptive sexual selection.

Mimicry appears to interact with mate preference in an even more fundamental way, which further contributes to reproductive isolation. Multiple studies have shown that *Heliconius* species and subspecies generally mate assortatively, with wing pattern serving as a critical cue in mate selection (McMillan *et al.* 1997; Jiggins *et al.* 2001, 2004; Naisbit *et al.* 2001; Kronforst *et al.* 2006b, 2007; Mavarez *et al.* 2006; Chamberlain *et al.* 2009; Melo *et al.* 2009; Merrill *et al.* 2011a,b, 2014; Finkbeiner *et al.* 2014). For example, *H. cydno* and *H. melpomene* rarely hybridize in nature, despite being broadly sympatric and partially interfertile (Naisbit *et al.* 2002), and that appears to be due, in part, to a strong preference for conspecific wing patterns (Jiggins *et al.* 2001; Jiggins 2008). Assortative mate preference based on color pattern also limits hybridization between *H. erato* and *H. himera* (Jiggins *et al.* 1997; McMillan *et al.* 1997; Merrill *et al.* 2014), as well as between *H. cydno* and *H. pachinus* (Kronforst *et al.*

2006b, 2007), pairs of closely related species that are otherwise interfertile. Hence, divergent mimicry phenotypes contribute to premating reproductive isolation as well. Perhaps surprisingly, the association between mimicry and mate choice extends even further because crosses between *H. cydno* and *H. melpomene*, as well as crosses between *H. cydno* and *H. pachinus*, reveal that mate preference is genetically linked to the dominant wing color cue distinguishing the hybridizing species: red *vs.* black in *cydno/melpomene* crosses (Merrill *et al.* 2011b) and white *vs.* yellow in *cydno/pachinus* (Kronforst *et al.* 2006b). This genetic linkage between preference and wing color has likely contributed to the recent *Heliconius* radiation because it facilitates the co-evolution of mimicry and mate preference and also maintains their association despite on-going hybridization among species.

Genetic Basis of Wing Patterning in *Heliconius*

Given that *Heliconius* wing patterns play such a central role in mediating critical aspects of their biology, as well as the fact that they stand out as adaptive traits that also influence speciation, it is not surprising that the genetic basis of *Heliconius* wing patterning has been the subject of considerable research. Here we briefly outline this work, starting with the original investigations of basic Mendelian genetics, all the way up to the latest discoveries that have identified the specific genes, and even the exact mutations in some cases, that control *Heliconius* wing-pattern diversity.

Mendelian genetics

Crossing experiments with *Heliconius* butterflies date back to at least 1954 (Beebe 1955), which is actually quite recent given that similar experiments in swallowtail butterflies began as early as 1902 (Leigh and Poulton 1909). A recent example of a *Heliconius* crossing experiment (Papa *et al.* 2013) is shown in Figure 3. The earliest work on *Heliconius* mimicry genetics (Beebe 1955; Turner and Crane 1962; Sheppard 1963; Emsley 1964; Turner 1971) culminated in the expansive treatment of Sheppard *et al.* (1985). This dense, >170-page paper provided a comprehensive summary of wing-pattern segregation based on 68 crosses (encompassing 1978 hybrid offspring) among eight color pattern races of *H. melpomene* and 69 crosses (1325 offspring) among eight races of *H. erato*. This early work revealed that much of the wing-pattern variation in both *H. melpomene* and *H. erato* is controlled by large-effect Mendelian loci that switch portions of the wing from one color to another. Sheppard *et al.* (1985) also identified a relatively large number of distinct loci in each species—11 loci in *H. melpomene* and 15 in *H. erato* —and showed that, despite some instances of linkage, many of the Mendelian switch loci appeared to be unlinked.

In synthesizing this previous work on *Heliconius* mimicry genetics and in incorporating newer observations (Nijhout and Wray 1988; Mallet 1989; Nijhout *et al.* 1990), Nijhout (1991) identified a total of 22 distinct mimicry loci in *H. melpomene* and 17 in *H. erato*. Similar work in *H. cydno* showed that this

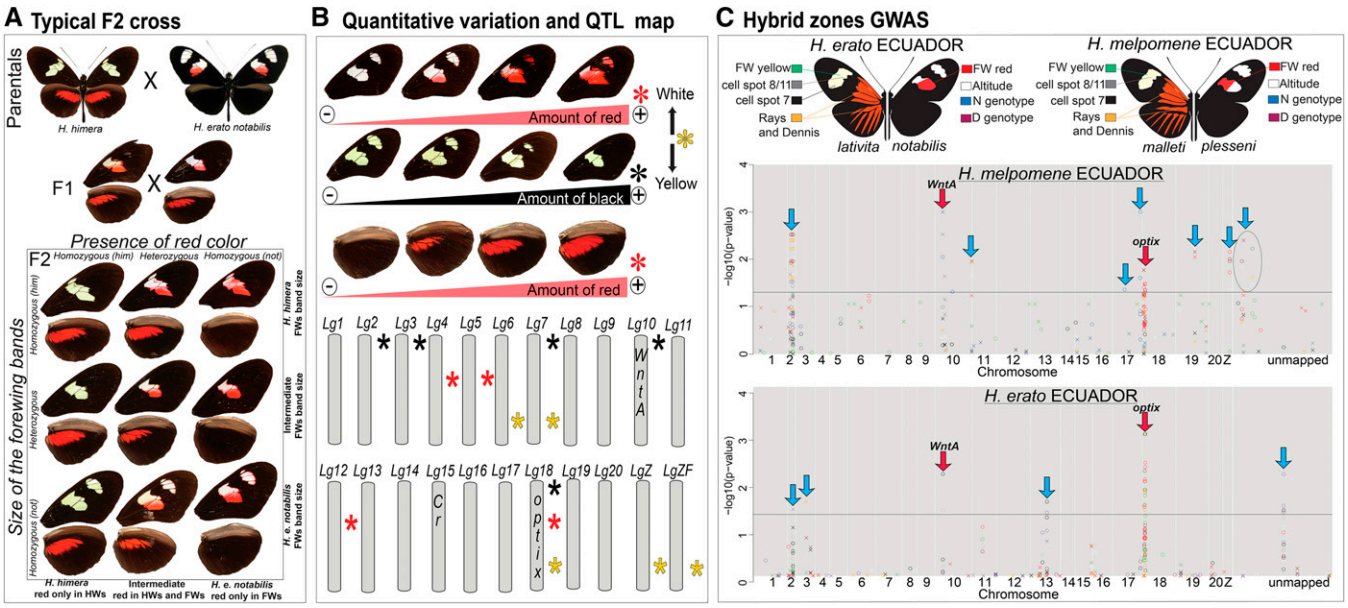

Figure 3 Mapping color-pattern variation in *Heliconius*: Mendelian segregation, quantitative variation, and genome-wide association. (A) An F_2 mapping family from a cross between sister species *H. himera* and *H. erato notabilis* shows Mendelian segregation of black (*Sd* = *WntA*) and red (*D* = *optix*) wing-scale distributions (Papa *et al.* 2013). (B) Quantitative wing-color variation and linkage group distribution of QTL for black, red, and yellow/white color-pattern variation (stars) in the same mapping family. Stars indicate the presence of at least one locus modulating black (black star), red (red star), or yellow/white (yellow star) pattern variation. (C) Genome-wide association study of wing-color-pattern variation in *H. melpomene* and *H. erato* Ecuadorian hybrid zones (Nadeau *et al.* 2014). The different colors of points represent individual SNPs associated with distinct pattern elements shown in the wing images above. Points above the lines represent significant associations. Red arrows indicate the positions of *optix* (*D* locus) and *WntA* (*Sd* locus). Blue arrows indicate the positions of putatively undescribed color-pattern loci, many of which are not shared between *H. melpomene* and *H. erato*.

species had some unique mimicry loci but shared others with *H. melpomene* (Linares 1996, 1997; Merchan *et al.* 2005). Importantly, Nijhout *et al.* (1990) tied color-pattern variation in *Heliconius* back to the highly conserved elements of the nymphalid ground plan. In contrast to previous work, which viewed *Heliconius* wings as a black background overlaid with color-pattern elements, inferences of homology offered by the nymphalid ground plan revealed that on *Heliconius* wings black and red regions are frequently the pattern with white and yellow as the background. Gilbert (2003) expanded on this pattern *vs.* background distinction by developing a unifying model of wing patterning in *Heliconius*. This model, which was based on decades of intra- and interspecific crossing experiments, combined with fundamental correlations among scale ultrastructure and pigmentation (Gilbert *et al.* 1988), characterized all observed wing-pattern variation in terms of three distinct elements: light colored (white or yellow) "windows," melanic or red "shutters" that overlay windows, and melanic "walls" that border the wing and are generally invariant. Gilbert's model also separated wing position from patterning locus, which are separable units that previously had been confounded, thereby disentangling allelic effects from epistasis.

Comparative genetic mapping

A long-term goal of research on *Heliconius* mimicry has been both to determine the genetic basis of adaptation and to infer the extent to which it is conserved over evolutionary time. The earliest crossing experiments in *Heliconius* characterized the very discrete genetic basis of wing patterning, but they were incapable of addressing the question of homology between species or even among different subspecies in many cases. This led to inflation in locus nomenclature, with presumably homologous mimicry loci being given different names in different species and subspecies (hence Nijhout's list of 39 loci in *H. melpomene* and *H. erato* alone). Given that hybridization is relatively common among *Heliconius* species, an initial step in tracing homology over evolutionary time came in the form of interspecific crossing experiments. Jiggins and McMillan (1997) analyzed wing-pattern segregation in crosses between *H. erato* and *H. himera*, Naisbit *et al.* (2003) in crosses between *H. melpomene* and *H. cydno*, and Gilbert (2003) among *H. melpomene*, *H. cydno*, and *H. pachinus*. In combination with the results from prior work, these three studies revealed that the same factors responsible for subspecific variation within *H. erato*, and within *H. melpomene*, also appeared to be responsible for the wing-pattern differences among closely related species. However, sister species, like subspecies, generally display divergent wing patterns, so the results largely reinforced the notion that across various young divergence times the same switch loci seem to control diversification. What about convergence? To address the genetic basis of convergence required comparisons between the two major *Heliconius* subclades because the majority of mimicry occurs between these two lineages. This, however, presented an obstacle because species from these two clades cannot interbreed. Comparative genetic mapping provided an initial step forward.

Genetic linkage maps with homologous markers offered the first glimpse of possible mimicry gene homology among convergent taxa. Beginning with Jiggins *et al.* (2005), Tobler *et al.* (2005), and Kapan *et al.* (2006), complete genetic linkage maps were published for the co-mimics *H. erato* and *H. melpomene*. While built on backbones of anonymous amplified fragment length polymorphism (AFLP) markers, which generally cannot be compared across species or even different mapping studies, the genetic maps of both species included an assortment of gene-based markers, allozymes, and microsatellite markers that could serve as anchors across taxa. These first studies were significant because they localized major mimicry switch loci to specific portions of chromosomes. The first of these loci to be mapped were *H. erato D* (red patterning), *Sd* (forewing melanin shuttering), and *Cr* (hind-wing melanin shuttering), and *H. melpomene Yb* and *Sb* (linked loci controlling hind-wing melanin shuttering) (Table 1). Kronforst *et al.* (2006a) showed that multiple mimicry loci with similar phenotypic effects mapped to homologous chromosomes in the *H. melpomene* and *H. erato* lineages.

Table 1 Summary of major *Heliconius* mimicry loci

Locus	Gene	Chromosome	Phenotype	*H. melpomene*	*H. cydno*	*H. erato*	*H. himera*	*H. numata*
D	*optix*	18	Red/orange at base of dorsal FW/HW	✓	NA	✓	✓	Modifier
B	*optix*	18	Red FW band	✓	NA	✓(*D*)	✓(*D*)	?
R	*optix*	18	Red/orange HW rays	✓	NA	✓(*D*)	✓(*D*)	NA
G	*optix*	18	Red at base of ventral FW/HW	✓	✓	✓(*D*)	✓(*D*)	?
Br	*optix*	18	Brown oval on ventral HW	NA[a]	✓	NA	NA	?
Ac/Sd	*WntA*	10	Distribution of melanin across FW	✓(*Ac*)	✓	✓(*Sd*)	✓(*Sd*)	Modifier
Fs/Ro	?	13	Distribution of melanin in upper FW	✓(*Fs*)	?[b]	✓(*Ro*)	?	?
Yb/Cr	?	15	Distribution of melanin on FW and HW	✓(*Yb*)	✓(*Yb*)	✓(*Cr*)	✓(*Cr*)	Modifier
Sb	?	15	Melanin along HW margin	✓(Sb)	✓(Sb)	✓(*Cr*)	NA	?
N	?	15	Shape of FW band	✓	✓	✓(*Cr*)	?	?
P	?	15	*H. numata* mimicry supergene	NA	NA	NA	NA	✓
K	?	1	White *vs.* yellow wing color	✓	✓	?	?	Modifier

FW, forewing; HW, hind wing.

[a] NA indicates that the phenotype does not exist in that species.

[b] A question mark (?) indicates the mimicry locus is currently not known to influence wing pattern in that species.

Specifically, they generated a genetic map using interspecific crosses between *H. cydno* and *H. pachinus*, close relatives of *H. melpomene*, and used it to localize the positions of three Mendelian mimicry loci: *Yb*, *Ac* (forewing melanin shuttering), and *G* (red wing spots). By using some of the same anchor loci from previous *H. erato* maps, Kronforst *et al.* (2006a) were able to subsequently show that *Yb* and *Cr* mapped to similar positions on homologous chromosomes, as did *Ac* and *Sd* and *G* and *D*. Joron *et al.* (2006b) explored this at a much finer scale and with an added twist, showing that *H. melpomene Yb* and *H. erato Cr* colocalized to within 1 cM of one another (1% recombination) and that this was precisely the same location as the single Mendelian "supergene" that controls all wing-pattern variation in *H. numata* (Table 1). In *H. melpomene*, red-wing patterning is thought to be controlled by tightly linked but separate loci, the *B* and *D* loci. Using a similar approach of comparative fine-mapping, Baxter *et al.* (2008) showed that *H. melpomene B/D* mapped to the same genomic location as *H. erato D*. It turns out this is the same location as the *H. cydno/pachinus G* locus (Chamberlain *et al.* 2011).

These comparative genetic mapping experiments took the critical first steps toward positionally cloning *Heliconius* mimicry loci, and they also provided preliminary evidence of probable homology among co-mimetic species. Indeed, much of the wing-pattern variation across the genus was quickly tracing back to just a handful of discrete genomic intervals. Beyond that, this research was instrumental in developing genomic resources in *Heliconius*. As part of their genetic mapping efforts, research teams generated bacterial artificial chromosome libraries for *H. melpomene* and *H. erato*, using these to generate targeted reference sequences across focal mimicry intervals, as well as expressed sequence tag (EST) data for annotation and analyses of gene expression (Papanicolaou *et al.* 2005; Joron *et al.* 2006b; Kapan *et al.* 2006; Pringle *et al.* 2007; Baxter *et al.* 2008; Papa *et al.* 2008b; Reed *et al.* 2008; Ferguson and Jiggins 2009; Ferguson *et al.* 2010; Wu *et al.* 2010; Surridge *et al.* 2011; Hines *et al.* 2012). These genomic resources ultimately led to the identification of the first *Heliconius* mimicry genes and the beginning of the *Heliconius* Genome Project.

Molecular characterization of mimicry loci

Optix: Comparative genetic mapping across *H. erato*, *H. melpomene*, and *H. cydno/H. pachinus* showed that various loci controlling red wing patterning (*D*, *B/D*, *G*) all mapped to the same genomic location, suggesting that species across the genus used the same gene or set of tightly linked genes to control red wing-pattern variation. The first surveys of population genetic variation among color-pattern races (Baxter *et al.* 2010; Counterman *et al.* 2010) or closely related species (Chamberlain *et al.* 2011) revealed striking signatures of genetic differentiation in this genomic interval, with a *kinesin* gene initially showing the strongest genotype–phenotype associations. This same gene also showed differential gene expression between divergent color-pattern phenotypes in both *H. melpomene* and *H. erato* (Baxter *et al.* 2010; Counterman *et al.* 2010).

It turns out that the population genetics and expression variation associated with this *kinesin* gene are relatively small in comparison to the adjacent gene, *optix*. Using a tiling array, Reed *et al.* (2011) examined expression of the entire 700-kb *D* locus interval in *H. erato*, comparing segments of individual wings that would eventually be red, black, and yellow. This revealed a striking pattern of *optix* expression associated with red patterning, an expression pattern further corroborated via *in situ* hybridization (Figure 4) and immunohistochemistry (Martin *et al.* 2014). Across all *Heliconius* and other closely related species, *optix* appears to be the critical gene specifying red wing patterning. Outside of this clade, *optix* does not appear to control wing patterning although it does appear to play an ancient, conserved role in specifying wing-coupling scales on ventral forewings and dorsal hind wings (Reed *et al.* 2011; Martin *et al.* 2014). Red-pattern variation seems to be the product of regulatory variation upstream of the *optix*-coding sequence because there is little coding sequence variation in the gene among *Heliconius* butterflies (Reed *et al.* 2011) and SNP association tests (Supple *et al.* 2013), as well as genome scans comparing the strength of genetic differentiation among closely related species (Nadeau *et al.* 2012), consistently point to the noncoding region between *optix* and *kinesin* (Figure 4). Interestingly, Pardo-Diaz and Jiggins (2014) recently resurrected the link between *kinesin* and red wing patterning by showing that both *optix* and *kinesin* contribute to red pigmentation. Unlike *optix*, which is associated with all red patterning, the *kinesin* gene appears to be expressed only in the red forewing band of the postman phenotype.

WntA: Similar to red patterning, melanic pattern variation on the forewing of various *Heliconius* species is Mendelian and has been shown to map to similar positions on homologous chromosomes (Kronforst *et al.* 2006a). Martin *et al.* (2012) showed that across *H. erato*, *H. melpomene*, and *H. cydno*, forewing melanic variation mapped to the gene *WntA*, and *WntA* expression on larval wing discs prefigured future adult melanin patterning (Figure 5). *WntA*, like *wingless* and other *Wnt* ligands, is an extracellular signaling molecule that presumably acts as a morphagen during wing-pattern specification. Heparin is known to potentiate *Wnt* signaling and Martin *et al.* (2012) showed that by injecting heparin sulfate into developing pupal wing discs, they were able to enhance melanization across the wing and produce allelic phenocopies (Figure 5). In line with the *optix* results, *WntA* shows little protein sequence change across all *Heliconius* species and color pattern forms, again implicating *cis*-regulatory changes in the evolution of novel wing-pattern phenotypes (Martin *et al.* 2012).

Recently, Gallant *et al.* (2014) stepped outside *Heliconius* on the butterfly phylogeny and found that *WntA* also controls a similar melanic shutter in *Limenitis* butterflies. Nonmimetic *L. arthemis arthemis* is black with white bands on both the fore- and hind wings while the subspecies *L. arthemis astyanax* mimics the toxic pipevine swallowtail, *Battus philenor*, and is

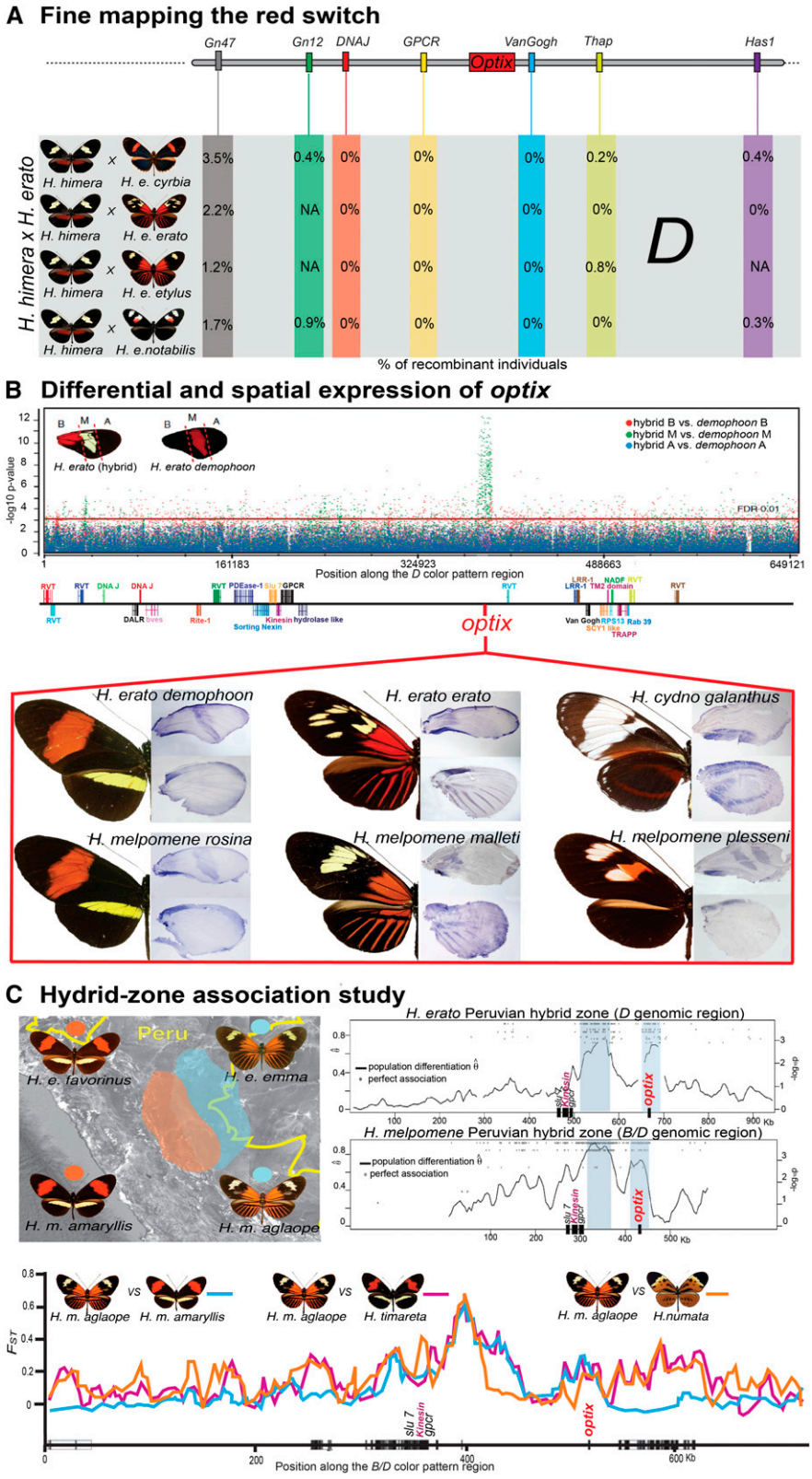

Figure 4 The molecular basis of red wing patterning in *Heliconius*. (A) Genetic mapping of red wing color-pattern variation (percentage of recombinants at several genes is shown) across multiple *H. erato* × *H. himera* families points to a narrow genomic interval containing the transcription factor *optix*. (B) Comparison of gene expression between three forewing color-pattern sections of two *H. erato* morphs using a *D*-locus tiling array suggests *optix* as the gene regulating red patterning (Reed *et al.* 2011). Messenger RNA expression (*in situ* hybridization) of *optix* on pupal wing discs of different *Heliconius* species spatially prefigures adult red wing patterning (Reed *et al.* 2011). (C) Targeted analyses of SNP associations and genetic differentiation (F_{ST}) in *H. erato* and *H. melpomene* Peruvian hybrid zones (geographic distributions shown in the map) suggest two genomic regions, one centered on *optix* and another upstream of *optix*, strongly associated with red wing color-pattern variation (Supple *et al.* 2013). Patterns of genetic differentiation across the *B/D* interval between *H. melpomene* subspecies and closely related species also reveal enhanced divergence in these two genomic regions (bottom) (Nadeau *et al.* 2012).

all black. Using a combination of fine-mapping, *in situ* hybridization, RNA-seq, and heparin injections, Gallant *et al.* (2014) showed that the Mendelian melanin switch in *Limenitis* is also controlled by *WntA*. Furthermore, by sequencing and comparing 30 *Limenitis* genomes, Gallant *et al.* (2014) identified 173 SNPs and a 9-kb LINE upstream of the *WntA*-coding sequence that were perfectly associated with wing-pattern phenotype, again pointing to *cis*-regulatory variation. Parallel

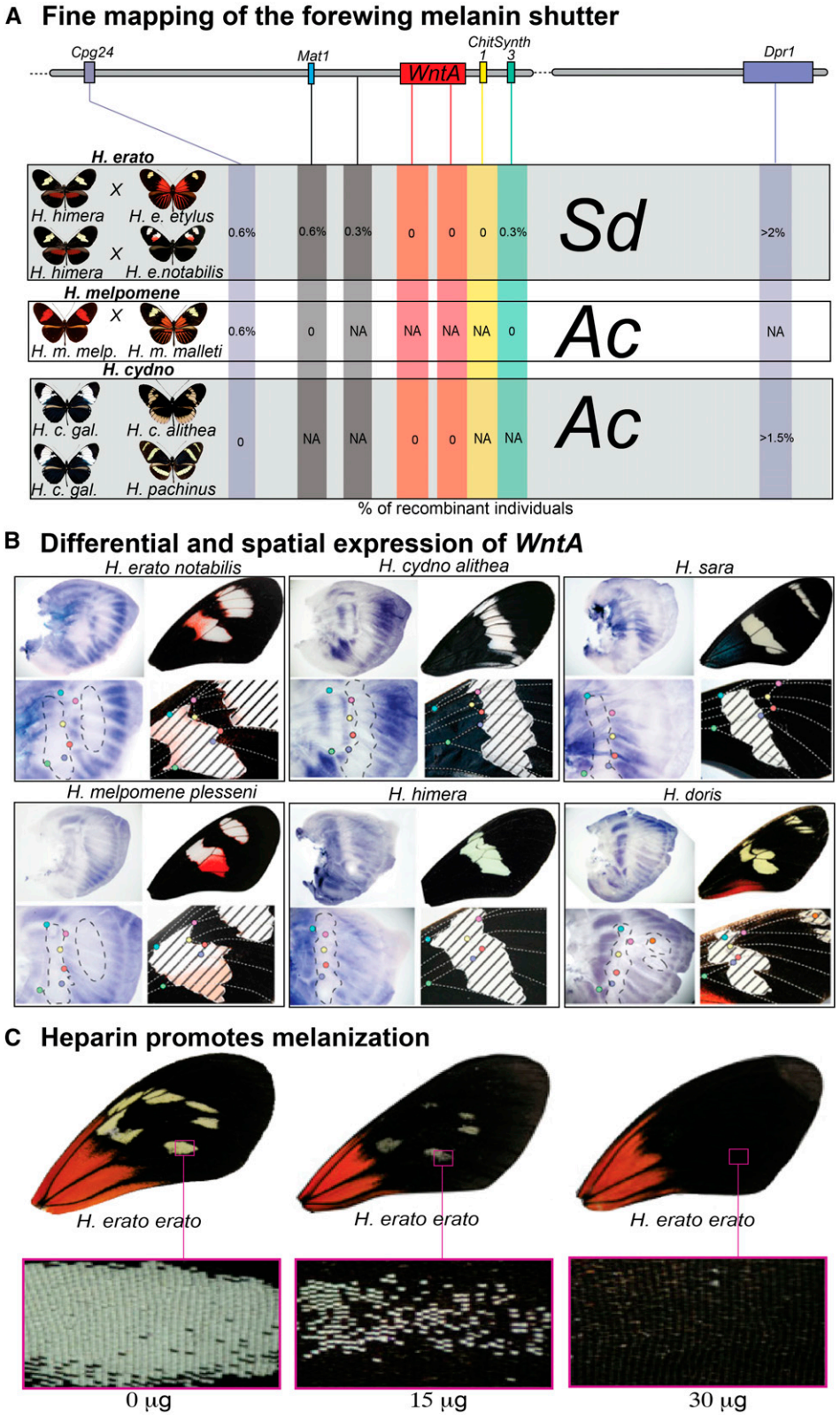

Figure 5 The molecular basis of melanin patterning in *Heliconius*. (A) Genetic mapping of forewing melanin variation across different families of several *Heliconius* species (percentage of recombinants at several genes is shown) points to a narrow genomic interval containing the gene *WntA* (Martin *et al.* 2012). (B) Spatial expression (*in situ* hybridization) of *WntA* on larval wing discs prefigures adult melanin patterning and confirms the role of *WntA* in forewing black-scale variation (Martin *et al.* 2012). (C) Pupal injection of heparin sulfate, which is known to extend *Wnt* signaling, enhances wing melanization (Martin *et al.* 2012), further supporting the idea that *WntA* controls melanin patterning in *Heliconius*.

analysis of 45 *Heliconius cydno* genomes identified a single 1.8-kb indel upstream of *WntA* that was perfectly associated with wing pattern, the position of which overlapped the position of the LINE in *Limenitis* (Gallant *et al.* 2014). Surprisingly, it appears that similar phenotypes have originated independently in *Heliconius* and *Limenitis* butterflies from functionally similar mutations targeting the same region upstream of *WntA*.

P locus supergene in H. numata: Few aspects related to mimicry genetics have received as much interest as supergene mimicry (Schwander *et al.* 2014; Thompson and Jiggins 2014). In a number of polymorphic species, the entire wing pattern is controlled by a single Mendelian locus, and this extreme genetic architecture has been dubbed a "supergene" (Fisher 1930; Clarke and Sheppard 1960; Charlesworth and Charlesworth 1975). There is a long history of work on supergenes in the context of both butterfly mimicry and self-incompatability loci in plants (Schwander *et al.* 2014; Thompson and Jiggins 2014). Clarke and Sheppard performed a large series of crossing experiments exploring supergene mimicry, primarily in *Papilio* swallowtail butterflies, and they envisioned supergenes as clusters of tightly linked loci brought together via interchromosomal translocation due to natural selection against maladaptive recombination (Clarke and Sheppard 1960; Thompson and Jiggins 2014). Charlesworth and Charlesworth (1975) explored the dynamics of supergene evolution using computer simulations and showed that the hypothesized translocation mechanism was unlikely because unlinked loci would not remain polymorphic in a population for very long. Rather, Charlesworth and Charlesworth (1975) proposed that distinct loci must be fairly tightly linked to begin with for natural selection to further reduce recombination and tighten them into a supergene.

Heliconius generally do not exhibit supergene mimicry, but there is one highly polymorphic species, *H. numata*, which does (Brown and Benson 1974). In a series of detailed molecular investigations, Joron *et al.* (2006b, 2011) characterized the *H. numata* supergene, the *P* locus, and provided the very first window into this long-term evolutionary enigma (Figure 6). Comparative fine-mapping among *H. numata* and other *Heliconius* species showed that the *P* locus maps to the location of a mimicry locus that is conserved across the genus (Joron *et al.* 2006b). More so, in *H. numata*'s close relative *H. melpomene*, this region contains three tightly linked but separable mimicry loci: *Yb*, *Sb*, and *N* (Joron *et al.* 2006b). This appears to be precisely in line with the results of Charlesworth and Charlesworth (1975); the supergene in *H. numata* may have evolved by the tightening of linkage among previously linked loci. Joron *et al.* (2011) discovered that chromosomal rearrangements were ultimately responsible for enhanced linkage at the *P* locus with various color-pattern morphs being associated with different chromosomal inversions (Figure 6C). Which of the genes contained in these inversions ultimately contribute to color pattern remains a mystery, but the inference is that multiple distinct elements in this region contribute to the phenotype and the inversion polymorphism greatly reduces recombination among these elements.

Modifiers: Much of the focus in the study of *Heliconius* mimicry has been on a handful of large-effect Mendelian switch loci. However, while certain major color-pattern elements segregate in a Mendelian fashion, overall color pattern is a complex trait ultimately controlled by allelic combinations at multiple unlinked large- and small-effect loci across the genome (Figure 3). This is apparent from multiple recent investigations that found widespread evidence for small-effect QTL and/or modifier loci influencing *Heliconius* wing-pattern mimicry (Baxter *et al.* 2009; Jones *et al.* 2012; Papa *et al.* 2013). Using a single cross between two *H. melpomene* races that differ in the size of their red forewing band, Baxter *et al.* (2009) found that at least 6 of 21 chromosomes influenced quantitative variation in this trait, including those now known to contain *optix* and *WntA*. Similarly, Jones *et al.* (2012) examined quantitative variation in crosses among *H. numata* morphs and found that 6 chromosomes, in addition to the supergene locus *P*, influenced color-pattern variation. In the case of *H. numata*, it is possible that many of these small-effect QTL will ultimately trace back to the major Mendelian switch loci present in other *Heliconius* species because 3 of the 6 chromosomes included those housing the *K*, *B/D* (*optix*), and *Ac* (*WntA*) loci. In the broadest survey of its kind, Papa *et al.* (2013) used a series of crosses between *H. himera* and different color-pattern races of *H. erato* to show that a number of the major switch loci previously thought to be distinct actually map to *optix* and *WntA*. Furthermore, Papa *et al.* (2013) showed that a substantial amount of variation not captured by these switch loci could be traced back to additional QTL scattered throughout the genome (Figure 3B). It is worth noting another source of complexity that has recently come to light: distinct but functionally similar color-pattern alleles in the same species, indicative of independent origins of the same phenotype or developmental drift over relatively short timescales (Maroja *et al.* 2012).

Overall, the current picture of *Heliconius* mimicry genetics may lend support to classic theoretical expectations. Punnett (1915), Nicholson (1927), and Turner (1977b, 1981) proposed a two-step model for the evolution of Müllerian mimicry whereby an initial large mutation would move a phenotype from one mimicry ring to another, after which additional smaller changes would refine mimetic resemblance. The combination of large-effect switch loci and small-effect modifiers that we see in the genetic control of *Heliconius* wing patterning is consistent with this model, but the order in which they occurred is an important aspect of the two-step model, which we currently know little about. It is interesting to note that, in a recent analysis of divergence across hybrid zones between color-pattern races of *H. erato* and *H. melpomene*, Nadeau *et al.* (2014) found strong divergence centered on large-effect mimicry loci in both species, but divergence associated with putative modifiers differed between species (Figure 3C). This result may indicate that large-effect loci are conserved among species but modifiers are not. In the end, we have come full circle in some respects in our view of *Heliconius* mimicry genetics; while originally believed to involve many switch loci, modern investigations have caused much of that variation to coalesce into a handful of loci but also have revealed the widespread action of unappreciated modifiers and quantitative variation.

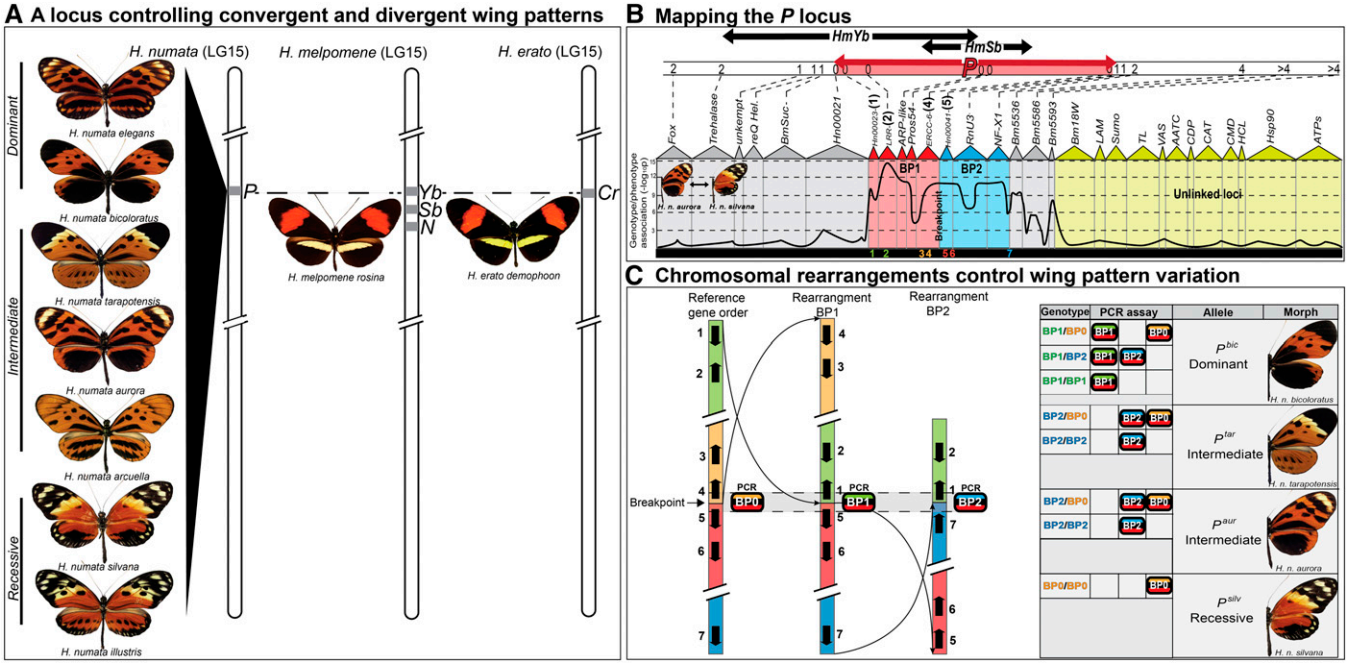

Figure 6 Characterizing the *H. numata* mimicry supergene. (A) A single Mendelian locus with multiple alleles (the *P* locus) controls wing-pattern diversity in *H. numata*. The *P* locus is positionally homologous to the *Yb-Sb-N* loci of *H. melpomene* and the *Cr* locus of *H. erato* (Joron *et al.* 2006b). (B) Fine-mapping and SNP associations narrow the *P* locus to a 400-kb interval spanning 31 genes and provide evidence of highly reduced recombination (red and blue areas) (Joron *et al.* 2011). Relative position of the genes (1–7) across the interval that were used to characterize genomic rearrangements is also shown. (C) Allelic variation at the *P* locus ultimately traces back to an inversion polymorphism with different wing-pattern morphs determined by distinct, nonrecombining haplotypes (Joron *et al.* 2011). PCR assay of the alternative breakpoints BP0, BP1, and BP2 (left) are perfectly associated with mimicry variation across four distinct morphs in eastern Peru (right).

The Molecules Matter

In an effort to characterize the mutational basis and evolutionary history of putatively adaptive phenotypic variation, evolutionary biologists are increasingly focusing on identifying the genes and causative molecular variation underlying traits of interest (Hoekstra and Coyne 2007; Nadeau and Jiggins 2010). Many of the major questions posed by the radiations and repeated instances of convergence seen in *Heliconius* ultimately rest on the identification and comparison of mimicry genes among species. For example, as noted above, biologists have long pondered the functional basis of supergenes and work on *H. numata*, and comparison to *H. melpomene* and *H. erato*, have provided the first insight into the molecular basis of supergene mimicry (Joron *et al.* 2006b, 2011). Similarly, our understanding of the molecular basis of mimicry in *Heliconius* provides genuine insight into a variety of additional evolutionary phenomena, including constraint and evolvability, the genetic basis of convergence, the potential of introgression to facilitate adaptation, the mechanisms of hybrid speciation in animals, and the process of ecological speciation.

Constraint vs. evolvability

Heliconius mimicry presents an enigma: the entire genus appears to use the same small number of large-effect switch loci, an apparent genetic constraint, yet this does not appear to constrain phenotype in any way as wing pattern is highly evolutionarily labile within and among species. In fact, there appears to be a virtually unlimited number of possible wing-pattern phenotypes available to them (Gilbert 2003). Potential evidence of genetic constraint appears at a deeper level, too, as the same narrow noncoding region upstream of the gene *optix* is most strongly associated with red wing patterning in both *H. erato* and *H. melpomene* (Supple *et al.* 2013). Furthermore, structural changes in the same or similarly located regulatory elements upstream of *WntA* appear to be responsible for melanic variation in both *Heliconius* and *Limenitis* butterflies (Gallant *et al.* 2014). Overall, the mutational targets available for color-pattern change appear to be narrow. While one might speculate if and how genetic or developmental constraints could limit evolutionary potential, *Heliconius* mimicry provides an example of apparently unlimited phenotypic potential operating over a constrained genetic system. This may suggest that genetic constraints are not imposed by mutational target size, and/or genetic constraints may not limit phenotypic evolution. *Heliconius* may circumvent apparent genetic constraints via the evolution of secondary modifiers or by modulating expression of the large-effect mimicry genes themselves. We currently have little information about the specific *cis*-regulatory elements that control *Heliconius* mimicry gene expression. However, it is likely that the architecture of these elements permits extensive phenotypic variation to emerge from each major switch locus.

Genetic basis of convergence

Examples of phenotypic convergence exist at many taxonomic levels in *Heliconius*, and having the genes responsible

for mimicry in hand finally allows us to dissect the evolutionary history of convergent/parallel evolution in the context of mimicry. At the smallest taxonomic scale—within species—we see in both *H. erato* and *H. melpomene* a perplexing scenario whereby geographically disjunct populations display nearly identical color patterns, which has led to speculation that similar color patterns may have arisen multiple times within each species (Brower 1994). For example, the red and yellow banded postman phenotype occurs throughout Central America, a large section of Brazil, and in isolated patches of Peru and Colombia (Figure 2). Genetic markers not linked to mimicry loci, such as mitochondrial DNA (Brower 1994, 1996b) and nuclear markers (Flanagan *et al.* 2004; Quek *et al.* 2010), consistently revealed phylogeographic patterns in both *H. erato* and *H. melpomene* that grouped subspecies by geography rather than color pattern, further fueling speculation of possible intraspecific convergence. In sharp contrast, however, sequence variation at the *optix* gene itself reveals a very different story, clustering subspecies by color pattern (Figure 7A) and showing that each wing pattern, including the postman phenotype, originated a single time in each species (Hines *et al.* 2011; Supple *et al.* 2013). This is a striking case in which analysis of the causative gene offered unique insight into an evolutionary history that was not visible in the rest of the genome (Turner *et al.* 1979; Hines *et al.* 2011; Supple *et al.* 2013). It is possible that the histories of different genomic segments are decoupled due to gene flow among *Heliconius* subspecies and closely related species.

At the other end of the taxonomic continuum is mimicry among the most distantly related *Heliconius* lineages, such as between *H. erato* and *H. melpomene*. This appears to be genuine convergence because even though there is evidence that these species are using the same genes, and possibly even the same regulatory regions, to generate matching color patterns (Supple *et al.* 2013), analysis of sequence variation at those genes shows no shared variation between species, indicating independent origins of co-mimetic phenotypes (Figure 7B). Between these two taxonomic extremes are co-mimetic species from the same *Heliconius* subclade, such as *H. melpomene* and *H. timareta*. In one of the more recent discoveries related to *Heliconius* mimicry, we now know that these closely related co-mimics use not only the same genes but also the exact same sequence variation to generate convergent wing patterns, not because of constraint or convergent molecular evolution, but because of adaptive gene flow between species.

Adaptive introgression

Brower (1996a) described a new species of *Heliconius*, *H. tristero*, that had the color pattern of *H. melpomene* but grouped with *H. cydno* based on DNA sequence and other morphological data. This was unexpected because *H. melpomene* and *H. cydno* are closely related, partially interfertile species, and their divergent color patterns have been shown to play an important role in mediating reproductive isolation. Giraldo *et al.* (2008), Merot *et al.* (2013), and Nadeau *et al.* (2014) have since revealed this to be a general phenomenon: there are populations of the *H. cydno* relative *H. timareta* mimicking *H. melpomene* all along the western side of the Andes mountains. Because hybridization between *H. melpomene* and the *H. cydno/timareta* clade is widespread, interspecific gene flow could be the source of their shared warning patterns. Indeed, Gilbert (2003) showed that much *Heliconius* color diversity can be recreated by interspecific hybridization. Another example where this may have occurred is *H. elevatus*, a species in the silvaniform clade that mimics *H. melpomene*, because there is evidence of hybridization and gene flow between these lineages as well (Dasmahapatra *et al.* 2007; Mallet *et al.* 2007; Kronforst 2008). Recent genomic analyses, including *de novo* assembly of the *H. melpomene* genome and targeted resequencing of mimicry loci from a variety of taxa, paired with analyses of SNP allele sharing, sequence divergence, and phylogenetic patterns (*Heliconius* Genome Consortium 2012; Pardo-Diaz *et al.* 2012; Smith and Kronforst 2013), point to adaptive introgression of color-pattern mimicry among *H. melpomene*, *H. timareta*, and *H. elevatus* (Figure 7C). It is worth noting that mimicry involves more than wing pattern: it potentially involves many aspects of ecology and locomotion, and it remains to be seen whether genes that contribute to these aspects of mimicry have also moved between species.

Hybrid speciation

Hybrid speciation is generally thought to be rare in animals (Mallet 2007; Mavarez and Linares 2008), but the process is facilitated when traits that mediate reproductive isolation are influenced in a direct way by hybridization (Jiggins *et al.* 2008). Divergent wing patterns generate multiple forms of reproductive isolation in *Heliconius*, both prezygotic and postzygotic. Since hybridization can transport color patterns between species (*Heliconius* Genome Consortium 2012; Pardo-Diaz *et al.* 2012), or recombine between species to produce totally new phenotypes (Gilbert 2003), this provides a mechanism by which hybridization may contribute to the evolution of reproductive isolation and the origin of species. There are multiple suspected cases of this phenomenon in the genus, the best documented of which is *Heliconius heurippa* (Salazar *et al.* 2005, 2008, 2010; Mavarez *et al.* 2006; Melo *et al.* 2009). *H. heurippa* has an intermediate, nonmimetic wing pattern that can be recreated by interbreeding red-banded *H. melpomene* and a yellow-banded species, either *H. cydno* or *H. timareta* (Mavarez *et al.* 2006). Furthermore, in mate choice trials, *H. heurippa* individuals prefer to approach, court, and mate with individuals that share their recombined wing pattern, as opposed to those of the parental species (Mavarez *et al.* 2006). Amazingly, experimentally recreated *H. heurippa*, produced by interbreeding *H. melpomene* and *H. cydno*, show similar assortative mate preference for the *H. heurippa* wing pattern (Melo *et al.* 2009). Detailed molecular genetic characterization further supports the hybrid origin of *H. heurippa*, showing that this species has a

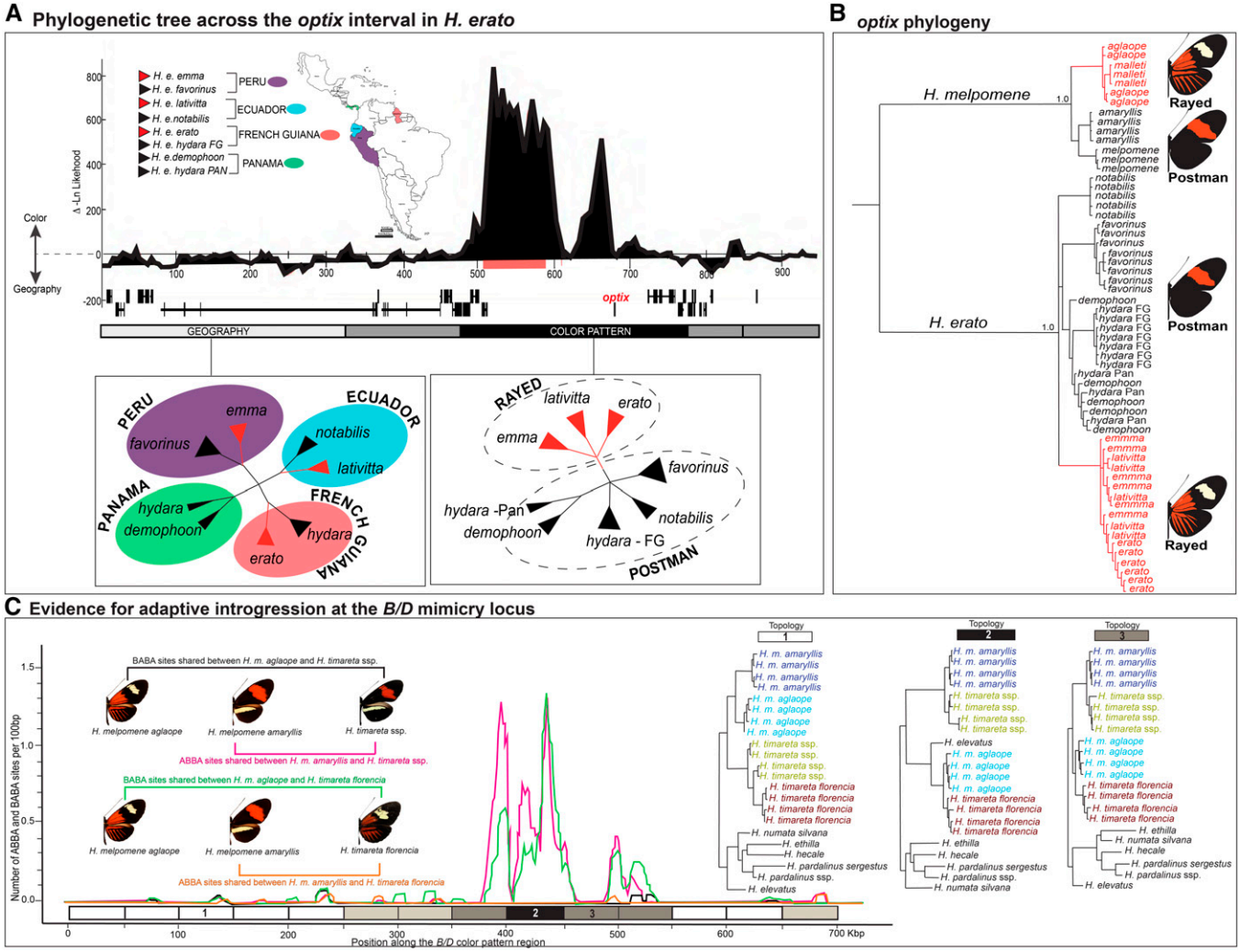

Figure 7 Tracing the evolution of *Heliconius* mimicry. (A) Genetic variation across most of the genome clusters *H. erato* races by geography, but the genomic region around *optix*, which controls red wing patterning, groups races by phenotype (Supple *et al.* 2013). A similar phenomenon occurs in *H. melpomene* (Hines *et al.* 2011). (B) However, wing patterns shared between *H. melpomene* and *H. erato* are due to convergent evolution because there is no shared genetic variation between these two distantly related species. (C) Wing-pattern mimicry has been passed among closely related co-mimics *H. melpomene*, *H. timareta*, and *H. elevatus* by interspecific hybridization (*Heliconius* Genome Consortium 2012). Evidence for adaptive introgression includes an enrichment of shared alleles (ABBA and BABA sites) near *optix* and phylogenetic clustering among phenotypes across species boundaries (topology 2).

genome largely derived from the *H. cydno/H. timareta* clade but with a contribution from *H. melpomene* near the *optix* and *kinesin* genes that generate its red forewing band (Salazar *et al.* 2008, 2010).

H. elevatus provides a second likely example of potential hybrid speciation. Genomic analysis places *H. elevatus* as a very recently diverged taxon nested within another species, *H. pardalinus*, but it appears to have recently acquired its entire wing pattern from *H. melpomene* (*Heliconius* Genome Consortium 2012). Such rapid, recent divergence associated with mimicry introgression is suggestive of a hybrid speciation scenario although more work needs to be done to clarify the details. Currently, we do not know how many times color-pattern alleles have moved between species or how many times this may have contributed to speciation. However, based on everything we do know about *Heliconius*, it could be quite common.

Ecological speciation

Ecological speciation is a phenomenon by which divergent ecological selection contributes to the evolution of reproductive isolation between populations, eventually leading to the origin of species. Owing to wing patterning and divergence for mimicry, *Heliconius* is likely to provide multiple examples of ecological speciation in action (Jiggins 2008). Recently, genome sequencing, combined with our detailed understanding of the genetic basis of wing patterning, has permitted a new take on the question of ecological speciation in *Heliconius*, again finding that color patterns appear to drive divergence between species. Using various genome-wide sequencing

approaches, from restriction site associated DNA (RAD) markers to full-genome resequencing, Nadeau *et al.* (2012, 2013), Kronforst *et al.* (2013), and Martin *et al.* (2013) examined patterns of divergence and gene flow among *H. melpomene*, *H. cydno*, and related species. Despite focusing on different taxa, geographic locations, and analytical methods, these studies all converged on a combined role for gene flow and selection in the speciation process. Notably, this work also found that the most recently diverged *Heliconius* taxa—subspecies, incipient species, and sister species—show the most pronounced divergence at color-pattern loci. This is consistent with the hypothesis that divergence for mimicry generates early reproductive isolation that eventually results in speciation. It is perhaps remarkable that lessons learned from decades of detailed behavioral and field studies are today born out in comparisons of the $>$ 100 *Heliconius* genome sequences thus far analyzed.

Conclusions

The study of butterfly mimicry has been interwoven with the theory of natural selection since its origin $>$150 years ago (Darwin and Wallace 1858). Identifying the molecules behind mimicry is now allowing us to test some long-standing questions about general evolutionary principles, and the answers so far are surprising. From here, research focused on *Heliconius* mimicry genetics is likely to progress in a variety of directions, moving both deeper into mechanistic details and expanding out in a more comparative context. At a functional level, we still lack an understanding of the molecular, cellular, and developmental mechanisms that link mimicry genes to mimetic wing patterns. At a comparative level, the surface has just been scratched, but the initial results are fascinating, revealing for example, that *optix* has only very recently been co-opted to a role in color patterning in *Heliconius* and relatives (Monteiro 2012; Martin *et al.* 2014) while *WntA* appears to have a much older, more fundamental role in butterfly wing patterning (Martin and Reed 2014). In addition, a notable strength of the *Heliconius* system, provided by a long history of excellent field work, is that we know so much about the amazing behavior, ecology, and biogeography of the many diverse species in the genus. Perhaps the most exciting future prospect is to take this newly acquired genetic and genomic information back to the field to explore age-old questions in a classic system using totally new techniques.

Acknowledgments

We thank the long and venerable line of *Heliconius* researchers whose discoveries have made it the wonderful and tractable research system it is today and the *Heliconius* Genome Consortium, in particular Jim Mallet, Chris Jiggins, and Owen McMillan, for pushing modern, comparative genomics work forward. It is notable that much of the *Heliconius* biology that we review here, including chemical defense and subsequent warning coloration and mimicry, traces back to their host plant associations and peculiar pollen-feeding behavior. This review is dedicated to Larry Gilbert, whose discovery of pollen feeding in *Heliconius* was published 43 years ago.

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