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SHORT REVIEW

Heliconius wing patterns: an evo-devo model for understanding phenotypic diversity

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Evolutionary Developmental Biology aims for a mechanistic understanding of phenotypic diversity, and present knowledge is largely based on gene expression and interaction patterns from a small number of well-known model organisms. However, our understanding of biological diversification depends on our ability to pinpoint the causes of natural variation at a micro-evolutionary level, and therefore requires the isolation of genetic and developmental variation in a controlled genetic background. The colour patterns of *Heliconius* butterflies (Nymphalidae: Heliconiinae) provide a rich suite of naturally occurring variants with striking phenotypic diversity and multiple taxonomic levels of variation. Diversification in the genus is well known for its dramatic colour-pattern divergence between races or closely related species, and for Müllerian mimicry convergence between distantly related species, providing a unique system to study the development basis of colour-pattern evolution. A long history of genetic studies has showed that pattern variation is based on allelic combinations at a surprisingly small number of loci, and recent developmental evidence suggests that pattern development in *Heliconius* is different from the eyespot determination of other butterflies. Fine-scale genetic mapping studies have shown that a shared toolkit of genes is used to produce both convergent and divergent phenotypes. These exciting results and the development of new genomic resources make *Heliconius* a very promising evo-devo model for the study of adaptive change.
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Heliconius butterflies: an emerging evo-devo model

Evolutionary developmental biology aims for a mechanistic understanding of the origins of phenotypic diversity. How are developmental pathways modified to produce evolutionary novelty? What are the genes that underlie evolutionary radiations or adaptive change? Do they share particular characteristics or modes of actions? The homeotic genes provide us with insights into major transitions in animal body plans and how genetic networks are modified in diverse taxa (Carroll *et al*, 2001; Davidson, 2001; Wilkins, 2002). In general, however, the evo-devo community has been slower to tackle the origins of recent evolutionary novelty (but see Bradshaw *et al*, 1998; Stern, 1998; Mundy *et al*, 2004; Colosimo *et al*, 2005; Gompel *et al*, 2005; Prud'homme *et al*, 2006). Such questions cannot be answered by studying a few distantly related model species. Instead, comparative studies are needed of closely related species that differ in traits of interest and require the development of new model systems that show great diversity among closely related species and forms but that are also amenable to evolutionary and ecological studies.

Butterfly wing patterns are excellent subjects for evo-devo studies because the patterns are structurally simple, highly variable, and, in many cases, the evolutionary and ecological significance of the pattern is well understood (see Brakefield *et al*, 1996; Beldade and Brakefield, 2002; McMillan *et al*, 2002; Beldade *et al*, 2005 for recent reviews). This is particularly true in the passion-vine butterflies, *Heliconius* (Nymphalidae: Heliconiinae), which combine extensive natural variation in colour pattern with a strong history of ecological and evolutionary research (eg, Benson, 1972; Gilbert, 1972; Brown, 1981; Mallet and Barton, 1989b; Joron *et al*, 1999; Jiggins *et al*, 2001; Kapan, 2001; Flanagan *et al*, 2004; Langham, 2004).

The group, composed of 40 species and hundreds of geographic variants across the Neotropics, shows significant variation in their wing patterns at every biological level from divergent species to sympatric colour morphs of the same species (Figure 1). The vivid colour patterns of *Heliconius* are adaptations that warn potential predators of the butterflies' unpalatability (Bates, 1862; Langham, 2004), presumably related to their evolutionary history in association with cyanogenic foodplants in the Passifloraceae (Brown, 1981; Engler *et al*, 2000). Nearly all *Heliconius* species participate in Müllerian mimicry association, such as the orange-rayed

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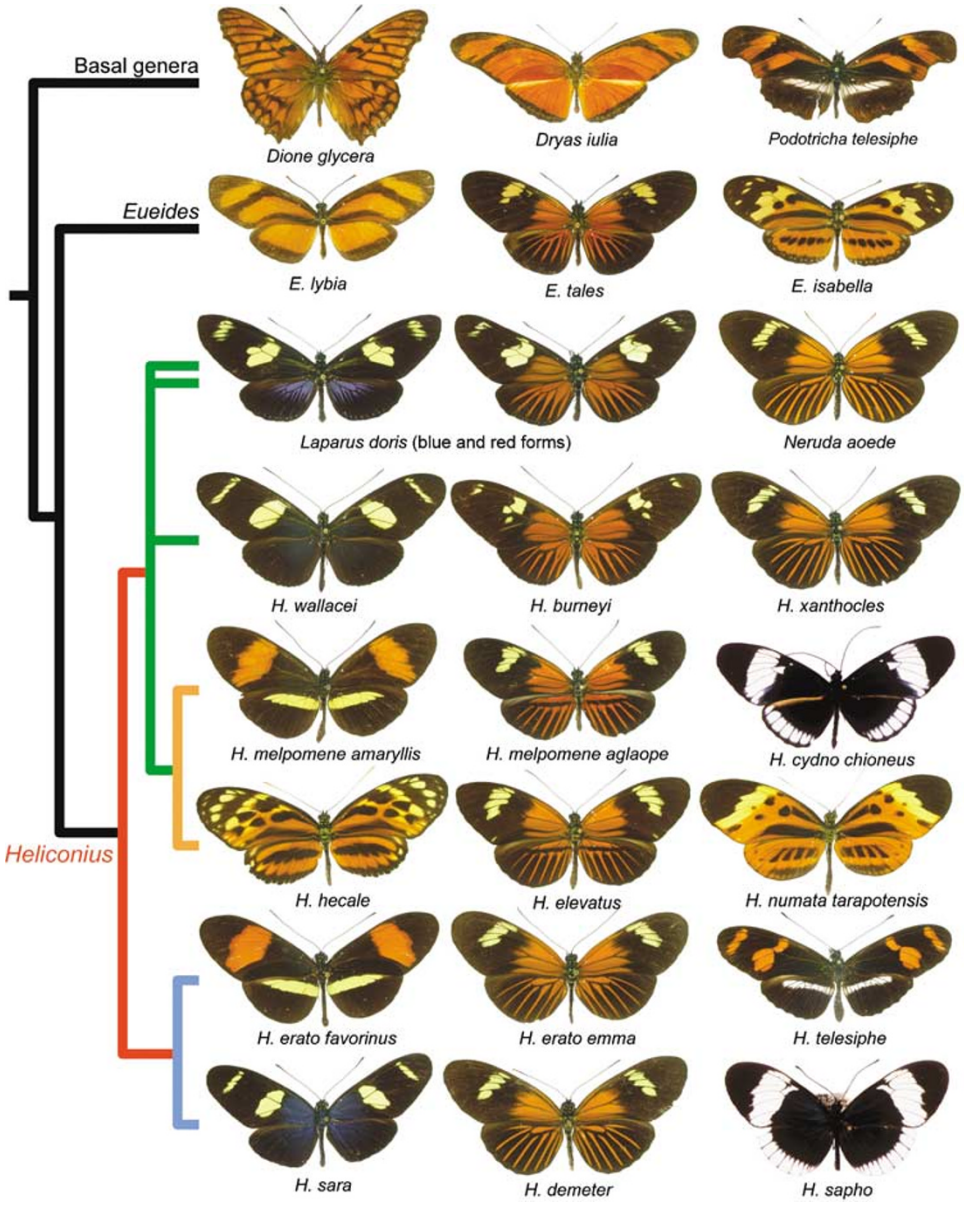

Figure 1 A sample of the morphological diversity of wing patterns in *Heliconius* and related genera. Each row represents a phylogenetic clade in the tribe Heliconiini. The phylogram on the left is a topology derived from mitochondrial and nuclear trees (Beltrán *et al*, 2002), highlighting the deeply diverged 'melpomene' (orange) and 'erato' (blue) clades of the genus. The phylogenetic position and consequent nomenclature of *Neruda* and *Laparus* are controversial and considered to fall within *Heliconius* on this figure (green). The figure highlights the rampant pattern diversification within clades and species, and mimicry between clades. Reconstruction of ancestral wing patterns is difficult for such rapidly evolving traits. It may be the case that the orange-rayed pattern is ancestral, in which case the other mimetic patterns are convergent derived patterns. However, it has also been suggested that ancestral *Heliconius* were more similar to the so-called 'postman' pattern of *H. melpomene amaryllis*, in which case the rayed pattern has evolved repeatedly.

Amazonian mimicry ring which involves up to nine species of Heliconiines, or with other butterflies such as the ubiquitous tiger-striped Ithomiinae (Nymphalidae) in lowland and premontane forests of tropical America (Brown and Benson, 1974; Brown, 1981; Figure 1). Geographic radiation in *Heliconius* colour patterns closely follows the geography of mimicry in local butterfly communities, many species occurring as a mosaic of sharply defined races across Central and South America. Parapatric races hybridise in narrow hybrid zones stabilised by frequency-dependent selection which are permeable to gene flow across the genome except around colour-pattern loci (Mallet, 1993). Such colour-pattern hybrid zones are known to move across geographic areas, possibly due to positive selection for new mimicry patterns or alternatively driven by allelic dominance (Mallet and Barton, 1989a; Blum, 2002).

Mimicry is common between members of diverged clades within *Heliconius* (Figure 1; note, for instance, the iridescent blue mimicry ring, or the black & white mimicry ring); in contrast sister species tend to diverge in pattern and mimicry associations, such as *H. melpomene vs H. cydno*, or *H. burneyi vs H. wallacei* (Figure 1; Beltrán, 2004). Indeed, *Heliconius* colour patterns are

used as mating signals, and play an important role in speciation (McMillan *et al*, 1997; Jiggins *et al*, 2001; Kronforst *et al*, 2006). The radiation in *Heliconius* colour patterns thus couples both divergent evolution and multiple independent cases of convergent evolution representing of varying evolutionary timescales. The fact that these patterns have clear functional significance in nature will link developmental diversity, within-population adaptation, and macroevolution.

For this review, we concentrate on the role that heliconiine butterflies can play as an emerging evo-devo model of phenotypic change. Our aim is to provide (i) an overview of the extant diversity of wing patterns in the group, (ii) review what is known about the genetic basis of this diversity, and (iii) highlight emerging research, research directions, and research tools that promise to make *Heliconius* a model system for studying the interface between development and adaptive change.

The genetic architecture of pattern variation in *Heliconius*

The natural diversity of colour patterns found among *Heliconius* species and races is determined by adaptive combinations of alleles at a surprisingly reduced set of genetic loci of large phenotypic effect. These genes are most likely developmental genes that regulate the spatial expression of downstream scale maturation pathways, thus controlling the development of morphology and pigmentation of the future scales which generate adult wing patterns (Gilbert *et al*, 1988; Nijhout, 1991). Although the molecular nature of these genes is unknown, gene action and interactions are well characterised: many years of crossing experiments between species and races have shown how a handful of loci control phenotypic shifts across large areas of the wing surface, changing the position, size and shape of red/orange/yellow and melanic patches on both the dorsal and ventral surfaces of the fore and hindwings (*H. numata*: Brown and Benson, 1974; *H. melpomene*, *H. erato*: Sheppard *et al*, 1985; Mallet, 1989; *H. erato*/*H. himera*: Jiggins and McMillan, 1997; *H. cydno*: Kapan, 1998; *H. cydno*/*H. melpomene*: Gilbert, 2003; Naisbit *et al*, 2003).

Alleles at major switch loci in *Heliconius* are natural variants, not laboratory generated mutants, and can be studied on a common genetic background by backcrossing pattern alleles between populations that are not genetically differentiated. *Heliconius* butterflies therefore offer an excellent opportunity to study the developmental and genetic basis of an adaptive radiation. Pattern variation is perhaps best understood in the two co-mimics *H. erato* and *H. melpomene*. The two species are distantly related, yet have undergone a parallel radiation into 23 colour pattern races (Turner, 1977). Although more than 20 different loci have been described in each radiation (Sheppard *et al*, 1985; Mallet, 1989; Jiggins and McMillan, 1997; Naisbit *et al*, 2003), geographic variation in wing pattern phenotype can be explained by allele changes at four to five loci of major effect. Pattern variation in *H. numata* provides perhaps one of the most striking examples of the broad action of major loci in *Heliconius* (Figure 2a and below). *H. numata* is closely related to *H. melpomene* and *H. cydno* ($\sim$5% mtDNA divergence; Beltrán *et al*, 2002), but has evolved to mimic large and highly distasteful Ithomiines (*Melinaea*, *Mechanitis*) and Danaines (*Lycorea*). Its wings are characterised by patterns of black spots and stripes on an orange and yellow background, and all the local and geographic pattern variation maps to a single pattern locus known as '*P*' (Brown and Benson, 1974; Joron, 2000). Hyperallelism is rampant and up to nine different alleles have been found to segregate in some populations (Figure 2b; Brown and Benson, 1974; Joron *et al*, 1999; Joron, 2000). The pattern of variation is therefore similar to the classic examples of polymorphism at colour-pattern 'supergenes' (clusters of tightly linked genes) in Batesian mimics such as *Papilio dardanus* and *P. memnon* (Turner, 1977).

Initially, the observation that adaptive variation in wing patterns in *Heliconius* was the result of a small number of major 'switch' loci was thought to be an unusual artefact of Müllerian mimicry selection, where the adaptive landscape was envisioned to be more rugged than that for most adaptive traits (Turner, 1988; Mallet, 1993; Coyne *et al*, 1997). However, although multiple loci are known to control morphologies under strong selection, such as some of the domestication traits in maize (Westerbergh and Doebley, 2002; Doebley, 2004), a growing number of studies on organisms ranging from plants to fish (eg monkeyflowers: Bradshaw *et al*, 1998; sticklebacks: Cresko *et al*, 2004; Colosimo *et al*, 2005) have shown that a small number of loci with large phenotypic effect often underlie adaptation, suggesting that the architecture of phenotypic evolution in *Heliconius* may be more typical of adaptive change than previously realised.

Macroevolutionary importance of *Heliconius* patterns

The genes that control wing patterns in *Heliconius* appear to be preserved across species boundaries. Reproductive barriers are often incomplete between closely related species, permitting interspecific crosses (Jiggins and McMillan, 1997; Gilbert, 2003; Naisbit *et al*, 2003). These studies demonstrate that colour-pattern differences *between* closely related species appear to be caused by allelic differences at same loci that are responsible for phenotypic differences *within* a species (Table 1). This is true even when speciation is coupled with a shift in mimetic alliance (Jiggins and McMillan, 1997; Naisbit *et al*, 2003), which occurs commonly in *Heliconius* (Beltrán, 2004). In combination with visual mate searching using wing colour signals, large shifts in pattern can lead to speciation (McMillan *et al*, 1997; Jiggins *et al*, 2001; Naisbit *et al*, 2001). Thus, there is a direct link between phenotypic shifts in pattern caused by just a few genetic changes and macroevolutionary diversification.

Rules and constraints on pattern formation

The radiation in the mimetic wing patterns of *Heliconius* butterflies provides an excellent model system for exposing the nature of constraints, bias, optimality, and chance in morphological change. Several authors have attempted to draw generalisations from the wealth of crossing data in *Heliconius* (eg, Turner, 1977; Sheppard *et al*, 1985). Notably Gilbert (2003), synthesising nearly 30 years of his own crossing work, identified a number of predictable dominance/epistatic effects in his crosses and defined three scale types based on pigment type and

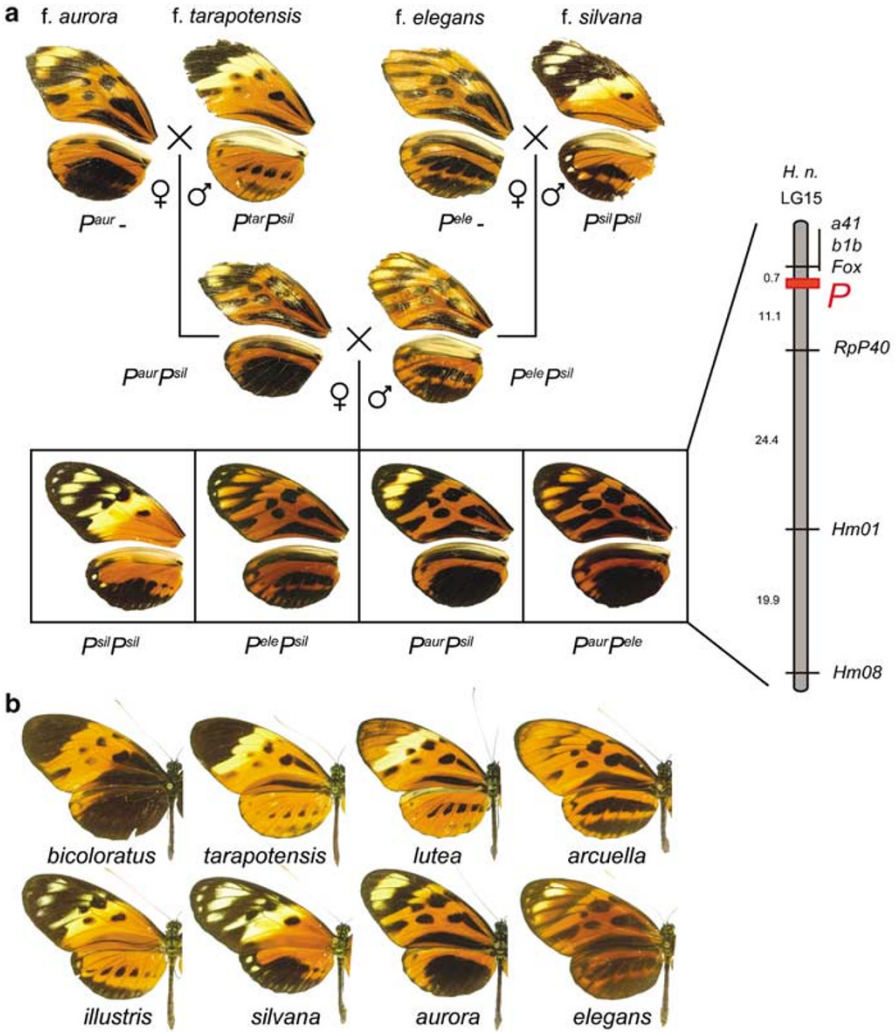

Figure 2 The genetic basis of polymorphism in *H. numata*. Crosses between sympatric forms show inheritance of the whole colour pattern at the single Mendelian locus *P*, and a largely linear dominance series of the *P* alleles which avoids non-mimetic heterozygotes (Brown and Benson, 1974; Joron, 2000). (**a**) F2-type cross (B502) between Peruvian forms, showing the recessive allele *silvana*, and the segregation of three alleles into four discrete phenotypes in the progeny. Melanin patches are usually (although variably) dominant, and orange is always dominant over yellow. However, melanin patches may be recessive to orange or yellow scales, for instance the black hindwing margin and black forewing patch of the form *silvana* ($P^{sil}P^{sil}$), suggesting regulation of dominance selected for mimicry (Joron, 2000). A linkage map derived from this and other broods was generated as outlined in Joron *et al* (in press), and shows the position of the *P* locus (red bar) relative to flanking molecular markers. Recombination distances are in Haldane cM. These markers show positional homology between *P* and the colour pattern complex locus *N*/*Yb*/*Sb* in *H. melpomene* (Joron *et al*, in press), and are now being used for positional cloning. (**b**) Diversity of alleles found in some populations of north-eastern Peru. Form *bicoloratus* (allele P^{bic}) is the top dominant and *silvana* (P^{sil}) the bottom recessive. Crosses and wild-caught recombinants suggest *P* to be a supergene (Brown and Benson, 1974), and some rare alleles such as *lutea* appear to be non-mimetic and may occur by recombination within the *P* locus. Allelic differences at the *P* locus are selected for mimicry of local *Melinaea* and *Mechanitis* butterflies (Nymphalidae: Ithomiinae). The single-locus inheritance of pattern in *H. numata* is likely a result of the atypical selection pressures associated with multiple mimicry (Brown and Benson, 1974; Joron *et al*, 1999).

scale morphology (Gilbert *et al*, 1988; Gilbert, 2003). Type I scales are white (pigmentless) or yellow (3-hydroxy-L-kynurenine); whereas Type II scales are black (melanin) and Type III scales are brown/red/orange (xanthommatin and di-hydro-xanthommatin). Alleles controlling type III scales are generally dominant to those controlling Type II scales, which are dominant to Type I scales. These patterns of dominance can also apply to inter-locus epistasis where two different loci influence the same wing region. Nonetheless, although generally true in *H. melpomene* and *H. erato* these generalisations do not apply to all *Heliconius*. For example, in *H. numata* melanin patterns are usually dominant over both orange and yellow (ie Type I < Type III < Type II), and in some cases yellow elements are even dominant to melanic ones, a complete reversal of the typical scheme (see Figure 2; Joron, 2000). Clearly, selection for mimicry can and does break the 'rules', highlighting the flexibility of butterfly wing patterns (Beldade *et al*, 2002b).

It has also been suggested that a common developmental process might constrain, or bias pattern evolution in *Heliconius*. At one extreme, it has been hypothesised that the precise and repeated convergent evolution between the two relatively distantly related co-mimics,

Table 1 Colour pattern genotypes of some races of *H. melpomene*, *H. heurippa* and *H. cydno*

Gene name	*B* *Red FW patch*	*D* *Red HW rays*	*Br* *Brown HW 'C'*	*N* *Yellow FW patch*	*Yb* *Yellow HW bar*	*Sb* *White HW margin*	*K* *White vs yellow*	*Ac* *FW discal spot*	*Vf* *FW vs colour*
H. m. malleti	b	D	br	N^N	Yb	Sb_1	K^Y	Ac	?
H. m. rosina	B	d	br	N^B	yb	Sb_1	K^Y	Ac	Vf_2
H. m. cythera	B	d	br	N^B	yb^{cy}	sb	K^W	Ac	Vf_2
H. heurippa	B	d	br	N^N	Yb_c	Sb_1	K^Y	Ac	?
H. c. chioneus	b	d	Br	N^N	Yb_c	Sb_3	K^W	ac	Vf_1
Linkage group	18	18	18	15	15	15	1	?	?

Abbreviations: FW, forewing; HW, hindwing, v., ventral.
Note that the major colour pattern changes are controlled by genes on just two linkage groups, 15 and 18 identified in *H. melpomene* (however, linkage relationships remain to be identified in *H. cydno* and *H. heurippa*). The genes on group 15 are very tightly linked (within 5 cM), while those on group 18 are loosely linked (within 30 cM; Sheppard *et al*, 1985).
The 'C' is a C-shaped pattern on the underside of the hindwing found in *H. cydno*. *H. heurippa* is a putative hybrid species which is morphologically and ecologically similar to *H. cydno* but shares two major patterning alleles with *H. melpomene*.

H. erato and *H. melpomene*, is caused by changes at homologous loci (Turner, 1984; Nijhout, 1991). However, there are notable differences in the exact nature of the phenotypes and their genetic control that has led others to argue that there is little homology between the species (Mallet, 1989). As mentioned above, the same pigment biosynthesis pathways are involved in both species, so questions about homology essentially relate to pattern formation prior to pigment production. However, the two species do not interbreed and the question of homology of genetic control between the two mimics is only now being addressed with molecular markers.

From patterns to genes

Application of molecular markers to crossing experiments

High-resolution linkage maps and the development of molecular markers transferable between species are allowing researchers to explore the architecture of convergent and divergent evolution in *Heliconius* and to test hypotheses about genetic homology between mimetic species. Mapping work in *Heliconius* takes advantage of the fact that controlled crosses between divergent colour pattern forms can be designed to follow the segregation of specific colour pattern alleles (Figures 2 and 3). This forward genetic approach has coupled Amplified Fragment Length Polymorphisms (AFLP) fingerprints with co-dominant anchor loci. AFLPs are a powerful technique for unexplored genomes (Mueller and Wolfenbarger, 1999; Parsons and Shaw, 2002) and, in *Heliconius* have allowed researchers to quickly home in on the regions of the genome that contain major colour pattern genes (Jiggins *et al*, 2005; Tobler *et al*, 2005; Kapan *et al*, in press). Indeed, there is now a tight association between AFLP markers and several major colour pattern loci in both *H. erato* and *H. melpomene*. For example, the *N*/*Yb*/*Sb* gene complex in *H. melpomene* and the *D* and the *Sd* locus in *H. erato* have all been localised and a number of tightly linked AFLP bands isolated (Jiggins *et al*, 2005; Kapan *et al*, in press; Figure 3). AFLP bands of interest can then be isolated, cloned and sequenced, and converted into co-dominant loci by designing primers that specifically amplify the AFLP fragment of interest (Beltrán, 2004). These 'targeted' AFLP loci work across different mapping families, facilitating finer precision mapping and are also an excellent source of probes for BAC libraries, which are now available for *H. erato*, *H. melpomene*, and *H. numata*.

In addition to AFLP markers targeted to colour pattern genes, the linkage maps are increasingly utilising co-dominant loci, which include microsatellites (Flanagan *et al*, 2002; Mavárez and González, 2006) and single copy nuclear loci (Beltrán *et al*, 2002; Kronforst, 2005; Papanicolaou *et al*, 2005), useful for anchoring and comparing maps from different crosses or species. In particular, a number of 'candidate' genes, chosen based on knowledge of gene action in other organisms, have now been mapped relative to the loci that cause pattern change in *Heliconius*. Candidate gene approach has been very successful in other organisms. For example, expression studies for genes known to be involved in *Drosophila* wing development revealed novel but related roles of such genes in pattern specification and variation in butterflies (Carroll *et al*, 1994; Brakefield *et al*, 1996; Brunetti *et al*, 2001; Beldade *et al*, 2002a). In *Heliconius*, however, this approach has allowed us to rule out most potential candidates by linkage mapping (Jiggins *et al*, 2005; Joron *et al*, in press; Kapan *et al*, in press). With the notable exception of tight linkage between *wingless* and the white/yellow colour switch locus *K* in *H. cydno* (Kronforst *et al*, 2006), several loci that are important in *Drosophila* wing development (*apterous*, *wingless*), in *Bicyclus* eyespot specification (*distal-less*, *hedgehog*, *patched*, *cubitus interruptus*), or in scale pigment synthesis (*vermilion*, *cinnabar*), are unlinked to pattern switch genes in one or more *Heliconius* species (see eg Jiggins *et al*, 2005; Tobler *et al*, 2005; Kapan *et al*, in press). These results can be disheartening, but they may imply, together with expression studies (Reed and Nagy, 2005), that novel or unexpected genes or pathways are involved in pattern specification.

The growing number of co-dominant markers that have been mapped in several *Heliconius* species also allows comparisons of gene order between species. Results so far indicate that gene order is well conserved across *Heliconius* (Joron *et al*, in press; Kapan *et al*, in press). Indeed, to date no conflicting linkage relationship has been found between *H. erato*, *H. melpomene*, and *H. numata*. The strongest support for the general conservation of linkage relationships within *Heliconius* comes from a cluster of ribosomal proteins (*RpL5*, *RpS5*, *RpL10a*, *RpS8*, *RpP0*), all of which map to the same linkage group and show conserved gene order in the three species

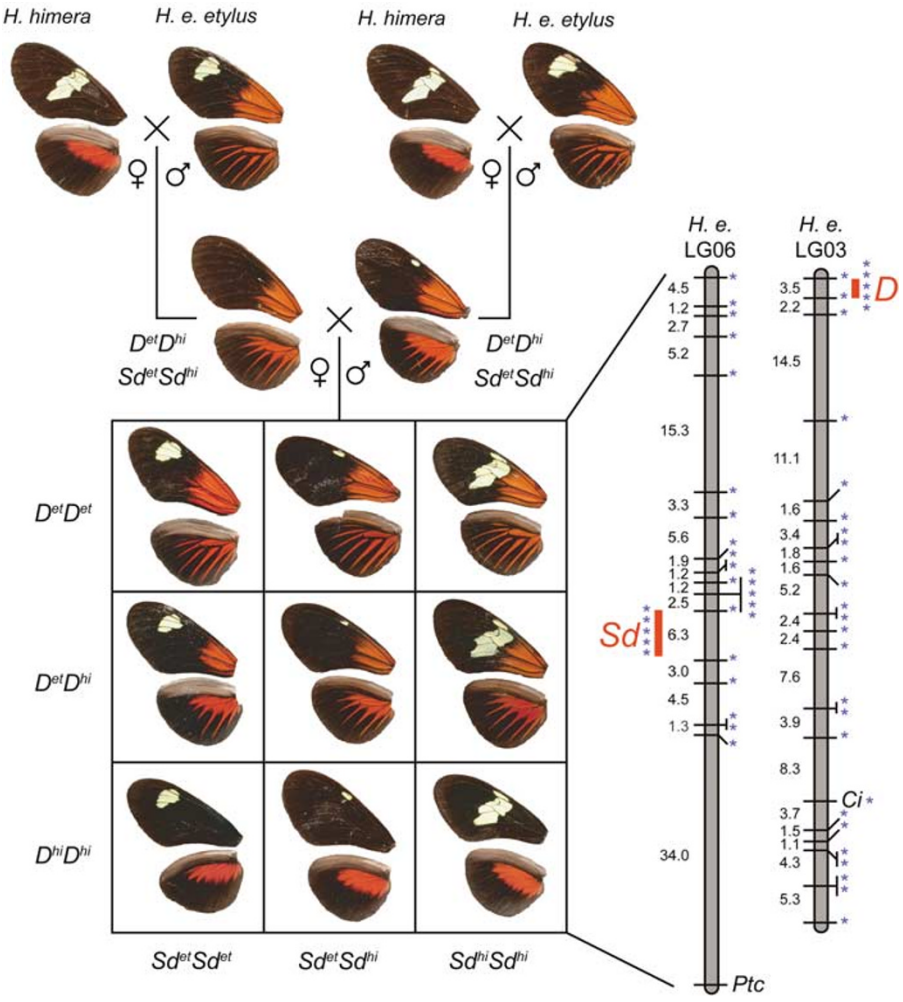

Figure 3 Segregation of two patterning loci in a single cross and their genomic position in *H. erato*. Segregating variation in a cross between *H. erato etylus* (rayed parent) and *H. himera*. The F1 individuals of this cross lack the forewing yellow band, possess an orange patch on the proximal part of the forewing and show both the characteristic rays and hindwing bar of the parental species. The effects of two major co-dominant loci segregating can be seen in F_2 offspring (Punnet square). Linkage maps of the two colour pattern linkage groups, LG03 and LG06, were generated as outlined in Kapan *et al* (in press). The blue stars represent AFLP markers on each chromosome. The red bar represents the interval across each chromosome where the colour pattern locus can be placed with high confidence. Bulk segregant analysis has generated a dense cluster of AFLP markers tightly linked to each of the two colour pattern loci, which are now being used for positional cloning.

(Jiggins *et al*, 2005; Joron *et al*, in press; Kapan *et al*, in press). Interestingly, the ribosomal cluster spans 47 cM in *H. melpomene vs* 32 cM in *H. erato*, which contrasts with the differences in genome size estimates, *H. erato*'s genome being 36% larger than *H. melpomene*'s (Jiggins *et al*, 2005). The apparently higher crossing-over frequency in *H. melpomene vs H. erato* is intriguing in light of evidence that *H. erato* may commonly act as the Müllerian model in this pair (Mallet, 1999; Flanagan *et al*, 2004). This suggests the interesting hypothesis that the genomic flexibility provided by increased recombination rate could help *H. melpomene* track pattern variation in *H. erato*. Efforts are now underway to develop and map many more anchor loci. Generating new anchor loci is now extremely efficient using EST sequences, with a primer design success rate of ca. 80% (ie 80% of primer pairs designed work in all three species; Papanicolaou *et al*, 2005). Furthermore, assigning loci to particular linkage groups is easy in Lepidoptera, because there is no crossing over during oogenesis (Suomalainen *et al*, 1973), and chromosomes are therefore inherited from the mother without recombination.

The linkage maps available for several *Heliconius* species and the high degree of conservation of gene order now allows a direct comparison of the genetic architecture underlying pattern evolution and a test of the long-standing hypothesis that homologous genes might be controlling mimcry in *Heliconius*. This comparative approach is yielding exciting results. Using a combination of 'targeted' AFLP markers and gene-based markers developed from initial genomic sequence, it has recently been demonstrated that *N/Yb/Sb* complex in *H. melpomene*, the *Cr* locus in *H. erato*, and the *P* locus in *H. numata*, all map to homologous 1 cM regions of the genome (Figure 2; Joron *et al*, in press). The *N/Yb/Sb* complex of *H. melpomene* and the *Cr* locus of *H. erato*

similarly affect the distribution of white and yellow patches on the fore and hind wing of both radiations and is one of four or five patterning loci that underlie convergent change in the wing patterns of the co-mimics (see Mallet, 1989; Jiggins *et al*, 2005; Kapan *et al*, in press). The observation that loci with similar phenotypic effects map to the same genomic region is the first direct support for the hypothesis that homologous genes or complex of genes regulate convergence in the two co-mimics. Additional, albeit less compelling, support for this hypothesis comes from emerging patterns of linkage at other major color pattern loci in the two radiations, such as the *D* locus in *H. erato* and the *D*/*B* complex in *H. melpomene* which have nearly identical phenotypic effects on red patterning in the two co-mimics and both map to the same end of homologous linkage groups (Joron *et al*, in press; Kronforst *et al*, unpublished). Similarly, the *Sd* locus in *H. erato* and the *Ac* locus in *H. cydno and H. melpomene*, both of which effect the pattern of melanic scales on the forewing, also map to the same linkage group (Kronforst *et al*, unpublished).

The observation that mimicry between *H. erato* and *H. melpomene* appears to be regulated by many of the same loci or suite of loci, as predicted by Nijhout (1991), might suggest that some developmental constraints are important in mimetic evolution in *Heliconius*. However, this sharply contrasts with the lack of similarity between the patterns of the *H. erato*–*H. melpomene* pair and those of *H. numata*. Despite strong evidence for genetic homology, there is no obvious phenotypic homology between the effects of allelic substitutions at *P* in *H. numata* and *N*/*Yb*/*Sb* in *H. melpomene*. The *P* locus regulates pattern diversity much more broadly by affecting the distribution of yellow, brown/orange, and black color patterns elements across the whole wing surface (Figure 2). Rather than constraints, these observations therefore demonstrate an extraordinary flexibility of homologous color pattern genes in *Heliconius*, *which* respond to a variety of selection pressures for mimicry of divergent color patterns. This conserved locus, or 'developmental hotspot' (*sensu* Richardson and Brakefield, 2003), is responsible for pattern variation in at least three species belonging to two diverged clades, and appears to play a disproportionate role in both divergent and convergent adaptive evolution in the genus. The architecture, identity, and mode of action of this genomic region remain to be characterised to better understand its role and flexibility.

Prospects for positional cloning of pattern genes

Of course, much of the mapping work in *Heliconius* is directed towards identifying the loci that control pattern variation. The increase in genomic resources including (1) large numbers of replicate crosses, (2) 'targeted' loci near colour pattern genes and (3) BAC libraries make this a realistic goal and one that is achievable within the next few years. Moving forward requires linking the recombination maps to physical maps of the corresponding region. In *H. melpomene* the AFLP marker linked to the *Yb* gene has been used to screen a BAC library and construct a 500 kb contig tightly linked to the patterning genes. Segregating variation at BAC end sequences in the broods has been used to determine the direction in which *Yb* lies and locate recombination breakpoints between the pattern genes and flanking markers. Candidate patterning genes will be found by sequencing the region within these physical boundaries and locating the open reading frames (ORF). Markers developed in *H. melpomene* from BAC sequences are simultaneously being used to narrow down the region containing the hypothesised homologues *P* in *H. numata* and *Cr* in *H. erato* in the same way. Having exhausted the mapping resolution of crosses in each species, the involvement of the gene(s) identified from this region in colour pattern variation will be tested using various methods: expression studies on developing wings (eg Reed and Nagy, 2005) can test the up- or downregulation of genes identified from the BAC sequences, while association studies using BAC-derived markers in wild populations (eg Colosimo *et al*, 2005) can take advantage of historical recombination around the colour-pattern locus to identify narrower regions associated to specific genotypes. More targeted reverse genetics methods aimed at disrupting or enhancing specific gene expression, such as germline transformation (Peloquin *et al*, 2000; Marcus *et al*, 2004) and especially RNA interference (Fabrick *et al*, 2004; Elefherianos *et al*, in press), have been successfully applied to lepidopteran species. Such techniques become increasingly transferable to diverse species (Marcus, 2005), and represent a promising way to test the involvement of genes in wing pattern phenotypes for species with rapid development such as *Heliconius*. The isolation of pattern genes is likely to be of major importance in our understanding of how the regulation at single loci can produce very different phenotypes. The comparative architecture and micro-synteny of targeted regions of the genome, such as that containing the complex loci *P* or *N*/*Yb*/*Sb*, will also tell us about the evolution of recombination patterns around genes under selection.

Indeed, the data already offer insights into how the 'supergene' in *H. numata* might have evolved. The pattern seen in *H. melpomene* and *H. erato* is one of geographic variation largely controlled by three to four clusters of tightly linked elements found on different chromosomes. One of these elements has taken over control of the entire pattern in *H. numata*, presumably facilitated by the fact that these regions already have major phenotypic effects on different parts of the wing in the ancestral species. There is also the possibility that the linked elements *Yb*, *Sb* and *N* found in *H. melpomene* might have been brought closer together to reduce the production of unfit intermediate genotypes in polymorphic *H. numata* populations. While the evolution of linkage between unlinked loci seems unlikely on theoretical grounds (Charlesworth and Charlesworth, 1975), a gradual reduction in recombination between already tightly linked elements seems more plausible. Thus, evolution of the *H. numata* supergene could have involved elements of both the 'macromutationist' and the 'gradualist' positions in this historical debate. Characterisation of the molecular basis of these genes will allow a direct test of these ideas.

From genes to pathways

From an evo-devo perspective, the major interest lies in linking the loci underlying pattern change in *Heliconius*, the so-called switch genes, with the pathways involved in wing pattern development. Identifying the pathways

or modules of genes involved in wing pattern development promises to open an entirely new set of questions. For instance, in cases of convergence, we can determine (i) the level in pathways where changes tend to occur, (ii) if certain types of molecules (signalling molecules, transcription factors, pigment enzymes, transporters, etc.) play a disproportionate role, and (iii) the nature of constraint and potential in different pathway elements for producing similar phenotypes.

Pattern formation on butterfly wings is envisioned to be a multistep process. In early development, genes involved in cell-signalling and signal transduction work together to 'pre-pattern' or specify the fate (colour and morphology) of the individual scale cells that pattern the wing. The final adult colour pattern is produced later in development when scale cells interpret this positional information and produce pattern-specific colour pigments (Nijhout, 1991). Work on the early stages of pattern formation in *Heliconius* (Reed and Serfas, 2004; Reed and Nagy, 2005) has not, as yet, yielded the striking association between gene expression patterns and wing pattern elements seen in the eyespots of other butterflies (Carroll *et al*, 1994; Brakefield *et al*, 1996; Brunetti *et al*, 2001). Nonetheless, these studies suggest that at least part of the difference in patterning between butterfly groups may be due to temporal changes in conserved pattern-formation processes. For instance, the *Notch/Distal-less* (*N*/*Dll*) pattern formation process, associated with intervein elements and particularly eyespots in *Bicyclus* or *Junonia*, is truncated in *Heliconius* and other species lacking eyespots (Reed and Serfas, 2004). This implies that pattern variation within Heliconiines might be associated with the regulation of earlier stages of the *N*/*Dll* pattern-formation process, or possibly involve distinct pathways altogether. Actually, the apparent differences in the developmental architecture between eyespots and the coloured bands of *Heliconius* are not entirely unexpected. Eyespots are highly localised pattern elements relative to the large patterns of *Heliconius*.

The recent discovery of a mutant *H. cydno* with greatly reduced wing veins further highlights the differences between eyespots and *Heliconius* patterns. This veinless mutant had a pattern that was very similar to the wild-type, implying that, unlike eyespots, *Heliconius* patterns develop independently of wing veins (Reed and Gilbert, 2004). The vein independence of the *Heliconius* patterns also seems to disprove Nijhout's (1991) hypothesis of a common 'nymphalid ground plan' in which the *Heliconius* patterns represent an expansion of vein-dependent pattern elements found in other nymphalid butterflies. The *Heliconius* patterns more probably result from a distinct, and unexplored, whole-wing proximodistal patterning system established in the larval wing disc (Reed and Gilbert, 2004).

Beyond candidate genes

The candidate gene approaches described above are largely based on inferences about gene actions and interactions gleaned from research on *Drosophila*. This research avenue has clearly yielded insights into the mechanism of pattern formation on butterfly wings. However, the development of scale-covered wings and the patterning system for pigmenting them are evolutionary innovations of the Lepidoptera (Nijhout, 1991) and must therefore involve either novel genes or novel functions for conserved genes. To identify genes expressed during wing formation, sequencing projects are currently underway in both *H. erato* and *H. melpomene*. We have a growing database of Expressed Sequence Tags (ESTs), the bulk of which come from wing disc cDNA libraries (Papanicolaou *et al*, 2005). To date, approximately 10 000 *Heliconius* ESTs have been clustered and annotated with hierarchical BLAST searches, putative protein translations, and gene ontology (GO) terms, and are publicly available at www.heliconius.org. ESTs are important source of loci known to be expressed during wing pattern development in *Heliconius* and this 'first-pass' has identified key members of both cell signalling and pigment synthesis pathways (Table 2; Papanicolaou, 2005). In addition, EST sequences are an important source of new PCR-based markers for linkage mapping and will allow AFLP maps to be compared both within and between *Heliconius* species (Papanicolaou, 2005; Joron *et al*, in press).

A significant step towards understanding the sequence of gene expression during wing development is now possible by turning the emerging EST data into a gene chip (DNA microarray) to use during the development of *Heliconius* developing wings. The wing discs of *Heliconius* are large and completely accessible to sampling throughout development. Furthermore, they do not undergo complex morphogenetic movements (like the evagination of *Drosophila* wing disks), and the shape and pattern of the developing wing imaginal disk maps directly onto that of the adult wing. These factors facilitate developmental research in pattern formation that, when coupled with the large natural variation in *Heliconius* wing patterns, promise insights into how gene expression varies between (i) different parts of the developing wing, (ii) different geographic variants of the same species, and (iii) different species with convergent morphologies. These data will form the foundation for uncovering the networks that connect patterning genes to the pigment synthetic pathways, and how these networks change during pattern evolution. At the most basic level, these data promises a new suite of candidate loci, whose expression patterns can be tested using quantitative reverse-transcriptase PCR and *in situ* hybridisation (following eg Reed and Nagy, 2005) and whose position relative to the known 'mimicry' genes can be readily mapped. Furthermore, since eyespot specification is shared by most Nymphalids and may have been lost or truncated in *Heliconius* (Reed and Serfas, 2004), and since various kinds of banding patterns are also common in many Nymphalids including *Bicyclus*, the developmental underpinning of the bold patterns of *Heliconius* will provide an interesting contrast to the formation of eyespots in *Junonia* and *Bicyclus* (Brakefield *et al*, 1996; McMillan *et al*, 2002; Beldade *et al*, 2002a; Monteiro *et al*, 2003).

Perspectives

Advances in genomic resources, including high-resolution maps, BAC libraries, EST scans, and gene chips, are now offering exciting possibilities for comprehensive analyses of colour pattern change in *Heliconius*. So far, research has focussed on a trio of species encompassing

Table 2 EST clusters with significant similarity (80 bits and above) to members of several signalling and biochemical pathways

Gene	*EST cluster in ButterflyBase*
Wingless signalling pathway	
Wingless [gi:139777]	HEC03937
Casein kinase II, alpha chain [gi:125270]	HEC00133
Casein kinase II beta subunit [gi:52788230]	HEC00944
cAMP-dependent protein kinase catalytic subunit (PKA C) [gi:125215]	HEC04717
Ras-like GTP-binding protein *Rho 1*[gi:350593]	HEC00468, HEC04729
Calmodulin-dependent calcineurin A2 subunit [gi:73920245]	HEC02823
Calcineurin B subunit isoform 2 [gi:12644421]	HEC05476
Notch signalling pathway	
Puff-specific protein *Bx42* [gi:728991]	HEC04926
CBF1 interacting corepressor [gi:81883502]	HEC05075
Hedgehog signalling pathways	
Cos2; costal 2 [gi:17137092]	HEC05086
cAMP-dependent protein kinase catalytic subunit (PKA C) [gi:125215]	HEC04717
TGF-beta signalling pathway	
Myoglianin [gi:4580679|]	HMC00374
Glass bottom boat [gi:112828]	HEC03961
Homologue of bone morphogenetic protein receptor, type I [gi:74967031]	HEC04653
DAD, daughters against *decapentaplegic* [gi:74763404]	HEC05233
Extra-macrochaeta [gi:30316332]	HEC03179
Ras-like GTP-binding protein *Rho 1*[gi|1350593]	HEC00468, HEC04729
Melanin and Ommochrome pathways	
Tryptophan 2,3-dioxygenase (*Vermilion*) [gi:137834]	HEC02951
Tyrosine 3-monooxygenase (*Pale*) [gi:29337193]	HEC00084
Yellow [gi:140623]	HEC00034
Brown [gi:115140]	HEC01555
DOPA decarboxylase [gi:13432098]	HEC03066
Henna [gi:61678477]	HEC00084
Ebony [gi:3286766]	HEC01015
Scarlet [gi:17647959]	HMC00270

Table modified from Papanicolaou (2005). All EST clusters are available from ButterflyBase (www.heliconius.org).

most aspects of colour pattern evolution including geographic diversification (in each of the three species), local polymorphism (*H. numata*), diverging mimetic associations between closely related species (*H. melpomene vs H. numata*) as well as convergent phenotypes between distantly related species (*H. erato vs H. melpomene*), and makes *Heliconius* an excellent model for comprehensive analyses of colour pattern evo-devo. One of the advantages of the *Heliconius* system is the potential for direct identification of pattern switch genes by positional cloning. This approach avoids any dependence on prior identification of candidate genes and promises to uncover the genes responsible for the *N/Yb/Sb* complex of *H. melpomene*, the *P* locus in *H. numata*, and the *Cr* locus in *H. erato* in the near future. The evidence for positional homology between pattern switch genes within *Heliconius* offers exciting possibilities for comparative studies of the pattern specification pathways and insights into to the evolution of genome complexity, synteny, recombination rates, and *cis*-regulatory change.

As new candidate loci emerge the challenge will be to carry out the experimental studies that will provide a more detailed picture of the networks that connect the switch genes of *Heliconius* to pigment synthesis pathways, and how these networks change during adaptive radiation. On a broader phylogenetic scale, a general mechanistic understanding of wing pattern formation will require an appreciation of the interplay of different patterning systems on the developing wing, such as eyespot and banding patterns. Integrating our knowledge of several kinds of pattern specification will permit a fuller understanding of pattern evolution and how developmental processes are shaped by selective pressures.

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