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Genes controlling mimetic colour pattern variation in butterflies

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Butterfly wing patterns are made up of arrays of coloured scales. There are two genera in which within-species variation in wing patterning is common and has been investigated at the molecular level, *Heliconius* and *Papilio*. Both of these species have mimetic relationships with other butterfly species that increase their protection from predators. *Heliconius* have a 'tool-kit' of five genetic loci that control colour pattern, three of which have been identified at the gene level, and which have been repeatedly used to modify colour pattern by different species in the genus. By contrast, the three *Papilio* species that have been investigated each have different genetic mechanisms controlling their polymorphic wing patterns.

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Introduction

Butterfly wing patterns are examples of evolutionary innovation that have fascinated scientists since the very inception of evolutionary theory [1]. The adaptive significance of these patterns has been established in many cases, and the main function is usually for defence against predators, for example as startle patterns [2], camouflage, or warning colours in chemically defended species [3]. Warning colours are also often shared between species, either through Müllerian mimicry, where multiple chemically defended species have the same pattern, increasing predator learning of these patterns [4], or through Batesian mimicry, where non-defended species copy the patterns of chemically defended species [5]. Wing colours and patterns can also function in mate choice and mate attraction [6], sometimes alongside an anti-predator function [3]. This dual function can lead to interesting evolutionary dynamics, for example the ability to function as 'magic traits' in speciation — causing both ecological divergence between populations with different patterns and reproductive barriers due to assortative mating [7].

Wing patterns in the butterflies and moths (Lepidoptera) are made up of arrays of coloured scales (Figure 1). These colours can be conferred either by pigments or by submicron-scale structures that produce interference colours (structural colour) or by a combination of these mechanisms [8]. Although the genetic pathways responsible for pigment production are fairly well characterised in most cases, virtually nothing is known about genes controlling structural colour. The most common pigment is melanin and the pathways producing this pigment from the amino acid precursor tyrosine are well known in insects [9,10]. Other butterfly wing pigments include ommochromes, pterins and flavonoids. The first two are synthesised from precursors tryptophan and guanosine triphosphate respectively, but the latter must be obtained from food plants [8].

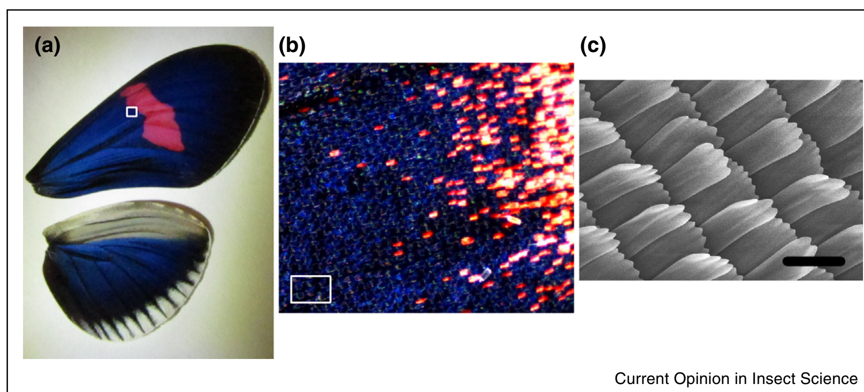
Although the genetic control of pigment production is reasonably well understood, these genes appear to be fairly conserved in evolutionary terms and contribute relatively little to the variation in wing pigmentation pattern observed in butterflies [11,12] or moths [13], at least over short evolutionary timescales. This contrasts with what is known in vertebrates [14–17] and to some extent also other insects [18,19], and suggests that on the lepidopteran wing there is a greater disconnect between the genes responsible for producing pigments and those responsible for the evolution of colour patterning.

There are two major butterfly groups in which genetic variation underlying pattern variation has been investigated, *Heliconius* and *Papilio* (Figure 2). Both of these show widespread within-species variation in wing pigmentation patterning related to mimicry. This variability has made them excellent systems for identifying genes controlling pattern variation.

The *Heliconius* 'Tool Kit'

As well as within-species variation in pigmentation patterning, the *Heliconius* butterflies have also been studied because of the often near-perfect mimicry between species. This mimicry has also made them an excellent system for studying the extent to which the same genes are used when evolving convergent traits [20]. Extensive genetic work on species within this genus (largely *H. erato*, *H. melpomene*, *H. cydno* and *H. numata*) has revealed a 'tool kit' of around five unlinked genetic loci (Figure 2)

Figure 1



Butterfly wing patterns are made up of arrays of coloured scales. **(a)** Wings of *Heliconius erato cyrbia*. The red and black colours are produced by melanin and ommochrome pigments respectively. The blue colour is due to scale nano-structure. **(b)** Magnification of the wing showing the scales. **(c)** Electron micrograph of wing scales. Bar indicates 50 μm. White boxes (in a and b) indicate approximate areas magnified (in b and c respectively).

that control almost all of the colour pattern variation in this group and that have been repeatedly used by different species to produce both convergent and divergent wing colour patterns [21–23,24•]. Over the last few years several of these have been pinned down to individual genes.

Optix

Fine-scale mapping and gene expression analyses have identified the transcription factor *optix* as being responsible for turning on and off most red, orange and brown colour pattern elements in *H. erato*, *H. melpomene* and *H. cydno* (Figure 2) [25]. In *Drosophila* the main function of *optix* is in controlling eye development [26]. However, the gene apparently took on a role in wing scale specification within the lepidoptera, initially controlling the development of specialised scales coupling together the fore-wings and hind-wings, and just within *Heliconius* has it taken on a role in colour patterning [27].

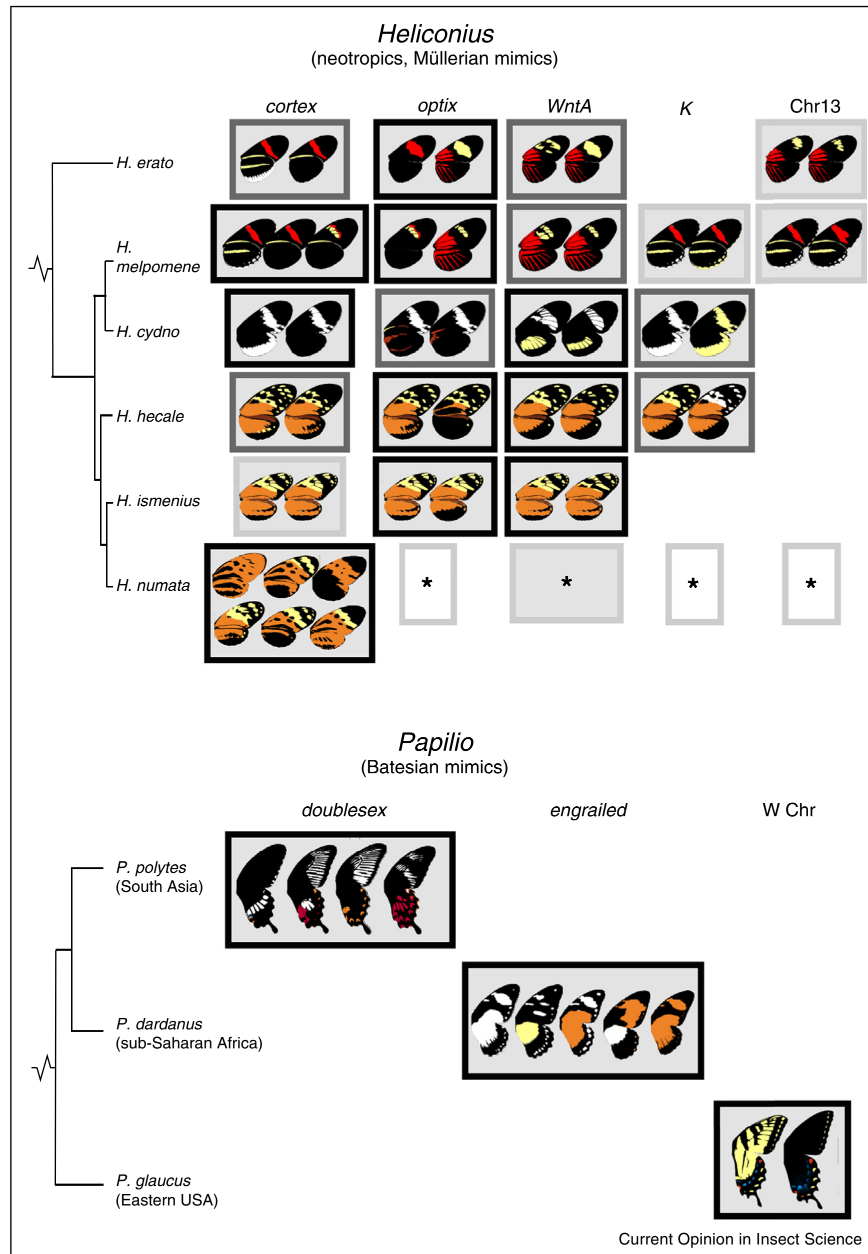
Population genomics approaches have identified a 65 kb interval ∼100 kb downstream of *optix* that likely contains cis-regulatory elements controlling red colour patterns in both *H. erato* and *H. melpomene* [28,29]. Detailed analysis of this region in *H. melpomene* has revealed two discrete regulatory modules, one 10 kb in length containing variants that control red patches at the wing bases ('dennis' patches) and one 25 kb in length controlling red 'rays' on the hind-wing (Figure 3b) [30••]. It seems likely that each of these modules contain one or more transcription factor binding sites that specify the expression pattern of *optix*. However, discovering exactly what the functional variants within these regions are will likely remain unresolved until transgenic techniques are developed in these species. It is also presumed that there is a third, currently unidentified, regulatory module for *optix*, which controls the presence of a red forewing band [22].

Cortex

A second major locus is responsible for switching on and off most white and yellow colour pattern elements in *H. erato*, *H. melpomene* and *H. cydno* (Figure 2) [21,23]. Interestingly this locus also overlaps with two inversions present in certain morphs of *H. numata*, which control quite different colour patterns of black, orange and yellow spots [31]. *H. numata* differs from most other *Heliconius* species in that multiple colour patterns are usually present within a single population and that all colour pattern variation is controlled by multiple alleles at single genetic locus with a strict dominance hierarchy between these alleles [32]. The gene *cortex* appears to be, at least partially, responsible for these colour pattern variants, with population genomics approaches mapping colour pattern variation within *H. erato*, *H. melpomene* and *H. numata* to within or near this gene and *H. melpomene* and *H. numata* showing colour-pattern-associated expression differences of *cortex* [33••].

Cortex belongs to a family of cell cycle regulators [34], which includes two genes that are highly conserved in all eukaryotes, *CDC20/fzy* and *cdh1/fzr*, and have a fundamental role in cell cycle progression [35]. *Cortex* itself appears to be insect specific and to have a much higher evolutionary rate [33••]. It seems likely that it could control scale cell colour through control of scale developmental rate, as melanic scales are known to develop at a slower rate than scales of other colours across a diversity of lepidoptera [36]. Indeed, the *cortex* gene also appears to regulate melanic pigmentation in the peppered moth, with the insertion of a transposable element in this gene producing the melanic form that proliferated during the

Figure 2

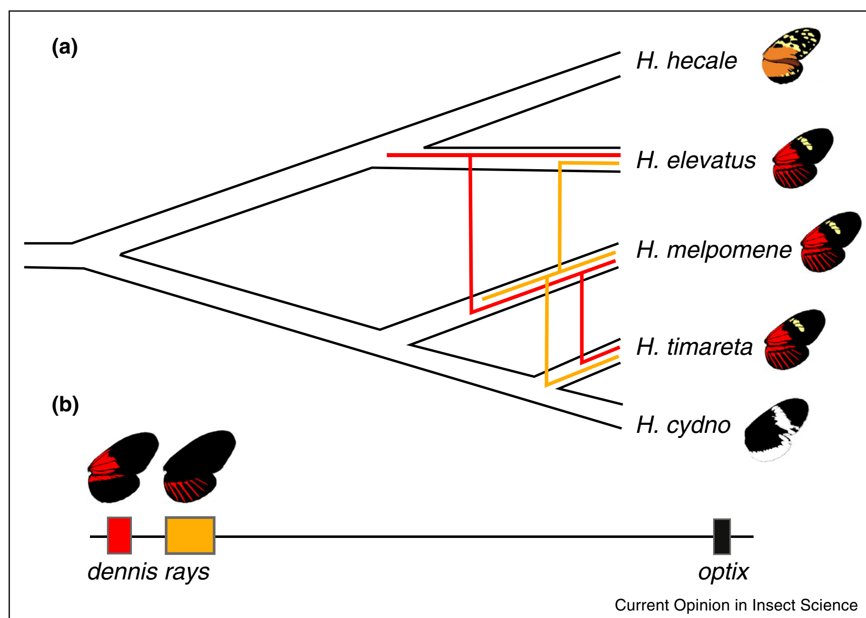


Genes controlling colour pattern in *Heliconius* and *Papilio*. Examples of the variation produced by each of the loci are shown for each species, the patterns differ more if the loci have a larger effect. Box colour also indicates effect size: black, large effect; dark grey, medium effect; light grey, minor effect. In some cases additional linked genes may be involved. *These loci have minor effects on phenotype in *H. numata* which are hard to represent pictorially, the size and fill shade of the boxes indicates the effect size. Based on information from [21,23,24•,25,33••,38,39,42,43,50•,54•,57,61,62].

industrial revolution [37•]. Therefore, it seems likely that cortex has a role in scale cell development and pigmentation across all lepidoptera.

Again, the precise functional variants of *cortex* causing differences in pigmentation patterning are unknown, but appear to be cis-regulatory rather than coding. *Cortex* has several 5′ untranslated exons (5′ UTRs) spanning a region of over 100 kb, suggesting a complex of dispersed regulatory elements [33••]. In addition to splicing variation of these 5′ UTRs, there are also alternative coding isoforms, some of which show associations with colour pattern. Further work is needed to understand if this splicing variation affects scale pigmentation.

Figure 3



Evolution of the 'dennis' and 'rays' regulatory modules of the *optix* gene in *Heliconius melpomene* and related taxa. **(a)** Evolutionary trees of dennis (red) and rays (orange) overlain on the species tree. **(b)** Schematic representation of the regulatory modules.
Source: Modified from [30••].

WntA

A third gene, *WntA*, controls several aspects of the size and shape of the colour pattern elements switched on and off by the previous two loci in both *H. erato* and *H. melpomene* (Figure 2) [38]. Unlike the previous two genes, some functional information does exist for this gene, with pharmacological treatments that enhance wnt signalling increasing the amount of melanin pigmentation on the wing and mirroring the natural effects of this locus [38]. On the other hand this locus has not been fine-mapped in the same detail as the previous two, so the location of functional sites is less clear. The evidence again seems to point to cis-regulatory variation, although mapping data places these closer to the coding region than is the case for the previous two genes [38,39], and coding variants have not been completely ruled out.

Like *cortex*, *WntA*'s role in wing patterning seems fairly ubiquitous, at least within the nymphalid butterflies [40]. Further, *WntA* also controls colour pattern differences between Batesian mimetic and non-mimetic populations of the admiral butterfly *Limenitis arthemis* in the eastern USA. In this species colour pattern variation shows a perfect association, again with the insertion of a transposable element, upstream of the coding exons of *WntA* [41•].

Other *Heliconius* loci

At least two other loci are known to control aspects of pigmentation patterning variation in *Heliconius*. Another locus controlling the shape of the forewing band has been found on *H. melpomene* chromosome 13 in both *H. erato* and *H. melpomene* (Figure 2) [39,42]. Further work is needed to identify the gene responsible, although the current mapping data implicates the *radial spoke head 3* gene [39].

The *K* locus controls a switch between yellow and white in *H. melpomene* and *H. cydno* (Figure 2) and has been mapped to a region of chromosome 1 that contains *wingless* [43], although the exact gene responsible is not known. Despite causing a simple switch in yellow pigment deposition it seems unlikely that the gene is involved in production of the yellow pigment since this is synthesised in the haemolymph, not *in situ* [44], and both yellow and white patterns can be present on the wing of a single individual with a particular *K* allele.

The importance of gene-exchange for *Heliconius* pattern variation

In addition to this tool-kit of loci that can be used flexibly to generate a wide range of patterns, gene exchange between species also appears to have played an important role in pattern evolution in this group [45]. There are now several well-supported cases of species that have gained novel wing patterns as a result of rare hybridisation events with other species, allowing introgression of colour pattern genes [45–47]. This mode of evolution is likely to be particularly effective, as it means that an entire locus,

containing multiple co-evolved mutations that have built up over evolutionary time, can be acquired instantaneously. It is also likely to particularly advantageous in *Heliconius*, where positive frequency dependent selection drives mimicry between species [4], so a species moving into a new area can rapidly join a mimicry ring by acquiring genes from other species already in that area.

However, gene-flow between species appears to be able to do more than just transfer existing patterns between species. In some cases it also appears to be able to generate novel patterns. Recent work has shown that the two distinct modules producing the 'dennis' and 'rays' pattern in Amazonian *H. melpomene* and *H. elevatus* have distinct evolutionary origins, with dennis arising first in the ancestor in *H. elevatus* and then being shared with *H. melpomene*, and rays arising later in *H. melpomene* and then being transferred in to *H. elevatus* [30••]. Therefore the current phenotype of both of these species is a chimaera of different patterns that evolved separately in each species with hybridisation acting to bring them together (Figure 3a).

Papilio supergenes

Within the swallowtail butterfly genus *Papilio*, female-limited Batesian mimicry has evolved multiple times, with males being non-mimetic and females mimicking other, chemically defended, species [48]. In several of these species the females are also polymorphic, often with a male-like non-mimetic morph and morphs that mimic either one or several toxic species [5]. The genes controlling the switch between different female morphs have often been described as 'supergenes' because of their ability to influence multiple aspects of the phenotype from a single genetic locus [49]. Two such genes, underlying female-limited polymorphism, have been identified (Figure 2). By contrast to the *Heliconius* system, the genes involved are not the same between different species, although both are transcription factors.

Papilio polytes

In this species there are multiple female morphs, including a non-mimetic male-like morph and three mimetic morphs resembling distantly related, toxic, *Pachliopta* swallowtails. Two teams independently mapped the female-limited polymorphism to the *doublesex* (*dsx*) gene [50•,51••]. This autosomal gene controls sexual dimorphism in all insects that have been investigated [52]. Fascinatingly, in at least one of the mimetic morphs, *dsx* is inverted relative to the ancestral orientation found in the non-mimetic morph [51••]. This has repressed recombination between the mimetic and non-mimetic alleles, allowing multiple sequence differences to accumulate.

As in other insects, there are multiple female-specific splicing isoforms of *dsx* in *P. polytes*, but the studies disagree on whether these are differentially expressed between morphs [50•,51••]. However, knockdown of *dsx* confirmed the functional role of this gene in specifying pattern and implied that coding or structural differences found in the gene could be important [51••]. Knockdown of the mimetic *dsx* allele produced a switch to a non-mimetic pattern, whereas knockdown of the non-mimetic allele in heterozygous individuals, which have the mimetic phenotype (it is dominant), produced no phenotypic effect, suggesting that changes in the expression level of *dsx* alone are insufficient to produce a change in colour pattern. Nevertheless, there must also be some regulatory component that prevents the mimetic *dsx* allele from affecting male phenotype.

Papilio dardanus

This species also has multiple mimetic female morphs, but in this case they mimic very distantly related nymphalid butterfly species and non-mimetic female morphs are less common [53]. Mapping and population genomics analyses have identified the gene responsible for switching between morphs as the autosomal gene *engrailed* [54•]. No inversions were present in the region, but one of the morphs had a duplication of *engrailed*, which could similarly act to reduce recombination and promote divergence between the alleles. In this case too, coding sequence changes are present and may have a functional role, although this remains to be tested. *Engrailed* expression patterns have previously been shown to correlate with adult wing patterns in the butterfly *Bicyclus anynana* [55], suggesting that the transcription factor may have a widespread role in regulating butterfly wing colour patterning.

Other *Papilio* species

Papilio memnon is similar to *P. dardanus* in having a large number of female morphs that are largely controlled by a single genetic locus [56], but the molecular genetics in this species has not been investigated. *Papilio [Pterourus] glaucus* has a mimetic and a non-mimetic female morph, largely controlled by a locus on the W chromosome, with a low frequency of a Z-linked modifier alleles coming from hybridisation with *P. canadensis* [57]. The fact that control is sex-linked in this species demonstrates that the genes involved are again distinct from those controlling polymorphism in *P. polytes* or *P. dardanus*.

Conclusions

'Supergenes' controlling butterfly colour were initially thought to be made up of multiple tightly linked genes [49]. However, in both investigated *Papilio* species only a single gene seems to be involved [51••,54•]. The situation in *Heliconius* is less clear. *H. numata*, the only species with classical supergene architecture, does have large inversions between different mimetic alleles, which lock together multiple genes [31]. Current evidence points to just one of these genes, *cortex*, as having a major effect on phenotype [33••], but it is still too early to say whether

other genes in the inversions might act with *cortex* to produce some of the phenotypes. Molecular investigation of other systems has also shown that in some cases supergenes can involve the action of multiple genes locked together in inversions [49,58,59].

Something that is clear is that these loci have not evolved through genomic rearrangements that have brought together previously unlinked genes from around the genome [60]. Indeed, in *Heliconius* the steps involved in building a supergene can be observed. The three major loci described above (*optix*, *corte*x and *WntA*) control most colour pattern variation not only in the co-mimetic species *H. erato* and *H. melpomene* but also in *H. hecale* and *H. ismenius*, which have spotted patterns like *H. numata* (Figure 2) [24•]. This, together with other studies [39], illustrates that each of these loci can have diverse effects on phenotype and that these effects can sometimes be overlapping and can vary in their magnitude. It is therefore not a great leap to see how accumulation of mutations concentrated at just one of these loci could take on broad phenotypic effects, with polymorphism at other loci being reduced. Indeed traces of this process can still be seen in *H. numata*, where variation linked to *wntA*, *optix*, *K*, and chromosome 13 was found to have minor effects on phenotype (Figure 2) [61].

This also demonstrates that the genetic variants in these systems are in fact the product of a, probably lengthy, process of refinement, that has likely led to a reduction in the number of loci controlling colour pattern. Selection will act against unfit recombinant phenotypes, and will be strongest in fully polymorphic populations and weaker (but still present) where morphs are parapatric [32]. Therefore, we need to be cautious about making inferences from these systems about the earliest stages of divergence and the distributions and effect sizes of the first mutations that were targeted by selection. It is likely that multiple mutations at each of these loci have led to the current polymorphic alleles, and evolution may also have been facilitated by mutations at unlinked loci, at which polymorphism was later lost due to selection [60].

A key remaining question is why the patterns of gene reuse are so different between *Helcionius* and *Papilio*, especially when, superficially, the workings of the different *Papilio* species seem so similar. One obvious possibility is the different forms of mimicry involved: *Heliconius* are Müllerian mimics, with different species converging on the same patterns, while the *Papilio* species mimic different, distantly related species. Maybe this is why the different *Heliconius* species use the same loci, while the *Papilio* species do not. However, the use of the same loci even in species that have very different patterns, like *H. melpomene* and *H. hecale*, suggests that this is not the whole story. Another plausible explanation could be the ubiquity of both colour pattern polymorphism and gene-flow throughout *Heliconius*. This could have helped to maintain polymorphism at the tool-kit genes, making them predictable targets for selection whenever a new colour pattern became favourable. By contrast, the polymorphic *Papilio* species are more sparsely distributed both geographically and on the phylogeny, perhaps making the evolution of mimicry somewhat more 'independent' between each species. This argument does not hold for other examples of gene re-use, however. For example, why the peppered moth and admiral butterfly have also used two of the *Heliconius* tool-kit genes [37•,41•], as these events are clearly evolutionarily distinct. Ultimately, the question of what drives patterns of gene use can only be answered by comparing more systems and understanding the genetic basis of further adaptive and polymorphic traits.

Acknowledgements

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