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Genetics of a color polymorphism in Heliconius doris

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Balancing selection refers to the maintenance of multiple phenotypic variants within a population. There are a number of proposed mechanisms explaining the origin and persistence of the evolution and genetics of polymorphisms, but they largely remain unresolved in the specific instances in which they occur. This study aims to identify the genetic basis of a polymorphism in the butterfly, *Heliconius doris*, which displays four distinct color patterns on the dorsal hindwings of individuals. While Mullerian mimetic theory proposes that phenotypes will converge on a common, aposematic phenotype, this is not the case in *Heliconius doris*. We identify an interval perfectly associated with the presence/absence of the red ray phenotype, and propose potential mechanisms and genetic architecture through which this polymorphism has been allowed to persist.

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NOMENCLATURE

CRE	Cis-regulatory Element
MYA	Million Years Ago
3-OHK	3-OH-kynurenine
CRISPR	Clustered Regularly Interspersed Short Palindromic Repeats
N50	Length of the shortest assembled sequence at 50% of total genome length
BWA	Burrows-Wheeler Aligner
GATK	Genome Analysis Toolkit
SNP	Single Nucleotide Polymorphism
QUAL	Quality of Variant Call
GQ	Genotype Quality
FST	Fixed Statistic
DXY	Absolute Divergence
π	Nucleotide Diversity
TWISST	Topology Weighting by Iterative Sampling of Sub-trees
SD	Standard Deviation
bp	Base Pairs
Mb	Megabases (1,000,000 base pairs)
Kb	Kilobases (1,000 base pairs)

CHAPTER I

GENETICS OF A COLOR POLYMORPHISM IN *HELICONIUS DORIS*

Introduction

Color polymorphism refers to the presence of multiple, discrete phenotypes within a population due only to genetic variation (Huxley, 1955). It has long been an area of interest in evolutionary biology for a number of reasons, but notably, because the variation was easily visible to the observer, and, therefore, served as a controllable indicator in the time before molecular genetics. Polymorphic systems have been reported in a number of taxa, and have been studied in the way of selection, speciation, and the maintenance of variation, to aposematism and mimicry (McKinnon & Pierotti, 2010).

There have been a number of observations of adaptive polymorphisms in populations of mimetic butterflies, where the varying wing patterns of one species often mimics the wing patterns of other species (O'Donald & Pilecki, 1970; Mallet & Gilbert, 1995; Constantino, 2005; Wilts *et al*., 2017). *Heliconius* butterflies have radiated into 400+ wing color pattern morphs across the Neotropic (Van Bellegham *et al*., 2017), and offer a suitable model to study the genetics of color polymorphisms. This is due to their display of Mullerian mimicry, where these unpalatable species have converged on phenotypes that serve as unique warning signals to predators, allowing them to equally benefit (Müller, 1879). Deviation from the local mimetic wing patterns would be selected against because predator recognition of toxicity is lost (Joron, 2005). The most well-known of these co-mimics are *Heliconius erato* and *Heliconius*

melpomene, which have diverged into over 25 geographic populations, each with distinct wing color patterns (Sheppard *et al*., 1985). However, other species, such as those within the *silvaniform* clade of *Heliconius*, have evolved polymorphic states within a single population. In some locations, the butterfly *Heliconius numata* has as many as seven different mimetic forms, copying those of local Ithomiine butterflies (Joron, 2005).

The research concerning the evolution of *Heliconius* mimicry has repeatedly implicated the same set of genes in the formation of color patterns: the transcription factor *optix* has been shown to be responsible for the deposition of red pigments (Reed *et al*., 2011); the *Heliconius* homolog to the signalling ligand *WntA* modulates melanin deposition in the forewing (Martin *et al*., 2012) and perhaps in an apparent epistatic interaction with *optix* in the hindwing (Concha *et al*., 2019; Van Bellegham *et al*., 2020); and cortex is associated with deposition and variation in yellow coloring (Nadeau *et al*., 2016). There is little divergence between species in the coding regions of these genes, and the observed variation has been attributed to changes within cis-regulatory elements (Nadeau *et al*., 2016; Van Belleghem *et al*., 2017; Lewis *et al*., 2019). A number of interdependent *optix* CREs are responsible for modulating red-patterning across multiple *Heliconius* species. It's suggested that the phenotypic convergence is due to an ancestral set of loci that are responsible for the co-evolution of mimetic red patterns in the erato and melpomene lineages (Lewis *et al*., 2019). In *H. erato* and *H. melpomene*, a simple allelic change around *cortex* is responsible for differences in yellow hindwing color between geographic populations (Nadeau *et al*., 2016). In *H. numata,* this same locus, *cortex*, controls a wide range of color polymorphisms through a series of chromosomal inversions that have been described as a color pattern "supergene" (Joron *et al*., 2011). Together, these studies show how it is possible for an ancestral suite of loci to facilitate convergence on only a few forms across a species radiation.

One of the lesser-studied members of the Heliconians is *Heliconius doris*. *H. doris* has only recently been placed within the Heliconius clade, as it was previously known as *Laparus doris*. Phylogenetically, *H. doris* appears to have diverged from *H. melpomene* around 10 MYA (Kozak, 2015). *Heliconius doris* is unique from *H. erato* and *H. melpomene*, in that it exhibits polymorphic color patterns across its entire range in South and Central America, instead of evolving geographic populations with distinct color pattern morphs. In Central America, *H. doris* is characterized by three different morphs having either red, blue or green rays with a black background on the dorsal hindwings, with each morph belonging to a distinct mimicry ring (Figure 1) (Constantino, 2005). In South America, *H. doris* local polymorphisms consist only of the red and blue forms; however the red rays are distinct from those of the Central American red morphs, and are characterized by the long needle-like rays more commonly found in South American *Heliconius*.

Figure 1 Dorsal Wing Patterns of *Heliconius doris* in Gamboa, Panama

The physical mechanisms with which these colored scales are produced have only recently been described in *H. doris* (Martin *et al*., 2014; Wilts *et al*., 2017). The ray pattern arises through a combination of pigmentary and structural features on the wing. Yellow and red coloration are derived from the pigments 3-OHK and dihydroxanthommatin, respectively. Blue rays are the product of cover scales with a blue-reflecting structure in the thin-film lower lamina, and are essentially modified melanin scales. The blue rays can vary in their shape and hue, and the role melanin plays in generating blue reflectance remains unclear. CRISPR/Cas9 knockouts of *optix* in *Junonia coenia* implicate the gene as a possible suppressor of structural iridescence (Zhang *et al*., 2017). Green patterning is unique in that it is derived from a combination of

pigmentary and structural coloration, where scales with blue-reflecting nanostructures are filled with 3-OHK (Wilts *et al*., 2017).

A previous crossing experiment of Panamanian *H. doris* from the Counterman lab predicts a pattern of inheritance for the color polymorphism. The experiment suggests that the presence/absence of red rays segregates at a single locus, with red being the dominant phenotype to both green and blue rays. Crosses between blue and green morphs suggest that green rays are dominant to blue rays, but the degree of yellow pigmentation and intensity of blue rays both appear to be highly variable (Counterman, unpublished results). The genetic architecture regulating the polymorphism in *Heliconius doris* is currently unknown. However, functional work in *H. doris* implicates the same color pattern genes found to regulate color patterns in other *Heliconius* species. Red rays are the product of *optix* pre-patterning, but green and blue rays are not (Martin *et al.*, 2014). While green rays contain 3-OHK, it is unknown whether the presence/absence of this pigment is regulated by *cortex*. The genetic basis of blue coloration in *Heliconius* lacks resolution. This study aims to resolve the genetics behind the color polymorphism in *H. doris* through genotype-by-phenotype associations.

Methods

Sampling & Sequencing

Heliconius doris butterflies were obtained from the Counterman lab crossing experiment and other lab collections with individuals from Panama (15), and South America (8) to undergo 100bp whole-genome Illumina sequencing at 10-15x coverage.

Table 1 Sampling of *Heliconius doris* Individuals

Morph	Location	Sample Size
Red	**Panama**	**5**
	South America	**3**
Blue	**Panama**	**5**
	South America	**5**
Green	**Panama**	**5**

Genotyping

The *H. doris* genome assembly used in this experiment consists of 13,861 scaffolds, with an N50 of 1.6Mb, and contains chromosome assignments from a synteny map built with *H. melpomene* (Cicconardi and Montgomery, unpublished results). Raw reads were aligned to the genome assembly using BWA v.0.7.17 (Li and Durbin, 2009) with default parameters and sorted with SAMtools v.1.9 (Li *et al*., 2009). Sample genotypes were called with GATK v.4.1.4.1 HaplotypeCaller, combined using the CombineGVCFs tool, and jointed genotyped with genotypeGVCFs with default parameters (Poplin *et al*., 2017). Genotypes were filtered for SNPs with RTGtools 3.10.1 (Cleary *et al*., 2014) and used downstream only if calls were present in at least three individuals per morph, twenty individuals overall, had Qual ≥ 30, GQ ≥ 25, minimum read depth ≥ 10, max read depth ≤ 100. Genotypes were then phased using Beagle 5.1 (Browning *et al*., 2018). This resulted in a list of 2,683,129 high-quality SNPs across the *Heliconius doris* genome assembly.

Population Genetic Analyses

SNPs were used to calculate various population genetic measures (F_{st}, D_{xy}, π) to look for regions of differentiation between morphs using Python scripts from Simon Martin's General Genomics GitHub repository (https://github.com/simonhmartin/genomics_general). Additionally, a two-tailed Fisher's exact test was conducted on these sites to look for perfect associations between genotype and phenotype. These were performed using comparisons that reflected the projected patterns of inheritance of red and green rays. All Central American morphs were compared to all South American morphs to test whether there is any clear evidence of divergence between populations that reflects the differences in color pattern morphs in these geographic regions. Central American red individuals were compared to South American red individuals to identify genomic intervals that could be candidate loci for the genetic basis of differences in the shape of the rays. Green individuals from Central America were compared to all blue individuals to look for regions of the genome that are potentially regulating the presence/absence of 3-OHK. Finally, independent comparisons of red and blue individuals within Central America and South America were conducted alongside comparisons combining all individuals of these morphs in order to determine whether potential candidate loci for the presence/absence of red rays are shared among these geographic regions.

Phylogenetic Analyses

Genotypes were run through phyml3.3.2 with a GTR model in windows of 25 and 50 SNPs using python scripts from the Simon Martin'sGenomics GitHub repository (https://github.com/simonhmartin/genomics_general). In order to identify potential differences in evolutionary history across the genome, these trees were then input into TWISST, a phylogenetic

weighting tool used to quantify the relative contribution of the various potential topologies of user-defined groups to a genomic interval (Martin and Van Belleghem, 2017).

Results & Discussion

Optix Association Shared among Central and South American Red Morphs

When comparing all red morphs to all blue morphs, ignoring geographic origin, Fisher's exact test identifies 41 SNPs perfectly associated with color pattern. While many of the SNPs are dispersed throughout the genome, there are two distinct peaks of divergence. The first of these contains 10 perfectly associated SNPs across an 8Mb interval located on chromosome 12. The haplotypes in this interval do not fit the expected inheritance pattern, where blue is expected to be a homozygous recessive genotype. Given the low number of variants, size and location of the observed peak, and genotype composition, this is not likely to be the causative variant for the presence/absence of red rays, but may indicate variation present between morphs in other areas of the wings.

The second peak of divergence between red and blue morphs appears on chromosome 18, containing 19 perfectly associated SNPs within a 7kb window, and is located ~111kb away from the color-patterning gene, *optix* (figure 2). Π measurements in this interval reflect the predicted pattern of inheritance, where there was no nucleotide diversity among blue morphs, but red morphs were heterozygous or homozygous for a variant nucleotide. TWISST produced a similar result in this region (Figure 3). Topologies that grouped red rayed individuals comprised a much higher proportion of weightings compared to those that grouped by geography; whereas in other regions of genome, the converse of this finding held true (Figure 4). These results reinforce previous studies that show *optix* is only expressed in the red rays of *H. doris* (Reed *et al*., 2011; Martin *et al*., 2014). It also recapitulates findings in other *Heliconius* species, where *optix* has

been found to be modulated by one or more cis-regulatory elements (Supple *et al*., 2013; Van Bellehem *et al*., 2017; Lewis *et al*., 2019), and it is likely this interval in *H. doris* is homologous. Further, as the association exists in spite of the geographic origin of samples, it is likely that the color polymorphism for the presence/absence of red rays predates any proposed divergence of Central American and South American populations.

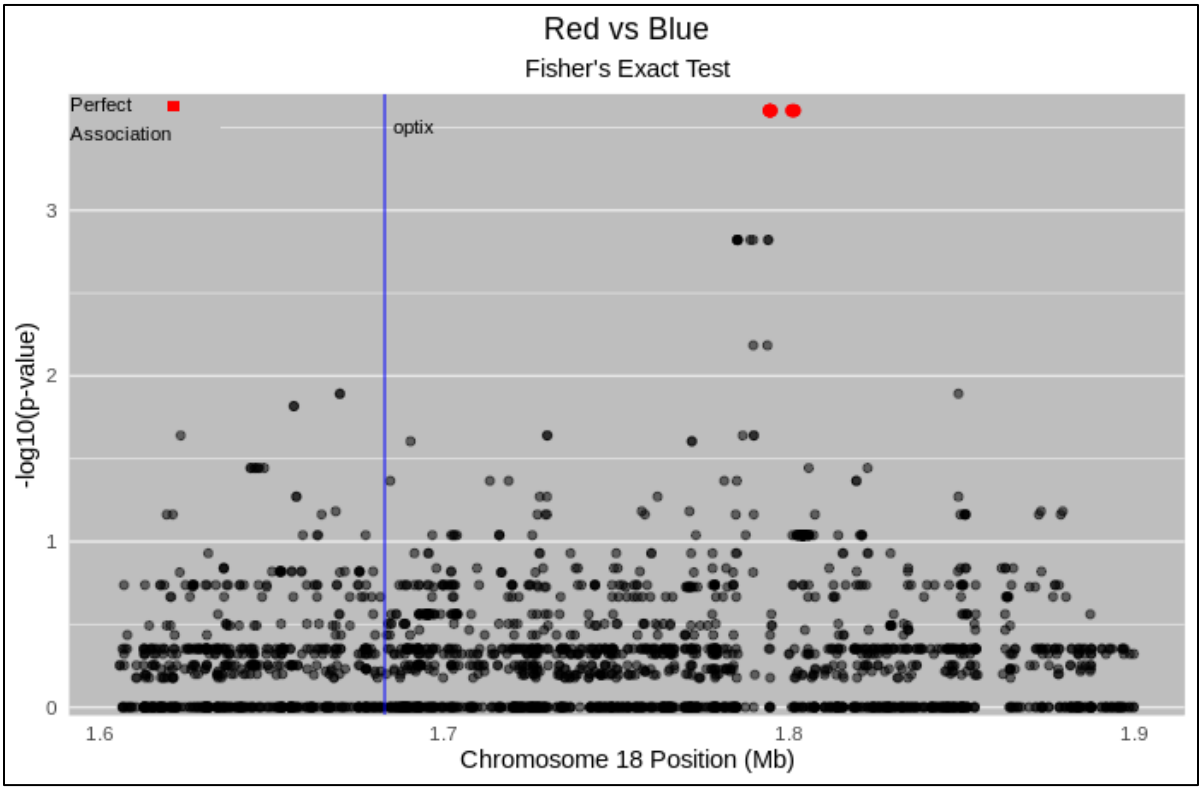

Figure 2 Genotype-by-Phenotype Association near *optix*

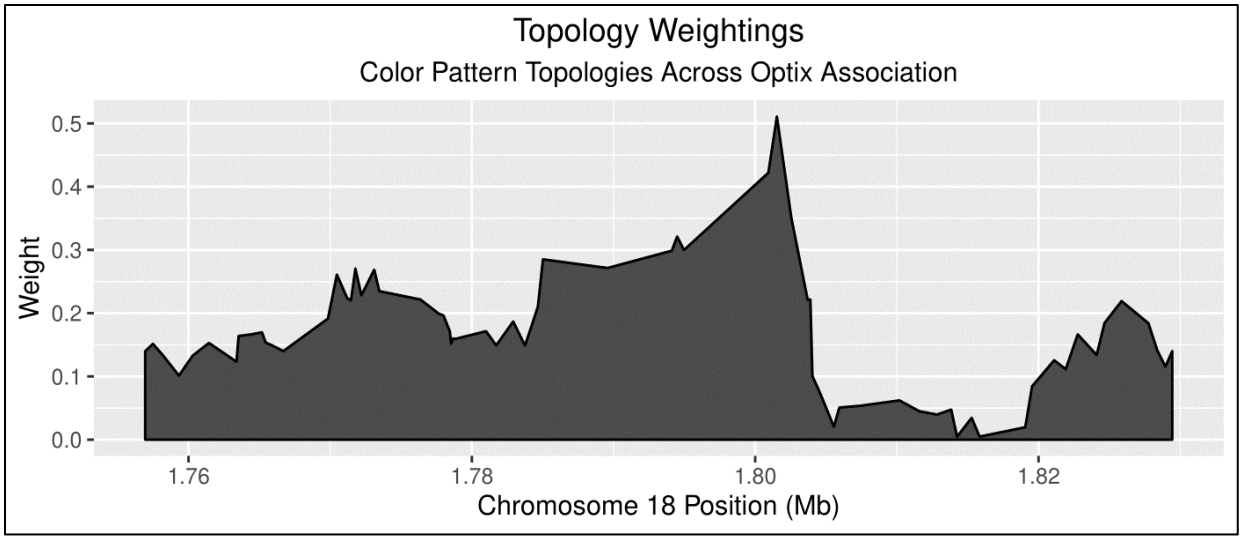

Figure 3 Relative Weighting of Color-Pattern Topologies at *optix* interval

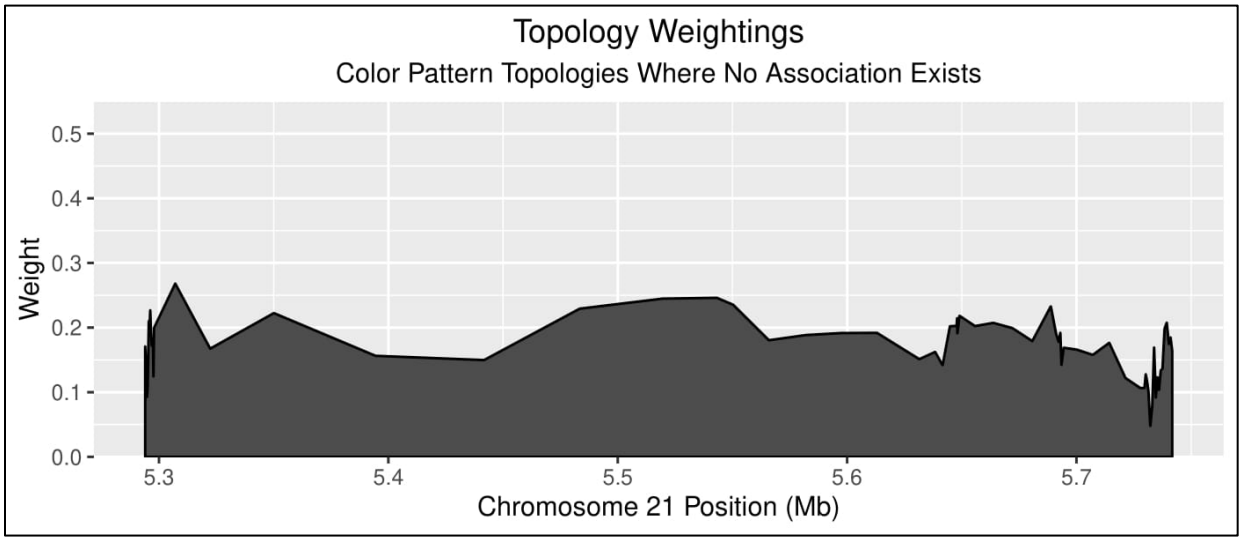

Figure 4 Relative Weighting of Color-Pattern Topologies on Chromosome 21

Pattern Differences between Central and South American Red Rays Lack Resolution

The distinct red morphs of Central and South America lack a clear genetic basis. Even when accounting for associations that are distinct between all Central and South American individuals, there are still greater than 30,000 perfect associations. Not only does this speak to a lack of clear divergence between these morphs, but between the two populations as a whole. It appears as though there is little evidence to suggest that, at present, there is selection driving isolation between Central and South American *H. doris*.

Genetics of Green Rays

Fisher's exact test between green and blue morphs shows 344 perfect associations across the entire genome, where nucleotide diversity is present in green individuals, but not blue. These SNPs are found on twenty out of twenty-one proposed chromosomes. While it has been shown that green rays are the result of 3-OHK deposition on blue-reflecting scales (Wilts *et al*., 2017), it is unclear whether the presence/absence of yellow is regulated by loci homologous to those of other *Heliconius* species: *Cr* in *H. erato*, *Yb/Sb/N* in *H. melpomene*, and *P* in *H. numata* (Joron *et al*., 2006). Due to both the size of this locus and diversity in effects between species, it is possible that the genome assembly lacks resolution in this region because chromosome assignments are based on a synteny map with *Heliconius melpomene*. However, due to failure to identify a single interval and the variation in pigmentation in the green rays, it is possible that the trait is subject to variation in regulation of a supergene locus, such as is the case of the inversions in the polymorphic *H. numata* (joron *et al*., 2011). Alternatively, multiple, interdependent modifier loci could be regulating the variation in these morphs, such as what's been observed in other *Heliconius* species (Lewis *et al*., 2019). Finally, there is evidence to suggest that chromosome number is variable both within and between *H. doris* populations, with numbers

varying between 21 and 28 chromosomes (Wesley and Emmel, 1975). However, sample sizes are limited, or completely absent, in some populations, such as those in Panama.

Origin and Maintenance of a Balanced Polymorphism

Despite the lack of clarity in the genetic basis of various *Heliconius doris* morphs, it is still possible to explore the origin and maintenance of the polymorphism. Considering the mimetic nature of *H. doris*, the question of how new morphs arise is challenging. This is because the number of mutations required to introduce entirely new variants would be expected to be restrained in intermediate stages due to negative selection against non-mimetic forms and/or genetic drift. However, there are a few proposed mechanisms for their origin that would overcome this initial setback: major-effect mutations, gene duplication, introgression, and incomplete lineage sorting (Llaurens *et al*., 2017; Lewis *et al*., 2019). Recent evidence suggests deep ancestry of *optix* CREs between *Heliconius erato*, *Heliconius melpomene*, and *Dryas iulia*, which diverged from the *Heliconius* clade ~25 MYA, and that co-evolution of these elements in *H. erato* and *H. melpomene* helped facilitate mimicry (Lewis *et al*., 2019). Given that *H. doris* falls near the root of the divergence between *H. erato* and *H. melpomene* (Kozak, 2015), the most parsimonious explanation would be incomplete lineage sorting of an ancestral *optix* CRE promoting the evolution of red rays.

In order to maintain the color polymorphism, there must be an underlying mechanism preventing the loss of low frequency variants. The following are a few mechanisms that have been proposed as potential explanations in polymorphic systems: negative frequency-dependent selection, heterozygote advantage, sexaul antagonism, and fluctuations in selection through time and/or space (Llaurens *et al*., 2017). Müllerian mimetic theory expects the most common phenotype to be driven to fixation through positive frequency-dependent selection (Merrill *et al*.,

2015) , but this is not the case with *H. doris*. In the polymorphic species, *H. numata*, the various morphs may be sustained due to a heterogeneous environment (Joron *et al*., 1999). No studies have been conducted to test a similar trend in *H. doris*, but the color polymorphism may be explained by a similar heterogeneity in selected forms over space and/or time. *H. doris* red rays have been suggested to mimic other *Heliconius* and *Parides* species. Green morphs resemble *H. hewitsonii*, *H. charitonia*, and others. The blue rays of *H. doris* mimic iridescent blue rays of *H. sara* and *H. wallacei*. The relative frequency of the morphs appears to reflect the presence of these proposed mimics, where red rays seem to predominate in northern stretches of its distribution, but blue is more common further south. However, *H. doris* is not always a perfect mimic of the proposed species, especially in the yellow forewing bar (Mallet, 1999). Perhaps this represents a concession in mimetic accuracy in favor of a higher capacity to spatio-temporally adapt to predominate local forms.

Summary & Conclusion

The evolution of color-patterns in *Heliconius* has been an area of interest for evolutionary biologists for roughly 150 years. The wings of these butterflies are striking examples of adaptive evolution that allow us to resolve the underlying mechanisms of both convergence and divergence. *Heliconius doris* uses a similar mechanism to that of other species to regulate the presence/absence of red rays within its populations. We identify a proposed causative variant for this color polymorphism, and show that its presence predates any segregation between Central and South American populations. This experiment aims to add to the body of work that shows the genetic architecture behind the co-evolution of *Heliconius* wing patterns is highly conserved, but also highly adaptable. While there are many *Heliconius* studies showing that an ancestral

color pattern toolkit can repeatedly evolve similar phenotypes, we show how this same suite of regulatory elements may also maintain multiple phenotypes within a single population.

Beyond the wings of a butterfly, this study provides further insight into the nature of balancing selection in general. While color polymorphisms remain to be fully understood, there is a growing body of work aiming to characterize innate features and mechanisms that contribute to their evolution. The experiment outlined here is presented in hopes of adding to that effort.

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