Published as: Villoutreix, Romain, de Carvalho, Clarissa F., Soria-Carrasco, Víctor, Lindtke, Dorothea, De-la-Mora, Marisol, Muschick, Moritz, Feder, Jeffrey L., Parchman, Thomas L., Gompert, Zach, & Nosil, Patrik. Large-scale mutation in the evolution of a gene complex for cryptic coloration. Science, 369 (6502). https://doi.org/10.1126/science.aaz4351

Large-scale mutation in the evolution of a gene complex for cryptic coloration

Short title: **Large-scale mutation of color genes**

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The types of mutations affecting adaptation in the wild are only beginning to be understood. In particular, whether structural changes shape adaptation by suppressing recombination or by creating new mutations is unresolved. Here we show that multiple, linked but recombining loci underlie cryptic color morphs of *Timema chumash* stick insects. In a related species, these loci are found in a region of suppressed recombination, forming a supergene. However, in seven species of *Timema* we find that a mega-base size 'supermutation' has deleted color loci in green morphs. Moreover, we find that balancing selection likely contributes more to maintaining this mutation than does introgression. Our results show how suppressed recombination and large-scale mutation can help package gene complexes into discrete units of diversity, such as morphs, ecotypes, or species.

Genes that control adaptive traits have now been identified for defensive armor in stickleback (*Eda* and *Pitx*)(*1, 2*), coat color in mice (*Agouti*)(*3*) and Soay sheep (*Tyrp1*)(*4*), beak size in Darwin's finches (*Hmga*)(*5*), flower color in phlox (*Chsd*)(*6*), and flowering time in *Arabidopsis* (*Fri*)(*7*), among other examples (*8*). However, our understanding of the genetics of adaptation remains far from complete. This is because the type, number, and sequence of mutations that cause variation in adaptive genes are often unknown, yet these details can affect evolutionary dynamics (*9, 10*). Moreover, adaptation often involves multiple genes (*11*), which potentially may interact. When this is the case, some gene combinations confer different fitness than others. This can lead to the evolution of co-adapted gene complexes and make it difficult to predict evolution from analyses of single genes alone (*9, 10*).

Our understanding of adaptation is particularly poor when genes in regions of suppressed recombination control traits because this complicates fine-scale genetic mapping (*12, 13*). Examples of multiple traits mapping to regions of suppressed recombination, for example due to structural variants such as chromosomal inversions, are accumulating across diverse insect, plant, bird, and fish taxa (*14-18*). Many of these cases are thought to represent 'supergenes', where suppressed recombination is favored to maintain adaptive gene combinations. This supergene hypothesis predicts that multiple genes control adaptive traits and that they are distributed throughout regions of suppressed recombination (Fig. 1).

Alternatively, structural variants create adaptive mutations (*14, 19*), such as inversion breakpoints that disrupt a reading frame or alter expression at a developmental switch gene. This mutation hypothesis predicts that genes affecting adaptive traits will be more localized, at regions like breakpoints. These

hypotheses are not mutually exclusive, but the processes involved may counter-act each other because reduced recombination can have a subsequent effect on suppressing mutation.

Here we find evidence related to each hypothesis, but also identify a large-scale deletion that eliminates dozens of genes, affecting multiple adaptive color loci (i.e., a 'supermutation', Fig. 1). We study wingless, phytophagous *Timema* stick insects, which rely on crypsis for protection against visual predators while resting on their host plants (*20-22*). Body coloration in many *Timema* species has evolved into green and brown (i.e., melanistic) morphs that approximate the colors of the leaves and stems of their hosts, respectively (Fig. 1). Green versus melanistic morphs of *T. cristinae* are distinguished by a major locus on linkage group eight (LG8 hereafter), named *Mel-Stripe* (*22, 23*) (Fig. 1). This locus spans ~10 mega-bases of sequence and exhibits suppressed recombination, putatively due to an inversion. Additionally, one edge of the locus exhibits a large-scale (~1 mega-base pair) insertion/deletion (indel) polymorphism, which harbors 83 predicted genes (Table S1).

Whether one, few, or many loci within *Mel-Stripe* affect color is unknown, as are the locations and identities of such loci. We aimed to resolve these issues here, in the context of the hypotheses above, including testing the core assumption of the supergene and supermutation hypotheses; i.e., that multiple linked loci, rather than a single developmental switch, affect trait variation. To achieve this, we studied ten of the eleven sexual species of *Timema* found in California, including all those in the Northern and Santa Barbara clades (Fig. 1, Northern clade = *T. poppensis, T. knulli, T. curi* 'spp.', *T. petita, T. californicum, T. landelsensis*. Santa Barbara clade = *T. cristinae*. Southern clade = *T. chumash, T. bartmani, T. podura*; nine species were used in both sequencing coverage and phylogenetic analyses, whereas data for coverage and phylogenetics was not collected for *T. podura* and *T. petita*, respectively).

We first collected genotyping-by-sequencing (GBS) data from thousands of individuals from nine *Timema* species distributed across the three clades found in California (*24*). We used this data to compare sequencing coverage between the indel region and the rest of LG8. This allowed us to test whether the indel represents an insertion or a deletion, and to quantify how widespread the mutation is (Fig. 1; Table S2-6). This revealed that sequencing coverage across the indel region is comparable to that for the remainder of LG8 for the melanistic morph in all species in the Santa Barbara and Northern clade, but reduced relative to the rest of LG8 for the green morph in all species in these two clades (including polymorphic species and those monomorphic for green)(Fig. 2). This pattern alone could represent an insertion in the melanistic morph, or a deletion in the green morph. However, if melanistic individuals in the Santa Barbara and Northern clade harbored an insertion, we would expect this region to be missing in the Southern California clade, resulting in low coverage in both morphs in this clade. In contrast, coverage across the indel region for both morphs in the Southern clade is comparable to that for the rest of LG8.

Our data thus support that the indel is a deletion in green individuals in the Santa Barbara and Northern clade (for convenience, we at times refer to this as 'the deleted region', with the understanding that it is not deleted in the Southern clade). Due to the observed low sequencing coverage of the deleted region for green morphs of different species, we hypothesize that this region controls color across species (Fig. 2), and that genes in this region maintain synteny among species (see linkage disequilibrium results below for further support for this claim).

To test the synteny prediction, we generated a new reference genome of a striped *T. cristinae* to compare to the melanistic reference. We did so via *de novo* assembly of Illumina data followed with Hi-C technology, yielding a chromosome level assembly. This revealed that the block of genes that is deleted in the green morphs from the Santa Barbara and Northern Clade form a contiguous segment in the genome of the striped *T. cristinae*, supporting the synteny hypothesis. Notably, *T. cristinae*, which exhibits the deletion, has three genotypes at color loci that occur at appreciable frequencies across the species range (GG – green homozygotes, MM – melanistic homozygotes, GM – heterozygotes) (*22*). Thus, the deletion does not have lethal effects.

Given these results, we performed genetic mapping of color in two species in the Southern clade, which lack the deletion. We collected GBS data for 531 *T. chumash* and 132 *T. bartmani* using standardized photos to quantify body color in the red to green color spectrum (a trait referred to as 'RG' hereafter) and in the green to blue color spectrum (a trait referred to as 'GB' hereafter). Single-nucleotide polymorphism (SNP) genome-wide association (GWA) mapping revealed that color maps to the ~1 mega base pair region that is deleted in northern California, and not to the larger region of suppressed recombination adjacent to it (Figs. 3, S2-4, see multi-locus mapping below for statistical support, Table S2, S5). Thus, color loci (or a color locus) were deleted in the Santa Barbara and Northern California clades, consistent with the supermutation hypothesis. We did detect two additional minor peaks of color association on LGs other than LG8. Examination of the striped *T. cristinae* reference confirmed that these peaks do not reside on LG8, and thus do not represent assembly errors in the melanistic reference. However, we focus subsequent analyses on LG8, which accounts for the overwhelming bulk of association.

We next inferred evolutionary history to better understand the dynamics by which *Mel-Stripe* evolved. This revealed four core points (Figs. 4, S8, S9). First, stochastic character state reconstruction revealed that the deletion most likely arose once in the ancestor of the Santa Barbara and Northern clades (posterior probability = 0.73), and almost certainly arose no more than twice (posterior probability > 0.99; Fig. S8). Second, as previously reported (*24*) there is no evidence for contemporary admixture inferred from a STRUCTURE-like analyses (Fig. 4, S9). Third, we inferred potential historical admixture at the *Mel-Stripe* locus in an explicitly phylogenetic context using TREEMIX (*25*). This revealed that a phylogenetic tree lacking admixture for this locus explained 98% of the covariance in allele frequencies (Fig. 4). Thus, historical admixture for the *Mel-Stripe* locus in *Timema* is low or absent. Fourth, allowing migration revealed two potential admixture events, involving the Santa Barbara and Northern clades and increasing the variance explained to 99.9%. Critically, these two migration events were from deeper branches in the phylogeny to two terminal branches such that they cannot explain overall color variation among species (Figs. 1,3). Moreover, these inferred admixture events would have been so ancient that the deletion polymorphism has been maintained for extended periods of time via processes other than introgression. Indeed, the chromosomal forms (i.e., morphs) of *T. cristinae* are ancient and maintained via long-term balancing selection (*22, 24*). Collectively, the weight of evidence implies that balancing selection is a more important mechanism for the maintenance of polymorphism in *Timema* than is introgression, contrasting other systems where introgression strongly affects evolution (e.g., mimetic *Heliconius* butterflies, Darwin's finches, *Helianthus* sunflowers, and African cichlids)(*18, 26-28*).

Finally, we sought to distinguish whether multiple, linked color genes or a single developmental switch control color, the former being a key assumption of both the supergene and supermutation hypotheses. We were drawn to *T. chumash* to test this assumption because it displays a wide range of color variation, in addition to green and brown including shades of yellow, pink, tan, beige, and blue (Fig. 1), and this species lacks the deletion observed in Northern green-colored *Timema*. This led us to speculate that *T. chumash* may exhibit recombination among multiple color loci. This hypothesis predicts low genetic structure in the region harboring color genes (i.e., a lack of morph-associated genetic clusters), with low linkage disequilibrium (LD) among color loci. As reported below, GBS data support these predictions, revealing that multiple, linked but recombining loci affect color (Figs. 3, Figs S2-5).

To infer the number of discrete genetic regions influencing color we employed a Bayesian multi-locus genome-wide association (GWA) mapping approach in GEMMA that accounts for linkage disequilibrium (LD) among SNPs (*29*). In contrast to past work in *T. cristinae* (*22*), we found evidence for multi-locus control of color in *T. chumash* (Figs. 3, S6-7). Specifically, the region that color maps to contains multiple, distinct peaks of phenotype-genotype association, generally separated from each other by several kilobase pairs. Some peaks were associated with variation in only one trait (red-green, RG or green-blue, GB), and accordingly the genetic correlation between RG and GB was moderate (R = -0.34, 95% CIs = -0.41, -0.26). These results suggest that a contiguous region controls color, but that control is multi-genic.

Our mapping approach allowed us to explicitly estimate the number of genetic variants (i.e., quantitative trait nucleotides, QTN) controlling each trait, by considering how often SNPs were retained as trait-associated across different Markov chain Monte Carlo (MCMC) steps in the GWA (the proportion of such steps is termed the posterior inclusion probability, PIP hereafter, Fig. 3). In the case of multi-genic control with recombination among loci, the one or few SNPs that best tag (via LD) each causal variant are expected to consistently be trait-associated across MCMC steps (i.e., exhibit high PIP values). Thus, PIP values across such SNPs sum to an estimate of the number of total causal variants (i.e., even if causal variants are not unambiguously identified, the number of such variants can be estimated).

This revealed that in the region that is deleted in Northern California, ~3-6 genetic variants control GB in *T. chumash* (4.4, posterior s.d.+- 1.3) and ~2-5 control RG (3.6 posterior s.d. +- 1.4; Fig. 3G-H, S6). In contrast, the number of causal variants predicted to be in the remainder of the *Mel-Stripe* locus in *T. chumash* was near zero (GB = 0.07 posterior s.d.+- 0.26; RG, 0.13 posterior s.d.+- 0.36). PIPs for SNPs in the deleted region for RG and GB were mostly uncorrelated (Pearson r = 0.085, 95% CI = -0.092, 0.256, $P = 0.35$). Nine SNPs had PIPs > 0.1 for RG (7.1%), eight for GB (6.3%), and four for both (3.1%); this overlap exceeds null expectations from a randomization test (expected = 0.6%, $P = 0.001$). Thus, while PIPs for most SNPs in this chromosomal region were not associated between the two different body colors, roughly half of the SNPs most associated with RG were also highly associated with GB.

Also consistent with a multi-genic model, effect sizes were moderate and fairly uniformly distributed among the most strongly color-associated SNPs (Fig. S6). Moreover, phenotypic color scores increasingly became more melanistic (defined by high scores for RG and low scores for GB) as the number of melanic-associated alleles an individual harbored increased (across the ten most strongly color-associated SNPs), and we did not detect evidence for epistasis (Fig. S7). Finally, linkage disequilibrium (LD) among the top color-associated SNPs was low, indicative of recombination between them (Fig. 3B; see also Fig. S10 for LD-based inferences on synteny). Thus, multiple linked but recombining variants affect GB and RG coloration in *T. chumash*.

While the identities of the genes causally affecting color remain to be resolved, several genes are promising candidates (Table S1), and their identities provide hints at how deletion of color loci could hypothetically lead to or maintain a green phenotype in *Timema*. One hypothesis is that the deletion prevents color pigment precursors from entering tegument color cells, and constrains the development of such cells. This could lead to transparency of the tegument, giving the insect a green appearance by exposing the green hemolymph underneath the tegument. Future tests of this idea are required.

Our collective results support the supermutation hypothesis, but they do not preclude a role for suppressed recombination, as these hypotheses are not mutually exclusive. Indeed, closer examination of the data from *T. bartmani* (which lacks the deletion) revealed suppressed recombination in the region harboring coloration loci as supported by genetic clustering by morph in principal components analysis, morph-associated haplotype blocks in phased structure analyses, high LD, and 'block-like' patterns of association in single-SNP GWAS (note that these patterns all contrast with *T. chumash*, which exhibits recombination among color loci, Figs. 3; S5). Thus, although large-scale mutation affected color loci in Northern California, a supergene scenario is likely in *T. bartmani* in Southern California. Our results highlight that whether the key role of structural variants in adaptation is to suppress recombination should be tested rather than assumed. Indeed, an example of a supergene controlling heterostyly in *Primula* involves not only suppressed recombination but also complex patterns of gene duplication (*30, 31*).

In conclusion, we demonstrate here multiple mechanisms controlling large evolutionary changes in *Timema* body coloration. While developmental switches via gene regulation have been described in other systems (*32*), our results indicate that in *Timema* discrete color morphs likely evolve via large-scale mutation of multiple genes. These mechanisms help explain rapid evolutionary leaps and

difficulties observing intermediate or transitional forms, thus helping resolve one of Darwin's greatest concerns with his theory of evolution by natural selection (*33*). Moreover, our results complement, but are distinct from, accumulating examples of introgression leading to evolutionary novelty. Collectively, our findings illustrate how the evolution of gene complexes can package variation into discrete units of biodiversity. Although our focus here was on color morphs, similar processes could underlay the evolution of other forms of diversity, such as ecotypes or species.

Figure 1. The genetics of coloration in *Timema* species and associated hypothesis testing. (A) Photographs of *T. cristinae*, which exhibit discrete green (left five individuals) and melanistic (right four individuals) color morphs. (B) Photographs of *T. chumash*, which exhibit a range of cryptic coloration. (C) Schematic of the *Mel-Stripe* locus on linkage group 8, including the indel polymorphism. (D) The three core hypotheses considered here. Grey bars are chromosomes and vertical black lines denote the breakpoint of an inversion. Red circles are causal variants affecting trait variation. The yellow box is a large-scale mutation, in this case, a deletion. See text for further details. (E) Phylogenetic relationships among *Timema* species (*24*) studied, and a summary of the core results. Vertical lines delineate the three main extant California clades (black line = Northern clade, grey line = Santa Barbara clade, red line = Southern clade). Green and brown filled circles depict green and melanistic morphs, respectively. Species with only one circle are monomorphic, and those with two circles are polymorphic. *T. podura* is found in California but not included, as data was not collected for this species.

Figure 2. Evidence for a large chromosomal insertion/deletion (i.e., indel) polymorphism in *Timema*. Shown are sequencing coverage values for a section of linkage group 8 for green versus melanistic individuals (mapped against a reference genome of a melanistic *T. cristinae*). Divergence from the reference genome is 24 million years ago for the Northern Clade (N), 30 million years for the Southern Clade (S), and zero for the Santa Barbara clade (SB)(*24*). Species monomorphic for green are: *T. curi* spp., *T. knulli, T. petita, T. poppensis*. All other species are polymorphic for green and melanistic individuals, as shown via the coverage results. Melanism is recessive such that melanistic individuals are likely homozygous. Shown is relative coverage as coverage (sequence depth) minus the mean across all 20 kilo-base windows. More specifically, we centered (subtracted the mean) and standardized (divided by the standard deviation) the read depth measures such that they had a mean of 0 and standard deviation of 1. The boxed region is the indel in the *T. cristinae* genome. *P*-values above each panel give the probability of observing coverage lower than that in *Mel-Stripe* more generally by chance based on a randomization test. Shown are results from a two-state Hidden Markov Model (green and brown = normal / high coverage state, grey = low coverage state). bp = base pair.

Figure 3. Evidence for multi-genic control of color variation in *T. chumash*. Panel (A) shows results of single-locus genome wide association (GWA) mapping of red-green (RG) color variation in *T. chumash*, without correction for population structure in GENABEL. For analogous results with correction for population structure, and for the green-blue (GB) trait see Figs. S2-4. The y-axis shows the negative $\log_{10}$ *P*-value (Neg. log p) for each test that a single-nucleotide polymorphism (SNP) is associated with color variation. A peak of association is evident in the deleted region (yellow shading), and not the remainder of the *Mel-Stripe* locus (grey shading). Panel (B) shows linkage disequilibrium (LD, measured as the squared correlation coefficient, r^2) between color-associated single nucleotide polymorphisms (SNPs) on LG8 in *T. chumash*, shown as a function of the base pair (bp) distances between such SNPs. Orange dots are SNPs with posterior inclusion probabilities (i.e., PIPs) > 0.4. LD between these SNPs is low, and no higher than between other SNPs (i.e., those with PIPs < 0.4, grey dots) in this genomic region. Panels (C) to (F) show results from multi-locus genome wide association (GWA) mapping in GEMMA, genome wide and for the scaffold showing the bulk of strong associations, i.e., scaffold 128 on LG8, zoomed into the 5-6Mbp position which represents the deleted region. PIP = posterior inclusion probability. Red-green (RG) color variation is shown with red dots and green-blue (GB) color variation by blue dots, with coloring restricted to linkage group (LG) 8 because it shows the bulk of association. pos = position, where bp = base pair. Panels (G)-(H) show the number of quantitative trait nucleotides (QTN) estimated to reside in the deleted region versus the remainder of *Mel-Stripe* for RG and GB, respectively (indel = 5.0-6.0 mega-base pairs on scaffold 128, *Mel-Stripe* excluding indel = 1 to 4,139,489 base pairs on scaffold 702 and 1 to 5.0 mega-base pairs on scaffold 128).

Figure 4. Inference of contemporary and historical admixture and introgression between *Timema* species. (A) Colored bars show the proportion of the genome for each individual inherited from each of two source populations (colored red versus blue) on the basis of the admixture model in ENTROPY (*34*). Species pairs shown are *T. chumash* vs. *T. podura* (locality = BMT, N = 73, SNPs = 4194), *T. podura* vs. *T. bartmani* (locality = BMCG3, N = 58, SNPs = 3108), *T. californicum* vs. *T. poppensis* (locality = SM, N = 53, SNPs = 2243). These were chosen because they are currently co-occurring (e.g., sympatric or parapatric) species pairs where the opportunity for admixture exists; other cases are shown in Figure S9. Data were taken directly from (*24*). (B) Historical admixture inferred from whole genome re-sequencing data analyzed in TREEMIX. Here, *T. chumash* was designated as the outgroup. The two migration edges for the graph explaining 99.9% of the variation in the population allele frequency covariance matrix are shown and colored to denote the extent of admixture. Arrows on these colored edges indicate the direction of introgression.

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ACKNOWLEDGEMENTS

We thank T. Reimchen, M. Joron, and D. Ayala for discussion and comments on previous versions of the manuscript, and T. Oakley for lab space. We thank Ó. Mira Pérez, L. Lloyd, J. Thakrar, F. Whiting and A. Štambuk for assistance with lab work. The support and resources from the Center for High Performance Computing at the University of Utah are gratefully acknowledged, as well as access to the High Performance Computing Facilities, particularly to the Iceberg and ShARC HPC clusters, from the Corporate Information and Computing Services at the University of Sheffield. **Funding.** The work was funded by a grant from the European Research Council (NatHisGen R/129639, https://erc.europa.eu/) and a fellowship from the Royal Society of London to PN. V. Soria-Carrasco was supported by a Leverhulme Trust Early Career Fellowship (ECF-2016-367). MM was supported by an SNSF Advanced Postdoc.Mobility fellowship (P300P3_147888). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. **Author contributions**. RV, CFC, ZG, and PN conceived the project. RV, CFC, VS, DL, MM, and PN collected data. RV, CFC, VS, MD, and ZG led data analysis, aided by all authors. All authors contributed to writing. **Competing interests**. The authors declare no competing interests. **Data and materials availability.** DNA sequences have been deposited in the NCBI SRA (accession numbers pending). Data processing and analysis scripts have been archived in Dryad Digital Repository XXXX. This includes bash scripts for DNA sequence alignment and variant calling, bash scripts for running GEMMA and TREEMIX, and R scripts for fitting hidden Markov models to the coverage data. SNP genotype matrices, population allele frequency data, and non-genetic data (i.e., color data for association mapping) were also archived in Dryad (accession numbers pending). Correspondence and requests for materials should be sent to patrik.nosil@cefe.cnrs.fr.

SUPPLEMENTARY MATERIALS

Link to online supplementary materials
Materials and methods
Supplementary text
Figs S1 to S10
Tables S1 to S7
References (35-80)