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**Selection on a genetic polymorphism counteracts ecological speciation in a stick insect**

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## Summary

The interplay between selection and aspects of the genetic architecture of traits (such as linkage, dominance, and epistasis) can either drive or constrain speciation [1–3]. Despite accumulating evidence that speciation can progress to ‘intermediate’ stages - with populations evolving only partial reproductive isolation - studies describing selective mechanisms that impose constraints on speciation are more rare than those describing drivers. The stick insect *Timema cristinae* provides an example of a system where partial reproductive isolation has evolved between populations adapted to different host plant environments, in part due to divergent selection acting on a pattern polymorphism [4, 5]. Here, we demonstrate how selection on a green/melanistic color polymorphism counteracts speciation in this system. Specifically, divergent selection between hosts does not occur on color phenotypes because melanistic *T. cristinae* are cryptic on the stems of both host species, are resistant to a fungal pathogen, and have a mating advantage. Using genetic crosses and genome-wide association mapping we quantify the genetic architecture of both the pattern and color polymorphism, illustrating their simple genetic control. We use these empirical results to develop an individual-based model that shows how the melanistic phenotype acts as a ‘genetic bridge’ that increases gene flow between populations living on different hosts. Our results demonstrate how variation in the nature of selection acting on traits, and aspects of trait genetic architecture, can impose constraints on both local adaptation and speciation.

## Highlights

- melanistic *T. cristinae* are found at moderate frequencies across populations
- multifarious selection acts to maintain this melanistic phenotype
- the presence of melanistic *T. cristinae* facilitates gene flow among populations
- selection and the genetic architecture of traits can constrain speciation

## eTOC Blurb

Comeault et al. show how differences in selection acting on traits, combined with the genetic basis of those traits, can constrain speciation. They show how melanistic stick insects are able to act as a ‘genetic bridge’ between populations, facilitating gene flow and constraining adaptive divergence. Their results provide a mechanistic example of phenotypic variation constraining speciation.

## Results and Discussion

Divergent selection is a fundamental evolutionary process that generates genetic differentiation among, but often reduces variation within, populations [6, 7]. By contrast, other forms of selection can act to maintain phenotypic and genetic variation within populations [8–11]. Research on individual forms of selection is extensive, but the evolutionary consequences of polymorphism maintained *within* populations on *among*-population evolutionary dynamics remains less explored [12].

Populations of the stick insect *Timema cristinae* have evolved traits that confer crypsis within, and are under divergent selection between, their two primary host-plant species [5]. Most notably, populations found on *Adenostoma fasciculatum* (a plant with small, needle-like leaves) have evolved an adaptive color pattern phenotype that consists of a white dorsal stripe on a green background (green-striped phenotype; Fig. 1a). By contrast, selection for crypsis on *Ceanothus spinosus* (a plant with broad, ovate leaves) favors green-unstriped individuals over green-striped individuals (Fig. 1a; [5]). Partial prezygotic reproductive isolation has evolved between populations as a consequence of divergent selection acting between hosts [4, 5]. However, adjacent populations on different hosts exhibit high gene flow [13] and low genomic differentiation (median  $F_{ST} \sim 0.01$ ; [14]), raising questions about the factors that maintain gene flow between populations.

A potential explanation for high gene flow is the presence of phenotypes that reduce levels of reproductive isolation (‘anti-speciation’ phenotypes). In addition to the two

green phenotypes described above, melanistic *T. cristinae* with dark, non-green, coloration are found at moderate frequencies across the range of *T. cristinae* (previously referred to as ‘grey’ or ‘red’ phenotypes [15]; mean [standard error] proportion of melanistic individuals within 12 populations sampled on *A. fasciculatum* = 0.12 [0.03] and 8 populations on *C. spinosus* = 0.11 [0.04]; Fig. 1b, Table S1b). Unlike the divergently selected pattern phenotypes, the frequency of melanistic individuals does not vary between host species (generalized linear mixed model [GLMM] with binomial error:  $Z = -0.37$ ,  $P = 0.71$ ; Fig 1b). These data suggest that melanistic *T. cristinae* are not subject to divergent selection between hosts. Here, we provide evidence that the melanistic phenotype in *T. cristinae* acts as an anti-speciation phenotype, imposing constraints on adaptive differentiation and speciation. Because the evolutionary processes we describe are general, our findings likely apply broadly to other systems.

### **Ecological relevance of color phenotypes**

Melanistic phenotypes have been shown to have wide-ranging fitness consequences in insects [16, 17]; here we investigate potential agents of selection acting on color in *T. cristinae*. Compared to green individuals we found that melanistic *T. cristinae* are: (1) more cryptic to avian predators when viewed against the woody tissue of both hosts (linear models [LMs] testing the effect of color phenotype on visibility against the woody tissue of *A. fasciculatum* and *C. spinosus*:  $F_{1,309} = 1097.2$ ,  $P < 0.0001$  and  $F_{1,309} = 300.5$ ,  $P < 0.0001$ , respectively; Fig. 2a; Table S2), (2) less likely to be infected by a fungal pathogen (quasi-likelihood generalized linear model [GLM] with Poisson-distributed error:  $t = -3.4$ ,  $P = 0.001$ ; Fig. 2b), and (3) more common in hot / dry climates (GLM

controlling for spatial autocorrelation [18]:  $P = 0.003$ ; Fig. 2c; Table S3). Conversely, melanistic individuals are less cryptic on leaves and less common in cool / wet climates (Fig. 2). Consistent with a lack of divergent selection acting on color phenotypes, crypsis in different plant microhabitats and effects of climate did not differ between host species (LMs and GLMs: host term,  $P > 0.05$ ).

Populations of *T. cristinae* are connected within a geographic mosaic of host plant species [19, 20] and geographically proximate populations experience high levels of gene flow ( $4N_e m = 36 - 642$ ; [13]). We tested whether phenotypic-dependent dispersal may contribute to this gene flow by conducting a mesocosm experiment measuring the propensity of individuals to move off of *A. fasciculatum* (SI). We found that melanistic individuals are 25% more likely to disperse off *A. fasciculatum* onto a soil substrate than green individuals (GLMM:  $Z = 2.56$ ;  $P = 0.011$ ).

### **Mating preferences of the color phenotypes**

Previous work in *T. cristinae* showing partial premating isolation between populations did not account for any effect of color. We analyzed 3554 no-choice mating trials (3320 previously published [4, 21]; SI) to test whether differences in mating success could contribute to the maintenance of the melanistic phenotype. We observed a 16% and 58% increase in mating success in trials where one or both of the individuals were melanistic, respectively (GLM contrasting trials containing two green individuals against those containing at least one melanistic individual:  $Z = 2.141$ ;  $P = 0.032$ ; Fig 2d). For trials that

included one melanistic individual, mating success was independent from whether the male or the female was melanistic ( $Z = 0.479$ ;  $P = 0.63$ ).

Cuticular hydrocarbons (CHCs) have been implicated in affecting mate choice in *Timema* [22] and other insects [16]. We found that CHC profiles differed between green and melanistic color phenotypes (MANOVA: color: partial  $\eta^2 = 0.014$ ;  $P = 0.043$ ; sex: partial  $\eta^2 = 0.644$ ;  $P < 0.001$ ; interaction: partial  $\eta^2 = 0.044$ ;  $P < 0.001$ ,  $N = 600$ ; SI), suggesting that CHCs affect mating success; however, experimental tests confirming this hypothesis are required.

### **Quantitative population divergence of the color polymorphism**

Results presented above (and illustrated in figure 2) indicate that the melanistic phenotype is maintained by a balance of many selective factors. If this is the case, we predict that genetic differentiation at the locus controlling color will be less than for neutral or divergently selected loci. We tested this prediction by asking whether the locus underlying color exhibits low differentiation relative to genome-wide expectations.

Because results from genetic crosses (SI) and genome-wide association mapping (GWA mapping; see following section) indicate that a single locus controls the green/melanistic color polymorphism with the green allele dominant to the melanistic allele, we estimated differentiation at the locus controlling color directly from the frequency of color phenotypes in 28 population pairs for which we have estimates of genome-wide  $F_{ST}$  (SI;  $F_{ST}$  from Nosil et al. [13]). We found that estimates of  $F_{ST}$  at the locus controlling color is below the 0.5% empirical quantile of genome-wide  $F_{ST}$  in 23 of the 28 pairwise

comparisons (Fig 3a). While the ideal analysis would directly compare  $F_{ST}$  at the SNPs underlying color to that of SNPs sampled from across the genome, we were unable to take this approach due to a lack of overlap between SNPs contained in our GWA mapping data set and those previously used to estimate genome-wide  $F_{ST}$  [13]. Nonetheless, error introduced by this approach is expected to be minor relative to the magnitude of difference we observe [23]. As such, this result provides further evidence that color is not subject to divergent selection between hosts.

### **Genetic architecture of pattern and color phenotypes**

Aspects of the genetic architecture of traits can influence the evolutionary response to selection, and speciation [24, 25]. For example, dominance relationships [25], divergent selection [26, 27], and balancing selection all influence patterns of variation observed within and among populations [28, 29]. However, we generally lack examples of how different forms of selection interact with trait genetic architecture to constrain speciation. To address this gap, we quantified aspects of the genetic architectures of color and pattern in *T. cristinae*.

Segregation of pattern and color phenotypes within 61 F1 families suggest that these traits are each controlled by a single locus with the unstriped allele dominant to the striped allele at the pattern locus and the green allele dominant to the melanistic allele at the color locus (SI; Table S4). Results from crosses were supported by multi-locus GWA mapping [30] carried out using 602 *T. cristinae* genotyped at >340,000 SNPs. Specifically, 95.25% (81.01 – 99.84%) and 49.89% (33.62 – 75.53%) of phenotypic



variation in the color or pattern polymorphism, respectively, could be explained by genotype (SI). Consistent with a genetic architecture of one or few loci of large effect, 94.73% (80.79 – 99.83%) and 70.80% (42.24 – 97.92%) of the variation explained by genotype, for color and pattern, respectively, was explained by a small number of large-effect SNPs (Fig 3b; SI). Likewise, genotypes at the top candidate SNPs identified by GWA mapping confirmed the dominance relationships inferred from crosses (Fig 3c; SI). A single SNP associating with color mapped to an intron of a gene encoding a cysteinyl-tRNA synthetase and two candidate SNPs associating with pattern were located within an exon of a gene lacking functional annotation and a non-coding genomic region, respectively.

All three candidate SNPs mapped to linkage group 8 (LG 8) of the *T. cristinae* genome (Fig. 3b; Fig. S1 for results from single-SNP GWA mapping). We therefore assessed whether color and pattern phenotypes are controlled by: (1) a single locus with three alleles, (2) separate but physically linked loci, or (3) two unlinked loci. Segregation of phenotypes in F1 families was inconsistent with genetic control by a single locus with three alleles (SI). Patterns of linkage disequilibrium (LD) support genetic control of two linked loci as LD between pattern and color candidate SNPs ( $r^2 = 0.038$  and  $0.041$ ) was elevated compared to genome-wide expectations (mean  $r^2$  of 1000 randomly sampled SNPs =  $0.0066$ ; SI). Current evidence therefore indicates that two linked genomic regions control the pattern and color polymorphisms.

**Effects of melanistic *T. cristinae* on evolutionary dynamics**

Our empirical results suggest three general patterns. First, unlike pattern phenotypes, the color phenotypes are not subject to divergent selection. Second, due to factors such as higher dispersal and mating success, melanistic individuals might facilitate gene flow among populations, acting as a ‘genetic bridge’. Third, locally maladaptive pattern alleles are able to ‘hide’ from selection in certain genotypes due to dominance relationships and epistasis (e.g., the stripe allele is not expressed in melanistic individuals).

To explore whether melanistic *T. cristinae* could facilitate gene flow, we parameterized an individual-based diploid model using previously published estimates of selection on pattern phenotypes [5], mating success [4], and migration [21], coupled with the empirical estimates of dispersal, mate choice, and genetic architecture we report here. Previous work in many systems has demonstrated how gene flow constrains adaptive divergence and speciation [31–33]. However, other than examples of phenotypic dependent dispersal and habitat choice [34], studies that explicitly focus on how selectively maintained intra-population polymorphisms might influence speciation are few. Thus, our goal was to test whether selectively maintained polymorphisms in general, and melanistic *T. cristinae* specifically, can constrain speciation.

Results from forward-time simulations support the ‘genetic bridge’ hypothesis: across a range of migration rates, the presence of melanistic individuals results in a decrease in levels of adaptive differentiation at the locus controlling pattern (Mann-Whitney *U* tests,  $P < 0.05$ ; left panel of Fig 4a; Table S7), and an increase in the frequency of inter-host mating (a measure of reproductive isolation; left panel of Fig 4c; Table S7). To a lesser

227 extent, and only in simulations with gross migration rates  $> 0.8\%$ , the presence of  
228 melanistic individuals also reduced differentiation at an unlinked neutral locus  
229 representing the neutral genome (left panel of Fig 4b; Table S7).

230  
231 Among simulations initiated with a melanistic allele frequency of 0.35 ( $n = 200$  per  
232 combination of parameter values) we found that after 1000 generations, the frequency of  
233 the melanistic allele was negatively correlated with levels of adaptive differentiation at  
234 the pattern locus and positively correlated with the frequency of inter-host mating (Fig 4a  
235 and 4c, respectively; Table S9). Weak and negative correlations were found between the  
236 frequency of melanistic alleles and levels of neutral differentiation (right panel of Fig 4b;  
237 Table S9).

238  
239 We next tested whether individual aspects of the melanistic phenotype drove the  
240 evolutionary effects presented above. To do this we simulated evolution under three  
241 ‘reduced’ models either: (1) enforcing equal dispersal between color phenotypes, (2)  
242 removing the mating advantage of melanistic individuals, or (3) making the fitness  
243 effects of alleles at color and pattern loci strictly additive (SI; Table S7). Reduced models  
244 (1) and (2) showed effects congruent with the full model (Table S7). In contrast to the  
245 other models, simulations carried out under the additive fitness scheme resulted in weak  
246 positive and weak negative correlations between the frequency of melanistic alleles and  
247 levels of adaptive differentiation and inter-host mating, respectively (Table S7). Thus,  
248 dominance and epistasis within and among color and pattern loci mediated the effects of  
249 the melanistic phenotype.

Our results provide a clear example of how selection that maintains variation within populations can constrain speciation. These results could be widely applicable because our simulations show how the evolutionary consequences of melanistic *T. cristinae* are not solely the result of individual aspects of our system (i.e., differences in dispersal or mating; reduced models 1 and 2 above, respectively). Rather, the effects of polymorphism on gene flow stem from the diverse effects of genetic architecture and the different selective processes maintaining phenotypic variation. While gene flow has long been recognized as a fundamental process constraining speciation [35], our results highlight how rates of gene flow can be influenced by interactions between both selection and genetic architecture. Similar processes could explain the porous species boundaries commonly reported in other plants and animals [36–39]. Finally, the genetic bridge we document here likely contributes to maintaining locally maladaptive pattern phenotypes in *T. cristinae*, which have been shown to have cascading effects on the richness of the co-habiting arthropod community [19]. Thus, our results suggest that selection and the genetic architecture of traits can have far-reaching effects on evolutionary, ecological, and community-level processes.

## **Experimental Procedures**

### **Ecological relevance of color phenotypes.**

*Crypsis in different plant microhabitats:* We used reflectance spectra (SI; Table S1a) to quantify the relative crypsis of *T. cristinae* color phenotypes when observed against each

of five microhabitats using the avian tetrachromatic color space method described by Endler and Mielke (40; SI).

*Rates of infection by a fungal pathogen:* We counted marks on the cuticle caused by infection with an unidentified fungus on 47 melanistic and 297 non-melanistic individuals collected from two populations (population codes: WTA [34.515833, -120.073150] on *A. fasciculatum* and OJC [34.533233, -119.246517] on *C. spinosus*; SI).

*Climate effects on the proportion of melanistic T. cristinae:* We compared observed frequencies of melanistic individuals within 20 populations across the range of *T. cristinae* (Table S1) with variables describing local climate. We tested for an affect of climate on the frequency of melanistic individuals by constructing GLMs for each of three climate PCs (derived from 19 bioclimatic variables; SI) while controlling for spatial autocorrelation.

### **Genetic architecture of pattern and color phenotypes.**

We carried out GWA mapping using Bayesian sparse linear mixed models (BSLMs) as implemented in the software *gemma* [31; SI]. Here we report estimates of genetic architecture as median values and 95% credible intervals (equal-tail posterior probability intervals [ETPPIs]).

In addition to hyperparameters, individual SNPs with posterior inclusion probabilities (PIPs) > 0.50 were identified as the top candidate SNPs tagging functional variant(s)

controlling phenotypic variation. To validate results from BSLMM analyses, we also carried out single-SNP GWA mapping (SI; Fig. S1). To determine dominance relationships of alleles at each candidate locus we used the  $d/a$  statistic [41, 42](SI).

We used the *T. cristinae* reference genome [14] to determine the genomic location of SNPs included in GWA mapping analyses. To measure LD among different classes of SNPs (SI) we calculated genotypic correlations ( $r^2$ , see SI for results using D') with the *r2fast* function of the *GenABEL* package in R [43].

#### **Modeling the effects of the melanistic polymorphism on evolutionary dynamics.**

We simulated evolution of *T. cristinae* populations using a spatially explicit, individual-based, diploid model with separate sexes (see SI for model and simulation details; Table S6 for parameters combinations used). For each simulation, we recorded (every 100 generations) rates of inter-host mating and levels of locus-specific differentiation ( $F_{ST}$ ) and allele frequencies at three loci: a neutral locus unlinked to all other loci, a color locus controlling whether individuals were green or melanistic, and a pattern locus loosely linked to the color locus ( $r = 0.1$ ) controlling whether green individuals were striped or unstriped. All loci were allowed to evolve over the course of 1000 generations.

#### **Data**

Spectral, fungal, mate-choice and CHC data tables have been deposited at <http://dx.dio.org/10.5061/dryad.qs113>. Source code and wrapper scripts for simulations

have been deposited at <https://github.com/flaxmans/timema>. Raw sequence reads have been deposited under NCBI BioProject accession: PRJNA284835, ID: 284835.

## Supplemental Information

Supplemental Information includes one figure, seven tables, and Supplementary Experimental Procedures and Results and can be found with this article online at <http://www.jneurosci.org>.

## Author Contributions

A.A.C., T.S., J.S., and P.N. designed research. All coauthors contributed to collection and analysis of data. A.A.C. and P.N. drafted the manuscript with input and comments from all coauthors.

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## Figure Legends

Figure 1. Pattern and color variation observed in populations of *T. cristinae*. (a) Variation in dorsal patterning (presence versus absence of the white dorsal stripe indicated by the arrow) segregates as a polymorphism within green-colored individuals and is under divergent selection between host plants. (b) The melanistic phenotype is found at moderate frequencies across populations of *T. cristinae* and does not vary in frequency between populations living on the two host plant species, *Adenostoma fasciculatum* and *Ceanothus spinosus* (Table S1 for population details).

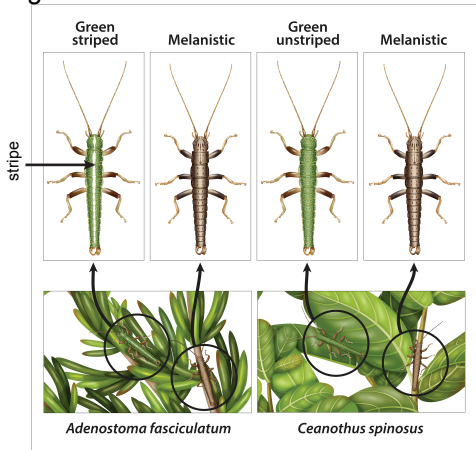
Figure 2. Ecological and sexual factors contributing to the maintenance of the color polymorphism. (a) Green color morphs of *T. cristinae* are more cryptic when viewed against leaf microhabitats whereas melanistic individuals are more cryptic in woody microhabitats (see also Table S2). (b) Melanistic *T. cristinae* show significantly fewer fungal infections than green individuals. (c) Melanistic *T. cristinae* are more frequent in populations found in warmer and drier climates (climate PC1) and there is an interaction between seasonality (climate PC2) and host plant (a random jitter has been applied to y-axis values to separate overlapping points; see also Table S3 for statistics). (d) The likelihood of mating in no-choice trials is greater when a pair consists of at least one melanistic individual (light and dark grey bars) than when two green individuals are paired (green bar).

Figure 3. Genetic divergence and genome-wide association mapping of the color and pattern polymorphisms. (a) Genetic divergence estimated for the locus controlling color ( $F_{ST-color}$ , in red) is less than the 0.5% empirical quantile of genomic divergence ( $F_{ST-genome}$ , lower black bars) in 23 of 28 pairwise population comparisons and greater than the 99.5% empirical quantile (upper black bars) of genomic divergence in three of 28 comparisons. (b) Multi-SNP genome-wide association mapping indicates genetic linkage of major loci affecting pattern and color phenotypes (see Table S5 for additional evidence of LD among color and pattern candidate SNPs). SNPs above the horizontal dashed line represent those with posterior inclusion probabilities (PIPs)  $> 0.50$ . (c) Mean phenotype (bars are 95% binomial confidence intervals computed using the *bincomf* function in R) for each genotype at the three candidate SNPs associated with variation in pattern (first two panels) and color (third panel). These SNPs are the three shown in panel (a) above the dashed line. Lower residual values for the pattern candidate SNPs represent less striped phenotypes whereas, for color, a phenotype score of 0 denotes a green phenotype and 1 denotes a melanistic phenotype (see Table S4 for results from classical genetic crosses).

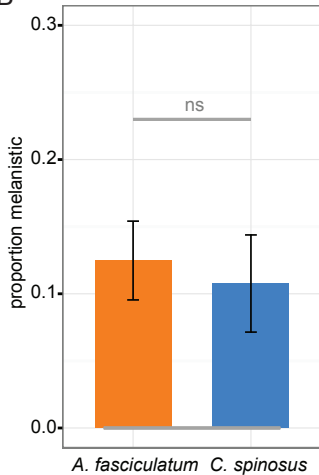
Figure 4. Simulated effects of melanism on adaptive divergence and speciation among populations of *T. cristinae*.  $F_{ST}$  at the pattern locus (a; left panel) and neutral locus (b; left panel) is significantly lower in simulations that included melanistic individuals. Among the simulations that included the melanistic phenotype there is also a negative relationship between the frequency of melanistic color alleles in the 1000<sup>th</sup> generation of a given simulation and  $F_{ST}$  at both the pattern and neutral locus (right panels of (a) and

(b), respectively; points are shaded with respect to the frequency of melanistic alleles in the 1000<sup>th</sup> generation). Simulations including melanistic individuals also had a higher frequency of inter-host mating (c; left panel) and there is a significant positive relationship between the frequency of melanistic alleles and the frequency of inter-host mating (c; right panel). Light shaded lines in the left column of panels represent results from 200 independent simulations run with the color locus fixed for the green allele (green lines) or with the starting frequency of melanistic alleles = 0.35 (grey lines). The two darker colored lines represent mean values across the same runs. Broken black lines in the right column of panels were generated by locally-weighted polynomial regression and Spearman's rank correlation ( $\rho$ ) is shown. Results are shown for simulations with a mean gross migration rate of 1.6%; however, qualitatively similar results were observed for runs with different migration rates (see Tables S7 and S8 for details and results from "reduced" models).

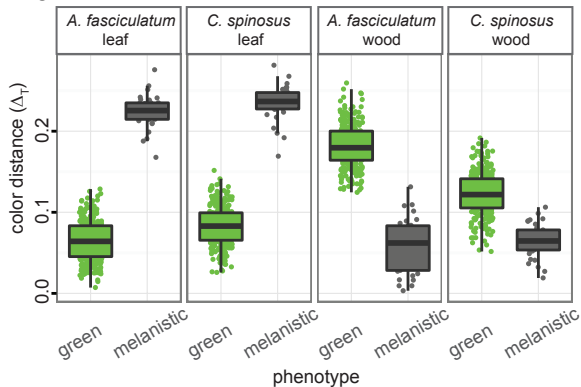
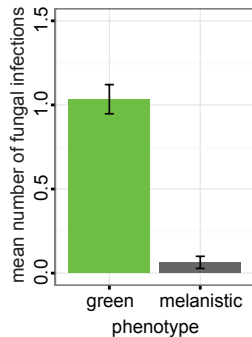
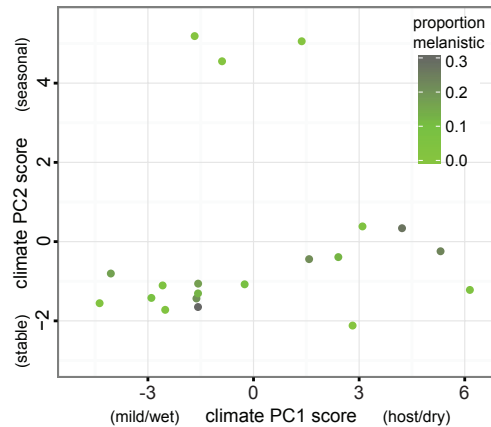
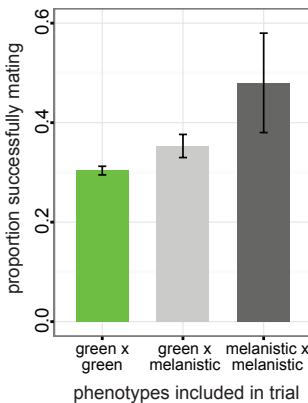
Figure 1

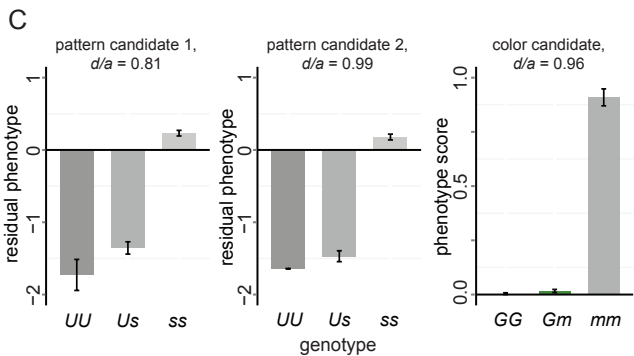
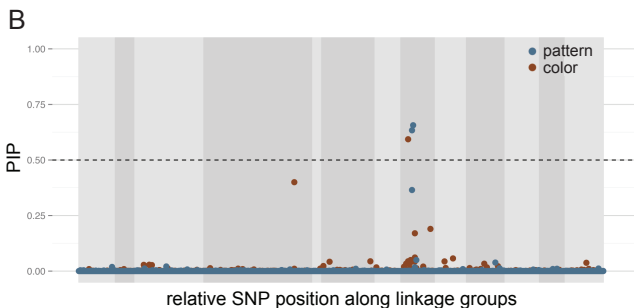
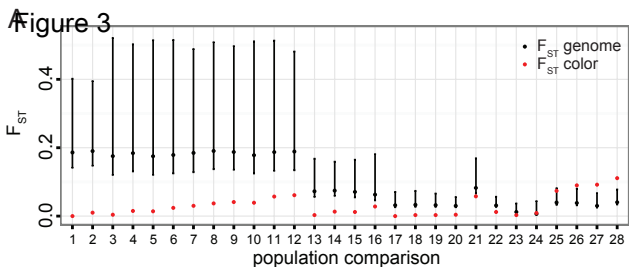


B





**Figure 2****B****C****D**



**Figure 4**

