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Exploring the molecular basis of monarch butterfly color pattern variation

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The monarch butterfly, *Danaus plexippus*, sequesters toxic cardiac glycosides from its milkweed host plant as a larva and then uses these compounds to defend itself from bird predators as an adult (Brower and Glazier, 1975; Reichstein et al., 1968). Like other examples of warning coloration, the bold orange wing pattern of *D. plexippus* helps deter predators by enhancing predator learning and distinguishing it from co-occurring palatable species. Wing pattern also serves a central role in mediating mimicry between *D. plexippus* and the viceroy butterfly, *Limenitis archippus* (Ritland and Brower, 1991). Across most of its range, the monarch displays a largely similar orange wing pattern, although there is variation in wing size, shape, and hue (Altizer and Davis, 2010; Davis et al., 2012; Dockx, 2007). However, on the Hawaiian island of Oahu, there is a rare, “white” form of *D. plexippus*, the *nivosus* morph, that dates back to the 1890’s (Vane-Wright, 1993) and has existed at a frequency ranging between 1%–8% (Stimson and Kasuya, 2000). Individual white monarchs have been reported from various locations across the broad geographic distribution of *D. plexippus* (Vane-Wright, 1993), but on Oahu the *nivosus* morph is maintained as a stable polymorphism. Previous work by John Stimson and colleagues has explored factors that may maintain this polymorphism as well as its Mendelian genetics (Stimson and Berman, 1990; Stimson and Kasuya, 2000; Stimson and Meyers, 1984), showing the *nivosus* phenotype segregates as a simple autosomal recessive trait.

Recently, Zhan et al. (2014) performed a comprehensive population genomic analysis of 101 *Danaus* genome sequences to explore the genetic basis of migration and color pattern variation in the monarch butterfly. As part of this analysis, we sequenced a number of Hawaiian monarchs, including white and orange samples and two F1 offspring. By scanning the genomes for allelic patterns consistent with the known Mendelian genetics, and then testing additional white and orange specimens reared or collected between 1984–1991, our analysis led us to a region centered on the gene DPOGS206617, which I annotated as a myosin gene. My original annotation was based on: (1) the presence of clustered IQ motifs, (2) myosin annotation of some BLAST hits, and (3) a predicted function of “myosin light chain binding” of a putative *Drosophila* ortholog (Franke et al., 2006). While many aspects of this gene made it look like a myosin, Hume (2015) has identified important myosin features that are lacking. So, what is DPOGS206617 and how might it generate color pattern variation in the monarch butterfly?

To better pinpoint the identity of DPOGS206617 in relation to myosin genes, I generated a gene tree among IQ motif containing proteins. The SMART database (<http://smart.embl-heidelberg.de/>) contains 17,616 IQ motif containing proteins, but I limited my analysis to

the 1,311 arthropod proteins. I downloaded full protein sequences, aligned them with Clustal Omega (Sievers et al., 2011), and inferred a maximum-likelihood tree using FastTree (Price et al., 2009; Price et al., 2010). I then annotated gene clusters based on information from *Drosophila melanogaster* (Figure 1). This analysis revealed that DPOGS206617 is not a myosin gene, but rather, groups with genes like *Cep97*, *abnormal spindle*, and *Cp110*. These genes all have well-characterized roles related to microtubules. *Cep97* and *Cp110* are interacting centrosomal proteins that play an important role in governing the switch between centriole and basal body (Bettencourt-Dias and Carvalho-Santos, 2008). Together, they are known to suppress the formation of cilia (Spektor et al., 2007) and *Cp110* controls centriole length (Schmidt et al., 2009), among other things (Avidor-Reiss and Gopalakrishnan, 2013). *Abnormal spindle* binds microtubule minus ends of both the spindle poles and the central spindle. This is thought to cross-link the minus ends and permit formation of the actin/myosin complex necessary for cytokinesis (Wakefield, 2001).

It is not immediately obvious how a putative microtubule-associated protein might function in the context of monarch wing pigment variation. However, this new clue points to cytoskeleton dynamics as a possible mechanism. Recently, Dinwiddie et al. (2014) explored the development of the actin cytoskeleton in butterfly wing scales, showing how it determines scale size and shape. As part of their analysis, Dinwiddie et al. (2014) inhibited actin polymerization during scale development with cytochalasin D, which yielded a variety of defects including misshapen and bent scales. While Dinwiddie et al. (2014) did not examine microtubules in butterfly scales, there is a well-characterized role of microtubules in shaping the development of the homologous structure in flies, the mechanosensory bristle (Bitan et al., 2010a). In the sensory bristle of *Drosophila*, stable, polarized microtubules, with the minus end distal to the cell, fill the shaft while dynamic, mixed-polarity microtubules exist at the tip (Bitan et al., 2012). Coordination between these two sets of microtubules determines bristle size and shape (Bitan et al., 2012). Furthermore, there is an interaction between microtubules and actin in the development of sensory bristles because microtubule mutants have both disorganized microtubules and poorly oriented actin bundles, resulting in shorter, thicker bristles that end with multiple tips (Bitan et al., 2010b).

The realization that DPOGS206617 may code for a microtubule-associated protein, combined with an emerging picture of what cytoskeleton changes might do to scale structure, prompted me to compare wing scales between orange and white monarchs. Strikingly, I found that the wing scales differ in dramatic ways (Figure 2). Broadly, while scales on orange monarch wings looked like normal, tiled butterfly wing scales, those in the grey patches on white monarchs were generally bent, crumpled, and did not lay flat against the wing surface. These scales also appeared more irregular in shape, particularly towards the tip. Indeed, the scales on white wings looked reminiscent of those presented in Dinwiddie et al. (2014) after inhibition of actin polymerization. It should be noted that the melanic scales that make up the dark regions on white monarch wings looked normal in comparison (Figure 2L). How might the observed differences in scale structure between orange and white wings lead to pigment variation? Interestingly, wing veins appear to be pigmented on white wings (Figure 2D), suggesting the orange pigment is still produced but not deposited in the scales. While still quite speculative, this is consistent with the idea that

the white monarch may result from reduced pigment or pigment precursor transport into the scale, as proposed by Zhan et al. (2014), but not because of a dysfunctional myosin but because of another cytoskeletal defect. Perhaps related to this, previous work has shown that the timing of scale development plays a major role in pigmentation (Koch et al., 2000), which may indicate the reduced pigmentation on white monarch wings results from developmental heterochrony.

An important, related issue concerns the mutational basis of the *nivosus* phenotype. Zhan et al. (2014) showed a portion of DPOGS206617 that contains single-nucleotide polymorphisms (SNPs) associated with wing color as well as reduced DNA sequence divergence, suggestive of long-term purifying selection. A closer look at the data yields some suggestive patterns related to possible causal mutations but no definitive answers. Notably, the focal region of DPOGS206617 contains an amino acid substitution, R219G, that is perfectly associated with wing color phenotype in our genome resequencing samples. *Cp110* is a coiled-coil protein and the predicted structure of wild-type DPOGS206617 suggests similar compact alpha-helices. The R219G substitution may have functional consequences because it disrupts a predicted alpha-helix, which may, in turn, impact higher order protein folding (Figure 3). Furthermore, there is a second amino acid substitution in this gene, I276V, that is also strongly, but not perfectly, associated with wing color in our genome resequencing samples. However, neither of these substitutions is very strongly associated with color in historical samples, although a synonymous substitution 17 bp away from R219G is. These results suggest at least two possible alternatives. First, it is possible that one or more functional mutations have yet to be discovered and these results reflect patterns of linkage disequilibrium in the region. Alternatively, the data raise the intriguing possibility that multiple mutations in the gene confer similar loss-of-function phenotypes and the frequency of these alleles has changed among white monarchs over time. More comprehensive sequencing of historical samples, as opposed to the SNP genotype spot checking we have done so far, should help tease this apart.

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Figure 1.
Maximum-likelihood tree showing relationships among the 1,311 arthropod IQ motif-containing proteins in the SMART database. Gene clusters are annotated based on *Drosophila melanogaster* and the long branch leading to the monarch color switch gene DPOGS206617 is indicated.

Figure 2.

Wing and wing scale images of orange (left column) and white (right column) monarchs. (A, B) Dorsal forewings with focal patch indicated where subsequent images were taken. (C, D) 10× magnification on Zeiss SteREO Discovery.V20. (E, F) 50× magnification on Zeiss SteREO Discovery.V20. (G, H) 100× magnification on Zeiss MERLIN SEM. (I, J) 200× magnification on Zeiss MERLIN SEM. (K, L) 100× magnification of melanic scales along wing veins.

Figure 3.
Protein structure prediction of DPOGS206617 using I-TASSER (Roy et al., 2010; Zhang, 2008). (A) Inferred structure with wild-type Arg residue at position 219. (B) Inferred structure with Gly residue at position 219.