CHAPTER TWO

The Genetic Basis of Pigmentation Differences Within and Between *Drosophila* Species

J.H. Massey, P.J. Wittkopp[1]
University of Michigan, Ann Arbor, MI, United States
[1]Corresponding author: e-mail address: wittkopp@umich.edu

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Abstract

In *Drosophila*, as well as in many other plants and animals, pigmentation is highly variable both within and between species. This variability, combined with powerful genetic and transgenic tools as well as knowledge of how pigment patterns are formed biochemically and developmentally, has made *Drosophila* pigmentation a premier system for investigating the genetic and molecular mechanisms responsible for phenotypic evolution. In this chapter, we review and synthesize findings from a rapidly growing body of case studies examining the genetic basis of pigmentation differences in the abdomen, thorax, wings, and pupal cases within and between *Drosophila* species. A core set of genes, including genes required for pigment synthesis (eg, *yellow*, *ebony*, *tan*, *Dat*) as well as developmental regulators of these genes (eg, *bab1*, *bab2*, *omb*, *Dll*, and *wg*), emerge as the primary sources of this variation, with most genes having been shown to contribute to pigmentation differences both within and between species. In cases where specific genetic changes contributing to pigmentation divergence

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were identified in these genes, the changes were always located in noncoding sequences and affected *cis*-regulatory activity. We conclude this chapter by discussing these and other lessons learned from evolutionary genetic studies of *Drosophila* pigmentation and identify topics we think should be the focus of future work with this model system.

1. INTRODUCTION

Heritable changes in DNA sequence within and among species explain much of life's diversity. Identifying these changes and understanding how they impact development to generate phenotypic differences remains a major challenge for evolutionary biology. A growing number of case studies have localized the specific genes involved in trait variation both within and among species, and some have described how individual mutations affect the developmental pathways underlying phenotypic differences. With a catalog of studies describing more than 1000 alleles contributing to morphological, physiological, or behavioral evolution of diverse traits in diverse species now available, researchers have begun to synthesize the genetic and developmental mechanisms underlying phenotypic evolution in search of genetic and molecular patterns that underlie the evolutionary process (Carroll, 2008; Kopp, 2009; Martin & Orgogozo, 2013; Stern & Orgogozo, 2008; Streisfeld & Rausher, 2011).

One finding from this synthesis is that different types of traits tend to evolve through different molecular mechanisms. For example, changes in *cis*-regulatory DNA sequences that regulate gene expression contribute to morphological differences within and among species more often than they contribute to differences in physiological traits, while the converse is true for changes in the amino acid sequence of proteins (Stern & Orgogozo, 2008, 2009). Another finding to emerge from this synthesis is that some traits have evolved multiple times independently using the same genetic changes (eg, xenobiotic resistance), whereas other traits have evolved similar changes using different mutations in the same gene (eg, coat color) or using different genes (Martin & Orgogozo, 2013). Differences in the genetic basis of phenotypic diversity also seem to exist within and between species, with changes in *cis*-regulatory sequences playing a larger role in interspecific than intraspecific differences (Coolon, Mcmanus, Stevenson, Graveley, & Wittkopp, 2014; Stern & Orgogozo, 2008; Wittkopp, Haerum, & Clark, 2008).

In this chapter, we examine patterns in the genetic and molecular mechanisms responsible for phenotypic evolution that emerge from focusing on a collection of studies investigating changes in a single trait within and among species in the same genus. Specifically, we review and synthesize the collection of case studies dissecting the genetic basis of body color (pigmentation) in *Drosophila*, emphasizing a comparison of genetic and molecular mechanisms that vary within and among *Drosophila* species. *Drosophila* pigmentation is an ideal trait for such an analysis because (i) pigmentation is one of the most variable traits within and among species (Kopp, 2009; Wittkopp, Carroll, & Kopp, 2003), (ii) much is known about the genes involved in pigment synthesis as well as those that control expression of these genes during *Drosophila* development (Kopp, 2009; Takahashi, 2013; True, 2003; Wittkopp, Carroll, et al., 2003), and (iii) specific genes and genetic changes have been identified as contributing to differences in *Drosophila* pigmentation that have evolved over multiple timescales and in multiple lineages (Table 1). These differences in pigmentation that have been dissected genetically include examples of trait divergence, convergent evolution, and evolutionary novelty.

2. DEVELOPMENT OF *DROSOPHILA* PIGMENTATION

In *Drosophila* (as well as in many other insects; True, 2003; Wittkopp & Beldade, 2009; Zhan et al., 2010), body color results from a combination of dark black and brown melanins as well as light yellow-tan and colorless sclerotins (True, 2003; Wittkopp, Carroll, et al., 2003; Wright, 1987). These four types of pigments are produced by a branched biochemical pathway that processes tyrosine obtained from the diet (Fig. 1). Tyrosine is first converted into DOPA (L-3,4-dihydroxyphenylalanine) by a tyrosine hydroxylase (TH) encoded by the *pale* gene. This DOPA is then converted into dopamine through a reaction catalyzed by the dopa decarboxylase enzyme encoded by the *Ddc* gene. Prior reviews have suggested that DOPA can also be polymerized into a black melanin through a process involving the Yellow protein (Kopp, 2009; Wittkopp, Carroll, et al., 2003), but recent data show that the formation of black pigment requires the function of *Ddc* (J.-M. Gibert, personal communication) and is thus likely produced from dopamine rather than DOPA, as has also been previously suggested (Riedel, Vorkel, & Eaton, 2011; Walter et al., 1996). Dopamine can then have one of four fates: it can be converted into a black melanin through a

Table 1 The Loci of Pigmentation Evolution in *Drosophila*

Level of Variation	Pigmentation Trait	Species	Gene(s)	Gene Function	Type of Mutation(s)	References
Within species	Abdominal	*D. melanogaster*	*bab*	TF	*cis*-Regulatory	Rogers et al. (2013)
Within species	Abdominal	*D. melanogaster*	*bab1*	TF	*cis*-Regulatory	Bastide et al. (2013)
Within species	Abdominal	*D. melanogaster*	*bab1*	TF	*cis*-Regulatory	Endler, Betancourt, Nolte, and Schlötterer (2016)
Within species	Abdominal	*D. melanogaster*	*bab*	TF	Unknown	Kopp, Graze, Xu, Carroll, and Nuzhdin (2003)
Within species	Abdominal	*D. melanogaster*	*bab1*	TF	*cis*-Regulatory	Bickel, Kopp, and Nuzhdin (2011)
Within species	Abdominal	*D. melanogaster*	*bab2*	TF	*cis*-Regulatory	Bickel et al. (2011)
Within species	Abdominal	*D. melanogaster*	*ebony*	Enzyme	*cis*-Regulatory	Johnson et al. (2015)
Within species	Abdominal	*D. melanogaster*	*ebony*	Enzyme	*cis*-Regulatory	Pool and Aquadro (2007)
Within species	Abdominal	*D. melanogaster*	*ebony*	Enzyme	*cis*-Regulatory	Rebeiz, Pool, Kassner, Aquadro, and Carroll (2009)
Within species	Abdominal	*D. polymorpha*	*omb*	TF	Unknown	Brisson, Templeton, and Duncan (2004)
Within species	Abdominal	*D. melanogaster*	*tan*	Enzyme	*cis*-Regulatory	Bastide et al. (2013)
Within species	Abdominal	*D. melanogaster*	*tan*	Enzyme	*cis*-Regulatory	Endler et al. (2016)
Within species	Abdominal	*D. erecta*	*tan*	Enzyme	*cis*-Regulatory	Yassin et al. (2016)
Within species	Abdominal	*D. polymorpha*	Two genes	Unknown	Unknown	Martinez and Cordeiro (1970)

Within species	Abdominal	*D. melanogaster*	84 genes	Multiple	*cis*-Regulatory	Dembeck, Huang, Magwire, et al. (2015)
Within species	Abdominal	*D. baimaii* *D. bicornuta* *D. burlai* *D. truncata*	Unknown	NA	Unknown	Ohnishi and Watanabe (1985)
Within species	Abdominal	*D. malerkotliana*	At least three QTL	NA	Unknown	Ng, Hamilton, Frank, Barmina, and Kopp (2008)
Between species	Abdominal	*D. melanogaster* *D. yakuba* *D. fuyamai* *D. auraria*	*bab*	TF	*cis*-Regulatory	Rogers et al. (2013)
Between species	Abdominal	*D. willistoni* *D. melanogaster*	*bab1*	TF	*cis*-Regulatory	Williams et al. (2008)
Between species	Abdominal	*D. auraria* *D. serrata*	*ebony*	Enzyme	*cis*-Regulatory	Johnson et al. (2015)
Between species	Abdominal	*D. americana* *D. novamexicana*	*ebony*	Enzyme	Unknown	Wittkopp et al. (2009)
Between species	Abdominal	*D. yakuba* *D. santomea*	*tan*	Enzyme	*cis*-Regulatory	Jeong et al. (2008)
Between species	Abdominal	*D. americana* *D. novamexicana*	*tan*	Enzyme	*cis*-Regulatory	Wittkopp et al. (2009)

Continued

Table 1 The Loci of Pigmentation Evolution in *Drosophila*—cont'd

Level of Variation	Pigmentation Trait	Species	Gene(s)	Gene Function	Type of Mutation(s)	References
Between species	Abdominal	*D. melanogaster* *D. subobscura* *D. virilis*	*yellow*	Unknown	*cis*-Regulatory	Wittkopp et al. (2002)
Between species	Abdominal	*D. melanogaster* *D. kikkawai*	*yellow*	Unknown	*cis*-Regulatory	Jeong, Rokas, and Carroll (2006)
Between species	Abdominal	*D. prostipennis* *D. melanogaster*	*yellow*	Unknown	*cis*-Regulatory	Ordway, Hancuch, Johnson, Wiliams, and Rebeiz (2014)
Between species	Abdominal	*D. yakuba* *D. santomea*	At least four QTL	NA	Unknown	Carbone, Llopart, deAngelis, Coyne, and Mackay (2005)
Between species	Abdominal	*D. yakuba* *D. santomea*	At least five QTL	NA	Unknown	Llopart, Elwyn, Lachaise, and Coyne (2002)
Between species	Abdominal	*D. arawakan* *D. nigrodunni*	Unknown	NA	Unknown	Hollocher, Hatcher, and Dyreson (2000)
Between species	Abdominal	*D. tenebrosa* *D. suboccidentalis*	At least two QTL	NA	Unknown	Bray, Werner, and Dyer (2014)
Within species	Thorax	*D. melanogaster*	*ebony*	Enzyme	*cis*-Regulatory	Miyagi, Akiyama, Osada, and Takahashi (2015)
Within species	Thorax	*D. melanogaster*	*ebony*	Enzyme	*cis*-Regulatory	Takahashi, Takahashi, Ueda, and Takano-Shimizu (2007)
Within species	Thorax	*D. melanogaster*	*ebony*	Enzyme	*cis*-Regulatory	Takahashi and Takano-Shimizu (2011)

Within species	Thorax	*D. melanogaster*	*ebony*	Enzyme	*cis*-Regulatory	Telonis-Scott, Hoffmann, and Sgro (2011)
Between species	Thorax	*D. guttifera* *D. melanogaster*	*wg*	Cell–cell signaling	*cis*-Regulatory	Koshikawa et al. (2015)
Between species	Wing	*D. biarmipes* *D. melanogaster*	*yellow*	Unknown	*cis*-Regulatory	Gompel, Prud'homme, Wittkopp, Kassner, and Carroll (2005)
Between species	Wing	*D. elegans* *D. gunungcola* *D. mimetica* *D. tristes*	*yellow*	Unknown	*cis*-Regulatory	Prud'homme et al. (2006)
Between species	Wing	*D. guttifera* *D. melanogaster*	*wg*	Cell–cell signaling	*cis*-Regulatory	Koshikawa et al. (2015)
Between species	Wing	*D. elegans* *D. gunungcola*	At least three QTL	NA	Unknown	Yeh and True (2014)
Between species	Pupariam	*D. virilis* *D. americana*	*Dat*	Enzyme	*cis*-Regulatory	Ahmed-Braimah and Sweigart (2015)

This table summarizes case studies that have examined the genetic basis of pigmentation differences within and between species of *Drosophila*.

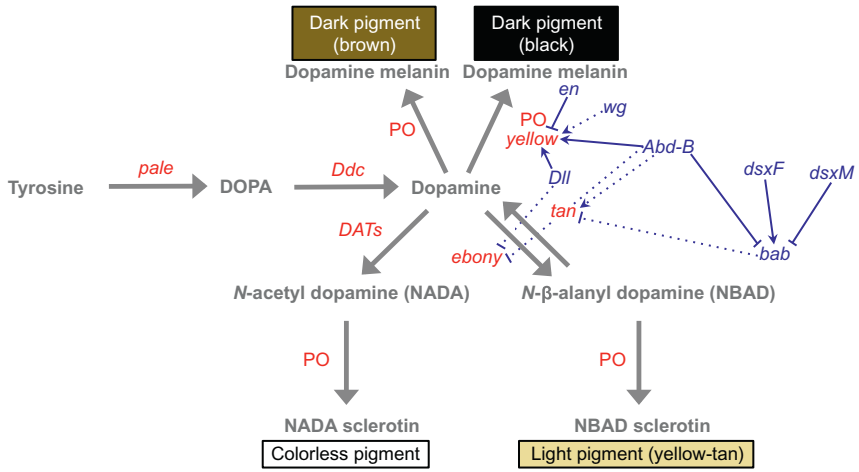

Fig. 1 Developmental and biochemical control of pigmentation in *Drosophila*. A simplified version of the biochemical pathway controlling pigment biosynthesis in insects is shown with regulators controlling expression of individual pigment synthesis genes in at least one *Drosophila* species overlaid. Genes colored *red* (placed next to thick arrows) are part of the pigment biosynthesis pathway, metabolites are colored *gray*, and *gray arrows* indicate chemical reactions during pigmentation synthesis. Genes colored *blue* (at ends of thin pointed and blunt arrows) are part of the regulatory network that directly (*solid arrows*) or indirectly (*broken arrows*) modulate enzyme expression during pigmentation development in *Drosophila*. *Pointed* and *blunt arrows* indicate positive and negative regulatory interactions, respectively. The pigment biosynthesis pathway is conserved among all *Drosophila*, but the regulatory relationships shown often function in only a subset of *Drosophila* species (Arnoult et al., 2013; Gompel et al., 2005).

process involving the Yellow protein and phenol oxidases (POs); converted into a brown melanin through a process involving POs, but not *yellow*; into a yellow-tan sclerotin through the activity of Ebony converting dopamine into beta-alanyl dopamine (NBAD) and POs polymerizing it into NBAD sclerotin, or into a colorless pigment through the activity of dopamine-acetyl-transferases (DATs) converting dopamine into *N*-acetyl dopamine (NADA) and POs polymerizing it into NADA sclerotin. One of these reactions, the conversion of dopamine into NBAD, is reversible, with the reverse reaction catalyzed by the Tan protein. Disruption of the *tan* gene reduces the production of dark melanins, indicating that the conversion of NBAD back into dopamine is a necessary step in the development of pigmentation. Changing relative expression levels of *yellow*, *ebony*, and/or *tan* can shift the balance between dark (black, brown) and yellow-tan pigments

as this branched biochemical pathway produces more of one type at the expense of the other (Wittkopp et al., 2009; Wittkopp, True, & Carroll, 2002).

Pigments produced by this biochemical pathway are deposited into the developing cuticle during late pupal and early adult stages (Kraminsky et al., 1980; Sugumaran, Giglio, Kundzicz, Saul, & Semensi, 1992; Walter et al., 1996; Wittkopp, Carroll, et al., 2003). The spatial distribution of these pigments is determined in a nearly cell autonomous manner by spatially regulated transcription of genes such as *yellow*, *tan*, and *ebony*. As discussed in detail later, changes in the expression patterns of these genes often underlie evolutionary changes in pigmentation. Genes regulating expression of these pigment synthesis genes are thus also potential targets for genetic divergence contributing to pigmentation diversity. Five transcription factors (*bric-a-brac* (*bab*), *abdominal-B* (*Abd-B*), *doublesex* (*dsx*), *Distal-less* (*Dll*), and *Engrailed* (*en*)) have been shown to regulate expression of pigment synthesis genes (*yellow*, *ebony*, *tan*) in *Drosophila* either directly (by binding to transcription factor binding sites located in enhancers controlling the gene's expression) or indirectly (by influencing abundance, activity, or binding of direct regulators; Fig. 1; Arnoult et al., 2013; Gompel et al., 2005; Jeong et al., 2006; Kopp, Duncan, Godt, & Carroll, 2000; Williams et al., 2008). For example, in at least one *Drosophila* species each, En (Gompel et al., 2005), Dll (Arnoult et al., 2013), and Abd-B (Jeong et al., 2006) have all been shown to directly bind to *yellow* enhancers, whereas Abd-B and Dsx (including both the male (dsxM) and female (dsxF) forms of dsx) have been shown to directly bind to enhancers of the *bab* gene (Williams et al., 2008). It is not yet known whether Bab proteins directly bind to enhancers of any pigment synthesis genes, but it is clear that Bab proteins affect expression of pigment synthesis genes in some manner (Kopp, 2009). Similarly, Wingless (Wg, a ligand for a signal transduction pathway) (Koshikawa et al., 2015; Werner, Koshikawa, Williams, & Carroll, 2010) has also been shown to influence expression of at least one pigment synthesis gene (Fig. 1), although questions remain about the precise molecular mechanisms by which it does so. Additional transcription factors with effects on abdominal pigmentation in *Drosophila melanogaster* have been identified in recent RNAi screens (Kalay, 2012; Rogers et al., 2014), but the ways in which they alter expression of pigment synthesis genes remain unknown. Elucidating the structure and complexity of the gene network regulating expression of pigment synthesis genes (and hence pigmentation) remains one of the biggest challenges for understanding the development and evolution of *Drosophila* pigmentation within and between species.

3. TISSUE-SPECIFIC REGULATION OF PIGMENTATION

Null mutations disrupting the function of proteins required for pigment synthesis such as TH, DDC, Yellow, Tan, Ebony, DATs, and POs alter pigmentation throughout the fly, whereas mutations in specific enhancers of these genes and mutations affecting transcriptional regulators of these genes typically alter pigmentation in only some parts of the fly. Evolutionary changes in pigmentation are often restricted to specific body parts, suggesting that such changes are likely to result from these latter types of mutations. One reason for this may be that null mutations in pigment synthesis genes often also alter behavior and/or other phenotypes in addition to pigmentation (Takahashi, 2013; True, 2003; Wittkopp & Beldade, 2009), making null mutations unlikely to survive in natural populations. The presence of tissue-specific enhancers for pigment synthesis genes coupled with differences in the sets of regulators that interact with each enhancer provide genetic mechanisms for overcoming these pleiotropic constraints and altering pigmentation independently in different body parts. Because the developmental control of pigmentation in different body regions often involves different regulatory genes, we have chosen to structure our review of the genetic mechanisms underlying pigmentation differences within and between *Drosophila* species by body part, examining the evolution of abdominal pigmentation, thorax pigmentation, wing pigmentation, and pupal pigmentation in *Drosophila* separately below.

4. ABDOMINAL PIGMENTATION

Abdominal pigment patterns (especially those on the dorsal side of the abdomen) are conspicuous and highly variable within and among species (Wittkopp, Carroll, et al., 2003; Rebeiz, Pool, et al., 2009; Rebeiz, Ramos-Womack, et al., 2009). It is not surprising then that most studies of genetic mechanisms underlying pigmentation differences in *Drosophila* have attempted to explain differences in intra- and interspecific abdominal pigmentation. These abdominal pigment patterns are displayed in a series of overlapping tergites that can vary in pigment color, pattern, and intensity among individuals and sexes in the same population, different populations, and different species (Kronforst et al., 2012; Wittkopp, Carroll, et al., 2003). For example, in *D. melanogaster* females, the most prominent abdominal tergites (A2–A6) show a "stripe" of dark melanins at the posterior edge of the

Fig. 2 Abdominal pigmentation in *D. melanogaster*. The dorsal abdomen of *D. melanogaster* is shown for wild-type adult females (*left*) and males (*right*). Note the dark pigment stripe visible at the posterior edge of abdominal segments A2–A6 in females and A2–A4 in males as well as the more complete melanization in tergites A5 and A6 of males relative to females.

segment as well as a peak of this dark color along the dorsal midline (Fig. 2, left). In male *D. melanogaster*, this pattern is seen in the A2, A3, and A4 tergites, but A5 and A6 are much more completely covered by dark melanins (Fig. 2, right). Sexually dimorphic pigmentation is absent in many species, however, with both sexes showing the same pigmentation pattern in all segments (Kopp et al., 2000). The pattern of pigmentation within each segment can also vary, with modifications to the shape of the stripe, unique patterns such as spots, and melanins distributed evenly throughout the abdomen as seen in different species (Wittkopp, Carroll, et al., 2003). Differences in abdominal pigmentation are generally assumed to result from adaptation, but the selection pressures responsible for the evolution of a particular pattern in a particular species remain unclear. Potential selection pressures proposed for divergent abdominal pigmentation include sexual selection resulting from mate choice as well as environmental factors that differ across gradients of altitude, latitude, temperature, humidity, and UV radiation (Bastide, Yassin, Johanning, & Pool, 2014; Brisson, De Toni, Duncan, & Templeton, 2005; Capy, David, & Robertson, 1988; Clusella-Trullas & Terblanche, 2011; Kopp et al., 2000; Matute & Harris, 2013; True, 2003; Wittkopp et al., 2011).

4.1 Genetic Basis of Abdominal Pigmentation Differences Within a Species

In *D. melanogaster*, the most studied of all *Drosophila* species, abdominal pigmentation often varies within and among populations. For example, in sub-Saharan Africa, *D. melanogaster* collected from low elevations showed lighter

abdominal pigmentation than *D. melanogaster* collected from high elevations (Pool & Aquadro, 2007); these differences persisted when rearing these flies in the lab, demonstrating that the differences in pigmentation were caused by genetic differences rather than phenotypic plasticity. Genetic analysis implicated one or more loci on the X and 3rd chromosomes in this pigmentation difference, and analysis of the pigment synthesis gene *ebony*, which is required for the synthesis of yellow-tan pigments and is located on the 3rd chromosome, revealed distinct haplotypes in populations from different altitudes that correlated with these differences in abdominal pigmentation. Nucleotide diversity levels within this region suggested that natural selection has elevated the frequency of dark *ebony* alleles in one of the populations sampled from Uganda, possibly facilitating adaptation to different altitudes (Pool & Aquadro, 2007). Further analysis identified a *cis*-regulatory element in this region that controls *ebony* expression in the abdomen and showed that the allele of this sequence from a lightly pigmented fly drives higher levels of *ebony* expression than the allele of this sequence found in a more darkly pigmented fly (Rebeiz, Pool, et al., 2009), consistent with Ebony's function in the synthesis of light-colored sclerotin (Walter et al., 1996). This region was also found to have recently accumulated multiple mutations in the Uganda population that appear to have given rise to an allele of large effect that contributes to divergence of abdominal pigmentation (Rebeiz, Pool, et al., 2009).

Genetic differences in *ebony cis*-regulatory sequences also appear to contribute to variable abdominal pigmentation in other populations of *D. melanogaster* and other species (Bastide et al., 2013; Dembeck, Huang, Magwire, et al., 2015; Endler et al., 2016; Johnson et al., 2015). For example, an association study using the Drosophila Genetic Reference Panel (DGRP) of *D. melanogaster* strains isolated from a population in Raleigh, North Carolina (Mackay et al., 2012) found a significant correlation between a noncoding variant located within a known *cis*-regulatory element of *ebony* and pigmentation variation within this population (Dembeck, Huang, Magwire, et al., 2015). Weak associations with noncoding SNPs in *ebony cis*-regulatory elements were also observed for European populations of *D. melanogaster* (Bastide et al., 2013; Dembeck, Huang, Carbone, & Mackay, 2015; Endler et al., 2016), with the most highly ranked SNP associated with *ebony* in Bastide et al. (2013) located in a sequence that inhibits *ebony* expression in male abdominal segments during development (Rebeiz, Pool, et al., 2009; Rebeiz, Ramos-Womack, et al., 2009). Outside of *D. melanogaster*, genetic variation linked to *ebony* has been shown to be

associated with polymorphic abdominal pigmentation within *Drosophila americana* (Wittkopp et al., 2009) and *Drosophila auraria* (Johnson et al., 2015). In this latter species, specific alleles of *ebony cis*-regulatory sequences were identified in light and dark individuals, and transgenic analyses of reporter genes were used to demonstrate the effects of these variable sites on *ebony* expression (Johnson et al., 2015). These *cis*-regulatory changes in *D. auraria* are located in a sequence that represses pigmentation in males (Johnson et al., 2015), but does not overlap with the male-specific enhancer (MSE) identified previously in *D. melanogaster* (Rebeiz, Pool, et al., 2009; Rebeiz, Ramos-Womack, et al., 2009).

The *tan* gene, which plays the opposite role of *ebony* in pigment synthesis, promoting production of dark brown melanin at the expense of yellow-tan sclerotin, also contributes to pigmentation variation within *Drosophila* species. In fact, the study that found evidence of an association between *ebony* genotype and abdominal pigmentation within the DGRP collection also identified multiple SNPs within noncoding regions near *tan* that were associated with differences in abdominal pigmentation in this population of *D. melanogaster* (Dembeck, Huang, Magwire, et al., 2015). Three of these noncoding SNPs were also found to be associated with abdominal pigmentation in European populations and an African population of *D. melanogaster* (Bastide et al., 2013; Endler et al., 2016). These SNPs were located within a *cis*-regulatory element known as the MSE (Jeong et al., 2008) that drives expression in *D. melanogaster* in the abdominal stripes as well as throughout the A5 and A6 abdominal segments with male-specific pigmentation. *tan cis*-regulatory evolution at the MSE was also recently implicated in a sex-specific color dimorphism involving abdominal pigmentation differences within *Drosophila erecta* (Yassin et al., 2016). This final case study is particularly interesting because ancient balancing selection was shown to likely be responsible for maintaining alternative alleles at the *tan* MSE and thus both light and dark morphs of female *D. erecta* (Yassin et al., 2016).

Genetic changes contributing to polymorphic pigmentation are not always caused by pigmentation synthesis genes such as *ebony* and *tan*; changes in regulatory genes upstream of the pigmentation synthesis pathway contribute to pigmentation differences segregating within a species as well. These sources of variation include genetic changes at the *bab* locus, a locus originally discovered to be an important regulator of abdominal pigmentation differences between sexes in *D. melanogaster* (Kopp et al., 2000). Null mutations in *bab* cause the development of a male-like pigmentation pattern in the A5 and A6 abdominal segments of female *D. melanogaster*, suggesting that

bab acts to repress male-specific abdominal pigmentation in females (Kopp et al., 2000). Using quantitative trait locus (QTL) mapping coupled with quantitative complementation tests to examine the genetic basis of abdominal pigmentation differences in a population of *D. melanogaster* from Winters, California, Kopp et al. (2003) found genetic variation at *bab* had a major effect on abdominal pigmentation differences in females. The *bab* locus includes two genes, *bab1* and *bab2*, each of which acts as a transcriptional regulator, and it was unclear in Kopp et al. (2003) if variation affecting *bab1* and/or *bab2* was responsible for variation in abdominal pigmentation. To address this uncertainty, Bickel et al. (2011) sequenced the *bab* region in multiple inbred lines from the California population and found that noncoding SNPs at both *bab1* and *bab2* were associated with abdominal pigmentation differences. Specifically, SNPs associated with pigmentation were found in the first intron of *bab1* and near the promoter region of *bab2*. In the DGRP collection, European populations, and an African population of *D. melanogaster*, only SNPs in the first intron of *bab1* were associated with abdominal pigmentation variation (Bastide et al., 2013; Dembeck, Huang, Carbone, et al., 2015; Dembeck, Huang, Magwire, et al., 2015; Endler et al., 2016). A *cis*-regulatory element controlling sex-specific expression of *bab1* in the A5–A7 segments in *D. melanogaster* males (repression) and females (induction) was also identified in the first intron of *bab1* (Williams et al., 2008) and overexpression of *bab1* during late pupal development was shown to be sufficient to suppress dark pigmentation (Salomone, Rogers, Rebeiz, & Williams, 2013), suggesting that the associated sites might alter pigmentation by altering expression of *bab1*. Indeed, Rogers et al. (2014) found that different alleles of this element were present in lightly and darkly pigmented *D. melanogaster* that drove different patterns of gene expression that correlate with pigmentation in the manner expected given *bab*'s role as a repressor of dark pigmentation. A small number of derived sequence changes were found to be responsible for these differences in *cis*-regulatory activity (Rogers et al., 2014). Genetic variation linked to another regulator of pigmentation, *omb*, has also been found to be associated with polymorphic body color in *Drosophila polymorpha*, but much less is known about this association, including whether coding or noncoding changes are more likely to be responsible for the association (Brisson et al., 2004).

Together, the studies described earlier demonstrate that genetic variation contributing to variable abdominal pigmentation within a species has repeatedly accumulated at noncoding regions near the *ebony*, *tan*, and *bab1* genes.

In fact, in the European *D. melanogaster* population studied in Bastide et al. (2013), 79% of the most strongly associated SNPs mapped to noncoding regions linked to *ebony*, *tan*, and *bab1*. Other loci also clearly contribute to polymorphic abdominal pigmentation, however (Dembeck, Huang, Carbone, et al., 2015; Dembeck, Huang, Magwire, et al., 2015; Ng et al., 2008), and some of these loci have recently begun to be identified in *D. melanogaster* (Dembeck, Huang, Carbone, et al., 2015; Dembeck, Huang, Magwire, et al., 2015). The developmental role that these newly identified genes (eg, *pinstripe*, *triforce*, *plush*, and *farmer*) play in pigment patterning remains unknown.

4.2 Genetic Basis of Abdominal Pigmentation Differences Between Species

Differences in pigmentation between species have evolved over longer timescales than differences in pigmentation within a species, suggesting that even phenotypically similar changes in pigmentation might have a distinct genetic basis within and between species (Orr, 2001). For example, different genes and/or different types of changes in the same genes might tend to contribute to phenotypic differences that have evolved over longer evolutionary timescales (Orr, 2001; Stern & Orgogozo, 2009). By directly comparing the genetic basis of intra- and interspecific pigmentation differences, we can better understand how the variants underlying polymorphism within a species give rise to divergence between species. In this section, we review what is known about the genetic basis of abdominal pigmentation differences between species.

The genetic basis of pigmentation differences between species can be dissected genetically using the same methods used to identify genes contributing to intraspecific polymorphism if two species with differences in pigmentation are closely related enough that they can still be crossed and produce viable offspring in the laboratory. One such species pair is *D. yakuba* and *D. santomea*, which are estimated to have begun diverging ~400,000 years ago (Cariou, Silvain, Daubin, Da Lage, & Lachaise, 2001) (Fig. 3). *D. yakuba* exhibits stripes of dark melanins in A2–A6 in both sexes as well as more complete dark pigmentation in segments A5 and A6 of males similar to *D. melanogaster* (Fig. 2B), whereas *D. santomea* lacks dark melanin in these regions in both sexes (Jeong et al., 2008). QTL mapping was used to identify regions of the genome contributing to abdominal pigmentation divergence between these two species. In Llopart et al. (2002), five QTLs were identified, one of which was on the X chromosome and

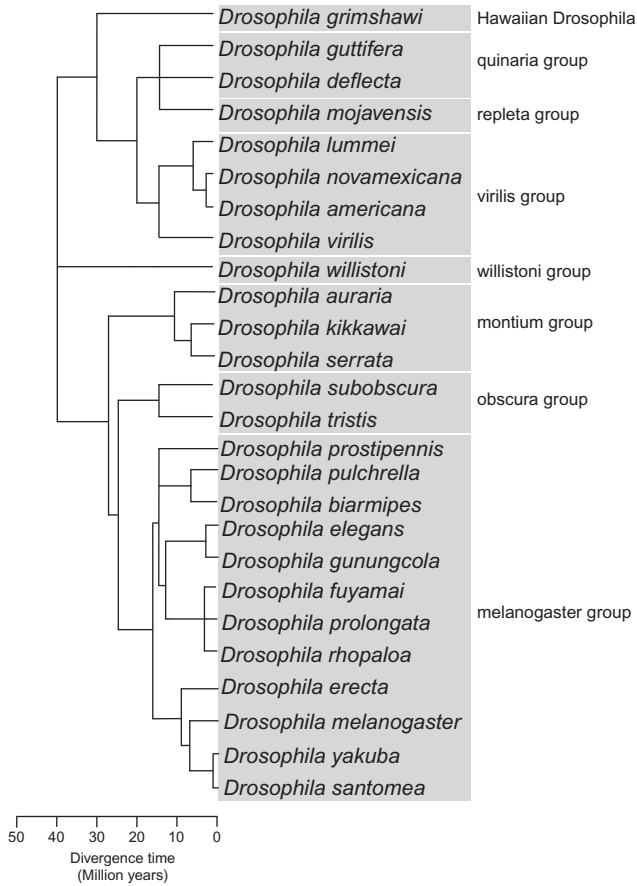

Fig. 3 Phylogeny of *Drosophila* species used to study the genetic basis of pigmentation evolution. Phylogenetic relationships shown were inferred using the online Interactive Tree of Life (iTOL) (Letunic & Bork, 2007, 2011), with branch lengths estimated using data from the online Time Tree website (Hedges, Dudley, & Kumar, 2006).

explained nearly 90% of the species differences. Using a slightly different phenotyping procedure, Carbone et al. (2005) identified four QTL, two located on the X chromosome (one with a much larger effect on pigmentation than the other) and two located on autosomes.

To identify the specific gene(s) that might be responsible for the X-linked QTLs contributing to pigmentation differences between *D. yakuba* and *D. santomea*, Jeong et al. (2008) took a candidate gene approach. Specifically, they examined the pigment synthesis genes *tan* and *yellow*, which were located within the large and small effect X-linked QTLs,

respectively, and found differences in expression of both genes that correlated with differences in abdominal pigmentation between *D. yakuba* and *D. santomea* (Jeong et al., 2008). Analysis of *yellow* and *tan* expression in F1 hybrids from reciprocal crosses showed that only the expression difference in *tan* was caused by *cis*-acting genetic changes on the X chromosome; the difference in *yellow* expression appeared to be caused by one or more *trans*-acting autosomal loci (Jeong et al., 2008). To further localize the genetic changes responsible for divergent *tan* expression and presumably pigmentation, transgenic reporter genes were used to compare enhancer activity of sequences from *D. yakuba* and *D. santomea* in *D. melanogaster*. Three distinct mutations within an MSE located 5′ of *tan* in the genome, each of which reduces *tan* expression, were found to have likely caused, in part, loss of abdominal pigmentation in *D. santomea* (Jeong et al., 2008). This role of *tan* in pigmentation divergence between *D. santomea* and *D. yakuba* was further supported by introgressing the *D. yakuba* allele of *tan* into *D. santomea* and directly demonstrating this gene's contribution to the evolution of abdominal pigmentation differences between these two species (Rebeiz, Ramos-Womack, et al., 2009).

A similar story has emerged for pigmentation differences between the interfertile sister species *D. americana* and *D. novamexicana*, which are also thought to have diverged approximately 400,000 years ago (Morales-Hojas, Vieira, & Vieira, 2008) (Fig. 3). *D. americana* has an overall dark body color typical for a member of the virilis species group, whereas *D. novamexicana* displays a derived light body color with greatly reduced abundance of dark melanins (Wittkopp, Williams, Selegue, & Carroll, 2003). Analysis of F1 hybrids from reciprocal crosses again showed a large contribution of the X chromosome to pigmentation divergence (Wittkopp, Williams, et al., 2003), at least some of which was attributable to loci linked to the *tan* gene (Wittkopp et al., 2009). Fine-scale genetic mapping confirmed that divergence at *tan* was indeed a contributor to pigmentation divergence and localized the functionally divergent sites within *tan* to the first intron (Wittkopp et al., 2009). Subsequent work has shown small, but significant differences in *cis*-regulatory activity of the *D. americana* and *D. novamexicana tan* alleles that presumably contribute to pigmentation differences (Cooley, Shefner, McLaughlin, Stewart, & Wittkopp, 2012). The contribution of *tan* to pigmentation divergence between these two species was further confirmed when the *D. americana tan* allele caused darker pigmentation than the *D. novamexicana tan* allele when each was put into a common *D. melanogaster* genetic background using transgenes

(Wittkopp et al., 2009). Variation linked to the *ebony* gene is also an important source of pigmentation divergence between these two species, with introgression of chromosomal regions containing *tan* and *ebony* from *D. americana* into *D. novamexicana* together explaining 87% of the difference in abdominal pigmentation seen between *D. americana* and *D. novamexicana* (Wittkopp et al., 2009). Effects of *ebony* have yet to be separated from linked loci, however, because *ebony* is located within a region of the genome inverted between these two species, recombination-based mapping is not possible. In all, genetic mapping between *D. americana* and *D. novamexicana* has identified five regions of the genome that contribute to the difference in abdominal pigmentation (Wittkopp et al., 2009; Wittkopp, Williams, et al., 2003).

Variation at *ebony* also appears to be important for abdominal pigmentation differences between the montium subgroup species *D. auraria* and *D. serrata* in the melanogaster group (Johnson et al., 2015), which last shared a common ancestor approximately as long ago as *D. melanogaster* and *D. simulans* (Nikolaidis & Scouras, 1996), that is ~1.5 million years ago (Cutter, 2008) (Fig. 3). In *D. auraria*, males have a stripe of pigment in each abdominal segment similar to *D. melanogaster*, but the more complete pigmentation of male abdominal segments is seen only on A6 rather than in A5 and A6 (Johnson et al., 2015). By contrast, males of *D. serrata* have an abdomen that is more yellow in color overall and lacks dark melanins almost completely in both A5 and A6 (Johnson et al., 2015). Using in situ hybridization, expression of *ebony* was found to be higher in the A5 and A6 segments of *D. serrata* than *D. auraria*, consistent with the role of *ebony* in the formation of yellow-tan sclerotins at the expense of dark melanins (Wittkopp, True, et al., 2002). This evolutionary change in expression appears to have resulted from changes in a *cis*-regulatory element located upstream of *ebony* that controls its expression in the A5 and A6 abdominal segments of males (Johnson et al., 2015).

Another montium subgroup species, *D. kikkawai*, which is estimated to have diverged from *D. melanogaster* ~20 million years ago (Prud'homme et al., 2006) (Fig. 3), has also lost the dark male-specific pigmentation in A5 and A6, but in this case, changes in a MSE of *yellow* that reduce its expression in these segments seem to have played a role (Jeong et al., 2006). Changes in *yellow* expression caused by *cis*-regulatory divergence have also been implicated in an expansion of male-specific abdominal pigmentation to include segments A3 and A4 in *D. prostipennis* relative to *D. takahashi*, two members of the oriental lineage in the melanogaster subgroup (Ordway et al., 2014) (Fig. 3). Interestingly, *D. prostipennis* also showed changes in

ebony and *tan* expression that correlated with the expanded male-specific pigmentation (decrease in *ebony* expression and increase in *tan* expression), but these changes in gene expression were found to be caused by divergence of *trans*-acting loci rather than *cis*-regulatory changes at *ebony* and *tan* (Ordway et al., 2014). Differences in the activity of *yellow cis*-regulatory sequences from *D. melanogaster*, *D. subobscura*, *D. willistoni*, *D. mojavensis*, *D. virilis*, and *D. grimshawi*, much more distantly related species (Fig. 3), that correlate with species-specific pigmentation were also observed when these *cis*-regulatory sequences were assayed in *D. melanogaster* (Kalay & Wittkopp, 2010; Wittkopp, Vaccaro, & Carroll, 2002) (Fig. 3). Observing these changes in *cis*-regulation and gene expression that correlate with divergent abdominal pigmentation for pigment synthesis genes *yellow*, *tan*, and *ebony* strongly suggests that these changes have contributed to pigmentation divergence, although their relative contributions in any individual case remain unknown.

Pigment synthesis genes are not the only source of abdominal pigmentation divergence between species; divergence in a transcription factor regulating expression of pigmentation genes, *bab1*, also plays a role in interspecific differences. In *D. melanogaster*, Bab1 expression represses development of dark pigmentation in segments A5 and A6 of males (Kopp et al., 2000). By contrast, in *D. willistoni*, a species without sexually dimorphic pigmentation in which males and females both have only a stripe of dark melanin near the posterior edge of each tergite, *bab1* is expressed in segments A2–A6 in both sexes (Kopp et al., 2000; Williams et al., 2008). Sex-specific differences in abdominal *bab1* expression seen in *D. melanogaster* were found to be controlled by a dimorphic *cis*-regulatory element containing binding sites for the transcription factors Abd-B and Dsx (Williams et al., 2008). Changes in the binding sites for these transcription factors as well as other changes in the *cis*-regulatory sequence were found to be responsible for the differences in *bab1 cis*-regulatory activity between *D. melanogaster* and *D. willistoni* (Williams et al., 2008). Divergence in this sexually dimorphic *cis*-regulatory element was also found to contribute to interspecific differences in *bab* expression that correlate with differences in female abdominal pigmentation among *D. melanogaster*, *D. yakuba*, *D. fuyamai*, and *D. auraria* (Rogers et al., 2013).

5. THORAX PIGMENTATION

Like abdominal pigmentation, thorax pigmentation varies widely in intensity and patterning within and among *Drosophila* species. Species like

D. guttifera, for example, possess distinctive stripes of black melanin along their thorax that *D. melanogaster* and most other *Drosophila* species lack (Koshikawa et al., 2015). In *D. melanogaster* populations, individuals often vary in the intensity of black and brown melanins that fill a "trident" pattern on the thorax, and variation in this pattern tends to follow altitudinal or latitudinal clines around the world (David & Capy, 1988; Parkash & Munjal, 1999; Telonis-Scott et al., 2011). Intensity of UV radiation was also recently shown to be a good predictor of thorax pigmentation in *D. melanogaster* for clinal variation in Africa, with more darkly pigmented flies found to inhabit regions with higher levels of UV radiation (Bastide et al., 2014). This finding suggests that increased levels of melanin in the thorax may play a protective role for *D. melanogaster* in the wild; however, *D. yakuba* shows the opposite relationship between the intensity of UV radiation and abdominal pigmentation (which is often correlated with thorax pigmentation; Matute & Harris, 2013; Rajpurohit & Gibbs, 2012), indicating that this is not a general relationship for all *Drosophila*. Regardless of the selective forces driving diversity of thorax pigmentation in *Drosophila*, the variety of pigment patterns seen within and among species provides the raw material needed to further investigate the genetic basis of phenotypic evolution.

5.1 Genetic Basis of Thorax Pigmentation Differences Within a Species

In natural populations of *D. melanogaster* and *D. simulans*, variation in a pigmented thorax trident pattern is often seen in which individuals differ in the intensity of darkness in trident shape and size (Capy et al., 1988; David & Capy, 1988). A similar darkening of this trident pattern is also readily observed in *D. melanogaster ebony* loss-of-function mutants (Lindsley & Zimm, 1992), suggesting that variation in *ebony* expression and/or activity might underlie this intraspecific diversity. Consistent with this hypothesis, Takahashi et al. (2007) found that a chromosomal region containing the *ebony* locus was most strongly associated with differences in trident pigmentation intensity between inbred lines of *D. melanogaster* isolated from West Africa and Taiwan. Complementation tests combined with differences in *ebony* expression levels between strains further suggested that regulatory changes at *ebony* contributed to these differences in trident pigmentation (Takahashi et al., 2007). Natural variation in trident intensity within a *D. melanogaster* population collected from Japan was also found to be associated with genetic variants in *ebony* enhancer regions located on the cosmopolitan inversion, *In(3R)Payne* (Takahashi & Takano-Shimizu, 2011).

Interestingly, none of the 19 nucleotide sites found to be in complete association with trident pigment intensity in this study overlapped with sites associated with differences in abdominal pigmentation in African populations described earlier (Pool & Aquadro, 2007; Rebeiz, Pool, et al., 2009). Genetic variants associated with thoracic pigmentation in this Japanese population do still appear to affect *cis*-regulation of *ebony*, however, because differences in relative allelic expression were observed for *ebony* in F1 hybrids produced by crossing lightly and darkly pigmented lines of *D. melanogaster* from this population (Takahashi & Takano-Shimizu, 2011). Variable sites located within an enhancer that drives expression in both the thorax and abdomen (Rebeiz, Ramos-Womack, et al., 2009) failed to cause differences in *cis*-regulatory activity when tested in a common genetic background using reporter genes, however (Takahashi & Takano-Shimizu, 2011). *cis*-Regulatory variation affecting *ebony* expression also seems to contribute to variable thoracic pigmentation observed among the DGRP lines of *D. melanogaster* used in the Dembeck, Huang, Magwire, et al. (2015) study of abdominal pigmentation, with the most strongly associated SNPs again unique to this population (Miyagi et al., 2015). Significant associations were also observed between genetic variants in known enhancers of *tan* and allele-specific *tan* expression levels, but not with variation in thoracic pigmentation (Miyagi et al., 2015). Taken together, these studies indicate that *ebony cis*-regulatory sequences are often variable in natural populations of *D. melanogaster*, with different genetic variants contributing to differences in thoracic pigmentation in different populations.

5.2 Genetic Basis of Thorax Pigmentation Differences Between Species

The best-studied difference in thoracic pigmentation between species is that seen between *D. guttifera* and *D. melanogaster*. In *D. guttifera*, a member of the quinaria species group (Fig. 3), males and females possess a distinct pattern of darkly pigmented stripes along their thorax in addition to the "polka-dot" deposits of black melanin seen on their abdomen and wings. To identify genes involved in the evolution of *D. guttifera* thoracic pigmentation, Koshikawa et al. (2015) examined the regulation of *wingless* expression, which was previously shown to be spatially correlated with the black polka-dots in the wings during development (Werner et al., 2010). After testing many noncoding sequences in and around *wingless* for activity in the thorax, an enhancer driving expression in this part of the body was finally located in an intron of the *Wnt10* gene, two genes away from *wingless* (Koshikawa

et al., 2015). This enhancer, called "gutTS" for *D. guttifera* thorax stripes, was sufficient to activate *wingless* expression during pupal stages of *D. guttifera* that mirrors the thoracic pigment stripes seen in adult *D. guttifera* (Koshikawa et al., 2015). In *D. melanogaster*, this *D. guttifera cis*-regulatory element drove weaker thoracic stripes, indicating that some *trans*-acting regulators of this *wingless* enhancer had diverged between species (Koshikawa et al., 2015). The orthologous enhancer from *D. melanogaster* was also tested for activity in both *D. melanogaster* and *D. guttifera* and failed to drive expression in thoracic strips in either species, indicating that *cis*-regulatory divergence had occurred between *D. melanogaster* and *D. guttifera* within the gutTS *wingless* enhancer (Koshikawa et al., 2015). These results suggest that the evolution of a novel *cis*-regulatory element affecting wingless expression contributes to the derived thoracic stripe pigment pattern seen in *D. guttifera*.

6. WING PIGMENTATION

D. melanogaster wings are evenly pigmented throughout the wing blade, but many other species of *Drosophila* (especially Hawaiian and Oriental species) have wing spots of dark melanins that vary in size, shape, and position on the wing (Edwards, Doescher, Kaneshiro, & Yamamoto, 2007; O'Grady & DeSalle, 2000; Prud'homme et al., 2006; Wittkopp, Carroll, et al., 2003). These darkly pigmented wing patterns are often sexually dimorphic and thought to be the result of sexual selection. Males that possess wing spots in the Oriental *melanogaster* species group, for example, perform an elaborate wing display behavior in front of females during courtship, whereas males without wing spots tend to perform courtship from behind the female (Yeh & True, 2006). Developmentally, these complex wing pigment patterns result from a two-step process in which (i) spatial prepatterns of enzymes involved in the pigmentation synthesis pathway are laid down in the developing wing during the *Drosophila* pupal stage and (ii) precursors for melanin such as dopa and dopamine are transported to the wing through the hemolymph and diffuse from the wing veins post-eclosion, polymerizing to form black and/or brown melanins in the shape of the enzymatic prepatterns (True, Edwards, Yamamoto, & Carroll, 1999). The precise size and shape of wing spots often varies within species, but the genetic basis of this variation has yet to be determined. Several studies have, however, elucidated genetic mechanisms underlying interspecific differences in wing spot size and patterning, and these are reviewed below.

6.1 Genetic Basis of Wing Pigmentation Differences Between Species

In the melanogaster group of *Drosophila*, several species possess a darkly pigmented male-specific spot at the distal tip of their wing. Phylogenetic reconstructions suggest that the common ancestor of the melanogaster group lacked a wing spot and that the current distribution of this trait in this species resulted from at least one gain followed by multiple losses in independent lineages (Prud'homme et al., 2006). The best studied of these spotted species is *D. biarmipes*, a member of the Oriental lineage within the melanogaster species group (Fig. 3), that has a single spot of dark pigmentation at the distal tip of the wing in males. This spot has been shown to be prefigured by expression of the Yellow protein and the absence of the Ebony protein during pupal stages (Wittkopp, True, et al., 2002). For *yellow*, the novel pattern of expression is caused by *cis*-regulatory changes in a preexisting wing enhancer of *yellow*, suggesting that *cis*-regulatory evolution at *yellow* contributed to the evolution of the wing spot pattern (Gompel et al., 2005). Further investigation revealed that *cis*-regulatory changes affecting *yellow* expression had arisen independently in multiple lineages, with different preexisting wing enhancers coopted to create the novel patterns of wing spot expression (Prud'homme et al., 2006). In the case of spot divergence between the two sister species *D. elegans* (spotted) and *D. gunungcola* (spotless), which are also members of the Oriental lineage of the melanogaster group (Fig. 3), the spot of *yellow* expression present in *D. elegans* is controlled by sequences orthologous to the spot enhancer in *D. biarmipes* and divergence of only a few nucleotides in this sequence is responsible for the loss of this *yellow* expression pattern (and presumably at least part of the wing spot) in *D. gunungcola* (Prud'homme et al., 2006). In another spotted species, however, *D. tristis*, which is a member of the obscura group, a wing spot prefigured by *yellow* expression has evolved using a novel *cis*-regulatory element that coopted a different preexisting wing enhancer of *yellow* (Prud'homme et al., 2006). Taken together, these studies suggest that the *cis*-regulatory sequences of *yellow* have evolved repeatedly to cause changes in gene expression that contribute to the gain and loss of wing spots in multiple *Drosophila* species (also reviewed in Monteiro & Das Gupta, 2016).

To better understand how *yellow* expression is regulated and evolves, Arnoult et al. (2013) performed an RNAi screen in a strain of *D. melanogaster* that carried a reporter gene reflecting activity of the *D. biarmipes* spot enhancer. Among the ~350 screened transcription factors,

five candidates emerged as potential activators of the *D. biarmipes* spot enhancer. One of these genes was *Distal-less* (*Dll*), which has previously been shown to be important for *Drosophila* wing development (Cohen, Wimmer, & Cohen, 1991). Using RNAi knockdown, overexpression, and electrophoretic mobility shift assays, *Dll* was shown to be both necessary and sufficient for driving activity of the *yellow* spot enhancer in the wings of *D. melanogaster* (Arnoult et al., 2013). Moreover, manipulating Dll expression in *D. biarmipes* itself lead to a gain and loss of wing pigmentation when Dll was over- and underexpressed, respectively (Arnoult et al., 2013). Neither changes in wing pigmentation nor *yellow* expression were observed when Dll expression was modified in *D. ananassae*, a species without a wing spot, indicating that the regulatory connection between Dll and *yellow* had evolved in the lineage leading to *D. biarmipes* since it last shared a common ancestor with *D. ananassae* (Arnoult et al., 2013). This regulatory link does not appear to be restricted to *D. biarmipes*, however, as correlations between Dll expression, *yellow* expression, and wing spots were also observed in *D. pulchrella*, *D. elegans*, *D. rhopaloa*, and *D. prolongata* (Arnoult et al., 2013). These data suggest an evolutionary trajectory in which Dll regulation of *yellow* was gained and then changes in Dll expression evolved to produce a variety of wing spot patterns. While the second step of this model remains to be tested, it is clear from these data that divergent expression patterns of *Dll* (as well as potentially other transcription factors) have contributed to the divergence of wing pigment patterns through the direct (and likely also indirect) modulation of genes in the pigmentation synthesis pathway (Monteiro & Das Gupta, 2016).

In other *Drosophila* species, wing pigmentation is not limited to males and involves more than a single spot. For example, in *D. guttifera*, both males and females develop a polka-dot pattern of 16 dark melanin spots and 4 melanized areas across their wings (Koshikawa et al., 2015; Werner et al., 2010). Yellow expression during pupal stages again mirrors the final adult wing pigment pattern (Werner et al., 2010), as does expression of Ebony expression, which is reduced in regions with wing spots (Gompel et al., 2005). To identify *cis*-regulatory regions of *yellow* responsible for this spotted expression pattern, noncoding regions surrounding *yellow* were tested for *cis*-regulatory activity using a reporter gene introduced into *D. melanogaster*. Unlike in other studies of *yellow cis*-regulatory elements (Arnoult et al., 2013; Gompel et al., 2005; Kalay & Wittkopp, 2010; Prud'homme et al., 2006; Wittkopp, Vaccaro, et al., 2002), the unique expression pattern of *D. guttifera yellow* could not be recapitulated by reporter genes in

D. melanogaster, indicating that changes in *trans*-regulatory factors controlling *yellow* expression in *D. guttifera* had diverged between these two species. Transforming these reporter genes into *D. guttifera* did, however, drive spotted patterns of expression similar to those seen for endogenous *yellow* (Werner et al., 2010). Through careful examination of the reporter constructs assayed in *D. melanogaster*, phenotypes observed in a spontaneous *D. guttifera* mutant, and prior knowledge of wing development, Werner et al. (2010) identified *wingless* as a potential regulator of *D. guttifera yellow*. Ectopic expression of *wingless* in *D. guttifera* resulted in ectopic wing pigmentation, providing evidence that *wingless* does indeed regulate wing spot pigmentation in *D. guttifera* (Werner et al., 2010). Additional reporter gene experiments using an orthologous spot enhancer from a closely related species lacking wing spots, *D. deflecta*, also showed that *D. guttifera* had evolved a novel pattern of *wingless* expression that contributed to the evolution of its polka-dotted wings (Werner et al., 2010).

The novel expression pattern of *wingless* in *D. guttifera* could have evolved through changes in its *cis*-regulatory sequences, changes in one or more *trans*-acting regulators of *wingless*, or both. To determine whether *cis*-regulatory changes were responsible for divergent *wingless* expression, Koshikawa et al. (2015) tested noncoding sequences in and around the *wingless* gene for *cis*-regulatory activity in pupal wings. A *cis*-regulatory element located 3′ of *D. guttifera wingless* was found to drive expression in *D. guttifera*-like spots near the distal tip of the wing, an activity that seems to have evolved by coopting activity of preexisting *cis*-regulatory elements driving expression in the cross-veins and/or wing margin (Koshikawa et al., 2015). Two more *cis*-regulatory elements that appear to drive novel patterns of *wingless* expression in *D. guttifera* were also identified more than 69 kb away from *wingless* in introns of the *Wnt10* gene (Koshikawa et al., 2015). Testing the activity of these *cis*-regulatory regions using transgenes inserted into *D. melanogaster* showed that changes in the *cis*-regulatory elements of *wingless* were largely sufficient to explain divergent *wingless* expression and presumably thus contribute to the evolution of novel wing pigmentation in *D. guttifera* (Koshikawa et al., 2015).

Because of the candidate gene approaches used to study the evolution of wing spots in the species described earlier, the contribution of *cis*-regulatory changes observed in *yellow* and *wingless* relative to changes that likely exist at other loci in the genome remain unknown. Two studies investigating the genetic basis of a difference in wing spot between interfertile species in the Oriental lineage of the melanogaster subgroup, *D. elegans* and

D. gunungcola (Fig. 3), begin to address this issue (Yeh & True, 2006, 2014). *D. elegans* has a male-specific wing spot of dark pigment similar to that seen in *D. biarmipes*, whereas its sister species *D. gunungcola* has no spots of dark pigment on its wing (Prud'homme et al., 2006). The similarity of wing spots seen in *D. biarmipes* and *D. elegans* is consistent with the proposed inheritance from a common ancestor that also had a wing spot (Prud'homme et al., 2006), suggesting that the roles of *yellow* (Prud'homme et al., 2006) and *Dll* (Arnoult et al., 2013) in the development of the *D. biarmipes* wing spot described earlier are likely conserved in *D. elegans*. Genetic mapping of loci contributing to the difference in wing spot between *D. elegans* and *D. gunungcola* identified three QTL affecting the wing spot (Yeh & True, 2006, 2014). Although each of these QTL encompasses many genes, the inclusion of *yellow* in one QTL and *Dll* in another is consistent with prior studies suggesting that divergence at these loci contributes to the loss of the wing spot in *D. gunungcola* (Arnoult et al., 2013; Prud'homme et al., 2006). The QTL overlapping *yellow* provides more circumstantial evidence that the *cis*-regulatory divergence of *yellow* identified between *D. elegans* and *D. gunungcola* using reporter genes (Prud'homme et al., 2006) impacts pigmentation. In addition, the QTL overlapping *Dll* suggests that differences in *Dll* expression might exist between *D. elegans* and *D. gunungcola* and be caused by *cis*-regulatory changes at *Dll* itself, similar to observations for divergent *wingless* expression in *D. guttifera* (Koshikawa et al., 2015). The third QTL does not include any obvious candidate genes.

7. PUPAL PIGMENTATION

In addition to the highly variable pigment patterns of the *Drosophila* abdomen, thorax, and wings, differences in pigmentation are also seen among some species in the pupal cases from which the adult flies emerge. For example, in the virilis group of *Drosophila* (Fig. 3), *D. virilis* has a distinctly darker pupal case color than its closest relatives, *D. americana*, *D. lummei*, and *D. novamexicana* (Stalker, 1942). The *D. virilis* pupal case appears almost completely black, whereas pupal cases in the other species are lighter shades of brown and tan (Ahmed-Braimah & Sweigart, 2015). The virilis species group is amenable to genetic dissection of this trait because *D. americana*, *D. novamexicana*, and *D. virilis* all produce fertile hybrids when crossed with each other (Heikkinen, 1992). Early studies investigating the genetic basis of this difference in pupal color between *D. virilis* and *D. americana* suggested that it was due to a large effect locus on chromosome

5 as well as other loci, possibly linked to chromosomes 2 and 3 (Stalker, 1942). To identify the molecular basis of pupal color divergence between *D. virilis* and *D. americana* more precisely, Ahmed-Braimah and Sweigart (2015) analyzed a backcross population between these two species and scored more than 30,000 recombinant offspring for pupal case color. This experimental design allowed them to identify an ~11-kb sequence on chromosome 5 that contributes to the difference in pupal case color. This region contains the first exon and noncoding regions of the *Dat* gene (Ahmed-Braimah & Sweigart, 2015). *Dat*, as described earlier and in Fig. 1, is required for the conversion of dopamine to NADA, which is then polymerized into a colorless pigment. Expression differences were observed for *Dat* at the onset of pupation between *D. americana* (high expression) and *D. virilis* (low expression; Ahmed-Braimah & Sweigart, 2015) that suggest reduced expression of *Dat* in *D. virilis* creates an excess of dopamine that allows production of more dark melanins and thus a much darker pupal case. Pupal expression of *Dat* in *D. novamexicana*, which has a lighter body color than *D. americana* but a similarly colored pupal case, was similar to that observed for *D. americana* (Ahmed-Braimah & Sweigart, 2015). Genetic variation linked to *Dat* did not explain any of the difference in body color between *D. americana* and *D. novamexicana*, consistent with prior work identifying *ebony* and *tan* as the primary drivers of divergent body color between these two species (Wittkopp et al., 2009).

8. LESSONS LEARNED FROM *DROSOPHILA* PIGMENTATION

With the rapid growth of studies identifying genes and genetic changes contributing to pigmentation differences within and between *Drosophila* species during the last 10 years, the time is ripe to step back and take an integrative look at the findings from these case studies. What have we learned about the genetic basis of pigmentation evolution and hopefully phenotypic evolution more generally? What questions remain unanswered?

First and foremost, we have learned that the same handful of genes have been modified over and over again in different lineages to give rise to polymorphic pigmentation within a species as well as divergent pigmentation between species (Table 1). A similar pattern has also been seen for other types of evolutionary changes (Martin & Orgogozo, 2013; Stern & Orgogozo, 2009), suggesting that evolutionary trajectories are sometimes

predictable. For pigmentation, genes harboring polymorphism and divergence that affects body color include genes that encode developmental regulators (blue in Fig. 4) as well as enzymes required for pigment biosynthesis (red in Fig. 4). The apparent reuse of these genes has likely been biased by the use of candidate gene approaches that limited analysis to these genes in some studies (Gompel et al., 2005; Johnson et al., 2015; Prud'homme et al., 2006; Werner et al., 2010; Wittkopp, Vaccaro, et al., 2002); however, the same conclusion emerges if only studies using unbiased genetic mapping approaches are considered (Bastide et al., 2013; Dembeck, Huang, Carbone, et al., 2015; Dembeck, Huang, Magwire, et al., 2015; Endler et al., 2016; Pool & Aquadro, 2007; Wittkopp et al., 2009). Despite this repeatability, the set of nine genes implicated in pigmentation diversity thus far is clearly not exhaustive; Dembeck, Huang, Carbone, et al. (2015) found SNPs in 84 loci that had significant associations with variable abdominal pigmentation in a single population of *D. melanogaster*.

A second lesson results from the striking consistency seen in the types of functional genetic changes observed in genes contributing to pigmentation diversity: *cis*-regulatory changes in noncoding sequences appear to be responsible for a gene's effects on pigmentation in all cases where the type of mutation is known (Table 1). This observation holds for both developmental regulators and genes in the pigment synthesis pathway (Table 1). *cis*-Regulatory changes have been proposed to be the predominant source of evolutionary change in genes with pleiotropic effects on multiple traits because they allow one function of the gene to be modified without affecting others (Carroll, 2008; Stern & Orgogozo, 2008; Wray et al., 2003). All of the genes implicated in pigmentation diversity thus far are indeed pleiotropic

Genes	Abdominal pigmentation	Puparium pigmentation	Thorax pigmentation	Wing pigmentation
bab1	Within and between species	–	–	–
bab2	within species	–	–	–
ebony	Within and between species	–	Within species	–
Dat	–	Between species	–	–
Dll	–	–	–	Between species
omb	Within species	–	–	–
tan	Within and between species	–	–	–
yellow	between species	–	–	Between species
wg	–	–	Between species	Between species

Fig. 4 The loci of pigmentation evolution. A summary of genes implicated in pigmentation differences within and/or between species is shown. Genes labeled in *blue* (*bab1*, *bab2*, *Dll*, *omb*, and *wg*) are regulators of pigmentation development. Genes labeled in *red* (*ebony*, *Dat*, *tan*, and *yellow*) are involved in the pigment biosynthesis pathway. All genetic changes identified as likely to be contributing to a pigmentation difference either within or between species thus far affect *cis*-regulatory sequences.

and are regulated by multiple *cis*-regulatory elements that subdivide their functions. In addition to pigmentation, *bab1* and *bab2* also affect development of mechanosensory organs (Godt, Couderc, Cramton, & Laski, 1993; Kopp et al., 2000); *ebony*, *tan*, *yellow*, and *Dat* also impact behavior (Drapeau, Radovic, Wittkopp, & Long, 2003; Shaw, Cirelli, Greenspan, & Tononi, 2000; True et al., 2005), and *Dll*, *omb*, and *wg* have widespread effects on development (Drysdale & FlyBase Consortium, 2008). The genetic basis of pigmentation differences in vertebrates reveals a different pattern, however, with changes in pigmentation attributed more equally to *cis*-regulatory changes and changes in amino acid sequence affecting protein function (Hubbard, Uy, Hauber, Hoekstra, & Safran, 2010).

A final message emerging from these studies is that intra- and interspecific sources of pigmentation diversity share some properties but not others. For example, nearly all genes shown to contribute to differences in abdominal pigmentation within a species also contribute to pigmentation differences that exist between species (Fig. 4). One notable exception is *yellow*. Changes in *yellow* expression often accompany changes in pigmentation between *Drosophila* species, but they have yet to be implicated in intraspecific variation. This might be because overexpression of *yellow* has more subtle effects on pigmentation than overexpression of *ebony*, *tan*, or *bab1* (Jeong et al., 2008; Salomone et al., 2013; Wittkopp et al., 2009; Wittkopp, True, et al., 2002), such that changes in *yellow* expression arising alone within a species are insufficient for altering pigmentation in most populations (but see Wittkopp, Vaccaro, et al., 2002). Genetic changes in the same *cis*-regulatory regions have been observed within and between species, but the scope of these changes differs. Within a species, genetic variants typically modulate activity of existing *cis*-regulatory elements, with different variants affecting *cis*-regulatory activity in different populations. By contrast, divergent sites that differ between species are much more likely to have given rise to a novel enhancer that coopts preexisting developmental regulators. Differences between alleles contributing to intra- and interspecifc pigmentation variation are not always apparent, however, as the alleles of *tan* and *ebony* contributing to divergent pigmentation in *D. novamexiana* were found to also contribute to clinal variation in pigmentation within *D. americana* (Wittkopp et al., 2009).

As illustrated in this chapter, detailed studies of pigmentation divergence within and among *Drosophila* species have provided an unprecedented look at the genetic mechanisms underlying phenotypic evolution over various timescales. There is still much more to be learned from studying this system,

however. For example, many QTLs contributing to pigmentation differences within and between species have been identified for which the causative genes remain unknown. Identifying these genes might alter our view of the types of genes most likely to harbor genetic changes affecting pigmentation. Many direct and indirect regulators of genes in the pigment synthesis pathway are also yet to be identified. Knowing the identity of these factors and the sequences they bind to will help us understand why some noncoding changes alter pigmentation while others do not. Important questions also remain about whether the complementary changes in expression of pigmentation genes such as *yellow* and *ebony* that are often observed between species have evolved through independent genetic changes or a single change affecting a shared regulator. Finally, improving our understanding of both the ecological functions of pigmentation in specific taxa and the pleiotropic effects of pigmentation genes will help us better understand the role natural selection might play in shaping the genetic basis of pigmentation evolution. Ultimately, understanding the genetic and molecular mechanisms underlying pigmentation diversity has the potential to answer questions not only about evolution but also about ecology, biochemistry, and neuroscience.

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