Moving Speciation Genetics Forward: Modern Techniques Build on Foundational Studies in *Drosophila*

Dean M. Castillo and Daniel A. Barbash[1]

Department of Molecular Biology and Genetics, Cornell University, Ithaca, New York 14853

ABSTRACT The question of how new species evolve has been examined at every level, from macroevolutionary patterns of diversification to molecular population genetic analyses of specific genomic regions between species pairs. *Drosophila* has been at the center of many of these research efforts. Though our understanding of the speciation process has grown considerably over the past few decades, very few genes have been identified that contribute to barriers to reproduction. The development of advanced molecular genetic and genomic methods provides promising avenues for the rapid discovery of more genes that contribute to speciation, particularly those involving prezygotic isolation. The continued expansion of tools and resources, especially for species other than *Drosophila melanogaster*, will be most effective when coupled with comparative approaches that reveal the genetic basis of reproductive isolation across a range of divergence times. Future research programs in *Drosophila* have high potential to answer long-standing questions in speciation. These include identifying the selective forces that contribute to divergence between populations and the genetic basis of traits that cause reproductive isolation. The latter can be expanded upon to understand how the genetic basis of reproductive isolation changes over time and whether certain pathways and genes are more commonly involved.

KEYWORDS *Drosophila*; hybrid incompatibilities; reproductive isolation; speciation; Flybook

Introduction: The History and Importance of Speciation Studies Using *Drosophila*

SPECIATION is the process where one lineage gives rise to distinct daughter lineages that eventually become separate species. The problem of how and why these lineages diverge was initially highlighted by Darwin in *On the Origin of Species* (Darwin 1895). Though our understanding of the speciation process, in terms of geographic context and phenotypic mechanisms, has grown immensely since Darwin, the speciation problem remains a central question in biology (Coyne and Orr 2004). In terms of the genetic basis of speciation, progress was made both by theoretical insights during the Modern Synthesis that formalized how selection operates on alleles in populations and more recently by technological advances such as next-generation sequencing (Provine 1971; Seehausen *et al.* 2014). The genus *Drosophila* has played a unique role in the study of speciation, by providing data that inspired seminal papers and ideas about speciation. The ease of conducting genetic studies and crosses in *Drosophila* has been critical to our understanding of the genetics of speciation and will continue to provide new opportunities moving forward (Noor and Feder 2006).

Early *Drosophila* speciation researchers focused on determining the genetic factors that contribute to reproductive isolation (Dobzhansky 1937b; Muller and Pontecorvo 1942; Tan 1946). Studies on these genetic factors then enabled Dobzhansky and Muller to formulate their theory of hybrid incompatibilities, which has been foundational to the study of speciation (Dobzhansky 1937a; Muller 1942). Further genetic studies involving backcrosses, F2s, and introgression mapping provided insight into the genetic basis of common patterns such as Haldane's Rule and the large effect of the X chromosome in causing hybrid incompatibility ("Large X-effect"), as well as the faster accumulation of male *vs.* female hybrid sterility and the genetic complexity of hybrid male sterility (Naveira and Fondevila 1991; Orr 1993; Wu and Davis 1993; Davis and Wu 1996; True *et al.* 1996; Tao *et al.* 2001, 2003b). Interestingly, other studies hinted that major-effect loci could be identified for at least some hybrid incompatibilities (Watanabe 1979; Hutter and Ashburner 1987; Sawamura *et al.* 1993).

In parallel, many early investigators made crosses to describe broad patterns of reproductive isolation across diverse *Drosophila* clades (Patterson and Stone 1952). As many different species were crossed, general patterns were detected in how reproductive isolation accumulates as a product of divergence time and species cooccurrence (Coyne and Orr 1989, 1997; Yukilevich 2012). The in-depth analysis of divergence and speciation in the desert *Drosophila* contributed to our knowledge of the range of mechanisms that cause reproductive isolation (Markow 1981, 1991; Etges 1992; Knowles and Markow 2001; Matzkin *et al.* 2006; Etges *et al.* 2007). Long-term studies of the ecological genetics and behavior of the Hawaiian *Drosophila* have demonstrated that sexual selection can generate rapid speciation (Carson and Kaneshiro 1976; Carson 1982).

Recent advances in molecular genetic techniques have enabled researchers to pinpoint individual loci and mutations contributing to phenotypes that are relevant to speciation. For example, using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas-9 to create specific mutations in different species backgrounds, Ding *et al.* (2016) identified a gene important for male courtship song (described in detail in *New Approaches to Determine the Genetic Basis of Reproductive Isolation*). Whole-genome sequencing studies are providing high-resolution analysis of population differentiation and interpopulation gene flow, which have been used to understand the evolutionary history of closely related species and to determine which regions of the genome may contain genes important for speciation (Garrigan *et al.* 2012; Kang *et al.* 2016). Ultimately, these tools and resources will help answer two of the major outstanding questions in speciation: (1) what are the selective forces that contribute to divergence between populations, and (2) what is the genetic basis of traits contributing to reproductive isolation. We suggest that a fruitful way forward will be to look at the within-species function of genes important for reproductive isolation combined with comparative studies of the genetics of reproductive isolation. This will allow direct connections between population-level processes and species divergence.

In this review, we first focus on a working definition of a species, discuss the implications that this definition has for thinking about divergence of genomic regions and individual loci, and outline how studies of molecular divergence are being applied in *Drosophila*. Next, we summarize the current major theories describing the speciation process, the forces generating reproductive isolation, and the evidence for each in *Drosophila*. We follow with a description of the diverse mechanisms and phenotypes that contribute to reproductive isolation in *Drosophila* and their genetic bases, where known.

Lastly, we discuss new approaches that can be leveraged in *Drosophila* to enhance our understanding of speciation and reproductive isolation.

The Biological Species Concept, Genic View of Speciation, and Population Genetic Patterns

The definition of a "species" has been historically controversial, but one of the most widely accepted is the Biological Species Concept (Mallet 2001; Coyne and Orr 2004). The Biological Species Concept works exceedingly well for *Drosophila* and for the study of the evolution of reproductive isolation, whereas other species concepts may have more utility for systematic or taxonomic studies. Under the biological species concept, a "species" is a group of natural populations that have the potential to interbreed and are reproductively isolated from other groups of populations (Mayr 1942). The importance of the biological species concept has been to place emphasis on the role that reproductive isolation plays in the speciation process. Reproductive isolation occurs when gene flow is limited between lineages either via geographical separation or reproductive barriers (Turelli *et al.* 2001). The two main classifications of reproductive barriers are prezygotic isolation, which occurs before a zygote is formed (such as mate discrimination or gamete incompatibility), and postzygotic isolation, which occurs in hybrids (hybrid lethality and sterility) (Coyne and Orr 2004).

A potential oversimplification of the original definition is that it emphasizes a complete lack of gene flow between species, a pattern that has not held up to genomic data (Feder *et al.* 2012; Cruickshank and Hahn 2014; Seehausen *et al.* 2014). Therefore, current interpretations suggest that what is important is the lack of gene flow at specific genomic regions that control reproductive isolation (Wu 2001; Coyne and Orr 2004). This "genic" view of speciation has important implications for what predictions can be made concerning population genetic patterns of species. For example, even though *Drosophila simulans*, *D. sechellia*, and *D. mauritiana* are reproductively isolated, have been historically geographically isolated, and are generally considered distinct species, genomic evidence showing shared derived nucleotide changes among these species suggests recent gene flow throughout the genome (Garrigan *et al.* 2012). The pattern of recent gene flow is difficult to disentangle from incomplete lineage sorting in these species, but nevertheless the data suggest a complex history of speciation (Pease and Hahn 2014). Within species, there is also evidence of recent admixture between partially reproductively isolated races, which may represent the earliest stages of divergence, such as the case of Cosmopolitan ("M-type") and African ("Z-type") races of *D. melanogaster* (Kao *et al.* 2015; Pool 2015; Bergland *et al.* 2016). Reproductive isolation between these races is strong and has been maintained in a putative secondary contact zone in the Caribbean and Southeastern US (Wu *et al.* 1995; Yukilevich and True 2008a,b). However, in both examples it is unclear whether these patterns of gene flow and admixture are influenced by

genes important to reproductive isolation such that the most differentiated genes contribute to reproductive isolation.

To link population genetic studies to speciation and reproductive isolation, the regions with low gene flow must harbor loci important for reproductive isolation. This has been challenging to demonstrate but one example is *D. pseudoobscura* and *D. persimils*, which are differentiated by several inversions that (1) suppress recombination and restrict gene flow between species and (2) harbor genes contributing to hybrid male sterility (Noor *et al.* 2001a). Going forward, population genomic approaches can be used with the *a priori* expectation that regions of low gene flow or high differentiation may contain genes for reproductive isolation. In the sister pair *D. silvestris* and *D. heteroneura*, Kang *et al.* (2016) sequenced many genomes of each species looking at patterns of differentiation (Fst and dxy) and selection (McDonald-Kreitman tests) to identify candidate loci contributing to reproductive isolation. A major caveat to this approach is that highly differentiated regions may also be consequences of adaptation or genome rearrangements. The strength of *Drosophila* systems is that well-controlled follow-up studies can be performed of candidate regions with crosses and genetic manipulation. The loci identified in *D. silvestris* and *D. heteroneura* have yet to be functionally tested, but the study provides a template for future efforts where implicating specific genes will provide information on the selective forces contributing to, and the genetic basis of, reproductive isolation.

Theoretical Studies of Speciation and the Forces Driving Species Divergence

Theoretical work describing speciation focuses on processes and traits that control fitness within populations and how they subsequently diverge between populations. A single unifying theory of speciation remains distant, but there are strong, well-supported models for the evolution of prezygotic or postzygotic reproductive isolation.

One such model of prezygotic isolation is the Lande–Kirkpatrick model of speciation by sexual selection, where a female allele arises that generates preference for a specific male trait. Over time, directional selection for the male trait and indirect selection for the female preference lead to linkage disequilibrium between these loci and the rapid evolution of the coupled male trait-female preference (Lande 1981; Kirkpatrick 1982; Kirkpatrick and Ravigne 2002). If this process occurs in different "directions" in different populations, the result can be reproductive isolation via prezygotic isolation (Figure 1). This model is well-supported by studies of sexual selection in *D. melanogaster* and Hawaiian *Drosophila* (Bateman 1948; Kaneshiro and Boake 1987; Boake *et al.* 1997; Promislow *et al.* 1998), which demonstrate that sexual selection is a strong driver of diversity (Markow 1996; Ritchie 2007). Further studies have documented sexual selection acting within populations on traits important to reproductive isolation, such as pigmentation, cuticular hydrocarbons
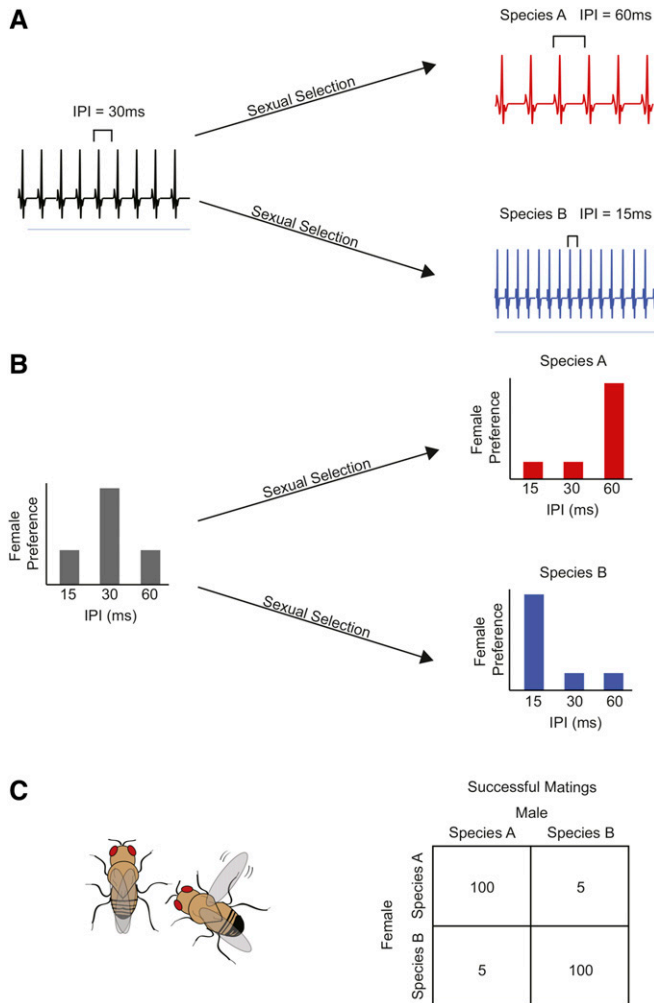
Figure 1 Sexual selection drives divergence of a male signal trait and female preference for that trait resulting in reproductive isolation. In this simplified example, *Drosophila* courtship song consists of pulse elements, with interpulse interval measured as the time between the start of pulses (Kyriacou and Hall 1980). (A) The interpulse interval (IPI) diverges between two lineages. (B) Female preference diverges to match the favored IPI in each species. (C) In mating trials conspecific matings are successful but heterospecific matings fail due to a mismatch of male courtship traits, including song, and female preference. ms, millisecond.

(CHCs), and male courtship song, an important step in connecting sexual selection to the evolution of reproductive isolation (Boake *et al.* 1997; Higgie and Blows 2007, 2008; Veltsos *et al.* 2012).

Sexual interactions can also put males and females into direct conflict over fitness leading to cycles of adaptation and counteradaptation (Arnqvist and Rowe 2005; Chapman 2006). This reciprocal selection via sexual conflict can generate reproductive isolation but with patterns distinct from the Lande–Kirkpatrick models (Parker and Partridge 1998; Arnqvist *et al.* 2000; Gavrilets 2000). *Drosophila* has provided useful data in support of sexual conflict leading to speciation because sexual conflict is well-documented for sperm competitive traits in *D. melanogaster* (Chapman 2006; Sirot

et al. 2009). These traits can contribute to reproductive isolation through conspecific sperm precedence (Price 1997).

Sexual interactions and mating preferences can also be influenced by the environment. In models of ecological speciation, traits important for sexual interactions experience selection from the environment or are altered as a consequence of adaptation to new environments (Schluter 2009). In a related model, known as sensory drive speciation, communication systems adapt to the local environment such that different environments alter the transmission and perception of sexual signals and lead to prezygotic isolation [Ryan *et al.* 1990; Endler and Basolo 1998; Boughman 2002; but see Fuller (2009)]. CHCs are often used for mate choice and species recognition in *Drosophila* and can be highly sensitive to environmental selection (Gibbs 2002; Chenoweth and Blows 2003; Foley and Telonis-Scott 2011). The pleiotropic function of CHCs in desiccation resistance and sexual signaling enables abiotic factors acting on CHCs to generate prezygotic isolation (Chung *et al.* 2014). Additional evidence for the ecological speciation model comes from subspecies in the *D. mojavensis* species complex. These subspecies show partial reproductive isolation that is correlated with differences in CHCs that result from the use of different host cacti as breeding sites and larval feeding substrates (Markow 1991; Stennett and Etges 1997). Sensory drive has not been explored in this system but the prediction from this model is that female perception of male CHCs should be associated with female ability to locate host plants. The relative strengths of sexual selection and ecological selection will ultimately determine the outcome of trait evolution for sexual signals.

Interactions with closely related heterospecifics can be a strong force driving the evolution of prezygotic isolation. When closely related species cooccur and have the potential to interbreed, selection can increase reproductive isolation if breeding with heterospecifics is costly, a process known as reinforcement (Servedio and Noor 2003). *Drosophila* studies have shown that reinforcement can act on both prezygotic isolation (Noor 1995) and postmating prezygotic (PMPZ) traits (Matute 2010), and can also have collateral consequences on interactions among populations within and outside of sympatry (Comeault *et al.* 2016; Humphreys *et al.* 2016). Together, these studies have provided a comprehensive framework to evaluate the wide-ranging effects of reinforcement and the multiple targets that reinforcing selection can act on.

The main theories developed to describe postzygotic isolation initially focused on the genetic basis rather than the evolutionary forces that would drive divergence. In what is now known as the Dobzhansky–Muller model of hybrid incompatibilities, postzygotic isolation is the result of negative epistatic interactions that occur in hybrids between alleles that have never otherwise been "tested" together (Figure 2; Dobzhansky 1937a; Muller 1942). *Drosophila* have been key to demonstrating this negative epistasis, first as interactions between chromosomes (Orr 1987; Pantazidis and Zouros 1988) and then as interactions between specific genes (Ting *et al.* 1998;
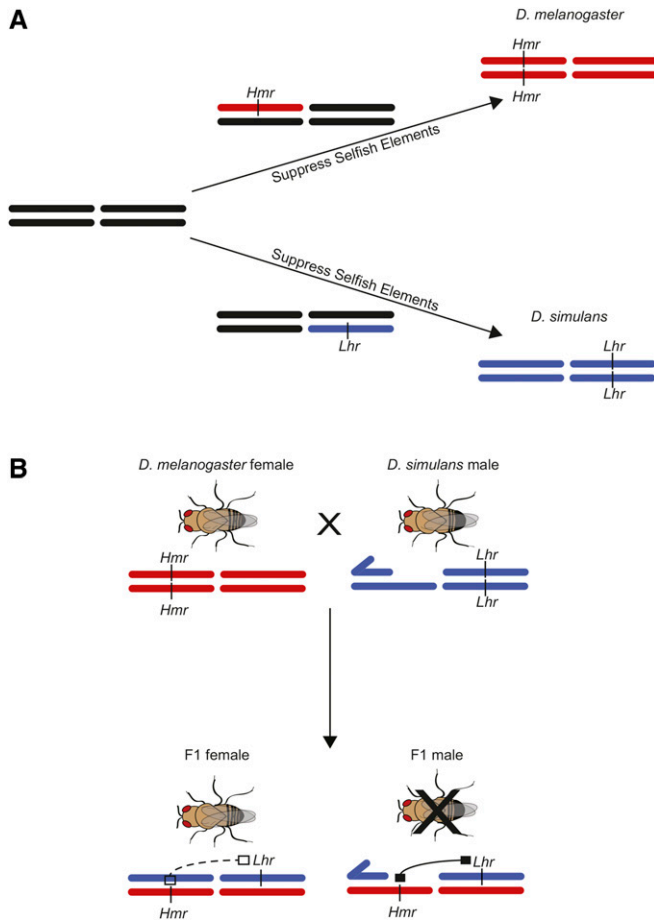
Figure 2 The Dobzhansky–Muller model of hybrid incompatibilities represented by the divergence of the *Hmr* and *Lhr* genes between *D. melanogaster* and *D. simulans*. (A) The *Hmr* and *Lhr* alleles diverge in opposite lineages and are never "tested" in the same genetic background. (B) When these two alleles are brought together, there are negative epistatic interactions resulting in lethality of the F1 hybrid male progeny (represented by the solid line, Brideau *et al.* 2006). Reduced viability and fertility also occurs in females due to this incompatibility (represented by dashed line, Barbash *et al.* 2000; Barbash and Ashburner 2003). Additional gene(s) contribute to this incompatibility though the nature of their interactions with *Hmr* and *Lhr* remain unknown (Phadnis *et al.* 2015).

Brideau *et al.* 2006). Variation in genome and chromosome structure that distinguishes species of *Drosophila* (Dobzhansky and Sturtevant 1938; Hsu 1952) prompted similar examination in many other taxa, eventually inspiring a chromosomal theory of speciation (Stebbins 1958; White 1978; Templeton 1981). In this model, fixed structural differences (such as inversions, translocations, and fusions) lead to hybrid sterility because recombination between divergent chromosomes produces unbalanced and unviable gametes. Theoretical difficulties in explaining how underdominant chromosomes fix in populations prompted models of chromosomal speciation to focus on other mechanisms besides underdominance. These new models incorporate observations that reduced recombination reduces gene flow and facilitates fixation of allelic differences that contribute to reproductive isolation (Barton 1979; Noor *et al.* 2001b; Rieseberg 2001). Other

models focus on how differences in heterochromatin and small RNA content can lead to hybrid incompatibilities (Michalak 2009; Brown and O'Neill 2010).

Selection to suppress the transmission of selfish elements, and particularly transposable elements (TEs), may be a strong and common force creating divergence between species and hybrid incompatibilities (Hurst and Schilthuizen 1998). As selfish elements are often in conflict with host fitness, models involving selfish elements invoke genetic conflict as the underlying evolutionary cause. Selfish elements can propagate through populations, which often leads to the evolution of suppressor alleles that mitigate their deleterious effects (Burt and Trivers 2005; Aravin *et al.* 2007). When two populations interbreed, the effectiveness of suppressors can become diminished and unleash the activity of selfish elements. Hybrid dysgenesis is such a process whereby TEs become activated and can lead to complete sterility in interpopulation crosses. Because of the strong phenotype, it seemed natural to speculate that a similar phenomenon might be a potent cause of interspecific hybrid incompatibility (Kidwell 1983), but early tests in *Drosophila* were negative (Hey 1988; Coyne 1989)

Kelleher *et al.* (2012) performed a comprehensive analysis of TEs in interspecific hybrids between *D. melanogaster* and *D. simulans* hybrids, examining their mRNA expression as a proxy for activity as well as their profile of piRNAs, small RNA molecules that function as *trans*-acting repressors of TEs. They found that the pattern of TE misexpression in hybrids does not reflect the presence of species-specific TEs, as predicted by the model of hybrid dysgenesis. Rather, TE misexpression and defects in piRNA pools resemble that seen in piRNA mutations within *D. melanogaster*. These findings led to the conclusion that TEs are an indirect, not direct, cause of hybrid incompatibility. TEs are proposed to drive adaptive evolution of piRNA regulatory genes within species, which leads to lineage-specific divergence of the regulatory complex. These coadapted gene complexes can then malfunction when brought together in hybrids (Ishikawa and Kinoshita 2009; Castillo and Moyle 2012; Satyaki *et al.* 2014).

This notion of selfish elements indirectly causing hybrid incompatibilities due to their propensity to drive adaptive evolution of host defense genes is supported by other studies that found that hybrid incompatibility genes encode proteins that localize to heterochromatin, the repeat-rich region of the genome where many selfish DNAs reside (Ting *et al.* 1998; Brideau *et al.* 2006; Bayes and Malik 2009; Thomae *et al.* 2013; Satyaki *et al.* 2014). Selfish elements have also been proposed to be the drivers of adaptive evolution of nucleoporin proteins that are implicated in hybrid lethality (Presgraves *et al.* 2003; Presgraves and Stephan 2007). The *Zhr* locus, which contains the 359 bp satellite (also known as the 1.688 g/cm³ satellite), is the sole known example of a repetitive DNA directly causing hybrid incompatibility (Sawamura and Yamamoto 1997; Ferree and Barbash 2009). This satellite is highly abundant in the X heterochromatin of *D. melanogaster* but largely absent from *D. simulans*. In hybrid embryos, this heterochromatic region mis-segregates leading to mitotic

defects and lethality. While satellite DNAs can evolve selfishly in genetic conflict with host fitness, there are also neutral explanations for differences in satellite DNA abundance between species. Trying to distinguish between these possibilities remains a major challenge (Maheshwari and Barbash 2011).

Mechanisms that Contribute to Reproductive Isolation and Their Genetic Basis

The evolution of reproductive isolation can be accomplished by numerous different traits that act as barriers to reproduction. Generally, these barriers are dichotomized into prezygotic *vs.* postzygotic, or alternatively premating *vs.* postmating (Coyne and Orr 2004). Here, we use the pre- *vs.* postzygotic distinction and include gamete interactions with prezygotic barriers to differentiate them from reproductive barriers that involve hybrid offspring. The relative prevalence of these reproductive barriers across evolutionary time has been studied in much detail in *Drosophila*, and these studies have been influential in prompting similar research across a wide range of taxa (Coyne and Orr 1989, 1997). The overarching conclusion from these meta-analyses is that prezygotic isolation evolves much more rapidly than postzygotic isolation, and that within postzygotic isolation male sterility evolves earlier than inviability and female sterility. This rapid evolution of prezygotic isolation is largely driven by species pairs that cooccur (*i.e.*, are sympatric), which highlights a role for geography, species interactions, and potentially reinforcement in the evolution of prezygotic isolation (Coyne and Orr 1989, 1997; Yukilevich 2012).

Prezygotic isolation

The traits that contribute to prezygotic isolation are incredibly diverse across the *Drosophila* phylogeny (Markow and O'Grady 2005), including genital morphology, song phenotypes, CHCs, and PMPZ incompatibilities. These traits reflect the nature of *Drosophila* sexual interactions where males spend considerable time courting and singing (Greenspan and Ferveur 2000; Ferveur 2010), hydrocarbons can predict male mating success (Cobb and Jallon 1990; Grillet *et al.* 2012), multiple mating is prevalent (Markow 1996), and diverging genital morphologies can cause mechanical mismatches that preclude copulation (Masly 2012; Yassin and Orgogozo 2013). The relative importance of a given signal varies in different clades (Markow and O'Grady 2005). Furthermore, these traits often act in concert and it is well-recognized that *Drosophila* courtship relies on multi-modal signaling (Greenspan and Ferveur 2000).

Most studies to date have focused on male courtship traits, as they are more conspicuous than traits underlying female preference. In this section, we highlight the diversity of these traits and provide updates for the genetic basis of song and CHCs [for a thorough review see Laturney and Moehring (2012)]. Though song and CHC phenotypes are the best genetically characterized prezygotic traits, the signaling modalities used in courtship differ greatly among species.

Future efforts to study the diversity of signal modalities during courtship will help focus studies of the genetics of female preference as well as trait reception and recognition.

The song of *D. melanogaster* consists of two main elements, sine song and pulse song (Ewing and Bennet-Clark 1968; von Schilcher 1976), with this basic structure elaborated on and highly differentiated among closely related species (Tomaru and Oguma 1994; Ritchie and Gleason 1995). However, across the *Drosophila* clade, the more general pattern is for species to have two types of pulse song, with some species producing no song at all (Suvanto *et al.* 1994; Gleason and Ritchie 1998; Oliveira *et al.* 2013). Song is not restricted to males, and in some clades male–female duets are an important aspect of courtship (Satokangas *et al.* 1994). The courtship song of *D. melanogaster* has been extensively studied with more than a dozen genes identified as contributing to song (Gleason 2005; Yamamoto and Ishikawa 2013). In contrast, only a few loci have been identified that also affect reproductive isolation. There is some correlation between song genes identified in *D. melanogaster* and causing interspecific differences [Lagisz *et al.* 2012; but see Stern (2014b)], but the resolution of most studies has been limited to QTL rather than genes (Williams *et al.* 2001; Gleason *et al.* 2002). The gene *slo* explains a difference between *D. simulans* and *D. mauritiana* in frequency of the sine song (Ding *et al.* 2016). While *slo* may not be important for speciation (it was determined to be a segregating variant within *D. simulans* rather than a fixed difference between species), the methods and techniques used provide a framework for moving forward in the genetics of these complex behaviors (see *New Approaches to Determine the Genetic Basis of Reproductive Isolation*).

CHCs are a diverse class of molecules produced in specialized cells called oenocytes and excreted onto the cuticle. CHCs have two distinct functions: they protect animals from desiccation and serve as pheromones that regulate behavioral interactions during mating (Gibbs 2002). CHCs play an important role in reproductive isolation for many *Drosophila* species (Cobb and Jallon 1990; Coyne 1996a,b; Liimatainen and Jallon 2007; Gleason *et al.* 2009; Grillet *et al.* 2012). Thus, these compounds are pleiotropic and are under selection from both sexual interactions and the abiotic environment (Foley and Telonis-Scott 2011; McGuigan *et al.* 2011). In fact, one gene, *mFas*, has been identified based on this pleiotropic nature, and contributes to CHC production and desiccation resistance in *D. serrata* (Chung *et al.* 2014). The hypothesized divergence in *mFas* between *D. serrata* and *D. birchi* is presumed to be driven by selection for desiccation resistance, with reproductive isolation occurring as a byproduct.

For diverse male traits to contribute to reproductive isolation, there must be loci expressed in females that control both species recognition and female choice, because ultimately females control copulation success in most species of *Drosophila* (Greenspan and Ferveur 2000). Unfortunately, female choice and mate preferences are severely understudied, especially at the genetic level, as compared to the male signal traits. Female choice is typically defined by how often females mate

with males that have certain trait combinations. The challenge for studying female choice is that, once an individual female mates, her preference/choice cannot be measured again with a different trait combination. Thus, many preference experiments can only be measured in isogenic lines, limiting the number of species that can be assayed. In the context of reproductive isolation, species differences may manifest as a suite of traits rather than a single one, making it difficult to disentangle which trait is important for species recognition.

Further study of the genetic basis of female choice is important to understand how female preference evolves since this trait is a key factor in reproductive isolation in many animals besides *Drosophila*. Currently, it is difficult to distinguish between the multiple ways that female preference evolves without uncovering the genetic basis. For example, female preference may evolve as a byproduct of selection acting on other traits such as female attractiveness (Chu *et al.* 2013) or instead may evolve when female preference is genetically linked to male traits that are under sexual selection (McNiven and Moehring 2013). There are several candidate genes within *D. melanogaster* that contribute to female acceptance or rejection of courting males (Nakano *et al.* 2001; Juni and Yamamoto 2009). Data on female preference during interspecific interactions are limited to mapping studies (Doi *et al.* 2001; Moehring *et al.* 2006; Chu *et al.* 2013). Only one specific gene, *desat2*, has been implicated in reproductive isolation via female preference. The identification of *desat2* in an association study (Fang *et al.* 2002) prompted a transgenic examination where *desat2* alleles from both *D. melanogaster* races were put into a common genetic background (Greenberg *et al.* 2003). However, a significant difference in female preference was only found between the homozygous transgenic lines that differed in *desat2* alleles. Furthermore, the effects were not observed when these transgenic alleles were crossed into other genetic backgrounds, creating doubt as to whether *desat2* or some artifact of the transgenic lines was responsible for differences in female preference (Coyne and Elwyn 2006).

Postmating prezygotic (PMPZ) isolation

PMPZ isolation is a common cause of reproductive isolation in *Drosophila* and can be either noncompetitive or competitive. Noncompetitive barriers include fertilization defects that reduce the total number of progeny produced in heterospecific crosses (Matute 2010; Ahmed-Braimah 2016). Competitive PMPZ barriers include sperm competition and selective sperm utilization, and thus only occur upon multiple mating in females (Price 1997; Dixon *et al.* 2003; Chang 2004; Levesque *et al.* 2010).

Noncompetitive PMPZ isolation due to fertilization defects includes two distinct phenotypes: failures in sperm storage in females and failures in fertilization after sperm enter the egg. Both have been observed in the *D. virilis* clade (Jennings *et al.* 2014; Ahmed-Braimah 2016) and the genetic basis of the sperm storage phenotype has been mapped (Sweigart 2010a; Ahmed-Braimah 2016). The failure to store sperm may result from incompatibilities between nonsperm ejaculate proteins

transferred from the male and proteins expressed in the female (Schnakenberg *et al.* 2011). Male ejaculate proteins have many effects on females (Sirot *et al.* 2009), including insemination reactions (Patterson 1946), which can have negative effects on the female reproductive tract and limit female reproduction and lifetime fitness (Knowles and Markow 2001).

Competitive PMPZ isolation, also known as conspecific sperm precedence, results from sperm competition between heterospecific and conspecific sperm (Price 1997; Dixon *et al.* 2003; Chang 2004; Levesque *et al.* 2010). The underlying mechanism is most likely differential storage and utilization by females of sperm from heterospecific *vs.* conspecific males (Lupold *et al.* 2013; Manier *et al.* 2013). Sperm competition has also been widely documented between individuals of the same species, and it is often assumed that the genetic basis underlying conspecific sperm precedence is the same as intraspecific sperm competition, given that both processes involve different sperm genotypes competing in a female reproductive tract. While some genes contribute to both phenotypes, it is not the case that all genes involved in intraspecific sperm competition act in reproductive isolation (Castillo and Moyle 2014; Civetta and Finn 2014). The lack of complete overlap in genes contributing to sexual selection and reproductive isolation may reflect fundamental distinctions between intraspecific and conspecific interactions. For example, conspecific sperm precedence usually occurs early in sperm storage, but intraspecific sperm competition occurs throughout sperm storage, release, and utilization.

Even though PMPZ barriers are common, their importance for reproductive isolation may be underappreciated (Marshall *et al.* 2002). It is difficult to infer that PMPZ isolation occurs in natural populations unless females are observed to mate with both heterospecifics and conspecifics, and all progeny are then genotyped to determine paternity. Even in a laboratory setting, most assays to detect reproductive isolation rely on single-pair matings.

Postzygotic isolation

Postzygotic isolation has been intensively studied in *Drosophila*, with many known examples of hybrid sterility and lethality (Patterson and Stone 1952; Coyne and Orr 1989). In addition to divergence of nuclear loci, the presence or absence of endosymbionts, in particular *Wolbachia*, can lead to postzygotic isolation due to cytoplasmic incompatibility (Shoemaker *et al.* 1999; Jaenike *et al.* 2006; Miller *et al.* 2010). The different genetic approaches used to study hybrid incompatibilities as well as descriptions of known hybrid incompatibility genes are well-described in several reviews (Presgraves 2010; Maheshwari and Barbash 2011). Here, we focus on several recent findings that illustrate advances in important and understudied aspects of hybrid incompatibilities. We discuss the new methodologies that facilitated these discoveries in the next section (*New approaches to Determine the Genetic Basis of Reproductive Isolation*)

Hybrid male sterility is the most prevalent type of postzygotic isolation in *Drosophila* (Coyne and Orr 2004), but the

underlying physiological causes are in general not known. High-resolution genetic studies have typically suggested that hybrid sterility is highly polygenic and involves complex epistatic interactions among genes, although there are exceptions (Sweigart 2010b). Evidence for complexity comes from introgression studies, where single introgressions often reveal the presence of multiple causal genes when a single large introgression is separated into multiple smaller introgressions by recombination. In a striking example, Lienard *et al.* (2016) analyzed a ∼9-kb introgression from *D. mauritiana* into *D. simulans* that causes almost complete sterility, and discovered that two separate protein-coding genes within it, *agt* and *taf*, each contribute to the sterility effect. Further experiments suggest that multiple substitutions within each gene are responsible for hybrid sterility.

Though not often considered, hybrid sterility can result from PMPZ phenotypes rather than reduction in the number or motility of sperm. Civetta and Gaudreau (2015) found that F1 hybrid males between *D. willistoni willistoni* and *D. willistoni quechua* produce motile sperm, but fail to transfer it to females when mated. Typical analyses of motile *vs.* nonmotile sperm to score hybrid sterility would fail to recognize this phenotype and it will be important to determine how common this barrier is compared to reductions in sperm number or motility for other species.

Within groups of closely related species, lineage-specific changes can modulate the effects of hybrid incompatibility. This has been highlighted in investigations where incompatibilities identified in one species pair are specifically tested in closely related species. For example, the lethal incompatibility involving the genes *Nup160* and *Nup96* (*Nup98-96*) was originally described between *D. melanogaster* and *D. simulans*. When subsequently examined in crosses between *D. melanogaster* and *D. mauritiana* or *D. sechellia*, the *D. melanogaster Nup96* incompatibility only occured in crosses with *D. sechellia* and not *D. mauritiana* (Barbash 2007). Similarly, sequence differences in *Nup160* between *D. simulans* and *D. mauritiana*, as well as differences in the autosomal background of these species, modulate the strength of hybrid inviability in crosses with *D. melanogaster* (Tang and Presgraves 2015).

Incompatibilities independently identified in related species can also be compared to explore lineage-specific effects. Two studies have identified loci that appear similar to the hybrid incompatibility locus *Zhr* (Sawamura *et al.* 1993), based on the fact that they are located in the heterochromatic base of the X chromosome and cause hybrid lethality. One locus, *hlx*, was identified in crosses between *D. mauritiana* and either *D. simulans* or *D. sechellia* (Cattani and Presgraves 2009) and another, *hhl*, contributes to incompatibility between *D. melanogaster* and *D. simulans* or *D. mauritiana* (Coyne *et al.* 1998; Cattani and Presgraves 2012). The potential allelism of the *hlx* and *hhl* loci was tested by Cattani and Presgraves (2012) with two alternative hypotheses resulting. The first is that each locus, while mapping to a similar region, is a separate gene because each is involved in different hybrid incompatibilities. The second hypothesis is that *hhl* and *hlx* are in fact alleles of *Zhr*, and lineage-specific differences in this region and in the

interacting partners have resulted in multiple complex incompatibilities. If correct, differences in the evolutionary histories of *D. simulans* and *D. mauritiana* might dictate how essential each hybrid incompatibility locus is for reproductive isolation with *D. melanogaster*.

New Approaches to Determine the Genetic Basis of Reproductive Isolation

Future prospects for the study of the genetics of speciation are highly promising given new technologies for introducing precise mutations into specific genetic backgrounds and abundant genomic data to discover causal mutations. Below, we highlight how these techniques are being used in novel ways to find genes that contribute to reproductive isolation.

Traditional recombination-based mapping of backcross progeny or introgressions has been very successful in identifying genomic regions associated with reproductive isolation, but typically has insufficient resolution to define the causal loci (Mackay 2001). These regions can now be dissected by incorporating dominant markers (either molecular or visible) into candidate regions using CRISPR-mediated recombination, and then scoring for meiotic recombination between these markers. This in turn creates smaller genomic regions that contain fewer candidate genes. The ease of transgenic and CRISPR experiments then allows multiple candidate loci and/or causal mutations to be feasibly tested for phenotypic effects on reproductive isolation. For example, Lienard *et al.* (2016) reduced a 1.26-Mb region that causes hybrid male sterility (HMS1) between *D. simulans* and *D. mauritiana* down to 9.2 kb in four generations of recombination. They then used transgenic experiments to further refine the region with complementation tests, identifying *agt* and *taf* as causal loci. In a similar approach, Ding *et al.* (2016) used QTL and introgression mapping to identify a 140-kb region that contributes to interspecific differences between *D. simulans* and *D. mauritiana* in the sine song carrier frequency used in male courtship. The efficiency of their introgression mapping was facilitated by a collection of *D. mauritiana* strains each containing a single transposon with a visible marker at a different site in the genome, allowing them to easily track the introgressions. They then used CRISPR/Cas-9 to insert additional visible markers at specific sites flanking the candidate 140-kb region. By generating recombinants between these markers, they obtained an extremely high mapping resolution, ultimately identifying a single causal gene, *slo*. To confirm this locus, they used CRISPR/Cas-9 to create mutations in both species and then conducted a reciprocal hemizygosity test (Stern 2014a), which provides unequivocal proof of a causal gene if its effects are recessive. The power of this test is that by creating deletions in the gene of interest in both strain backgrounds, one can create F1 progeny that are identical except at the deletion.

F1 hybrids are lethal or sterile in some models, such as with *D. melanogaster*. This has been overcome by Phadnis *et al.* (2015), who identified the gene *gfzf* as a dominant

suppressor of hybrid incompatibility from *D. simulans* using a forward genetic screen and whole-genome sequencing. In crosses between *D. melanogaster* females and *D. simulans* males, F1 male hybrids die as larvae. After mutagenizing the *D. simulans* males, they recovered several adult F1 males that must be carrying a mutation inherited from the *D. simulans* parent that suppresses lethality. The causal locus rescuing viability in these individuals was then identified by comparing whole-genome sequences and looking for shared mutations in a single gene, because the rescued males are sterile and thus cannot be further analyzed or mapped by conventional genetic approaches.

Differential expression analysis is a complementary tool to mapping studies for identifying candidate loci. This method can be applied to both prezygotic and postzygotic isolation, but is not without caveats. For prezygotic isolation, the most common approach is to look for changes in expression in females that are presented with either conspecifics or heterospecifics for courtship or mating, with the hypothesis that differentially expressed genes are important for female preference and mate recognition (Bailey *et al.* 2011; Immomen and Ritchie 2012). The main caveat of this approach is that many genes are differentially expressed and not all of them have previously described roles in mating decisions, so functional studies must still be used to validate the strongest candidates. For postzygotic isolation, several studies have documented correlations between the misregulation of gene expression in hybrids and hybrid sterility [reviewed in Landry *et al.* (2007)], but this correlation does not always hold for other reproductive barriers (Barbash and Lorigan 2007; Wei *et al.* 2014). Recent studies comparing sibling progeny from crosses that produce both sterile and nonsterile hybrids suggest that misregulation may not be specific to the sterile hybrids (Civetta 2016). These results question the assumption that misregulated genes are directly involved in the sterility or inviability phenotype and supports the alternative explanation that misregulation is at least sometimes symptomatic of divergent gene regulation between parents.

The ability to test genetic function in other nonmodel systems (*i.e.*, species not closely related to *D. melanogaster*) will likely rely on CRISPR/Cas-9 to make allelic substitutions and precise deletions in specific genetic backgrounds (Bono *et al.* 2015). In some species, there are further challenges, such as large inversions that prevent the production of viable recombinants with related species. One solution is to create rearrangements to colinearize chromosomes, as has been accomplished in other systems (Kraft *et al.* 2015).

Can an Integration of Intraspecific and Interspecific Research Increase Understanding of Speciation?

The genetic basis of speciation

Given the new tools discussed in the preceding section, our efforts should now turn to developing research programs that achieve a synthesis of the speciation process and its genetic basis. We propose that a comparative approach aimed at

determining the genetic basis of reproductive isolation in multiple cases within a clade is the most valuable way forward, particularly where one can analyze divergence between subspecies, nascent species, and older genetically divergent species. A comparative approach can help answer three outstanding questions in speciation: (1) how does the genetic basis of reproductive isolation change over time, (2) are certain pathways and genes more commonly involved in reproductive isolation, and (3) does stronger isolation require more genes and therefore does the genetic architecture become more complex over time? Integrating these questions can also provide insight into whether the effect size of individual genes changes over time as more genes potentially contribute to reproductive isolation.

One main challenge in implementing a comparative approach, beyond the immense effort required, is in careful experimental design and interpretation of the results. For example, the most used methods for mapping loci important for reproductive isolation either focus on a specific trait known to act as a reproductive barrier (Coyne 1996a; Etges *et al.* 2007; Cande *et al.* 2012; Ding *et al.* 2016) or map reproductive isolation naïvely with respect to specific traits, most commonly for female preference where it is unknown which signal traits females are responding to (Laturney and Moehring 2012). These methods work for a single-species pair, but may fail in a comparative analysis because the comparative approach must account for the reproductive barriers that contribute to reproductive isolation in each set of species studied. Asking whether certain pathways or genes are commonly involved only makes sense if the same specific barrier to reproduction, such as visual or chemical signals, is operating in each species pair. Focusing on specific reproductive barriers will require fine-scale analysis of which traits drive reproductive isolation across large species comparisons, data that are currently lacking. We suggest that reproductive isolation should be mapped in parallel with candidate traits such as pigmentation, song, or CHC profiles for each population. All traits and their contributions to reproductive isolation can then be compared across species to determine whether single or multiple traits are important at different divergence times (Yukilevich *et al.* 2016). To interpret comparative results, it will also be essential to understand how candidate genes, both independently and in combination, contribute to reproductive isolation. For example, it has been proposed that the number of loci contributing to reproductive isolation and the complexity of the interactions among these loci increases nonlinearly over time, often termed the "snowball effect" (Orr and Turelli 2001), but this does not answer whether a more complex genetic basis is necessary for stronger reproductive isolation. This issue may be particularly relevant for prezygotic isolation because multiple traits can contribute that have completely distinct genetic bases.

Given the power of the comparative approach and its success in other systems, such as wing patterning in *Heliconius* butterflies (Joron *et al.* 2006; Reed *et al.* 2011; Belleghem *et al.* 2017), it is surprising that it has not been implemented for

reproductive isolation. Several logistical and historical factors may contribute to this lack of comparative data. Relatively few *Drosophila* species have yet been the focus of high-resolution genetic analysis, and the comparative approach cannot be applied to the set of genes that have been identified to contribute to postzygotic isolation either because sets of genes have been identified that trace back to a single speciation event (typically involving *D. melanogaster*), or only a single gene has been identified in a clade (for example, *D. pseudoobscura*). The power of comparative genetic studies requires many species and independent species comparisons within a single clade (Moyle and Payseur 2009; Wang *et al.* 2013).

However, there is one interesting case of genes involved in both interspecific and intraspecific prezygotic isolation in the *D. melanogaster* subgroup (Figure 3). The desaturase genes *desatF* (*Fad2*) and *Desat2* are required for the production of CHCs in *Drosophila*, which function in desiccation resistance and act as pheromones that regulate mating behavior (Cobb and Jallon 1990; Gibbs 2002). The gene *desatF* encodes an enzyme that is responsible for the production of female-specific CHCs (Chertemps *et al.* 2006). *desatF* likely contributes to female CHC differences between *D. simulans* and *D. sechellia* and between *D. simulans* and *D. melanogaster* because *D. simulans* does not express *desatF* (Legendre *et al.* 2008). As a consequence, *D. simulans* males and females are monomorphic for particular CHCs, including 7-tricosene, whereas in the other two species males produce 7-tricosene and females produce the diene 7,11-heptacosadiene (Gleason *et al.* 2009). These dienes are important for reproductive isolation because males from species that are monomorphic for 7-tricosene court females that lack high amounts of 7-tricosene at a significantly reduced rate compared to the rate at which they court conspecifics (Cobb and Jallon 1990). Thus, *desatF* likely plays a direct role in causing prezygotic isolation.

The gene *desat2* is closely related to *desatF* but operates more upstream in the CHC production pathway (Figure 3). There are two alleles of *desat2* in *D. melanogaster* populations; the functional allele is rare on a global scale but occurs at high frequency in populations from southern Africa. Females from these populations, often termed Z-type populations for their discovery in Zimbabwe, have a CHC profile that contains a specific diene, 5,9-heptacosadiene (Coyne *et al.* 1999). The more common *desat2* allele found in most cosmopolitan populations (M-type populations) throughout the world has an 18-bp deletion in the regulatory region (Fang *et al.* 2002). This deletion reduces expression of the gene and female CHC profiles are instead dominated by the compound 7,11-heptacosadiene (Dallerac *et al.* 2000). There is prezygotic isolation between the Z-type populations and M-type populations, as Z-type females strongly reject M-type males (Wu *et al.* 1995). The prezygotic isolation and CHC difference are both strongly correlated with the specific *desat2* allele (Fang *et al.* 2002). To directly test the role of *desat2*, a transgenic experiment introduced both the Z-type and M-type *desat2* alleles into a common M-type background (Greenberg *et al.* 2003). When these genotypes were tested for prezygotic isolation, the females carrying the Z-type
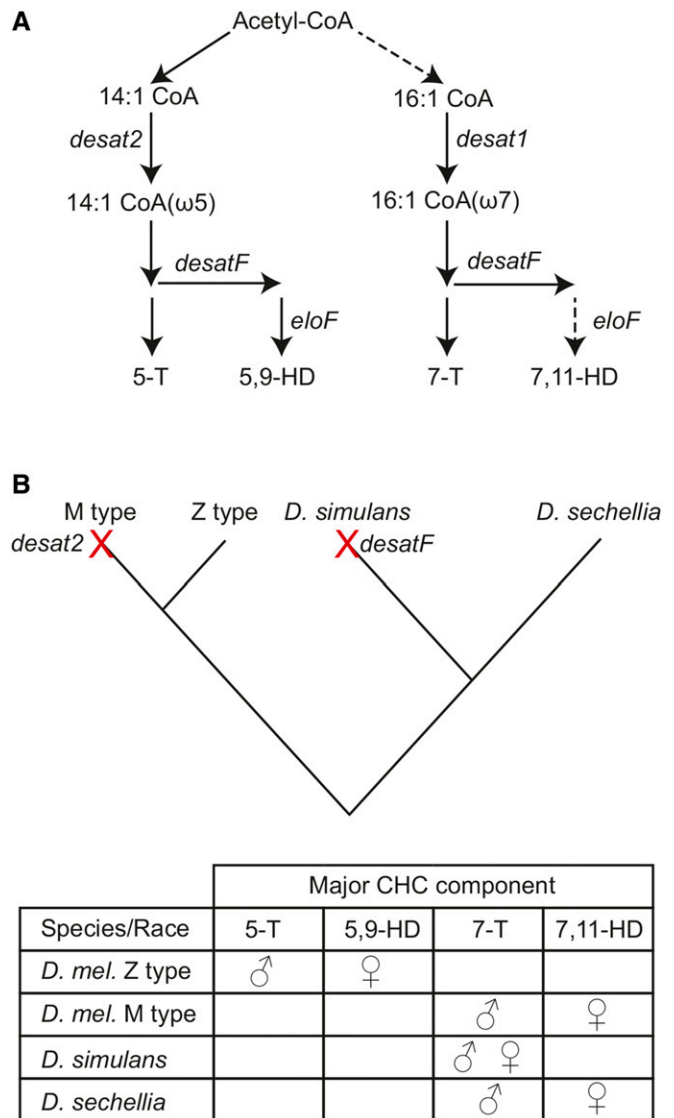

Figure 3 Changes in two desaturase genes produce differences in cuticular hydrocarbons (CHCs) that lead to reproductive isolation. (A) A simplified diagram of CHC synthesis in *D. melanogaster*. (B) Known changes in desaturase genes are mapped to a phylogeny. (C) Lineage-specific changes in desaturase genes result in differences in major CHC components between the species. The change in *desatF* in the *D. simulans* lineage is from a functional to nonfunctional allele based on the inferred ancestral state, depicted as red "X" on the phylogeny. Similarly, the change in *desat2* in the M-type *D. melanogaster* is to a nonfunctional allele. Both *desatF* and *desat2* likely originated from ancestral duplication events from *desat1*. The retrotransposition that generated *desatF* and the tandem duplication that resulted in *desat2* predate the split of the *Drosophila* and *Sophophora* clades (Fang *et al.* 2009).

allele rejected M-type males in the same way that wild-type Z-type females reject M-type males (Coyne and Elwyn 2006). However, the transfer of CHCs between the different races does not appear to affect premating isolation (Coyne *et al.* 1999). Furthermore, CHC profiles were only moderately changed in the transgenic experiment and did not recapitulate the profiles of the two races. Therefore, the current data suggest that even though *desat2* is pleiotropic, either its roles in female preference

and CHC production may be independent or it may interact with other unknown genes. This highlights the necessity for functional analyses of genes implicated in reproductive isolation to determine precisely how they contribute to reproductive isolation.

Even though both genes are desaturases, it is interesting that they contribute to reproductive isolation through two distinct pathways: female CHC production that elicits male courtship (*desatF*) or female preference possibly independent of CHC production (*desat2*). CHCs are important components for reproductive isolation throughout the *D. melanogaster* subgroup (Cobb and Jallon 1990), and large changes in the abundance or presence/absence of a particular CHC can be caused by a single locus (Ferveur 1991; Coyne 1996a, 1999). In comparison the genetic basis of female preference is complex and involves many genes and possibly epistatic interactions (Hollocher *et al.* 1997). The comparison of *desatF* and *desat2* has been important to identify a group of genes that contributes to reproductive isolation, but this comparison is limited because these genes appear to contribute to reproductive isolation through different mechanisms. To address the larger comparative goal outlined here, it is necessary to identify the genetic basis of a single component of reproductive isolation (*i.e.*, female preference, CHC differences, male song, or other courtship parameters) across multiple species pairs.

To implement a comparative approach on a large scale, a reasonable starting point would be to choose candidate genes that have been identified through null mutations in a single species as affecting a trait important for isolation, and then testing for a role in reproductive isolation. However, it remains to be determined how frequently such null mutants reflect phenotypes relevant to reproductive isolation. For example, in at least two studies, candidate genes affecting intraspecific sexual interactions were identified from null mutant alleles but failed to contribute to interspecific reproductive isolation [male song (Gleason and Ritchie 2004), and sperm competition (Castillo and Moyle 2014)]. It is unclear if this reflects differences in the genetic architecture of traits important for within-species processes *vs.* reproductive isolation, or if it instead reflects the distinct nature of null alleles *vs.* segregating allelic differences. Perhaps there is more promise in identifying candidate genes for reproductive isolation from studies that use a combination of genetic association tests with natural allelic variants and follow-up tests with null mutants or RNAi. For example, Dembeck *et al.* (2015) identified single nucleotide polymorphisms that contribute to quantitative differences in the suite of CHCs within *D. melanogaster*. They then validated that these genes produce changes in CHC profiles using RNAi. Interestingly, a few of the genes contribute to the production of CHCs that were previously identified as important for reproductive isolation between *D. melanogaster* races (Z and M) in an independent study (Grillet *et al.* 2012).

Integration of sexual selection and prezygotic reproductive isolation

The hypothesis that sexual selection is a main driving force of prezygotic isolation (see *Mechanisms that Contribute to Reproductive Isolation and Their Genetic Basis*) is often proposed, but a direct link between these processes has limited empirical support (Ritchie 2007; Castillo and Moyle 2014). One reason is that it is typically much easier to demonstrate reproductive isolation than to show which traits, and potential interactions among traits, are responsible for the isolation. Only a handful of studies have evaluated whether traits involved in reproductive isolation are also under sexual selection within a population (Boake *et al.* 1997; Higgie and Blows 2008; Veltsos *et al.* 2012). For example, head width is significantly correlated with *D. heteroneura* male mating success and is also used in species discrimination between *D. heteroneura* and *D. silvestris* (Boake *et al.* 1997). Similarly, CHC differences contribute to reproductive isolation between populations of *D. montana*, and divergent selection operates between populations. In the Vancouver population, mating success increases when specific CHCs are present, while these same compounds cause reduced mating success in the Oulanka population (Veltsos *et al.* 2012). Studies that aim to connect sexual selection and reproductive isolation will be valuable in a comparative framework to understand whether similar traits are under sexual selection across a clade and in which species pairs divergent sexual selection has contributed to reproductive isolation.

Determining the precise phenotypes involved in prezygotic isolation and their genetic basis will further allow us to understand how these traits evolve. When phenotypes are complex and/or can be decomposed into smaller constituent phenotypes, different genes may be under different selective pressures or be pleiotropic. For example, the full complement of CHCs may be important for both sexual selection and reproductive isolation, but different CHC biosynthesis genes may be important for only one of these processes. Only by identifying the actual genes that produce specific CHCs will we be able to assess their pleiotropic properties, as in the example described above of the *mFas* gene in *D. serrata* (Chung *et al.* 2014). Lastly, some genes affect both male and female phenotypes. This has the potential for genetic coupling of traits in both sexes that are important to mate choice and may help us understand why prezygotic isolation evolves so rapidly (McNiven and Moehring 2013).

Can integration help us understand the evolution of postzygotic isolation?

A fundamental aspect of postzygotic isolation is that most of the known hybrid incompatibility loci act as gain-of-function mutations that only manifest in hybrids (Maheshwari and Barbash 2011). For example, the presence, not the absence, of the genes *Hmr* and *Lhr* cause hybrid inviability in crosses between *D. melanogaster* and *D. simulans*. Both genes encode proteins that localize to heterochromatin and form a complex that represses transcripts derived from satellite DNA and TEs (Thomae *et al.* 2013; Satyaki *et al.* 2014). Even though some families of TEs are misregulated in hybrid progeny, misregulation does not likely cause hybrid lethality (Thomae *et al.* 2013; Satyaki *et al.* 2014). Likewise, the

presence of *OdsH* causes hybrid sterility between *D. simulans* and *D. mauritiana*, while its absence only mildly affects fertility [within *D. melanogaster*, Sun *et al.* (2004); its phenotype within *D. simulans* and *D. mauritiana* is unknown]. Similarly, the presence of the *Zhr* locus causes embryonic lethality in *D. melanogaster/D. simulans* hybrids, while its absence has not been rigorously analyzed but is clearly not required for full viability in *D. melanogaster* (Sawamura *et al.* 1993). Thus, it is important to ask whether the comparative approach, or which specific aspects, will be important to understanding the evolution of hybrid incompatibility.

The way forward for implementing the comparative approach for postzygotic isolation is to identify hybrid incompatibility genes in more species pairs, particularly in more recently diverged lineages. Contrasting the genetic basis of hybrid incompatibilities between younger and older lineages will determine if the genes that have been identified (mostly in older species pairs) contribute to reproductive isolation at early stages of divergence. The genetic architecture in older species is complex with many genes involved (Noor *et al.* 2001a; Tao *et al.* 2003a). In contrast, some systems have a relatively simple genetic basis, for example sterility between *D. virilis* and *D. americana* (Sweigart 2010b). In fact, the *D. virilis* clade may be an ideal clade for comparing the genetic basis of hybrid incompatibilities, because crosses can be completed between both close and distantly related species and incompatibilities can be detected in F2 or backcross progeny (Patterson and Stone 1952; Heikkinen and Lumme 1991, 1998; Sweigart 2010b).

The assumption that we made above for applying the comparative approach for prezygotic isolation was that similar forces acting within species (*i.e.*, sexual selection) directly contribute to divergence and reproductive isolation. From this, we would predict that traits and genes that are important to sexual selection within species are candidates for causing reproductive isolation. If we apply this analogy to postzygotic isolation, we should see evidence for hybrid incompatibilities segregating within species and for a shared genetic basis of incompatibilities within and between species. These two observations are interconnected if segregating incompatibilities eventually lead to between-species incompatibilities. The majority of previously identified hybrid incompatibility genes (*Hmr*, *Lhr*, *OdsH*, and *Zhr*) either do not have strong negative effects within species or have mutant phenotypes distinct from their hybrid incompatibility phenotypes; therefore, the expectation of a shared genetic basis for within- and between-species incompatibilities is less certain. Phadnis *et al.* (2015) found that the *D. simulans* allele of the gene *gfzf* contributes to hybrid lethality in hybrids with *D. melanogaster*. They note that this gene had previously been identified as a suppressor of the *prune-Killer of prune* incompatibility that segregates within *D. melanogaster* (Provost *et al.* 2006). It is still yet to be seen if the function of *gfzf* is similar in the within- *vs.* between-species incompatibility and whether this supports the hypothesis that there are only so

many pathways that can generate dominant inviability suppressors (Phadnis *et al.* 2015). If the function of *gfzf* is similar in both situations then it will be important to determine if the other players, *prune* and *Killer of prune* (*awd*), also interact with known hybrid incompatibility genes in *D. melanogaster* and *D. simulans*.

Within-species incompatibilities do appear to be segregating within *D. melanogaster* (Lachance and True 2010; Corbett-Detig *et al.* 2013). Lachance and True (2010) identified inviability and sterility effects in lines that combined X chromosomes with autosomes from different populations. Corbett-Detig *et al.* (2013) first looked for evidence of negative epistasis by analyzing patterns of transmission distortion in a panel of recombinant inbred lines. They then made F1 crosses between the parents that originally generated the recombinant lines to confirm the presence and negative effect of these incompatibilities on fitness (Corbett-Detig *et al.* 2013). In both studies, the effects that they identified had an epistatic dependency similar to the Dobzhansky–Muller model of hybrid incompatibility between species.

If incompatibilities segregate within populations, we need to understand the fate of these incompatibilities by identifying the underlying genes and determining whether these incompatibilities can eventually lead to reproductive isolation between species. Segregating incompatibilities should follow a predictable trajectory if they will ultimately contribute to reproductive isolation between species in a manner consistent with the Dobzhansky–Muller model. The intermediate phase is polymorphism for between-species incompatibility (Cutter 2012). Polymorphic incompatibilities occur when the presence/absence of the incompatibility between species depends on the specific genotype of the parents used in a cross. Polymorphism for factors contributing to hybrid male sterility has been identified in two very recently diverged systems. In crosses between *D. mojavensis* and *D. arizonae*, the magnitude of hybrid sterility depends on which *D. mojavensis* subspecies is used in the cross (Reed and Markow 2004). Similarly, in hybrids between two subspecies of *D. bipectinata*, the level of hybrid male sterility varies substantially based on which particular line is used (Kopp and Frank 2005). In both of these cases, some strains produce hybrids that are fertile, suggesting the possibility that the polymorphisms segregating for hybrid incompatibility reflect intraspecific variation that could indeed eventually contribute directly to interspecific incompatibility. In contrast, while there is variation in the strength of hybrid inviability between *D. melanogaster* and *D. simulans* that depends on the genetic background of the lines used, essentially all hybrid progeny remain sterile (Watanabe *et al.* 1977; Barbash *et al.* 2000; Matute *et al.* 2014). In this example, where reproductive isolation is fixed, genetic background effects may be trivial and reflect the kind of small effect loci (modifier alleles) that contribute to almost all quantitative traits (Chandler *et al.* 2013).

Conclusions

Drosophila has contributed a vast amount of empirical data to the study of speciation and inspired many theoretical studies. We suggest that in the future the following areas and research programs will be particularly insightful.

1. Genome scans of divergence across populations and more sophisticated modeling to identify diverged regions contributing to reproductive isolation.
2. More sampling of intraspecific incompatibilities and partially isolated behavioral races within species, and further study of their genetic bases.
3. Increased attention to species groups hitherto classified as nonmodel, with a particular focus on those with multiple species pairs.
4. Increased understanding of the genetic basis of behavioral traits that are potentially important for premating isolation.

Acknowledgments

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