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The genetics of speciation by reinforcement

Daniel Ortiz-Barrientos
Louisiana State University and Agricultural and Mechanical College

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Daniel Ortiz-Barrientos
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ABSTRACT

Reinforcement occurs when natural selection strengthens behavioral discrimination to prevent costly interpopulation matings, such as when matings produce sterile hybrids. This evolutionary process can complete speciation, thereby providing a direct link between Darwin's theory of natural selection and the origin of new species. My dissertation presents the first study on the genetics of reinforcement. This study is framed in a conceptual body that explains how genomic architecture, selection and recombination, interact to facilitate divergence in the presence of gene flow. In addition, in my dissertation I produced a dense recombination map for *D. pseudoobscura*, which together with the genome sequence opens many possibilities for classic population genetic and genomic analyses in this system.

I examine a case of speciation by reinforcement in *Drosophila*. I present the first high-resolution genetic study of variation within species for female mating discrimination that is enhanced by natural selection. I show that reinforced mating discrimination is inherited as a dominant trait, exhibits variability within species, and may be influenced by a known set of candidate genes involved in olfaction. My results show that the genetics of reinforced mating discrimination is different from the genetics of mating discrimination between species, suggesting that overall mating discrimination might be a composite phenomenon, which in *Drosophila* could involve both auditory and olfactory cues. Examining the genetics of reinforcement provides a unique opportunity for both understanding the origin of new species in the face of gene flow and identifying the genetic basis of adaptive female species preferences, two major gaps in our understanding of speciation.

CHAPTER I

INTRODUCTION

The Origin of New Species

Millions of species inhabit Earth. From the Cambrian explosion of body plans to recent fish radiations in African lakes, earth has continually produced a vast number of organisms with a variety of morphologies, ecologies and behaviors. Man has always wondered about the origin of species, but it was only in the last few centuries that we began to understand the problem of speciation (e.g., Rice and Hostert 1993; Schluter 1998; Servedio and Noor 2003; Shaw 2001; Templeton 1994; Turelli et al. 2001). The fundamental approaches to understanding speciation have come from genetics, systematics and ecology. Together, these approaches have provided clues about the molecular mechanisms (Barbash et al. 2003; Presgraves et al. 2003), the geographic patterns (Templeton et al. 1995) and individual interactions (e.g., Feder et al. 1988; Nosil et al. 2002) that take place during speciation. My dissertation uses a genetic approach to understand a behavior that contributes to the speciation process between two taxa.

During speciation, groups of individuals stop exchanging genes (species are defined in my dissertation using the biological concept: “species are groups of interbreeding natural populations that are reproductively isolated from other such groups “ (Mayr 1963)). Many events can trigger this phenomenon. For instance, geographic barriers may prevent migration between populations (Mayr 1942). Likewise, new resources might become available in a population and disruptive selection acting on variation for resource use may split the population in two groups (Doebeli and Dieckmann 2000; Feder et al. 1988; Fry 2003; Hawthorne and Via 2001). In either case, the exchange of genes is prevented to some extent, but the key issue is how this process can become irreversible. In particular, how do diverging taxa persist in the face of gene flow? A major focus of my dissertation is to understand how species can persist in the face of gene exchange after coming back into contact.

Many traits can contribute to the separation of two hybridizing species. One of such traits is hybrid fitness reduction (Dobzhansky 1951). This category can come in different flavors: sometimes hybrids cannot reproduce, other times they do not reach sexual maturity as they die early in development. These traits, hybrid sterility and hybrid inviability, have been among the most heavily studied in the speciation field (Coyne and Orr 1988; Fishman and Willis 2001; Noor et al. 2001b; Rieseberg 1999; Wu and Davis 1993). Another trait that can contribute to the persistence of species is mate choice. If matings only take place between organisms of the same taxon, then hybrids will not be produced, and the probability of species fusion is eliminated (e.g., Moehring et al. 2004; Noor 1999; Sætre et al. 1997; Wu et al. 1995). Mate choice, mostly present in organisms with elaborate behaviors, may be the most important trait contributing to the origin of new species in higher organisms, yet it is still poorly understood. A major goal in my dissertation is to understand the genetics of mate choice. I use a system of species in which

certain individuals mate more often with individuals with the same species, but other individuals sometimes fail to discriminate.

Mate choice, hybrid sterility and hybrid inviability may evolve under the influence of a variety of evolutionary forces. Darwin claimed that natural selection is the major force driving the origin of species (although he did not explain how this force would originate new species) (Darwin 1859). Recent studies have shown that natural selection can prevent gene exchange directly or indirectly. Indirect selection acts on traits that incidentally produce reproductive isolation rather than on traits that cause reproductive isolation itself (Presgraves et al. 2003). Direct selection, on the other hand, acts precisely on traits that reduce interspecies gene exchange to result in reproductive isolation between taxa (i.e., direct selection strengthens mate choice, or reinforces, for instance, the abortion of otherwise sterile/ inviable zygotes) (Ortiz-Barrientos et al. 2004). Strengthening of barriers to gene flow can occur during sympatric speciation (i.e., speciation without geographic barriers) or during speciation by reinforcement (i.e., the process by which natural selection increases reproductive isolation in response to maladaptive hybridization). For my dissertation *I* use a system of study that appears to have speciated by the direct action of natural selection on mate choice in response to maladaptive hybridization.

Speciation by Reinforcement

Direct selection for mating discrimination in the face of maladaptive hybridization, a phenomenon usually called reinforcement, is one of the most debated modes of speciation (Butlin 1987; Dobzhansky 1940; Fisher 1958; Howard 1993; Servedio and Noor 2003). Starting with Fisher and Dobzhansky, reinforcement received great attention and it was considered as a necessary step to complete the speciation process. However, many noted theoretical problems that could affect the viability of the reinforcement hypothesis. For example, Felsenstein (1981) produced a seminal work in which he showed that recombination opposed speciation. He demonstrated that recombination would break apart any genetic association between traits that contribute to hybrid fitness reduction or mating discrimination, thus preventing divergence. The second chapter of my dissertation is a review paper that addresses this issue. *I* examine the interplay between recombination and factors contributing to reproductive isolation in hybridizing species. I provide both a synthesis of the theoretical and empirical literature on the role of genomic architecture as a key player on the persistence of species diverging in the face of gene flow.

Recent empirical and theoretical work has provided strong support for the existence and plausibility of reinforcement in nature. For example, Liou and Price (1994) demonstrated that reinforcement could complete the speciation process if sexual selection was incorporated into reinforcement models. Similarly, Kelly and Noor (1996) showed that reinforcement was likely to occur if hybridizing species could share genes that would enhance their mating discrimination, thus resulting in

strong assortative mating. Finally, in a series of papers, Servedio and Kirkpatrick extended the theory by showing the most conducive situations for the existence of reinforcement (see Servedio and Noor 2003 for a review). In spite of this progress, these models may lack realism because they do not incorporate empirical genetic data and assume many parameters. The fourth chapter of my dissertation investigates the genetic basis of speciation by reinforcement in the fruit fly *Drosophila pseudoobscura*. I show that reinforced discrimination is a dominant trait, it maps to areas of the genome that have been extensively exposed to gene flow in the past, and genes on both the X chromosome and autosomes contribute to the phenotype.

Fish, insects, frogs, birds, plants and marine organisms have been investigated for evidence of reinforcement (Noor 1995; Nosil et al. 2003; Pfennig 2003; Rundle et al. 2000; Sætre et al. 1997). These studies were driven by classic and innovative ways to detect reinforcement in nature. For example, reinforcement predicts that mating discrimination is stronger in individuals from areas of overlap of two species than in individuals from areas where only one species is present (Howard 1993; Servedio and Noor 2003). This prediction was confirmed in an extensive meta-analysis of data from *Drosophila* (Coyne and Orr 1989) and produced excitement to investigate other systems. In one case, European hybridizing flycatchers have marked plumage differences (presumably affecting mate choice) in areas of sympatry but not in allopatry (Sætre et al. 1997) 1997). Similarly, Nosil and colleagues (2002), working with *Timema* walking sticks, have not only shown that the classic prediction of reinforcement applies to hybridizing taxa of this genus, but also that reinforcement is stronger when the rate of hybridization is intermediate (Nosil et al. 2003). This is exactly what reinforcement theory predicts since natural selection for reduced gene exchange between hybridizing species is not swamped by high levels of gene flow. In my dissertation, I use *Drosophila pseudoobscura* and *D. persimilis* as model organisms for studying reinforcement. These species occasionally hybridize, produce hybrid sterile male offspring and show stronger female mating discrimination in areas of overlap than in areas where only one species occurs.

Reinforcement in *D. pseudoobscura*

Drosophila pseudoobscura provides a unique system to study reinforcement (Noor 1995). This fruit fly lives in deciduous forests along the west coast of north and Central America and shares a fraction of its range with its sibling species, *D. persimilis*. These species share similar behavioral and ecological features, occasionally hybridize in nature, and discriminate against mating with each other. Behavioral experiments have repeatedly shown that mating discrimination is stronger in *D. pseudoobscura* females that co-occur with *D. persimilis* than in those that are isolated from *D. persimilis* (Noor 1995; Ortiz-Barrientos et al. 2004). This provides an ideal setting for studying genetic differences between highly and basally discriminant females. In my dissertation, I use a QTL mapping approach to

localize regions of the genome that contribute to differences in mating discrimination levels between flies derived from sympatric and allopatric regions of the species.

Many traits contribute to the reproductive isolation between *D. pseudoobscura* and *D. persimilis*. Remarkably, all traits that prevent the exchange of genes between these species (e.g., hybrid sterility and inviability, mating discrimination, hybrid male courtship dysfunction) map to regions of the genome that are chromosomally rearranged between the two species (Noor et al. 2001c). These rearrangements (i.e., inverted chromosomal regions) are fixed between species and are thought to prevent gene exchange by their antirecombinational effects on hybrid offspring. In chapter four of my dissertation, I ask whether genes contributing to mating discrimination strengthened by natural selection (reinforced mating discrimination) map to inverted regions of the genome.

A major challenge for speciation studies is the identification of genes that prevent species mixing. Although a handful of genes for reproductive isolation have been isolated, in all cases they correspond to genes contributing to differential fertilization, hybrid inviability, or hybrid sterility (Adam et al. 1991; Barbash et al. 2000; Presgraves et al. 2003; Ting et al. 1998). However, we have yet to identify the genes responsible for behaviors that prevent gene exchange before fertilization takes place. These genes are likely major players in the speciation process, and their identification would open numerous avenues of research in the speciation field. A major finding of my dissertation is the localization of very small chromosomal regions (some containing up to five genes only) responsible for increased mating discrimination between *D. pseudoobscura* and *D. persimilis*.

The recent completion of the genome sequence of *D. pseudoobscura* (Richards et al. 2005) has brought this model organism to the forefront of evolutionary studies. This has permitted the creation of a dense recombination map and the characterization of genomic regions involved in evolutionarily important traits. In the third chapter of my dissertation, I present the first high-density molecular recombination map for *D. pseudoobscura.* I provide detailed information for recombination rates for four chromosome arms of its genome, primer information for ~150 microsatellite markers, and compare these findings with some recently obtained for regions of the *D. persimilis* genome.

Prospects

My dissertation opens new avenues of research for a variety of speciation studies. First, the identification of genes for reinforced mating discrimination will certainly be possible. Both the high recombination rates observed in *D. pseudoobscura* and the ease of producing large backcross populations will make possible the construction of recombination transgenics (i.e., replacing, via recombination, the gene of interest in a foreign genetic background). Also,

candidate genes identified via this process can be cloned and used in transformation experiments where their direct effects on mating discrimination can be assessed. This project is currently underway. Specifically, I have produced *D. pseudoobscura* lines with an allopatric genetic background that contain selected sympatric chromosomes previously identified as contributors to reinforcement in this system. In the very near future, we will find speciation genes that contribute to mating discrimination, which we believe to be essential to the speciation process between *D. pseudoobscura* and *D. persimilis*.

Second, my dissertation will make tests of basic theories of speciation possible. For example, we will be able to check if *D. pseudoobscura* and *D. persimilis* share some of the same discrimination alleles (see conclusions). This test, never done before, is fundamental to understanding speciation cases where gene flow is present during divergence. As such, these results will not only advance our understanding of reinforcement, but also sympatric speciation. I have already created *D. persimilis* lines homozygous for regions of *D. pseudoobscura* contributing to reinforcement and behavioral experiments are already being conducted.

Third, my dissertation is a starting point for understanding the genetics of a behavior (i.e., mate recognition). Behavioral traits are very poorly understood, yet they influence, for instance, survivorship, mate acquisition and, food localization in many animals. As we discover the genes for reinforcement we are discovering genes for a behavioral trait itself. The molecular genetics of these genes will provide new insights into perception mechanisms acting on insects.

Finally, my development of a detailed molecular linkage map of *D. pseudoobscura* will facilitate a wide variety of molecular evolution or population genetic tests. No comparable molecular linkage map exists in *Drosophila* outside of the *D. melanogaster* species group, and a large number of researchers have already begun to flock to *D. pseudoobscura* since the genome sequence became available.

Experimental science driven by theory is a fulfilling approach to understand nature. Model organisms are particularly suited for this endeavor although they are a limitation in themselves. Experimental work framed in a comparative approach is necessary to cure these weaknesses. I expect to use this approach in the future, engage in new systems of study, and contribute to our understanding of the origins of biodiversity on earth.

CHAPTER II

RECOMBINATION AND THE DIVERGENCE OF HYBRIDIZING SPECIES*

* Reprinted with permission of Springer. Citation: Daniel Ortíz-Barrientos, Jane Reiland, Jody Hey, and Mohamed A.F. Noor, Recombination and the Divergence of Hybridizing Species, Genetica, Volume 116, Issue 2 - 3, Nov 2002, Pages 167 - 178

Theoretical models have suggested that recombination can oppose species formation or the persistence of hybridizing species in numerous ways (see table 2.1). For example, in a classic theoretical model of hybridizing subpopulations, Felsenstein (1981) showed that linkage disequilibrium between loci conferring adaptation to different environments and a locus for assortative mating substantially favors the divergence of these subpopulations through fixation of alternate alleles. If recombination breaks the allelic association between mate choice and adaptation, speciation does not proceed. Empiricists also noted the ideas of species acting as “coadapted gene complexes” (Mayr 1963), "fields for recombination" (Carson 1975), or bearing “supergenes” of tightly linked loci that confer fitness advantages in specific environments (Anderson et al. 1975). In any of these cases, recombination between different types could break apart these complexes, resulting in the formation of unfit progeny

In recent times, several authors have presented empirical evidence of reductions in recombination or evolved genetic correlations possibly contributing to species formation or persistence (e.g., Hawthorne and Via 2001; Noor et al. 2001c; Rieseberg 2001). Interestingly, although these many authors, early and recent, have reached similar conclusions, their rationales differ on *why* such genetic associations may aid the speciation process. In this article, we review the empirical results suggesting the importance of genetic associations on speciation, and we review some similar suggestions from theoretical studies of related disciplines (e.g., sexual selection).

The fundamental association between recombination and speciation is rarely noted. If species are considered to be entities capable of exchanging genes, or populations within which adaptive variants can spread, then the complete absence of recombination (defined in this paper as the mixing of genetic material due to either independent assortment of chromosomes or crossing over within chromosomes) in hybrids between two taxa would by definition cause speciation. Two completely non-recombining genomes could come together in a heterozygous form, but introgression could not occur from one taxon into another because of the absence of any form of recombination (see figure 2.1). Any adaptation within one taxon could not spread into the other, as would be true of two species that produce completely sterile hybrids. This extreme example illustrates how recombination is intrinsically tied to speciation in the population genetic sense.

Means by which Genetic Associations Can Arise

Recombination can be reduced among loci in many ways, thus producing genetic correlations among alleles or phenotypes. Crossing over is reduced between physically proximate loci or loci in centromeric (e.g., Lambie and Roeder 1986; Nachman and Churchill 1996; Payseur and Nachman 2000) or telomeric regions (e.g., Carpenter 1979). Similarly, chromosomal rearrangements (e.g., inversions) may effectively impede crossing over along the rearranged region in

heterozygotes through the lack of recovery of recombinant progeny. Genomic rearrangements are widely known and reported from natural populations of numerous species (Anderson et al. 1991; Li et al. 1997; Rieseberg et al. 1999; Shaw et al. 1979; Wallace and Searle 1994). However, evolutionary processes such as runaway sexual selection, genetic drift, admixture, or nonadditive fitness interactions among loci may also produce genetic correlations among loci independent of genome organization. During runaway sexual selection, a cyclic coevolution of two alleles with sex-specific effects produces a genetic correlation between female preferences and preferred male characters (Fisher 1958; Kirkpatrick 1982; Lande 1981), whereas genetic drift and admixture produce non-random associations among alleles at many loci by reducing the sample of possible genotypes (Hartl and Clark 1997).

The processes that create genetic correlations may facilitate speciation by allowing combinations of alleles among genes contributing to adaptation or reproductive isolation to persist in hybridizing taxa. To understand their effects on the evolution of reproductive isolation, we will focus on cases of sympatric speciation and secondary contact after speciation has begun in allopatry (e.g., speciation by reinforcement) since the concept of hybridizing taxa is applicable to either case despite the difference in when gene flow occurs.

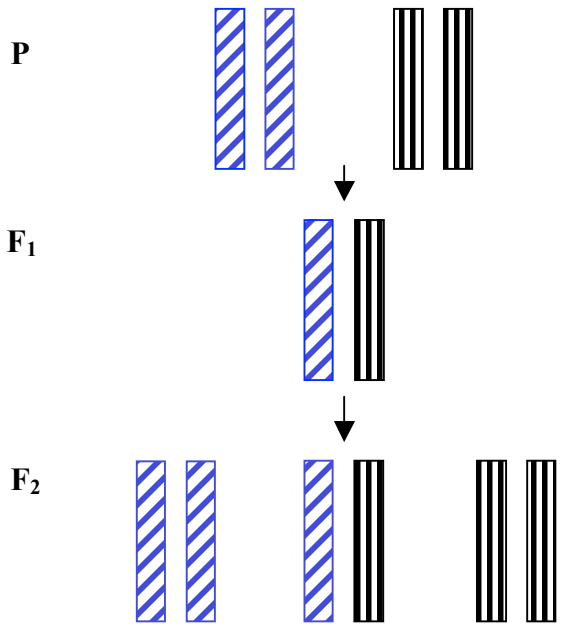

Figure 2.1 Two non-recombining genomes are not able to mix their genetic material since an f_1 individual can only produce parental types of offspring identical to itself after crossing with another f_1 or backcrossing.

Table 2.1 Studies examining the effect of recombination between adaptation and/or reproductive isolation components on the course of divergence.

Study	Geography	Fitness or reproductive isolation components	Effect of free recombination
Barton and Turelli 1991, Kirkpatrick 1982, Lande 1981	Sympatry	Female preference and sexual male trait	Approach to equilibrium can be retarded.
Barton and Bengtsson 1986	Hybrid zone	Viability selection	Reduces the strength of the barrier to gene flow
Felsenstein 1981, Barton and Hewitt 1985	Sympatry, hybrid zone	Premating and postmating	Stable polymorphism, divergence less likely
Gregorius and Steiner 2001	Not specified	Viability selection and mating	Modifier locus of mating does not increase in frequency, and divergence does not occur
Hostert 1997	Laboratory sympatry	Premating and postmating	No reinforcement
Kirkpatrick and Barton 1997	Sympatry	Coadapted traits	Decreases indirect selection
Li et al. 1997	Laboratory hybridization	Fitness	Destruction of supergenes
Liou and Price 1994	Hybrid zone	Female preference and sexual male trait	Decreases divergence when there is significant hybrid fitness
Sanderson 1989	Cline	Fitness component and fitness modifier	Modifier that is favored in both races is hindered by recombination
Servedio 2000	Hybrid zone	Female preference and sexual male trait	Reinforcement less likely in two-island model
Trickett and Butlin 1994	Sympatry	Premating and postmating, female preference and sexual male trait	Divergence less likely

In this paper, we classify the mechanisms that create genetic correlations among loci as structural and population-based mechanisms (table 2.2). In the presence of a structural mechanism, recombination is reduced due to genome organization. Structural mechanisms may involve a single gene that has pleiotropic effects on the phenotype, or more than one locus contributing to a phenotype. Population-based mechanisms include sexual selection, genetic drift, or recent admixture. Below, we discuss many of these mechanisms with respect to speciation, with emphasis on structural mechanisms and discuss some recent studies that exemplify the interplay between recombination and components of reproductive isolation.

Table 2.2 Some means by which genetic correlations can arise.

1. Structural
A. Linkage due to pleiotropy
b. Linkage due to proximity
c. Linkage due to chromosomal rearrangements
i. Additive model
ii. Negative epistatic model
2. Populational
a. Sexual selection
b. Genetic drift
3. Allopatry

Genetic Associations due to Allopatry

Although this mode of speciation does not involve gene flow, allopatry is the simplest means by which nonrandom associations among alleles may appear. After populations cease to exchange migrants (see figure 2.2), new gene variants are fully restricted from recombining between populations. Ignoring segregating ancestral polymorphisms, loci in genetic equilibrium within populations would be initially in complete disequilibrium between populations if they were to come into contact again. Subsequently, recombination would be restored and genetic associations would dissipate with time. Below, we discuss the means by which genetic associations may persist in the presence of gene flow.

Pleiotropy or Linkage due to Proximity

Speciation is facilitated when loci conferring traits undergoing disruptive selection, such as by specialization to different hosts, are physically linked to or identical to loci conferring mating discrimination (e.g., Felsenstein 1981; Rice and Salt 1990). Because recombination would be suppressed between these loci or effects, the genetic correlation among alleles of particular types would persist

longer in the face of hybridization. In the course of habitat specialization, habitat preference should become associated with habitat performance, as organisms should tend to choose habitats in which they will have the highest fitness (e.g., Berlocher and Feder 2002; Diehl and Bush 1989; Thompson 1994; Via 1990). In addition, as offspring develop on the hosts their parents preferred, they would undoubtedly be subjected to selection pressures of performance on that host, and host performance and preference will become even more associated. Sympatric speciation may then accompany this habitat divergence if mating only occurs in the preferred habitat, hence associating mate preference with habitat preference. This linkage of habitat performance, habitat preference, and assortative mating may contribute to the frequent proposed sympatric divergence of phytophagous insects.

To observe the predicted pattern of linkage associated with sympatric divergence, there must be genotype-based habitat preference differences within species or between diverging species (e.g., Taylor and Powell 1978), and this preference should be associated with host performance. For example, patterns consistent with genetic linkage of host performance and assortative mating have been observed in closely related pea aphids that specialize on different hosts (Hawthorne and Via 2001). In these aphids, host performance is positively correlated with contypic mate choice, and discriminant individuals typically exhibit poor fitness on hosts used by the other type. Interestingly, quantitative trait loci for host performance and assortative mating (through habitat choice) map to the same genomic regions, suggesting, albeit not proving, physical linkage between loci. This genetic architecture would potentially increase individual fitness and would tend to spread easily in the population, perhaps completing the speciation process. Hawthorne and Via (2001) suggest that this type of genetic architecture may be common in taxa that have speciated under divergent natural selection, as is speculated for other phytophagous insect species (Singer 1986)

In a variety of other organisms, however, the genetic relationships between host performance and preference are not as clear as suggested by Hawthorne and Via (2001). In some cases, genes affecting, for example, larval performance on hosts are different from those affecting adult oviposition preferences. This lack of association has been observed in brown plant hoppers (Sezer and Butlin 1998) and swallowtail (Hagen and Scriber 1995; Thompson et al. 1990): in the latter case, genes affecting oviposition preference but not those for host-related performance map to the sex chromosome.

Similarly, the relationship between host performance and preference is absent when studying an adaptive trait in the species *Drosophila simulans* and *D. sechellia*. These species are largely allopatric, though they now co-exist on one island of the Seychelles, likely due to a recent secondary contact. *D. Sechellia* has specialized on and prefers to oviposit on the fruit of *Morinda citrifolia*. This fruit is toxic to *D. Simulans* larvae, and *D. Simulans* females actively avoid ovipositing on it. In contrast to the situation above, the limited genetic data available suggest that

the oviposition preference for and adaptation to morinda fruit are not linked in *D. Sechellia*: the region of the genome with the strongest effect on oviposition preference is on the 2nd chromosome (Higa and Fuyama 1993), while this chromosome has only a very weak effect on resistance (Jones 1998). As these species probably have come into contact only recently, no linkage is expected under the model above, and the data provide a good contrast to the results from studies of sympatric pea aphids.

In summary, we can conclude that the evolutionary relationship between host performance and preference is somewhat unclear, but the little genetic evidence for its existence suggests that it is sometimes associated with a genetic architecture that favors divergence in sympatry. This genome architecture is characterized either by pleiotropy or close linkage between genes for host performance and preference. Hence, recombination does not impede divergence.

Linkage due to Chromosomal Rearrangements

Chromosomal rearrangements, such as chromosomal inversions or translocations, may allow factors conferring adaptation or reproductive isolation to be genetically correlated when not physically proximate along chromosome arms. The effect of such rearrangements would be analogous to physical linkage or pleiotropy, as offspring of heterozygotes would possess the entire rearranged region from one or the other taxon. If these rearranged regions bear alleles under divergent selection or conferring reproductive isolation, rearrangements could potentially suppress the opposing effect of recombination to sympatric speciation or reinforcement described in the theoretical models referenced in table 2.1. One might predict that, in general, most homosequential regions of the genome would tend to introgress more easily between hybridizing species than rearranged regions because of incomplete linkage to alleles conferring adaptation, mating discrimination, or hybrid dysfunction. Rearranged regions may sometimes be completely linked to such alleles, and introgression will be more limited.

These expectations have been demonstrated in empirical studies. For example, rates of gene flow are higher between homosequential than between rearranged chromosomes in sunflower hybrid zones (Rieseberg 1999). Rieseberg et al. (1999) examined three hybrid zones of two sunflower species inhabiting Nebraska. Assuming that homosequential regions of the genome would introgress between species, Rieseberg et al. (1999) were able to estimate the deviation from expected numbers of introgressed markers occurring in these hybrid populations. Remarkably, they found that most markers in homosequential regions of the genome tended to be observed at neutral expected frequencies, whereas markers from regions bearing inversion or translocation differences between the species were almost always underrepresented in the hybrid zone. These rearranged segments were also commonly associated with pollen sterility.

Similar results were obtained from genetic studies of hybrid sterility, sexual isolation, and other barriers to gene exchange between the hybridizing species *Drosophila pseudoobscura* and *D. persimilis*: all effects mapped primarily or exclusively to regions bearing fixed inversion differences between the species (Noor et al. 2001b; Noor et al. 2001c). Sequence analyses also suggest that these inverted regions do not introgress between these two species as well as homosequential regions (Machado et al. 2002; Wang et al. 1997). These results suggest that (1) gene pools are semi-permeable, (2) chromosomal rearrangements may prevent gene flow across large genomic regions, and (3) chromosomal rearrangements may play an important role in the genetic isolation of species in the presence of hybridization.

Two recent non-mutually-exclusive explanations have been proposed whereby chromosomal rearrangements, such as inversions, may facilitate the persistence of hybridizing species, hence explaining the results described above. We call these the "additive model" and the "negative epistatic model" for simplicity. Each is described in turn.

Additive Model

The additive model (Rieseberg 2001) suggests that chromosomal rearrangements prevent gene flow between hybridizing species by summing the effects of genes conferring adaptation or hybrid dysfunction across large regions of the genome. Similarly, multiple adaptive or isolating alleles within inversions would reduce further the possibility of gene flow for all loci in the inverted regions. Recombination is effectively suppressed across rearranged regions in heterozygotes (hybrids), the fitness effects of the alleles at the individual loci are summed, and the entire region behaves as a single allele possibly under very strong selection. Hence, when hybridizing taxa differ in gene arrangement, and the rearranged regions contain multiple genes conferring adaptation or reproductive isolation, then gene flow can be substantially reduced or prevented across a large fraction of the genome.

Negative Epistatic Model

Noor et al. (2001c) have forwarded another explanation for why chromosomal inversions may aid the speciation process. This model focuses on hybrid dysfunctions in particular, and it rests on two additional assumptions, each of which has been supported through empirical data. First, the genetic incompatibilities that produce hybrid sterility are typically asymmetric: an allele from species a will produce hybrid sterility in the genetic background of species b, but the alternate allele at the same locus from species b will not necessarily produce hybrid sterility in the genetic background of species a (see Johnson 2000). This assumption may be supported by the work on *Odysseus*, which produces sterility when introgressed from *Drosophila mauritiana* into *Drosophila simulans* (Perez et al. 1993), but several introgressions in the other direction are fertile (Palopoli and

Wu 1994). It is also supported by the frequent observation of f_1 hybrid male sterility in one direction of hybridization but not in the opposite. Second, the model assumes that many loci possess alleles that can confer hybrid male sterility, consistent with high-resolution genetic data from the *D. simulans* - *D. mauritiana* group (Wu and Hollocher 1998).

Noor et al. (2001b) suggested that large chromosomal rearrangements produce a symmetric hybrid male sterility effect from asymmetric genetic incompatibilities. After hybridization, recombination can eventually tie together alleles from the two species not conferring hybrid sterility onto the same chromosome in homosequential regions. This recombinant chromosome will be fully fertile, and introgression can occur. In contrast, when hybrids inherit entire rearranged regions from one species or the other, either of these regions will often possess alleles that confer sterility in the foreign genetic background. Because recombination does not occur, each arrangement will continue to be associated with hybrid male sterility in the foreign genetic background in succeeding generations. In classical genetics terms, the alleles from each species that do not cause sterility are trapped in repulsion phase and cannot come together into coupling phase. Hence, reduced recombination via rearrangements prevents introgression of these regions into the foreign species. This process therefore allows the hybridizing species to persist, as complete fusion cannot occur.

This model works best if hybrid sterility is caused by negative epistatic interactions between loci on two or more chromosomes rearranged between the hybridizing taxa. For example, in *D. pseudoobscura* and *D. persimilis*, the strongest negative interactions that cause sterility occur between loci on the inverted XL and the inverted second chromosomes. Had only one of these chromosomes been inverted relative to the other species, then the loci with which the remaining inverted region interacted may have recombined to eliminate the sterility phenotype, and fusion would still occur.

The Strength of Barriers to Gene Flow

Now let us suppose recombination is restored after secondary contact. During the initial formation of the hybrid zone, recombination will be restored varying with the number of genes that contribute to fitness reduction in hybrids. Genes may contribute to fitness reduction because they are incompatible in heterospecific genetic backgrounds or because they have undergone adaptive divergence in the previously isolated populations. The rate at which neutral markers will introgress into a heterospecific background is a function of the recombination rate in the chromosomal region where this marker resides, the deleterious effects to which neutral markers are linked, and their distance along the chromosome from the selected genes (Barton and Bengtsson 1986). This can be mathematically expressed as $I_{10} = v_i \alpha r$, where I is a matrix representing the number of genes that will go from population 1 into population 0 (effective migration rate); v_i

is the number of neutral markers in a chromosome carrying *I* deleterious effects, and αr is the probability that the neutral marker will recombine away from the deleterious effects. This computation is done over all possible hybrid backgrounds and is subsequently used to generate a mathematical recursion describing the barrier strength to gene flow generated by n selected loci.

Barton and Bengtsson's (1986) general results are consistent with the verbal arguments presented in this paper and add to our understanding of the process of speciation in several ways. First, their results demonstrate that the relationship between recombination and selection intensity greatly affect the maintenance and strength of barriers to gene flow. Second, recombination will be prevented across much of the genome if multiple genes contribute to hybrid fitness reduction, so neutral markers are more likely to be linked to barriers to gene flow. As a consequence, early speciation events are characterized by differential introgression between hybridizing populations. Finally, we can infer from the results of Barton and Bengtsson (1986) that chromosomal rearrangement bearing genes reducing hybrid fitness are likely to present a powerful barrier to gene flow since any neutral marker contained within the rearrangement will be less likely to recombine away from alleles conferring deleterious effects.

Dynamic Mechanisms

Correlations evolve between preferences and fitness traits or between preferences and preferred characters in various sexual selection models. These correlations can sometimes be favored by reduced recombination among traits. The effect of recombination has been studied primarily in the context of runaway sexual selection. Runaway sexual selection results in the joint evolution of a preference and a preferred trait in the absence of direct viability or fertility selection on the preference locus (Fisher 1958; Kirkpatrick 1982; Lande 1981). Lande (1981) showed that female preferences evolve as a correlated response to selection on males. This process occurs through a genetic correlation between the loci independent of their linkage relationships, and this disequilibrium is maintained by selection. However, limited recombination can accelerate the approach to equilibrium in some sexual selection models, though it may not affect the final equilibrium condition (Barton and Turelli 1991; Kirkpatrick 1982). Similarly, one study noted that having a lower recombination rate between female preference genes and male trait genes can enhance the effectiveness of runaway sexual selection (Otto 1991). This is especially true if a rare allele affecting female preferences arises within a population in which a male trait is maintained as an overdominant polymorphism. The rare preference allele will easily spread in the population by association with heterozygotes at the male trait locus if recombination is low between the loci (Otto 1991). Reducing recombination between coadapted fitness alleles may also increase the force of indirect selection on female preference (Kirkpatrick and Barton 1997), and decreased recombination between male trait and female preference loci may increase the likelihood of

speciation by runaway sexual selection (Takimoto et al. 2000; Trickett and Butlin 1994).

Linkage or associations of loci contributing to preferences and different fitness components may also have an impact on the dynamics of "good genes" sexual selection. A variety of sexual selection studies have suggested that offspring of attractive males have high fitness through enhanced growth, fecundity, viability, or attractiveness (Hamilton and Zuk 1982; Kotiaho et al. 2001; Roulin et al. 2000; Welch et al. 1998). In these situations, preferred male characters are positively correlated with fitness, and the associations among these loci are generally thought to enhance the progress of sexual divergence and speciation. As with runaway sexual selection, recombination can retard the evolution of this correlation, so if these characters are initially positively associated, as by linkage, sexual selection will be more efficient.

However, recombination does not necessarily retard the progress of sexual selection. The generally positive effect of reduced recombination on sexual selection may be more applicable to the allo-sympatric scenario depicted in figure 2.2 than sympatric divergence. In the allo-sympatric case, the initial disequilibrium between alleles that must remain together will generally be positive, thus facilitating persistence in the face of gene flow (e.g., Kirkpatrick and Ravigné 2002). However, when divergence begins in sympatry, the initial disequilibrium between alleles that must spread together may be positive or negative. If these alleles are in repulsion phase, then reduced recombination may initially impede or prevent the progress of sexual selection.

Also, in contrast to many sexual selection studies, (Brooks 2000) found a *negative* correlation between male attractiveness and offspring survival to maturity in guppies. Brooks noted that genes for ornamentation have been mapped to the nonrecombining y-chromosome of guppies, which would place them in tight linkage with several genes that affect fitness. Sexual selection may be efficient at spreading y-linked preferred male characters because all male offspring inherit the preferred trait. However, because much of the y-chromosome is nonrecombining, deleterious y-linked alleles cannot be shed by recombination, so deleterious alleles can accumulate at other loci and hitchhike via this sexual selection. Hence, in these guppies, the benefit of mating with attractive males is opposed by reduced offspring survival following such matings and sexual selection is impeded by the absence of recombination. This circumstance may be fairly unusual for most sexual species, though, as it relies on a large, almost-completely-nonrecombining region bearing the loci that confer preferred male characters.

Effects of Recombination on Evolutionary Studies

Speciation is the process by which recombination between genomes of subpopulations is minimized through time due to strict allopatry, accumulation of

genomic incompatibilities, or adaptive divergence. It is generally accepted to have occurred when two gene pools can come into contact and yet remain distinct from each other. This process of divergence is sometimes, but not always, gradual, and it may involve phases in which introgression occurs in some parts of the genome between the divergent populations. The porosity of this process has important implications for our understanding of modes of speciation because the unit of study becomes those portions of the genome that fail to recombine between diverging taxa.

Similar ideas have been put forward through the years (Barton and Hewitt 1985; Carson 1975; Harrison and Bogdanowicz 1997), albeit without the genetic data now available. For example, Carson (1975) suggested that the diploid chromosomal system provides a "field for genetic recombination" where only a portion of the field is amenable to exchange between species. The remaining chromosomal fraction would consist of balanced blocks of genes under strong natural selection precluded from recombining with another species since unfit offspring would be produced. Thus, in hybridizing species, introgression would occur only outside the balanced blocks. Although Carson's concept is consistent with our description of the events immediately following hybridization, it has conceptual differences from our suggestion of how species originate. While we suggest that recombination is reduced through time by accumulation of genomic incompatibilities between hybridizing species or adaptive divergence, Carson implies that the reduction in recombination is a fixed measure that defines the species itself. Under Carson's analogy the reduction in recombination is never completed and gene flow eventually obliterates incipient species. As a result, Carson suggests species are likely to originate through a genomic disorganization mediated by bottlenecks that shift the gene pool from one coadapted block of genes to another block, and he concludes that sympatric speciation and reinforcement are not likely to occur. Our model does not make such assumptions and is consistent with various modes of speciation with gene flow.

In retrospect, the extent of introgression between species will greatly depend on recombination rates as well as the genetic architecture of the adaptations, mate choice, or incompatibilities that have accumulated and differentiate the taxa. Incompatibilities may be distributed across large fractions of the genome or may be concentrated in few regions. Their effects may be extended or localized based on the organization of the genome in which they are located. Additionally, genetic incompatibilities may hitchhike with other alleles contributing to adaptation or mate choice if they are closely linked, are included in chromosomal rearrangements together, or co-segregate together more often than expected due to coevolution.

As described above, several studies have found that traits involved in adaptive or reproductively isolating differences between diverging taxa map preferentially to regions of the genome with reduced recombination (Feder 1998;

Hawthorne and Via 2001; Noor et al. 2001c; Rieseberg et al. 1999). Some of this tendency may come from intrinsic biases in the way genetic mapping studies are performed (Noor et al. 2001a), but the weight of evidence suggests that such regions may truly harbor a disproportionate number of such alleles. An interesting avenue of research would be to experimentally induce chromosomal rearrangements in two homosequential species, allow them to hybridize in the laboratory for many generations, and see if this tendency can be reproduced experimentally.

Recombination and Phylogenies of Closely Related Species

Varying rates of introgression between diverging taxa can greatly complicate phylogenetic analyses. This complication reduces (or eliminates) fixed genomic DNA sequence differences between hybridizing taxa, or it may cause sequences from some regions of the genome to suggest one phylogenetic relationship while others suggest different relationships. Recombination is intrinsically tied to these complications, as regions where recombination is effectively eliminated will yield similar phylogenies when studied. For example, if loci that cannot introgress (due to adaptation or reproductive isolation) are within regions inverted between hybridizing taxa, the lack of introgression of these loci will be extended to all other genes in this inverted region.

An empirical example of this suggestion comes from research on the *Drosophila pseudoobscura* group, comprised of the two subspecies *D. p. bogotana* (bog) and *D. p. pseudoobscura* (ps) and the sibling species *D. persimilis* (per). Bog and ps are estimated to have diverged approximately, 150,000 years ago (Schaeffer and Miller 1991), while ps/bog and per diverged approximately 500,000 years ago (Aquadro et al. 1991; Wang et al. 1997). Ps and per co-occur and hybridize in nature (Dobzhansky 1973; Powell 1983), while bog is allopatric to the other two taxa. All components of reproductive isolation map primarily or exclusively to the fixed inversion differences on the X and 2nd chromosomes (Noor et al. 2001b; Noor et al. 2001c).

Recently, Machado et al. (2002) sequenced several loci of these three taxa and constructed phylogenies based on these sequences. Sequences of loci within the fixed inversion differences clearly distinguished ps/bog from per, as predicted from numerous other characters, while loci across most of the remainder of the genome yielded poor phylogenetic resolution. Interestingly, when mitochondrial DNA sequences were examined, ps and per appeared to be much more closely related to each other than either was to bog. As the mitochondrion is not associated with any known barriers to gene exchange in these species (Hutter and Rand 1995; Noor 1997), it has freely introgressed between ps and per (powell, 1983), while other parts of the genome, and especially those within fixed inversion differences, have not. Hence, sequences from the different parts of the genome suggested dramatically different phylogenetic relationships among these taxa.

However, we need to be cautious when inferring phylogenetic relationships based on genes that putatively cannot introgress between species. Factors conferring hybrid sterility today may have evolved their sterility effect subsequent to the speciation process and may have introgressed between species earlier in evolutionary divergence. Thus, present-day incompatibilities may not necessarily reflect the speciation history of these taxa.

One possible such misinterpretation comes from a phylogenetic study of the *Odysseus* gene, which confers sterility in hybrids of D*rosophila simulans* (sim) and *D. mauritiana* (mau). Early phylogenetic studies of these two species and their sister species, *D. sechellia* (sec), had yielded numerous potential relationships (see Kliman et al. 2000), many of which conflict with each other. Ting et al. (2000)studied this triad using the *Odysseus* gene sequence, suggesting that it should not introgress between sim and mau, and should therefore present a more accurate representation of species relationships. Gene flow between sim and mau (e.g., Ballard 2000) may have contributed to the complications in the earlier phylogenetic studies.

Ting et al. (2000) found many shared-derived sites (synapomorphies) in *Odysseus* sequences that would cluster sim with mau more than either to sec, and they argue that this phylogeny more likely represents the species phylogeny since *Odysseus* cannot introgress between sim and mau. However, they fail to consider three aspects of this argument. First, if *Odysseus* confers sterility only between sim and mau, why were synapomorphies not noted in other genes? If gene flow is occurring at much of the remainder of the genome between sim and mau, and sim and mau are the in-group species relative to sec, then sim and mau should cluster even more tightly when introgressing genes are studied than when *Odysseus* is examined. Their argument would have applied only if sim and sec clustered together, as was originally suspected (Palopoli et al. 1996). Second, *Odysseus* confers sterility in one genetic background: there is no consistent fitness consequence for introgression of the *D. simulans* allele into *D. mauritiana* (Palopoli and Wu 1994), which is what is assumed to have occurred with the mitochondrial DNA sequences of these species (Ballard 2000). Hence, unidirectional gene flow may have been possible at *Odysseus* in the recent past, and their study is not necessarily more conclusive than studies of the many other loci that also presently show fixed differences between these species (see Kliman et al. 2000). Finally, as described above, we do not know when *Odysseus* acquired its hybrid sterility effect. An estimated, 120 genes may contribute to sterility in these species (Wu and Hollocher 1998), and *Odysseus* may have been among the last to evolve its effect on hybrid fertility. The phylogenetic relationship based on *Odysseus* gene sequences is not compelling.

Recombination and Species Concepts

One question that remains unresolved by a focus on recombination is the question of how to identify species. If species are evolutionary entities within which

gene exchange occurs and between which gene exchange does not occur, then most speciation genetic studies are necessarily addressing properties of partial species. Thus, for example, *D. pseudoobscura* and *D. persimilis* are only partially reproductively isolated and have been exchanging genes in nature. This partial isolation permits genetic research, but the subjects of that research are, in a critical genetic sense, not complete species. Two sorts of conceptual entanglements are associated with this uncertainty. First, since we cannot easily do genetics on completely isolated taxa, we cannot fully address whether the incomplete stages that we study are representative of early stages of speciation. Second, the presence of natural gene flow between purported "species" creates a context that is without the reproductive isolation that inspires such investigation.

The path through this particular species muddle is to see that, throughout this paper and others like it, the critical focus is not on the distinction between two species, but rather the presence of barriers to recombination. It is the origin of such barriers that permits diversity to accrue between entities. We may think of those entities as species, but it is even more useful to think of them as gene complexes (Mallet 1995). When the subject of investigation is envisioned as recombination, per se, then questions regarding the degree of distinction between entities that might engage in recombination fall by the wayside.

Consider Dobzhansky's case for the concept of a mendelian population: a reproductive community that shares a common gene pool (Dobzhansky 1951). Mendelian populations need not be completely distinct, and indeed have no particular necessity for distinction, and they can be nested within one another. Dobzhansky devised the idea to help biologists think more about the factors that affect gene movement, and less about whether or not particular populations warrant some systematic status. If we adopt this viewpoint, and consider the degree and circumstances of recombination to be the focus of inquiry, then we can study the origins of biological diversity without regard to question, about whether or not the organisms we study belong to one or two species.

Conclusions

Recombination can retard species formation or persistence in numerous ways that researchers are only now beginning to understand. Although theoretical studies have varied recombination rate to investigate its effects, empiricists are just beginning to examine its role in speciation and species persistence in natural systems. Its effect appears nontrivial, and its implications span many evolutionary issues, such as the genetics of sexual isolation, phylogenetics, and the nature of species. Indeed, when reduced recombination is considered, previously controversial modes of speciation such as sympatric speciation and reinforcement become more plausible. Future speciation studies should consider recombination as a fundamental variable in the process and how it can impact their findings.

CHAPTER III

A RECOMBINATIONAL PORTRAIT OF THE *D. PSEUDOOBSCURA* GENOME

The fruit fly *Drosophila pseudoobscura* is one of the best model systems for studies of adaptation and speciation. This species is perhaps best known for its rich inversion polymorphism on the third chromosome. Third chromosome arrangements bear striking longitudinal clines across the species range and are usually associated to life history traits like fecundity (Anderson et al. 1991). Cline frequencies are maintained despite apparently extensive gene flow among populations (Noor et al. 2000; Prakash et al. 1969; Schaeffer and Miller 1992) suggesting that natural selection operates on these inversions. This species has also been studied extensively with respect to the genetic basis of traits associated with reproductive isolation, such as hybrid sterility and sexual isolation (Dobzhansky 1934; Noor et al. 2001b; Noor et al. 2001c; Orr 1987). For example, known traits contributing to the reproductive isolation between *D. pseudoobscura* and its sibling species *D. persimilis*, map primarily or exclusively to fixed inverted regions of their genomes (Noor et al. 2001b; Noor et al. 2001c). Their genetic identity is maintained via recombination suppression in these regions, which effectively lock the species into always producing sterile offspring.

Much of the progress in studying adaptation and speciation in *D. pseudoobscura* came from genetic mapping studies that employed either morphological mutant markers or a moderate number of microsatellite markers. Recently, the genome sequence of *D. pseudoobscura* has become available (Richards et al. 2005), and this provides an opportunity for developing a higher resolution molecular linkage map of the species. A high-resolution linkage map using variable molecular markers combined with the genome sequence can dramatically facilitate a variety of genetic and evolutionary efforts, such as high-resolution QTL mapping, and comparisons of regional recombination rate and variation.

Several early recombination maps are available for *D. pseudoobscura*. For example, Anderson (1993) provided a linkage map based on 63 morphological markers and allozymes across the genome, Orr (1995) identified linkage relationships for 23 markers on the X chromosome, and Noor et al. (2000) created a molecular map of the genome using 24 microsatellite markers. Although these maps have advanced our knowledge of the adaptive history and speciation in *D. pseudoobscura*, new genetic maps incorporating molecular markers can facilitate high-resolution genome scans to compare with the physical sequence map, hence allowing mapping of phenotype to single genes. Here, we provide a dense recombination map and information on regional rates of recombination for four chromosome arms of *D. pseudoobscura*. We also present preliminary information for recombination rates across the second chromosome of *D. persimilis* and compare them to *D. pseudoobscura*. Finally, we present primer sequences for ~150 microsatellite markers, of which ~ 70 have been shown to be variable within *D. pseudoobscura*, and ~40 between species. Our map, albeit not final, in conjunction with the recently sequenced genome of *D. pseudoobscura*, will facilitate population genomic analysis and will take us one step closer to the

identification of genes involved in adaptation and speciation and understanding the evolutionary forces acting on them.

Materials and Methods

Fly Rearing and Lines

Lines of *Drosophila pseudoobscura* were established from individuals collected in Mather, CA, in 1997 and Flagstaff, AZ, in 1993, both of which bear the "arrowhead" arrangement on the third chromosome. The *D. persimilis* lines were established from flies collected in Mt. St. Helena, ca, in 1993 (line MSH1993) and 1997 (line MSH3). All lines were maintained under a constant temperature (20 °c) and humidity (85%) regime in diurnal/nocturnal cycles of 12h and reared on a mixture of agar, dextrose, and yeast.

Recombination Maps

Pure Species Maps

Recombination maps were obtained by estimating recombination fractions in f_2 backcross populations between species-specific isofemale lines. Each parental line was tested for allelic differences in microsatellite loci previously published (Noor et al. 2000) or extracted from the *D. pseudoobscura* genome sequence (Richards et al. 2005), and scored for 70 microsatellite markers. The *D. pseudoobscura* map was made by backcrossing f_1 females of the cross between two lines to the Flagstaff 1993 line (see Ortiz-Barrientos et al. 2004). The *D. persimilis* map was made by backcrossing f_1 females of the cross between two lines to the MSH1993 strain. We used polytene chromosome preparations of f_1 progeny to confirm these strains also bear the same third chromosome arrangement. For each species, approximately 275 flies were genotyped for such microsatellites (see appendix C) and a multipoint-linkage approach, as implemented in Mapmaker version 3.0 (Lander et al. 1987), was used to generate recombination maps for chromosomes X, 2 and 4 from *D. pseudoobscura*, and the second chromosome of *D. persimilis*. We did not investigate the third chromosome despite its complete assembly because analyses of it would be complicated by the inversion polymorphisms within these species. Recombination distances are reported in Kosambi centimorgans, and recombination rates in Kb/cM.

Backcross Hybrids Maps

Recombination maps from two backcrosses between *D. pseudoobscura* and *D. persimilis* were also analyzed. These backcrosses have been previously described by (Noor et al. 2001b) and correspond to a f2 backcross to *D. persimilis* and a f2 backcross to *D. pseudoobscura*. Recombination maps and units were estimated and reported as above.

Physical Map Information

The recently published and assembled *D. pseudoobscura* genome sequence was used to anchor our recombination map to the physical map. The genome comes in groups of assembled contig whose links have been obtained computationally, cytologically, or by using known recombination distances between markers in such groups. For detailed information see Richards et al. (2005). We use our recombination data to predict linkage between contig groups and to infer gap sizes between them. Our estimates are based on average recombination rates obtained by dividing the genetic size of a linkage group by its physical size.
Polymorphism data

Published DNA sequence data from *D. pseudoobscura* were obtained from the literature (Hamblin and Aquadro 1999; Machado et al. 2002). Nucleotide polymorphism data from noncoding regions were used to test whether DNA sequence polymorphism, GC content and/ or codon usage positively correlated with recombination rates.

Results

D. pseudoobscura Assembly of Contig Groups

We used our recombination map to link groups of contigs from the incomplete assembly of the *D. pseudoobscura* X and fourth chromosomes (see appendices a-c). Richards et al. (2005) have already connected groups from the second chromosome and our linkage relationships generally support their assemblage. Below, we describe major findings regarding the sequence assembly provided by our recombination map.

The left arm of the X chromosome (XL) is composed of several contig groups. We were able to link group XL1a to group XL1e. These major groups appear to link to two transitional small groups that link XL to the right arm of the X chromosome (XR). Group XL3a and group XL6 link to groups XL1e and XR6, respectively. This group from XR is linked to other major groups XR9-XR8, that finally link to group XR3a. These groups provide an uninterrupted linkage group for the whole X chromosome anchored to a great fraction of the physical map for the chromosome (see appendix a), though some smaller groups may have been missed. The estimated total size of the contig groups for the X chromosome is 52mb. However, the estimated size of the X chromosome map based on inferred number of base pairs separating markers is ~42.5mb. We have also linked two groups from the fourth chromosome, group 1 and group 5, via markers DPS4032 and DPS4034, respectively. These two groups span ~7mb and there is an estimated gap of ~2mb between DPS4032 and DPS4034. The estimated size of the contig groups for the fourth chromosome is ~7.5mb, while the inferred gap-based map is ~9.6mb.

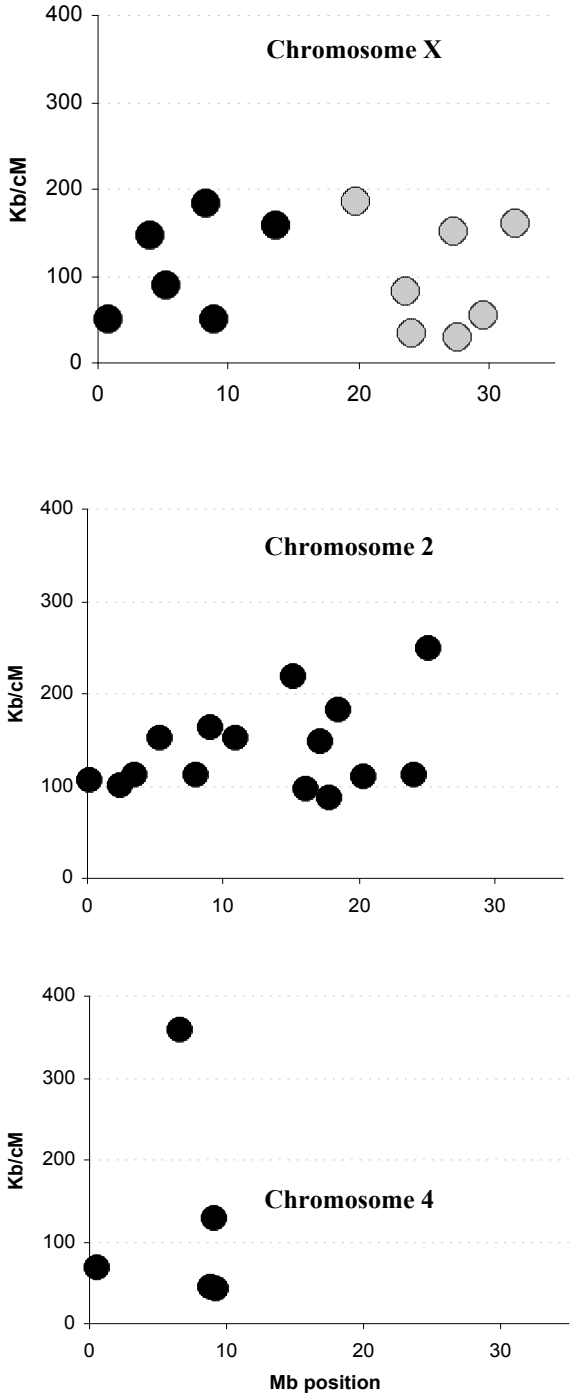

Figure 3.1 Distribution of recombination rates (Kb/cM) over physical chromosomal locations (Megabases) of *D. pseudoobscura.*

We have found several discrepancies between our recombination links between markers and their locations according to DNA sequence assembly. On the second chromosome, our linkage analyses suggested that DPS2014 and

DPS2027 would occur in the opposite order of the DNA sequence assembly (see appendix A). Mapmaker also inferred the opposite order for markers DPSX037n and DPSX021 on the XR chromosome arm. We also forced the assembly-based order into the linkage analyses and analyzed accordingly on both the X and second chromosomes.

In addition to differences between the assembly and the recombination maps, we found that some published *in situ* hybridization data does not match our findings or the physical assembly. Based on our map assembly, gene Hsp82 (group XR6, base 4,030,963) is closer to the centromere than marker DPSX009 (group XR6, base 4,925,036). This order does not match with the order reported by Machado et al. (2002), but it agrees with Kovacevic and Schaeffer's (2000) inferred order. Similarly, marker DPS2002, a marker assumed to be inside a region on the second chromosome inverted between *D. pseudoobscura* and *D. persimilis* (Machado et al. 2002), appears to be outside the inversion on the telomeric side (M.A.F. Noor, personal communication).

Recombination Portrait

D. pseudoobscura

We estimated recombination rates for chromosomes x, 2 and 4 of *D. pseudoobscura* (see appendices c-e). Figure 3.1 shows the distribution of Kb/cM at various positions along these chromosomes. Recombination rates average 135 Kb/cM across the three chromosomes and do not vary dramatically within each chromosome. Mean recombination rates for intervals we examined across all chromosome arms are XR: 113.7 Kb/cM, XL: 100.2 Kb/cM, 2^{nd}: 153.04 Kb/cM, 4^{th}: 143.93 Kb/cM and are not significantly different from each other (Wilcoxon signed rank test, $p>0.1$). Despite the general uniformity of recombination rates across the genome, we found a few regions with notably different recombination rates than the rest of the genome: the fourth chromosome, assuming the sequence assembly is correct, contains a region of comparatively low recombination rate (~360 Kb/cM), and the X and fourth chromosomes bear a couple of regions with very high recombination rates (~50Kb/cM). As expected, genetic distance correlates with physical distance (see figure 3.4). The second chromosome is the largest assemblage with ~200cm spanning 29mb (see appendix A). Both the X and fourth chromosomes are not fully assembled, and thus, our absolute estimates are preliminary.

Comparative Portraits

We also compared the recombination landscape of the fully assembled second chromosome between *D. pseudoobscura*, *D. persimilis*, and hybrid f2 backcrosses between the two species. We focus this comparative analyses on just the markers or regions surveyed in both species, so the higher density of markers we studied in *D. pseudoobscura* does not artifactually inflate its apparent recombination rate. The average recombination rate of the second chromosome is

significantly higher in *D. pseudoobscura* than *D. persimilis* (Wilcoxon signed rank test, p=0.043). Hybrid recombination rates are similar to each other irrespective of the direction of the backcross (Wilcoxon signed rank test, p=0.686). Hybrid recombination portraits also reflect the presence of a region with dramatically low recombination rate. This region is known to encompass a fixed inverted segment between *D. pseudoobscura* and *D. persimilis* (Tan 1935). When this region is excluded (region contained between markers DPS2001 and *bcd*) from analysis, recombination rates in hybrids are significantly higher than in either parental (Wilcoxon signed rank test p=0.0165 for *D. pseudoobscura* and p=0.0171 for *D. persimilis*).

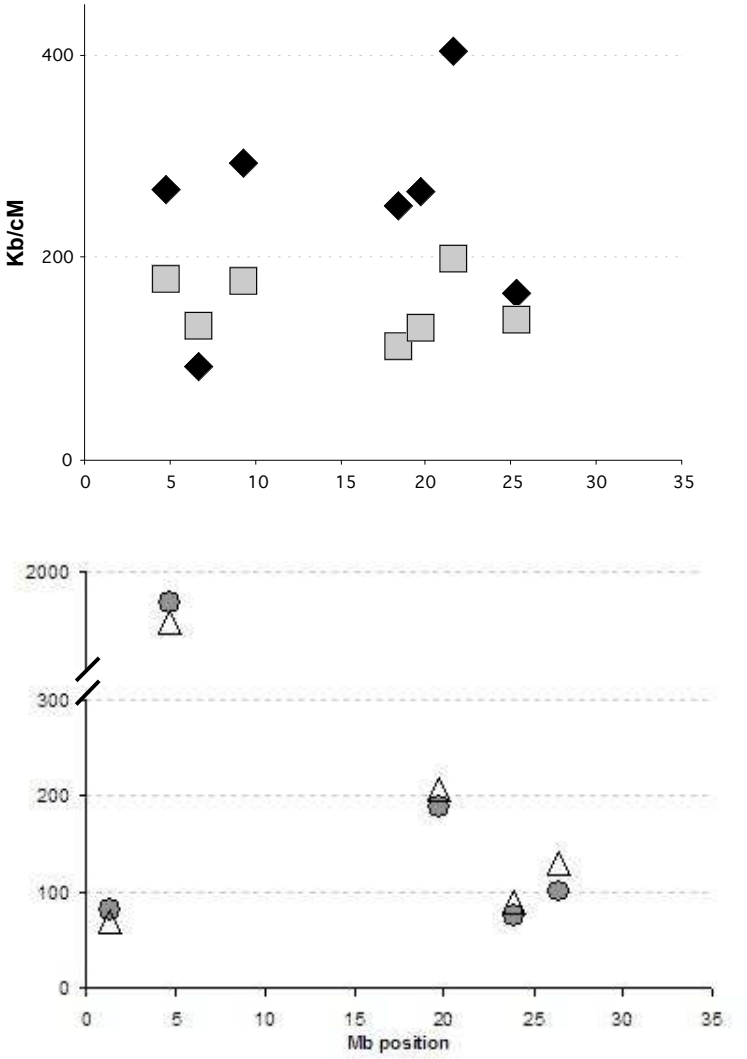

Figure 3.2 Comparative recombination portrait of the (a) *D. pseudoobscura* (gray squares), *D. persimilis* (black diamonds), and their hybrids (b), second chromosome. Hybrid maps were derived from f2 backcrosses to either *D. persimilis* (triangles) or *D. pseudoobscura* (circles).

Discussion

We have built a molecular marker-based recombination map of *Drosophila pseudoobscura* and described recombination rate variation across the genome. We relate our recombination map to the published genome sequence assembly, and address some inconsistencies among earlier maps. For example, the reported genetic size of some of the *D. pseudoobscura* chromosome arms varied among previous studies. Anderson (1993) reported the genetic size of the second chromosome to be 101 cM, and Hamblin and Aquadro (1999) similarly reported its size as 128 cM. In contrast, Noor et al (2000) reported a genetic size of ~204 cM for the same chromosome. Our data, using many more markers, shows that the second chromosome has a genetic size of ~202 cM, which agrees remarkably well with Noor et al (2000).

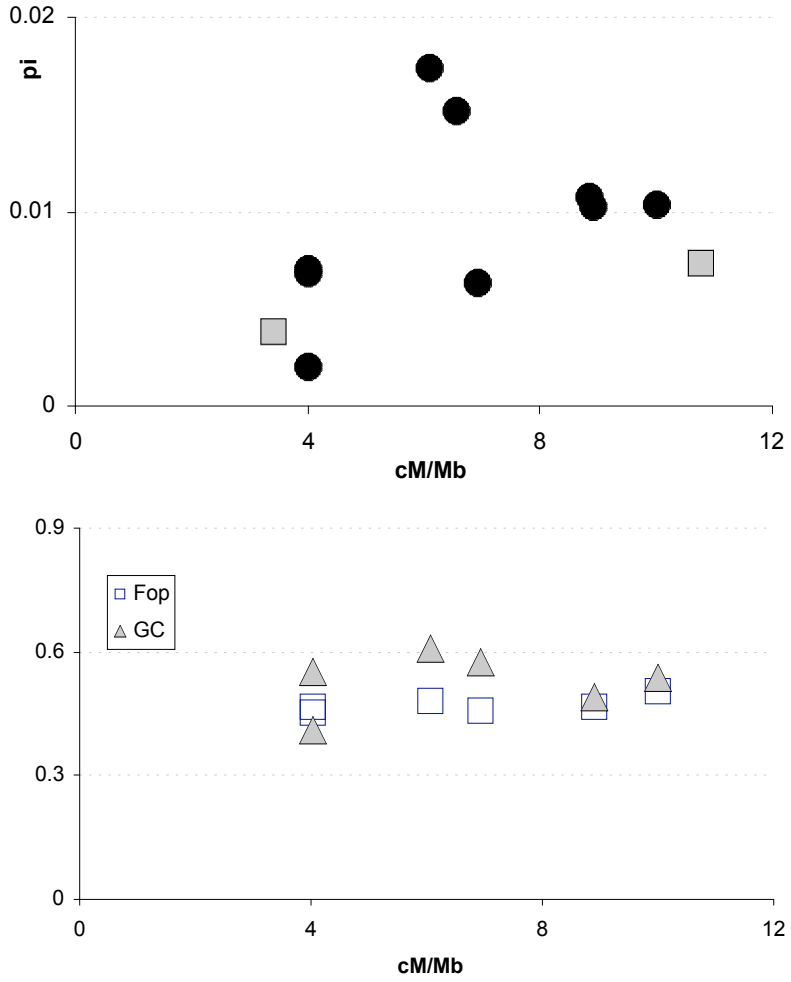

Figure 3.3 The effects of recombination rates on (a) nucleotide diversity (black circles is *D. pseudoobscura* and gray squares is *D. persimilis*), (b) optimal codon usage (fop) and GC content (only for *D. pseudoobscura*).

Contig Assembly

We connected a few contig groups previously assembled by Richards et al. (2005). Both the X chromosome and the fourth chromosome became available to the community in groups of contigs whose links were unknown. Our markers, many of them in a subset of such contig, allow us to estimate linkage relationships between them, therefore to orient and order some of the contigs available.

One predicts that contig groups are contiguous if the total size of the contig groups is greater than or equal to the inferred size of the chromosome. We inferred gaps between markers using the average recombination rate for the genome (mean recombination rate is ~133 Kb/cM with a standard error of ~13 Kb/cM) and found that the X chromosome contig group size is ~10mb, longer than the inferred gap-based size of X Mb. This suggests that many of the contig groups comprising the X chromosome may already overlap. This situation might be possible if the quality of the sequence close to the edges of the group decays or if repetitive DNA is located in those regions. This explanation assumes that recombination rates do not vary much across the genome, an assumption generally supported by our data.

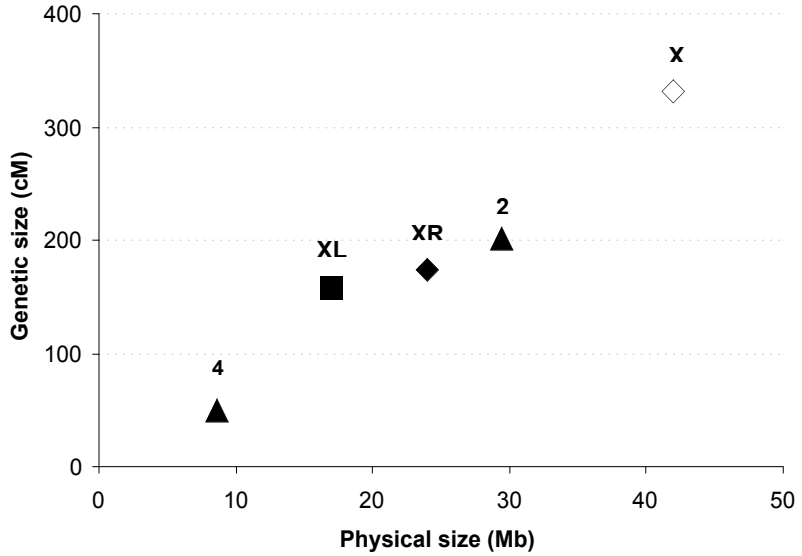

Figure 3.4 Correlation of genetic and physical size for *D. pseudoobscura* chromosomes. Data for the X chromosome is shown for both the entire chromosome (hollow) and its individual arms (filled).

Alternatively, one can argue that recombination in the unconnected regions is unusually suppressed. Because, in general, regions of low recombination tend to bear more repetitive DNA sequence than regions of high recombination, it might occur that assemblage of sequences in such regions is more difficult. This would

also artificially deflate our estimate of base pairs separating markers in distinct contig groups. The second chromosome provides a unique opportunity to check if estimates of gaps highly correlate with true gaps between markers. We found a strong correlation between these two forms of gaps (Pearson correlation coefficient = 0.998). This suggests that gap inference is reasonably accurate and provides support for the idea that at least some X chromosome gaps do not exist.

The fourth chromosome data is easier to interpret since the two markers linking the contig are nearly at the ends of each contig. The total size of the two groups of contig is ~2mb, shorter than the inferred size for the total linkage group. This suggests that there is still 2mb of DNA sequence not assembled between chromosome four groups 1 and 5.

Recombination Rates

We found that recombination rates are quite uniform across the genome of *D. pseudoobscura*. For example, second chromosome recombination rates range from 4.0 to 11.3 cM/Mb with a mean of ~7.5 cM/Mb and a standard error over the mean of just 0.7 cM/Mb. This uniformity was previously observed in another study that provided recombination rates for the second chromosome of *D. pseudoobscura* (Hamblin and Aquadro 1999). We found evidence for modest suppression of recombination at centromeric regions for the X chromosomes (the average recombination rate between markers spanning the centromeric region is 185 Kb/cM compared to the average recombination rate for the whole chromosome of 106 Kb/cM). It is interesting to note that the X chromosome is metacentric and resulted from a fusion between an ancestral autosome and one of its current sex-linked arms. Although more data is needed around the centromeric region, the lack of strong recombination suppression around their junction might suggest that the centromere has not yet been fully formed. More data is needed around centromeric and telomeric regions to fully reveal large-scale recombination patterns across the genome.

Recombination rates are higher in *D. pseudoobscura* compared to many other organisms, including its close relative *D. persimilis*. Average recombination rates in *D. pseudoobscura* can be almost four times larger than those observed in *D. melanogaster* (Kliman and Hey 1993; True et al. 1996) (e.g., the average recombination rate of the X chromosome in *D. pseudoobscura* is ~13.8 cM/Mb, while in *D. melanogaster* is ~3.46 cM/Mb). Similarly, in humans, average chromosomal recombination rates range from 0.96 to 2.11 cM/Mb (Kong et al. 2002). More strikingly, mouse average chromosomal recombination rates range only from 0.35 to 0.72 cM/ Mb (Nachman and Churchill 1996). These differences may come from variation in density and intensity of recombination hot spots between species (Nachman 2002).

Although we did not find significant differences in recombination rates among chromosomes, it was apparent that the X chromosome has a slightly higher

mean recombination rate than the other chromosomes. This observation is interesting since, in sexual organisms, the X chromosome has half the chance to recombine than any autosome (assuming equal sex ratios). It is possible that recombination rates may increase over time on the X chromosome to compensate for such imbalance. This hypothesis predicts that a recently evolved x-linked chromosome should have lower recombination rates than an ancestral x-linked chromosome. The right arm of the X chromosome in *D. pseudoobscura* is homologous to the third chromosome of *D. melanogaster*, while the left arm of both species has remained x-linked for a period longer than the species age. Although the difference in recombination rates between these two arms is not significant (possibly due to lack of power), recombination rates appear to be smaller in XR vs. Xl yet higher on XR than on the autosomes. Interestingly, the *D. melanogaster X* chromosome also shows a higher average recombination rate (3.46 cM/Mb) than two autosomes (2^{nd}, 2.71 cM/Mb and 3^{rd}, 2.35 cM/Mb). However, in mouse and rat the X chromosome has the lowest recombination rate (jensen-seaman et al. 2004).

We observed that recombination rates were much higher in hybrid backcross populations than in either parental species in collinear regions of the second chromosome. One possibility that may apply to the difference in recombination rates between hybrids and pure species in the *D. pseudoobscura* system is the interchromosomal effects produced by chromosomal rearrangements (Schultz and Redfield 1951). It has been observed that recombination rates tend to be higher in collinear regions of individuals heterozygous for chromosomal inversions. Hybrid females of *D. pseudoobscura* and *D. persimilis* are heterozygous for 3-4 large paracentric inversions, so it could be expected that these inversions are altering recombination rates in their genomes.

The high levels of recombination rates (sometimes just 50Kb/cM) observed in *D. pseudoobscura*, and even higher levels in hybrids, should prove very useful for mapping studies. Many genetic studies are limited to big chromosomal segments and subsequent elaborated molecular genetic analyses. Although this problem can be alleviated with enormous sample sizes, it is often true this is often unfeasible for study. Our findings imply that mapping traits in *D. pseudoobscura* will not require as many individuals to be scored relative to the numbers required in other fruit flies or other model organisms. This feature, in conjunction with the variety of interesting traits found in this system, will boost even more *D. pseudoobscura* as a prime model organism for studies of adaptation and speciation.

The Effect of Recombination on DNA Sequence Variation

The repeated observation of a correlation between nucleotide sequence variability and level of recombination suggests that the efficacy and impact of natural selection can be affected by regional variation in recombination rates (Begun and Aquadro 1992). Natural selection will reduce variability in regions of low recombination because linked polymorphism is eliminated whenever a

beneficial mutation sweeps in the population (MaynardSmith and Haigh 1974) or a deleterious mutation is selected out (Charlesworth et al. 1993). In either case, only the favored variants (usually one or very few) residing in the selected chromosomes remain in the population while the rest are eliminated. The size of the genomic region affected will correlate inversely with the rate of recombination. As a consequence, regions of high recombination should harbor more polymorphism than regions of low recombination.

We noted a nonsignificant positive correlation between nucleotide sequence variability and recombination rates in *D. pseudoobscura*. The lack of significance may come from the limited data available (see Hamblin and Aquadro 1999), as we do not have data from regions of very low recombination. This deficiency makes difficult to test the hypothesis that the intercept of the regression model is not significantly different from zero (i.e., nucleotide sequence variation is minimized as recombination is eliminated). Hamblin and Aquadro (1999) also noted uniformly high recombination rates across this chromosome in *D. pseudoobscura* (albeit without access to a sequence assembly), even in pericentromeric regions. This uniformity makes difficult studying the interaction between natural selection and recombination because regions with contrasting recombination environments are limited.

We also report the variation of GC content and optimal codon usage with respect to recombination rates of the second chromosome. None of these variables have a detectable correlation with recombination rates and may not affect the observed trend between nucleotide variability and recombination rates. However, due to small sample size, we were not able include GC content and optimal codon usage as covariates in linear models for published data.

Effective Population Size

Our data also allow us to estimate the effective population size of *D. pseudoobscura*. Previous estimates of N_e have ranged from several thousands to 4.5×10^6 individuals (Crumpacker and Williams 1973; Dobzhansky and Wright 1941; Powell et al. 1976; Schaeffer 1995). These estimates were obtained from lethals within populations, estimates of dispersion rates or mutational and recombination parameters. $4N_ec$ has been estimated to be 487.3 for *adh* in *D. pseudoobscura.* Accordingly, the average recombination rate of the genome is (1/ (127306 Bp / 0.01 recombination events) = 7.856×10^{-8} recombination events per base pair and c = (0.5) (7.8125×10^{-8} events per base pair) (3.2×10^3 base pairs) = 1.25×10^{-4} recombination events per base pair in females. Thus, from our results, $4N_ec/4c$ = 487.3/(4)(1.25×10^{-4}) = 974600. Our estimate of recombination rate is ~7 fold higher than a previous one by Chovnick, Gelbart and Mccarron (1977). This leads to the 4-fold difference between Schaeffer's (1995) estimate and ours. However, this data is still consistent with the role of genetic drift not being nearly as strong as envisioned by Dobzhansky and Wright (1943).

Prospects

In the near future twelve species of *Drosophila* will have their genomes sequenced. This presents a great opportunity for molecular evolutionary genomic studies, and for gaining deeper understanding on the genetics of speciation and adaptation. Ideally, we will have access to both physical and genetic maps for all these species. This would provide opportunities to check with great power the effects of reduced recombination in the efficacy of selection. The production of dense recombination maps will also help in solving two of the great-unsolved problems in evolutionary biology: the genetics of adaptation and speciation. QTL mapping is one of the main approaches to study the genetics of species differences and traits contributing to reproductive isolation. Ultimately, identifying the genes responsible for these differences and traits will allow to explore issues like the size of an adaptive change, the number of adaptive changes separating two species, and ultimately the order of events leading to adaptation and the magnitude of their effects. Similarly, the genetics of speciation will surely find rejuvenated with the identification of genes for hybrid sterility and mating discrimination.

CHAPTER IV

THE GENETICS OF SPECIATION BY REINFORCEMENT*

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During reinforcement, mating discrimination is strengthened by natural selection in response to maladaptive hybridization between closely related taxa (Dobzhansky 1940; Fisher 1958). Although reinforcement was a contentious issue in the past (Butlin 1989; Howard 1993; Noor 1999) recent theoretical work has identified the most favorable conditions for its existence (Kelly and Noor 1996; Kirkpatrick and Servedio 1999; Liou and Price 1994; Servedio 2000; Servedio and Kirkpatrick 1997), and empirical data have provided potential examples of its occurrence in nature (Noor 1995; Nosil et al. 2003; Pfennig 2003; Rundle and Schluter 1998; Sætre et al. 1997).

Theoretical work on reinforcement shows that reproductive isolation may be strengthened when either the same (one) or different (two) alleles conferring mating discrimination spread in the emerging species (Felsenstein 1981). In two-allele models, alleles conferring mating discrimination spread if they become genetically correlated with alleles reducing hybrid fitness. However, the evolution of such a correlation is opposed by recombination because alleles conferring discrimination in a given species do not confer discrimination in the other species. Consequently, two-allele models require either very strong selection, or tight linkage (e.g., physical or via chromosomal rearrangements) between alleles conferring mating discrimination and alleles reducing hybrid fitness (Kirkpatrick and Ravigné 2002). In contrast, one-allele models are not opposed by recombination because alleles conferring mating discrimination reduce hybridization in the genetic background of both species (Kelly and Noor 1996; Servedio 2000) and so may be more conducive to reinforcement. Unfortunately, empirical data concerning these two models of speciation are lacking (see Ortíz-Barrientos et al. 2002; Servedio and Noor 2003).

In addition to providing fundamental information for theoretical models, discerning the genetics of reinforcement will also develop our understanding of both the physiological basis of and forces governing changes in female preference. Furthermore, because the strengthening of female preference is driven by natural selection, the genetics of reinforcement will provide unique insights into the genetics of adaptation, another unsettled issue in evolutionary biology. Finally, high-resolution genetic studies of reinforcement can identify candidate speciation genes with effects on mating discrimination, information almost nonexistent in speciation studies (but see Ritchie and Noor 2004). Here, we address these many fundamental issues in speciation by examining a case of reinforcement in *Drosophila* and present for the first time a high-resolution genetic study of variation within species in female mating discrimination, including a set of candidate reinforcement genes and a discussion of the evolutionary implications of our findings.

The North American fruit flies *Drosophila pseudoobscura* and *D. persimilis* hybridize in nature and produce sterile male hybrids. While *D. pseudoobscura* occurs alone in non-coastal western United States and Central America, the two

species co-occur in California and the Pacific Northwest. Males court females from both species indiscriminately (Noor 1996), but females mate preferentially with individuals from the same species. The strength of this discrimination is not homogeneous across the species' geographic range: in a previous study, Noor (1995) showed that *D. pseudoobscura* females derived from populations where *D. persimilis* was absent exhibited weak mating discrimination (hereafter, "basal mating discrimination") while females derived from populations where *D. persimilis* is present exhibited strong mating discrimination (hereafter "reinforced mating discrimination"). This difference in mating discrimination is likely the evolutionary consequence of maladaptive hybridization where the two species coexist: reinforcement has strengthened mating discrimination in the *D. pseudoobscura* populations co-occurring with *D. persimilis*. These observations and the recent completion of the genome sequence of *D. pseudoobscura* (Richards et al. 2005) make these species an ideal system to genetically dissect the enhancement of mating discrimination in sympatry.

Although the genetics of reinforcement has not been studied in *D. pseudoobscura,* or in any system, the genetic basis of other traits contributing to the species' reproductive isolation (i.e., hybrid sterility and basal mating discrimination) is known in detail. All traits contributing to reproductive isolation between *D. pseudoobscura* and *D. persimilis,* including traits for basal discrimination, map primarily or exclusively to regions bearing fixed chromosomal inversion differences between the species (Noor et al. 2001b; Noor et al. 2001c). This result is consistent with a two-allele model of speciation in which the reduction in recombination between alleles for hybrid unfitness (i.e., hybrid sterility) and mating discrimination creates the necessary genetic correlations to advance divergence in the presence of gene flow. However, we do not know whether the genetic basis of reinforced mating discrimination corresponds to this picture, and specifically, whether chromosomal inversions are fundamental to this process. Comparing these genetic architectures will provide the most comprehensive view yet on the genetics of mating discrimination contributing to the formation of new species in the face of interspecies gene flow.

Materials and Methods

Our approach is based on measuring in one species the effects of substituting chromosomal segments from a highly discriminant genome into a less discriminant genome. In particular, we (1) test for within-species variation in the genetic architecture of female mating discrimination (f_1 male-parent backcrosses), (2) identify chromosomal regions contributing to reinforced mating discrimination (f_1 female-parent backcross) and compare them to regions conferring basal mating discrimination, and (3) provide a set of candidate genes for increased mating discrimination.

Fly Rearing and Lines

D. persimilis flies were collected in 1993 from Mt. St. Helena, California. *D. pseudoobscura* flies were collected from Mather, California (1997), Mt. St. Helena, California (1997), Flagstaff, Arizona (1993 and 1997), and Mesa Verde national park, Colorado (2001). Isofemale lines were established by rearing the offspring of individual females previously mated in the wild. All lines were maintained under a constant regime of temperature (20 °c) and humidity (85%) in diurnal/nocturnal cycles of 12 h and reared on a mixture of agar, dextrose, and yeast.

Reinforced Mating Discrimination in Sympatry

Pairs of *D. pseudoobscura* isofemale lines from each of two populations were crossed: Mather (1997) 52 _ 10 (California, sympatric with *D. persimilis*) and Flagstaff (1997) 16 _ 17 (Arizona, allopatric to *D. persimilis*), respectively. Virgin f_1 females from these crosses as well as *D. persimilis* males were routinely collected during afternoons and confined for 8 days. On the morning of the eighth day, individual females were confined with individual *D. persimilis* males. The rationale of this no-choice design is based on behavioral observations suggesting that females tend to copulate more often in the presence of single males than when multiple males approach them (Noor, unpublished data). Therefore, no-choice experiments should provide a more conducive setting for mating. The flies were observed for 10 min. If the male attempted fewer than three copulations, the pair was not scored, and the data were discarded. Otherwise, the pair was scored for successful copulation versus not (the male must have been on the back of the female for at least 1 min—the average copulation duration in *D. pseudoobscura* is 3 min). These protocols are the same used in Noor et al. (2001a). We performed fisher exact tests to evaluate differences among *D. pseudoobscura* lines sympatric versus allopatric to *D. persimilis*. Comparisons between allopatric and sympatric populations were performed both for outbred lines and inbred lines and only between lines that were tested for the phenotype at the same time, thus controlling for environmental error. Our comparisons between the two allopatric lines and between the two sympatric lines were not temporally controlled, and therefore may have been subject to some environmental heterogeneity. We used pairs of *D. pseudoobscura* inbred lines that significantly differed in their degree of female mating discrimination against *D. persimilis* in our mapping experiments (see below).

The heritable basis of increased mating discrimination in sympatry

We measured the frequency of matings with *D. persimilis* males of f_1 females resulting from crosses between sympatric and allopatric *D. pseudoobscura* lines. If f_1 females discriminated as strongly as the parent derived from sympatry, then we concluded that higher (reinforced) mating discrimination was inherited as a dominant trait. Fisher exact tests where performed to evaluate this hypothesis (see table 4.1.)

Testing for Male Discrimination

D. persimilis males were tested against *D. pseudoobscura* females from the Mather 17 and Flagstaff 1993 strains. We measured the time to first attempted copulation, the number of attempted copulations, and the time between the first attempt to copulate and copulation itself. Analysis of variance was conducted to test for a difference between treatments.

Mapping Approach

Microsatellite markers include those reported previously and 100 more that were developed by scanning contig sequences produced by the *D. pseudoobscura* genome project (Noor et al. 2000; Richards et al. 2005). Microsatellites were tested for fixed allelic differences between *D. pseudoobscura* lines Mather 17 and Flagstaff 1993. All primer information, both for informative and non-informative markers, will be published elsewhere and is available upon request. A recombination map with an average distance of 15 cM between markers was produced using the female-parent backcross (see below) and the multipoint-linkage approach implemented in Mapmaker version 3.0 (Lander et al. 1987).

Male-parent Backcross

Two male-parent backcrosses (n_1 = 900 and n_2 = 600 flies) were used to determine the chromosomal basis of reinforced mating discrimination and its natural within-species variation. Crossing over does not occur in male *Drosophila,* and they thus transfer whole chromosomes to their offspring (see figure 4.1). Each f_1 backcross female was scored for mating (as above), and its DNA was subsequently extracted. Lines used in each backcross were: for backcross 1, Mather (California) 17 and Flagstaff (Arizona) 1993, and for backcross 2, Mt. St. Helena (California) 7 and Mesa Verde (Colorado) 17. We consider strains derived from California as sympatric and strains derived from Arizona or Colorado as allopatric to *D. persimilis*.

We used microsatellite markers to score the origin of each chromosomal segment in backcross hybrid females. To determine the chromosomal contributions from each chromosome, we performed analyses of variance in which the dependent variable was mating discrimination and the independent variables the origin of each chromosome.

Female-parent Backcross

Once we determined the chromosomal effects and their variation for mating discrimination, we scored an additional 1,500 females derived by backcrossing Mather 17 _ Flagstaff 1993 f_1 females to Flagstaff 1993 males (see figure 4.1) for mating discrimination against *D. persimilis*. Single-marker analyses and Composite Interval Mapping (CIM) (Zeng et al. 1999) were used to map QTLs for reinforced mating discrimination. Both approaches consistently identified the same regions. When implementing CIM, forward-backward stepwise regressions were used to search for target QTLs over 2-cM intervals while simultaneously fitting partial

regression coefficients for background markers with a window size of 15 cM. We tested for epistatic interactions between significant QTLs using multiple interval mapping (Zeng et al. 1999). In all cases, procedures were carried out as implemented in QTL cartographer (Basten et al. 1999). Significance threshold values were obtained by permutation analysis as described by Doerge and Churchill (1996) .

Results

Female Discrimination is Dominant and Reinforced in Sympatry

Table 4.1 shows that *D. pseudoobscura* females derived from sympatry (with *D. persimilis*) exhibited stronger mating discrimination against *D. persimilis* males than did *D. pseudoobscura* females derived from allopatry. This pattern holds for both inbred and outbred lines. Also, our data show that both sympatric-derived lines and allopatric-derived lines vary considerably in their degree of discrimination ($p < 0.001$ for sympatric inbred lines, and $p = 0.0006$ for allopatric inbred lines), suggesting some within-population variation in female mating discrimination, both basal and reinforced.

Table 4.1 Matings of *D. persimilis* males to *D. pseudoobscura* females derived sympatry or allopatry. Each comparison involves either a sympatric versus an allopatric line of *D. pseudoobscura,* or f_1 females (allopatric _ sympatric) versus sympatric lines. Probability values were derived from fisher's exact tests using geography (allopatric versus sympatric) and copulation occurrence (yes versus no) as variables. O, outbred lines; i, inbred lines; f_1, first generation offspring from crossing the *D. pseudoobscura* sympatric and allopatric line.

Geography	Female	Total	Mated	Probability
Allopatric (o)	Flagstaff	115	48	
Sympatric (o)	Mather	115	26	<0.002 (allopatric vs. Sympatric)
F_1		106	20	0.5136 (sympatric vs. F1)
Allopatric (i)	Flagstaff	100	78	
Sympatric (i)	Mather	100	48	<0.001 (allopatric vs. Sympatric)
F_1		96	44	0.5637 (sympatric vs. F1)
Allopatric (i)	Mesa Verde	105	34	
Sympatric (i)	Mt. St. Helena	104	9	<0.001 (allopatric vs. Sympatric)
F_1		105	8	0.8061 (sympatric vs. F1)

Apparent reinforced mating discrimination could result from behavioral differences in *D. persimilis* males when exposed to sympatric or allopatric *D. pseudoobscura* females. To exclude this possibility, we measured the copulation latency and number of attempted copulations by *D. persimilis* males towards *D. pseudoobscura* females derived from sympatry or allopatry, and found no significant differences between groups (copulation latency, p = 0. 736, n = 138; attempted copulations, p = 0. 937, n = 110). Finally, we investigated the mode of inheritance of the phenotype and observed that f_1 females from crosses between sympatric and allopatric flies discriminated as strongly as their sympatric parent, suggesting that reinforced female mating discrimination is inherited as a dominant trait in both inbred and outbred lines (see table 4.1). This f_1 female mating discrimination is restricted to pairings with *D. persimilis* males, as f_1 females mate readily with conspecifics (data not shown). Taken together, these results suggest that reinforced mating discrimination in *D. pseudoobscura* is exclusive to females derived from sympatric areas to *D. persimilis*, is inherited as a dominant trait, and is not markedly affected by inbreeding.

Within-species Variation in Reinforced Female Mating Discrimination

We investigated within-species variability in reinforced discrimination by estimating the chromosomal contributions to mating discrimination between two pairs of *D. pseudoobscura* populations. In each case, we performed a male-parent backcross in which a mixture of whole chromosomes from sympatry and allopatry (f_1 genome) was substituted into an allopatric background (f_2 backcross genome) (see figure 4.1, left panel). Each male-parent backcross was also replicated with the reciprocal f_1 cross between parental strains, thus ruling out any maternal effects and providing insight into the effect of the X chromosome on mating discrimination.

Our two backcrosses identified different chromosomes as affecting reinforced mating discrimination (binomial test of proportions for effects of all chromosomes, $p < 0.01$). For example, sympatric X and fourth chromosomes derived from Mather, California (male-parent backcross 1), contribute significantly to reinforced mating discrimination ($p < 0.0001$ for X chromosome, $p < 0.005$ for fourth chromosome, n of approximately 1,000 for all markers), while the same chromosomes show no detectable effect on reinforced mating discrimination when derived from Mt. St. Helena, California (male-parent backcross 2, p = 0.2297, n = 600 for all markers) (see figure 4.2a and 4.2b). In contrast, the second chromosome shows the opposite relationship between the two backcrosses. The third chromosome shows marked effects on reinforced mating discrimination in both backcrosses, although at this level of resolution, it is impossible to tell whether they carry the same alleles in both sympatric populations. Figure 4.2c shows the genome composition of backcross females between Flagstaff, Arizona (allopatric), and Mather, California (sympatric), and their respective frequency of matings with *D. persimilis* males. The strongest effect is observed when both sympatric X and

fourth chromosomes are substituted in an allopatric background, and no significant epistatic interactions were detected between chromosomes.

These results suggest that different alleles for reinforced mating discrimination are segregating within sympatric populations of *D. pseudoobscura* despite extensive gene flow within and between populations (Noor et al. 2000; Schaeffer and Miller 1992).

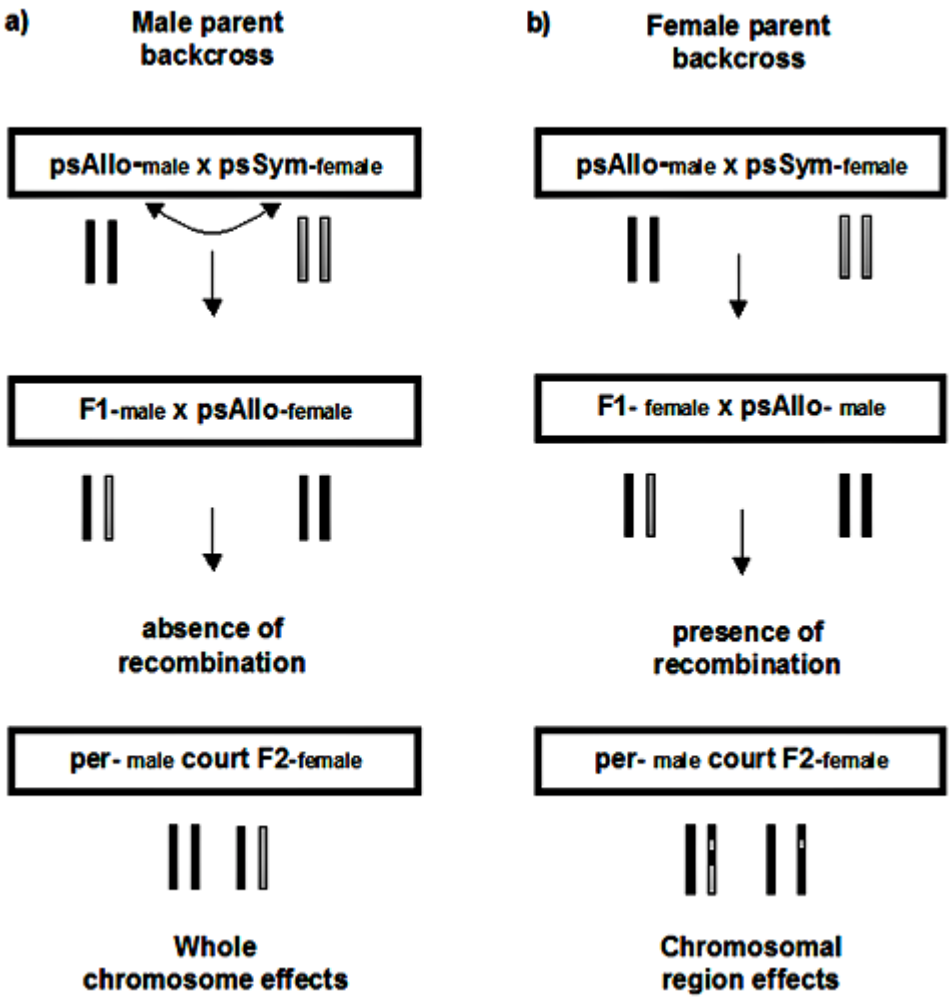

Figure 4.1 Experimental design to substitute chromosomes or chromosomal regions derived from sympatry into an allopatric background and measure their effect on mating discrimination. F_1 male-parent backcrosses (a) allow measurements of whole chromosome effects, while f_1 female-parent backcrosses (b) measure specific chromosomal region effects. Curved arrow represents the reciprocal backcross of the one shown.

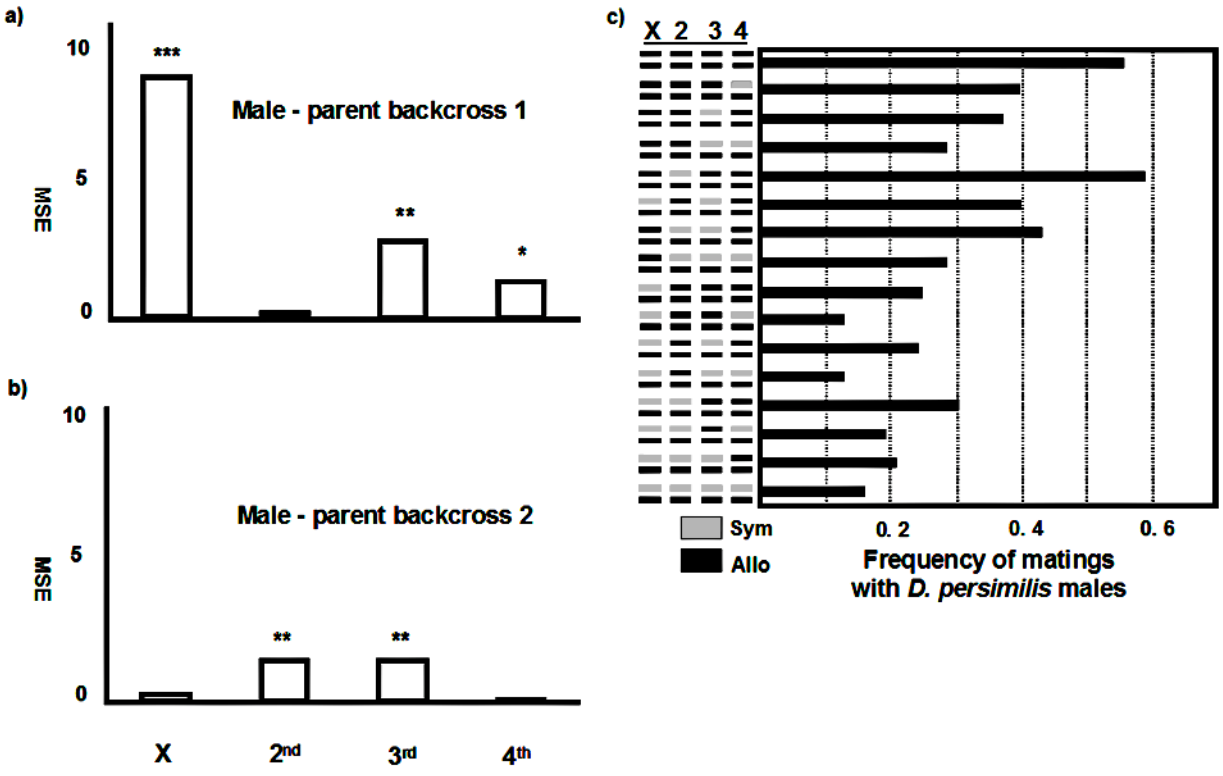

Figure 4.2 Mean square chromosomal effects on mating discrimination. (a) Male-parent backcross 1 shows the effects of substituting chromosomes derived from Mather, California (sympatry), into a background derived from Flagstaff, Arizona (allopatry). (b) Male-parent backcross 2 shows the effects of substituting chromosomes derived from Mt. St. Helena, California (sympatry), into a background derived from Mesa Verde, Colorado (allopatry). *, $p < 0.005$; **, $p < 0.001$; ***, $p < 0.0001$. (c) Combined chromosomal contributions to female mating discrimination. Small bars on the left represent chromosomes (x, 2, 3, and 4), while long bars on the right show the frequency of matings of backcross females with *D. persimilis*.

Fine-mapping the Genes Causing Reinforcement

We measured female mating discrimination against *D. persimilis* males in 1,500 f_2 individuals derived from a female-parent backcross between a line derived from Mather, California (sympatric line), and a line derived from Flagstaff, arizona (allopatric line), and genotyped 275 to 1,500 individuals for 70 markers dispersed along the four major chromosomes in *D. pseudoobscura*. Our initial single-marker analyses revealed significant associations between reinforced mating discrimination and three regions defined by markers located on the right and left arms of the X chromosome (XR and XL, respectively) (XR marker X021, $p < 0.0001$, $n = 1,129$; XL marker X002, $p = 0.02$, $n = 1,293$) and the fourth chromosome (4034 marker, $p < 0.0001$, $n = 1,434$). We were not able to detect an

effect of any single region on the third chromosome even though nine markers were surveyed. Effects identified on XR and chromosome 4 reinforced mating discrimination when the sympatric allele was present (positive), while the effect from XL was negative. After our initial scan, we used CIM to account for any inflated estimates in the absence of background correction. In addition, several markers were genotyped around the X021 and 4034 regions with the goal of refining the segments containing the quantitative trait loci (QTLs). Figure 4.3 shows the major results from CIM, and confirms our previous observations: one major QTL was identified on XR around X021, and a suggestive one close to the telomere of XL. In addition, one major QTL was found on the fourth chromosome. These results validate our previous findings using male-parent backcross females and provide a high-resolution definition of regions contributing to reinforced mating discrimination.

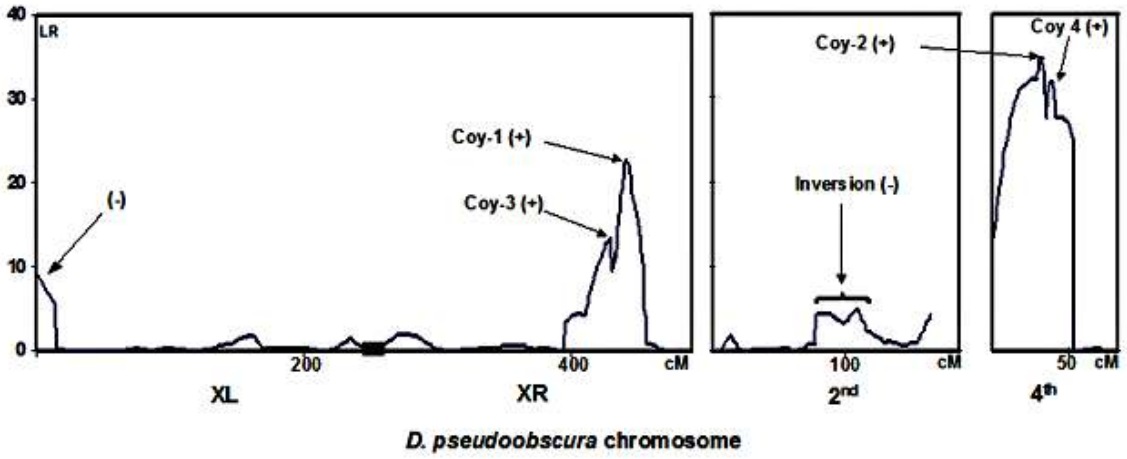

Figure 4.3 QTLs and candidate genes for reinforced mating discrimination. Each panel shows CIM estimations of chromosomal region effects on mating discrimination. Arrows point to major QTL locations and are named after their candidate genes. The direction of the chromosomal region effect on mating discrimination is shown in parentheses. The y-axis, LR, is the ratio of the likelihood value under the null hypothesis of no QTL to the likelihood value under the hypothesis that there is a QTL in a given interval of adjacent markers. The likelihood ratio significance threshold reflecting a type *I* error of 0.05 is 11.5s the indicated inversion is a fixed chromosomal inversion differentiating *D. pseudoobscura* and *D. persimilis.*

X chromosome: Candidate Genes coy-1 and coy-3.

A more careful examination of the X021 region showed that the QTL location (CIM lod score = 5. 16), hereafter referred to as *coy-1,* is estimated to lie between two additional markers, X021-a1 and X021-a4 (these markers are physically separated by 390 Kb and by a recombination fraction of 4.5 centi-morgans (cM)). According to the recently obtained genome sequence of *D.*

pseudoobscura, there are seven genes between these two markers, one of which, *bru-3,* and accounts for one-third of the sequence length of this region. In addition, CIM also identified another QTL, hereafter referred to as *coy-3,* located between markers X021 and X021-b2, although with a weaker effect (CIM lod score = 2. 84). There are approximately 200 Kb and 30 genes between these markers and a recombination fraction of 3.5 cM. Finally, a third QTL was found near the XL telomere and, in contrast to the X021 region, showed a negative and weak additive effect (CIM lod score = 2. 45). We tested this model for the X chromosome using multiple interval mapping and found that the strongest support is for *coy-1,* followed by *coy-3.* We were unable to recover any support for the QTL on XL. No epistatic interactions were detected among any QTLs.

Chromosome 4: Candidate Genes coy-2 and coy4.

Dissection of the 4034 region using CIM split the effect into two QTLs for reinforced mating discrimination; we refer to them as *coy-2* and *coy-4,* respectively. These QTLs show the strongest effects (CIM lod scores of 7.7 and 7, respectively). As with *coy-1* and *coy-3,* these QTL are additive and contribute positively to the degree of mating discrimination of f_2 females. *Coy-2* is located next to marker 4034-a8. This marker is within a 300-Kb region homologous to a *D. melanogaster* region containing a p-element insertion disrupting normal olfactory behavior (see discussion for details) (Anholt et al. 2001; Anholt et al. 2003). The *D. melanogaster* region contains 30 genes of which at least ten have known or predicted olfactory functions. The primary candidate gene for the disrupted olfactory behavior in the p-element mutant is *cg13982*. Interestingly, the *D. melanogaster* p-element mutation up-regulates expression of *bru-3,* suggesting a possible functional link between the candidate genes *coy-1* and *coy-2.* The second QTL in this region, *coy-4,* is defined by two markers, 4003 and 4032, on each side of 4034. CIM places the QTL between 4003 and 4034, an approximately 200-Kb region containing only nine genes. Five of these nine genes are a conglomerate of UDP-glycosyltransferases, genes preferentially expressed in the *Drosophila* antenna and coding for biotransformation enzymes involved in detoxification and olfaction (Wang et al. 1999). However, a more careful examination of the genes shows that their sequence overlap results from the inability of blast homology searches to distinguish the members of this gene family, suggesting that there may be only one or few UDP-glycosyltransferase genes here. Consequently, the number of candidate genes in the region may be reduced from nine to five genes, at least one of which is involved in olfaction. As before, we tested this model using multiple interval mapping and recovered significant support for *coy-2* under stringent conditions and no evidence of significant epistasis among previously identified QTLs.

Based on these results and those for the X chromosome, we suggest that the strongest evidence for QTLs contributing to reinforced discrimination in sympatry lies with *coy-1* and *coy-2,* and that *coy-3* and *coy-4* are suggestive QTLs.

Discussion

We have provided the first genetic dissection of an adaptive female preference involved in speciation by developing a QTL map for discrimination variation in *Drosophila pseudoobscura*. The resolution of our approach is novel to genetic studies of behavioral discrimination in that we have surveyed the genome with 70 microsatellite markers for chromosomal regions contributing to increased mating discrimination and have narrowed some of these regions to intervals containing as few as five genes. The role of these genes in reinforcing mating discrimination is supported by indirect evidence from *D. melanogaster* mutants: two of the major QTLs identified in our mapping experiments bear genes identified in smell impairment screenings of p-element mutants (Anholt et al. 2003). A gene in one of these intervals, CG13982 (*D. melanogaster* chromosome 2L), appears to up-regulate a second gene located in the other interval, *bru-3* (*D. melanogaster* X chromosome). Furthermore, we have shown that the chromosomal contributions to reinforced mating discrimination vary among strains of *D. pseudoobscura*. Finally, the chromosomal effects on mating discrimination are inherited in a dominant fashion, consistent with general theories on the evolution of adaptive characters (Haldane 1924). Below, we discuss these results in the context of several evolutionary hypotheses of reinforcement and speciation.

TheGenetics of “Basal” versus “Reinforced” Female Mating Discrimination

Most genetic studies of female preference and sexual isolation have utilized between-species genetic crosses or non-hybridizing allopatric populations (e.g., Moehring et al. 2004). Some of these studies suggest that female preference is a polygenic character (Ting et al. 2001), while other researchers have found a very simple genetic basis for female discrimination (Doi et al. 2001). A study of another behavioral trait, response to odorants, showed that many genes contribute to olfaction, and epistasis plays a fundamental role in determining the specificity of odor identification (Anholt et al. 2001; Anholt et al. 2003). We expect the genetics of reinforced female mating discrimination to bear some similarities to the genetics of overall female species preferences and/or traits involved in response to olfactory cues.

Available genetic data on “basal” female mating discrimination in *D. pseudoobscura* (between-species crosses using a *D. pseudoobscura* line derived from areas allopatric to *D. persimilis*) show that all QTLs for this trait map unequivocally to two inverted chromosomal regions separating it from *D. persimilis* (Noor et al. 2001c), one on XL and one on chromosome 2. This result suggests that the regions we localized as contributing to reinforced mating discrimination (on XR and chromosome 4) are distinct from those previously identified as contributing to basal discrimination. Hence, chromosomal inversions may have been crucial in allowing these species to persist in sympatry (Noor et al. 2001c), but the rearranged regions might not have contributed directly to the subsequent reinforcement of mating discrimination. This idea is consistent with data showing

that a region *(*DPS4003*)* just 400 Kb away from the QTL identified on the fourth chromosome seems to have introgressed recently between *D. pseudoobscura* and *D. persimilis* (Machado et al. 2002).

This result supports either a one-allele mechanism, perhaps controlling the genetics of variation within species for female mating discrimination if there was not strong assortative mating before sympatry, or possibly a two-allele mechanism, if reinforcement took place after sympatry and strong assortment had already evolved. The definitive test will be to determine whether introgressing the different *D. pseudoobscura* alleles into *D. persimilis* affects female discrimination in the same manner.

Female Mating Discrimination is a Composite Trait

These "layers" of female discrimination (see figure 4.4) are intimately related to the genetic differences being evaluated. Genes localized within fixed chromosomal regions inverted between *D. pseudoobscura* and *D. persimilis* are responsible for the first layer, basal discrimination. In contrast, genes localized outside those inverted regions cause the second layer, reinforced mating discrimination. Basal discrimination appears to stem mostly from female responses to acoustic "courtship song" signal differences between *D. pseudoobscura* and *D. persimilis*. This is suggested by both a strong correlation in mating success of backcross hybrids with song parameters (Williams et al. 2001) and in playback experiments with wingless flies (M. Lineham, M. A. F. Noor, and M. Ritchie, unpublished data). Even though we cannot discard fine-tuning of the acoustic receiver signaling system in sympatric females, the nature of the candidate genes we identified suggests that olfactory responses might play a major role in the second layer of female preference. Non-auditory cues conferring reinforced discrimination are also suggested by behavioral data collected by Mark Lineham and Michael Ritchie (personal communication) showing that the rejection exercised by *D. pseudoobscura* females towards *D. persimilis* male song is the same in lines derived from sympatry and allopatry, even though females from the two regions clearly show differences in mating discrimination (Noor 1995). Finding different genetic architectures for traits involved in speciation is expected under models based on selection on many traits (Rice and Hostert 1993). These traits may be a composite response of behavioral traits, as exemplified in this study, or ecology and behavior, as evidenced by Timema walking sticks, in which traits conferring ecological adaptation and traits contributing to mating discrimination act in conjunction to increase the overall level of sexual isolation between hybridizing populations (Nosil et al. 2003).

Our results suggest, albeit not conclusively, that reinforced mating discrimination is related to differences in response to olfactory cues. We have shown here that candidate regions on the fourth chromosome bear an unusual excess of olfactory genes, and some of these have been associated with specific olfactory responses in other *Drosophila*. Further, the fact that we mapped

reinforced discrimination to two interacting gene regions involved in olfaction (bearing *bru*-3 and *cg13982*) was striking in this regard, supporting a potential role of olfactory response in reinforcement in these species.

We also observed differences among strains in the genetic architecture of reinforced mating discrimination. Such variation in genetic control may be common when populations exchanging genes differ in phenotype because of selection. Multiple alleles from different loci may have increased in frequency because of selection for discrimination, and these alleles sometimes spread into allopatry or are replaced by the allopatric alleles in sympatry. When sampling from single lines, we capture only a fraction of the genetic variation in mating discrimination, and sometimes a high-discrimination allele is even sampled from allopatry (as we observed in the QTL on XL). This observation should be typical in many QTL mapping studies that utilize strains within species with extensive gene flow among populations, as in the many studies of *D. melanogaster* variation.

In brief, these results show that basal and reinforced discrimination are different, species discrimination in *D. pseudoobscura* is a composite trait, and there is genetic variation within species in reinforced mating discrimination.

Reinforced Mating Discrimination is Inherited as a Dominant Trait

Recessive adaptive mutations are often lost before selection can screen their effects on the phenotype. Conversely, adaptive mutations that are visible to selection in a heterozygous state will be available for selection even at very low frequencies. Therefore, we expect that most adaptive mutations reaching high frequencies in a population are dominant (Haldane 1924 but see ; Orr and Betancourt 2001).This process is commonly referred to as Haldane's sieve and predicts that alleles for mating discrimination that increase in frequency in response to selection should be dominant. Our study shows that f_1 female offspring of crosses between allopatric and sympatric populations of *D. pseudoobscura* are as reluctant to mate with *D. persimilis* males as are *D. pseudoobscura* females from sympatric populations. This result implies that high discrimination can be expressed in heterozygous individuals, suggesting a dominant basis for the phenotype. In contrast, "basal" female mating discrimination seems to be a recessive trait (f_1 female hybrids from crosses between allopatric *D. pseudoobscura* individuals and *D. persimilis* do not discriminate against *D. persimilis* males). Taken together, these results are consistent with genetic differences between basal and reinforced female mating discrimination and with general predictions from Haldane's sieve theory.

Conclusions

This is the first study to provide a detailed description of the genetic basis of speciation by reinforcement. We conclude that, in *D. pseudoobscura,* (1) high discrimination in sympatry is inherited in a dominant fashion, (2) there is within-

species variability for high female mating discrimination as evidenced by the different genetic architectures recovered in the male-parent backcross experiments, (3) there are multiple genes, possibly involved in olfaction, contributing to enhanced female mating discrimination, (4) some candidate genes for reinforcement identified here have been previously identified in p-element mutant screenings for smell impairment in *D. melanogaster,* (5) the genetic architecture of basal female mating discrimination is different from that of reinforced mating discrimination, and (6) inversions seem to play no direct role in creating or maintaining the genetic differences directly responsible for increased female mating discrimination in sympatry. However, these inversions seem to play a crucial role, as evidenced by previous studies (Noor et al. 2001c), in maintaining the identity of hybridizing species and thus providing time for selection to reinforce their sexual isolation. These results have broad evolutionary implications, as discussed above, and open exciting new avenues of research to understand the genetics of an adaptive behavioral trait involved in speciation.

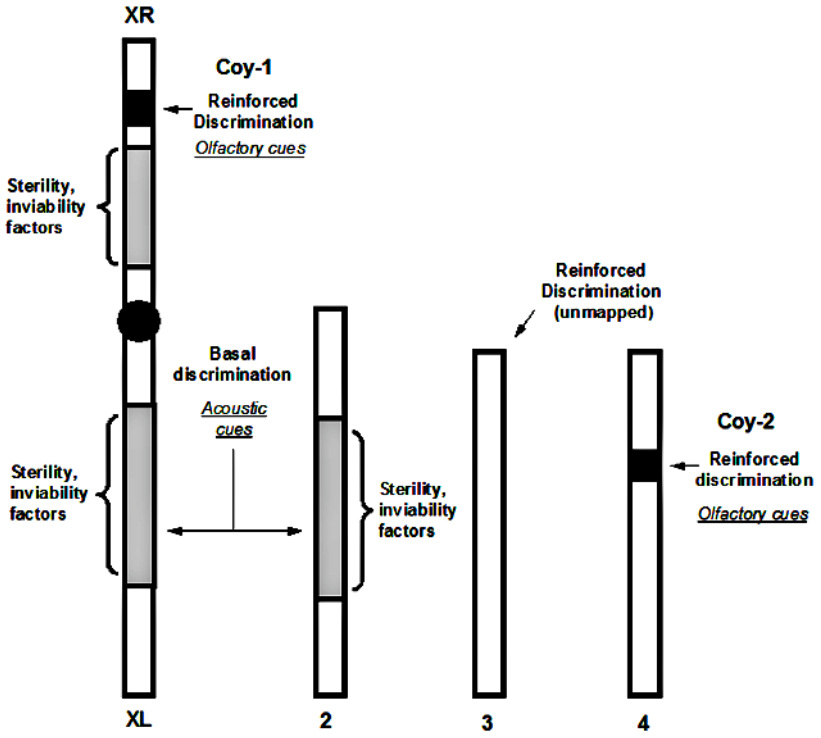

Figure 4.4 Genomic distribution of genetic factors preventing gene flow between *D. pseudoobscura* and *D. persimilis.* Gray boxes denote fixed chromosomal inversions separating *D. pseudoobscura* and *D. persimilis*. Black boxes denote the genomic locations of QTLs for reinforced mating discrimination. Note that the third chromosome also confers high discrimination in sympatry, but no particular QTLs have been identified for this chromosome.

CHAPTER V

CONCLUSIONS

Here I provide conclusions derived from data and concepts obtained in previous chapters of my dissertation. Although each chapter has its own conclusions section, there are many inferences that only become possible when all information is taken into account simultaneously. In general, my dissertation studies the genetics of reinforcement framed in the interaction between recombination and gene flow. My dissertation provides data relevant to the conceptual foundation of speciation (chapter II), the genome of *D. pseudoobscura* (chapter III), and the genetics of reinforced mating discrimination in *D. pseudoobscura* (chapter IV). This research is allowing others and me to develop new projects (e.g., genetic test of one- vs. two allele model of speciation, identification and characterization of reinforcement genes) that eventually will provide the best evolutionary and genetic picture yet of reinforcement.

The genetics of reinforcement in *D. pseudoobscura* appears to be a mixture of genetic architectures. The first one partially controls introgression via the inexorable production of sterile male hybrids, while the second controls the overall probability of mating with the same species. The genetic architecture controlling introgression is localized exclusively to inverted regions of the genome, while the genetics of reinforced discrimination occurs outside the inverted regions. This is interesting because it suggests that the interaction between recombination and gene flow is different for each genetic architecture. On one hand, gene flow via recombination possibly purged many genetic incompatibilities between *D. pseudoobscura* and *D. persimilis* outside the inverted regions, thus restricting species identity to a fraction of their genome. On the other hand, the genetics of reinforcement appears to be insensitive to gene flow.

The genetics of reinforcement in *D. pseudoobscura* may also be a mixture of phenotypic responses. Females may discriminate conspecific from heterospecific males based on both acoustic and olfactory cues. This is interesting because selection on many traits may result in stronger behaviors. In other words, there may be limits on the ability of females to distinguish conspecific from heterospecific males based on a single cue. It would be interesting to test this hypothesis based on selection experiments for only one cue while controlling for others. Finally, because one of the possible cues involved in reinforced discrimination is olfactory, this might suggest that ecological factors might play an important role in species discrimination (i.e., host preference and its effects on both body and odor composition). The ecology of this system plus its connection to the genetics of reinforcement is badly needed.

The genetics of reinforcement appear to be also a mixture of dominant and recessive responses. *D. pseudoobscura* x *D. persimilis* f_1 hybrid females are not discriminant, while allopatric X sympatric f_1 females are as discriminant as their mothers. Recessive effects between species are partially responsible for introgression between *D. pseudoobscura* and *D. persimilis*. Introgression between species purges genetic incompatibilities, except those inside the inverted regions.

Also, introgression creates the mosaic nature of their genomes and indirectly provides a window of gene flow that apparently has acted on the genetics of reinforced mating discrimination. Dominant effects, on the other hand are characteristic of reinforced mating discrimination. This, as discussed in chapter iii, this is consistent with the idea that natural selection screens more easily dominant alleles in high frequency than recessive alleles.

The genetics of reinforcement will be ultimately understood best when we isolate and examine the genes responsible for enhanced mating discrimination. The recombination landscape of *D. pseudoobscura* suggests that we will be able to achieve this goal. Recombination rates in *D. pseudoobscura* are uniform and pretty high compared to other organisms. The constructions of recombination transgenics should be possible and therefore the identification of candidate genes. Once these genes are identified, classic transgenic experiments and population genetic analysis should reveal the genes causing reinforcement and the forces shaping their variation between and within species.

LITERATURE CITED

Adam, D., W. Maueler, and M. Schartl. 1991. Transcriptional activation of the melanoma inducing *Xmrk* oncongen in *XIphophorus*. Oncogene 6:73-80.

Anderson, W. W. 1993. Linkage map of *Drosophila pseudoobscura in* S. J. O'Brien, ed. Genetic Maps: Locus Maps of Complex Genomes. Cold Spring Harbor Laboratory Press, Plainview, NY.

Anderson, W. W., J. Arnold, D. G. Baldwin, A. T. Beckenbach, C. J. Brown, S. H. Bryant, J. A. Coyne, L. G. Harshman, W. B. Heed, D. E. Jeffery, L. B. Klaczko, B. C. Moore, J. M. Porter, J. R. Powell, T. Prout, S. W. Schaeffer, J. C. Stephens, C. E. Taylor, M. E. Turner, G. O. Williams, and J. A. Moore. 1991. Four decades of inversion polymorphism in *Drosophila pseudoobscura*. Proc. Natl. Acad. Sci. USA 88:10367-10371.

Anderson, W. W., T. Dobzhansky, O. Pavlovsky, J. R. Powell, and D. Yardley. 1975. Three decades of genetic change in *Drosophila pseudoobscura*. Evolution 29:24-36.

Anholt, R. R., J. J. Fanara, G. M. Fedorowicz, I. Ganguly, N. H. Kulkarni, T. F. C. Mackay, and S. M. Rollmann. 2001. Functional Genomics of Odor-guided Behavior in *Drosophila melanogaster*. Chem. Senses 26:215-221.

Anholt, R. R. H., C. L. Dilda, S. Chuang, J.-J. Fanara, N. H. Kulkarni, I. Ganguly, S. M. Rollmann, K. P. Kamdar, and T. F. C. Mackay. 2003. The genetic architecture of odor-guided behavior in Drosophila: epistasis and the transcriptome. Nat. Genet. 35:180-184.

Aquadro, C. F., A. L. Weaver, S. W. Schaeffer, and W. W. Anderson. 1991. Molecular evolution of inversions in *Drosophila pseudoobscura*: The amylase gene region. Proc. Natl. Acad. Sci. USA 88:305-309.

Ballard, J. W. O. 2000. When one is not enough: introgression of mitochondrial DNA in Drosophila. Mol. Biol. Evol. 17:1126-1130.

Barbash, D. A., J. Roote, and M. Ashburner. 2000. The *Drosophila melanogaster Hybrid male rescue* gene causes inviability in male and female species hybrids. Genetics 154:1747-1771.

Barbash, D. A., D. F. Siino, A. M. Tarone, and J. Roote. 2003. A rapidly evolving MYB-related protein causes species isolation in Drosophila. Proc. Natl. Acad. Sci. USA 100:5302-5307.

Barton, N., and B. O. Bengtsson. 1986. The barrier to genetic exchange between hybridising populations. Heredity 56:357-376.

Barton, N. H., and G. M. Hewitt. 1985. Analysis of Hybrid Zones. Ann. Rev. Ecol. Syst. 16:113-48.

Barton, N. H., and M. Turelli. 1991. Natural and sexual selection on many loci. Genetics 127:229-255.

Basten, C. J., B. S. Weir, and Z.-B. Zeng. 1999. QTL Cartographer. Department of Statistics, North Carolina State University, Raleigh, NC.

Begun, D. J., and C. F. Aquadro. 1992. Levels of naturally occurring DNA polymorphism correlate with recombination rates in *D. melanogaster*. Nature 356:519-520.

Berlocher, S. H., and J. L. Feder. 2002. Sympatric speciation in phytophagous insects: Moving beyond controversy? Annu. Rev. Entomol. 47:773-815.

Brooks, R. 2000. Negative genetic correlation between male sexual attractiveness and survival. Nature 406:67-70.

Butlin, R. 1987. Speciation by reinforcement. Trends Ecol. Evol. 2:8-13.

Butlin, R. 1989. Reinforcement of premating isolation. Pp. 158-179 *in* D. Otte and J. A. Endler, eds. Speciation and Its Consequences. Sinauer Associates, Inc., Sunderland, Mass.

Carpenter, A. T. C. 1979. SYNAPTONEMAL COMPLEX AND RECOMBINATION NODULES IN WILD-TYPE DROSOPHILA MELANOGASTER FEMALES. Genetics 92:511-541.

Carson, H. L. 1975. The genetics of speciation at the diploid level. Am. Nat. 109:83-92.

Charlesworth, B., M. T. Morgan, and D. Charlesworth. 1993. The Effect of Deleterious Mutations on Neutral Molecular Variation. Genetics 134:1289-1303.

Chovnick, A., W. Gelbart, and M. McCarron. 1977. Organization of the Rosy locus in Drosophila melanogaster. Cell 11:1-10.

Coyne, J. A., and H. A. Orr. 1988. Patterns of speciation in *Drosophila*. Evolution 43:362-381.

Coyne, J. A., and H. A. Orr. 1989. Patterns of speciation in *Drosophila*. Evolution 43:362-381.

Crumpacker, D. W., and J. S. Williams. 1973. Density, dispersion, and population structure in Drosophila pseudoobscura. Ecol. Monogr. 43:499-538.

Darwin, C. 1859. The origin of species. Random House, Inc., New York.

Diehl, S. R., and G. L. Bush. 1989. The role of habitat preference in adaptation and speciation. Pp. 345–365 *in* D. Otte and J. A. Endler, eds. Speciation and its consequences. Sinauer Associates, Sunderland, Massachusetts.

Dobzhansky, T. 1934. Studies of hybrid sterility. I. Spermatogenesis in pure and hybrid *Drosophila pseudoobscura*. Z. Zellforch. Microsk. Anat. 21:169--221.

Dobzhansky, T. 1940. Speciation as a stage in evolutionary divergence. Am. Nat. 74:312-321.

Dobzhansky, T. 1951. Genetics and the Origin of Species. Columbia University Press, New York.

Dobzhansky, T. 1973. Is there gene exchange between *Drosophila pseudoobscura* and *Drosophila persimilis* in their natural habitats? Am. Nat. 107:312-314.

Dobzhansky, T., and S. Wright. 1941. Genetics of natural populations. V. Relations between mutation rate and accumulation of lethals in populations of *Drosophila pseudoobscura*. Genetics 26:23-51.

Dobzhansky, T., and S. Wright. 1943. Genetics of natural populations. X. Dispersion rates in *Drosophila pseudoobscura*. Genetics 28:304-340.

Doebeli, M., and U. Dieckmann. 2000. Evolutionary branching and sympatric speciation caused by different types of ecological interactions. Am. Nat. 156:S77-S101.

Doerge, R. W., and G. A. Churchill. 1996. Permutation tests for multiple loci affecting a quantitative character. Genetics 142:285-294.

Doi, M., M. Matsuda, M. Tomaru, H. Matsubayashi, and Y. Oguma. 2001. A locus for female discrimination behavior causing sexual isolation in *Drosophila*. Proc Natl Acad Sci USA 98:6714-6719.

Feder, J. L. 1998. The apple maggot fly, Rhagoletis pomonella: Flies in the face of conventional wisdom about speciation? Pp. 130-144 *in* D. J. Howard and S.

H. Berlocher, eds. Endless Forms: Species and Speciation. Oxford University Press, New York.

Feder, J. L., C. A. Chilcote, and G. L. Bush. 1988. Genetic differentiation between sympatric host races of the apple maggot fly *Rhagoletis pomonella*. Nature 336:61-64.

Felsenstein, J. 1981. Skepticism towards Santa Rosalia, or Why are there so few kinds of animals? Evolution 35:124-138.

Fisher, R. A. 1958. The Genetical Theory of Natural Selection. Dover, New York.

Fishman, L., and J. H. Willis. 2001. Evidence for Dobzhansky-Muller incompatibilities contributing to the sterility of hybrids between *Mimulus guttatus* and *M. nasutus*. Evolution 55:1932-1942.

Fry, J. D. 2003. Multilocus models of sympatric speciation: Bush versus Rice versus Felsenstein. Evolution 57:1735-1746.

Gregorius, H.-R., and W. Steiner. 2001. Reinforcement of genetic coherence in a two-locus model. BMC Evolutionary Biology 1:2.

Hagen, R. H., and J. M. Scriber. 1995. Sex chromosomes and speciation in tiger swallowtails. Pp. 211-227 *in* J. M. Scriber, Y. Tsubaki and R. C. Lederhouse, eds. Swallowtail Butterflies: Their Ecology and Evolutionary Biology. Scientific Publishers, Gainesville, Florida.

Haldane, J. B. S. 1924. II. A mathematical theory of natural and artificial selection. Trans. Camb. Phil. Soc. 23:19-41.

Hamblin, M. T., and C. F. Aquadro. 1999. DNA sequence variation and the recombinational landscape in *Drosophila pseudoobscura*: A study of the second chromosome. Genetics 153:859-869.

Hamilton, W. D., and M. Zuk. 1982. Heritable true fitness and bright birds: A role for parasites. Science 218:384-387.

Harrison, R. G., and S. M. Bogdanowicz. 1997. Patterns of variation and linkage disequilibrium in a field cricket hybrid zone. Evolution 51:493-505.

Hartl, D. L., and A. G. Clark. 1997. Principles of population genetics. Sinauer, Sunderland.

Hawthorne, D. J., and S. Via. 2001. Genetic linkage of ecological specialization and reproductive isolation in Pea Aphids. Nature 412:904-907.

Higa, I., and Y. Fuyama. 1993. Genetics of Food Preference in Drosophila-Sechellia .1. Responses to Food Attractants. Genetica 88:129-136.

Hostert, E. E. 1997. Reinforcement: A new perspective on an old controversy. Evolution 51:697-702.

Howard, D. J. 1993. Reinforcement: Origin, dynamics, and fate of an evolutionary hypothesis. Pp. 46-69 *in* R. G. Harrison, ed. Hybrid Zones and the Evolutionary Process. Oxford University Press, New York.

Hutter, C. M., and D. M. Rand. 1995. Competition between mitochondrial haplotypes in distinct nuclear genetic environments: *Drosophila pseudoobscura vs. D. persimilis*. Genetics 140:537-548.

Johnson, N. A. 2000. Speciation: Dobzhansky-Muller incompatibilities, dominance and gene interactions. Trends in Ecology & Evolution 15:480-482.

Jones, C. 1998. The genetic basis of *Drosophila sechellia*'s resistance to a host plant toxin. Genetics 149:1899-1908.

Kelly, J. K., and M. A. F. Noor. 1996. Speciation by reinforcement: A model derived from studies of *Drosophila*. Genetics 143:1485-1497.

Kirkpatrick, M. 1982. Sexual selection and the evolution of female choice. Evolution 36:1-12.

Kirkpatrick, M., and N. H. Barton. 1997. The strength of indirect selection on female mating preferences. Proc. Natl. Acad. Sci. USA 94:1282-1286.

Kirkpatrick, M., and V. Ravigné. 2002. Speciation by natural and sexual selection: Models and experiments. Am. Nat. 159:S22-S35.

Kirkpatrick, M., and M. R. Servedio. 1999. The reinforcement of mating preferences on an island. Genetics 151:865-884.

Kliman, R., and J. Hey. 1993. Reduced natural selection associated with low recombination in Drosophila melanogaster. Mol. Biol. Evol. 10:1239-1258.

Kliman, R. M., P. Andolfatto, J. A. Coyne, F. Depaulis, M. Kreitman, A. J. Berry, J. McCarter, J. Wakeley, and J. Hey. 2000. The Population Genetics of the Origin and Divergence of the Drosophila simulans Complex Species. Genetics 156:1913-1931.

Kong, A., D. F. Gudbjartsson, J. Sainz, G. M. Jonsdottir, S. A. Sigurdardottir, J. Barnard, B. Hallbeck, G. Masson, A. Schlien, S. T. Palsson, M. L. Frigge, T. E. Thorgeirsson, J. R. Gulcher, and K. Stefansson. 2002. A high-resolution recombination map of the human genome. Nat. Genet. 31:241-247.

Kotiaho, J. S., L. W. Simmons, and J. L. Tomkins. 2001. Towards a resolution of the lek paradox. Nature 410:684-686.

Kovacevic, M. K., and S. W. Schaeffer. 2000. Molecular population genetics of X-linked genes in Drosophila pseudoobscura. Genetics 156:155-172.

Lambie, E. J., and G. S. Roeder. 1986. REPRESSION OF MEIOTIC CROSSING OVER BY A CENTROMERE (CEN3) IN SACCHAROMYCES CEREVISIAE. Genetics 114:769-789.

Lande, R. 1981. Models of speciation by sexual selection on polygenic traits. Proc. Natl. Acad. Sci. USA 78:3721-3725.

Lander, E. S., P. Green, J. Abrahamson, A. Barlow, M. J. Daly, S. E. Lincoln, and L. Newburg. 1987. MAPMAKER: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174-181.

Li, Z., S. R. M. Pinson, A. H. Paterson, W. D. Park, and J. W. Stansel. 1997. Genetics of hybrid sterility and hybrid breakdown in an intersubspecific rice (*Oryza sativa* L.) population. Genetics 145:1139-1148.

Liou, L. W., and T. D. Price. 1994. Speciation by reinforcement of premating isolation. Evolution 48:1451-1459.

Machado, C. A., R. M. Kliman, J. A. Markert, and J. Hey. 2002. Inferring the history of speciation from multilocus sequence data: the case of *Drosophila pseudoobscura* and its close relatives. Mol. Biol. Evol. 19:472-488.

Mallet, J. 1995. A species definition for the Modern Sythesis. Trends Ecol. Evol. 10:294-299.

MaynardSmith, J., and J. Haigh. 1974. The hitch-hiking effect of a favourable gene. Genet. Res. 23:23-35.

Mayr, E. 1942. Systematics and the Origin of Species. Columbia University Press, New York.

Mayr, E. 1963. Animal Species and Evolution. Belknap Press, Cambridge, Mass.

Moehring, A. J., J. Li, M. D. Schug, S. G. Smith, M. deAngelis, T. F. C. Mackay, and J. A. Coyne. 2004. Quantitative Trait Loci for Sexual Isolation Between Drosophila simulans and D. mauritiana. Genetics 167:1265-1274.

Nachman, M. W. 2002. Variation in recombination rate across the genome: evidence and implications. Curr Opin Genet Dev 12:657-63.

Nachman, M. W., and G. A. Churchill. 1996. Heterogeneity in Rates of Recombination Across the Mouse Genome. Genetics 142:537-548.

Noor, M. A. 1995. Speciation driven by natural selection in *Drosophila*. Nature 375:674-675.

Noor, M. A. F. 1996. Absence of species discrimination in *Drosophila pseudoobscura* and *D. persimilis* males. Anim. Behav. 52:1205-1210.

Noor, M. A. F. 1997. Genetics of sexual isolation and courtship dysfunction in male hybrids of *Drosophila pseudoobscura* and *D. persimilis*. Evolution 51:809-815.

Noor, M. A. F. 1999. Reinforcement and other consequences of sympatry. Heredity 83:503-508.

Noor, M. A. F., A. L. Cunningham, and J. C. Larkin. 2001a. Consequences of Recombination Rate Variation on Quantitative Trait Locus Mapping Studies: Simulations Based on the Drosophila melanogaster Genome. Genetics 159:581-588.

Noor, M. A. F., K. L. Grams, L. A. Bertucci, Y. Almendarez, J. Reiland, and K. R. Smith. 2001b. The genetics of reproductive isolation and the potential for gene exchange between *Drosophila pseudoobscura* and *D. persimilis* via backcross hybrid males. Evolution 55:512-521.

Noor, M. A. F., K. L. Grams, L. A. Bertucci, and J. Reiland. 2001c. Chromosomal inversions and the reproductive isolation of species. Proceedings of the National Academy of Sciences of the United States of America 98:12084-12088.

Noor, M. A. F., M. D. Schug, and C. F. Aquadro. 2000. Microsatellite variation in populations of *Drosophila pseudoobscura* and *Drosophila persimilis*. Genet. Res., Camb. 75:25-35.

Nosil, P., B. J. Crespi, and C. P. Sandoval. 2002. Host-plant adaptation drives the parallel evolution of reproductive isolation. Nature 417:440-443.

Nosil, P., B. J. Crespi, and C. P. Sandoval. 2003. Reproductive isolation driven by the combined effects of ecological adaptation and reinforcement. Proc. R. Soc. Lond. B 270:1911-1918.

Orr, H. A. 1987. Genetics of male and female sterility in hybrids of *Drosophila pseudoobscura* and *D. persimilis*. Genetics 116:555-563.

Orr, H. A. 1995. A new linkage map of the *D. pseudoobscura* X chromosome. Drosoph. Inf. Serv. 76:127-128.

Orr, H. A., and A. J. Betancourt. 2001. Haldane's Sieve and Adaptation From the Standing Genetic Variation. Genetics 157:875-884.

Ortiz-Barrientos, D., B. A. Counterman, and M. A. F. Noor. 2004. The Genetics of Speciation by Reinforcement. PLoS Biology 2:e416.

Ortíz-Barrientos, D., J. Reiland, J. Hey, and M. A. F. Noor. 2002. Recombination and the divergence of hybridizing species. Genetica 116:167-178.

Otto, S. P. 1991. On evolution under sexual and viability selection: A two-locus diploid model. Evolution 45:1443-1457.

Palopoli, M. F., A. W. Davis, and C.-I. Wu. 1996. Discord between the phylogenies inferred from molecular *versus* functional data: Uneven rates of functional evolution or low levels of gene flow? Genetics 144:1321-1328.

Palopoli, M. F., and C.-I. Wu. 1994. Genetics of hybrid male sterility between Drosophila sibling species: a complex web of epistasis is revealed in interspecific studies. Genetics 138:329-341.

Payseur, B. A., and M. W. Nachman. 2000. Microsatellite Variation and Recombination Rate in the Human Genome. Genetics 156:1285-1298.

Perez, D. E., C.-I. Wu, N. A. Johnson, and M.-L. Wu. 1993. Genetics of reproductive isolation in the *Drosophila simulans* clade: DNA marker-assisted mapping and characterization of a hybrid-male sterility gene, Odysseus (Ods). Genetics 134:261-275.

Pfennig, K. S. 2003. A test of alternative hypotheses for the evolution of reproductive isolation between spadefoot toads: Support for the reinforcement hypothesis. Evolution 57:2842-2851.

Powell, J. R. 1983. Interspecific cytoplasmic gene flow in the absence of nuclear gene flow: Evidence from *Drosophila*. Proc. Natl. Acad. Sci. USA 80:492-495.

Powell, J. R., T. Dobzhansky, J. E. Hook, and H. E. Wistrand. 1976. Genetics of Natural Populations XLIII. Further studies on Rates of Dispersal of *Drosophila pseudoobscura* and Its Relatives. Genetics 82:493-506.

Prakash, S., R. C. Lewontin, and J. L. Hubby. 1969. A molecular approach to the study of genic heterozygosity in natural populations. IV. Patterns of genic variation in central, marginal and isolated populations of *Drosophila pseudoobscura*. Genetics 61:841-858.

Presgraves, D. C., L. Balagopalan, S. M. Abmayr, and H. A. Orr. 2003. Adaptive evolution drives divergence of a hybrid inviability gene between two species of Drosophila. Nature 423:715-719.

Rice, W. R., and E. E. Hostert. 1993. Laboratory experiments on speciation: What have we learned in forty years? Evolution 47:1637-1653.

Rice, W. R., and G. W. Salt. 1990. The evolution of reproductive isolation as a correlated character under sympatric conditions: Experimental evidence. Evolution 44:1140-1152.

Richards, S., Y. Liu, B. R. Bettencourt, P. Hradecky, S. Letovsky, R. Nielsen, K. Thornton, M. J. Hubisz, R. Chen, R. P. Meisel, O. Couronne, S. Hua, M. A. Smith, P. Zhang, J. Liu, H. J. Bussemaker, M. F. van Batenburg, S. L. Howells, S. E. Scherer, E. Sodergren, B. B. Matthews, M. A. Crosby, A. J. Schroeder, D. Ortiz-Barrientos, C. M. Rives, M. L. Metzker, D. M. Muzny, G. Scott, D. Steffen, D. A. Wheeler, K. C. Worley, P. Havlak, K. J. Durbin, A. Egan, R. Gill, J. Hume, M. B. Morgan, G. Miner, C. Hamilton, Y. Huang, L. Waldron, D. Verduzco, K. P. Clerc-Blankenburg, I. Dubchak, M. A. F. Noor, W. Anderson, K. P. White, A. G. Clark, S. W. Schaeffer, W. Gelbart, G. M. Weinstock, and R. A. Gibbs. 2005. Comparative genome sequencing of Drosophila pseudoobscura: Chromosomal, gene, and cis-element evolution. Genome Res. 15:1-18.

Rieseberg, L. H. 2001. Chromosomal rearrangements and speciation. Trends Ecol. Evol. 16:351-358.

Rieseberg, L. H., J. Whitton, and K. Gardner. 1999. Hybrid zones and the genetic architecture of a barrier to gene flow between two sunflower species. Genetics 152:713-727.

Rieseberg, L. H., Whitton, J., and K. Gardner. 1999. Hybrid zones and the genetic architecture of a barrier to gene flow between two sunflower species. Genetics 152:713-727.

Ritchie, M. G., and M. A. F. Noor. 2004. Evolutionary genetics: Gene replacement and the genetics of speciation. Heredity 93:1-2.

Roulin, A., T. W. Jungi, H. Pfister, and C. Dijkstra. 2000. Female barn owls (Tyto alba) advertise good genes. Proceedings of the Royal Society of London Series B-Biological Sciences 267:937-941.

Rundle, H. D., L. Nagel, J. W. Boughman, and D. Schluter. 2000. Natural selection and parallel speciation in sympatric sticklebacks. Science 287:306-308.

Rundle, H. D., and D. Schluter. 1998. Reinforcement of stickleback mating preferences: Sympatry breeds contempt. Evolution 52:200-208.

Sætre, G.-P., T. Moum, S. Bures, M. Kral, M. Adamjan, and J. Moreno. 1997. A sexually selected character displacement in flycatchers reinforces premating isolation. Nature 387:589-592.

Sanderson, N. 1989. Can gene flow prevent reinforcement? Evolution 43:1223-1235.

Schaeffer, S. 1995. Population genetics in Drosophila pseudoobscura: A synthesis based on nucleotide sequence data for the Adh gene. Pp. 329-352 *in* L. Levine, ed. Genetics of Natural Populations: The Continuing Importance of Theodosius Dobzhansky. Columbia University Press, New York.

Schaeffer, S. W., and E. L. Miller. 1991. Nucleotide sequence analysis of Adh genes estimates the time of geographic isolation of the Bogota population of Drosophila pseudoobscura. Proc. Natl. Acad. Sci. USA 88:6097-6101.

Schaeffer, S. W., and E. L. Miller. 1992. Estimates of gene flow in *Drosophila pseudooscura* determined from nucleotide sequence analysis of the alcohol dehydrogenase region. Genetics 132:471-480.

Schluter, D. 1998. Ecological causes of speciation. Pp. 114-129 *in* D. J. Howard and S. H. Berlocher, eds. Endless Forms: Species and Speciation. Oxford University Press, New York.

Schultz, J., and H. Redfield. 1951. Interchromosomal effects on crossing over in Drosophila. CSH Symp. Quant. Biol. 16:175-197.

Servedio, M. R. 2000. Reinforcement and the genetics of nonrandom mating. Evolution 54:21-29.

Servedio, M. R., and M. Kirkpatrick. 1997. The effects of gene flow on reinforcement. Evolution 51:1764-1772.

Servedio, M. R., and M. A. F. Noor. 2003. The role of reinforcement in speciation: Theory and data. Ann. Rev. Ecol. Evol. Syst. 34:339-364.

Sezer, M., and R. K. Butlin. 1998. The genetic basis of oviposition preference differences between sympatric host races of the brown planthopper (*Nilaparvata lugens*). Proc. Roy. Soc. Lond. B 265:2399-2405.

Shaw, D. D., P. Wilkinson, and C. Moran. 1979. A comparison of chromosomal and allozymal variation across a narrow hybrid zone in the grasshopper Caledia captiva. Chromosoma 75:333-51.

Shaw, K. L. 2001. The genealogical view of speciation. J. Evol. Biol. 14:880-882.

Singer, M. C. 1986. The definition and measurement ofoviposition preference in plant-feeding insects. Pp. 65-94 *in* J. R. Miller and T. A. Miller, eds. Insect-Plant Interactions. Springer-Verlag, New York,.

Takimoto, G., M. Higashi, and N. Yamamura. 2000. A deterministic genetic model for sympatric speciation by sexual selection. Evolution 54:1870-81.

Tan, C. C. 1935. Salivary gland chromosomes in the two races of *Drosophila pseudoobscura*. Genetics 20:392-402.

Taylor, C. E., and J. R. Powell. 1978. Habitat choice in natural populations of *Drosophila*. Oecologia 37:69-75.

Templeton, A. R. 1994. The role of molecular genetics in speciation studies. Pp. 455-477 *in* B. Schierwater, B. Streit, G. P. Wagner and R. DeSalle, eds. Molecular Ecology and Evolution: Approaches and Applications. Birkhauser Verlag, Basel, Switzerland.

Templeton, A. R., E. Routman, and C. A. Phillips. 1995. Separating population structure from population history: A cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. Genetics 140:767-782.

Thompson, J. N. 1994. The Coevolutionary Process. University of Chicago Press, Chicago.

Thompson, J. N., W. Wehling, and R. Podolsky. 1990. Evolutionary genetics of host use in swallowtail butterflies. Nature 344:148-150.

Ting, C.-T., A. Takahashi, and C.-I. Wu. 2001. Incipient speciation by sexual isolation in Drosophila: Concurrent evolution at multiple loci. Proc Nat Acad Sci USA 98:6709-6713.

Ting, C.-T., S.-C. Tsaur, and C.-I. Wu. 2000. The phylogeny of closely related species as revealed by the genealogy of a speciation gene, *Odysseus*. Proc. Natl. Acad. Sci. USA 97:5313-5316.

Ting, C.-T., S.-C. Tsaur, M.-L. Wu, and C.-I. Wu. 1998. A rapidly evolving homeobox at the site of a hybrid sterility gene. Science 282:1501-1504.

Trickett, A. J., and R. K. Butlin. 1994. Recombination suppressors and the evolution of new species. Heredity 73:339-345.

True, J. R., J. M. Mercer, and C. C. Laurie. 1996. Differences in Crossover Frequency and Distribution Among Three Sibling Species of Drosophila. Genetics 142:507-523.

Turelli, M., N. H. Barton, and J. A. Coyne. 2001. Theory and speciation. Trends Ecol. Evol. 16:330-343.

Via, S. 1990. Ecological Genetics and Host Adaptation in Herbivorous Insects - the Experimental-Study of Evolution in Natural and Agricultural Systems. Annual Review of Entomology 35:421-446.

Wallace, B. M. N., and J. B. Searle. 1994. Oogenesis in Homozygotes and Heterozygotes For Robertsonian Chromosomal Rearrangements From Natural-Populations of the Common Shrew, Sorex-Araneus. Journal of Reproduction and Fertility 100:231-237.

Wang, Q., H. Gaiti, and C. W. Pikielny. 1999. Preferential expression of biotransformation enzymes in the olfactory organs of *Drosophila malanogaster*, the antennae. J. Biol Chem. 274:10309-10315.

Wang, R. L., J. Wakeley, and J. Hey. 1997. Gene flow and natural selection in the origin of *Drosophila pseudoobscura* and close relatives. Genetics 147:1091-1106.

Welch, A. M., R. D. Semlitsch, and H. C. Gerhardt. 1998. Call duration as an indicator of genetic quality in male gray tree frogs. Science 280:1928-1930.

Williams, M. A., A. G. Blouin, and M. A. F. Noor. 2001. Courtship songs of *Drosophila pseudoobscura* and *D. persimilis*. II. Genetics of species differences. Heredity 53:157-161.

Wu, C.-I., and A. W. Davis. 1993. Evolution of postmating reproductive isolation: The composite nature of Haldane's Rule and its genetic bases. Am. Nat. 142:187-212.

Wu, C.-I., and H. Hollocher. 1998. Subtle is nature: The genetics of species differentiation and speciation. Oxford University Press, New York.

Wu, C.-I., H. Hollocher, D. J. Begun, C. F. Aquadro, Y. Xu, and M.-L. Wu. 1995. Sexual isolation in *Drosophila melanogaster:* A possible case of incipient speciation. Proc. natl. Acad. Sci. USA 92:2519-2523.

Zeng, Z.-B., C.-H. Kao, and C. J. Basten. 1999. Estimating the genetic architecture of quantitative traits. Genet. Res., Camb. 74:279-289.

APPENDIX A

Recombination Rates in *Drosophila pseudoobscura*

Chromosome X

Recombination rates between markers (cm1), cumulative recombination rate (cm2), mean recombination rates (bases/cM), estimated gap between markers (gap1), physical distance between markers (delta-bases) for markers along the X chromosome of *D. pseudoobscura*. Location shows the direction of the group sequence.

	Marker	Group	Location (bases)	Gap1	Delta-bases	Bases	Cm1	Cm2	Bases/cM
1	DpsX008	Xlgroup1a	7482817		811904	811904	15.9	15.9	51063
2	DpsX055	Xlgroup1a	6670913	1682157.6		2494062	15.8	31.7	
3	DpsX046	Xlgroup1e	12316097		1429761	3923823	9.7	41.4	147398
4	DpsX002	Xlgroup1e	10886336		1241067	5164890	13.8	55.2	89932
5	DpsX035	Xlgroup1e	9645269		3108750	8273640	16.9	72.1	183950
6	DpsX022	Xlgroup1e	6536519		660917	8934557	12.9	85	51234
7	DpsX056	Xlgroup1e	5875602		4614317	13548874	29.1	114.1	158568
8	DpsX031	Xlgroup1e	1261285	2171899.7		15720773	20.4	134.5	
9	DpsX023	Xlgroup3a	2508535	2459357		18180130	23.1	157.6	
10	DpsX036	Xrgroup6	4293648		1527569	19707699	8.2	165.8	186289
11	DpsX043	Xrgroup6	5821217	2395477.6		22103177	22.5	188.3	
12	DpsX047	Xrgroup9	2756260		1406474	23509651	17	205.3	82734
13	DpsX007	Xrgroup9	4162734		478980	23988631	13.7	219	34962
14	DpsX048	Xrgroup9	4641714	1565045.4		25553676	14.7	233.7	
15	DpsX024	Xrgroup8	1150746		1595908	27149584	10.5	244.2	151991
16	DpsX030	Xrgroup8	2746654		428196	27577780	14.9	259.1	28738
17	DpsX011	Xrgroup8	3174850		1854781	29432561	33.2	292.3	55867
18	DpsX037n	Xrgroup8	5029631		2452188	31884749	15.2	307.5	161328
19	DpsX021	Xrgroup8	7481819	2533883		34418632	23.8	331.3	
20	DpsX052	Xrgroup3a	1185942	7866485		42285117	--	--	
						Average:	17.4		106466

Mean of means recombination rate: ~106 Kb/cM
Grand mean recombination rate: ~128 Kb/cM

Chromosome 2

Recombination rates between markers (cm1), cumulative recombination rate (cm2), mean recombination rates (cM/Mb or ~Kb/cM) for markers along the 2nd chromosome of *D. pseudoobscura*.

	Marker	Location	~Kb/cM	Cm1	Cm2	Cm/Mb
1	Dps2028	1515879	106.16	13.4	13.4	9.420
2	Dps2014	2938447	341.96	2.2	15.6	2.924
3	Dps2027	3690753	100.04	10.6	26.2	9.996
4	Dps2017	4751214	112.98	16.7	42.9	8.851
5	Dps2019	6637997	152.03	17.6	60.5	6.578
6	Dps2018	9313692	111.93	8.7	69.2	8.934
7	Dps2026	10287490	164.39	11.9	81.1	6.083
8	Dps2011	12243695	152.98	27.8	108.9	6.537
9	Dps2022	16496624	219.84	4	112.9	4.549
10	Dps2021	17375999	96.60	10.7	123.6	10.352
11	Dps2024	18409605	147.83	4.8	128.4	6.764
12	Dps2012	19119203	88.51	7	135.4	11.298
13	Bcd	19738796	182.52	10.2	145.6	5.479
14	Dps2031	21600547	109.76	33.6	179.2	9.111
15	Dps2015	25288491	112.29	9.9	189.1	8.905
16	Gld	26400179	248.88	12.4	201.5	4.018
17	Dps2016	29486344	--		--	--
	Total:	30711475	Average:	12.6		7.487

Mean of means recombination rate: ~143 Kb/cM
Grand mean recombination rate: ~149 Kb/cM

Chromosome 4

Recombination rates between markers (cm1), cumulative recombination rate (cm2), mean recombination rates (bases/cM), estimated gap between markers (gap1), physical distance between markers (delta-bases) for markers along the 4th chromosome of *D. pseudoobscura*. Location shows the direction of the group sequence.

Marker	Group	Group size	Location (bases)	Gap1	Delta-bases	Bases	Cm 1	Cm 2	Bases/cM
Dps4024	3	11635473	575484		499597	499597	7.3	7.3	68438
Dps4023	3		75887	1050694		1550291	14.0	21.3	
Dps4033	1	5302587	5068643		5031784	6582075	14.0	35.3	359413
Dps4032	1		36859	2015029		8597103	4.2	39.5	
Dps4034	5	2329291	2327441		202847	8799950	4.6	44.1	44097
Dps4003	5		2124594		286041	9085991	2.2	46.3	130019
Dps4a8	5		1838553		156116	9242107	3.7	50.0	42194
Dps4a4	5		1682437			10924544	--	--	
					1.235277		6.36		143931

Mean of means recombination rate: ~144 Kb/cM
Grand mean recombination rate: ~208 Kb/cM

Chromosome 2 in *D. pseudoobscura* and *D. persimilis*

Recombination rates between markers (cm1), cumulative recombination rate (cm2), mean recombination rates (cM/Mb or ~Kb/cM) for markers along the 2nd chromosome of *D. pseudoobscura* (ps) and *D. persimilis* (per).

Markers ps	Markers per	Location	Delta-bases	per cM	per Kb/cM	ps cM	ps Kb/cM
Dps2014	Dps2014	2938447	1812767.000	6.8	266.583	10.1	179.482
Dps2017	Dps2017	4751214	1886783.000	20.3	92.945	14.2	132.872
Dps2019	Dps2019	6637997	2672918.000	9.1	293.727	15.2	175.850
Dps2018	Dps2_1109i	9310915		3.0		8.0	
Dps2026	Dps2_138b			19.7		26.9	
Dps2022	Dps2026			3.1		3.9	
Dps2021	Dps2_3447a	17301481	1108124.000	4.4	251.846	9.9	111.932
Dps2024	Dps2024	18409605	1329191.000	5.0	265.838	10.2	130.313
Bcd	Bcd	19738796	1861751.000	4.6	404.728	9.4	198.059
Dps2031	Dps2031	21600547	3687940.000	22.3	165.378	26.5	139.168
Dps2015	Dps2015	25288487		--	--	--	--

APPENDIX B

DNA Sequence Variation on the *Drosophila pseudoobscura* Chromosome 2

DNA sequence variation on the *D. pseudoobscura* 2nd chromosome and its relationship to recombination rates. Location refers to gene position on *D. pseudoobscura*, *pi* is gene nucleotide diversity for noncoding regions.

Gene	Interval	Location	Ps Kb/cM	Ps pi	Per pi
Mlc1	3_4	3801326	100	0.0104	
Dps2001	4_5	4807929	113	0.0108	0.0073
Dps2002	5_6	8068766	152	0.0152	0.0038
Xdh	7_8	11247000	164	0.0174	
Bcd	12_14	19738796	144	0.0063	
Rh1	15_16	26204786	112	0.0103	
Rh3	16_17	27681000	249	0.0070	
Trop1	16_17	27988789	249	0.0020	
Dps2003	16_17	29200493	249	0.0068	0.0038

APPENDIX C

Primer List for Microsatellite Markers in the *D. pseudoobscura* Genome

Chromosome X

Marker	Primer f	Primer r
Dps2032	Ataaaccgacatcaccgccatc	Acgagacctcctctttcgctca
DpsX054	Atcaaccggtgaattagcagca	Agacggaggagcactttgtttgt
DpsX038	Tgtggctcgtgtcagtgattttc	Aatggcactgcagcagcaac
DpsX033	Cccatccgtttgaatgctaaatt	Atacttggtggcggccctt
DpsX005	Acggcaacggtacttgaatc	Gttttgattccaggcgtgat
DpsX004	Aagtacttcattttgtcttgg	Cgtgcgcgcttataattctt
DpsX032	Tcgaattcgtagagcgggtgtt	Atctcagctggcgactgttgtagt
DpsX059	Tgaaagttgagctcacgcac	Cggatcttcgatgaacaaca
DpsX039	Aatgctcgaatgctgtttcgg	Caagagctctctcgtagcgaaaatt
DpsX055	Aacgatggtggcgaggctta	Ggatcaattgtgcccactctttt
DpsX041	Aatctgagccgtagagaatgaccaa	Aaatgcccgcaaacgagct
DpsX028	Catggtcccctcgttgtttga	Acacacatacacaggcacggg
DpsX008	Ccacagcgtagtgagcagat	Tttccttctgtgtgttggca
DpsX045	Caatggaacgacagcggatg	Ttctcacacctttggagttcctgt
DpsX031	Gctcaaggactcgttagcgtagaa	Acaagccagcaatacaggaagtca
DpsX003	Gcctacagtgagagctgcct	Tggggagtggacttatctcg
DpsX060	Tgagtgtgtgccacagtgtt	Gtagtcgttgtcgctgcc
DpsX056	Gattgctcgttttatgaatgccac	Atcggtaggcgatcgatctattg
DpsX022	Gccaaagttgaagggtccaga	Tgctgcactccgttcgataac
DpsX034	Aggagatgcactcggtactcgg	Ggactgagcaaacaaatgctcgta
Runt	Ccctgccacaagtaacaagc	Agacaaaaggggcaggtatc
DpsX035	Tggaattgtggcttgcagtca	Aaagccaagccaaagccaag
DpsX001	Gaatctctctcctgttgcgg	Ccacactcgctttcccata
DpsX002	Attcttgtcgctctgttggc	Tcagctgcgtaacaatctgg
DpsX046	Aaatcgcagcggcattgac	Aaatgcagagcaagtacacgcatc
Permicro2	Gctccttttcggttgt	Gactgccgttagagatagacctgatgac
Permicro	Gggaccttgtctgaggtttctc	Ggggacttggttaacaatatggaaacg
DpsX023	Ccatgtggacgctttctagca	Agagagaaggcaaacggagca
DpsX052	Aaaaccccgacactaagccatct	Gcggcgtatgcgcaataatt
DpsX010	Aaaaggccttattgtagttg	Agagattctcacccaccatg
DpsX017	Gccatgcacgatactttcaa	Ctcgcatcggtttattggaa
DpsX006	Agccagctctgtggtctgtt	Aaaacggtttcattgttgcc
DpsX015	Cgaggcgaagttgaatcaaa	Cagctcaatgccaatcaatg
DpsX070	Cctggatttgctaaaggtaagcga	Gcgagtcaaaatctcagactggc
E74a	Agagacagctcctgctcctg	Actcgggccgattttagttt

DpsX036	Gtgataactcgaaatttgggcca	Gcatgaaagagccaagttgacaa
DpsX018	Aaagcacacagggaattgtct	Ttgtttggcctcctcttcact
DpsX043	Aaatcagcgcctaccctcgtt	Gagctgccaactattttgcatcc
DpsX014	Atgtgtatctgtgcatgtgca	Actccacacccaaggaacaat
DpsX024	Tttgtgaggcagcagcagc	Ttcgtcctccatcctcattcg
DpsX030	Gctaacacacactcgcgcaca	Tgcacactgtgatggccaaat
DpsX011	Tgtgtattagtgtgcgcctgt	Tcggcttaaaaaacctgcaa
DpsX012	Tatgtccctgtgtgcgtgtgt	Acagcacttgcttttgctga
DpsX058	Tatcgcgggcagtcgtttagt	Cctgcaacgaattgatctcaagtg
DpsX044	Gatgactcaaccacagctcatgaa	Cacctgcatgcatttcttgtttc
DpsX061	Cgccaattaatttggtggaa	Gaattgcgatctgtgaaggtc
DpsX025	Tctgatgtcgccaccaaccat	Aaacactaagcgccgcttttc
DpsX037n	Acgagaatggtagaaggaggaatcc	Aaaggcctgatgctcctggtt
DpsX049	Actgcgggatgactatagcatcaa	Taaagtgcaagtcgcgctgc
DpsX021a	Tccttggtgcagatgctcatg	Aacagcaggcatacaccaggc
DpsX021a2	Tccttggtgcagatgctcatg	Aacagcaggcatacaccaggc
DpsX021a4	Ccgagcctttacagatgaactttatg	Ccctgttggtagcttactgttaatttc
DpsX021a1	Gaaatcgacaaatcgctgactga	Gccatatgagagcgttcgttca
DpsX021a3	Gaacctgagcggattcgaaaatt	Caagatcaacttgttgcggacaa
DpsX021a5	Ctctccagcatttacagagtcatgtt	Ccgagcaacagcatagttgtgtagat
DpsX021a6	Agcagccgcagacactcagat	Gcaaacgcttagccaaacgg
DpsX021	Gaaattaattcacattctctggcg	Aatgagctcgacaattccgc
DpsX021b2	Gagctaagccgatttcctccct	Tgccaacaacagacagccga
DpsX021b1	Cccatattaaaggtaattgattgcc	Tccaagacgtgtctacagtcgaaa
DpsX019	Gccctcacaaaaggagtgaaa	Atccctcggtcgacatcttt
DpsX016	Aagcagcacgacgagagcat	Atgaaccccaactctcagtca
DpsX050	Acacaccgaagtggaactggaagt	Tgcatccaccatccaccattt
DpsX062	Cagagacagccccaaagaga	Ttagtggcacaaacagacgg
DpsX013	Tccgcaaagtactcggcttat	Ttttgtccgtgtgcctgc
DpsX047	Cgcaaattgctgtccattcagt	Caaagttatgtggcttggcagttg
DpsX029	Actgtgtgcctgggtgaacct	Gcccagctgagctttcagctt
DpsX007	Cactcgaggttattgaacgg	Aatctatggcgggttctaag
DpsX048	Ggaaatgattcagctgctggg	Ccgagctaatcaaattaccagacg
DpsX009	Tcaggaaaagaacagcagca	Cgccacagcaaatcaactta
DpsX063	Gctctgctctggacagcc	Tgcgttgcctgataaaacct
DpsX051	Acggcgacaccttggtttg	Atctgcatctctgtgctcgcat

Chromsome 2

Marker	Primer f	Primer r
Dps2005	Attgattggggctacgtgtc	Gctaacccaatgatgaggga
Dps2028	Tcagcctccgcttcgattg	Cgcctacctcgtacctatacagcat
Dps2020	Cagaaagagccggtgaggtg	Catgtcgccagctctagcaca
Dps2029	Tctgctctcatagtggcggtttt	Agctgcttggcgtttaacaaca
Dps2014	Cccgtaccacccataggatattc	Ctcgcgctgtcaattggttaat
Dps2027	Agctgctgcgaatttggctc	Gtgtttcgtctagcgaacttctgg
Mlc	Caacagaatgttgccaacagc	Acgcctttgggatctcgaac
Clone102	Gcgcattatttgcaggcggc	Ttcaaatgaactcacatccacccac
Dps2017	Acctcgcttaccattttcctcca	Gggaaatttgtgcagcttgtga
Dps2001	Caaagacagagccaaagcct	Tgggcattaaagtgcaatca
Dps2034	Cgtgtcagccaaatggcgt	Caaagctttcgtgcaatgcttc
Dps2007	Tgcggagagagtttgtgaga	Gaactacagccagcgagagg
Dps2019	Ggacaggccacgttgaaatgt	Ttcaggggttgagggttgcta
Dps2030	Cgtggtgtgccacaagcaa	Agaatggaaatcggagattgcag
Dps2002	Acatccgcatccacatacg	Cgtcctgccaaagtgtttct
Dps2033	Ggcatcaggtgcacatctttaattc	Cacgtacgccacgcgtttt
Dps2_2803a	Ggtaacccgaggtcagcgtc	Caatccattcaatcggaggcaaacactac
Dps2_1109a	Cggaatgggacagagattgaagcc	Tttctccaagtcacgctctcaaagtatcc
Dps2_1109i	Tgtcagccatcgtgggcatc	Caacagcgtctaacgcttgtaattggc
Dps2018	Aggccaagcagcacagcaat	Agaggcaggaaggatatacacaagc
Dps2_1109b	Ccaactgacattgcgtaatgatgatgc	Cctggttggtttaatgagctggc
Dps2_1109j	Cccatcgtatcccttgatgaaaaccc	Gagcaacaacagcagccagagatac
Dps2_1109k	Tgcattcatttccattcacccgc	Gacaaattttccgctctgccacc
Dps2_1109g	Cctgccacacccaaagaaaagag	Gtacacgcacggcaatcatcc
Dps2_1109l	Aatccaccaaatttgtctctcgtacacac	Cttgtgtgtggcacgtgtgtc
Dps2_1109m	Cggctaacacagagcctgc	Cgctttcaacgcccaaaaatcaac
Dps2_1109n	Ctgctgatatcagcagtaagaacgttctg	Ggcattaggcagcgtcgac
Dps2_1109h	Gctgcctgacatggatagacgc	Ccactcgctcattcccttgtagc
Dps2_1109f	Cggcagccgcaaaaaacc	Atgtccgctcctgcaacc
Dps2013	Agcagcgcctgaactgattg	Actgagaattgtcacggctttgtg
Clone114	Tttatgcgcttggaaattgaggtc	Ggcagagcggcacttcagc
Dps2026	Cagactcttactacgagcacggaga	Gcaaatatccttgaagcagatgca
Dps2026	Cagactcttactacgagcacggaga	Gcaaatatccttgaagcagatgca
Dps2011	Acttgtctgcagctgtcagacaga	Aattgcactttgcgctgatg
Dps2_138b	Ggctcgtaaataaacatccagaggaacc	Gtatttgtcacgtctttgtggtggc
Dps2022	Ggcgcaaggtccttttttgt	Tcccgataccgacgaaacatt
Dps2_138a	Gcacattgatgatgagctccatcc	Ttcaccctaggccacataaatctcac
Dps2_138c	Cagaaacgaattgaaaatcgcacatgcac	Gctctctttcactcactatgtgcgtc
Dps2_138d	Cgatacgaagagcataatggcataagctc	Ctggtgctgtcgtttcatgtttaagtttc

Dps2_3447c	Caactcttcttcgttaagccacaagc	Ccgtttgtggttttcctggcattac
Dps2_3447a	Accagactccataattcgctacgtattttc	Tctctaggtgcgtgtttatttagtgtcaatttac
Dps2021	Tgaaatgggaaaacggcatc	Ggcatcggaaacaacaccttc
Dps2_3447b	Atcgtgctacggtggttgtcc	Ctcccttacatgggtaacctttatctgc
Dps2024	Gtgtccaaatcccacgcagat	Atgctccaaatggccgatg
Dps2012	Gtatgtgttgtgcagctttggc	Agtgcgcgtgtagaactctgtg
Clone222	Aggaatggctaaggtacgttcc	Aaagcggcgtttgtctgc
Bcd	Ccaggctcagggccagcgcc	Gcatctgatgcggcacgtgg
Dps2031	Tgttgacaatttggcgataccc	Gctgcctcatttgcattggtt
Dps2025	Tgggcgatgttcaagtgtcaa	Attatggaagcgatcgaagcg
Dps2006	Tttatcatgtgcccgagtga	Tcgctttaactcgtttcgct
Dps2_6581b	Cgttctacttcttgagtggagcaacatc	Ggtcatgtggcatgtgtaggc
Dps2015	Gccacgttctacttcttgagtgga	Ggcgctcgttgagttacgttg
Dps2_6581a	Ctagagagaaaaggagaccgcgac	Gcttgtcatcaacatcgttatattgttacggc
Dps2_6581c	Cctgcagagggaatgcacgatac	Cggcatcttatcacaccatcgcc
Rh1	Ggcaaccaccagcgaggccg	Gcttttagatattggaggcaag
Gld	Ttcacacccctgagcacaag	Gtcttcattgctgccgttgc
Dps2_534l	Aattataccggcaccaccagcc	Aaaggatgaaccttcctccaggtgtac
Dps2_534k	Ctgtaagccatattttaatggcattccacac	Cctgccgtctactgtaactgtatctc
Dps2_534g	Ccgtagaacgtgccacacg	Agaccaatgcgccatgtgc
Dps2_534j	Acgaagcggaaaagcagaatgc	Ggtccgtttagttttccacaatctctcc
Dps2_534i	Cagtcactcttccactcatttactggc	Cgttaagcgcgtgtatatctgtcgc
Dps2_534d	Tttcttcaaacgaacgtttcctcgtcc	Gggtaaagcagctgccacc
Dps2_534f	Caattttgcattcacatagcaacactaggac	Gtgttcggcaggaaaacggaaac
Dps2_534e	Gtcttgtcattcaattgttttcgactgtgtc	Ggttagcgactggttggtgc
Dps2_534h	Ggtgcatgccaccagatgc	Gtcacacgcggttcagatctgc
Dps2_534b	Ccgcacatgtcataaagaatgatgacg	Cgaattaacttcaaacaggcgtgaataatgc
Dps2_534	Ctcattcaaagcttcggctttccac	Gcacatgaatattcccggagacc
Dps2_534c	Tcgtcatcaagtattatgctgatccttgc	Agccttctgtttaaactctgaactcgaaag
Dps2035	Ccattccacagggcaaaacaa	Gttgcctgtctacttgcactctgtc
Trop1	Gattaccttgttcttatgtggc	Cgagattgatgatatttggcag
Dps2_3773	Ccactgctgttcatagtccgaaaatgc	Gctgccagttagttagcaattgcaac
Dps2_2395	Ccaaagccaacttttcagttgccaac	Tatgtgtgatttatggatttaaaactgtatacttaatatgccc
Dps2_2395d	Catgctacttacaacagccaaagagcc	Caaaacgtggcatcgccaagag
Dps2_2395b	Cctcacgagtgtatccgcacttc	Gtttgagacgaacgggagatgctc
Dps2_2395c	Gaactggaaaattgcatggccacc	Catgccacacagtgagtggc
Dps2004	Ggtacccaaagccaatctca	Acgtcctgttgaaagccact
Dps2003	Catttcaagcagaagacgca	Cctcgggtattatttcgggt
Dps2016	Gctgaatcgcgtttttggcatc	Atgatttcacgaaggtggg
Dps2_1193	Cactctggcatgcatataggccac	Ctgcagtttttgtggcagtggc
Dps2_1206	Gggcggttgctgatgcttttc	Aagccaacaatttgccatgctcc

Chromosome 3

Marker	Primer f	Primer r
Dps3005	Ggtccagaataaatgcccaa	Aactgcattgccaaacacaa
Dps3020	Aagccaagccagaccgacaa	Ccaaaccggttaggttaggcatt
Dps3026	Atttcataccgttccagggcg	Ccgttgatgcagcgctatttt
Engrailed	Ccttctccagcgagcaat	Tgtaaatattttggtgcaaatatga
Dps3021	Ggcaaaagtgtatctaattgctcga	Ctacttgtcagagccatacatcagct
Dps3027	Tggctgatgagcaaaacatgtgt	Tgcagcattagacagactcagcg
Dps3007	Ttaagcagatgggggatgag	Tttgcaagggcactaaaagc
Dps3001	Gggaaaccataagaaaatgcc	Gtacatgaatcggctacggg
Dps3022	Gttggtcgaaaaccctcatcaatt	Gcatggacatccctatccatttgt
Dps3002	Gagtccccaaaatccgaaac	Cccacaacggacagaaaaat
Dps3008	Ggatgattgaagggctgaca	Ttgataaattgccccacaca
Dps3003	Ggcccgaaaataaaacaaca	Ctgcactctctttccccctt
Dps3028	Tctttaagtggcatacttccatcca	Gcttaccacatgccaaaccaga
Dps3006	Caagtacggcaaggatttgg	Tgttgcctacacatttccca
Dps3029	Gaaacagtggctctggctttgg	Ttccagcagagctgtgtgcct
Dps3023	Gaacatgagcgagccactgctat	Tgttatacgatcgcgacgtttca
Dps3024	Gtgcctgtgtgagtgagagcgt	Ttccatgtgcctttggctttg
Dps3004	Tgaacgtggtgggtgtagaa	Gtgacaaagaggaggtccca
Dps3025	Gtgagtgtgccagggcttgaa	Ctcgttgagccgatttccatg

Chromosome 4

Marker	Primer f	Primer r
Dps4032	Aataacccgatgtcaaagagcagc	Taaacttgacgaaaggtgtgagcg
Dps4020	Actgccttgaaccactctccg	Cttgtgcctgtgtgtccgataag
Dps4028	Aaaacgactcacgagcaaag	Gagcgctttagcatagggtc
Dps4033	Cctgcatttgtccgttcttcatt	Cagtgtcagagttccagagagcca
Dps4029	Ttggaaggtattttcgtacagag	Gctcacttctacggggtttc
Dps4021	Cccacgctcttctcactgtatgtg	Acaaactttggaggcctactgga
Dps4035	Agttctccgctgtgttgctggt	Ttccgagtgtgccagtatgtgagt
Dps4022	Ttttgcgcgatagcactttgg	Gccgctgcttgactattatattgc
Dps4023	Tcagcacactgctcggcaa	Tgctcgcagacaaatggaaaa
Dps4024	Tctcagtgcagagaccacctcc	Aatacacaatagcggcagcaaca
Dpp	Ctgatgttgcagagcacgat	Tctttctttttcctcgtcgc
Dps4025	Ggacacgacacgcaaactcataa	Gttgtaaatgtggcgtgggagtat
Dps4002	Taccgtatgcaacccagctt	Cggaatgcactctgctgata
Dps4026	Actgaacgaggcagattgtgtttg	Atttggtcaggcagtgcactctt
Dps4030	Tgaactttcattgagcttctgc	Agcggaatgtactcaccgag
Dps4001	Gtctgctgcgattaaaagcc	Cggcaggcggtataaaaata
Dps4031	Cagcaggcgaatgatttcta	Gcatggtgattgtacagcga
Dps4027	Ggaggagagtacagtcctgttgcc	Tccgccatgtccatgtcct
Dps4003	Ttctgtccgctgcagccctc	Tatcaagccatcttctgcac
Dps4034	Gccacgaatcccaagtcttaacat	Ggtaaggtgcgacgaggcttact

APPENDIX D

Letter of Permission

February 23, 2005
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Title: recombination and the divergence of hybridizing species
Authors: Daniel Ortíz-Barrientos, Jane Reiland, Jody Hey, and Mohamed A.F. Noor. Archive: volume 116, issue 2 - 3, nov 2002, pages 167 – 178

I need to include the entire text and all figures. I will somewhat rearrange the article to conform to the dissertation consistency guidelines required by the university. The literature cited will be rearranged for consistent style (alphabetical by author). The article will otherwise remain unaltered. A footnote is inserted at the start of the dissertation chapter acknowledging permission from *Genetica* to use the article.

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I will promptly address any issues that may arise. Thank you for your assistance with this matter.

Sincerely,

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Dr. Daniel Ortiz-Barrientos
Louisiana State University
107 Life Sciences Building
Baton Rouge, LA 70803
USA

Van Godewijckstraat 30
3311 GX Dordrecht
The Netherlands
tel +31 (0) 78 65 76-000
fax +31 (0) 78 65 76-254
www.springeronline.com

17/02/2005

Re: Genetica 116 (2): 167-178, 2004, (Ortiz-Barrientos, Reiland, Hey,& Noor)

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Daniel Ortiz-Barrientos was born in Medellín, Colombia, to Jorge Ortiz and Pilar Barrientos, and is married to Cristina Lopez-Gallego. He went to Universidad de Antioquia to study biology and had his first research experience in human population genetics under the guidance of Dr. Andres Ruiz-Linares. After graduating in 1998, he went to London to continue his studies in human population genetics and during this period he develop interests on speciation, adaptation and molecular evolution. He started his doctoral studies in the fall of 2000 at Louisiana State University under the supervision of the superb Dr. Mohamed Noor and will receive the degree of Doctor of Philosophy in May 2005. Daniel is deeply interested in understanding both the genetic and ecological factors contributing to the origin of the species. After graduation, Daniel will begin a postdoctoral position with Loren Rieseberg, a plant evolutionary geneticist also in love with the origin of the species.