



## The nature of genetic variation in sex and reproduction-related genes among sibling species of the *Drosophila melanogaster* complex

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### Abstract

Much is known about the biology of *Drosophila melanogaster*. As a model organism, a comprehensive understanding of its development, physiology and reproduction has been acquired. As a result, a broad variety of transferable genetic tools and information has allowed sibling species of the *D. melanogaster* complex to emerge as an important speciation model system. By comparing *D. melanogaster* with its close relative, *Drosophila simulans*, as well as its other sibling species, we are beginning to understand the nature of genetic changes during the early stages of speciation. In general, we find that genes and traits involved in sex and reproduction are more variable. A large assortment of genes and traits that are involved in various aspects of mating and fertility reveal diagnostic differences between these sibling species. Sex and reproduction-related (SRR) genes are, on average, more diverged than genes with no apparent reproductive function. Furthermore, SRR genes appear more permissive at opting in novel function. These results follow a general trend observed in other taxa and demonstrate the preferential involvement of SRR genes in reproductive isolation and species formation.

### Introduction

#### *D. melanogaster* species complex as a speciation model system

Understanding the processes by which species are formed is one of the most fundamental objectives in evolutionary biology. Yet despite its importance, speciation has largely remained a mystery until recently. For nearly a century after Darwin (1859) originally framed the speciation problem, the study of speciation did not significantly advance. Only now is speciation beginning to be understood in a unified manner. The coupling of traditional genetic tools with recent advances in molecular biology is offering solutions to the problem of speciation. Still, much of our comprehension about speciation arises from a strong focus on reproductive isolation, initiated during the Synthesis years (Dobzhansky, 1937; Mayr, 1942). The parity between speciation and reproductive isolation

remains a successful legacy from this period although increasingly more emphasis is currently being placed on populational aspects of the speciation process (see Howard & Berlocher, 1998).

Perhaps the most successful approach in understanding how reproductive isolation evolves has been to genetically dissect the conspicuous differences between closely related species and their hybrids. Consequently, the genetic bases of many phenotypic differences have been thoroughly studied in a variety of traits among sibling species. Thus, the realization that mechanisms of reproductive isolation may lead to speciation has allowed species formation to become an experimentally amenable problem. In one class of differences, incompatibilities found in the interspecific hybrids (i.e., inviability and sterility) allow one to track the nature of the genetic changes that cause reproductive isolation. Over the years, increased interest in such phenomena as Haldane's rule (the preferential involvement of the heterogametic sex

in F<sub>1</sub> hybrid incompatibilities) across a wide spectrum of taxa has led to the development of new tools and methodologies that permit reproductive isolation to be comprehensively analyzed. For example, Dobzhansky (1936) pioneered some of the first studies of hybrid male sterility in the two sibling species of *Drosophila*, *D. pseudoobscura* and *D. persimilis*, by using a series of backcrosses to track sterility factors to particular chromosomes. He subsequently demonstrated that the X-chromosome has a large effect on hybrid sterility which lead to the development of various genetic models of species divergence (see Coyne, 1992).

Using this approach, it becomes apparent that the major limitation in studying speciation lies in the availability of genetic tools that have been developed in a given experimental system. Fortunately, the genetic armament of *D. melanogaster* has allowed such studies to be performed in a more comprehensive manner. *D. melanogaster* has become an important model organism in many biological fields that have been able to utilize its large collection of mutant phenotypes, its well developed molecular techniques, and most recently, its sequenced genome. Most importantly for speciation studies, *D. melanogaster* is part of a species clade which includes the three sibling species, *D. simulans*, *D. mauritiana*, and *D. sechellia* (Lachaise et al., 1986). As a consequence, many genetic tools and procedures can easily be transferred between these species. Furthermore, since each of these species possesses a unique origin, a whole range of speciation models including biogeography (Lachaise et al., 1988), demography (Kliman et al., 2000), and heterospecific gene flow (Kliman et al., 2000; Ballard, 2000) can effectively be studied.

To date, species of the *D. melanogaster* complex are considered the most examined speciation model system (Kliman et al., 2000). Accordingly, a broad range of differences from morphology to molecules have accumulated in the literature. Studying the differences between species of this complex has become a powerful approach in understanding the type of genetic changes that occur in the early stages of species divergence (see Capy, 2004) and will ultimately provide answers about the origin of species. In this paper, we show that the reproductive system is a common component of the many differences that exist between sibling species of the *D. melanogaster* complex. In particular, we report on this component's high variability and address its implications to speciation.

## Functional basis of speciation

Both hybrid incompatibilities and species-specific differences play an important role in the development of reproductive isolation between incipient species and studying such differences between species and their hybrids have offered valuable insights into the speciation process. In *The Genetic Basis of Evolutionary Change*, Lewontin (1974) asks the following important questions, "What is the genetic difference between two species? .. Is the reproductive isolation a result of differentiation of a few loci only?" (p. 163). While these questions allude to the number of loci involved in various isolating mechanisms, such questions may also be asked along a qualitative front. Specifically, a *functional* understanding of such genes involved in species isolation may prove more productive in our attempts to elucidate mechanisms of speciation. In particular, we may ask the questions, "What functional class constitutes the subset of genes important in speciation? And why would this particular genetic subset be preferentially involved in speciation?" Fortunately, current advances in genomics have made such queries addressable.

One functional approach to classify genes differentiates between sexual and nonsexual genetic systems (see Singh, 2000). We note that such a classification may be imperfect in some cases as numerous genes and functions have effects in both sexual and nonsexual systems (in fact many of these functionally overlapping genes/traits are species-specific as in the case of secondary sexual characters). However, differences in fitness components – between fertility and viability – have already been utilized (but paid less attention to until recently) in theoretical and empirical population genetics (Prout, 1971; Kingsolver et al., 2001), thus setting a precedent in the sex versus nonsex dichotomy.

This classification also makes sense in light of the recent focus on sexually selected traits. One relevant development has been the extension of sexual selection to traits other than the usual secondary sexual characters (Eberhard, 1996; Civetta & Singh, 1998a). Previous examples focussed on classical morphological traits involved in precopulatory courting. This extension increases the number of traits on which sexual selection could act upon. For example, Eberhard (1985) demonstrated that male genitalia, directly involved in copulation, are extremely diverged in a variety of animal taxa and proposed that this diversity is generated by sexual selection. Proteins

involved in fertilization such as *Drosophila* accessory gland proteins (Aguadé, Miyashita & Langley, 1992; Clark et al., 1995; Tsaur & Wu, 1997) are also highly diverged and sexual selection, particularly sperm competition, may be the causal factor. Because sexual conflict and sexual coadaptation involves the coevolution of male and female traits, female traits/genes are also expected to be highly diverged. Civetta and Singh (1995), using two-dimensional electrophoresis, demonstrated that proteins from male and female reproductive tracts are more diverged between closely related species of *Drosophila* than are proteins from other sampled tissue.

An accumulating number of examples of sex and reproduction-related (SRR) genes have been found to be rapidly evolving among a wide range of species (Singh & Kulathinal, 2000) and allude to such genes comprising a different genetic component. The use of an extended or 'broad-sense' concept of sexual selection (Civetta & Singh, 1999) allows us to abandon the classical view of speciation as simply the gradual divergence of allopatric taxa. Sexual selection may represent an important force that increases the rate of speciation. The greater fitness component found in sexual systems within species (Prout, 1971; Hoekstra et al., 2001; Kingsolver et al., 2001) may translate into the phenotypic variation we observe between species. Thus, a new and encompassing view of speciation, based on the SRR component of the gene pool, is being formed.

### Sexual traits and species differentiation in the *D. melanogaster* complex

Evidence is mounting that a variety of traits involved in sex and reproduction are evolving rapidly in a variety of taxa. The use of sibling species from the *D. melanogaster* complex has especially been important in uncovering evidence that SRR traits/genes are preferentially involved in speciation. Table 1 shows multiple examples of species-specific differences that have been observed between members of this species complex. On a molecular level, two-dimensional electrophoresis on reproductive tract proteins first demonstrated the rapid evolution of SRR genes. Such experiments using sibling species of *D. melanogaster* found that, on average, testis proteins evolve more rapidly than proteins from other sampled tissues (Coulthart & Singh, 1988; Thomas & Singh, 1992). In particular, these studies found that between

Table 1. Examples of SRR trait differences among species of the *D. melanogaster* complex

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Reproductive morphology
Genital arch shape (Coyne & Kreitman, 1986; Liu et al., 1996)
Sex comb tooth number (Coyne, 1985)
Sperm morphology (Karr & Pitnick, 1996; Joly et al., 1997)
Testes length/area (Civetta & Singh, 1998b)
Hybrid male sterility
Common postzygotic mechanism (Bock, 1984; Lachaise et al., 1986)
Differences in severity of F <sub>1</sub> hybrid sterility (Kulathinal & Singh, 1998)
Rapid evolution of sterility gene, <i>OdsH</i> (Ting et al., 1998)
Divergence of testis-expressed proteins (Civetta & Singh, 1995)
Mating behavior
Courtship song and display (Greenspan & Ferveur, 2000; Moulin et al., 2001)
<i>period</i> evolution (Kyriacou & Hall, 1986)
Pheromonal profile (Jallon & David, 1987; Coyne, Crittenden & Mah, 1994)
Duration of mating (Coyne & Kreitman, 1986)
Sperm competition
Accessory gland protein evolution (Clark et al., 1995; Begun et al., 2000)
Sperm precedence (Price, Coyne & Dyer, 1999; Civetta & Clark, 2000)

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sibling species of *Drosophila*, which are morphologically similar but produce sterile male hybrids when crossed (Lachaise et al., 1986; Kulathinal & Singh, 1998), 20% of all variable testis protein spots revealed an absence of a detectable homolog in one of the species. These may represent proteins that have diverged significantly, possess varying levels of gene expression, or are novel. Furthermore, highly diverged testis proteins, for the most part, were found to be less polymorphic among individuals within species (consistent with rapid diversifying selection) and in many cases differed in levels of gene expression between species. In a subsequent study, using a larger sample of proteins, tissue comparisons and species groups, it was found that not only were male reproductive tissues rapidly evolving, but female reproductive tissues (i.e., ovaries) were also highly diverged between sibling species (Civetta & Singh, 1995).

Associated with the high divergence of reproductive proteins is the prevalence of hybrid male

Table 2. Varying degrees of male sterility in interspecific F<sub>1</sub> hybrids of the *D. melanogaster* complex<sup>a</sup>

Female parent	Male parent			
	<i>mel</i>	<i>sim</i>	<i>sec</i>	<i>mau</i>
<i>mel</i>	–	Male absent	Male absent	Male absent
<i>sim</i>	Aspermic	–	Postmeiotic	Postmeiotic
<i>sec</i>	Aspermic	Postmeiotic	–	Postmeiotic
<i>mau</i>	Aspermic	Premeiotic	Premeiotic	–

<sup>a</sup> Data compiled from Lachaise et al. (1986) and Kulathinal and Singh (1998).  
*mel*, *D. melanogaster*; *sim*, *D. simulans*; *sec*, *D. sechellia*; *mau*, *D. mauritiana*.

sterility in many taxa, particularly *Drosophila*. In fact, Haldane's rule (Haldane, 1922) appears to be a universal phenomenon in the genus *Drosophila* (Bock, 1984) and hybrid male sterility is, in most cases, the first incompatibility to evolve (Wu, 1992; Wu & Davis, 1993). The latter rule is certainly true among F<sub>1</sub> hybrids of the *D. melanogaster* complex (Lachaise et al., 1986). But even hybrid male sterility manifests different phenotypes depending on the species crossed and these sterility phenotypes relate directly to the severity of defects in spermatogenesis. For example, crosses between females of any of the three sibling species of the *D. simulans* clade to a *D. melanogaster* male will produce spermless F<sub>1</sub> male progeny with sometimes atrophied testes (Lachaise et al., 1986). (The reciprocal cross, i.e., to a *D. melanogaster* mother, will result in lethal male hybrids.) In crosses between sibling species of the *D. simulans* clade, a variety of sterility phenotypes, that are clearly consistent within genotypes, are found among the F<sub>1</sub> progeny (Kulathinal & Singh, 1998). The most severe sterility is observed when crossing a *D. mauritiana* mother to either a *D. simulans* or *D. sechellia* father – F<sub>1</sub> males contain testes in which the spermatocytes do not progress beyond meiosis (Table 2). This extreme sterility phenotype is most likely caused by an X-linked or cytoplasmic factor originating from *D. mauritiana*. In contrast, other F<sub>1</sub> hybrids from this clade progress to at least a post-meiotic stage of spermatogenesis. These observations indicate the rapid evolution of numerous male reproductive proteins and/or highlight the sensitivity of reproductive systems to genetic perturbation.

A growing number of sex-specific characters involved in mating and fertility are also observed to reveal species-specific differences (Table 1). Traits affecting copulation and fertility which include such primary sexual traits as testis and sperm length (Joly,

Bressac & Lachaise, 1995; Karr & Pitnick, 1996) and secondary traits such as sex combs (Coyne, 1985) and genital arch morphology (Lachaise et al., 1986; Liu et al., 1996) have been found to be highly diverged between species of the *D. melanogaster* complex. Genes that affect mating behavior such as *period* (involved in *Drosophila* mate song rhythm; Ritchie & Kyriacou, 1994) as well as those involved in sperm competition as exemplified by such accessory gland proteins as *Acp26Aa* and *Acp70A* in *Drosophila* (Cirera & Aguadé, 1997; Tsaur & Wu, 1997; also see Begun et al., 2000), have also been demonstrated to be rapidly evolving. Accessory glands are associated with the male testis and are necessary for successful fertilization. In a large-scale EST comparison, Swanson et al. (2001) found significantly higher divergence, in

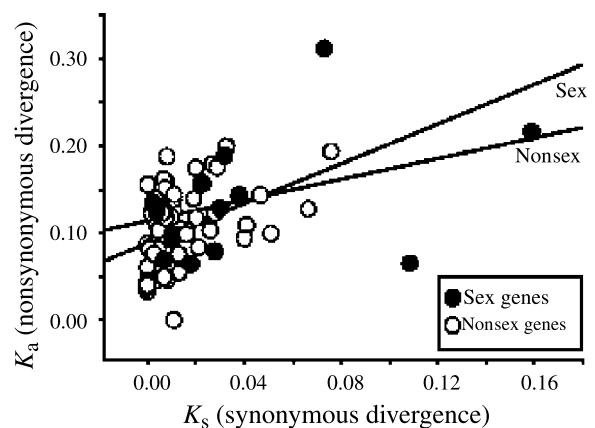


Figure 1.  $K_s$  versus  $K_a$  between orthologs of *D. melanogaster* and *D. simulans*. Both partial and complete sequences from 75 orthologous loci of the sibling species pair were aligned and divergences estimated.  $K_s$ , synonymous substitutions per synonymous site;  $K_a$ , nonsynonymous substitutions per nonsynonymous site. Divergences are calculated by the method of Nei and Gojobori (1986). Genes are classified as sex if they were sex-specific in expression pattern or possess a sex-specific *D. melanogaster* mutant phenotype.

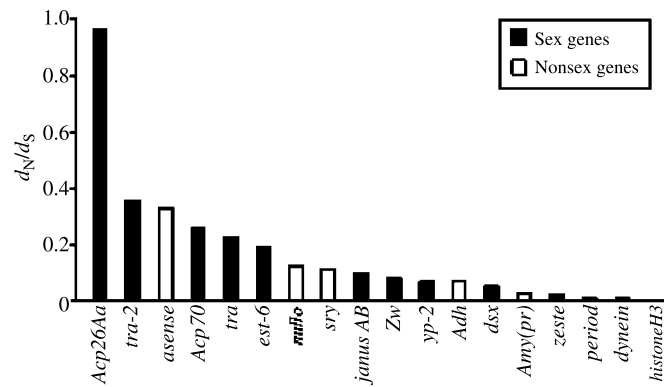


Figure 2. Estimates of the parameter  $d_N/d_S$  between orthologs of the *D. melanogaster* complex. Only loci that had been sequenced in all four species of the *D. melanogaster* complex are listed. Loci and statistical tests based on various models using a codon substitution model and maximum likelihood are found in Appendix A. Loci are classified as sex genes based on either one of two criteria: (1) the phenotypic mutant in *D. melanogaster* possesses a sex-specific effect, (2) its mRNA has been solely isolated from a sex-specific tissue or cDNA library.

terms of  $d_N/d_S$  (see below), in accessory gland sequences between *D. melanogaster* and *D. simulans* compared to sequences not expressed in accessory glands.

As Table 1 shows, many of the differences between *D. melanogaster* and its sibling species are *reproductive* in nature. In an attempt to quantify the rate of divergence among SRR genes, we utilized the large set of genes that have been sequenced in both *D. melanogaster* and *D. simulans*. From 75 aligned orthologs we classified genes as either sex or nonsex. Sex genes possessed phenotypic mutants from *D. melanogaster* that were sex-specific in their effects and/or sex-specific expression patterns. Sex genes ( $n = 14$ ) were, on average, more diverged, at least in terms of nonsynonymous substitutions per site ( $K_a$ ), than genes with no discernable sex-specific effect (Figure 1;  $K_a$ , one-tailed Student's  $t$ -test,  $P = 0.018$ ;  $K_s$ , one-tailed Student's  $t$ -test,  $P = 0.051$ ). Additionally, among 18 loci that have been sequenced in all four species of the *D. melanogaster* complex,  $d_N/d_S$  was estimated using a codon substitution model and maximum likelihood approach (Yang, 1997). Basically,  $d_N/d_S$  is an estimated model parameter that reflects the relative proportion of nonsynonymous to synonymous substitutions per site and may be higher owing to lower selective constraints or positive Darwinian selection. Sex genes were found to have a higher mean  $d_N/d_S$  compared to nonsex genes. However, we remark that this difference is not statistically significant owing to the large variance in  $d_N/d_S$  among sex genes (Figure 2; mean sex- $d_N/d_S = 0.20$  v.s. mean nonsex- $d_N/d_S = 0.11$ ;  $P = 0.18$ ).

### Novel SRR genes in the *D. melanogaster* subgroup

Novel evolutionary innovations that are involved in sex and reproduction abound in the literature and sexual selection has been suggested to direct the evolution of such novelty (see Andersson, 1994). For example, Carson (1997) observed a number of newly evolved sexual characters between closely related species of Hawaiian *Drosophila*. In members of the *D. planitibia* subgroup, the distribution of foreleg cilia, which are used in copulation, have diverged between species, most likely due to sexual selection. Thus, sexual selection may be an important force in the generation of morphological innovation.

Parallel to the evolution of sexual trait novelty, a growing number of molecular studies are discovering genes that have recently appeared *de novo* and have evolved a novel function in reproductive tissue. Many of these genes also show high rates of nucleotide substitution. One classic example demonstrated the rapid fixation of 13 amino acids in a testis-specific isoform of cytochrome c in mouse (Carlson et al., 1977). This particular isoform is coded by a gene that was duplicated from the relatively conserved, but ubiquitously expressed, cytochrome c gene.

The *D. melanogaster* complex holds striking evidence of reproductive genes newly evolving. For example, *Sdic* has recently evolved *de novo* in the *D. melanogaster* lineage (Nurminsky et al., 1998). This gene has been demonstrated to be a fusion between two neighboring loci and functions specifically in *D. melanogaster* sperm as an axonemal

dynein subunit. *Sdic* possesses a novel testis-specific promoter derived from a protein-coding region and contains a new protein-coding exon derived from an intron. Interestingly, a selective sweep has been thought to have taken place in *Sdic* (Nurminsky et al., 1998; Kulathinal et al., 2003a). Other cases of genes evolving a novel testis function have also been demonstrated in the *D. melanogaster* subgroup. The newly evolved *ocnus* locus is found in members of the *D. melanogaster* subgroup but not among species of the *D. obscura* group (Parsch et al., 2001) and is testis-specific in expression. After identifying *Odysseus* as a factor involved in hybrid male sterility between *D. simulans* and *D. mauritiana*, Ting et al. (1998) demonstrated the rapid divergence of its homeodomain, which may coincide with novel function in the *D. mauritiana* testes. *jing-wei* represents another example of a gene which was demonstrated to result from recent duplications in *Drosophila* (Long, Wang & Zhang, 1995). In most of the examples above, novel function was acquired in the testis and high rates of evolutionary change were evident.

The sex determination gene, *transformer*, has also been recently found to possess coding regions that have evolved *de novo* among species of the *D. melanogaster* complex through duplication events (O'Neil & Beloté, 1992; Kulathinal et al., 2003b). *D. melanogaster* contains a tandem duplication of 13 amino acids which adds a third arginine-serine (RS) domain – important for protein-protein interaction – to the *tra* protein. Most surprising is another independent tandem duplication of 74 amino acids (almost 30% of the total protein) that occurred solely in the *D. sechellia* lineage. This duplication adds two additional RS domains and drastically alters the protein structure of this important developmental gene. Neither *D. simulans* nor *D. mauritiana* possess either of these insertions. The consequence of such duplication events to the overall sexual phenotype is currently being pursued experimentally.

The consistent appearance of novelty in sexual systems may indicate that these systems contain large yet concealed amounts of genetic variation and furthermore, such systems can tolerate large genetic perturbations. However, many more examples must be observed to validate this evolutionary pattern. Duplication events and rapid gene evolution are a major evolutionary source of new protein function (Ohno, 1970) and it remains an intriguing supposal that sexual systems may be an important field for evolutionary novelty to occur.

## Variability of sexual systems

High variability has generally been found among sexual systems in species of the *D. melanogaster* clade. One important consequence of highly variable sexual systems is that they may serve as depots of genetic variation which allow for the introduction of evolutionary novelties and eventually, adaptive mechanisms of evolutionary change. Such flexible genetic systems increase the probability that nonlethal genetic mutations accumulate, thereby increasing the appearance of phenotypic innovations.

While sexual selection may be driving much of the SRR gene divergence, the high degree of genetic variability found among SRR genes may also be attributed to selectively neutral changes. But the classification of this variation as neutral does not imply that these alterations are functionless. They simply represent alternative forms that are (nearly) equally fit or acceptable, in terms of survival and reproduction of the organism (Kimura, 1968, 1983). This important qualifier of neutral theory differs from previous misconceptions which suggest that amino acid substitutions that are absolutely impartial to the action of natural selection can be considered 'genetic junk'. This latter term represents a misnomer which prevents us from further understanding the potential importance of transient neutral polymorphisms as a source of heritable genetic material. As Kimura stated,

"We should not overlook the possibility that some of the 'neutral' alleles may become advantageous under an appropriate environmental condition or a different genetic background; thus, neutral mutants have a latent potential for selection. This means that polymorphic molecular mutants, even if selectively neutral under prevailing conditions of a species, can be the raw material for future adaptive evolution. To regard random fixation of neutral mutants as 'evolutionary noise' is inappropriate and misleading." (Kimura, 1983, p. xiii)

The presence of greater genetic variation in sexual systems offers a number of important consequences for the development of phenotypic diversity. Less selective constraints on genes involved in sex and reproduction will generate a larger pool of genetic variation thereby increasing the evolutionary potential of that genetic system. The addition of such selective mechanisms as sexual selection, may then drive the rapid divergence of sexual traits. Furthermore, the rapid fixation of alleles between species, whether caused by

selection or drift of neutral alleles plays an important role in the evolution of reproductive isolation. According to the Dobzhansky–Muller incompatibility model of speciation (Dobzhansky, 1937; Muller, 1942; also see Orr, 1995), any acceleration of the fixation process within populations will accelerate the production of incompatibilities in the hybrid. This model demonstrates the importance of variable sexual systems in the generation of organic diversity at both micro- and macro-evolutionary scales.

## Conclusions

The following three lines of evidence observed between sibling species of the *D. melanogaster* complex suggest that a high degree of genetic variability is found among *sex* and *reproduction-related* genetic systems: (1) SRR traits are preferentially involved in species differences, (2) an increasing number of SRR genes have been found to evolve more rapidly, and (3) SRR traits/genes have a greater propensity for being recruited for novel function. These observations suggest, in general, that sexual systems not only maintain greater variation but are also major drivers of evolutionary change. In conjunction with sexual selection, the rapid evolution of genes and traits that comprise variable sexual systems is argued to be an important factor in speciation and the generation of the diversity of life.

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## References

- Aguadé, M., N. Miyashita & C.H. Langley, 1992. Polymorphism and divergence in the *Mst26A* male accessory gland gene region in *Drosophila*. *Genetics* 132: 755–770.
- Andersson, M.B., 1994. *Sexual Selection*. Princeton University Press, Princeton, NJ.
- Ballard, J.W., 2000. Comparative genomics of mitochondrial DNA in members of the *Drosophila melanogaster* subgroup. *J. Mol. Evol.* 51: 48–63.
- Begun, D.J., P. Whitley, B.L. Todd, H.M. Waldrip-Dail & A.G. Clark, 2000. Molecular population genetics of male accessory gland proteins in *Drosophila*. *Genetics* 156: 1879–1888.
- Bock, I.R., 1984. Interspecific hybridization in the Genus *Drosophila*. *Evol. Biol.* 18: 41–70.
- Capy, P. & P. Gibert, 2004. *Drosophila melanogaster*, *Drosophila simulans*: so similar yet so different. *Genetica* 120: 5–16.
- Carlson, S.S., G.A. Mross, A.C. Wilson, R.T. Mead, L.D. Wolin, S.F. Bowers, N.T. Foley, A.O. Muijsers & E. Margoliash, 1977. Primary structure of mouse, rat, and guinea pig cytochrome c. *Biochemistry* 16: 1437–1442.
- Carson, H.L., 1997. Sexual selection: a driver of genetic change in Hawaiian *Drosophila*. *J. Hered.* 88: 343–352.
- Cirera, S. & M. Aguadé, 1997. Evolutionary history of the sex-peptide (*Acp70A*) gene region in *Drosophila melanogaster*. *Genetics* 147: 189–197.
- Civetta, A. & R.S. Singh, 1995. High divergence of reproductive tract proteins and their association with postzygotic reproductive isolation in *Drosophila melanogaster* and *Drosophila virilis* group species. *J. Mol. Evol.* 41: 1085–1095.
- Civetta, A. & R.S. Singh, 1998a. Sex-related genes, directional sexual selection, and speciation. *Mol. Biol. Evol.* 15: 901–909.
- Civetta, A. & R.S. Singh, 1998b. Sex and speciation: Genetic architecture and evolutionary potential of sexual versus nonsexual traits in the sibling species of the *Drosophila melanogaster* complex. *Evolution* 52: 1080–1092.
- Civetta, A. & R.S. Singh, 1999. Broad-sense sexual selection, sex gene pool evolution, and speciation. *Genome* 42: 1033–1041.
- Civetta, A. & A.G. Clark, 2000. Correlated effects of sperm competition and postmating female mortality. *Proc. Natl. Acad. Sci. USA* 97: 13162–13165.
- Clark, A.G., M. Aguadé, T. Prout, L.G. Harshman & C.H. Langley, 1995. Variation in sperm displacement and its association with accessory gland protein loci in *Drosophila melanogaster*. *Genetics* 139: 189–201.
- Coulthart, M.B. & R.S. Singh, 1988. High level of divergence of male-reproductive-tract proteins between *Drosophila melanogaster* and its sibling species, *D. simulans*. *Mol. Biol. Evol.* 5: 182–191.
- Coyne, J.A., 1985. Genetic studies of three sibling species of *Drosophila* with relationship to theories of speciation. *Genet. Res.* 46: 169–192.
- Coyne, J.A., 1992. Genetics and speciation. *Nature* 355: 511–515.
- Coyne, J.A. & M. Kreitman, 1986. Evolutionary genetics of two sibling species, *Drosophila simulans* and *Drosophila sechellia*. *Evolution* 40: 673–691.
- Coyne, J.A., A.P. Crittenden & K. Mah, 1994. Genetics of a pheromonal difference contributing to reproductive isolation in *Drosophila*. *Science* 265: 1461–1464.
- Darwin, C.R., 1859. *On the Origins of Species*. John Murray, London.
- Dobzhansky, Th., 1936. Studies on hybrid sterility. II. Localization of sterility factors in *Drosophila pseudoobscura* hybrids. *Genetics* 21: 566–570.
- Dobzhansky, Th., 1937. *Genetics and the Origin of Species*. Columbia University Press, New York.
- Eberhard, W.G., 1985. *Sexual Selection and Animal Genitalia*. Harvard University Press, Cambridge, MA.
- Eberhard, W.G., 1996. *Female Control: Sexual Selection by Cryptic Female Choice*. Princeton University Press, Princeton, NJ.
- Greenspan, R.J. & J.F. Ferveur, 2000. Courtship in *Drosophila*. *Annu. Rev. Genet.* 34: 205–232.
- Haldane, J.B.S., 1922. Sex-ratio and unisexual sterility in hybrid animals. *J. Genetics* 12: 101–109.
- Hoekstra, H.E., J.M. Hoekstra, D. Berrigan, S.N. Vignieri, A. Hoang, C.E. Hill, P. Beerli & J.G. Kingsolver, 2001. Strength and tempo of directional selection in the wild. *Proc. Natl. Acad. Sci. USA* 98: 9157–9160.

- Howard, D.J. & S.H. Berlocher (eds), 1998. *Endless Forms: Species and Speciation*. Oxford University Press, New York.
- Jallon, J.-M. & J.R. David, 1987. Variations in cuticular hydrocarbons among the eight species of the *Drosophila melanogaster* subgroup. *Evolution* 41: 294–302.
- Joly, D., C. Bressac & D. Lachaise, 1995. Disentangling giant sperm. *Nature* 377: 202.
- Joly, D., C. Bazin, L.-W. Zeng & R.S. Singh, 1997. Genetic basis of sperm and testis length differences and epistatic effect on hybrid inviability and sperm motility between *Drosophila simulans* and *D. sechellia*. *Heredity* 78: 354–362.
- Karr, T.L. & S. Pitnick, 1996. The ins and outs of fertilization. *Nature* 379: 405–406.
- Kimura, M., 1968. Evolutionary rate at the molecular level. *Nature* 217: 624–626.
- Kimura, M., 1983. *The Neutral Theory of Molecular Evolution*. Cambridge University Press, New York.
- Kingsolver, J.G., H.E. Hoekstra, J.M. Hoekstra, D. Berrigan, S.N. Vignieri, C.E. Hill, A. Hoang, P. Gibert & P. Beerli, 2001. Strength of phenotypic selection. *Am. Nat.* 157: 245–261.
- Kliman, R.M., P. Andolfatto, J.A. Coyne, F. Depaulis, M. Kreitman, A.J. Berry, J. McCarter, J. Wakeley & J. Hey, 2000. The population genetics of the origin and divergence of the *Drosophila simulans* complex species. *Genetics* 156: 1913–1931.
- Kulathinal, R.J. & R.S. Singh, 1998. Cytological characterization of premeiotic versus postmeiotic defects producing hybrid male sterility among sibling species of the *Drosophila melanogaster* complex. *Evolution* 52: 1067–1079.
- Kulathinal, R.J., S.A. Sawyer, C.D. Bustamante, D.I. Nurminsky, R. Ponce, J.M. Ranz & D.L. Hartl, 2003a. Selective sweep in the evolution of a new sperm-specific gene in *Drosophila*, in *Selective Sweeps*, edited by D.I. Nurminsky. Landes Company, Austin, TX (in press).
- Kulathinal, R.J., L. Skwarek, R.A. Morton & R.S. Singh, 2003b. Rapid evolution of the sex-determining gene, *transformer*: Structural diversity and rate heterogeneity among sibling species of *Drosophila*. *M.B.E. Mol. Biol. Evol.* 20: 441–452.
- Kyriacou, C.P. & J.C. Hall, 1986. Interspecific genetic control of courtship song production and reception in *Drosophila*. *Science* 232: 494–497.
- Lachaise, D., J.R. David, F. Lemeunier, L. Tsacas & M. Ashburner, 1986. The reproductive relationships of *Drosophila sechellia* with *D. mauritiana*, *D. simulans*, and *D. melanogaster* from the Afrotropical region. *Evolution* 40: 262–271.
- Lachaise, D.L., M.-L. Cariou, J.R. David, F. Lemeunier, L. Tsacas & M. Ashburner, 1988. Historical biogeography of the *Drosophila melanogaster* species subgroup. *Evol. Biol.* 22: 159–226.
- Lewontin, R.C., 1974. *The Genetic Basis of Evolutionary Change*. Columbia University Press, New York.
- Liu, J., J.M. Mercer, L.F. Stam, G.C. Gibson, Z.B. Zeng & C.C. Laurie, 1996. Genetic analysis of a morphological shape difference in the male genitalia of *Drosophila simulans* and *D. mauritiana*. *Genetics* 142: 1129–1145.
- Long, M., W. Wang & J. Zhang, 1995. Origin of new genes and source for N-terminal domain of the chimerical gene, *jingwei*, in *Drosophila*. *Gene* 238: 135–141.
- Mayr, E., 1942. *Systematics and the Origin of Species*. Columbia University Press, New York.
- Moulin, B., F. Rybak, T. Aubin & J.M. Jallon, 2001. Compared ontogenesis of courtship song components of males from the sibling species, *D. melanogaster* and *D. simulans*. *Behav. Genet.* 31: 299–308.
- Muller, H.J., 1942. Isolating mechanisms, evolution, and temperature. *Biol. Symp.* 6: 71–125.
- Nei, M. & T. Gojobori, 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Mol. Biol. Evol.* 3: 418–426.
- Nurminsky, D.I., M.V. Nurminskaya, D. DeAguiar & D.L. Hartl, 1998. Selective sweep of a newly evolved sperm-specific gene in *Drosophila*. *Nature* 396: 572–575.
- Ohno, S., 1970. *Evolution by Gene Duplication*. Springer, New York.
- O'Neil, M.T. & J.M. Beloté, 1992. Interspecific comparison of the *transformer* gene of *Drosophila* reveals an unusually high degree of evolutionary divergence. *Genetics* 131: 113–128.
- Orr, H.A., 1995. The population genetics of speciation: The evolution of hybrid incompatibilities. *Genetics* 139: 1805–1813.
- Parsch, J., C.D. Meiklejohn, E. Hauschteck-Jungen, P. Hunziker & D.L. Hartl, 2001. Molecular evolution of the *ocnus* and *janus* genes in the *Drosophila melanogaster* species subgroup. *Mol. Biol. Evol.* 18: 801–811.
- Price, C.S.C., J.A. Coyne & K.A. Dyer, 1999. Sperm competition between *Drosophila* males involves both displacement and incapacitation. *Nature* 400: 449–452.
- Prout, T., 1971. The relation between fitness components and population prediction in *Drosophila*. I. The estimation of fitness components. *Genetics* 68: 127–149.
- Ritchie, M.G. & C.P. Kyriacou, 1994. Reproductive isolation and the *period* gene of *Drosophila*. *Mol. Ecol.* 3: 595–599.
- Singh, R.S., 2000. Toward a unified theory of speciation, in *Evolutionary Genetics: From molecules to morphology*, edited by R.S. Singh & C.B. Krimbas. Cambridge University Press, London.
- Singh, R.S. & R.J. Kulathinal, 2000. Sex gene pool evolution and speciation: a new paradigm. *Genes. Genet. Syst.* 75: 119–130.
- Thomas, S. & R.S. Singh, 1992. A comprehensive study of genic variation in natural populations of *Drosophila melanogaster*. VII. Varying rates of genic divergence as revealed by two-dimensional electrophoresis. *Mol. Biol. Evol.* 9: 507–525.
- Ting, C.-T., S.-C. Tsaur, M.-L. Wu & C.-I. Wu, 1998. A rapidly evolving homeobox at the site of a hybrid sterility gene. *Science* 282: 1501–1504.
- Tsaur, S.-C. & C.-I. Wu, 1997. Positive selection and the molecular evolution of a gene of male reproduction, *Acp26Aa* of *Drosophila*. *Mol. Biol. Evol.* 14: 544–549.
- Wu, C.-I., 1992. A note on Haldane's Rule: hybrid inviability versus hybrid sterility. *Evolution* 46: 1584–1587.
- Wu, C.-I. & 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.
- Yang, Z., 1997. PAML: a program package for phylogenetic analysis by maximum likelihood. *Comput. Appl. Biosci.* 13: 555–556.