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THE EVOLUTION OF SEX-RELATED TRAITS AND SPECIATION IN *DROSOPHILA*

By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

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Doctor of Philosophy

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SEX-RELATED TRAITS AND SPECIATION

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ABSTRACT

The amount of genetic change associated with species differentiation, and the phenotypic characteristics of such changes between closely related species, are crucial elements to the problem of speciation. This thesis describes an analysis of morphological, protein and DNA sequence divergence between closely related species that show a common pattern of higher interspecific divergence for sex-related traits (i.e. constituents of the genitalia, traits involved in mating, fertilization, or sex differentiation) than non sex-related traits.

Proteins expressed in gonadal tissues (testis and ovary) showed a higher interspecific divergence than proteins expressed in nongonadal tissues (brain and malpighian tubule) and a positive significant correlation was found between testis protein divergence and postzygotic isolation when closely related species were compared. An analysis of internal as well as external traits morphology revealed high divergence in testis length and area and genital arch area between species of the *melanogaster* complex. Similar levels of variation within species and non significant differences in trait asymmetry were found between sex-related and non-sex related traits. The analysis of interspecific hybrid morphology revealed between species allele complementation in non sex-related traits, while sex-related traits displayed signs of disrupted genetic interactions. A preliminary survey of DNA sequence divergence showed a high ratio of nonsynonymous to synonymous substitutions for sex-related genes. Such an elevated ratio proved to be the result of a high proportion of nonsynonymous substitutions between closely related species. Synonymous substitutions did not show any translational selective constraints that may have reduced the proportion of such changes between species.

The results presented in this thesis do not support speciation models that have proposed the disruption of selectively stabilized sex-related traits during speciation. Instead, they suggest that the high interspecific divergence of sex-related traits may be due to directional selection.

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J. B. S. Haldane taught me something on how to face sickness. Here goes a short fragment of his poem "Cancer's a funny thing".

> " I know that cancer often kills,
> but so do cars and sleeping pills;
> and it can hurt one till one sweats,
> so can bad teeth and unpaid debts.
> A spot of laughter, I am sure,
> often accelerates one's cure;
> so let us patients do our bit
> to help the surgeons make us fit"

My family was always supportive. I am particularly grateful to my father who was by my side whenever I needed him. I know how difficult it was for him not to try to drag me back to Argentina when my health was deteriorating.

Finally, I want to dedicate this thesis to my mother, Ana Maria Juliana Teruel, who saw me take my first steps as a Ph.D. student. I wish she could have seen this work completed.

THESIS ORGANIZATION

This thesis is comprised of a total of five chapters. The first chapter provides a general introduction together with the objectives and findings of the studies. Chapter 2 consists of a paper published in *Journal of Molecular Evolution*, chapter 3 has been submitted for publication to *Evolution* and chapter 4 has been accepted for publication in *Molecular Biology and Evolution*. The last chapter (chapter 5) provides a general discussion and final comments. All the work in the paper and manuscripts (chapters 2 to 4) that constitute the major body of the thesis was done by A. Civetta under the supervision of Dr. R. S. Singh.

Chapter 1: **General introduction, objectives and findings.**

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CHAPTER 1

GENERAL INTRODUCTION

The word species refers to an entity with unique characteristics, and the existence of different entities or kinds implies discontinuity. Biologists have been able, without the need of any elaborate techniques, to recognize the discontinuity or patchy nature of life in the wide variety of plants and animals that populate our planet. This has been such an obvious observance that a typological concept of species has been embraced since the time of the ancient Greeks. Different explanations were attempted to shed light on how such a wide variety of fauna and flora had originated, but most biologists and philosophers prior to the nineteen century adhered, in one way or another, to creationist dogma. Their work was then directed to classify organisms, to recognize the order that revealed creation.

Darwin (1859) did not accept that the order within and the gaps between biological species were an inherent property of nature, and he proposed the theory of natural selection to describe the major force generating gradual changes. However it remained unclear how the morphological, behavioural or functional gaps could have arisen as a simple byproduct of selection. With the rediscovery of Mendel's law of inheritance and the rise of the mutation school of thought, biologists interested in evolution became aware of the importance of understanding the genetic basis of speciation and at the same time, they dismissed the role of selection (Provine 1971). Selection alone was seen as incapable of forming new species by acting on continuous variation, and ideas on the origin of species focused on the discontinuous effect of major mutations (Provine 1971).

With the advent of the modern synthesis, authors such as Huxley (1942),

Dobzhansky (1951), and Mayr (1963) firmly opposed speciation models based on major mutations. A single mutation causing isolation between species must propagate through a population before it could become established, but its propagation would be certainly opposed by selection. The neodarwinian theories of speciation combined Mendelian genetics and Darwinian evolutionary mechanisms. They emphasized the necessity of changes in the gene pool through natural selection and the need for isolation between newly arisen species. Although crossing experiments, combined with the use of phenotypic markers, shed some light on the genetic basis of speciation in terms of chromosomal effects (Dobzhansky 1936), fine molecular tools to dissect the nature of such genetic changes were not available. Speciation models then focused mainly on geographical aspects, as it became clear that evolution was possible without isolation, but speciation nearly impossible without it.

But the controversy on the amount and nature of the genetic changes that is required to explain species formation remained an unsolved problem and it was not until the 1960's, when gel electrophoresis became an available laboratory technique, that the first attempts to answer questions pertaining to the genetic basis underlying the observed gaps between species could be attempted.

The earliest studies of genetic divergence between species using gel electrophoresis did not consider what proportion of the genetic differences was simply variation within a species (See Hubby and Throckmorton 1965). In this sense, the work of Prakash (1969) and Prakash *et al.* (1969) were pioneer studies as they were the first to show what proportion of changes between species were polymorphisms of the kind found within species. The comparisons between *D. pseudoobscura* - *D. persimilis* (Prakash 1969) and *D. pseudoobscura* - *D. pseudoobscura bogotana* (Prakash *et al.* 1969) showed that approximately half of the 24 loci sampled were monomorphic for the same allele in

both species. The other half showed only differences in gene frequencies and in most cases (approximately 75%) the most common allele was the same in both species. Similar results were found in other studies and have been summarized by Lewontin in his book " The genetic basis of evolutionary change" (Lewontin, 1974). But the result obtained by Prakash *et al.* (1969) became more relevant to the problem of speciation when Prakash (1972) demonstrated that crosses between *D. pseudoobscura* males and *D. pseudoobscura bogotana* females, so far considered members of the same species, rendered sterile male hybrids. The two populations became species *in statu nascendi*.

The results obtained in the allozyme electrophoresis surveys that expanded from 1965 to 1974 led Lewontin to conclude:

> " The first stage of speciation, the acquisition of primary reproductive isolation in geographical solitude, does not require a major overhaul of the genotype and may result from chance changes in a few loci". (Lewontin 1974, p. 185).

A few loci is a relative term although an important one as it suggested that previous hypothesis on the genetic basis of speciation based on the reorganization of the whole gene pool (Mayr, 1963) may have been erroneous. However, these early studies were based on a relatively small number of loci (less than 30). In a large scale survey including 112 loci, only 7% of the loci examined showed complete divergence (i.e. no shared alleles) between *D. melanogaster* and *D. simulans* and the level of genetic differentiation between species was dependant on the type of loci sampled (i.e. enzymes or abundant proteins) (Choudhary *et al.* 1992). This result confirmed that the analysis of structural genes coding for enzymes would not provide an accurate picture of the genetic changes required for speciation. What was needed was to link genetic changes to species reproductive isolation. Given the improvement in the number of genetic markers available

from *D. melanogaster* and its sibling species, a line of work revised the experiments designed by Dobzhansky in 1936 in an attempt to map chromosomal segments and eventually single genes responsible for the interspecific hybrid male sterility that characterize crosses between closely related species (Coyne 1984; Coyne and Orr 1989). However, another question remained to be answered: What are those "few loci" mentioned by Lewontin (Lewontin 1974). In other words, what is the genetic basis (i.e. major *vs.* minor genes) and phenotypic effects (in terms of morphology, function and behaviour) of such genes? The work in this thesis focus on this second question and for reasons of simplicity I will refer to this problem as "the genetic nature" of speciation.

1.1 THE GENETIC NATURE OF SPECIATION: A FIRST GLANCE TO AN ANSWER.

In 1975, O'Farrell described a new method called high-resolution two-dimensional gel electrophoresis (2DE) which allowed the separation of a mixture of proteins in a single gel slab. This technique separated proteins on the basis of charge and molecular weight properties. Singh and Coulthart (1982) and Coulthart and Singh (1988a) showed that previous differences in estimates of within and between species variation between the conventional one dimensional gel electrophoresis (1DE) techniques and the new 2DE methodology were probably more a result of the structural and functional characteristics of the proteins sampled than a simple byproduct of the techniques' sensitivities. These studies showed a substantial variation in polymorphism based on the proteins' site of expression and the developmental stage sampled (Singh and Coulthart 1982), and similar levels of heterozygosity between proteins surveyed by 1DE techniques and the polymorphic proteins found in 2DE analysis (Coulthart and Singh 1988a).

If allozyme electrophoresis proved not to be the right path to a proper

understanding of the genetic basis of speciation, since different results in terms of polymorphism and divergence could be found depending on what kind of proteins were sampled, then the question became which sub-sample of proteins are more closely linked to the speciation event. Any attempt to resolve the biochemical or molecular aspects of speciation would require an analysis of loci that can be linked to phenotypic characteristics associated with species isolation. The fact that *Drosophila* sibling species of the *melanogaster* species complex differ drastically in the shape of the posterior lobe of the male genital arch (Stutervant 1920; Coyne 1983), that interspecific hybrid breakdowns show a bias towards male sterility (Bock 1984), and the differences in genetic variability based on the protein's site of expression (Singh and Coulthart 1982) pointed to the need for an analysis of male-reproductive tract protein divergence between closely related species. In comparisons between *D. melanogaster* and *D. simulans*, Coulthart and Singh (1988b) found a higher proportion of unique proteins in male reproductive tract than wing disc samples. Although these two species had diverged for a considerable period of time and were not certainly in the first steps of species formation, the authors speculated on a possible link between divergence of proteins expressed in the male reproductive tract and reproductive isolation (Coulthart and Singh 1988b). This possible link was strengthened when a more extensive survey including different tissues and developmental stages from the four species of the *melanogaster* complex showed once again a high divergence for proteins expressed in male reproductive tissues (testes and accessory gland) (Thomas and Singh 1992).

However, the four species of the *melanogaster* complex covered only two distinct levels of postzygotic isolation, species pairs that render all hybrid inviable or sterile and species pairs that produce F_1 sterile males. In chapter 2 I present a survey of protein divergence in different gonadal and non-gonadal tissues. The study includes species of the

virilis group and the *melanogaster* complex. The *virilis* group has the advantage of providing, within the same species group, a wide variety of reproductive isolation levels. Although this approach does not reveal the polypeptides specifically related to reproductive isolation, an association between tissue-expressed protein divergence and reproductive isolation could be established. Another unsolved question was whether the high divergence found for proteins expressed in the male germ-line tissues is simply a consequence of a male reproductive breakdown at the onset of speciation. The fact that proteins in the female germ-line (i.e. ovaries) showed as much divergence as male gonadal tissues, despite the fact that females were usually fertile in crosses between the species surveyed, suggested a possible role of sexual selection in building up the interspecific divergence of these molecular sex-related traits (chapter 2).

In the section that follows I briefly summarize evidence obtained from other studies that further suggest a tight association between molecular and phenotypic reproductive traits and speciation, as well as the role played by sexual selection in the divergence of such traits during species formation.

1.2. REPRODUCTIVE TRAITS, SEXUAL SELECTION AND SPECIATION.

1.2.1 Non-molecular evidence

Several studies on estimates of genetic divergence, analysis of phenotypic breakdown in hybrids obtained between closely related species, or description of species - specific behavioural and morphological characters provide a framework to the question on the nature of the genetic changes underlying speciation, and they all suggest a possible link between sex-related traits and speciation.

For example, the biological species concept states that the gene exchange between species should be limited or prevented by *reproductive* isolating mechanisms (Dobzhansky

1951 ; Mayr 1963). The word *reproductive* becomes crucial as it suggests that any deviation (either behavioural, physiological, or developmental) from the repertoire of mating and procreation may cease or restrict gene exchange and eventually lead to speciation.

The early studies of Dobzhansky and Pontecorvo on the breakdown of phenotypic characteristics in hybrids obtained between closely related species suggest a possible role of genes affecting genitalia at the early stages of species formation. The phenotypic analysis of the sterile male hybrids that result from the cross between *D. pseudoobscura* and *D. persimilis* showed that besides sterility, the progeny resulting from the cross *D. pseudoobscura* male x *D. persimilis* female had atrophied testes (Dobzhansky 1934). Pontecorvo (1943) showed that, regardless of the direction of the cross, testes were atrophied in the sterile male progeny obtained from crossing *D. melanogaster* and *D. simulans*. In both Dobzhansky and Pontecorvo studies, the interspecific hybrids were phenotypically normal except for their sterility and atrophied testes.

Haldane observed that "when in the F_1 offspring of two different animal races one sex is absent, rare, or sterile, that sex is the heterozygous sex" (Haldane 1922). Within mammals and *Drosophila*, where males are the heterogametic (heterozygous) sex, the majority of F_1 hybrids are sterile (100% and 93%, respectively) (Wu and Davis 1993). Among lepidoptera and birds, where females are the heterogametic sex, the proportion of viable and sterile hybrids is more even (70% and 62% sterile for lepidoptera and birds respectively) (Wu and Davis 1993). This observation lead Wu and Davis to suggest an explanation to Haldane's rule based on a faster evolution of male reproductive (sterility) components (Wu and Davis 1993).

So far, these observations relate only to postmating barriers. When premating

barriers are key components of interspecific isolation, male secondary sexual traits or the mate-recognition system are the most diverged between related species. A thoroughly studied example are the Hawaiian *Drosophila* group species. *D. heteroneura* and *D. sylvestris* are capable of producing F_1 viable and fertile hybrids, however the males of the species differ drastically in the shape of the head which is used in male - male competition by *D. heteroneura* males (Spieth 1981). The courtship songs in Hawaiian *Drosophila* species show evolutionary innovations, such as high frequency sounds and abdominal vibrations, with respect to continental *Drosophila* species (Hoy *et al.* 1988). Differences in the mating sequence between two closely related species of the *planitibia* subgroup of the Hawaiian *Drosophila* group, *D. sylvestris* and *D. planitibia*, seem to complicate female acceptance of heterospecific males. The key components in the females rejection of heterospecific males lay in the last steps of the courtship dance that involved the movement of males under the "head under wing" position and the time spent in wing and leg vibration (Hoikkala and Kaneshiro 1993). Speciation in a recently colonized area such as the Hawaiian islands, could be a simple byproduct of ecological divergence. However, the observation that mating traits are the most diverged between closely related species have been used to support the role played by sexual selection.

There are two possible explanations for the role played by sexual selection in the divergence of mating systems and preferred male traits. One relies on the perfection of species recognition systems by stabilizing selection favouring a tight coupled system of signals and responses. For example, Kaneshiro and Boake (1987) have suggested that the loss, through founder events, of behavioural components of an stabilized signal-response chain system in the ancestral species may have promoted speciation. The existence of intraspecific constraints in mate recognition signals has been used as an indication of the possible role of stabilizing selection in the establishment of species isolation (Gerhardt

1982; Butlin *et al.* 1985). A mate recognition system under stabilizing selection should be invariant within species.

Alternatively, the mating recognition signals have been proposed to be a flexible system where males may evolve rapidly to match female preferences outside their original species range. Some evidence on the flexibility of females preferences come from studies in which females preferred heterospecific signals added to homospecific ones (Ryan and Rand 1993), or females recognize traits from ancestral or extant species (Ryan and Rand 1995). The existence of such flexibility in female preferences is not in agreement with previous models of speciation proposing that mate-recognition systems should be well buffered within species but disrupt easily during speciation events (Carson 1985; Paterson 1985; Kaneshiro and Boake 1987).

Among organisms that reproduce through internal fertilization, the morphology of genitalic structures such as claspers, modified anal fins, intromittent chelicerae, penes, and spermatophores show rapid and divergent evolution between species (Eberhard 1985). Eberhard (1985) suggested that these structures are not simply required for sperm transfer and that their complexity is an indication of their function as internal courtship devices under sexual selection by female choice. Eberhard and Cordero (1995) have extended this proposal to seminal substances in the ejaculate (chemical genitalia) that influence the mating behaviour and reproductive physiology of the females (Eberhard and Cordero 1995).

1.2.2 Molecular evidence

An increase in the number of genes sequenced provides an opportunity to study the molecular nature of sex-related gene evolution (i.e. genes involved in mating recognition, fertilization, spermatogenesis, or involved in sex determination). These genes

have been independently sequenced and analyzed by different authors in different organisms, and they show an interesting pattern of high divergence between related species.

For example, *fus-1* is a gene involved in mate recognition in *Chlamydomonas* species. Ferris *et al.* (1996) have shown that the *fus-1* gene is the first of approximately 100 genes in *C. reinhardtii* to show no codon bias. Ferris and Goodenough (1997) have recently characterized the first sex-determination gene in *Chlamydomonas*. The sequence composition of the *minus* dominance gene (*mid*) was similar to the one previously obtained for the mate recognition gene *fus-1*. The GC content was similar in both coding and non-coding (i.e. flanking and introns) regions of the *mid* gene, and codon bias was extremely low compared to other genes of *C. reinhardtii*. These results have lead Goodenough to suggest a strong mutational pressure as responsible for the rapid evolution of sex-related genes in this group (Goodenough pers. com.; see also Ferris *et al.* 1996).

Among abalone species, the sperm gene *lysin* is known to be involved in fertilization by creating a hole in the egg vitelline envelope. This gene has a higher proportion of nonsynonymous substitutions (i.e. those that change the amino acid sequence in the protein product) (K_a) than synonymous (K_s) changes among closely related species (i.e. species pairs with $K_s < 20\%$) (Lee *et al.* 1995). Nonsynonymous and synonymous changes were not influenced by nucleotide or codon usage bias, and hence their high K_a / K_s ratio have been suggested to be the result of positive selection rather than mutational bias or selective constraints on the rate of synonymous changes (Lee *et al.* 1995). An eighteen KDa protein of yet unknown function that coats the acrosomal process of the abalone sperm also has a high proportion of nonsynonymous to synonymous changes in comparisons between five species of California abalone, suggesting intense positive selection affecting the evolution of this protein (Swanson and Vacquier 1995).

In a sequence analysis study of a gamete recognition gene (*bindin*) between three species of sea urchins, Metz and Palumbi (1996) found an unusually high ratio of polymorphic and fixed replacement to silent substitutions. The observation that most amino acid substitutions accumulated preferentially in a 40 codon domain of the gene, and that these changes were mostly non-conservative in terms of charge and polarity lead the authors to suggest positive selection favouring both the polymorphism within species and the differences between species. Metz and Palumbi (1996) entertained the idea that selection of the polymorphisms detected within species coupled with population isolation may trigger differentiation of the species-specific gamete recognition system, and eventually lead to speciation.

Male accessory gland secretions in *Drosophila* affect females receptivity to remating and increases egg-laying (Chen *et al.* 1988). Some of the accessory gland genes (*Acp26A*, *Acp29B*, *Acp36DE* and *Acp53E*) have also been linked to sperm displacement (Clark *et al.* 1995). Only *Acp26A* gene sequence divergence have been analyzed between closely related species of the *melanogaster* complex. The gene showed high levels of nonsynonymous substitutions between the four species of the *melanogaster* complex and divergence of cleavage sites in polymorphic alleles of *D. melanogaster* and its sibling species (Aguade *et al.* 1992). Esterase-6 is mainly expressed in the ejaculatory duct of *D. melanogaster, D. simulans* and *D. mauritiana* (Stein *et al.* 1984), it is transferred during copulation to the females affecting their reproductive behaviour (Scott 1986), and it has shown a higher replacement to synonymous site divergence between *D. melanogaster* and *D. simulans* (Karotam *et al.* 1993).

Among mammals, the sequence of the homeobox gene *Pem* proved to be the one showing the highest divergence between rat and mouse in a homeodomain region (Maiti *et al.* 1996). According to the authors, the extremely high proportion of nonsynonymous

substitutions in the amino-terminal half of the homeodomain, which might act as a species-specific protein-interaction domain, could be an indicative of the adaptive nature of its interspecific divergence. Although Maiti *et al.* (1996) found no direct evidence that *Pem* regulates reproductive physiology, the gene was selectively expressed in reproductive tissues such as testis, epididymis and ovary.

Gene sequences involved in sex-determination have also been shown to be rapidly evolving between related species. Among nematodes, the sex-determination genes transformer (*tra-1* and *tra-2*) have revealed the lowest amino acid identity out of fifteen gene sequences compared between *Caenorhabditis elegans* and *C. briggsae* (de Bono and Hodgkin 1996; Kuwabara and Hodgkin 1996). In *Drosophila*, the gene *transformer* (*tra*) acts as a switch in the path of sexual differentiation. In a sequence comparison between *D. melanogaster*, *D. simulans*, *D. erecta*, *D. hydei* and *D. virilis*, the protein sequence of *transformer* was poorly conserved between the species analyzed (O'Neill and Belote 1992). Finally, rapid evolution and positive Darwinian selection have been suggested for the *Sry* sex-determination gene in mammals based on nonsynonymous to synonymous changes between species (Whitfield *et al.* 1993; Tucker and Lundrigan 1993). Pamilo and O'Neill (1997) reevaluated the evolution of the *Sry* gene in primates, rodents and bovids taking into account the phylogenetic relationship of the species being compared and the different regions in the gene (i.e HMG box and terminal regions). Only the terminal sequences of the *Sry* gene of apes had a frequency of nonsynonymous substitutions higher than expected from the neutral hypothesis ($K_a = K_s$), which suggested positive selection at the protein level (Pamilo and O'Neill 1997).

At least one example from plants seems to suggest that the pattern of rapid sex-related gene evolution may extend beyond the animal kingdom. The example comes from a sequence analysis of the self-incompatibility locus which functions in the recognition of

pollen. A comparison between alleles from four species of *Solanaceae* revealed high sequence diversity with a homogeneous synonymous rate but a heterogeneous proportion of nonsynonymous changes among different regions of the gene. An excess of nonsynonymous changes were found in sites that are assumed to be linked to protein function (Clark and Kao 1991).

It should be clear that other genes, unrelated to reproduction, are highly diverged between species. Particularly interesting as a group are genes involved in the mammalian immune system, such as the major histocompatibility complex (Hughes and Nei 1988), interleukin-3 (Burger *et al.* 1994), immunoglobulins (Hughes 1997) and defensin (Hughes and Yeager 1997). These genes show a high proportion of nonsynonymous substitutions between distantly related species such as rodents and humans suggesting a strong selective regime favouring a diversifying strategy. In chapter 4, sex-related genes (i.e. genes involved in mating recognition, fertilization, expressed in the genitalia, or involved in sex determination) are shown to be a fast evolving group, however the rapid burst of nonsynonymous substitutions is noteworthy between species such as *D. melanogaster* and *D. simulans* that have diverged for a short period of time (approximately 2.5 Myr., see Lachaise *et al.* 1988). It is possible that sex genes undergo rapid divergent evolution during speciation events and slow down between speciation events (see chapter 4).

1.2.3 Extending the role of sexual selection

In the preceding paragraphs, no distinction was made between mating (precopulatory) and copulatory devices as copulation itself is part of the mating courtship required to sire offspring. Eberhard (1996) provides several examples in diverse groups of animals where copulatory and postcopulatory behaviours such as biting, rubbing,

vocalizations, postcopulatory displays, postcopulatory feeding, genitalic movements, etc. seem to be still part of the courtship repertoire to increase female's receptivity.

Sexual selection is the result of the differential mating ability among males, and such differences are based on courtship. However, studies of sexual selection have been traditionally limited to secondary sexual traits and behavioural components of the mating (precopulatory) system. If receptivity by the female is not guaranteed by the time copulation has started, both copulatory and postcopulatory devices are still important in order to assure females receptivity of the sperm and fertilization. Therefore, all components of the reproductive strategy (chemical, behaviour, morphology) are expected to be influenced by sexual selection. Studies in beetles (Bella 1992), grasshoppers (Wade *et al.* 1994), crickets (Gregory and Howard 1994), *Drosophila* (Clark *et al.* 1995), birds (Birkhead and Møller 1992), and rodents (Dewsbury 1984) show that female choice or male - male competition are still part of the reproductive strategy even after sperm has been transferred to the female. These few examples in a diverse group of organisms suggest that sexual selection is not to be restricted to the pre-copulatory stages leading to mating.

Considering the examples provided in sections 1.2.1 and 1.2.2, it seems also possible to entertain the hypothesis that sexual selection has been involved in shaping the evolution not only of secondary or behavioural sexual traits but also other physiological, behavioural, or developmental aspects that relate to sex but are not necessarily involved in mating.

1.4 OBJECTIVES AND FINDINGS

The purpose of this thesis has been to test the idea that the nature of loci responsible for speciation may be intimately linked to sex traits in its wider conception

(development, behaviour, reproduction, genitalia). This thesis deals mainly with molecular (chapter 2 and 4) and morphological (chapter 3) divergence between species and it is suggested that such divergence has been shaped by directional selective pressures at the onset of speciation.

The analysis of interspecific divergence in sex-related traits in different species of *Drosophila* was carried out to answer the following questions: 1) Is there a link between the high protein divergence previously found in male reproductive tissues (Coulthart and Singh 1988b, Thomas and Singh 1992) and reproductive isolation? 2) Is the high divergence only a result of breakdown of male fertility and so limited to proteins found in the male gonads? 3) Is the high sexual traits (morphological and molecular) divergence a result of stabilizing selection and episodic disruption, high mutability, or directional selection? 4) How do sexual and non-sexual "gene pools" interact in the interspecific hybrids between closely related species? Does their complementation suggest similar underlying genetic architectures (i.e. number of genes, nature of genes, and their interactions)?

Chapter 2 deals with the first two questions and two dimensional gel electrophoresis was used as it allows the survey of a large number of proteins in one tissue sample. Testis, ovary, brain and malpighian tubules samples obtained from the four species of the *melanogaster* complex and nine species of the *virilis* group were analyzed for protein divergence (scored as proportion of unique spots) between pair of species. The *virilis* group was chosen since it provides species pairs that range from completely viable and fertile interspecific progeny to complete postzygotic isolation, as well as a good range of premating levels of isolation. Only the proteins expressed in testes showed a positive and significant correlation with postzygotic isolation between closely related species

suggesting a possible role for male-related traits in speciation. In fact, the correlation observed is most probably an underestimate as not all proteins analyzed are necessarily linked to postzygotic isolation barriers. Both ovary and testis proteins showed similar and high levels of divergence which indicate that the previously found high divergence for male reproductive tract proteins (Coulthart and Singh 1988a; Thomas and Singh 1992) may not be the sole effect of the divergence of male fertility components as suggested by the predominant male fertility breakdown suffered by interspecific hybrids. Such high male and female reproductive tract protein divergence lead us to hypothesize a possible role of sexual selection at the level of reproductive tract molecules. Both results combined suggest the possibility of sex-related traits being an example of key species-specific traits and adaptations in the sex molecular milieu.

Chapter 3 extends the previous finding of high male reproductive tract protein divergence to morphology. Sexual traits not directly involved in mating, such as the area of the posterior lobe of the genital arch and testes length and area showed high divergence between the four species of the *melanogaster* complex. Interestingly, comparisons of intraspecific variation and interspecific divergence as well as traits asymmetry did not support previous models of speciation suggesting that sexual traits should be constrained by stabilizing selection within species but easily disrupted by founder events that may trigger speciation (Carson 1985; Kaneshiro and Boake 1987). Non-sexual traits in interspecific hybrids were overdominant. Wright (1977) suggested that such behaviour may be a consequence of a defective regulation of growth in the interspecific hybrids. However, non-sexual traits were less or equally asymmetric in hybrids when compared to the parental species, suggesting proper allele complementation due to a less diverged genetic system. On the contrary, interspecific hybrid sexual traits were additive or

dominant and more asymmetric than the parental species, suggesting that the sex-trait gene pool had diverged to a point of upsetting major genes or the epistatic control of the sex-related phenotypes.

Finally, chapter 4 is a survey of sex and non-sex related gene sequence divergence between species pairs of the *Drosophila* genus as well as two closely related species of nematode. Once again, the results obtained support a high interspecific divergence for sex-related traits between closely related species (*D. melanogaster* - *D. simulans*) reaching a plateau between more distantly related species (*D. melanogaster* - *D. pseudoobscura*). The high divergence was the product of a rapid burst of nonsynonymous substitutions (i.e. those that change the amino acid sequence in the protein product). We propose directional selection during the early stages of speciation as the key force responsible for the high nonsynonymous sequence divergence. However we are aware of the limitations due to the small number of genes and taxa that were surveyed and the possibility that different groups of organisms may render alternative results based on their reproductive characteristics.

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CHAPTER 2

HIGH DIVERGENCE OF REPRODUCTIVE TRACT PROTEINS AND THEIR ASSOCIATION WITH POSTZYGOTIC REPRODUCTIVE ISOLATION IN *DROSOPHILA MELANOGASTER* AND *DROSOPHILA VIRILIS* GROUP SPECIES

This chapter has been published in Journal of Molecular Evoltion. In this paper, two dimensional gel electrophoresis was used to score interspecific protein divergence in different tissue samples. Proteins expressed in gonadal tissues (testis and ovary) showed higher divergence than proteins expressed in nongonadal tissues (malpighian tubules and brain), and only protein expressed in testis showed a significant positive correlation with postzygotic reproductive isolation when closely related species were considered.

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High Divergence of Reproductive Tract Proteins and Their Association with Postzygotic Reproductive Isolation in *Drosophila melanogaster* and *Drosophila virilis* Group Species

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Abstract. The possible association between gonadal protein divergence and postzygotic reproductive isolation was investigated among species of the *Drosophila melanogaster* and *D. virilis* groups. Protein divergence was scored by high-resolution two-dimensional electrophoresis (2DE). Close to 500 protein spots from gonadal tissues (testis and ovary) and nongonadal tissues (malpighian tubules and brain) were analyzed and protein divergence was calculated based on presence vs absence. Both testis and ovary proteins showed higher divergence than nongonadal proteins, and also a highly significant positive correlation with postzygotic reproductive isolation but a weaker correlation with prezygotic reproductive isolation. Particularly, a positive and significant correlation was found between proteins expressed in the testis and postzygotic reproductive isolation among closely related species such as the within-phylad species in the *D. virilis* group. The high levels of male-reproductive-tract protein divergence between species might be associated with F_1 hybrid male sterility among closely related species. If so, a lower level of ovary protein divergence should be expected on the basis that F_1 female hybrids are fully fertile. However, this is not necessarily true if relatively few genes are responsible for the reproductive isolation observed between closely related species, as recent studies seem to suggest. We suggest that the faster rate of evolution of gonadal proteins in comparison to nongonadal proteins and the association of that rate with postzygotic reproductive isolation may be the result of episodic and/or sexual selection on male and female molecular traits.

Key words: *Drosophila* — Two-dimensional electrophoresis — Gonadal protein divergence — Postzygotic reproductive isolation — Speciation — Hybrid sterility

Introduction

Since the pioneering work of Hubby and Throckmorton (1965), numerous studies of enzymes and abundant proteins have been done to learn about the nature and the levels of protein divergence among closely related species and their significance for mechanisms of speciation (Lewontin 1974; Ayala 1975; Throckmorton 1977). The majority of the studies have been based on genes coding for enzymes, and there is some evidence that the level of variation at gene-enzyme loci may not be representative of all loci in the genome. For example, Singh and Rhomberg (1987) and Choudhary and Singh (1987) showed that all loci showing complete divergence between *D. melanogaster* and *D. simulans* were enzymes and none were abundant proteins. Selander and Johnson (1973) and Harris et al. (1977) showed a wide range of variation among proteins, with some abundant soluble (serum) proteins being more polymorphic than others. These results suggested that the analysis of structural genes coding for enzymes may not give an accurate picture of the level of protein divergence and brought our attention to the consideration of different classes of proteins.

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Two-dimensional electrophoresis (2DE) has the advantage of allowing a simultaneous analysis of a large number of abundant proteins on a single gel. The technique allows one not only to analyze hundreds of proteins but also to sample different kinds of tissue proteins. Coulthart and Singh (1988a–c) used this technique to estimate genic variation within species and to compare levels of divergence between *D. melanogaster* and *D. simulans*. Male-reproductive-tract proteins showed low polymorphism within species but high divergence between species (Coulthart and Singh 1988a,b). Polymorphism within species was significantly lower for testis than accessory gland proteins (Coulthart and Singh 1988c). Generally, secretory or extracellular proteins, such as those from hemolymph, accessory gland, and serum, show high heterozygosity as well as high divergence (Selander and Johnson 1973; King and Wilson 1975; Singh and Coulthart 1982; Coulthart and Singh 1988a; Thomas and Singh 1992). The high divergence between species and low heterozygosity within species for the testis proteins may suggest strong purifying selection within species and disruptive selection between species. Alternatively, it is also possible that reproductive tract proteins evolve at a rapid rate during speciation and the low heterozygosity detected within *D. melanogaster* and *D. simulans* is a result of recent fixation events.

There is other indirect evidence to suggest a possible link between gonadal divergence and postzygotic reproductive isolation. Crossing experiments between closely related species of the genus *Drosophila (melanogaster* complex) produce sterile hybrid males which are somatically normal except for their gonads. Dobzhansky and Beadle (1936) performed transplantations of larvae gonads between hybrids and parental species (*D. pseudoobscura* and *D. persimilis*) and showed that the improper functioning or atrophy of the gonads in the hybrids is determined within the gonad itself and not by an interaction between the gonad and other parts of the body (autonomy). Animal genitalia, particularly male genitalia, have been claimed to be very complex in form and show higher diversification between species than other traits (Eberhard 1985). The high divergence of male genitalia and the autonomy of gonadal functions together with the high divergence of testis proteins suggest that testis-expressed genes may be preferentially involved in the development of reproductive isolation.

The objectives of the present study were twofold. The first was to make a comparison of protein divergence in testis and ovary proteins. It has been hypothesized that the high testis-protein divergence detected among species of the *melanogaster* group may be associated with postzygotic reproductive isolation (Thomas and Singh 1992). If divergence is related to the development of hybrid male sterility then ovary proteins should not show high divergence as hybrid females resulting from crosses among closely related *Drosophila* species are fertile. The second was to test whether gonadal protein divergence between species is correlated with postzygotic reproductive isolation. If high divergence of testis proteins is related to the postzygotic reproductive isolation, species pairs showing only prezygotic isolation should not show high testis divergence, and a positive correlation would be expected between levels of testis protein divergence and postzygotic reproductive isolation.

We have utilized species of the *Drosophila melanogaster* and *D. virilis* groups. Considering species pairs from different groups may introduce a bias in an attempt to correlate divergence with reproductive isolation, as pairs of sibling species from different *Drosophila* groups vary considerably in their levels of genetic divergence (Ayala 1975; Singh 1990). The *virilis* group species has the advantage of offering, within the same group, a wide range of variation in levels of reproductive isolation (Throckmorton 1982; Coyne and Orr 1989).

In this report, we present results which show that both ovary and testis proteins are highly diverged between species, and that only testes protein divergence correlates with postzygotic reproductive isolation among species that have diverged for a short period of time.

Materials and Methods

Drosophila *Stocks and Sample Preparation.* The *melanogaster* group species were *D. melanogaster* (Oregon R), *D. simulans* (Townsville), and *D. mauritiana* (LG24) obtained from Dr. Jean David and *D. sechellia* (Robertson) from Dr. Jerry A. Coyne. The *virilis* group species stocks were obtained from the Drosophila Species Resource Center and were as follows: *D. americana* (0951.0), *D. americana texana* (1041.0), *D. lacicola* (0991.0), *D. lummei* (1011.1), *D. montana* (1021.0), *D. novamexicana* (1031.12), *D. virilis* (1051.48), *D. borealis* (0961.0), and *D. flavomontana* (0981.0). Stocks were maintained at 24°C in 250-ml bottles containing banana medium under a 12:12-h light-dark cycle.

Newly hatched adults were sexed and kept in separate vials containing banana medium. Organs were dissected from 4–6-day-old adults in Ringer's solution (Cheney and Shearn 1983) and placed in 40 µl of sample buffer (Hochstrasser et al. 1988). The number of tissues per sample varied for different organs and species groups. For the *melanogaster* group, eight pairs of testes and five pairs of ovaries were used, while three pairs of testes and two pairs of ovaries were used for the *virilis* group as well as ten brains and ten malpighian tube (M.T.). In all cases, the testis samples also contained the seminal vesicle and the vas defferens. The samples were stored at −70°C until electrophoresis. Prior to electrophoresis, cell disruption and debris separation were performed by freeze/thaw cycles and two rounds of centrifugation at 14,000 rpm for 20 and 10 min, respectively. After the second centrifugation, 30 µl of supernatant of each sample was loaded per gel.

Two-Dimensional Electrophoresis. The first dimension was run vertically in 4.5% acrylamide tube-gels (1.5 mm in diameter and 18 cm in length). The gel solution used and the procedure followed were as described in Zeng and Singh (1993a). Isoelectrofocusing was carried out at room temperature in a Hoefer model GT tube-gel apparatus, with the lower reservoir containing 0.06 M phosphoric acid and the upper reservoir containing 0.02 M sodium hydroxide. Gels were run at a

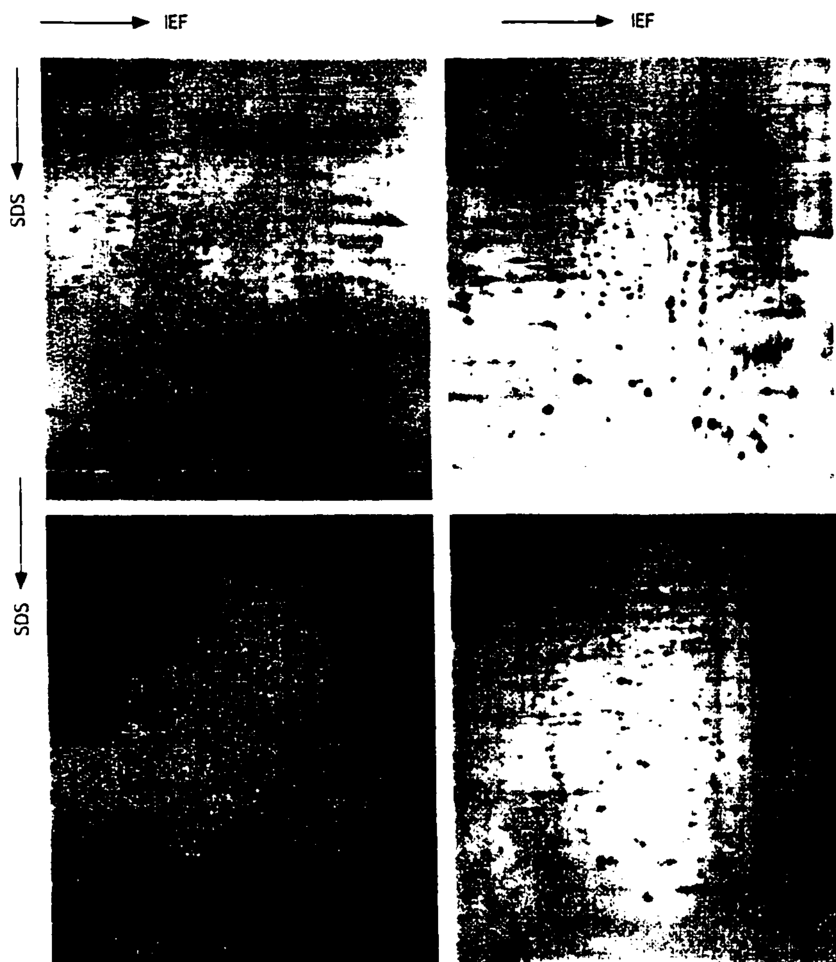


Fig. 1. 2D gels showing protein spots in adult male testes **(upper left)**, adult female ovaries **(upper right)** of *Drosophila simulans*, adult malpighian tubules of *Drosophila novamexicana* **(lower left)**, and adult brain of *Drosophila americana* **(lower right)**. Only well-resolved protein spots were scored.

constant voltage of 200 V for 2 h, at 500 V for 5 h and 1,000 V for 10 h. When isoelectrofocusing was completed, the gels were removed from the tubes with air pressure and soaked in transfer solution (Zeng and Singh 1993a). Tube-gels were carefully loaded on top of 12% acrylamide second-dimension gels (20 cm × 16 cm × 1.5 mm) prepared in a Bio-Rad multi-gel casting chamber. The second-dimension separation was carried out in a Protean II multicell apparatus from Bio-Rad. Gels were run vertically at 10-mA constant current per gel for approximately 16 h in a cooling chamber. A Tris-glycine buffer was used in both lower and upper reservoirs for the second dimension (0.05 M Tris; 0.38 M glycine; 0.0035 M SDS). The silver staining protocol was performed following the procedure described by Coulthart (1986) which was adapted from Merril et al. (1981) and Morrisey (1982).

Gel Scoring and Data Analysis. Gels were scored visually using a light box. Two gels were scored by marking shared and unique (present only in one of the gels) well-resolved protein spots. The homology of spots between species was assigned by relative position in both dimensions and the appearance of the spots (shape, size, and color).

Comparison of 2D protein patterns with the aim of detecting protein divergence requires high reproducibility of the gels (Zeng and Singh 1993a). Changes in the pattern might be due to either technical (e.g., sample protein concentration, differences between ampholyte preparations, staining resolution) or biological reasons (e.g., age, growing conditions). In order to reduce experimental variation only well-resolved gels were compared and read twice. A second set from different samples of the same *Drosophila* strain was used in dubious situations to confirm the presence/absence of protein spots. Undesirable biological variation was reduced by sampling individuals of approximately the same age that grew at the same temperature, light-dark, and culture medium conditions.

Well-resolved gels were read twice and average number of common and unique spots were used for the estimation of protein divergence. $D = 1 - F$, F being $2n_{xy}/(n_x + n_y)$ where n_x and n_y are the number of protein spots in species x and y, respectively, and n_{xy} is the number of protein spots shared by both species x and y (Sneath and Sokal 1973). Spicer (1991) used the simple matching coefficient (S_{sm}) (Sokal and Sneath 1963; Sneath and Sokal 1973) as a measure of protein identity and $-\ln S_{sm}$ as a measure of protein distance analogous to Nei's D (Nei 1972). $S_{sm} = n_{xy}/n$, where n is the total number of characters (protein spots) scored. Spicer (1991) compared a constant number of protein spots among species pairs ($n_x = n_y$) so that $2n_{xy}/(n_x + n_y) = n_{xy}/n$; in other words, our F is equal to Spicer's S_{sm}. In order to compare our results to those of Spicer (1991), we also used $-\ln F$ as a measure of protein distance.

The D values obtained and their associated errors (Tables 1–3) were calculated from the two replicate readings of the same gel-pairs. The estimates of protein divergence for pairwise species comparisons among members of the *melanogaster* group were based on nearly 450 protein spots each detected for both testis and ovary samples (Fig. 1). Among the *virilis* group species, the estimates of divergence were based on approximately 500 protein spots scored in species comparisons for the different tissues analyzed, with the exception of M.T. samples in which approximately 400 spots were scored (Fig. 1).

The prezygotic and postzygotic reproductive isolation indices (RI) between species analyzed in this work were obtained from previously published data (Coyne and Orr 1989).

Table 1. Prezygotic and postzygotic reproductive isolation indices (RI) and estimates of pairwise protein divergence (±SD) between the *melanogaster* group species[a]

Spp. pairs	RI (pre)	RI (post)	Testis	Ovary
si-ma	0.607	0.5	0.120 (±0.010)	0.147 (±0.018)
si-se	—	0.5	0.155 (±0.009)	0.147 (±0.038)
ma-se	—	0.5	0.143 (±0.008)	0.140 (±0.032)
me-si	0.914	1.0	0.251 (±0.009)	0.212 (±0.014)
me-ma	0.883	1.0	0.232 (±0.024)	0.219 (±0.019)
me-se	—	1.0	0.265 (±0.027)	0.225 (±0.002)
Average			0.194 (±0.062)	0.181 (±0.041)

[a] me = *D. melanogaster;* si = *D. simulans;* ma = *D. mauritiana;* se = *D. sechellia*

Results

High Divergence of Reproductive-Tract Proteins

The difference in average protein divergence for testis and ovary samples among species of the *melanogaster* group was not significant (Wilcoxon's signed-rank test. $T_s = 4$; $N = 6$; $P > 0.05$) (Table 1) and the same is true for the *virilis* group ($T_r = 90$; $N = 25$; $P > 0.005$) (Tables 2 and 3). Table 1 shows not only similar overall species divergence for both testis and ovary samples, but also a remarkable similarity between the estimates of protein divergence for testes and ovaries on a pairwise species basis. The estimates of divergence involving *D. melanogaster* are twice as large as those among the other three species, a result which is in agreement with the suggestion that *D. melanogaster* is the oldest species in the *melanogaster* complex. Chromosomal, morphological, molecular, and hybridization data support the separation of *D. melanogaster* from the *simulans* clade (*D. simulans, D. sechellia,* and *D. mauritiana*). However, the phylogenetic relationship among the members of the *simulans* clade is still an unresolved issue due to the inconsistency between the different trees obtained using alternative data characters (Lachaise et al. 1988). Our data on testis proteins support a closer relationship between *D. simulans* and *D. mauritiana,* whereas distances estimated from ovary data do not support any particular relationship and leave the triad unresolved (Fig. 2).

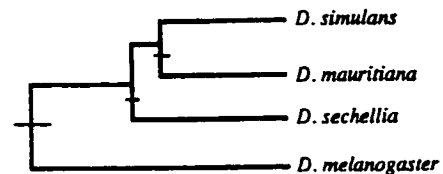
In Tables 2 and 3, the prezygotic and postzygotic isolation indices are presented along with the proportion of protein divergence for different tissues between species of the *virilis* group belonging to either the same phylad (Table 2) or different phylads (Table 3). In agreement with previous results from our laboratory (Thomas and Singh 1992), the divergence for brain tissue is much lower than the estimate for the other tissues. The average protein divergence between phylads appears to be nearly twice as large as the grand average within phylad for all tissues.

Among *virilis* group species (Tables 2 and 3), the levels of protein divergence among testis, ovary, brain and M.T. differ significantly (Kruskal-Wallis rank test, $X^2_3 = 13.8$; $P < 0.01$). In order to discern which tissue-pairs comparisons were responsible for the significant departure among tissues, a Wilcoxon two-sample test was performed (Table 4). Only the divergence between testis-brain and ovary-brain were significant ($X^2_1 = 11.9$; $P < 0.001$ and $X^2_1 = 8.7$; $P < 0.01$, respectively). However, it could be argued that the Kruskal-Wallis and Wilcoxon two-sample tests are more appropriate for completely randomized designs. In our data, protein divergence in different tissues was estimated between species pairs chosen according to their degree of reproductive isolation. Then, the species pairs considered can be treated as blocks.

We tested the significance of protein divergence among tissues by applying Friedman's method to blocks consisting of those species pairs for which protein divergence in all tissues had been scored (data from Tables 2 and 3). This test confirmed that protein divergence among testis, ovary, brain and M.T. differ significantly ($X^2_3 = 15$; $P < 0.01$). A comparison between pairs of tissues through a Wilcoxon signed-rank test revealed significant differences between testis and M.T., testis and brain, ovary and brain, and M.T. and brain samples ($T_r = 0$; $N = 6$; $P < 0.05$). From these two tests we can conclude that testis-ovary and ovary-M.T. protein divergence do not differ significantly from each other, whereas testis-brain and ovary-brain do (Table 4).

The high level of divergence detected for M.T. is not surprising. These tubules perform an excretory function.

Testis data:



Ovary data:

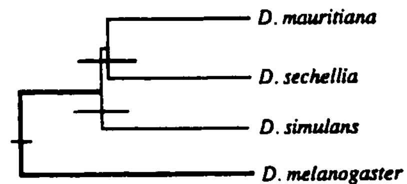


Fig. 2. Phenogram (UPGMA) obtained using the distance values in Table 1. *Thin lines* crossing the branching ones represent the standard deviation associated with the genetic distance estimates. In the case of ovary data, the distance values (D ± SD) among the members of the *simulans* clade overlap each other, and the relationship between the three members of the clade remains equivocal (represented by *thin lines*).

Table 2. Prezygotic and postzygotic reproductive isolation indices (RI) and estimates of pairwise protein divergence (±SD) between species from the *virilis* group belonging to the same phylad[a]

Spp. pair	RI (pre)	RI (post)	Testis	Ovary	M.T.	Brain
virilis phylad						
am-no	0.465	0.00	0.172 (±0.047)	0.133 (±0.005)	0.149 (±0.009)	0.071 (±0.006)
am-vi	0.748	0.00	0.116 (±0.001)	0.130 (±0.026)	0.101 (±0.029)	0.096 (±0.006)
no-vi	0.493	0.00	0.178 (±0.026)	0.135 (±0.020)	0.145 (±0.001)	0.100 (±0.022)
am-tx	0.242	0.00	0.147 (±0.009)	0.170 (±0.011)		
lu-vi	—	0.00	0.160 (±0.005)	0.163 (±0.020)		
tx-vi	0.749	0.00	0.172 (±0.008)	0.219 (±0.031)		
no-tx	0.444	0.00	0.144 (±0.017)	0.127 (±0.005)		
am-lu	—	0.50	0.190 (±0.004)	0.216 (±0.020)		
lu-tx	—	0.50	0.157 (±0.016)	0.202 (±0.009)		
Average			0.159 (±0.02)	0.163 (±0.040)		
montana phylad						
la-mo	0.954	0.00	0.158 (±0.008)	0.136 (±0.001)		
bo-mo	—	0.50	0.181 (±0.005)	0.154 (±0.016)		
fl-mo	—	0.50	0.182 (±0.004)	0.152 (±0.021)		
fl-la	—	0.50	0.179 (±0.018)	0.161 (±0.006)		
bo-fl	—	1.00	0.189 (±0.012)	0.138 (±0.002)		
bo-la	1.000	—	0.172 (±0.006)	0.154 (±0.014)		
Average			0.180 (±0.006)	0.152 (±0.008)		
Grand average			0.166 (±0.019)	0.159 (±0.030)	0.131 (±0.027)	0.089 (±0.016)

[a] am = *D. americana;* tx = *D. americana texana;* bo = *D. borealis;* fl = *D. flavomontana;* la = *D. lacicola;* lu = *D. lummei;* mo = *D. montana;* no = *D. novamexicana;* vi = *D. virilis*

Table 3. Prezygotic and postzygotic reproductive isolation indices (RI) and estimates of pairwise protein divergence (±SD) between species from the *virilis* group belonging to different phylads[a]

Spp. pair	RI (pre)	RI (post)	Testis	Ovary	M.T.	Brain
mo-vi	0.895	0.50	0.294 (±0.020)	0.252 (±0.012)	0.230 (±0.012)	0.147 (±0.017)
mo-tx	0.985	0.50	0.256 (±0.015)	0.265 (±0.027)		
mo-no	1.000	0.75	0.264 (±0.002)	0.250 (±0.014)	0.196 (±0.015)	0.154 (±0.029)
la-tx	0.992	0.75	0.301 (±0.020)	0.271 (±0.033)		
la-vi	0.717	0.75	0.274 (±0.004)	0.291 (±0.017)		
fl-vi	—	0.75	0.295 (±0.011)	0.275 (±0.011)		
am-mo	0.992	1.00	0.284 (±0.002)	0.258 (±0.008)	0.189 (±0.006)	0.128 (±0.011)
mo-lu	—	—	0.254 (±0.011)	0.248 (±0.022)		
am-la	1.000	—	0.275 (±0.019)	0.238 (±0.006)		
la-lu	—	—	0.253 (±0.005)	0.228 (±0.004)		
Average			0.275 (±0.018)	0.258 (±0.019)	0.205 (±0.022)	0.143 (±0.014)

[a] Nomenclature as in Table 2

They lie in the body cavity surrounded by hemolymph and there is extensive transport of substances from the hemolymph to the M.T. (Wessing and Eichelberg 1978). Singh and Coulthart (1982) and Thomas and Singh (1992) have previously shown high levels of polymorphism and divergence for proteins expressed in the hemolymph for species of the *melanogaster* complex.

Throckmorton (1982) suggested that species of the *virilis* phylad changed more slowly than did species of the *montana* phylad. His argument was based on chromosome and protein changes as well as the fact that the members of the *virilis* phylad retained a higher crossability among themselves than those of the *montana* phylad. More recent studies based on immunological dis-

Table 4. Results obtained from paired comparisons among protein divergence in different tissues (*D. virilis* group) using Wilcoxon's two-sample test (complete randomized design) and Wilcoxon's signed-rank test (blocks design) are shown[a]

Tissue compared	Wilcoxon two-sample	Wilcoxon signed-rank
Testes *vs* ovary	NS	NS
Testes *vs* M.T.	NS	$p < 0.05$
Testes *vs* brain	$P < 0.001$	$p < 0.05$
Ovary *vs* M.T.	NS	NS
Ovary *vs* brain	$P < 0.01$	$p < 0.05$
M.T. *vs* brain	NS	$p < 0.05$

[a] NS = nonsignificant results

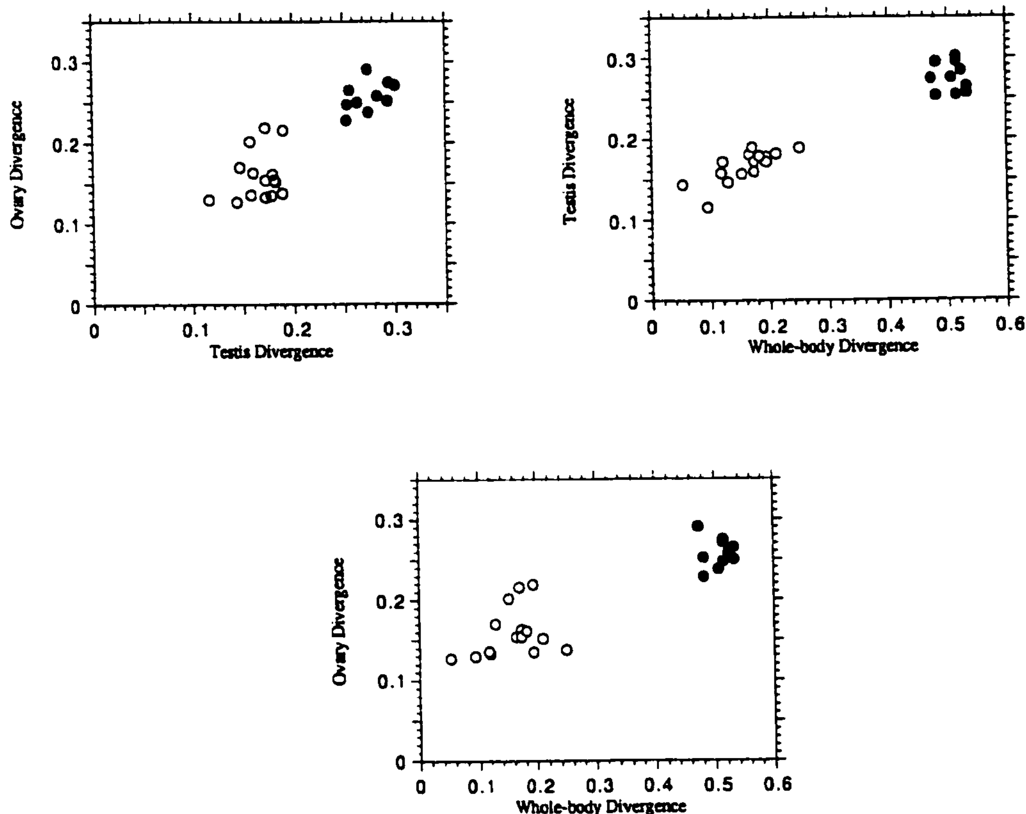


Fig. 3. Correlation between protein divergence ($D = -\ln F$ for testes and ovaries data; $D = -\ln S_{sm}$ for Spicer's whole-body data) in the *virilis* group species estimated from different tissue samples. *Open circles* are data points for species pairs belonging to the same phylad. *Solid circles* represent species pairs from different phylads. The whole-body protein data is from Spicer (1991).

tances of 6-phosphogluconate dehydrogenase (Reinbold and Collier 1990) and 2DE proteins (Spicer 1991) showed nonsignificant differences in evolutionary change between the two phylads. The differences in levels of protein divergence between the two phylads are not significant for either testes ($X^2_1 = 3.81$; $P > 0.05$) or ovaries ($X^2_1 = 1.48$; $P > 0.05$) (Wilcoxon two-sample test). Thus, our results support the previous findings of Reinbold and Collier (1990) and Spicer (1991).

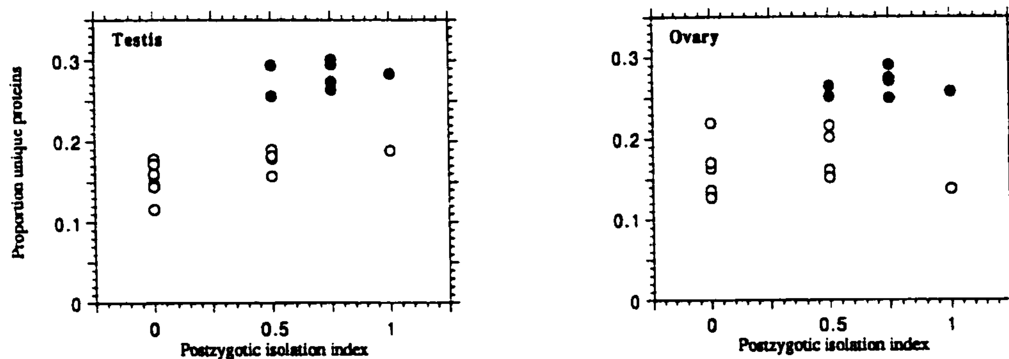
In Fig. 3, protein divergence between species pairs of the *virilis* group is expressed as $-\ln F$. The estimate, $-\ln F$, is used in order to compare our results for testes and ovaries with previously published results from whole-body 2DE protein analysis, where $-\ln S_{sm}$ was used as an estimate of divergence (Spicer 1991). Protein divergence estimated from different samples (testis, ovary, and whole-body) highly correlate with each other. However, this may be simply the correlation between the two groups considered (within phylad and between phylads). In all cases, pairwise species comparisons within the same phylad show lower divergence than those between members of different phylad. The protein divergence among species of the same phylad is slightly smaller for whole-body proteins. When more distantly related species pairs (from different phylads) are considered, protein divergence values for whole-body are higher than those for testis and ovary samples (Fig. 3).

Association Between Reproductive-Tract Protein Divergence, and Prezygotic and Postzygotic Reproductive Isolation

In Tables 1–3, the prezygotic and postzygotic isolation indices as well as the values of protein divergence obtained for the different species pairs are presented. Unfortunately, an analysis of the correlation between gonadal protein divergence and reproductive isolation indices for the *melanogaster* group species is not reliable as only two postzygotic isolation classes (RI = 0.5; RI = 1.0) are covered and estimates of prezygotic isolation are only available for three species pairs among the six species pairs sampled (Table 1).

In Fig. 4, postzygotic and prezygotic isolation indices are plotted against protein distance for the different tissues analyzed (data from Tables 2 and 3). In order to detect whether the level of divergence was correlated to the isolation indices, Kendall's coefficients of rank correlation were estimated. Both testis and ovary divergence correlated significantly with postzygotic isolation ($\tau = 0.84$; $N = 21$; $P < 0.001$ and $\tau = 0.64$; $N = 21$; $P < 0.01$). The correlation of testis divergence to prezygotic isolation indices was also significant ($\tau = 0.40$; $N = 15$; $P < 0.05$), whereas ovary divergence was not significant. These results suggest that protein divergence in testis as well as ovary samples strongly correlate with postzygotic

a)



b)

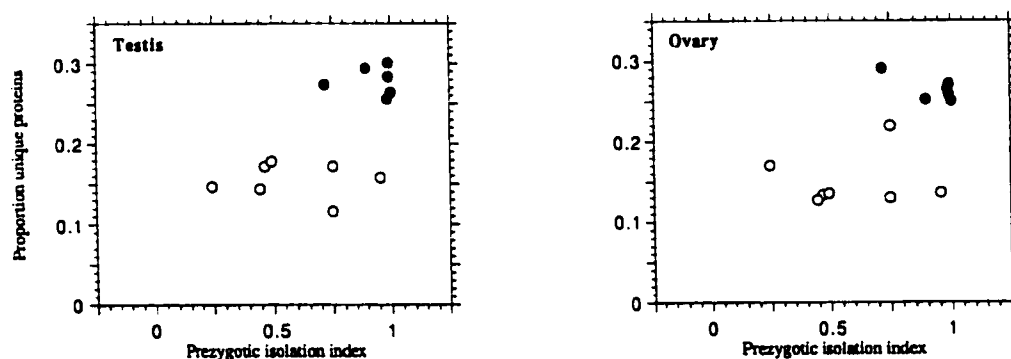


Fig. 4. Correlation between the proportion of unique proteins detected in testis and ovary samples and the level of (a) postzygotic reproductive isolation among the *virilis* group species and (b) prezygotic reproductive isolation among the *virilis* group species. *Solid and open circles* as in Fig. 3.

reproductive isolation indices. (The estimates for brain and M.T. are too few for statistical analysis.) However, the time span since speciation is generally related to the level of postzygotic isolation, with older species pairs having attained stronger isolation. The species compared in this study belong to either the *virilis* or the *montana* phylad. (See Throckmorton 1982; Spicer 1992.) Species pairs belonging to the same phylad (Table 2) have similar protein divergence values among themselves, and lower protein divergence than those belonging to different phylads (Table 3), independent of their postzygotic isolation indices. Moreover, species pairs from the same phylad, having the same postzygotic reproductive isolation as species pairs from different phylads, have lower D values (Tables 2 and 3). So, the correlations obtained between postzygotic isolation index and protein divergence seem to be biased by the time elapsed since speciation.

A partition of the correlation analysis for species pairs of same or different phylads showed nonsignificant correlations for ovary or whole-body proteins. This result supports the hypothesis that the overall correlation detected between postzygotic isolation and reproductive tract protein divergence is due to the amount of time since speciation. For testis proteins, however, the correlation is significant for closely related (same phylad) species pairs comparisons ($\tau = 0.59$; $N = 14$; $P < 0.05$) (Fig. 5), but nonsignificant for species pairs that have diverged for a longer period of time (species from different phylads of the *virilis* group species).

Discussion

The Role of Reproductive Tract Proteins in Reproductive Isolation

Previous genetic models of speciation have put greater emphasis on the amount of genetic changes involved in speciation. These models suggest either a temporary depletion and major reorganization of the gene pool (Mayr 1963), disorganization of polygenic balances (Carson 1975, 1982), or changes in a genetic complex with a few major genes and epistatic modifiers (Templeton 1980, 1981). However, there has been no clear description of

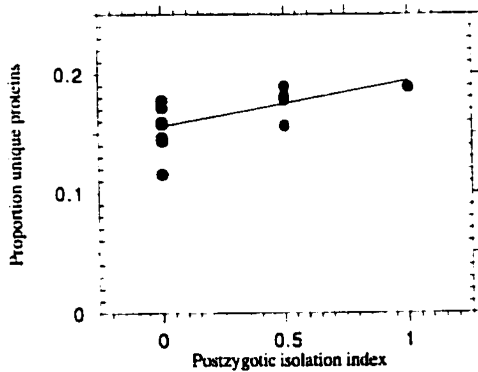


Fig. 5. Correlation between the proportion of unique proteins in testis and postzygotic reproductive isolation among species pairs belonging to the same phylad (either *virilis* or *montana*).

the nature of the genes that might be specifically involved in speciation. In an attempt to fill this gap, an alternative model of speciation was proposed (Singh 1990), emphasizing the role of genes affecting primary reproductive traits in the development of reproductive isolation. This idea was based on two key observations: (1) Male reproductive tract proteins show low variation within species but high divergence between species (Coulthart and Singh 1988a–c), and (2) among species of the genus *Drosophila* where species hybrids of both sexes are produced, the hybrids look phenotypically normal except for their genitalia. These observations provide a basis to entertain the hypothesis that genes affecting reproductive traits in general might be changing faster during speciation than genes affecting basic metabolism (i.e., housekeeping genes).

The present results for species of both the *melanogaster* and *virilis* groups show that both testis and ovary proteins have high but similar levels of protein divergence. Furthermore, the protein divergence in these tissues strongly correlates with postzygotic reproductive isolation. Although these correlations may suggest a role for reproductive tract proteins in postzygotic reproductive isolation, they should be treated with caution. Species pairs that have diverged for a longer time have both higher protein divergence and higher reproductive isolation than closely related species. Any positive correlation between reproductive isolation and protein divergence could simply be a consequence of both variables being dependent on time since species divergence.

Protein divergence is expected to increase with time since species divergence. If prezygotic and postzygotic isolation increase over time at a similar rate, time should equally affect the association between protein divergence and postzygotic isolation as well as protein divergence and prezygotic isolation. The association between time and reproductive isolation is difficult to test as our estimates of time since speciation are approximate. However, in the *virilis* group, species can be arranged in three distinctive groups based on their time since divergence. Group 1 contains three species, *D. americana*, *D. americana texana*, and *D. novamexicana*, which are closest amongst themselves and are believed to have diverged from each other for 2 to 5 Myr (Spicer 1991). Group 2 compares *D. lummei* and *D. virilis* with respect to species in group 1, and species of the *montana* phylad among themselves (approximately 6–9 Myr) (Spieth 1979; Spicer 1991). Group 3 contains species belonging to different phylads (*virilis* and *montana*) which have diverged for more than 15 Myr (Throckmorton 1977, 1982; Reinbold and Collier 1990; Spicer 1991). In Fig. 6, prezygotic and postzygotic isolation is plotted for these three groups. Although prezygotic isolation appears to originate earlier than postzygotic isolation (see also Coyne and Orr 1989), both seem to have evolved at a similar rate between species belonging to group 1 and 2 as the slopes in the graph are quite similar. Prezygotic isolation seems to slow down as compared to postzygotic isolation between groups 2 and 3. Group 1 and 2, showing similar rate of change for both types of reproductive isolation, contain species pairs that show significant positive correlation only between testis protein divergence and postzygotic isolation (Figs. 4 and 5). However, clustering species in three groups could be considered arbitrary, and doing so results in too few points remaining for comparison, so our conclusions become more speculative.

However, if only species pairs that have diverged for a shorter period of time but cover a good range of reproductive isolation values (both prezygotic and postzygotic) are considered (Fig. 4, open circles), the only case in which a significant correlation between divergence and postzygotic reproductive isolation is seen is protein divergence in testes (Fig. 5).

At this point, it should also be pointed out that the significant positive correlation detected between protein divergence in testes and postzygotic reproductive isolation is not necessarily indicative of causation. Proposing male reproductive tract protein divergence as a causal factor of speciation could be premature at this point. It would be of interest to score reproductive tract protein

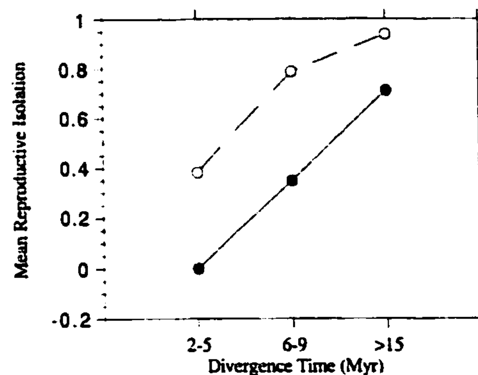


Fig. 6. Mean postzygotic reproductive isolation (*filled circles*) and mean prezygotic reproductive isolation (*open circles*) among species of the *virilis* group plotted against three distinctive species group classes that have diverged for different periods of time.

divergence among species pairs covering a wider range of time since speciation to confirm whether the significant correlation between reproductive tract protein divergence and postzygotic isolation is a more generalized phenomenon limited to species that have diverged for short periods of time.

If genes expressed in the male reproductive tract do play some role in the establishment of reproductive isolation between species of the *Drosophila* genus, then the association between reproductive isolation and male reproductive tract proteins will be dependent on the number of genes involved in the development of reproductive isolation. Data from crossing experiments between closely related *Drosophila* species (Coyne 1992; Wu et al. 1993) and a combination of crossing experiments with molecular genetic approaches (Perez et al. 1993; Zeng and Singh 1993b) seem to point to the involvement of a relatively small number of genes in the development of reproductive isolation. If so, they would not necessarily affect the overall levels of divergence of reproductive tract genes, and the answer for the high divergence of reproductive tract proteins and its association with postzygotic isolation should be looked for elsewhere.

Different Rates of Protein Evolution

Since Kimura (1968, 1969) introduced the concept of constancy of evolutionary rate, the tendency has been toward a relaxation of this postulate. The differences in DNA divergence between different taxa or among genes have been attributed to factors such as intrinsic differences in mutation rates due to DNA repair mechanisms (Britten 1986), episodic changes in mutation rate (Gillespie 1984), embryogenic patterns (Powell et al. 1986), and differences in the fraction of nucleotides free to vary (Palumbi 1989). However, the number of genes analyzed in these studies is limited to one or at most a few. In this report, we considered general divergence over hundreds of proteins sampled in different tissue environments instead of examining particular genes or proteins.

Based on the results obtained from the present data and previously published data (Thomas and Singh 1992), we can safely state that reproductive tract proteins have evolved faster than brain proteins in both the *virilis* and *melanogaster* group species. (The brain tissue provides a baseline against which other tissues can be compared.) The level of divergence for testis and ovary proteins is also higher, but not significantly, than that obtained for M.T. proteins.

The high divergence detected for species in both the *melanogaster* and *virilis* groups agree with the idea that genitalic morphological characters evolve at a faster rate in the short term following speciation (consider hybrids between closely related species that are phenotypically normal except for their genitalia), but that in the long term, both general morphology and germline traits become diverged (Fig. 3). However, we should be wary of accepting this generality until more data from different taxa covering a wider time-span since speciation event become available.

High Rates of Reproductive Tract Protein Divergence: Neutral vs Selective Hypothesis

In order to explain the high divergence detected for testes and ovaries it could be suggested that the majority of proteins produced by these tissues are secreted with the sperm or the eggs and hence they are all, similar to hemolymph, accessory gland, and M.T., secretory proteins. In order to clarify this argument, sperm- and egg-specific protein divergence should be measured independently from reproductive tract proteins that are not sperm or egg specific. However, a clear difference exists in the levels of variation within *D. melanogaster* and *D. simulans* for the truly secretory tissues and testes. We know that the levels of protein variation are lower in testis proteins than secretory ones in these two species (Singh and Coulthart 1982; Coulthart and Singh 1988c). According to the neutral model of molecular evolution, interspecific divergence and intraspecific variation would be correlated, so this low level of intraspecific variation and high divergence for testis proteins between species seems to reject a high neutral mutation rate as an explanation for the high divergence. However, more data on intraspecific variation for gonadal proteins in species of the *melanogaster* complex are needed.

It is possible that the low level of genetic variation is the result of recent fixation events (during speciation) or purifying selection (within species) coupled with episodic fixation of alleles (between species) by natural selection. Extending the analysis of polymorphism to gonadal proteins among more distantly related species in the *melanogaster* group may allow us to differentiate between these two hypothesis. If purifying selection is the main force maintaining low variation in reproductive traits, then more distantly related species should also show low genetic variation. Otherwise, we would expect the distantly related species to have recovered from the loss of genetic variation over time, in accordance with the neutral model of molecular evolution. Marshal (1983 and pers. com.) analyzed the morphology of male genitalic structures within and between species belonging to different genera of the Sphaeroceridae family (diptera). He detected an interesting pattern of uniformity of genitalic structures within species but high divergence between species, suggesting strong stabilizing selection within species and rapid (adaptive) divergence between species.

Alternatively, sexual selection by female choice has been invoked in order to explain the rapid and divergent evolution of male morphological structures involved in

copulation (Eberhard 1985). The data on morphological differences of reproductive structures among species of the *Drosophila* genus are so far concordant with the general pattern described for insects' male genitalia and male genitalic products involved in copulation. Among species of the *melanogaster* group, the only reliable characteristic for species recognition is the shape of the male genital arch. Variation in sperm structure and size is quite large in the *Drosophila* genus (Joly et al. 1991). However, two aspects that do not fit quite well with this previous picture should be noticed. First, in a recent study of the *nannoptera* group (*Drosophila*), sperm storage organs in females showed substantial differences among species and exhibit a positive relationship with sperm morphology (Pitnick and Markow 1994). The other interesting pattern in the *Drosophila* group is that internal reproductive organs not directly related with copulation seem to show extensive variation among species. Ovaries highly differ in the mean number of ovarioles (egg chamber) (Mahowald and Kambysellis 1980), differences in the color of the testis sheath can be used to distinguish species of the *D. melanogaster* complex (Coyne 1985), and among species of the *nannoptera* group, extensive variation in testis length, volume, and dry weight have been recently described (Pitnick and Markow 1994). It would be informative to conduct a larger and more detailed study of both female and male internal reproductive tract organs in *Drosophila* to test whether their morphology is in fact rapidly evolving, as seems to be the case for ovary and testis proteins.

Although it seems unlikely that internal structures such as ovaries and testes, or their protein products, may be under the influence of sexual selection, it is possible that gene products involved in mating and/or fertilization may be indirect targets of sexual selection. Clark et al. (1995) have found a significant association between accessory gland specific proteins and the ability of males to resist sperm displacement. Previous studies have shown that substances in the seminal fluid transmitted in the ejaculate are responsible for the refractory period following copulation (Boswell and Mahowald 1985; Kalb et al. 1993). Hence, the concept of sexual selection could be extended to both molecular and morphological traits not directly involved in copulation.

Although 2D-electrophoresis data have the advantage of hundreds of proteins being scored per gel and hence a trend based on a large sample size can be established, the data is genetically more static than direct DNA sequence analysis of tissue-specific expressed genes when it comes to discerning the reasons for the patterns of variation and divergence. Until now, there is evidence for translational control of gene expression for a small gene family (*Mst84D*, *Mst87F*, and *Mst98C*) involved in spermatogenesis (Schafer et al. 1990; Kuhn et al. 1991) and for janusB (*janB*), a gene transcribed in premeiotic spermatocytes (Yanicostas et al. 1989). Unless a larger and more detailed analysis of different genes and gene regions is performed, it will be difficult to establish the role played by selective forces in the history of these genes. High rates of molecular evolution have been suggested for two different genes specifically expressed in the male reproductive tract (*Acp26A* and *janB*), and in both cases strong selection in or near the region analyzed has played a major role in their history (Aguade et al. 1992; Veuille et al. 1994). Whether the trend of higher divergence in reproductive tract proteins is due to these proteins suffering major changes between species in their mode of regulation (translational control), function (e.g., secretory), structural characteristics (protein-protein, protein-DNA interactions), or as a result of being targets of adaptive evolution during speciation, remains an open question.

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CHAPTER 3

SEX AND SPECIATION: GENETIC ARCHITECTURE AND EVOLUTIONARY POTENTIAL OF SEXUAL *VERSUS* NON-SEXUAL TRAITS IN THE SIBLING SPECIES OF THE *DROSOPHILA MELANOGASTER* COMPLEX

This chapter has been submitted for publication to Evolution. Morphological analysis of sexual traits not directly involved in mating showed high between species divergence as well as significant between strains variation. The phenotypic distribution of sexual traits among interspecific hybrids was additive or dominant and the trait's asymmetry higher than the parental species. Interspecific non-sexual traits were overdominant and less to equally asymmetric with respect to the parental species.

ABSTRACT

In an attempt to understand the genetic architecture and evolutionary potential during speciation of traits that are part of the male reproductive system (genitalia and gonads), we have studied phenotypic divergence between species of the *Drosophila melanogaster* complex and their hybrids. Internal as well as external, sexual and non-sexual traits were analyzed with respect to genetic variation and trait asymmetry between strains within species, genetic divergence between species, and dominance and asymmetry in species and hybrids. The variation within species was significant among sexual traits and only external traits were less asymmetric than internal ones, suggesting that sexual traits are not strongly constrained within species. Three main findings show that sexual traits are most divergent between species. (1) Testis length and area, and the area of the posterior lobe of the genital arch (sexual traits), showed the highest proportion of variation between species. (2) Linear discriminant functions with the highest components associated to sexual traits were better predictors of species identity. (3) Testis length and area revealed a departure from a linear relationship between members of the species group. Comparison between species and their hybrids showed two main results. (1) Sexual traits showed higher asymmetry in species hybrids than the parental species, and (2) sexual traits showed additivity or dominance whereas non-sexual traits showed overdominance (with the exception of malpighian tubules length). These results suggest that sexual traits have undergone more changes presumably involving selection on modifier genes and as a result, tend to show higher divergence and stronger hybrid breakdown between species than non-sexual traits. We propose that sexual selection in the *broad-sense*, affecting all aspects of sexuality, may be responsible for the diversified appearance of sexual traits among closely related species, and that the genetic architecture underlying sexual traits may be more prone to disruption during the early stages of speciation.

Key Words: Sex, Speciation, Sexual selection, Dominance, Homeostasis, *Drosophila*.

INTRODUCTION

The characterization of species as separate entities depends on some form of distinctiveness measured in genetic and/or phenotypic terms. The biological species concept and the geographic theories of speciation (Mayr 1963; Dobzhansky 1970) place special emphasis on reproductive isolation in the process of speciation and hence a large proportion of studies have focused on the identification of mechanisms that can restrict gene flow among closely related species. This was particularly the case before species divergence could be studied at the genetic level. With the increasing emphasis on the genetic architecture of the speciation process (Mayr 1963; Carson 1975, 1982; Templeton 1980, 1981), there has been a growing shift towards identifying characters or mechanisms that would help keep species together as distinct clusters or unique gene pools. These have led both Paterson (1985) and Carson (1985) to suggest that speciation models that emphasized the role played by morphological and behavioural traits linked to mating components of sexual reproduction. Both authors predicted that these traits should be well-stabilized within species but would be the first to disrupt during the early stages of speciation, leading to rapid divergence and the establishment of new species-specific characteristics.

However, traits linked to mating components of reproduction are not the only ones that show species-specific characteristic. Among different animal groups, external male genitalia has been a useful taxonomic character at the species level. Eberhard (1985) surveyed major groups of animals, in which either primary or secondary external male genitalia were used to distinguish species (see Tables 1.1 and 1.2 in Eberhard 1985). Eberhard focused on morphological traits directly involved in copulation, excluding internal organs such as testes and accessory glands, and proposed sexual selection as the

driving force responsible for the high interspecific divergence detected in male genitalia (Eberhard 1985). Recently, Eberhard and Cordero (1995) have tried to extend the role of sexual selection to seminal products that might influence female reproductive behaviour and physiology as well as to female post-copulatory mechanisms for male discrimination (cryptic mate choice).

The high divergence of reproductive traits is certainly not restricted to external organs. Variation in sperm structure and size is quite large among different species of the *Drosophila* genus (Joly *et al.* 1991). The extremely long sperm found in some species of *Drosophila* such as *D. bifurca* and *D. hydei*, and the extreme divergence of such trait between species, are a puzzle for evolutionary biologists and explanations have been attempted based on post-fertilization sperm contribution to the zygote (Bressac *et al.* 1994), sperm competition (Pitnick and Markow 1994; Joly *et al.* 1995), and species-specific sperm-egg interactions (Karr and Pitnick 1996).

Testes length also shows high divergence among *Drosophila* species. A pictorial representation of the testis morphology by Patterson and Stone (1952; see Figs. 1 to 18) describes a range in length from species with highly coiled and long testes such as *D. virilis* and *D funebris* to extremely short testes such as *D. pseudoobscura*. However, most studies have focused on comparisons in sperm length with some mention of testes length. For example, Pitnick (1996) has shown that *D. melanogaster* and *D. bifurca* only varied four times in body mass but by more than forty times in testis and sperm length. Species such as *D. bifurca* and *D. hydei* represent one phenotypic extreme with respect to sperm length and it is then possible that the high divergence could be emphasized in comparisons between these species and distantly related ones. Few studies in *Drosophila* have looked at sperm length in comparison to other morphological traits among closely related species. One study that compared sperm, testes and thorax length among the four species of the

nanoptera group showed that these closely related species differ by four times in testis and sperm length but are almost identical in thorax length (Pitnick and Markow 1994).

Studies at the molecular level have also shown a trend of high divergence of reproductive traits among *Drosophila* species. Protein divergence using two-dimensional gel electrophoresis have shown that the male reproductive tract has a higher proportion of unique protein spots than wing disc and brain (Coulthart and Singh 1988; Thomas and Singh 1992). More recently (Civetta and Singh 1995), similar levels of protein divergence among species belonging to the *D. melanogaster* and *D. virilis* group have been found for proteins expressed in both male (testes) and female (ovaries) gonadal tissues. The divergence is higher for gonadal (testes and ovary) than non-gonadal (brain and malpighian tubules) expressed proteins, although the difference was only statistically significant for gonads *vs.* brain comparisons (Civetta and Singh 1995). Interspecific sequence divergence for *Acp26A*, a gene specifically expressed in the male accessory gland of *D. melanogaster* and its sibling species, have shown an extremely high level of non-synonymous substitutions between the four species of the *melanogaster* complex (Aguade *et al.* 1992). *Esterase 6*, a gene that is mainly expressed in the ejaculatory duct of *D. melanogaster*, *D. simulans* and *D. mauritiana*, showed a higher replacement to silent site divergence between *D.melanogaster* and *D. simulans* than seven out of ten loci compared (Karotam *et al.* 1993). Interestingly, the locus with the highest number of replacement to silent substitutions in Karotam's comparison was *transformer*, a gene involved in sex determination previously shown to have a high degree of divergence when coding regions were compared among five *Drosophila* species (O'Neil and Belote 1992).

We can hypothesize that if sexual traits (i.e. those traits that are part of the genitalia but are not necessarily involved in reproduction) play a major role in the early stages of speciation, then these traits should show higher divergence than non-sexual traits

between closely related species. Presently, there are two hypothesis to explain how these changes may occur. According to Paterson (1985) and Carson (1985), the high divergence observed among mating components of the reproductive system is the consequence of a direct disruption of a genetically buffered system, both authors predict that these sexual traits should be invariant within species. Alternatively, the high divergence of sexual traits between species could be the result of directional selection during the early stages of speciation, with plenty of genetic variation remaining within species. We can also entertain the hypothesis that high divergence between species should lead to incompatible interactions of genes responsible for sexual traits morphology in the hybrids, eventually leading to sexual traits breakdown.

We have used the four species of the *melanogaster* complex and measured interspecific morphological divergence for both external and internal sexual and non-sexual traits (Table 1). The sexual traits analyzed, testes and the posterior lobe of the genital arch, are traits not directly involved in copulation. In order to test whether the between species morphological divergence is the result of the disruption of a genetically buffered system, levels of asymmetry between the different sides of a character were measured. When spatially repeated characters are studied, asymmetry has been regarded as an indicator of developmental homeostasis (See Palmer and Strobeck 1986; Parson 1990). Finally, interspecific hybrid breakdown and interspecific genetic interactions of sexual and non-sexual traits were analyzed by both patterns of dominance and comparisons of trait asymmetry between parental species and their hybrids.

Our results extend the previous observations that sexual traits not directly involved in mating show high divergence between closely related species of *Drosophila* (Coulthart and Singh 1988; Thomas and Singh 1992; Civetta and Singh 1995). This high divergence, combined with the variation found within species and the pattern of traits asymmetry,

suggest that the evolution of sexual traits may be driven by directional sexual selection rather than stabilizing selection and episodic allele fixation (Paterson 1985; Carson 1985). The latter scenario was also considered by Coulthart and Singh (1988), whose two-dimensional electrophoresis results for male reproductive tract proteins showed low variation within and high divergence between species. Finally, the different nature of the dominance relationship found for sexual and non-sexual traits, and the higher interspecific hybrid asymmetry of sexual traits, indicate that species-specific gene interactions is an important aspect of the sexual traits' genetic architecture.

MATERIALS AND METHODS

Drosophila Cultures and Stock Maintenance

All the stocks used in this study, except for Oregon R and *D. mauritiana* LG24 (provided by Dr. J. David), are currently available from the Bowling Green Species Resource Center and the stock numbers are given in brackets. Three different strains of each of the four species of the *melanogaster* complex were studied. *D. melanogaster* strains were Oregon R, Peru (0231.1), and Australia (0231.3). *D. simulans* Colombia (0251.2), California (0251.163), and South Africa (0251.164). *D. mauritiana* LG24, 72 (0241.3), and 207 (0241.5) (all from Mauritius island). *D. sechellia* 21 (0248.6), 22 (0248.2), and Robertson (0248.7) (all from Seychelles island). The strains were maintained at 22°C under a 12:12 hour light-dark cycle.

Flies used in our analysis were the progeny obtained from mating ten virgin females to males in a 1:1 sex ratio in vials containing approximately 5 ml of banana medium. In order to avoid crowding, females were allowed to lay eggs and the adults transferred to a new vial whenever the count of eggs per vial was around 30. Eggs were

allowed to develop to adult stage at 22°C under a 12:12 hour light-dark cycle. Newly hatching adults were anesthetized with CO_2 and sexed. The males were transferred to vials containing fresh banana medium, where they remained for five days until collected for dissection. Between 10 and 15 individuals from each strain or cross were measured for the different traits analyzed.

Morphological Measurements

The traits studied were chosen to compare sexual *vs.* non-sexual characters that were internal as well as external (Table 1). The external non-sexual traits were wing length and width, tibia length and femur length. The external sexual trait was the area of the posterior lobe of the genital arch. The internal non-sexual traits measured were the length and area of the stalk connecting the malpighian tubule to the pyloric region of the ventriculus (referred from now on as malpighian tubule). Testis length and area were used as the internal sexual counterpart. The fly organs were dissected from each individual fly on a slide containing a drop of Drosophila Ringer's solution (Ashburner 1989a). Wings, legs and genital arches were flattened by covering them with an 18 mm^2 coverslip and heating the slide until the solution boiled. Testes and malpighian tubules were transferred and stretched in a drop of liquid paraffin . A video camera (Hitachi VK-C150) connected to a dissecting microscope and a Macintosh computer were used to capture images of the different preparations. Quick Image™ software package was used for image capturing, and NIH Image for measurement of the different morphological traits. Measurements were taken by using either a straight or free-hand mouse-controlled line selection.

Wing length (WL), wing width (WW), tibia length (TiL), and femur length (FeL) were measured as in Long and Singh (1995), but the femur measurement did not include the trochanter. The posterior lobe of the genital arch (GA) was demarcated by a line

across its base, and its area was assessed (Coyne 1983). Testes length (TL), testes area (TA), malpighian tubules length (MTL) and malpighian tubules area (MTA) were measured as shown in figure 1. Data was obtained in pixels and then converted into millimetres by scaling with a micrometer. Measurements were taken from both sides of a trait and its mean was used as an individual score.

Analysis of phenotypic variation

Analysis of variance (ANOVA) of the data obtained from each trait measurement was used to examine the amount of variation associated to species and strains within species. The analysis was done using JMP version 2.0.2 (SAS 1989) for the Macintosh. For malpighian tubules length and area, where only *D. simulans* (California and Colombia), *D. mauritiana* 72 and *D. sechellia* 21 were scored, measurements were analyzed by using a single-factor ANOVA model:

$$Y_{ij} = \mu + Species_i + \varepsilon_{ij}$$

The other character measurements were analyzed by a nested ANOVA model with species and strain within species effects.

$$Y_{ijk} = \mu + Species_i + Strain_{j[i]} + \varepsilon_{ijk}$$

Both normal quantile plots and the Shapiro-Wilk statistic (Shapiro and Wilk 1965) were used to check for departures from normality. The genital arch area and malpighian tubules area data showed significant deviations from normality ($W = 0.93$; $p = 0.0005$, and $W = 0.92$; $p = 0.0142$ respectively). A normal distribution was recovered by logarithmic

transformation of both GA and MTA data (W= 0.98 ; p= 0.633, and W= 0.96; p= 0.278 respectively).

The quantitative variables (trait measurements) showing the lowest correlation among themselves were standardized and retained as independent predictors of species membership. From these set of variables, linear combinations (canonical variables) that summarize between species variation were obtained by a linear discriminant function analysis using SYSTAT 5.0 (SYSTAT 1990) for the Macintosh.

Dominance effect

Only *D. simulans* (California and Colombia), *D. mauritiana* 72 and *D. sechellia* 21 were used for interspecific crosses. The dominance relationship of the parents to their respective hybrids was scored as h, where

$$h = \frac{\dot{Y} - ((\bar{Y}_A + \bar{Y}_B) / 2)}{|\bar{Y}_A - \bar{Y}_B| / 2}$$

$\dot{Y}$ being the mean phenotypic value of the interspecific hybrid obtained from the cross between species, $\bar{Y}_A$ the mean phenotype for species A, and $\bar{Y}_B$ the mean phenotype for species B.

A multidimensional representation of the total traits measurement variation among species of the *simulans* clade (all strains) and the hybrids was summarized by a principal component analysis using JMP version 2.0.2 (SAS 1989) for the Macintosh. From this analysis, the minimum number of independent linear combinations (components) of the standardized measurements that explained the major part of the variation were retained. Only individuals from which GA, TL, TA, TiL, FeL, WL and WW were measured, were

included in the analysis. MT data was not included as these measurements came from a different set of individual flies.

Analysis of asymmetry

D. simulans (California and Colombia), *D. mauritiana* 72, *D. sechellia* 21 and their respective interspecific hybrids were assessed for levels of asymmetry of sexual and non-sexual internal as well as external morphological traits. Wing length, tibia length, the area of posterior lobe of the genital arch, testes length and area, and malpighian tubules length and area were analyzed. When flies were dissected, the two sides of an internal trait could not be distinguished as left or right. Thus, comparisons of asymmetry among all traits and between parental species and hybrids were done by using the absolute difference between sides of a trait. Three replica measurements of each side of a trait were taken since differences in asymmetry may be very small and affected by measurement error.

RESULTS

Phenotypic variation within and between species

The variance components obtained from the analysis of variance among the four species of the *melanogaster* complex showed that sexual traits have a lower between strains within species variation than non-sexual traits (Table 2). However the results were significant for all traits, suggesting that sexual traits have no major constraints in terms of variation within species. The between species comparisons revealed higher sexual than non-sexual traits variation, but the results were significant for all the traits analyzed.

An inspection of the trait's mean phenotypic values obtained for the species suggests a different trend in the divergence of sexual and non-sexual traits. Non-sexual

traits were higher in mean value for *D. melanogaster* than the three species of the *simulans* clade, and *D. sechellia* appeared slightly smaller than the others. Instead, sexual traits showed *D. sechellia* closer to *D. melanogaster* in mean value, although for testes area *D. sechellia* was closer to *D. simulans* (Table 3).

The product-moment correlations were significant and very close to perfect linearity for wing length and width ($r = 0.977$; $p < 0.01$), tibia length and femur length ($r = 0.953$; $p < 0.01$), malpighian tubules length and area ($r = 0.949$; $p < 0.05$), and testes length and area ($r = 0.843$; $p < 0.01$) (Fig. 2). However, *D. sechellia* seemed to fall off from a linear relationship between testes length and area suggesting that testes differences among these closely related species involved a slight change in shape besides the change in size that is common to all traits (Fig. 2). When a linear function was fitted to the testes data, *D. sechellia* strains did not behave as outliers (i.e. standardized residuals were lower than 2) but the standardized residuals were consistently in the negative range (Fig. 3). In figure 3, the standardized residuals show an irregular distribution around the linear fit, but the unique pattern of positive deviations for *D. melanogaster* - *D. simulans* and negative for *D. sechellia* - *D. mauritiana* seen for testes measurements supports a change in shape during the evolution of testes morphology in the species of the *melanogaster* complex.

The shape of the posterior lobe of the genital arch has been the only reliable morphological character used to classify the four species of the *melanogaster* complex (Ashburner 1989b), and its mean area was the measurement that most clearly distinguished the four species in this study (Table 3). The slightly higher divergence for sexual than non-sexual traits detected from the analysis of variance suggested that perhaps sexual traits as a whole could be used as predictors of species membership. However, the efficiency of a particular trait to predict species membership could be influenced by correlation to other traits. Given the high correlation detected for measurements taken on

the same character, the number of variables was reduced or combined in order to re-define variables with the lowest correlation among themselves. Malpighian tubules were excluded as data was not obtained from *D. melanogaster* and only one strain of *D. mauritiana* and *D. sechellia* were measured. The multicollinearity among variables was tested based on estimates of the variance inflation factor (VIF), a function of the multiple correlation coefficient (R_i) of an ith given variable and the remaining variables (Afifi and Clark 1990). Tibia length divided by femur length (TiL / FeL), wing length divided by wing width (WL / WW), testes length (TL), and the area of the posterior lobe of the genital arch (GA) gave the lowest VIF estimates, indicating independence among themselves (TL = 1.16; GA = 1.10; WL / WW = 1.11; TiL / FeL = 1.03).

Linear combinations of these four variables that summarize the variation between the grouping variables (species) were obtained by a discriminant function analysis. The total data set was reduced as individuals for which a measurement was missing were dropped from the analysis. The first two discriminant functions (V_i) showed the stronger correlations with the grouping factor (species) and they explained 99 percent of the between species variation (Table 4). GA was the original variable with the highest loading associated to the first discriminant function, and TL had its highest discriminant coefficient associated with the second function. The highest coefficients for the third function were for WL / WW and TiL / FeL (Table 4). A plot of the first two discriminant function scores obtained for each individual gives the maximum possible separation among the species. The first function scores separated all the species in the sample except for partial overlapping between *D. sechellia* and *D. melanogaster* (Fig. 4a). This result is in agreement with the separation obtained between the same species based on the size of the posterior lobe of the genital arch (Liu *et al.* 1996). D. *mauritiana/ simulans* and D. *melanogaster/ sechellia* were separated by the second discriminant function which had the

highest coefficient associated with TL (Fig. 4a). Figure 4b shows that the function with high coefficients for WL / WW and TiL / FeL (V_3) did not discriminate between species.

Species hybrids: genetic dominance in sexual vs. non-sexual traits

The size traits (wings and legs) showed a consistent pattern of overdominance as the hybrids had mean phenotypic values higher than both parents (Appendix and Fig. 5). Malpighian tubules measurements showed average underdominance (length) or overdominance (area) (Fig. 5), although the error associated to these measurements made the conclusions uncertain (Appendix). Sexual traits behaved additively in average value (Fig. 5), but testes length and area showed a tendency towards dominance of the male parental species (Appendix). For testes length, this tendency was greater for the crosses between *D. simulans* and *D. mauritiana*, and *D. sechellia* female *D. simulans* male. *D. sechellia* female x *D. mauritiana* male and *D. simulans* female x *D. sechellia* male were closer to additivity (Appendix).

In order to represent the dominance relationship between parental species and hybrids, the original variables were transformed into uncorrelated variables by a principal component analysis. Two main criteria were used to decide the number of components to be retained from this analysis. First, almost 88% of the total variance is explained by the first three components (Table 5). Second, a plot of the variance (eigenvalue) associated with each component shows a break between the steep slope of the main factors to be considered and the trailing off of the remaining factors (scree plot) (Fig. 6).

Wing and leg measurements had the largest coefficients, and hence the highest association, with the first component (PC1), testes with the second component (PC2), and genital arch with the third component (PC3) (Table 5). The individual scores obtained from each component were plotted against each other and are shown in figure 7. The first

component (PC1), in which the bulk of the variance is due to non-sexual traits, showed overdominance as the hybrid scores laid on the positive range and beyond the distribution of the parental species. The hybrid scores for testes (PC2 axes) were additive in mean value, but closer to the male parental species. Genital arch area scores (PC3 axes) were additive.

Thus, the results showed that the phenotypic distributions of non sexual traits (malpighian tubules, legs and wings) in the hybrids are beyond the parental range, whereas sexual traits were either additive or dominant.

Species hybrids: homeostasis of traits

In order to compare asymmetry across traits, the absolute difference between sides was divided by the traits size. The size of the trait was estimated as the average between sides. A logarithmic transformation of these measurements homogenized the variances across traits (O'Brien, Brown-Forsythe and Levene tests: $F_{(6, 716)} < 1.0$, $p > 0.1$) but their distributions remained skewed.

Internal traits were consistently more asymmetric than external characters, and no pattern was detected based on the sexual *versus* non-sexual classification of traits (Fig. 8). It could be argued that a trait's asymmetry may be a consequence of measurement error. However, no association was observed between the degree of trait's asymmetry and differences among replicate measurements. For example, one of the least asymmetric traits (tibia length, see Fig. 8) showed the largest difference between replicas (Kruskal-Wallis test: $X^2_2 = 1.32$; $p = 0.518$).

For each trait, differences in asymmetry between parental species and their hybrids were compared by using the logarithmic transformation of the absolute difference between

sides. Sexual traits were always significantly higher for interspecific hybrids than parental species, although the difference was only borderline significant for testes area (Mann-Whitney test: $Z= -1.90$; $N_H= 65$, $N_P= 38$; $p= 0.057$) (Fig. 9a). Except for malpighian tubules length, non-sexual traits showed a higher parental than hybrid asymmetry but the differences were not significant for wing length (Mann-Whitney test: $Z= 1.19$; $N_H= 63$, $N_P= 49$; $p= 0.234$) and malpighian tubules area (Mann-Whitney test: $Z= -1.34$; $N_H= 64$, $N_P= 38$; $p= 0.179$) (Fig. 9b).

DISCUSSION

Sexual-traits: interspecific divergence and sexual selection

Our results showed a slightly higher divergence for sexual than non-sexual traits between species of the *Drosophila melanogaster* complex, and sexual traits were better predictors of species' distinctness than non-sexual traits. Our previous results on protein divergence among species of the *Drosophila* genus showed the same pattern; proteins expressed in testes and ovaries were more diverged among species than non-reproductive tract proteins (Civetta and Singh 1995). The emerging picture from DNA sequence analysis also shows that sex-related genes, that is, genes expressed in the male genitalia, involved in sex determination, or linked to mating behaviour, have large number of amino acid replacements between species (Aguade *et al.* 1992; O'Neil and Belote 1992; Karotam *et al.* 1993). This pattern seems to cross over the boundaries of the *Drosophila* genus (Whitfield *et al.* 1993; Tucker and Lundrigan 1993; Lee *et al.* 1995; Swanson and Vacquier 1995).

Why do sexual traits coincide in a common theme of high interspecific divergence? A few explanations can be attempted.

According to the neutral model of evolution (Kimura 1983), the high divergence of male sexual traits may be seen as the result of a higher mutation rate or a less constrained selection regime. Based on such a model, variation within species should positively correlate with the variation detected between species. In this paper this trend is reversed, sexual traits showed lower variation within but higher divergence between species than non-sexual traits. Interestingly, Patterson and Stone's description of the male and female reproductive system of different *Drosophila* groups (1952, see figs. 1 to 18) indicates that species with highly coiled and long testes have also long female seminal receptacles, whereas species with short testes have short seminal receptacles. A positive correlation between the length of the seminal receptacle and sperm length has been reported among species of the *Drosophila nanoptera* group, and the seminal receptacle was the only female reproductive tract trait that showed significant divergence between species (Pitnick and Markow 1994). Similar results have been found for passerine birds, where sperm length and the length of female sperm storage tubules are positively correlated (Briskie and Montgomerie 1992). The co-evolving nature of these male and female traits does not seem to support neutrality as a possible explanation for the divergence detected between species.

Alternatively, Paterson's recognition species concept (Paterson 1985) and Carson's organization theory of speciation (Carson 1985) predict that traits responsible for holding species together should be well coadapted and subject to stabilizing selection. Then, low variation within species is predicted for such characters since any deviation from the norm becomes detrimental. Our results do not support this prediction as testes length and area, and the area of the posterior lobe of the genital arch, showed significant variation within species. The results seem more in line with models of speciation by sexual selection that require within species variation (Lande 1981; Kaneshiro 1989; Iwasa and Pomiankowski

1995), and with studies that show high variability for sexually selected traits in *Drosophila* (Carson and Lande 1984), crickets (Cade 1981; Hedrick 1988), fish (Houde 1992), seaweed flies (Wilcokson *et al.* 1995), etc. Although Paterson's prediction may still apply to mating behavioural traits, a review by Cade (1984) showed high heritability for such traits which indicates considerable genetic variation underlying mating behaviour.

The theory of sexual selection was originally proposed to explain the evolution of any extravagant male organ, its advantage being in exclusive relation to mating and reproduction (Darwin 1871). Sexual selection triggers a run-away mechanism of rapid divergence on the male trait as well as the females preference (Fisher 1958). Eberhard and Cordero (1995) have recently attempted to extend the concept of sexual selection to "chemical genitalia", that is seminal products that induce physiological and behavioural changes in the females. These products will undergo rapid changes as a result of female choice in favour of substances with better stimulating properties. Rice (1996) has shown strong intersexual coevolution in selection experiments with *Drosophila melanogaster*, and he has suggested that such a process might contribute substantially to genetic divergence among populations, becoming eventually an important factor in the process of speciation. It is possible that male sex-related structures, at both morphological and molecular levels, may be directly or indirectly (hitchhike effect) affected by directional sexual selection.

Secondary sexual traits have shown higher levels of asymmetry than other morphological characters, and it has been proposed that directional sexual selection on secondary sexual traits may act on modifier genes that reduce developmental control leading to rapid divergence and increased asymmetry (Møller 1993; Pomiankowski and Møller 1995). However, the level of asymmetry is also dependent on how selection prevents a phenotype from random deviation from its norm. A poorly canalized trait may

also be highly asymmetric. Our results do not show sexual traits being more asymmetric than non-sexuals, and the only pattern appears in terms of internal *versus* external. The phenotypes with the lowest asymmetry were wing and tibia length, both traits are probably under strong stabilizing selective pressures as random variations between sides in these traits may affect locomotion and dispersal. On the other side, malpighian tubules length had the highest asymmetry, and there are no obvious reasons as to why fluctuations between sides in the length of the stalks connecting the malpighian tubule to the pyloric region of the ventriculus should affect the fitness of the organism.

Species hybrids and the genetic architecture of sexual vs. non-sexual traits

Except for malpighian tubules length, the mean phenotypic values for non-sexual traits of interspecific hybrids were constantly above the range of the parental species. Such phenomenon has been well known and Dobzhansky (1952) coined the term *luxuriance* to distinguish it from heterosis resulting from crosses between different varieties of the same species. Wright (1977) also refers to *luxuriance* and suggests that it may have more to do with the defective regulation of growth in the interspecific hybrids than a complementary action of the parental genes. A phenotype resulting from a misregulation of growth should be poorly buffered and then more asymmetric than the parental forms. Our results for non-sexual traits showed not only a phenotypic distribution of the hybrids beyond the parental species, but also less asymmetry than the parental species. This is what has been observed for heterozygote hybrids obtained from crosses between different strains (For reviews see Palmer and Strobeck 1986; Parson 1990). Both the overdominant effect and the lower asymmetry in the interspecific hybrids than the parental species suggest allele complementation in the hybrids as a result of a less diverged genetic system among species.

Sexual traits did not show overdominance. The posterior lobe of the genital arch showed an intermediate phenotype in interspecific hybrids, whereas testes showed an average additive effect but a trend towards paternal dominance. Before attempting an explanation for the different phenotypic behaviour of the two sex traits analyzed it should be noted that similar differences in phenotypic behaviour of sexual *vs.* non-sexual traits can be found in previous studies that focus on other aspects of species morphological characteristics. Coyne (1983) and Liu *et al.* (1996) showed that the genital arch in male hybrids between *D. simulans* and *D. mauritiana* is intermediate in size between those of their parents. Coyne (1985) showed that the number of male sex comb tooth is intermediate in hybrids between *D. simulans* and *D. mauritiana*. Coyne *et al.* (1991) carried on an analysis of the genetic basis of morphological differences among the species of the *melanogaster* complex. Their measurements of tibia and femur length showed higher hybrid than parental species phenotypic values, whereas ovariole number and genital arch area behaved additively. The pattern looks constant throughout the literature and it is possible that the additivity *vs.* overdominance behaviour might pertain to sexual *vs.* non-sexual traits independent of maleness.

Although the interspecific hybrid testes morphology was additive on average, this trait showed a less consistent pattern than the others. The slight differences between hybrids for the phenotypic distribution of testes may relate to the complexity of its genetic basis. Joly *et al.* (1997) made an attempt to elucidate the genetic basis of sperm and testes length through backcross analysis between *D. simulans* and *D. sechellia* and the use of phenotypic markers. They obtained the same result of testes length additivity we observed for the F_1 hybrid between *D. simulans* female and *D. sechellia* male but the backcross to *D. simulans* males showed a complicated range of phenotypes that, with the exception of one particular backcross type, ranged from the average distribution between the parental

species to the average phenotype of the original *D. sechellia* male parent. The study suggested the existence of autosomal factors on both arms of chromosome 3 of *D. sechellia* interacting with *D. sechellia* chromosome 2 as responsible for the enhanced length of the testes in the backcrossed male progeny (Joly *et al.* 1997). The dominance effect seen in our study may be the result of either similar autosomal effects or Y chromosome effects. Interestingly, only in crosses involving *D. sechellia* male, the hybrids remained closer to additivity suggesting that the Y chromosome of *D. mauritiana* and *D. simulans*, but not *D. sechellia*, may have an effect on the length of testes. The genetic basis of testes morphology seem quite complex, and it will require further genetic analysis to properly elucidate it.

Sexual traits showed higher interspecific hybrid asymmetry than parental species. This result suggests that sexual trait genes have lost their ability to properly interact in the hybrids. In a system under intense directional selective pressure and hence subject to rapid changes, negative pleitropic side effects are expected that would need to be buffered through the evolution of epistatic modifiers. The higher asymmetry found in the hybrids may indicate an upsetting of the epistatic control of the sexual phenotype so that the negative pleiotropic effects arise. This situation seems to be enhanced in hybrids between more distant species such as *D.melanogaster* and *D. simulans*, where testes become atrophied (Pontecorvo 1943). It is possible that the complex testes phenotypic behaviour of the different hybrids between the three species of the *simulans* clade represents a first step towards phenotypic breakdown.

Conclusion: the evolutionary potential of sexual traits in speciation

It could be argued that postmating barriers, such as hybrid sterility, might not be the first step in speciation. For example, *D. heteroneura* and *D. silvestris* , two well

recognized species of the Hawaiian group, are interfertile in the lab but are strongly isolated in mate-choice experiments (Kaneshiro 1976; Carson 1978). Interestingly, the two species differ in two male secondary sexual characters, one involved in male-male competition (the head) (Spieth 1981) and the other which function relates to the stimulation of the female's abdomen during courtship (the male tibial cilia) (Carson *et al.* 1982). This kind of observations have generated the proposal of speciation models that emphasize the role of the behavioural components of the reproductive system as key traits responsible for species identity (Carson 1985; Paterson 1985).

On the other hand, Haldane's observation that in the F_1 offspring of two different species the heterogametic sex is rare, absent or sterile (Haldane 1922) have become a keystone for those attempting a resolution of the speciation problem. Wu and Davis (1993) have shown that in groups such as *Drosophila* and mammals, crosses between species almost always result in hybrid male sterility, whereas hybrids females are either inviable or sterile among lepidoptera and birds. The result suggest that the high proportion of hybrid male sterility in *Drosophila* and mammals is not only a condition brought about by the heterozygote nature of the sex chromosomes in the males. The diminished hybrid male fertility may also be the result of an easier disruption of the male genitalia during the early stages of speciation.

Based on these previous observations, the high between species divergence of sexual morphological traits not directly involved in copulation, their higher asymmetry in hybrids than parental species, their F_1 hybrid dominance relationship, and our previous result showing high between species divergence of proteins expressed in the male reproductive tract that are not necessarily involved in reproduction (Coulthart and Singh 1988; Thomas and Singh 1992; Civetta and Singh 1995), we suggest that *all aspects of sexuality undergo rapid changes during speciation*. Regardless of whether pre or

postmating barriers establish isolation between species, the common theme seems to be the major role played by sexual traits in the establishment of such barriers.

Finally, the high divergence between species coupled with the within species variation found for the sexual traits analyzed in this study, and the fact that sexual traits were not less asymmetric than non-sexual traits, argue in favour of their role in speciation through directional sexual selection coupled with population isolation rather than strong stabilizing selection and episodic random perturbations of the genome though founder events. Sexual selection has been traditionally linked to behavioural or morphological characters directly involved in male-male competition, courtship or mating (Andersson 1994). We propose a distinction between this original *narrow-sense* sexual selection definition and a broader selective process affecting all aspects of sexuality (molecular and morphological) not directly linked to courtship or mating (i.e. *broad-sense* sexual selection).

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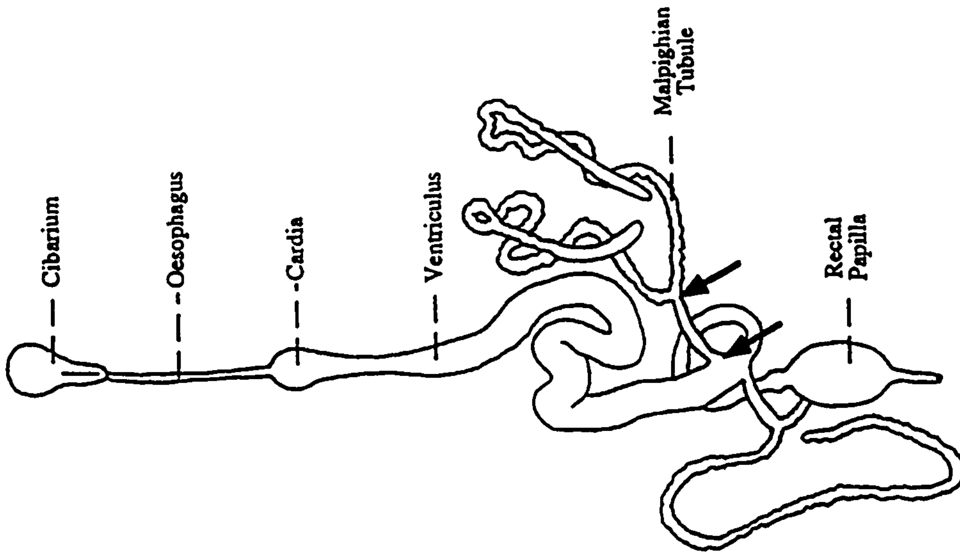
Figure 1: A diagram of testes (left) and the digestive system (right) is shown. Pair of arrows delineate the portion of the testes and malpighian tubules for which length and area were measured. The diagram of the digestive system is modified from Demerec (1950).


Cibarium
Oesophagus
Cardia
Ventriculus
Malpighian Tubule
Rectal Papilla

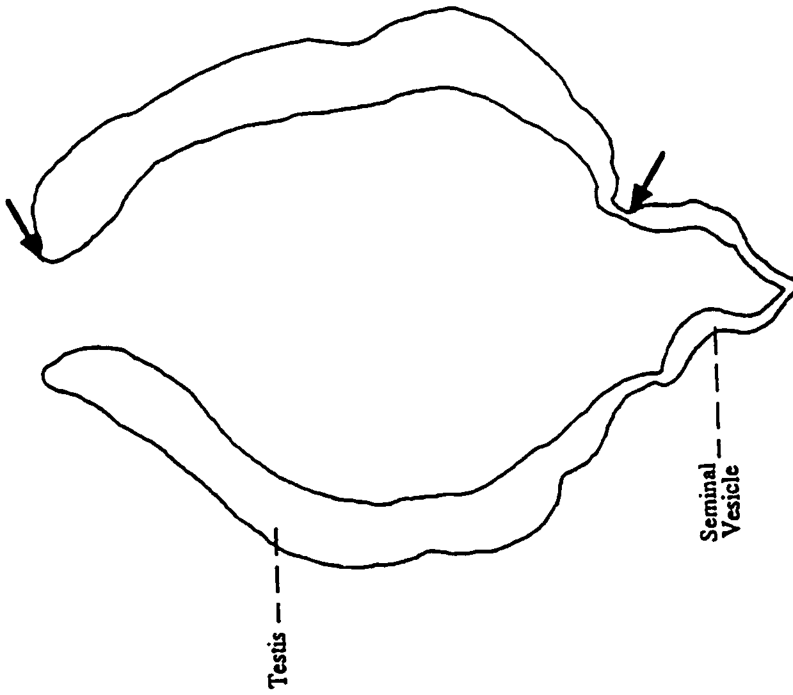
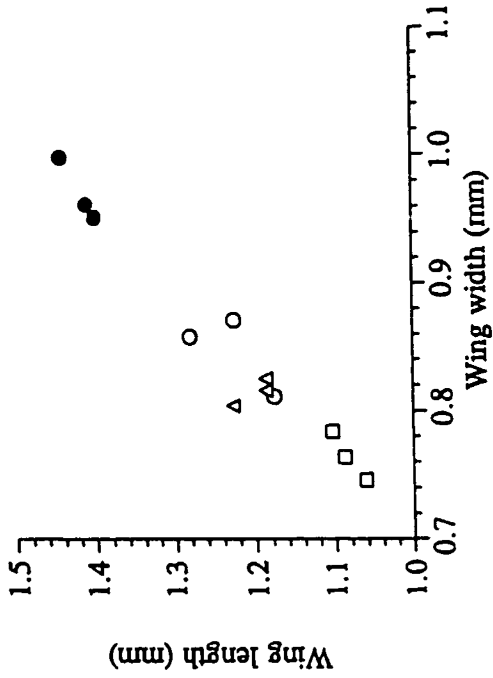
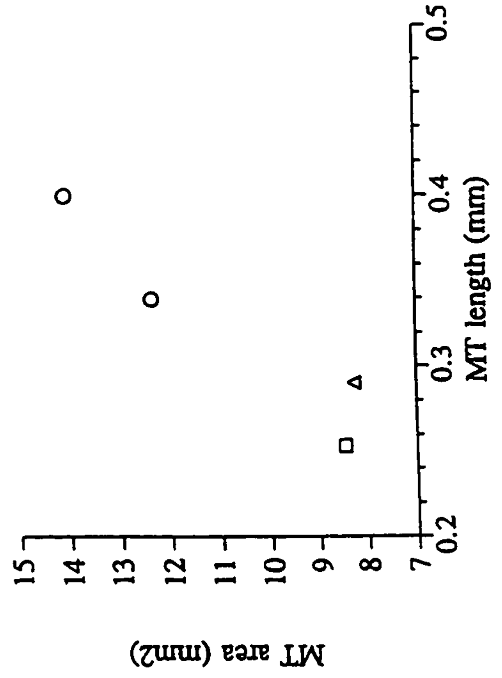
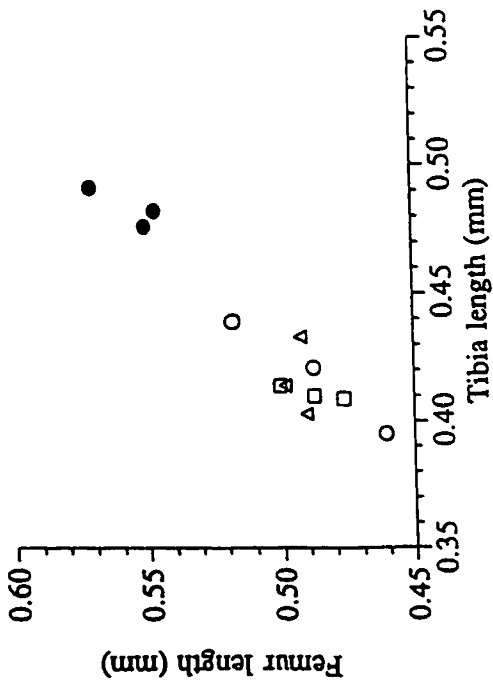

Testis
Seminal Vesicle

Figure 2: Correlation between measurements taken on the same trait. Filled circles represent the mean phenotypic value obtained for different lines of *D. melanogaster*, open circles, *D. simulans*, triangles, *D. mauritiana*, and squares, *D. sechellia*.


Femur length (mm)
Tibia length (mm)
0.60
0.55
0.50
0.45
0.35
0.40
0.45
0.50
0.55


Wing length (mm)
Wing width (mm)
1.5
1.4
1.3
1.2
1.1
1.0
0.7
0.8
0.9
1.0
1.1


Testes area (mm²)
Testes length (mm)
200
180
160
140
120
100
80
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4

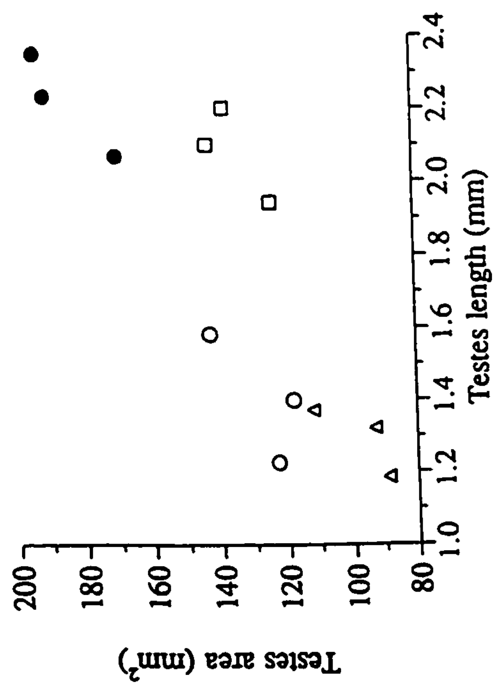
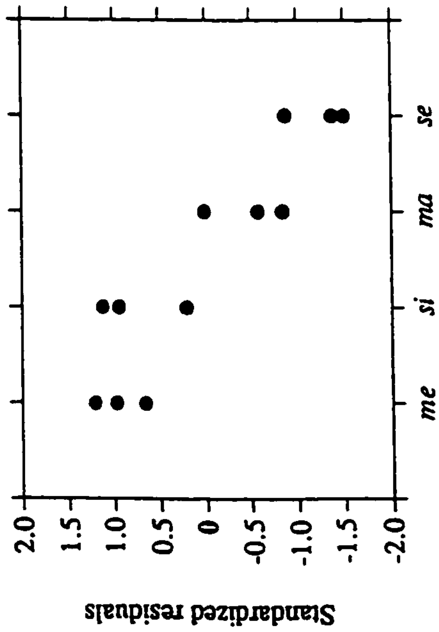
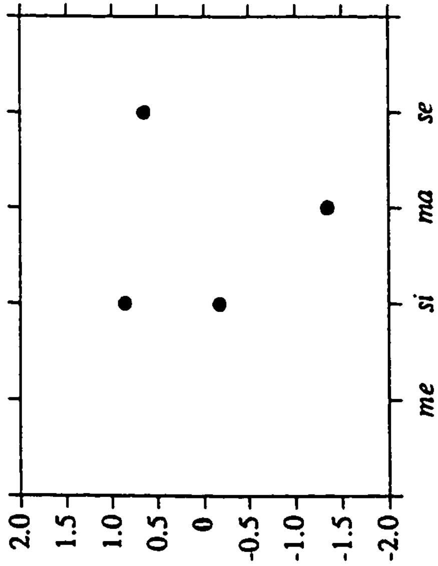

MT area (mm2)
MT length (mm)
15
14
13
12
11
10
9
8
7
0.2
0.3
0.4
0.5

Figure 3: Standardized residuals obtained from linear regressions between **a)** testes length and area, **b)** malpighian tubules length and area, **c)** wing length and width, and **d)** tibia length and femur length . The standardized residuals show departures from a perfect linear fit (0) for the different lines of the four species of the *melanogaster* complex. Abbreviations for species are *me*= *D. melanogaster*, *si*= *D. simulans*, *ma*= *D. mauritiana*, and *se*= *D. sechellia*.

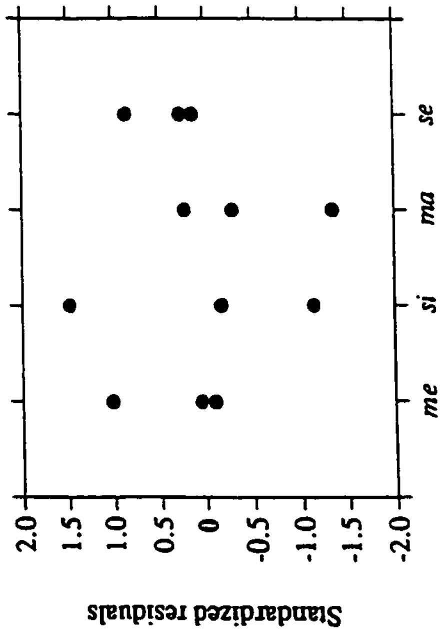
a)



b)



c)



d)

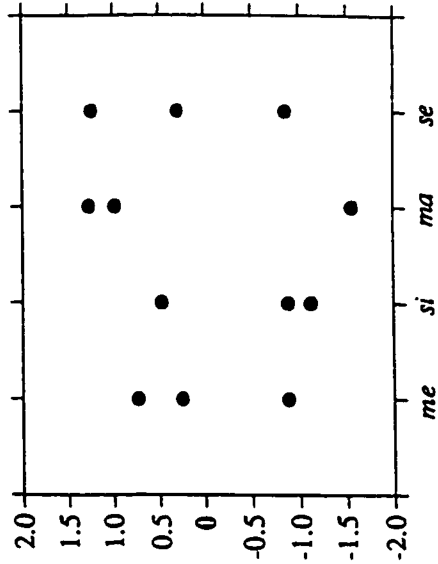
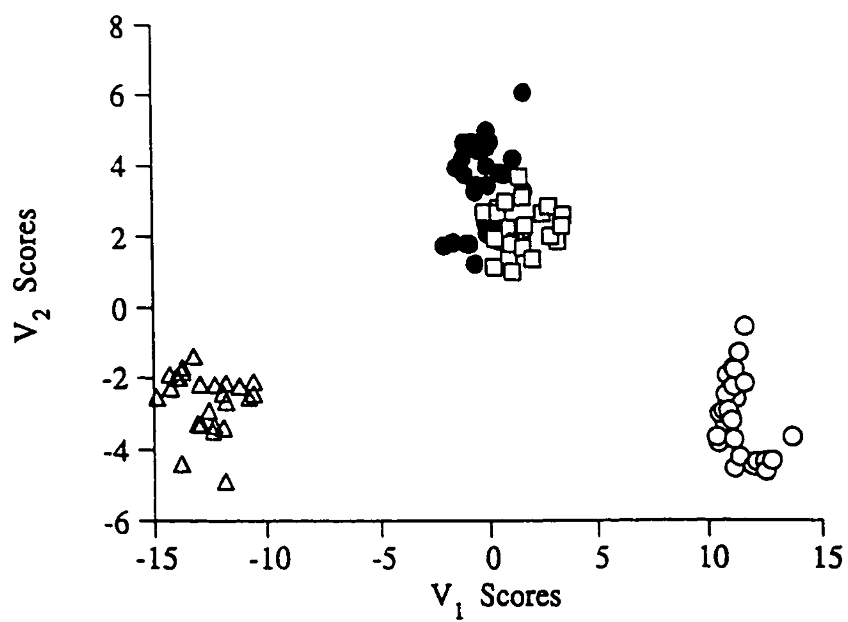


Figure 4: Discriminant function scores obtained from individuals belonging to the four species of the *melanogaster* complex. The symbols used for the different species are as in figure 2. **a)** Individual scores for the first (V_1) *versus* second (V_2) dicriminant function. **b)** Individual scores for the first *versus* third (V_3) dicriminant function.

a)



b)

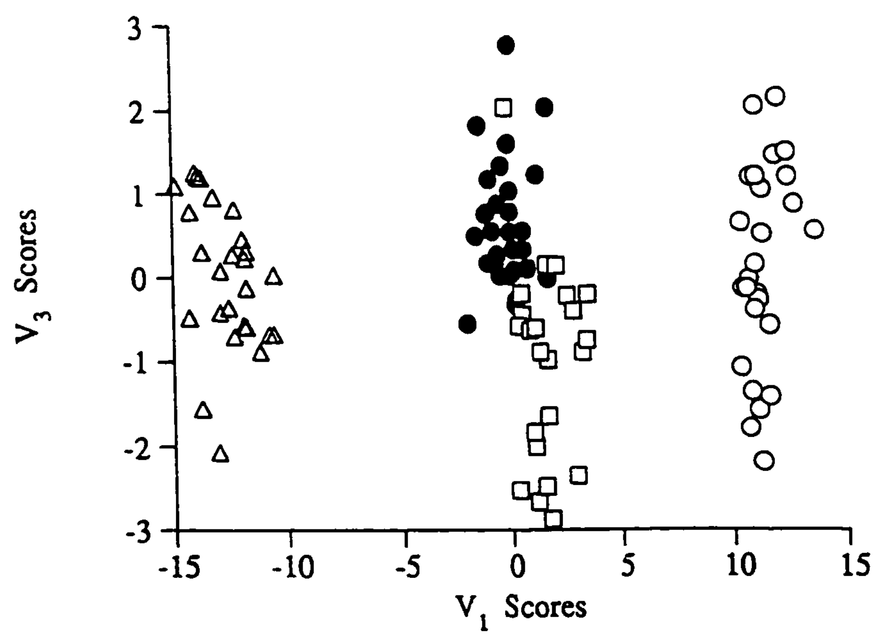


Figure 5: Dominance relationship (h) between species and their respective hybrids for the sexual (filled bars) and non-sexual (crossed bars) traits analyzed. The abbreviations used for traits are explained in the Material and Methods section. Means ± standard error are shown.

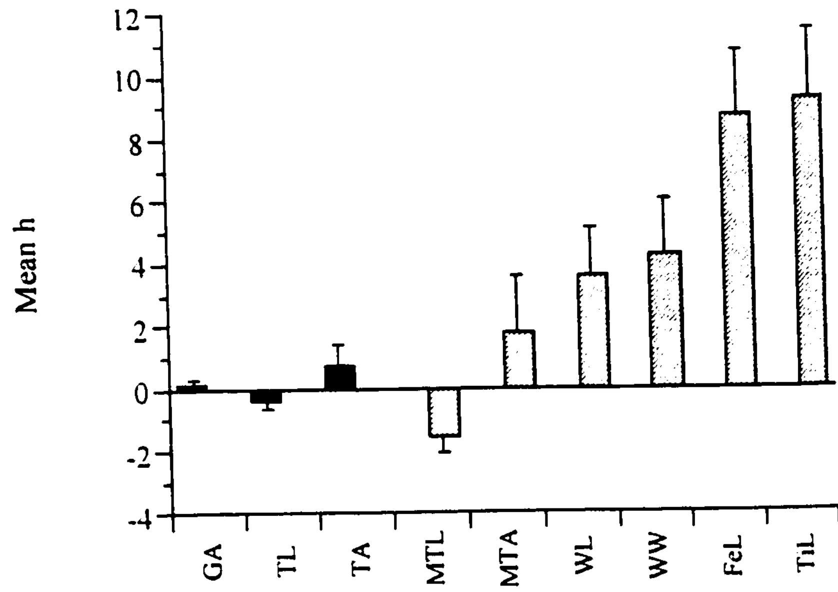

Mean h
12
10
8
6
4
2
0
-2
-4
GA
TL
TA
MTL
MTA
WL
WW
FeL
TiL

Figure 6: Eigenvalues associated to each of the components obtained from the principal component analysis.

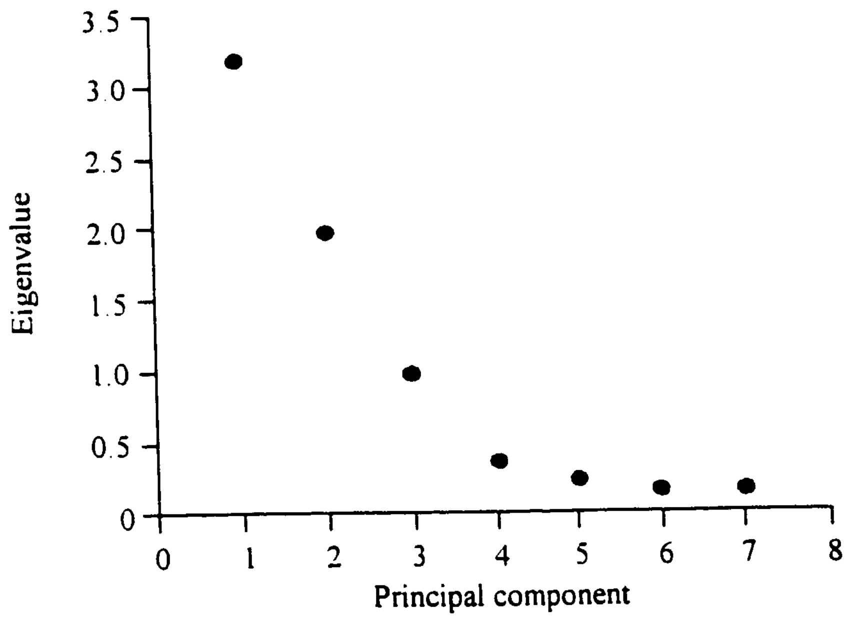
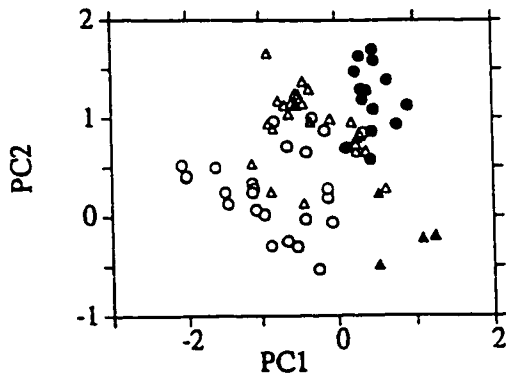
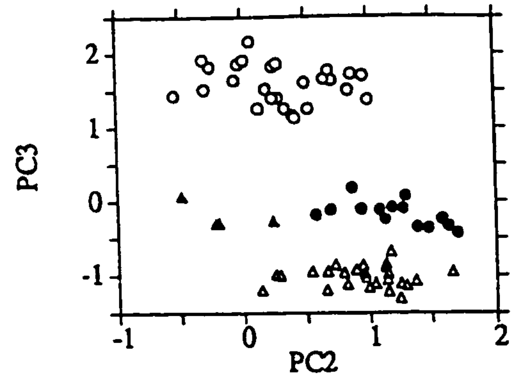

3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Eigenvalue
0
1
2
3
4
5
6
7
8
Principal component

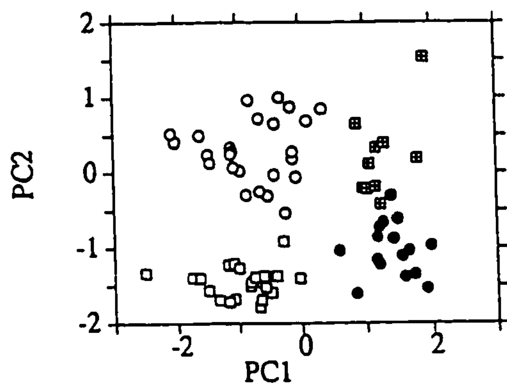
Figure 7: Plot of individual scores obtained from the principal components analysis for species and hybrids. The symbols used for the different species are as in figure 2. Interspecific hybrids are represented by using filled parental species symbols, where the shape of the symbol coincides with that of the female parental species.

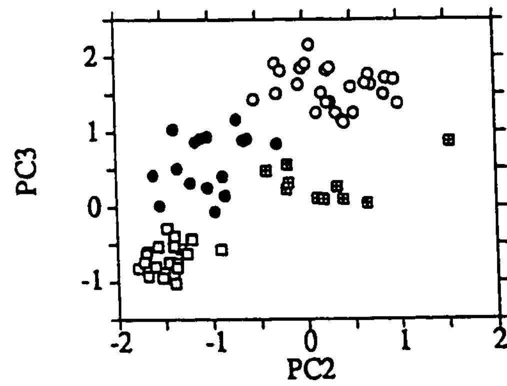
a) *D. simulans x D. mauritiana*



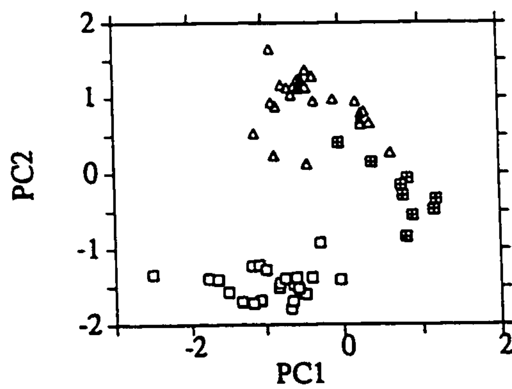


b) *D. simulans x D. sechellia*





c) *D. sechellia x D. mauritiana*



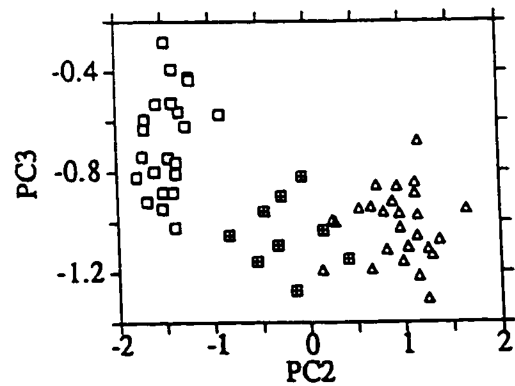
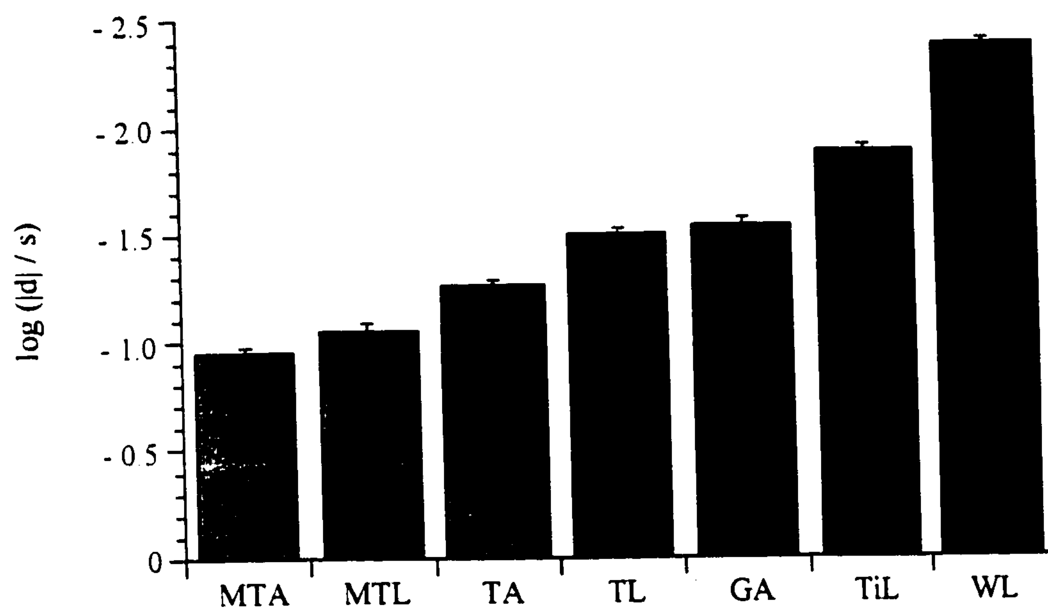


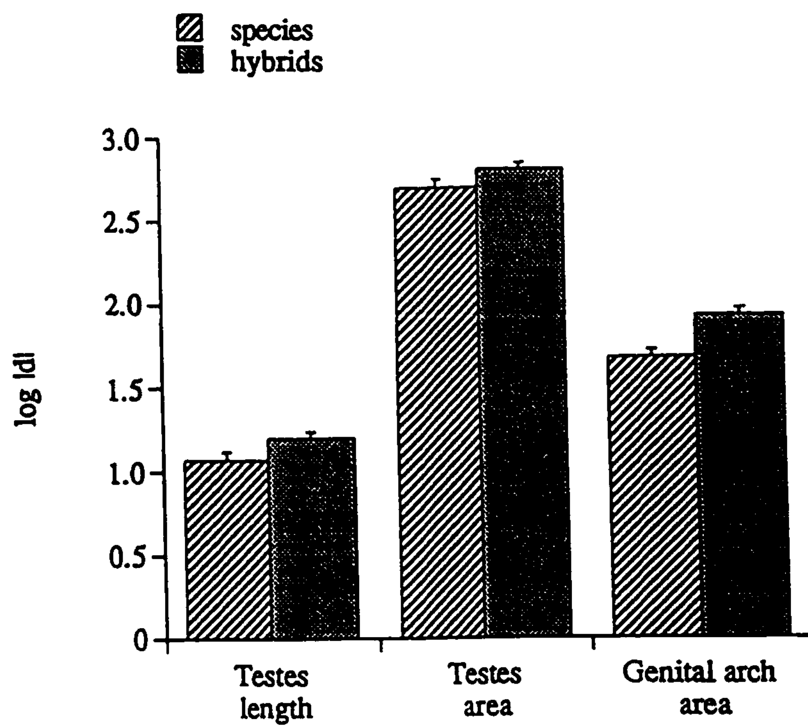
Figure 8: Comparison of the level of asymmetry associated to each trait. In order to compare across traits, the absolute difference between sides (|d|) was divided by an estimate of size as the average between sides (s). Variances of the asymmetry estimates were homogenized by a logarithmic transformation. The abbreviations used for traits are explained in the Material and Methods section. Means ± standard error are shown.



log (|d| / s)
- 2.5
- 2.0
- 1.5
- 1.0
- 0.5
0
MTA
MTL
TA
TL
GA
TiL
WL

Figure 9: Measurement of asymmetry (log |d|) in species and hybrids for various sexual (**a**) and non-sexual (**b**) morphological traits. Means ± standard error are shown.

a)



b)

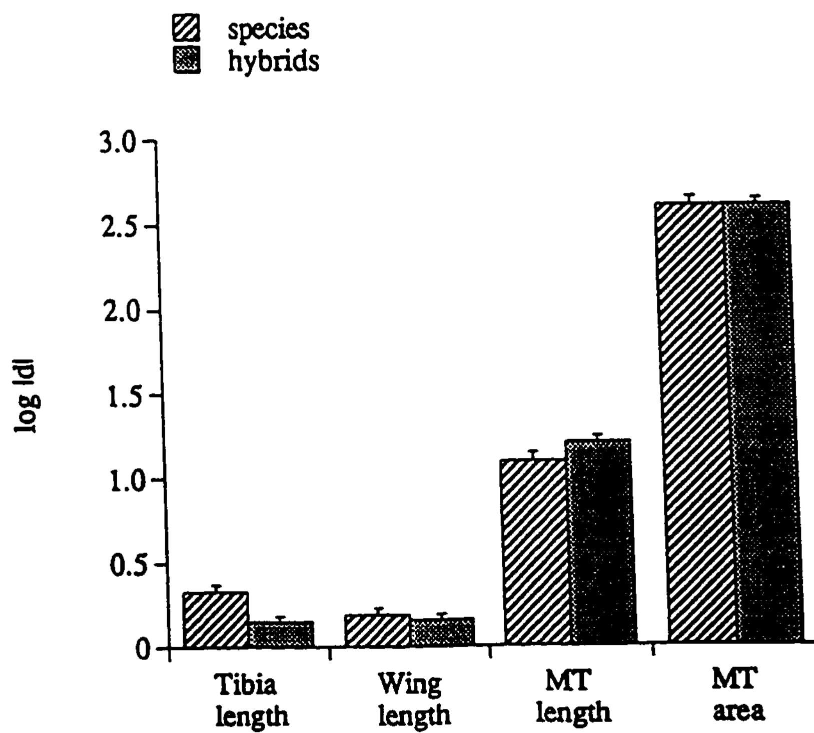


Table 1: A classification of the different traits measured.

Traits	*Sexual*	*Non-sexual*
External	**Genital arch** (area)	**Tibia and Femur** (length) **Wing** (length and width)
Internal	**Testis** (length and area)	**Malpighian Tubule** (length and area)

Table 2: Proportion of variance between strains within-species, and between species obtained from the analysis of variance (ANOVA) of each trait among the species of the *melanogaster* complex.

Trait	Variance components	
	Between strains within species	Between species
Sexual		
Testes length	0.0788	0.8885
Testes area	0.0974	0.8318
Genital arch area	0.0088	0.9867
Non-sexual		
Tibia length	0.1296	0.7232
Femur length	0.1619	0.6071
Wing length	0.1095	0.6920
Wing width	0.1620	0.6071
Malpighian tubules length	n/a	0.5475
Malpighian tubules area	n/a	0.6424

n/a: not available

Table 3: Traits mean phenotypic value obtained for each of the species of the *melanogaster* complex. Length units are in millimetres, and area units in millimetres^2. Standard errors and sample size are given in brackets.

Trait	*melanogaster*	*simulans*	*mauritiana*	*sechellia*
Sexual				
Testes length	2.2 (0.02; 44)	1.4 (0.02; 45)	1.3 (0.02; 45)	2.1 (0.02; 41)
Testes area	185 (2.5; 44)	128 (2.1; 45)	98 (2.0; 44)	135 (2.1; 41)
Genital arch area	3.8 (0.06; 30)	12.4 (0.15; 27)	1.3 (0.04; 27)	4.7 (0.10; 24)
Non-sexual				
Wing length	1.420 (0.0054; 43)	1.228 (0.0075; 45)	1.202 (0.0051; 45)	1.085 (0.0055; 45)
Wing width	0.969 (0.0042; 42)	0.847 (0.0046; 45)	0.815 (0.0034; 45)	0.765 (0.0047; 45)
Tibia length	0.483 (0.0033; 44)	0.418 (0.0035; 44)	0.417 (0.0026; 45)	0.411 (0.0029; 45)
Femur length	0.556 (0.0036; 44)	0.489 (0.0043; 45)	0.494 (0.0023; 45)	0.488 (0.0036; 45)
Malpighian tubules length	n/a	0.371 (0.0156; 19)	0.290 (0.0163; 8)	0.253 (0.0130; 10)
Malpighian tubules area	n/a	13.3 (0.65; 19)	8.3 (0.55; 9)	8.5 (0.76; 10)

n/a: not available

Table 4: Results obtained from discriminant analysis. Each V_i is a variable defined by the linear combination of the trait variables. The canonical squared correlations are between the V_i variables and a corresponding linear function that results from the combination of the grouping variables (species). The eigenvalues show the variation among species explained by each linear function V_i. For each linear combination (V_i), a coefficient (or loading) is associated to each trait.

	Discriminant functions		
	V_1	V_2	V_3
Squared Correlation	0.986	0.894	0.257
Eigenvalue	75.34	8.57	0.35
Proportion of total variation	0.89	0.10	0.01
Variable		**Coefficients**	
GA	1.07	-0.06	0.02
TL	0.20	1.03	0.01
WL / WW	0.30	0.21	0.86
TiL / FeL	-0.16	0.03	0.69

Table 5: Results obtained from the principal component analysis on the species and hybrids data. The eigenvalues show the variance in the data explained by each component. The value a_{ij} is the coefficient of the jth variable for the ith principal component. Only coefficients higher than 0.4 are bolded.

	Principal components		
	PC1	PC2	PC3
Eigenvalues	3.18	1.96	0.98
Percent of variation	45.4	28.0	14.0
Cumulative percent	45.4	73.4	87.4
Traits		**a_{ij}**	
Testes length	-0.121	**0.658**	-0.117
Testes area	0.159	**0.630**	0.167
Genital arch	0.294	0.168	**0.781**
Wing length	**0.474**	-0.281	0.115
Wing width	**0.517**	-0.122	0.059
Tibia length	**0.460**	0.097	**-0.401**
Femur length	**0.411**	0.197	**-0.413**

Appendix: Traits mean phenotypic value obtained for parental species and their hybrids. The name of the parental strains used in the interspecific crosses are abbreviated as *si* (Ca)= *D. simulans* California, *si* (Co)= *D. simulans* Colombia, *ma* (72)= *D. mauritiana* 72, and *se* (21)= *D. sechellia* 21. For hybrids, the name of the female is followed by the name of the male parental strain. Standard errors and sample size are given in brackets. Units are millimetres for length and millimetres^2 for area.

a) *D. simulans* x *D. mauritiana*.

Trait	*si* (Ca)	Ca x 72	72 x Ca	*ma* (72)	Co x 72	*si* (Co)
Sexual						
TL	**1.58** (0.025; 15)	1.23 (0.021; 10)	1.63 (0.030; 8)	**1.18** (0.021; 15)	1.15 (0.016; 5)	**1.40** (0.012; 15)
TA	**143** (2.6; 15)	103 (3.8; 10)	150 (3.5; 8)	**89** (2.1; 14)	83 (3.3; 5)	**118** (2.5; 15)
GA	**12.2** (0.04; 10)	5.7 (0.08; 10)	5.7 (0.07; 4)	**1.4** (0.02; 8)	5.8 (0.08; 4)	**11.7** (0.12; 8)
Non-sexual						
TiL	**0.42** (0.005; 15)	0.44 (0.002; 11)	0.46 (0.004; 8)	**0.41** (0.003; 14)	0.45 (0.004; 9)	**0.39** (0.003; 15)
FeL	**0.49** (0.003; 15)	0.53 (0.005; 10)	0.53 (0.004; 8)	**0.50** (0.003; 14)	0.54 (0.005; 9)	**0.46** (0.003; 15)
WL	**1.23** (0.008; 15)	1.24 (0.006; 11)	1.29 (0.009; 8)	**1.19** (0.004; 15)	1.24 (0.003; 9)	**1.18** (0.007; 15)
WW	**0.87** (0.005; 15)	0.90 (0.005; 10)	0.91 (0.005; 8)	**0.82** (0.003; 15)	0.88 (0.004; 9)	**0.81** (0.004; 15)
MTL	**0.34** (0.018; 9)	0.23 (0.014; 11)	0.24 (0.014; 12)	**0.29** (0.016; 8)	0.29 (0.013; 10)	**0.40** (0.056; 10)

MTA	**12.4** (0.78; 9)	10.0 (0.68; 11)	9.7 (0.50; 12)	**8.3** (0.55; 9)	12.6 (0.76; 10)	**14.1** (0.81; 10)

b) *D. simulans* x *D. sechellia*

Trait	*si* (Ca)	Ca x 21	21 x Ca	*se* (21)	21 x Co	Co x 21	*si* (Co)
Sexual							
TL	**1.58** (0.025; 15)	1.74 (0.023; 9)	1.60 (0.045; 9)	**2.10** (0.022; 15)	1.52 (0.048; 6)	1.75 (0.024; 15)	**1.40** (0.012; 15)
TA	**143** (2.6; 15)	174 (5.2; 9)	115 (5.1; 9)	**141** (2.3; 14)	132 (8.0; 6)	155 (4.4; 15)	**118** (2.5; 15)
GA	**12.2** (0.04; 10)	9.7 (0.09; 8)	9.6 (0.11; 9)	**4.4** (0.07; 15)	na	**9.8** (0.10; 9)	**11.7** (0.12; 8)
Non-sexual							
TiL	**0.42** (0.005; 15)	0.47 (0.003; 9)	0.47 (0.003; 9)	**0.41** (0.007; 15)	0.45 (0.005; 6)	0.45 (0.007; 15)	**0.39** (0.003; 15)
FeL	**0.49** (0.003; 15)	0.56 (0.005; 9)	0.57 (0.004; 9)	**0.49** (0.007; 15)	0.66 (0.008; 6)	0.66 (0.010; 15)	**0.46** (0.003; 15)
WL	**1.23** (0.008; 15)	1.28 (0.006; 8)	1.28 (0.015; 9)	**1.09** (0.010; 15)	1.26 (0.007; 6)	1.22 (0.008; 15)	**1.18** (0.007; 15)
WW	**0.87** (0.005; 15)	0.92 (0.004; 8)	0.89 (0.004; 9)	**0.76** (0.008; 15)	0.89 (0.007; 6)	0.86 (0.006; 15)	**0.81** (0.004; 15)
MTL	**0.34** (0.018; 9)	0.25 (0.010; 11)	na	**0.25** (0.013; 10)	na	0.27 (0.016; 10)	**0.40** (0.056; 10)
MTA	**12.4** (0.78; 9)	10.2 (0.55; 11)	na	8.5 (0.76; 10)	na	10.8 (0.75; 10)	**14.1** (0.81; 10)

c) *D. mauritiana* x *D. sechellia*

Trait	*ma* (72)	21 x 72	*se* (21)
Sexual			
TL	**1.18** (0.021; 15)	1.62 (0.054; 9)	**2.10** (0.022; 15)
TA	**89** (2.1; 14)	129 (4.7; 9)	**141** (2.3; 14)
GA	**1.4** (0.02; 8)	2.8 (0.08; 9)	**4.4** (0.07; 15)
Non-sexual			
TiL	**0.41** (0.003; 14)	0.45 (0.005; 9)	**0.41** (0.007; 15)
FeL	**0.50** (0.003; 14)	0.55 (0.005; 9)	**0.49** (0.007; 15)
WL	**1.19** (0.004; 15)	1.20 (0.008; 9)	**1.09** (0.010; 15)
WW	**0.82** (0.003; 15)	0.84 (0.005; 9)	**0.76** (0.008; 15)
MTL	**0.29** (0.016; 8)	0.23 (0.010; 10)	**0.25** (0.013; 10)
MTA	**8.3** (0.55; 9)	9.3 (0.48; 10)	8.5 (0.76; 10)

CHAPTER 4

SEX-RELATED GENES, DIRECTIONAL SEXUAL SELECTION AND SPECIATION.

This chapter has been accepted for publication in Molecular Biology and Evolution. This chapter shows an analysis of nuclear gene DNA sequences from closely related species. The sequences analyzed were retrieved from GenBank and classified based on the functional characteristics of their final protein products. A high ratio of nonsynonymous to synonymous substitutions was found for genes affecting mating behaviour, fertility, spermatogenesis, or sex determination (i. e. sex-related genes). This result was more pronounced between closely related species. The high ratio of nonsynonymous to synonymous substitutions was due to an elevated proportion of nonsynonymous changes. The results suggest that directional sexual selection has shaped the evolution of sex-related genes during the early stages of speciation.

ABSTRACT

Reproductive isolation and speciation can result from the establishment of either premating or postmating barriers that restrict gene flow between populations. Recent studies of speciation have been dominated by a molecular approach to dissect the genetic basis of hybrid male sterility, a specific form of postmating reproductive isolation. However, relatively little attention has been paid to the evolution of genes involved in premating isolation and genes generally involved in other sex-related functions (e.g. mating behaviour, fertilization, spermatogenesis, sex determination). We have assembled DNA sequences from fifty-one nuclear genes and classified them based on their functional characteristics. The proportion of nonsynonymous to synonymous nucleotide substitutions were compared between *D. melanogaster*, *D. simulans* and *D. pseudoobscura*, as well as between *C. elegans* and *C. briggsae*. We found a high ratio of nonsynonymous to synonymous substitutions for sex-related genes (i.e. genes involved in mating behaviour, fertilization, spermatogenesis, or sex determination). The results suggest that directional sexual selection has shaped the evolution of sex-related genes at least during the early stages of speciation. It is possible that directional selection becomes relaxed after reproductive isolation has been completed between more distantly related species (e. g. *D. melanogaster* and *D. pseudoobscura*). However, a saturation in the number of nucleotide substitutions since the time of species split may mask any sign of directional selection between distantly related species.

Keywords: Sex genes, *Drosophila*, sexual selection, speciation.

INTRODUCTION

The problem of the genetic basis of speciation has been traditionally approached by attempts to map genes that influence interspecific hybrid male sterility (Dobzhansky 1936; Coyne 1984; Perez and Wu 1993) or by molecular analysis of genetic divergence between closely related species (Lewontin 1974; Hey and Kliman 1993). The first approach directly deals with genes responsible for reproductive isolation. However, it only focuses on a particular type of postzygotic reproductive isolation (i.e. interspecific hybrid male sterility), and it is restricted to species that are crossable in laboratory conditions. The second approach has the advantage of being applicable to a wide variety of organisms and it is not restricted to a single type of reproductive isolation barrier. However, it has been used mainly for phyletic inference, and the genes are typically sampled randomly with no regard to their physiological effects.

It has been previously shown that the establishment of premating barriers between closely related species relies on the modification of courtship and visual and /or chemical mating cues (Kaneshiro and Boake 1987; Coyne, Crittenden, and Mah 1994). Among *Drosophila*, most hybrids from crosses between closely related species are viable but sterile (Bock 1984). Wu and Davis (1993) have shown that this trend is not only a consequence of the heterogametic condition of males but it may also be influenced by a fast evolution of the male reproductive system.

The early evolutionary breakdown of mating recognition or fertility between species, together with the high divergence found for morphological (Eberhard 1985; Joly et al. 1991) and molecular (Coulthart and Singh 1988; Thomas and Singh 1992; Civetta and Singh 1995) components of the male genitalia has led us to entertain the hypothesis of a major role for genes affecting various aspects of reproduction during speciation (Singh

1990; Singh and Zeng 1993). However, the role played by drift and selection during the evolution of such traits remains obscure. For example, Dobzhansky (1951) suggested that during secondary contacts, selection would particularly reinforce differences in mating and reproductive traits that have arisen as a consequence of genetic divergence between allopatric populations. Such selective pressure would strengthen reproductive isolation and lead to speciation. Other models of speciation have predicted that mating and fertilization traits should remain well buffered within species, and that the escape from such selective constraints will be achieved by a founder event strong enough to disrupt previous genetic balances (Carson 1985; Paterson 1985).

Several studies on the molecular evolution of single genes have independently reported fast interspecies divergence for sex-related genes. The pattern seems common to a diverse group of organisms (Lee, Ota, and Vacquier 1995; Metz and Palumbi 1996; Tsaur and Wu 1997; Ferris et al. 1997; Sutton and Wilkinson 1997). However, it remains unclear how these genes differ in their pattern of evolution from other non-sex related genes, and how the fast between species divergence relates to speciation. Here we have taken an approach that combines a survey of DNA gene sequences from closely related species that are rapidly accumulating in public databases (i.e. GenBank), together with methods of sequence analysis that have become a strong tool to discern the role played by different evolutionary forces during DNA sequence evolution.

We have collected and analyzed gene sequences retrieved from GenBank for which complete nucleotide coding and amino acid sequences were available. A total of fifty-one nuclear genes were compared between species, including seventeen genes between *D. melanogaster* and *D. simulans*, twenty-two between *D. melanogaster* and *D. pseudoobscura*, and twelve between *C. elegans* and *C. briggsae*. Genes were classified into four groups (*catalytic activity*, *developmental*, *sex-related*, and *others*) based on

single characteristics that allowed the grouping of as many genes as possible. When a common grouping characteristic could not be found, genes were labeled *others*. Any gene product functionally related with aspects of mating behaviour, fertility, or involved in sex differentiation, was categorized as *sex-related*. We have deliberatively kept the categories broad to include as many genes as possible per group. Overlaps between groups were considered in the analysis since genes could sometimes be included in more than one group.

Contrary to models of speciation that predict that mating and fertilization traits should be well buffered within species (Carson 1985; Paterson 1985), we show a lack of selective constraints on the evolution of sex-related genes (i.e. genes involved in mating behaviour, fertilization, spermatogenesis, or sex determination). The results suggest a main role of directional sexual selection in the evolution of sex-related genes during the early stages of speciation.

MATERIALS AND METHODS

Data collection

Amino acid and nucleotide coding sequences were collected from GenBank from pairs of species from *Drosophila* and *Caenorhabditis*. These genera were chosen based on the number of gene sequences available from closely related species. The search for genes involved four steps. 1) A logical *and* boolean search combined the name of the species in the genus for which the largest number of genes had been sequenced (i.e. *melanogaster*, *elegans*) and the word *sex* or *mating*. 2) The amino acid sequence of such genes were used in a BLAST-p search for similar sequences in closely related species. 3) The closest species in which similar genes were found was then searched for the total number of genes sequenced. 4) Each one of these genes was searched in *D. melanogaster* or *C. elegans*.

The genes surveyed were classified into *catalytic activity*, *developmental*, and *others* based on their functional characteristics. Genes that related to sex, either due to site of expression, sex differentiation, or behaviour, were labeled as *sex-related*. This information was obtained from the Swiss-prot database. Overlaps between gene groups were considered in the analysis. Table 1 shows an example on how genes compared between *D. melanogaster* and *D. simulans* were classified based on their products' main functional characteristics.

The following genes were analyzed for *D. melanogaster* and *D. simulans* (the gene symbol and gene accession numbers are given in brackets): Accessory gland protein (*Acp26A*, X70888/ X70899), alcohol dehydrogenase (*Adh*, M17831/ M36585), amylase (*Amy*, L22716-7/ D17733-4), andropin (*Anp*, X56726), decapentaplegic (*dpp*, M30116/ U63854), esterase (*Est-6*, M15961/ L34264), heat shock protein (*Hsp83*, X03810/ X03811), myosin (*Mlc-1*, M10125-K01567/ L08051), period (*per*, M11969/ L07826), phosphogluconate dehydrogenase (*Pgd*, M80598/ U02288), phosphoglucose isomerase (*Pgi*, L27554/ L27548), refractory (*ref(2)P*, X16993/ U23930), spalt (*sal*, X57474/ M21227), superoxide dismutase (*Sod*; X13780/ X15685), transformer (*tra*, M17478/ X66930), vermilion (*v*; M34147/ U27204), white (*w*, X51749/ U64875). The *andropin* gene was compared between *D. melanogaster* and *D. mauritiana*, the *D. mauritiana* sequence was kindly provided by Dr. A. G. Clark.

The genes analyzed between *D. melanogaster* and *D. pseudoobscura* were: Adenine phosphoribosyl transferase (*Aprt*, L06280/ L06281), alcohol dehydrogenase (*Adh*, U64521), amylase (*Amy*, U20330), bicoid (*bcd*, X14458/ X55735), decapentaplegic (*dpp*, U63856), esterase (*Est-5*, M55907), exuperantia (*exu*, S72757/ L22553-4), glucose dehydrogenase (*Gld*, M29298/ M29299), glycerol-3-phosphate dehydrogenase (*Gpdh*, J04567/ U59682), glycinamide ribotide transformylase (*Gart*, J02527/ X06285), heat

shock protein (*Hsp83*, X03812), janus (*jan*, M27033/ S77099), myosin (*Mlc-1*, L08052), opsin (*Rh*, K02315-M12896 -M17718/ X65877-9), period (*per*, X13878), ribosomal protein (*Rp49*, X00848/ S59382), rosy (*ry*, Y00308/ M33977), runt (*run*, X56432/ U22357), toll (*Tl*, M19969/ L25390), tumor supression (*l(2)*, X05426/ X73259), ultrabithorax (*Ubx*, X05723/ X05179), urate oxidase (*Uro*, X51940/ X57113).

The names and GenBank accession numbers of the genes analyzed between *C. elegans* and *C. briggsae* are: Acetylcholinesterase (*ace-1*, U58731/ U41846), beta tubulin (*tbb*, X15242/ U55260), cell death (*ced-9*, L26545/ L26546), cytochrome (*cyt-1*, L26545/ L26546), globin (*glb*, S66164/ U48289), gut esterase (*ges-1*, M96145/ M96144), heat shock protein (*Hsp83*, M26604/ M26906), mechanosensory (*mec-4*, U58726/ U53670), myosin (*myo-d*, M59940/ U05000), transposon (*tc*, X01005/ X07827-M64308), transformer (*tra*, M93256-M91371/ U60649-U59879), uncoordinated (*unc*, U32854/ U45326).

Gene sequence analysis

Only complete coding sequences were included in the analysis. The amino acid and nucleotide coding sequences of similar genes obtained from the database search were aligned using CLUSTALW (version 1.4) (Thompson, Higgins, and Gibson 1994). MEGA (version 1.0) (Kumar, Tamura, and Nei 1993) was used to obtain general nucleotide composition information. The proportion of synonymous substitutions per synonymous site (K_s) and non-synonymous substitutions per non-synonymous site (K_a) were obtained by using the method developed by Li, Wu, and Luo (1985), and Nei and Gojobori (1986). Although both methods gave similar results, Li's method (Li, Wu, and Luo 1985) was preferred over Nei and Gojobori approach (Nei and Gojobori 1986) as it considers unequal rates of nucleotide substitutions, and it gives a higher weight to evolutionary

pathways that involved only synonymous substitutions than those involving amino acid changes.

Codon usage bias was calculated by the effective number of codons (ENC), where alternative codons for a given amino acid are considered as analogous to alleles of a gene (Wright 1990).

RESULTS

Testing the role of selection: Patterns of nonsynonymous to synonymous substitutions in different genes between species of Drosophila

An excess of nonsynonymous changes above the number of synonymous substitutions has been previously regarded as an indication of positive selection (Whitfield Lowell-Badge, and Goodfellow 1993; Tucker and Lundrigan 1993; Lee, Ota, and Vacquier 1995; Swanson and Vacquier 1995; Metz and Palumbi 1996; Pamilo and O'Neill 1997). But, unless genes have remained under the influence of directional selection since the time of species formation, the accumulation of random mutations may obscure any pattern of directional selection at the onset of speciation.

Sex-related genes grouped into any other functional class had the highest K_a / K_s ratio when compared to any other gene within that class (fig. 1). In comparisons between *D. melanogaster* and *D. simulans*, sex-related genes showed a higher K_a / K_s ratio than any other group of genes (fig. 1a). Gene sequence comparisons between *D. melanogaster* and *D. pseudoobscura*, a species pair with a divergence time approximately ten times longer than that between *D. melanogaster* and *D. simulans* (Russo, Takezaki, and Nei 1995), showed a higher K_a / K_s ratio for developmental genes (fig. 1b). These results suggest a possible different time effect on the accumulation of nucleotide substitutions in

sex-related and developmental genes, but does not explain what mechanisms are responsible for the elevated K_a / K_s ratio observed.

The high K_a / K_s ratios could be the consequence of a selective constraint on the proportion of synonymous substitutions that have accumulated between species. In that case, sex-related and developmental genes should show a reduced proportion of synonymous substitutions and a similar proportion of nonsynonymous changes when compared to the remaining genes in the total sample. This was not found here. The proportion of nonsynonymous changes was significantly higher for only sex-related genes than non-sex related genes between the two more closely related species *D. melanogaster* and *D. simulans* (Mann-Whitney test: $Z= 2.70$; $N_{ns}= 13$, $N_s= 7$; $p= 0.007$). Developmental genes showed only a significantly higher proportion of nonsynonymous substitutions than non-developmental genes in the comparison of *D. melanogaster* and *D. pseudoobscura* ($Z= 2.18$; $N_{nd}= 23$, $N_d= 6$; $p= 0.03$).

Codon bias in Drosophila melanogaster: Are sex-related and developmental genes under purifying selection at the translational level?

The observation that *Drosophila* genes show no correlation between base composition at silent sites (i.e. introns and flanking regions) and fourfold degenerate sites in coding regions, has been used to suggest that synonymous substitutions in different genes of *Drosophila* have been affected by different selective constraints rather than mutational bias (Moriyama and Hartl 1993). Sex-related genes appear to be under a less stringent selective constraint at synonymous sites than non-sexual genes, since their effective number of codons (ENC) tended to be closer to values expected for random codon usage (fig. 2a-b). A set of 383 *D. melanogaster* genes, for which ENC values were calculated (Kliman and Hey 1993), were grouped based on information on their functional

characteristics obtained from the SwissProt database. We found a significantly higher ENC for both *sex-related* and *developmental* genes than *catalytic activity* and *other* genes (Tukey-Kramer multiple pairwise comparison: $p < 0.05$).

The significantly lower codon bias found for sex-related and developmental genes indicates an absence of selective constraints at synonymous sites in these genes, even though the high K_a / K_s ratio observed among sex-related and developmental genes suggests that some form of selection acts on their protein products.

Directional sexual selection and the evolution of sex-related genes between closely related species of Drosophila

It is possible that directional sexual selection on gene products associated with the reproductive system may have increased the proportion of nonsynonymous substitutions over random synonymous changes at the time of species split. These changes would be favored provided that they avoid the production of less fit hybrids between early diverging species. Once isolation is completed, the proportion of nonsynonymous to synonymous nucleotide substitutions is expected to level off, either by a relaxation of directional selection at nonsynonymous sites or a saturation in the number of both synonymous and nonsynonymous nucleotide substitutions that have accumulated since the time of speciation. In figure 3, the proportion of nonsynonymous changes between *D. melanogaster – D. simulans* and *D. melanogaster – D. pseudoobscura* gene comparisons are plotted against the proportion of synonymous changes between these species pairs. The proportion of synonymous changes is taken as a general indicator of time since species divergence. A logarithmic function was slightly better than a linear function in explaining the amount of variation in the proportion of changes at nonsynonymous *versus* synonymous sites for sex-related genes (logarithmic: $R^2 = 0.65$; linear: $R^2 = 0.59$) (fig. 3).

Although the logarithmic function has no better statistical support than a linear fit, the pattern is consistent with the significantly higher proportion of nonsynonymous substitutions found among sex-related genes between the two closely related species *D. melanogaster* and *D. simulans*.

The high K_a / K_s ratio observed among developmental genes seems to be the result of a different evolutionary history. The high proportion of nonsynonymous substitutions for developmental genes was significant only when distantly related species were compared and the relationship between K_a and K_s followed an exponential function (exponential: R^2= 0.79; linear: R^2= 0.57) (fig. 3). Such pattern may result from an increase in the number of nonsynonymous differences among genes that have saturated their proportion of nucleotide substitutions at synonymous sites, or a relaxation of selective constraints at nonsynonymous sites between the more distantly related species *D. melanogaster* and *D. pseudoobscura*.

The evolution of sex-related genes between two species of the genus Caenorhabditis

Our analysis of sex-related genes in nematodes also shows a high K_a / K_s ratio for sex-related genes when compared to non-sex related genes (Z= 2.10; N_{ns}= 12, N_s= 2; p= 0.036) (fig. 4). For the comparison between *C. elegans* and *C. briggsae*, the elevated ratio found for the sex determination genes *tra-1* and *tra-2* seems to have resulted from an elevated proportion of both nonsynonymous and synonymous substitutions. However, the K_a values for these genes are almost two times larger than the ones obtained for other genes with high K_a values, whereas the K_s for *tra-1* and *tra-2* are only slightly higher than the values obtained for other non sex-related genes with a high K_s. This may indicate a burst of nonsynonymous substitutions in sex-related genes at the time of species split. However, the *transformer* gene could also be labeled as a developmental gene. More

conclusive evidence on the forces shaping the high divergence observed for the sex-related genes in nematodes would require a larger sample size together with gene sequences from more closely related species that could reveal the pattern of gene sequence evolution through time.

DISCUSSION

Both the biological species concept (Mayr 1963; Dobzhansky 1970) and the species recognition concept (Paterson 1985) emphasize the importance of reproductive traits during the process of speciation. Both concepts suggest that deviations from a general physiological or behavioural reproductive norm would genetically isolate individuals from their parental species. Although the biological species concept has been criticized due to its limitation to sexual organisms, Maynard Smith (Maynard Smith, Dowson, and Spratt 1991; Maynard Smith 1995) has suggested that the reproductive isolation concept can be extended to asexual organisms. Asexuals such as bacteria do not fuse gametes, nor do they go through meiosis, but they still exchange genes through a variety of mechanisms. Interestingly, mutants defective for genes of the mismatch repair system or induction of the SOS response produce a higher proportion of recombination between different species of bacteria (Matic, Rayssiguier, and Radman 1995). Similar results were found in yeast (Hunter et al. 1996). These results suggest a main role for genes that relate to aspects of reproduction (or simply recombination) as inducers or inhibitors of gene flow between species.

Both morphological and behavioural data suggest that sex and reproduction are the main targets of change during speciation, and it could be assumed that such traits should be under strong stabilizing selection as to prevent deviation from the reproductive norm of the species. This kind of scenario, where founder events are required to break the

strong selective constraints within species and trigger speciation, was strongly advocated by Carson (1985) and Paterson (1985). If the genes related to aspects of mating and reproduction are under strong stabilizing selection and their divergence is due to random perturbations of the whole genome by founding events, then it is expected that closely related species should be less divergent at mating and reproduction-related genes than genes under less stringent selective constraints (e.g. non house-keeping genes). However, our study of DNA sequence divergence between closely related species of *Drosophila* shows exactly the opposite pattern. The analysis of nonsynonymous and synonymous substitutions and codon bias suggest a unique pattern of directional selection shaping the evolution of sex-related genes in the early stages of speciation. The signature of directional selection is not evident between more distantly related species (*D. melanogaster* - *D. pseudoobscura*), and it is possible that directional selection has been relaxed after reproductive isolation was completed. Alternatively, any pattern of directional selection could be masked by the accumulation of multiple substitutions since the time of speciation.

Studies on the molecular evolution of sex-related genes in groups other than *Drosophila* have also supported directional selection. Among marine invertebrates, directional selection acting on the fertilization gene *lysin* and the gamete recognition gene *bindin* has been suggested from studies on the proportion and distribution of nonsynonymous substitutions. Similar explanations have been offered for the evolution of the sex-determination gene *Sry* in mammals (Whitfield, Lowell-Badge, and Goodfellow 1993; Tucker and Lundrigan 1993), and for the intriguing high interspecific nucleotide and amino acid sequence divergence found for a homeobox gene (*Pem*) that is expressed in reproductive tissues and that has been suggested to regulate spermatogenesis and sperm maturation (Maiti et al. 1996; Sutton and Wilkinson 1997).

The mate recognition gene *fus1* and the sex-determination gene *mid* have shown to be unique among *Chlamydomonas* genes in that they show no codon bias (Ferris, Woessner, and Goodenough 1996; Ferris and Goodenough 1997). This result has led the authors to speculate that a stronger mutational pressure could be responsible for the rapid between-species evolution of these two genes. An analysis of sequence polymorphism within *C. reinhardtii* and sequence divergence between this species and other species of the genus *Chlamydomonas* has revealed a pattern of almost no genetic variation but high divergence for both *fus1* and *mid* genes (Ferris et al. 1997). Among the six *C. reinhardtii* strains analyzed, only one nonsynonymous change in the *fus1* gene and two synonymous changes in *mid* were found. Ferris et al. (1997) suggested strong purifying selection within species but rapid divergence between species. However, the low polymorphism between strains becomes puzzling given the lack of selective constraints at the translational level suggested by the lack of codon bias in *fus1* and *mid*.

In conclusion, sex-related genes show an increased proportion of nonsynonymous to synonymous substitutions. This result is more pronounced among closely related species. The lack of selective constraints at synonymous sites and the significantly higher proportion of nonsynonymous substitutions between closely related species support that at least in *Drosophila*, and perhaps in other invertebrates and mammals, the evolution of sex-related genes has been driven by directional selection at the time of species formation.

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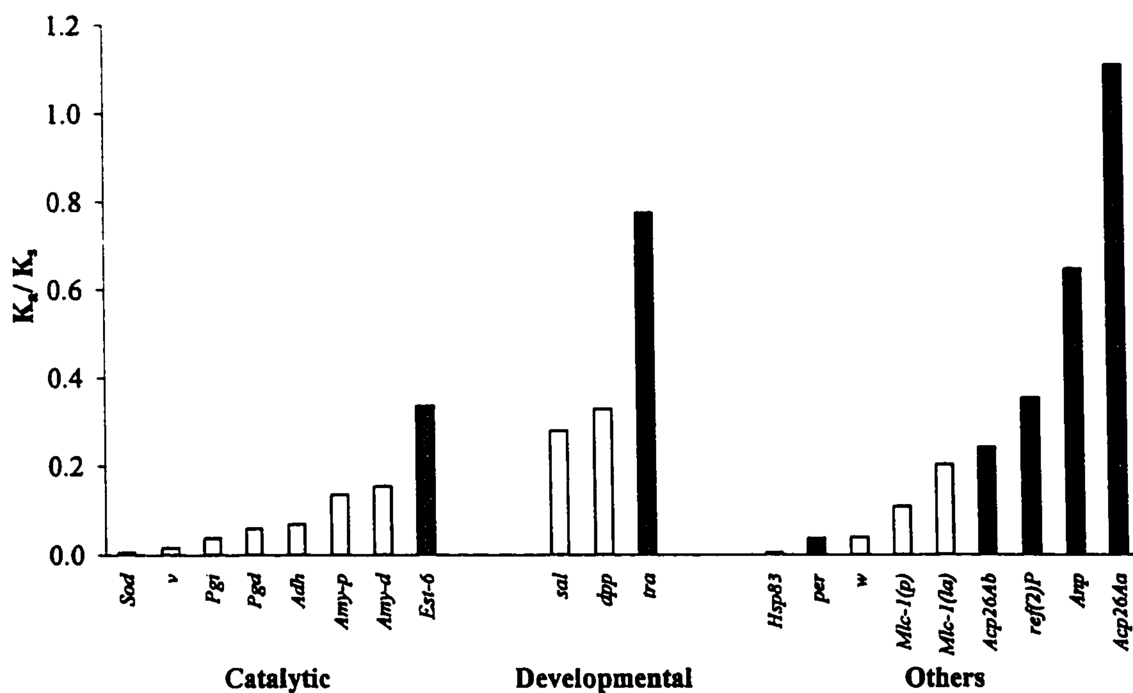
Figure 1. Proportion of nonsynonymous to synonymous substitutions in genes compared between closely related species of *Drosophila*. The genes surveyed are grouped into three major groups based on their functional characteristics (i. e. Catalytic activity, developmental, and others). Genes with a *sex-related* function are plotted as *black bars*. Sex-related genes had the highest K_a / K_s ratio when compared to any other genes within major class. **a)** Sex-related genes showed a higher average K_a / K_s ratio than any other group of genes when sequences were compared between closely related species (*D. melanogaster* - *D. simulans*). **b)** The average K_a / K_s ratio was higher for developmental genes in comparisons between the more distantly related species pair (*D. melanogaster* - *D. pseudoobscura*).

a) *D. melanogaster - D. simulans*



b) D. melanogaster - D. pseudoobscura

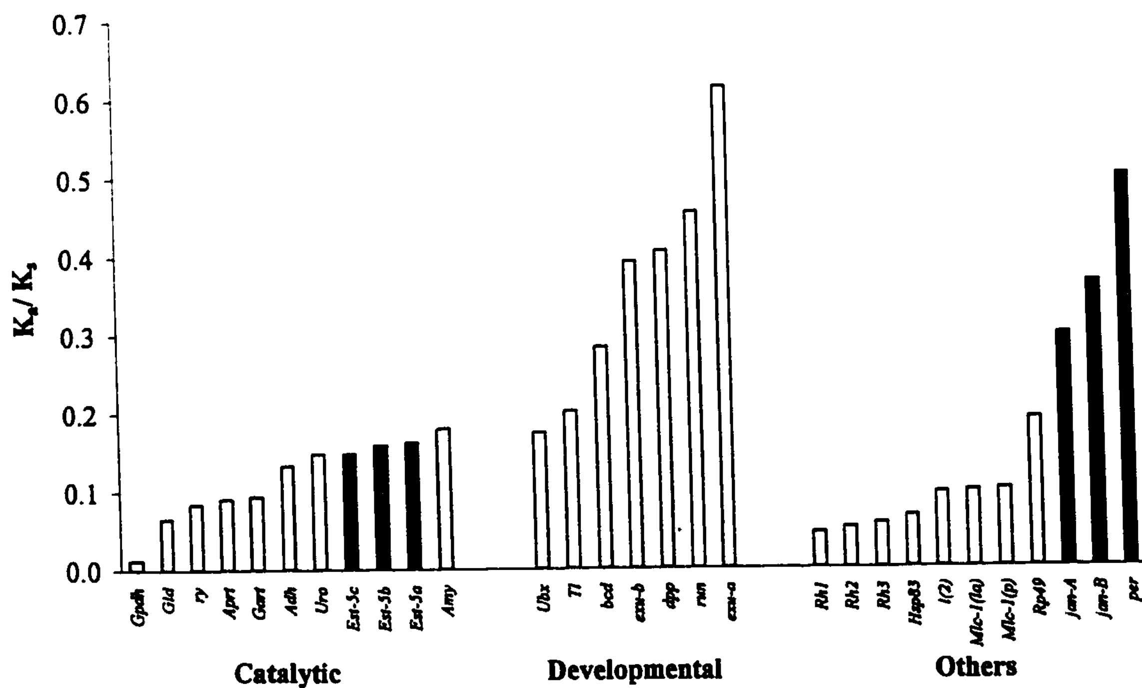
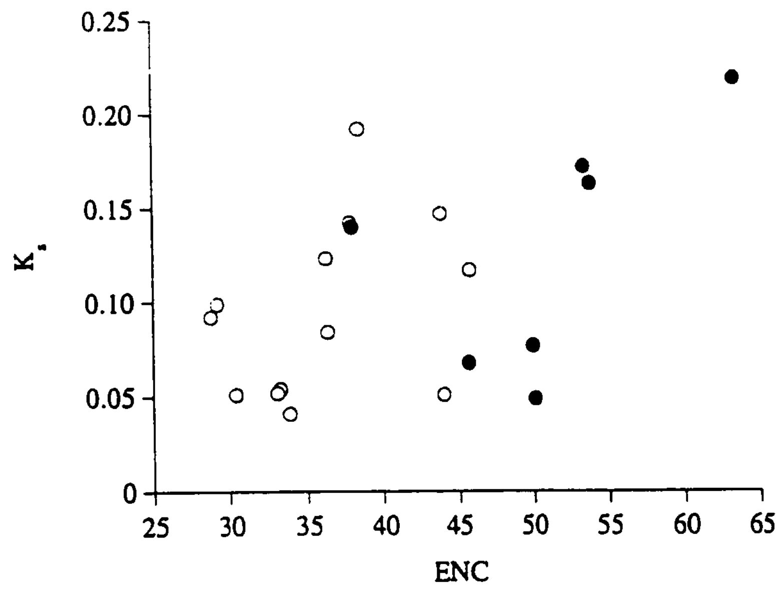


Figure 2. Correlation between the proportion of synonymous substitutions (K_s) and the effective number of codons (ENC) between *D. melanogaster* and *D. simulans* (**a**), and *D. melanogaster* and *D. pseudoobscura* (**b**). Filled circles were used for sex-related genes and open circles for others. Filled squares were used for genes that are sex-related in only one of the species analyzed.

a) *D. melanogaster / D. simulans*



b) *D. melanogaster / D. pseudoobscura*

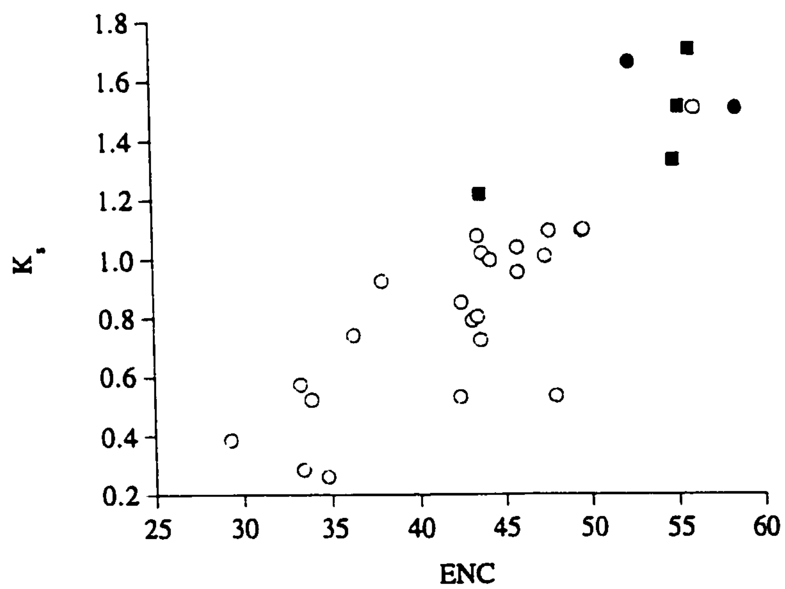
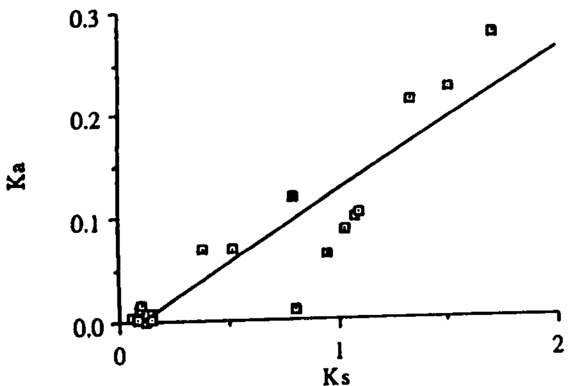
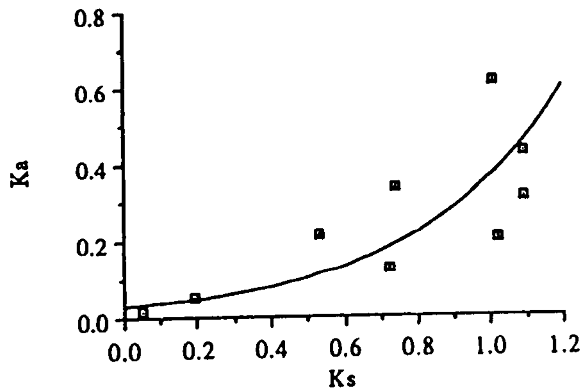


Figure 3. Proportion of nonsynonymous *versus* synonymous substitutions for each gene analyzed between *Drosophila* species. Different models were fit to the four gene groups. R^2 values represent the proportion of variation explained by each model. The gene groups *Catalytic activity* and *Others* followed a linear relationship between nonsynonymous (K_a) and synonymous (K_s) substitutions. The two groups with the highest K_a / K_s ratio behaved differently. *Sex-related* genes showed a high proportion of non-synonymous changes between closely related species followed by a plateau (logarithmic function), whereas *Developmental* genes showed a slow rate of nonsynonymous changes between closely related species and a latter increase between more distant species (exponential function).

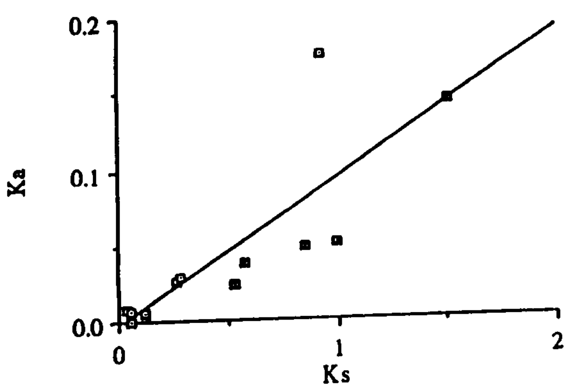
Catalytic activity $y = -1.62e\text{-}2 + 0.14\,(x)$ $R^2 = 0.81$



Developmental $y = 2.75e\text{-}2 * 10^{1.12\,(x)}$ $R^2 = 0.79$



Others $y = -4.09e\text{-}3 + 9.75e\text{-}2\,(x)$ $R^2 = 0.67$



Sex-related $y = 0.34 + 0.28 * \log(x)$ $R^2 = 0.65$

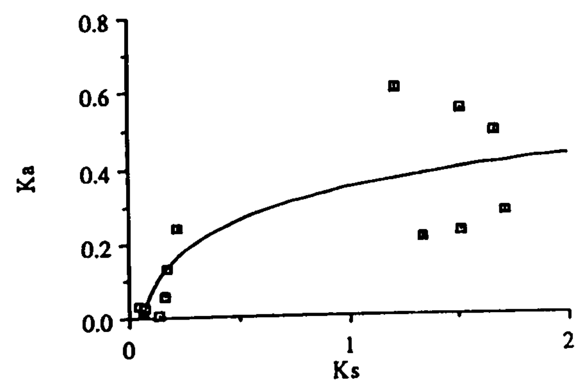


Figure 4. Proportion of nonsynonymous to synonymous substitutions for each gene analyzed between *Caenorhabditis* species. Open bars were used for *Catalytic activity* genes, crossed bars for *developmental* genes, dotted bars for *others*, and filled bars for *sex-related* genes. Full gene names and accession numbers are given in the materials and methods section.

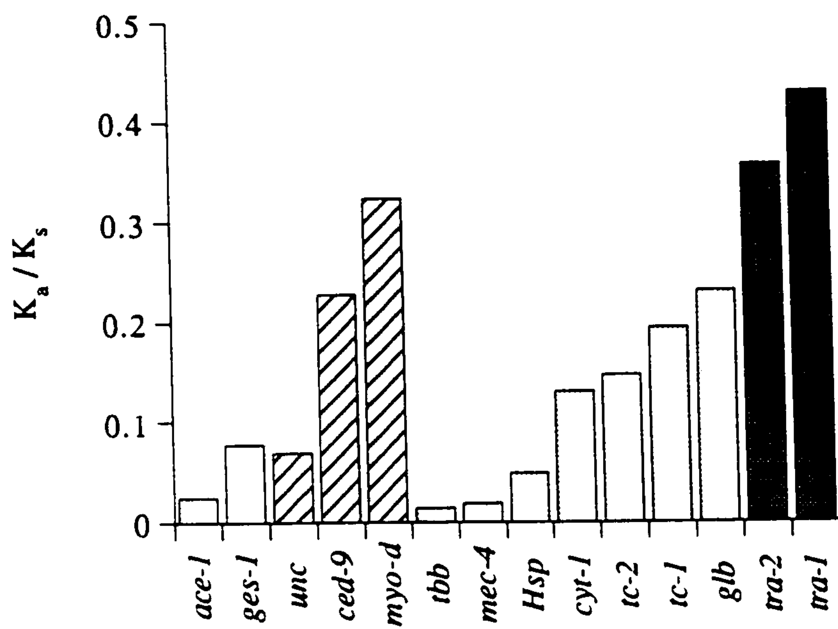

0.5
0.4
0.3
0.2
0.1
0
K_a / K_s
ace-1
ges-1
unc
ced-9
myo-d
tbb
mec-4
Hsp
cyt-1
tc-2
tc-1
glb
tra-2
tra-1

Table 1: Functional classification of genes analyzed between *D. melanogaster* and *D. simulans*. This information is mainly available from the Swiss Prot database. Sex-related characteristics are bolded. Gene products with no common major grouping characteristics were labeled as others.

Gene	Acc[a]	Main group	Description
Acp26A	P10333 P10334	Sex-related	This protein is transferred from male to female's hemolymph during **mating**. It **affects egglaying and behaviour** after mating. Tissue specificity: Main cells of the **accessory glands of males**.
Adh	P00334	Catalytic	Belongs to the short-chain dehydrogenases/reductases family.
Amy	P08144	Catalytic	Cofactor: Binds a calcium ion required for its activity. Belongs to the family of glycosyl hydrolases.
And	P21663	Sex-related	**Male-specific peptide** with moderate activity against Gram-positive bacteria. Structurally related to cecropins. It is **induced in response to mating**. Tissue specificity: **Ejaculatory duct of adult**

			males.
Dpp	P07713	Developmental	This protein acts as an extracellular morphogen to establish at least two cellular response thresholds within proper development of the embryonic dorsal hypoderm. Expressed in the imaginal discs. Associated with establishment of the proximal-distal axis of the appendages, and midgut mesoderm.
Est-6	P08171	Sex-related	Transferred from the ejaculatory bulb of males to the female upon copulation, plays an important role in the **reproductive biology**. Specifically expressed in the **ejaculatory bulbs of males**. Belongs to the type carboxylesterase/ lipase family.
Hsp83	P02828	Others	Molecular chaperone. It has ATPase activity (by similarity). Belongs to the heat shock protein HSP90 family but it is also expressed at normal growth temperatures. It is developmentally expressed during oogenesis.

Mlc-1	P06742 P06743	Others	Muscle protein. The two alternative forms are produced by alternative splicing of the same gene.
per	P07663	Sex-related	Involved in the generation of biological rhythms. The *per* mutants show aberrant rhythms that change components of the **male courtship** song.
Pgd	P41572	Catalytic	Oxireductase. Pathway: hexose monophosphate shunt.
Pgi	P52029	Catalytic	Involved in glycolysis and in gluconeogenesis.
ref(2)P	P14199	Sex-related	Implicated in sigma Rhabdovirus multiplication and necessary for **male fertility**.
sal	P39770	Developmental	Required for the establishment of the posterior-most head and the anterior-most tail segments of the embryo. First expressed at blastoderm and later in restricted areas of embryonic nervous system as well as in the developing trachea.

Sod	P00444	Catalytic	Destroys radicals which are produced within the cells and are toxic to biological systems.
tra	P11596	Sex-related	Regulates female somatic **sexual differentiation**. The sexual regulation of transformer occurs through alternative RNA splicing.
v	P20351	Catalytic	Controls the level of potentially harmful free tryptophan in the hemolymph. First step in the brown eye pigment biosynthesis.
w	P10090	Others	Part of the membrane-spanning permease system necessary for the transport of pigment precursors into pigment cells responsible for eye color. Integral membrane protein. Belongs to the ATP-binding transport protein family.

[a] Accession number

CHAPTER 5

GENERAL DISCUSSION

The molecular and morphological data presented in chapters 2 to 4 show a common theme of high divergence between species for traits that relate to sexuality and a possible link between this divergence and reproductive isolation. Although the work in this thesis has focused on closely related species in the genus *Drosophila* (see Chapters 2 - 4), I have also attempted to show the generality of the high divergence for sex-related traits by surveying and analyzing data from diverse taxa (see General Introduction and chapter 4). In the following sections, I discuss the need for a redirection of studies on the genetic basis of speciation towards the "sexual component" of the gene pool, the evolutionary forces that may have shaped the divergent characteristic of sex-related traits, and the genetic architecture of these traits between related species.

5.1 THE "SEX" GENE POOL: A KEY PLAYER IN SPECIATION?

Most models of speciation acknowledge the need for a population split to trigger the disruption of the gene pool that may end in speciation. According to Mayr (1963) individuals in a population share epigenetic systems and homeostatic devices which prevent peripheral isolates from diverging from the central range of the species. Mayr envisioned such a tight genetic cohesion in the parental population that isolation and adaptive divergence in a new habitat were seen as not strong enough to disrupt the parental genetic system. He needed to invoke a chance effect (genetic drift), as a

consequence of a founding event, that could break the genetic homeostasis and trigger a "genetic revolution". Finally, selective pressures in the new environment would establish new balanced genetic systems (Mayr 1963). Other models that followed also focused on the need for founding events to trigger speciation but did not support a major reorganization of the gene pool (Carson 1982, Templeton 1981). Carson (1982) stressed the importance of recognizing an *open* genetic system consisting of genes that freely recombine and have no major viability effects and a *closed* genetic system of genes that are restricted from recombination. Only the disorganization by founder events and reorganization by new stabilizing selective pressures of *closed* systems could lead to speciation. Templeton (1981) envisioned a genetic architecture of a few major genes with epistatic modifiers, and suggested that the disruption of coadapted gene complex could be achieved by a founder event induced drift of major genes.

Carson's views are of particular interest to the findings of this thesis. He not only attempted a distinction between different components of the gene pool (i.e. *open* and *closed* genetic systems), but he also suggested a need to link such systems to sexual traits.

> "The principal [balanced] system that is affected by the disorganization reorganization cycle ...is the very complex coadaptational system involved in mate recognition, sexual behaviour, and sexual selection." (Carson 1982, p. 424).

This conclusion was based on studies on the mating behaviour and morphology analysis of secondary sexual traits of species of the Hawaiian *Drosophila* group. Both Carson (1982, 1985) and Patterson (1985) recognized the divergent nature of sexual traits but limited their models to those directly involved in mate recognition and fertilization. They both agree in the role played by stabilizing selection in developing balances for the genes underlying such traits.

In the previous chapters, I have attempted to extend the role of sex-related traits in speciation to all aspects of sexuality, with the emphasis being placed on the physiological, behavioural, developmental and cellular environmental characteristics of the different gene products. I have also argued against the role played by stabilizing selection on the evolution of such traits. **First**, such strong stabilizing selective requirements will predict low phenotypic variation within species. In chapter 3, I presented results that show high variability within species for sex-related traits such as testes length and area and the area of the posterior lobe of the genital arch. It might be argued that Carson's and Patterson's predictions only apply to sex traits that are directly linked to mating behaviour or secondary sexual traits, however high within species variability has been found for such traits in a wide variety of organisms (see references in Chapter 3). **Second**, if morphological sexual traits were strongly constrained by stabilizing selection they should be less asymmetric than other traits, such a pattern was not found (chapter 3). **Third**, close genetic balances built up by strong stabilizing selection would become extremely difficult to disrupt. This is the reason why Carson (1982), as well as Mayr (1963) and Templeton (1981), borrowed Wright's adaptive landscape metaphor and the need for drastic stochastic events (i. e. founder events) to induce a displacement from an adaptive peak that could lead to speciation (Wright 1931).

I will also borrow Wright's adaptive landscape concept to show that if the genetic system that needs to be disrupted during speciation is under strong stabilizing selection then traits influenced by such systems should show low divergence between newly arisen species. For reasons of simplicity, imagine the ancestral species as composed of a sex-related (closed according to Carson's terminology) and a non-sex related gene pool. In figures 1 and 2 these two genetic systems are represented as balls resting on their respective adaptive peaks in the topography, and maintained at such peaks by selective

pressures. According to Carson's and Patterson's views, the selective forces would keep the sex-related genetic system strongly tied to its adaptive peak, but other systems will be more loosely linked. If drift shakes such a topography, then non-sexual traits that were more loosely attached to the peak are expected to end further apart in the topography from its original position, and hence the potential new species should be more divergent in their non-sexual characteristics than in their sex-related attributes (Fig. 1). The genetic system under strong stabilizing selection is the least likely to respond to drift. This makes such a genetic system not only less divergent but speciation become less likely since only populations of newly arisen species that do not have the key components of their gene pool tied strongly enough could be disrupted and moved into a new species domain (Fig. 1).

I do not deny that founding events may be capable of moving populations across adaptive valleys. However, the ability of founding events to disrupt well adapted gene complexes and to trigger reproductive isolation remain unclear and have been strongly criticized (Barton and Charlesworth 1984; Barton 1989; Coyne *et al.* 1997). I alternatively propose that peak shifts in an adaptive landscape would be highly dependant on the landscape's changeability as a consequence of environmental fluctuations (this allows the inclusion of many more potential speciation factors than just founding events). These changes would constantly modify the direction of selection. The candidate components of the genetic systems to generate reproductive isolation would be those more vulnerable to such selective changes. I propose that the sex gene pool system would be prone to move from its original adaptive peak in the ancestral population to a more distant, derived peak (Fig. 2). Such a shift would explain the high within species variability and the potential capacity to trigger speciation. The landscape for the non-sex components of the gene pool

would be less affected by the directional selective pressures encountered in the new environment and hence the changes would be, if any, minimal (Fig. 2).

5.2 WHY ARE SEX-RELATED TRAITS HIGHLY DIVERGENT?

In the previous section, I have expanded the views held by Carson (1982, 1985) and Patterson (1985) on the potential role of mate recognition and mating behavioural components in speciation to *all aspects of sexuality*. I have also argued in favour of directional selection rather than selective constraints and founding events as responsible for such divergence.

The divergent selection leading to speciation could be a consequence of the diverse ecological environments encountered in allopatry and reproductive isolation could simply evolve as a by-product of population divergence in allopatry. If so, sexual traits involved in mating and reproduction become particularly important during secondary contacts between the divergent populations as selection against hybridization is expected to reinforce sex traits divergence and strengthen isolation (Dobzhansky 1951). This model strongly restricts the feasibility of speciation as it requires both isolation and secondary contacts between populations which can only occur in restricted areas of their distribution. Even if the contact occurs, a situation of negative heterosis arises that would make extinction of one of the divergent populations or fusion of both populations very plausible alternatives to complete reproductive isolation (Templeton 1981; Spencer *et al.* 1986).

Sexual selection directly shaping the divergent characteristics of sexual traits in a runaway fashion becomes an attractive alternative. The original sexual selection model proposed by Fisher is a two-step process. First, there is an advantage for a male trait due entirely to natural selection, then the trait becomes more frequently chosen by females. This triggers a continuous development of the preferred male trait and at the same time

strengthens female preference, creating linkage between the male and female characters and starting an accelerating paced evolutionary process (Fisher 1958). In Lande's model of Fisherian sexual selection, the male traits most likely to be exaggerated are those under weak stabilizing selection and subject to relatively large variance in female's preferences. This also makes such traits more likely to enter an unstable phase of equilibrium that may lead to extreme divergence and sexual isolation (Lande 1981). Lande's scenario becomes an attractive alternative to speciation models that limit speciation to very strict demographic conditions such as founder events or complete isolation and reinforcement during secondary contacts. The evolution of females mating preferences can greatly increase the adaptive variation in male phenotypes along a continuous cline and trigger speciation (Lande 1982).

The early models of sexual selection (Lande 1981; Kirkpatrick 1982) assumed that females preferences were neutral and evolved as a correlated response to selection on male traits, the lines of equilibria that resulted from such assumptions could be easily perturbed by random forces such as genetic drift. This idea may have lead to an over-stress of stochastic events in speciation models induced by sexual selection (Lande 1981; Nei *et al.* 1983; Wu 1985). However, the neutral nature of female preferences seems misleading (see Kirkpatrick 1987; Eberhard 1996). Other models were developed that incorporated selection on mating preferences and mutational bias on the male character (Kirkpatrick 1985, 1987; Pomiankowski 1988; Bulmer 1989; Iwasa and Pomiankowski 1991; Pomiankowski and Iwasa 1991, 1993). Females are capable of imposing a strong selective pressure on the male trait by favouring male characteristics that may increase their viability or fecundity. Mutational bias is an expected consequence from selecting an extreme male trait, any new mutation in such an elaborated trait would most probably cause its deterioration. The mutational bias idea closely resembles Fisher's counterbalancing

selection (Fisher 1958). A model of sexual selection that considered these two factors simultaneously showed the unstable and cyclic nature of the evolution of traits under sexual selection, which might explain the high within and between species variability that is characteristic of sexual traits (Iwasa and Pomiankowski 1995). This model does not take into account drift or major changes in selective pressures and proposes that the rapid divergence in male traits between closely related species may be a consequence of unstable cycles falling out of step with one another (Iwasa and Pomiankowski 1995).

The previous models are important in showing sexual selection as a causing factor for the extreme male traits diversity between closely related species and its potential role in speciation. However, with the exception of Lande (1982), most models disregard a crucial characteristic of sexual selection: Sexual selection, as opposed to natural selection, would continuously favour innovation. A male trait would cease to be favoured by sexual selection as soon as it becomes established in a population. In this sense, sexual selection is continuously changing directions and such a fluctuating nature makes it a good candidate to trigger fast divergence and speciation.

Until recently most of the discussion has been restricted to mating behaviour or the morphology of secondary sexual traits. This restriction is probably a direct consequence of Darwin's original definition of sexual selection as responsible for shaping extravagant male organs as a consequence of male - male competition and mating preferences (Darwin 1871). However, increasing evidence seems to extend the scope of sexual selection to internal morphological traits, chemical and molecular aspects of the genitalia (Eberhard and Cordero 1995; Eberhard 1996; Chapters 2-4). In this thesis, the high divergence of proteins expressed in female tissues (i.e. ovary) among pairs of species that have not evolved hybrid female sterility suggest that such fast paced evolution may be linked to a male-female reproductive runaway process rather than adaptive divergence built up during

isolation (Chapter 2). In other words, sexual selection rather than adaptive (natural selection) divergence and possibly reinforcement through secondary contacts shape the evolution of sex-related traits. Morphological sex-traits show no sign of being under strong stabilizing selection. They show extensive variation within species and no major constraints to avoid developmental asymmetry (Chapter 3). Finally, the analysis of the patterns of nucleotide changes between species can reveal aspects of the role played by drift and selection in their evolution. The relationship between nonsynonymous and synonymous substitutions suggest selective forces as responsible for shaping the fast divergence observed in sex-related genes, and selection seems to be directional and strong among closely related species (Chapter 4). Based on these results I have previously attempted to extend the original definition of sexual selection (see Chapter 3): *Narrow sense* sexual selection is used for the original concept that refers to selection of mates; *Broad sense* sexual selection is used for *all aspects* of sexuality that are not necessarily linked to courtship and mating.

5.3 THE GENETIC ARCHITECTURE OF SEX-RELATED TRAITS

It is well established that gene products interact to produce a final phenotype. However, the way they interact and the way single genes behave when producing a phenotype could greatly differ from phenotype to phenotype. I would use an analogous example to illustrate this idea. Imagine an orchestra playing one of the Brandenburgh concertos, where a violinist plays the main melody. If we silence all the instruments in the orchestra but the violin, we would still recognize the concerto. The same violinist could also play in a quartet where a pianist has the leading part. We can say that the violinist is one of the "major" components of the orchestra and a supporting instrument in the quartet. Genes behave in a similar way to the violinist, they can be major genes supported

in their task to produce a particular phenotype (the Brandenburgh concerto) by a group of modifiers (the orchestra), and at the same time they could have pleiotropic effects (the violinist's different jobs) as a modifier gene for a different phenotype (the quartet).

Selection is expected to have an impact on the gene pool and its organization. When stabilizing selection favours intermediate phenotypes, as might be the case in the non-sexual traits studied in Chapter 3, then genes directly linked to the production of the phenotype (major genes) would be expected to evolve towards a homozygous state by the elimination of alleles that produce departures from the optimum. Dominance relationships will be favoured in order to mask deleterious variants. Genes acting as epistatic modifiers are expected to be more polymorphic so that they can balance in either direction the possible effect of deviant mutations. This could be seen as a negative epistatic system (i. e. a gene that deviates from the intermediate phenotype in a positive direction, let's say an increase in size, will be favoured in a predominating small size gene pool. Directional selection, on the other hand, is expected to build up a system that favours new variants moving the phenotype away from its current state, and positive epistatic modifiers pulling the phenotype towards the extremes.

If sex-related traits have undergone more directional changes at the onset of species formation than non-sexual traits (see chapter 4 of this thesis), then two non-mutually exclusive outcomes would be expected in the phenotype of the interspecific hybrids: 1) The two sets of alleles of the major genes would lack the ability to interact and mask the deleterious effects on the trait's size, and / or 2) the hybrid nature of the epistatic modifiers would fail to render an extreme phenotype in either of the parental directions. This will lead to an intermediate to dominant behaviour of the trait in the hybrid. If non-sexual traits have a slower rate of change during speciation, alleles from the different species might still conserve their ability to mask deleterious effects that may have drifted

to fixation at the time of speciation in one or the other parental species. If such was the case, non-sexual traits would show overdominance in the interspecific hybrids. These differential predictions were observed in this study (Chapter 3) and they have also been reported in other studies that analyzed sex and non-sex related morphological traits between closely related species (Coyne 1983, 1985; Coyne *et al.* 1991; Liu *et al* 1996).

However, traits under directional selection are expected to show signs of genetic and environmental stress due to selection against modifiers that control development (lack of control against phenodeviants). High fluctuating asymmetry in bilateral traits has been used as an indicator of the lack of developmental homeostasis (For reviews see Palmer and Stobeck 1986; Parson 1990). Secondary sexual traits have been a good example of traits showing large degrees of fluctuating asymmetry (Møller 1990; Møller and Hoghlund 1991; Anderson 1994). However, the levels of asymmetry of the secondary sexual traits may vary among species depending on whether the trait acts as an indicator of "good genes" or simple attractiveness (Ridley 1992). The sex-related traits studied in chapter 3 were not more, or less asymmetric than non-sexual ones. This could simply be a consequence of such traits not being directly influenced by female preference, or that the directional selective pressure has only been strong at the onset of speciation (see chapter 4) giving time for such traits to evolve their own species-specific homeostatic controls. The last alternative seems feasible, not only due to the results presented in chapter 4, but also from the higher sexual traits asymmetry in interspecific hybrids between closely related species (chapter 3). In the progeny obtained from crosses between closely related species, the hybrid nature of the genes controlling the sexual traits homeostasis may upset the traits' development leading to asymmetry (Chapter 3) and eventually atrophy (Pontecorvo 1943).

5.4 CONCLUSIONS

A few final comments are provided in point form. Rather than a simple enumeration of the particular results, these comments relate to the theoretical implications of this thesis to future studies of speciation.

1. Speciation does not require a major divergence of all components of the gene pool. There is a need for a distinction of its constituent parts.
2. The gene pool can be broadly divided into a sex-related system (i.e. genes involved in mating recognition, fertilization, spermatogenesis, or involved in sex determination) and a non sex-related system (i.e. genes involved in development, metabolic physiology, and viability). The divergence of the sex-related gene system plays a key role at the onset of species formation
3. The sex-related gene system evolves by directional sexual selection rather than by destabilization of selective constraints through founding events.
4. The effect of directional selection seems to be stronger at the early stages of species differentiation.
5. The effect of directional selection on sex-related traits has an impact on the evolution of its underlying system of genetic interactions (i.e. genetic architecture).

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Figure 1. A modification of Wright's adaptive landscape (Wright 1931). The sex-related components of the gene pool are represented by filled circles and the non-sex related components by dotted circles. Species A represents the ancestral species adaptive topography from which a new founder population may be shifted (arrows) into a new species domain.

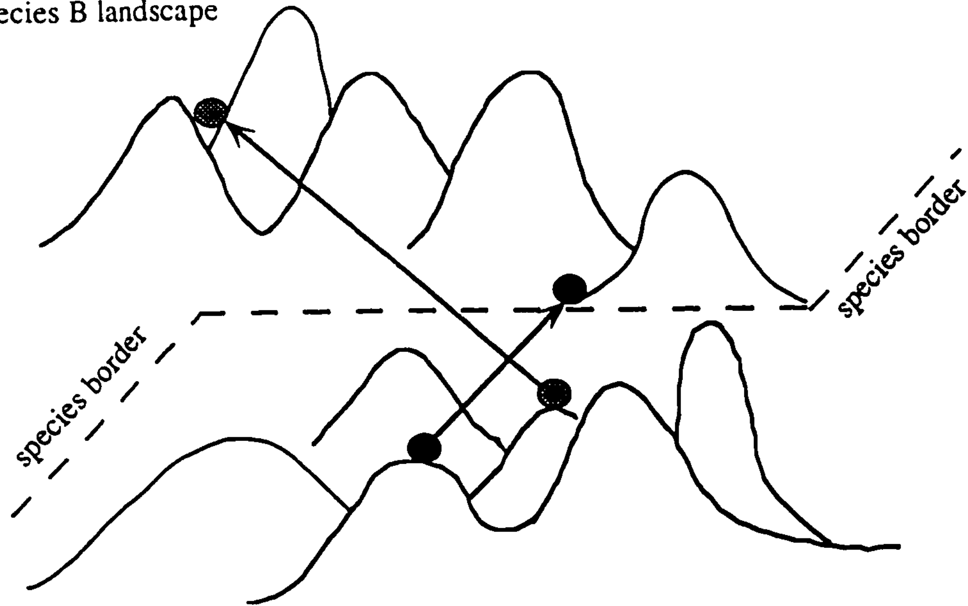
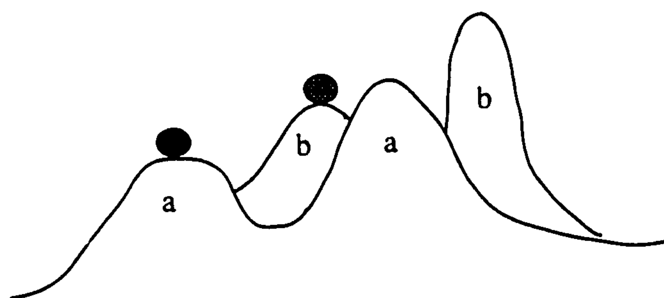
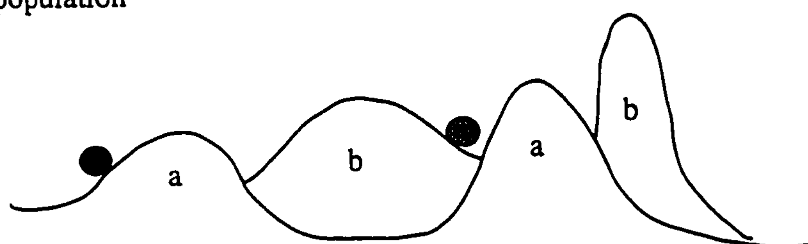

species B landscape
species border
species border
species A landscape

Figure 2. A modification of Wright's adaptive landscape (Wright 1931). The sex-related components of the gene pool are represented by filled circles and the non-sex related components by dotted circles. The ancestral sex-gene pool landscape (1a) goes through a more drastic modification in derived populations (2a) than the non-sexual components of the gene pool (1b, 2b). Such fluctuations may eventually lead to speciation (3) and a higher divergence in sex-related (full bar) than non-sex related (dashed bar) components of the gene pool.

1. Ancestral population



2. Derived population



3. Derived species