HALDANE'S RULE AND THE GENETIC BASIS OF HYBRID MALE STERILITY IN *DROSOPHILA SIMULANS*, *DROSOPHILA MAURITIANA* AND *DROSOPHILA SECHELLIA*

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ABSTRACT

Genetic analysis of hybrid sterility and inviability has recently become a successful experimental approach to pursue the problem of speciation. In the present study, classical genetic analyses and high resolution two-dimensional gel electrophoresis (2DE) have been used to investigate the genetic basis of hybrid male sterility in three sibling species of the *Drosophila melanogaster* species subgroup. The genetic basis of Haldane's rule, i.e., the preferential hybrid sterility and inviability of heterogametic sex, is also examined. The *D. sechellia* Y chromosome was shown to have no effect when placed in the background of *D. simulans*. This shows that the X-Y interaction hypothesis can not be a general explanation of Haldane's rule. Cytoplasmic factors were shown to have no effect on hybrid male sterility between *D. simulans* and *D. mauritiana* and between *D. simulans* and *D. sechellia*. An asymmetric X-autosome interaction provides the best explanation of hybrid male sterility between *D. simulans* and *D. sechellia*. The asymmetric model of X-autosome interaction can also explain the asymmetries (unidirectionality) in reproductive isolation commonly observed between related species.

Testis protein divergence on X chromosome was shown to be smaller than that on autosomes between these species. This

implies that Haldane's rule may not be accounted for by a faster evolution of the X chromosome. The number of genes involved in hybrid male sterility between *D. simulans* and *D. sechellia* was shown to be small by using 2DE. A few *D. sechellia* specific testis proteins were identified to be consistently associated with male sterility.

A general method for detecting major hybrid male sterility genes has been introduced. By using this method, a single Mendelian factor of *D. mauritiana* which produces complete male sterility in the background of *D. simulans* was identified and mapped to the X chromosome.

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PREFACE

The major body of the thesis (Chapters 3-5) consists of two papers that have been published in *Genetics* and a manuscript submitted to *Proceedings of National Academy of Sciences, U.S.A.* for publication. The author is responsible for all the work presented in the two published papers and the submitted manuscript.

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CHAPTER 1

INTRODUCTION

The genetic basis of species formation is one of the most challenging problems in evolutionary biology. While the genetic basis of speciation is usually meant to include both the genetic changes that lead to reproductive isolation and changes that lead to species-specific adaptation, the genetic analysis of reproductive isolation between related species is the favored experimental approach to pursue this problem. Post-zygotic isolation involves well defined characters, i.e., hybrid sterility and inviability, which are especially suitable for genetic investigation. More than 70 years ago, Haldane (1922) drew attention to the peculiar nature of species hybrid sterility and inviability and pointed out 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". (Note: by "heterozygous sex", Haldane referred to heterogametic sex) This observation is known as Haldane's rule. Systematic genetic studies of hybrid sterility and inviability on various groups of *Drosophila* species pioneered by Dobzhansky (1936) and followed by others (reviewed in Coyne and Orr 1989) have revealed a related general pattern in post-zygotic reproductive isolation: "The genes having the greatest

effect on hybrid sterility and inviability are X-linked." (Coyne and Orr 1989). The two patterns are remarkably consistent and are therefore called "two rules" of speciation (Coyne and Orr 1989). In spite of the consistency of the two "rules", their genetic basis (or bases), which is crucial to the understanding of the process of speciation, is still unclear.

The knowledge of the number (few vs. many) and nature (major vs. minor genes) of genes causing reproductive isolation is very important for our understanding of speciation. It can help us discriminate among various theories of speciation. Of course, a complete genetic picture of the speciation process would require examination of individual loci causing reproductive isolation, determination of their normal function and explanation of their evolutionary changes. The first step in this process would be to identify and localize genes with important effects on interspecific isolation. In this study, the genetic basis of Haldane's rule and hybrid male sterility in three sibling species in the *Drosophila melanogaster* species subgroup have been investigated.

1.1 Genetic basis of Haldane's rule

Haldane's rule holds both in situations where males are the heterogametic sex (*e.g.*, in dipterans and mammals) (Haldane 1922, Gray 1954, Bock 1984) and in situations where the females are the heterogametic sex (*e.g.*, in birds, lepidopterans) (Haldane 1922, Gray

1958). In *Drosophila*, for example, Bock (1984) listed 144 cases of interspecific hybridizations that produced one sterile and one fertile sex in offsprings and all but one of these crosses yielded sterile males (XY) and fertile females (XX). A summary of hybridization results in different species groups is presented in Table 1.1. There are only 19 out of 340 cases that violate Haldane's rule.

Such a striking general phenomenon has been known for more than 70 years, but intensive debate on its genetic basis is still going on. Dobzhansky (1936) pioneered the investigation of the genetic basis of hybrid male sterility in the sibling species of *Drosophila pseudoobscura*. In his studies, a handful of morphological markers were used to identify the species origin of chromosomal segments in the backcross progenies, and to correlate them with male fertility. His studies and subsequent studies on various groups of *Drosophila* species using the same or similar methods have revealed a second general pattern in post-zygotic isolation: The X chromosome has the largest effect on hybrid sterility and inviability (Coyne and Orr 1989). This empirical generalization is based on numerous genetic studies of hybrid sterility and inviability in different groups of *Drosophila* including the *obscura* (Dobzhansky 1936, Wu and Beckenback 1983, Orr 1987), the *melanogaster* (Coyne 1984, Coyne and Kreitman 1986), the *virilis* (Orr and Coyne 1989), and the *repleta* groups (Hennig 1977, Zouros, Lofdahl and Martin 1988). These studies, most of which are on hybrid male sterility, show that a replacement of the X chromosome or marked segments of the X

TABLE 1.1

Conformity of species hybridizations to Haldane's rule[a]

Group	Trait	Hybridizations with asymmetry[b]	Number obeying Haldane's rule
Drosophila	Fertility	202	199
	Viability	23	14
Mammals	Fertility	25	25
	Viability	1	0
Birds	Fertility	30	30
	Viability	23	21
Lepidoptera	Fertility	15	15
	Viability	40	36

[a] The data in the table were compiled by Wu and Davis (1993).

[b] Only one sex of the hybrids is sterile or inviable.

chromosome by that of a foreign species usually have a disproportionately greater effect on hybrid male sterility than an autosomal replacement of similar size.

The search for the genetic basis of Haldane's rule started ever since the rule was formulated, and a number of explanations have been offered since then. A traditional explanation for Haldane's rule is the X-autosome imbalance hypothesis (Dobzhansky 1937a). This explanation is based on the assumption that "many species have a balance of genes in the X chromosome and the autosomes peculiar to themselves and different from other species" (Dobzhansky 1937b). As female hybrids (Note: For convenience, I use male for heterogametic sex and female for homogametic sex) have a complete homospecific set of autosomes and an X chromosome from each parental species, the X-autosome balance remains undisturbed. In male hybrids, however, the X chromosome corresponding to the paternal set of autosomes is missing, so the genic balance between the autosomes and the X chromosome is perturbed and such an imbalance in hybrid males (heterogametic sex) results in sterility or inviability.

The X-autosome imbalance hypothesis predicts that the homogametic sex would be as much affected as the heterogametic sex, if the former sex could be made to have the same level of X-autosome imbalance as the latter. This prediction was tested by Coyne (1985) in the species pair *Drosophila simulans* and *D. mauritiana* (or *D. sechellia*). He crossed *D. simulans* females with

attached-X chromosomes to *D. mauritiana* (or *D. sechellia*) males and obtained hybrid females that are homozygous for the X chromosomes from one species (*D. simulans*) and heterozygous for the autosomes from both parental species. These females have the same degree of X-autosome imbalance as the sterile hybrid males from this species pair but are, nevertheless, fertile. Thus he concluded that the X-autosome imbalance can not explain Haldane's rule. However, just because X-autosome imbalance does not cause hybrid female sterility does not mean that hybrid male sterility is also not caused by such an imbalance, because the developmental pathways involved in male and female fertility are very much different. A better test of the hypothesis should be on inviability, as developmental pathways involved in male and female inviability are likely to be more similar than those involved in male and female sterility (Wu and Davis 1993).

By using the same method as used by Coyne (1985), Orr (1993) tested the inviability aspect of the X-autosome imbalance hypothesis in species pair *Drosophila simulans* and *D. melanogaster* (or *D. teissieri*). The results of the test are exactly what is predicted by the X-autosome imbalance hypothesis, *i.e.*, hybrid females that are homozygous for the X from one species are as inviable as hybrid F_1 males bearing the same X. Orr (1993) interprets his results as being due to the composite nature of Haldane's rule (Wu and Davis 1993), *i.e.*, the X-autosome imbalance explains the inviability aspect but not the sterility aspect of Haldane's rule. However, I would consider an

alternative interpretation to be that Coyne's (1985) test is inconclusive and Orr's (1993) test provides strong evidence supporting the X-autosome imbalance hypothesis (For detailed discussion on this point, see Chapter 6).

Another explanation for Haldane's rule suggested by Haldane (1932) and invoked by Coyne (1985) is the X-Y interaction hypothesis, which explains the preferential heterogametic hybrid sterility and inviability by the genic interactions between X and Y chromosomes (or "Z" and "W" chromosomes where females are heterogametic) from different species. This hypothesis has been shown to be invalid by two independent tests [Johnson et al. 1992, Zeng and Singh 1993a (Chapter 3)]. We have shown that male flies with *D. simulans* X chromosome and *D. sechellia* Y chromosome are completely fertile (see Chapter 3 for details). In addition, the X-Y hypothesis does not explain Haldane's rule in cases where males are XO.

One of the most comprehensive explanations for Haldane's rule is that of Charlesworth, Coyne and Barton (1987), subsequently extended by Coyne and Orr (1989). Recessive advantageous mutations will more often be fixed if they are on sex chromosomes than if they are on autosomes. As a result, sex chromosomes evolve more rapidly than autosomes. The model of faster evolution of sex-linked genes has been shown to explain both Haldane's rule and the large effect of the X chromosome on post-zygotic isolation with two additional assumptions (Coyne and Orr 1989). The first assumption is

that genes causing sterility or inviability in the hybrid background have a selective advantage in pure species. The second assumption is that substantial portion of genes have sex-specific expressions. These genes cause sterility or inviability in the hybrid of one sex, but also enjoy selective advantage in the pure species of the same sex. This model has considerable appeal because the logic that genes with recessive advantage would evolve faster when they are X-linked is unassailable. However, there is little evidence for the two arbitrary assumptions, and there are some problems associated with this model as well.

First, it does not explain why characters other than hybrid sterility and inviability are not also strongly influenced by sex-linked genes (Charlesworth, Coyne and Barton 1987; Coyne and Orr 1989). Morphological differences between species of *Drosophila* do not involve the X chromosome more than the autosomes (Charlesworth, Coyne and Barton 1987), and pre-zygotic isolation is not unduly influenced by the sex chromosomes (Coyne 1989). Second, it is possible that the large X effect is an artifact due to the nature of Dobzhansky's (1936) backcross analysis which has been used for most studies showing the great effect of the X chromosome (Wu and Davis 1993). In this backcross analysis, the interspecific replacements of chromosomes or segments of chromosomes are performed on one copy only. The replacements of the X are hemizygous, but the replacements of the autosomes are heterozygous. Both dominant and recessive effects of X chromosome

can be revealed by this analysis, but only dominant effects of autosomes can be revealed. The effects of autosomes have not been fully revealed by such an analysis. A more proper analysis would be to compare hemizygous replacements of the X with homozygous replacements of the autosomes. This type of comparisons have revealed large effects of the autosomes as well as the sex chromosomes in the studies of Hennig (1977) and Pantazidis and Zouros (1988). Two lines of reasoning, however, militate against the large effect of the X being solely artifactual. First, Coyne and Orr (1989) present some data suggesting that there may also be a large effect of the X for the few cases of female sterility. Here the comparison is between heterozygous replacements of both X chromosome and autosomes. Second, if the apparent large effect of X were an artifact, a large effect of X would also be expected for the morphological and behavioral traits when studied using the same backcross analysis.

A central prediction of this model is that X chromosome evolve faster than autosomes. This prediction was tested in *Drosophila simulans*, *D. mauritiana* and *D. sechellia* [Zeng and Singh 1993b (Chapter 4)]. The proportion of divergent testis proteins associated with the X chromosome was found to be no larger than expected on the basis of the genome size of the X chromosome (for details, see Chapter 4).

The meiotic drive model, which has been extensively debated (Frank 1991a,b; Hurst and Pomiankowski 1991; Coyne, Charlesworth

and Orr 1991; Johnson and Wu 1992; Coyne and Orr 1993; Charlesworth et al. 1993; Pomiankowski and Hurst 1993), attempts to explain Haldane's rule as the consequence of the divergence of meiotic drive system (Frank 1991a,b; Hurst and Pomiankowski 1991). This model assumes that sex-linked driving elements arise often in natural populations but intraspecific drive and sterility are prevented by the rapid evolution of suppressors. Diverging populations will acquire different suppressors. Thus hybrids with heterospecific chromosomes may be sterile due to the action of two different driving systems each not suppressed. The meiotic drive model has been theoretically examined (Coyne, Charlesworth and Orr 1991) and empirically tested (Johnson and Wu 1992; Coyne and Orr 1993) and shown to be invalid.

Jablonka and Lamb (1991) proposed a model that is based on greater conformational changes of sex chromosomes than autosomes during gametogenesis. Their main emphasis is on the epigenetic effects and X-Y interactions. However, as discussed above, X-Y interaction is often not involved in hybrid sterility (Johnson et al. 1992; Zeng and Singh 1993a).

It seems that none of the available models can completely explain Haldane's rule. It remains a question whether or not Haldane's rule is a composite rule and there is no unitary explanation (Wu and Davis 1993) or simply that we have not yet found the right explanation.

1.2 Epistatic interactions underlying hybrid sterility and inviability

Hybrid sterility or inviability results when two sets of genes from different species are brought together into hybrid individuals. As each of the two sets of genes function normally in the parental species the incompatibility (hybrid sterility or inviability) must necessarily be a result of epistatic interactions of certain genes from the two species. There are many possible incompatible interactions between heterospecific chromosomes, and most of them have been observed. The most common form of interaction is the X-autosome interaction which has been observed in species pairs *Drosophila pseudoobscura / D. persimilis* (Dobzhansky 1936), *D. pseudoobscura pseudoobscura / D. p. bogotana* (Prakash 1972), *D. hydei / D. neohydei* (Schafer 1978), *D. virilis / D. lummei* (Heikkinen and Lumme 1991) and *D. simulans / D. sechellia* (Zeng and Singh 1993a). In species pair *D. hydei / D. neohydei* (Schafer 1978), interactions between different autosomes and between the Y chromosome and autosomes have also been found. Interactions between Y chromosome and autosomes have also been observed in species pair *D. virilis / D. lummei* (Heikkinen and Lumme 1991). A well established case of interactions between autosomes and Y chromosome is in the species pair *D. mojavensis* and *D. arizonensis* (Vigneault and Zouros 1986; Zouros, Lofdahl and Martin 1988; Pantazidiz, Galanopoulos and Zouros 1993). Interaction between

heterospecific sex chromosomes has been reported (Curtis, Langley and Trewern 1980; Coyne 1985; Orr 1987). However, these studies were carried out by comparing fertility between backcross progeny which had highly heterogeneous chromosome background. These comparisons are liable to alternate interpretations. For example, based on virtually the same results, Dobzhansky (1936) and Orr (1987) have reached opposite conclusions regarding the role of the Y chromosome in hybrid male sterility. Incompatible interactions involving cytoplasm have also been reported to affect hybrid male and female sterility (Orr 1987, 1989a).

1.3 Identification and mapping of hybrid male sterility genes

Identification and mapping of hybrid male sterility genes have been the main focus in the studies of reproductive isolation. The first study on detecting genes responsible for hybrid male sterility was carried out by Dobzhansky (1936), who used morphological markers to identify foreign chromosome segments linked to the markers in backcross progenies, and to examine the effects of the segments on male fertility. The outcome of his and subsequent studies on various groups of *Drosophila* species (Coyne 1984; Coyne and Kreitman 1986; Orr 1987, 1989a; Khadem and Krimbas 1991) turned out to be virtually the same: every marker used is associated with hybrid male sterility and the largest effects are associated with X-linked

markers. For example, Coyne (1984), and Coyne and Kreitman (1986) used five markers on five chromosomal arms and studied hybrid male sterility between *D. simulans* and *D. mauritiana* (or *D. sechellia*). All the five markers were associated with hybrid male sterility with the X-linked marker having the largest effect.

Another method to identify and map major genes linked to X-linked markers is the backcross introgression which is basically an extension of Dobzhansky's backcross analysis approach. In this method, the marked chromosome segments of one species are introgressed into the background of another species by continuous backcrosses (Coyne and Charlesworth 1986, 1989; Pantazidis and Zouros 1988; Orr 1989b; Pantazidis, Galanopoulos and Zouros 1993; Perez et al. 1993; Wu and Backenback 1983; Naveira and Fontdevila 1986, 1991a,b). The putative hybrid male sterility genes are then mapped by recombination analysis (Coyne and Charlesworth 1986, 1989; Pantazidis and Zouros 1988; Orr 1989b; Pantazidis, Galanoloulos and Zouros 1993) or by other genetic and molecular means (Orr 1992; Perez et al. 1993). The first marker assisted introgression study was carried out by Wu and Beckenback (1983), who identified three X-linked hybrid male sterility genes between *Drosophila pseudoobscura* and *D. persimilis*. Coyne and Charlesworth (1986, 1989) introgressed three regions of the X chromosome from *D. mauritiana* (or *D. sechellia*) to *D. simulans*, and identified and mapped (by a maximum likelihood method) three hybrid male sterility genes. One of the three gene is closely linked to *forked* locus and has been

mapped at a finer scale by Perez et al. (1993) using some molecular markers around the region. Another example is the *SMF* (sperm motility factor) identified and mapped on *Drosophila mojavensis* fourth chromosome by Pantazidis and Zouros (1988), Pantazidis, Galanoloulos and Zouros (1993). This gene causes compete male sterility in the presence of *D. arizonensis* Y chromosome.

In spite of the successful application of this introgression approach, it suffers from two general problems. One is that the introgressed regions are usually very large and it is not known if the segments carry major genes or many polygenes. The other problem is that it is limited by the availability of markers. I used a simple method which is free of these problems (see Chapter 5 for details). In this method, individual major hybrid male sterility genes, or very tightly linked gene complexes, are introgressed from one species into the background of another species by continuous backcrosses. This is done by selecting females, each generation, which are heterozygous for the sterility gene (and which therefore produce both fertile and sterile sons in equal proportions) and backcrossing them to males of one of the parental species. No marker is required for this introgression, which makes the method one of general use.

By applying this method to species pair *Drosophila simulans* / *D. mauritiana* a major gene (or gene complex) has been identified and mapped to the *D. mauritiana* X chromosome.

1.4 The project

1.4.1 The species

The Melanogaster complex, part of the *Drosophila melanogaster* species subgroup, consists of four species -- *D. melanogaster*, *D. simulans*, *D. mauritiana* and *D. sechellia*. They are closely related species. Three of the four species, *D. simulans*, *D. mauritiana* and *D. sechellia*, are closer to each other than to *D. melanogaster*. These three species were then chosen to study the genetic basis of reproductive isolation.

D. simulans is cosmopolitan and it was discovered in 1919 (Sturtevant 1919). *D. mauritiana* was discovered in 1974 (Tsacas and David 1974 cited in Lachaise et al. 1986). It is restricted to the island of Mauritius, 960 km east of Madagascar. *D. sechellia* was discovered in 1981 (Tsacas and Bachli 1981 cited in Lachaise et al. 1986). It is restricted to three islands in the Seychelles, about 1000 km north east of Madagascar (Lachaise et al. 1986). *D. mauritiana* is the only species of subgroup which lives on the island of Mauritius. *D. sechellia* is the only species of the subgroup which lives on the three islands in the Seychelles, although *D. simulans* has been found in other islands in the Seychelles.

The three species are very closely related sibling species. The only diagnostic morphological difference is the shape of the male genital arch (Coyne 1983; Coyne and Kreitman 1986). The

chromosome banding sequences of the three species are identical (Lemeunier and Ashburner 1976, 1984).

Crosses among the three species produce fertile female and sterile male hybrids (Lachaise et al. 1985; Lee and Watanabe 1986), therefore, conforming Haldane's rule. Fertile females can backcross with any of their parental species. Male backcross progeny are of variable fertility (Lachaise et al. 1986; Coyne 1984; Coyne and Kreitman 1986). All these features make the three species excellent material for studying the genetic basis of hybrid male sterility and Haldane's rule, as classical genetic analyses can be employed.

1.4.2 The objectives

The genetic analysis of hybrid male sterility is expected to answer the following questions: 1) How many genes (few vs. many) are involved in determining hybrid male sterility? 2) What type of genes (major vs. minor) are involved in hybrid male sterility? 3) How do these genes cause hybrid male sterility? 4) What is the basis of Haldane's rule?

These questions are not expected to be answered within a single thesis dissertation. What I outline below, is a collection of my work which provides partial answers to some of these questions and eliminates possible answers of others.

1.4.3 The findings

Chapter 3 of the thesis deals with the effects of the Y chromosome and the cytoplasmic factors on hybrid male sterility and a test of the X-Y interaction hypothesis of Haldane's rule. An introgression of the *D. sechellia* Y chromosome into the background of *D. simulans* revealed no effect on male sterility. This demonstrates that the X-Y interaction is not involved in the male sterility of *D. simulans* (females) x *D. sechellia* (males) hybrids. In both species pairs *D. simulans* / *D. mauritiana* and *D. simulans* / *D. sechellia*, interspecific cytoplasm replacement has shown that the cytoplasmic factors are also not involved in hybrid male sterility. The exclusion of the Y chromosome and the cytoplasm and the consideration of the large effects of the X chromosome (see review in Coyne and Orr 1989) make the X-autosome interaction the most plausible hypothesis for hybrid male sterility between *D. simulans* females and *D. sechellia* males. This X-autosome interaction has been shown to be of an asymmetrical type. An asymmetric X-autosome interaction model has been proposed to explain the often observed unidirectional hybrid sterility and inviability in closely related species.

The classical genetic analyses combined with high resolution two dimensional gel electrophoresis (2DE) have been used to assess the number of genes involved in hybrid male

sterility and to detect proteins associated with hybrid male sterility (Chapter 4). By comparing 2DE protein profiles of the two parental, F_1 hybrid and backcross males, I was able to partition the divergent proteins to different chromosomes and show that the X chromosome has not diverged more than the autosomes. This provides a direct test of the model of fast evolution of the X chromosome and shows that Haldane's rule may not be accounted for by this model. My backcross analysis and 2DE protein assay show that hybrid male sterility is not caused by a large number of genes, instead a relatively small number of genes are involved with a few having large effects. A number of proteins have also been identified to be consistently associated with hybrid male sterility.

In Chapter 5, I describe a general method for detecting major hybrid male sterility genes which does not depend on use of morphological markers. Using this method, a single Mendelian factor (a single gene or gene complex) of *D. mauritiana*, which produces complete hybrid male sterility when introgressed into the background of *D. simulans*, was identified and mapped to the X chromosome.

CHAPTER 2

MATERIALS AND METHODS

2.1 *Drosophila* Stocks

Seven isofemale lines of *D. simulans*, two mutant marker strains of *D. simulans*, one *D. simulans* stock with attached-X chromosomes, nine isofemale lines of *D. mauritiana* and two *D. sechellia* strains were used in this study. They are listed in Table 2.1, with information on their origins and sources from which they were obtained.

2.2 Fly Culture

All the *Drosophila* stocks listed in Table 2.1 were cultured at 24-25°C and a diurnal photic cycle of 12 hour light and 12 hour dark. The composition of the culture medium used is described in Table 2.2. Cultures were reared in 8-dram glass vials.

2.3 Interspecific Hybridizations

D. simulans females are easily crossed to *D. mauritiana* males,

TABLE 2.1

List of *Drosophila* stocks used in this study

Species stock	Obtained from
D. simulans isofemale lines:	
Brazzaville S3	Dr. Jean David
Cape Town 2-5	"
Seychelles 7	"
South France #30	"
South France 25	"
Town Swille 10	"
Tunisia 44	"
D. simulans mutant marker strains:	
0251.16 f^2	National Drosophila Species Resource Centre
0251.17 lz^s male/X^XY yw female	"
0251.41 f; nt pm; st e	Dr. Jerry Coyne
D. mauritiana isofemale lines:	
mauritius LG 5	Dr. Jean David
mauritius LG 8	"
mauritius LG 12	"
mauritius LG 24	"
mauritius LG 26	"
mauritius LG 44	"
mauritius LG 58	"
mauritius LG 61	"
mauritius LG 69	"
D. sechellia stocks:	
seychelles #12	Dr. Jean David
Robertson	Dr. Jerry Coyne

TABLE 2.2

Drosophila culture medium

Agar	20g
Water	1.8 litre
Bananas (medium)	1.5
Corn syrup	2 tablespoons
Sugar	1.5 tablespoons
Yeast	60g

Agar is dissolved in water by heating to boil. The remaining ingredients are mixed in a blender and then added to the boiling agar solution. The mixture is heated to boil and then allowed to cool. When the medium is cooled to 40 °C, 36mL tegosept (10g Methyl p-hydroxy benzoate in 100mL 95% ethanol) is added. After proper mixing of the tegosept with the medium, about 8 ml of the medium is poured into individual vials using a 50 ml syringe.

whereas the reciprocal cross is not easily made (Lachaise et al. 1986). The reciprocal cross was attempted a number of times with two lines from each species, no offspring was obtained. *D. mauritiana* females reject *D. simulans* males.

The pre-mating isolation between *D. mauritiana* females and *D. simulans* males is, however, not so strong that successful mating is impossible. There is a low probability (~1%) of successful mating (Lee and Watanabe 1987). Three strategies were used to increase the probability of successful mating between *D. mauritiana* females and *D. simulans* males. The strategies are (1) to try a number of different lines of each species, (2) to use a high ratio of males to females in the cross, and (3) to isolate the virgin females and males for a longer time before putting them together.

D. simulans females X *D. mauritiana* males:

This cross was easily made. Large number of female and male offsprings were obtained.

D. mauritiana females X *D. simulans* males:

Six lines of *D. simulans* and 9 lines of *D. mauritiana* listed in Table 2.1 were tried for this cross. A total of 50 out of 54 possible crosses were made between these 15 lines. Only 4 of the 50 combinations tried produced F_1 hybrids. Surprisingly, when the F_1 males and females were mated, one of the four successful combinations produced F_2 offspring. This can be due to either strain

differences or contamination. One of the three remaining combinations which produced F_1 but not F_2 progeny is *D. mauritiana* mauritius LG 24 females X *D. simulans* Town Swille 10 males. The successful mating between this two lines is reproducible and the two strains of the two species were used for the studies described in Chapters 3-5.

Other crosses:

D. simulans females were crossed with *D. sechellia* males of strain sechelles # 12 easily, however, the reciprocal cross is difficult. Only one out of seven *D. simulans* lines used in this cross produced a few offspring and this is not reproducible. *D. mauritiana* females of strain mauritius LG 24 were also successfully crossed to *D. sechellia* males of the strain sechelles # 12.

Town Swille 10 of *D. simulans*, mauritius LG 24 of *D. mauritiana* and sechelles # 12 of *D. sechellia* were used in most of the crosses described in this thesis.

2.4 Protein Electrophoresis: Tissue Dissection and Sample Preparation

2.4.1 Solutions

Ringer's solution used for tissue dissection and solution used for sample preparation are given in the following.

Ringer's solution:

Add 7.5g NaCl, 0.35g KCl and 0.21g $CaCl_2$ into 1 litre H_2O.

Sample solution (DTT-CHAPS-Urea sample solution):

DTT	0.1 g (1% w/v)
CHAPS	0.4 g (4% w/v)
Urea	5.4 g (9 M)
Ampholyte 3-10 or 3.5-10	0.5 ml (2% v/v)
H_2O	6 ml

Dispensed into 0.5 ml aliquots and stored for up to 6 months at -70°C.

Reference: Hochstrasser, et al. (1988).

2.4.2 Sample preparation:

Young male flies were separated from females and aged for 5 to 6 days before dissection. Individual etherized flies were mounted on a glass slide with a drop of Ringer's solution. Under a dissection microscope, a pair of forceps were employed to hold the fly and a fine needle with bent tip was used to tear the yellow testes apart from the abdomen. Testes were cut off and collected in a drop of Ringer's solution on a separate slide.

A collection of seven, 14 or 21 pairs of testes were placed in 40µl, 80µl or 120µl sample solution which was thawed and placed on

ice. The tissues were dissolved in the sample solution quickly by a brief vortex mixing and stored at -70°C until electrophoresis. Just prior to electrophoresis, samples were alternately thawed and frozen by immersion in liquid nitrogen and a 37°C water bath, for five freeze/thaw cycles. After the last thaw, samples were centrifuged at 10,000 rpm for 20 minutes. The supernatants were transferred to new tubes and centrifuged for another 10 minutes at 14,000 rpm. Thirty µl supernatant of each sample was loaded for electrophoresis.

2.5 High Resolution Two-Dimensional Gel Electrophoresis (2DE)

Two dimensional gel electrophoresis is a very powerful technique for simultaneously resolving hundreds of polypeptides. Because of the difficulty in achieving adequate reproducibility and truly high resolution, the technique has successfully been practised in a relatively small number of laboratories (Hanash and Strahler 1989). There has been considerable amount of effort put in modifying O'Farrell's (1975) method to apply to Drosophila reproductive tract proteins (Coulthart 1986) and the resolution and reproducibility have been significantly improved (Coulthart and Singh 1988a, b, c). However, the earlier application of this technique did not totally eliminate some of the common problems of 2DE. After enormous effort, the technique has been improved with increased resolution and reproducibility. The separation has been dramatically

increased and as a consequence the number of protein spots that can be resolved on a single gel has been increased from about 400 to over 1000 using the same tissue.

2.5.1 The 2DE method

In 2DE the proteins are separated based on their intrinsic chemical charges (isoelectric focusing; IEF) in the first dimension and further separated according to their molecular size in the second dimension. As proteins are separated by two independent properties of proteins, they are well separated in the form of spots on a gel.

The first dimension--isoelectric focusing:

When protein samples are loaded on top of a tube gel containing a pH gradient generated by carrier ampholytes, the proteins move in the electric field when electricity is applied. A protein will keep moving until it reaches a point on the gel where the pH is the same as the pI value of the protein. This is termed as "steady state". When a protein is close to its steady state, it carries very few charges and, so, it moves very slow in the electric field. High resolution of the IEF gel requires that most of the proteins are in their steady state. To allow most proteins reach their steady state, a strong electric field and a long running time are necessary. That is why a high voltage and a long time is applied in the first dimension of 2DE.

A well formed and stable pH gradient is critical in IEF. Carrier ampholytes are what is used in generating a pH gradient. Ampholytes are composed of isomers and homologues of aliphatic polyaminopolycarboxylic acids with pI values ranging from 2.5 to 11. Some commercially available ampholyte mixtures may contain hundreds of such molecular species (Dunbar 1987).

The second dimension--SDS-PAGE:

SDS polyacrylamide gel electrophoresis is a well established technique which is almost directly applied in 2DE. Proteins with identical pI resolved by IEF are further separated according to their molecular sizes.

2.5.2 Solutions for 2DE

2.5.2.1 Solutions for the first dimensional gel

Acrylamide/Bis (30%, 2.67% C) stock (monomer stock):

Total volume	50ml	100 ml
Acrylamide	14.6 g	29.2 g
Bis	0.4 g	0.8 g
H_2O	30 ml	60 ml
Dissolve and add H_2O to	50 ml	100 ml

Filter and store in an amber bottle at 4°C.

IEF gel solution (4.5%):

Total volume	20 ml	40 ml
Urea	10.0 g	20 g
Monomer stock (30%)	3 ml	6 ml
CHAPS	0.3 g	0.6 g
Nonidet P-40	0.1 ml	0.2 ml
Ampholytes		
5-7 (Bio-Rad)	0.8 ml	1.6 ml
3-10 or 3.5-10 (Bio-Rad)	0.2 ml	0.4 ml
H_2O	7.5 ml	15 ml

Stored in 4 ml aliquots at -70°C.

To cast gels:

Warm up to room temperature; degas for 3 min.; and add:

TEMED	5 µl
10% Ammonium Persulfate	10 µl

Swirl 4-5 times and cast the gels.

Electrolytes:

Catholyte: 20 mM NaOH made by adding 1 ml 10 N NaOH in 500 ml H_2O. Freshly made for each use.

Anolyte: 6 mM H_3PO_4 made by adding 1.8 ml concentrated H_3PO_4 in 2.6 litres of H_2O. Freshly made for each use.

2.5.2.2 Solutions for second dimensional gel and transfer of IEF gel to second dimensional gel

SDS-PAGE monomer stock (40%):

Acrylamide (Bio-Rad)	195.0 g
Bis (Bio-Rad)	5.0 g
H_2O	to 500 ml

Stored at 4°C in an amber bottle.

Resolution gel buffer stock (Tris-HCl buffer):

Tris (Bio-Rad)	91.5 g (1.51 M)
1 N HCl	100-110 ml
H_2O	to about 400 ml
Dissolve and add:	
1 N HCl	to pH 8.8 (25°C)
H_2O	to 500 ml

Store at 4°C in an amber bottle.

10% ammonium persulfate:

Dissolve 1g ammonium persulfate (Bio-Rad) in water and bring to 10 ml.

This solution can be stored for 2 days at 4°C.

Second dimensional running buffer:

	Upper buffer	Lower buffer
Tris	24 g	120 g
Glycine	115.2 g	576 g
ddH_2O	to 4 litres	to 20 litres
Sodium azide		4 g

Running buffer is stored at 4°C.

The upper buffer can be stored for 3 months and the lower buffer can be reused for 3 months.

12% gel solution:

Total volume	120 ml (2 gels)	400 ml (7 gels)
Monomer stock (40%)	36 ml	120 ml
Tris-HCl, 1.51 M, pH 8.8	30 ml	100 ml
H_2O	53.7 ml	180 ml
Mix, filter and degas (with stirring bar) for 5 to 7 min.; add:		
TEMED	0.05 ml	0.15 ml
Ammonium persulfate	0.3 ml (10%)	0.1 g

Gently swirl 8-10 times and pour the gels.

Transfer solution:

Total volume	70 ml
Tris-Hcl, 0.5 M, pH 8.8	10 ml
SDS, 10%	20 ml
Bromphenol blue, 0.05%	4 ml
ddH_2O	36 ml

2.5.2.3 Solutions for silver staining and procedure

Solutions used in silver staining:

Solution number	Solution composition
1	95% ethanol/acetic acid/water (52.5:5:42.5 v/v/v)
2	10% ethanol
3	5 mg/litre DTT
4	0.1% (w/v) A_gNO_3
5	3% (w/v) Na_2CO_3 + 0.5 ml/litre (w/v) formaldehyde
6	2.3 M citric acid
7	0.75% (w/v) Na_2CO_3

Comments:

1. Solution 4 and solution 5 are freshly made.
2. Formaldehyde is added to solution 5 just before use.
3. Other solutions can be stored for up to 6 months.

Procedure of silver staining:

Step	Solution Number	Time	Comments
1. Fix	1	2 to 3 days	No shaking
2. Wash	2	1 hr.	Shaking
3. Wash	2	30 min.	Shaking
4. Wash	Water	30 min.	Shaking
5. Wash	Repeat Step 4		
6. Reduce	3	30 min.	No water rinse
7. Silver binding	4	30 min.	Shaking
8. Wash	Water	10 sec.	Float gel in tray with hand shaking
9. Wash	5	10 sec.	Float gel in tray with hand shaking Minimal finger pressure on gel when draining
10. Wash	Repeat Step 9		
11. Develop	5	5-10 min.	Shaking
12. Stop	6	5-30 min.	15 ml per 300 mL solution 5
13. Store	7	indefinite	

Comments:

1. In steps 1, 2, 3, and 4, 250 ml of solutions are used; in steps

5, 6, 7, 8, 9, 10, and 11, 300 mL of solutions are used.

2. Following each step (except step 6), the gel is rinsed with distilled water.

2.5.3 Procedures of 2DE

The first dimension:

Six capillary tubes (18 cm long by 1.4 mm in inner diameter) were cast simultaneously in a glass vial (8.5cm long and 2cm in diameter) with 4 ml gel solution. The glass vial with gel solution and the capillary tubes was placed in a cylinder made from two 50 ml centrifuge tubes. The cylinder was then slowly filled with water, the water pushed the gel solution into the six capillary tubes evenly. The length of all the six gels was the same and was adjusted to 16 cm by adding in or taking out the water from the cylinder. Each tube was overlaid with 5 µl water by a 50 µl Hamilton micro syringe. The gels were left overnight to polymerize.

Hoefer tube gel apparatus was used for the first dimensional gel electrophoresis. Four or six gels were run in the same gel apparatus at room temperature. A programable power supply (1000/500 Microprocessor Controlled Power Supply, Bio-Rad) was used to provide a constant voltage of 200 volts for two hours, followed by 500 volts for five hours, and then 1000 volts for ten hours. After completion, the gels were extruded from the tubes and soaked in 165 µl transfer solution on a strip of Parafilm. The tube

gels were then immediately run in the second dimension.

The second dimension:

Seven slab gels of size 20mm X 16mm X 1.5mm (the inner glass plates are of size 20cm X 20cm and the outer plates are of size 22.3cm X 20cm) were cast by using PROTEAN II Multi-Gel Casting Chamber (Bio-Rad). Four or six gels were run simultaneously in the PROPEAN II 2-D Multi-Cell gel apparatus (Bio-Rad). A constant current of 10 milli-amperes per gel was applied and run for about 16 hours or until the dye reached the bottom edge of the gels. The gels were then silver stained by the procedure given in section 2.5.2.3.

2.5.4 Improvement of 2DE

2DE has been used in our lab for quite a few years, but the high resolution has not been achieved until recently. There are a number of problems which cause poor resolution and reproducibility. I spent almost a year in seeking for solutions to the problems. Fortunately, the problems have been gradually solved and the resolution of the technique has been increased by 100% to 200% (in terms of protein spots) with great reproducibility.

2.5.4.1 Problems of 2DE

Based on O'Farrell's (1975) method, Coulthart (1986) made some modifications and applied this technique to the study of male reproductive tract proteins of *Drosophila*. There are some problems of this application, though a lot of effort have been put on the technique. The first problem is degradation of pH gradient of IEF gel at the basic end. The pH gradient of about 2 cm of the basic end of the IFE gel is degraded or there is no pH gradient at the basic end of the IEF gel. As a result no spots but a few smears are present at the basic end of the second dimensional gel. The proteins in this area of the gel could not be resolved.

The second problem is that too many horizontal smears show on the second dimensional gel after silver staining. The high density of spots plus many horizontal smears on the upper part of the gel make it impossible to score even a small proportion of the spots unambiguously.

The third problem is poor resolution of high molecular weight proteins. The spots are big with different shapes. Big size of a spot is formed by a number of proteins which have similar mobility and therefore move together. Different shapes of spots are simply due to overlapping of neighbouring spots. Big sizes and shapes of spots result in poor resolution as well as difficulty in comparing different gels.

The fourth problem is loss of low molecular weight proteins. Much fewer spots in the bottom part of the gel are detected than that in the middle or upper part of the gel.

2.5.4.2 Solutions to the problems

Several and repeated experiments were conducted to solve the various problems mentioned above. As numerous steps are involved in this technique and almost any step can affect resolution and reproducibility, the whole process of 2DE is a very delicate and sensitive experimental system. Experimentation on one or two factors could hardly solve all the problems. In addition, it is not easy to find out the factors which may affect resolution more and therefore should be tested first. Experimentation and attentive observation have led to a high resolution of the technique and they are described in the following.

2.5.4.2.1 Sample preparation

Heavy smears and big spots result in poor resolution, which have a number of possible causes.

1) Too much sample loaded on a IEF gel would cause poor separation in IEF gel and therefore produce smears and large spots in second dimensional gel.

2) If sample is too concentrated the large proteins are not well dissolved, and a poor separation on IEF gel would result.

3) Denaturants may not work well in sample and/or gel. This leads to poor dissolution of proteins in sample.

4) Sample may not be centrifuged well and the undissolved pellets in the sample is loaded onto gels.

5) Unbroken tissue causes poor dissolution.

A number of steps in sample preparation have been changed in response to the five possible causes. Among the changes, reduction of sample amount gives the best effects on resolution. The sample amount for a gel has been reduced from 10 pairs male-reproductive-tracts (including testes, accessory glands and vas deferens) to 7 to 8 pairs of testes. The sample amount I use is about one third of what was used in Coulthart's (1986) study, but the number of spots detected on my gel is two to three times as many as in the previous study.

There are also some other changes in sample preparation.

1) Sample solution has been changed from lysis buffer (used in O'Farrell's protocol) to DTT-CHAPS-Urea sample solution (Hochstrasser, et al. 1988). The major change is that the detergent Nonide P-40 has been replaced by CHAPS.

2). The time of centrifugation has been changed from 10 minutes to 30 minutes. After 20 minutes of centrifugation, the supernatant is transferred to new centrifugal tubes and centrifuged for another 10 minutes.

The optimum sample amount was decided by running gels with different sample amounts. If sample is not enough, very few spots would be detected. On the other hand, if sample is too much, there

would be a poor resolution and a degradation of pH gradient at basic end of IEF gel. The effects of poor resolution and pH gradient degradation resulting from sample amount is dependent on running conditions (temperature, voltages and time) in IEF (details will be discussed later in this chapter). So optimum sample amount is decided by two-factor-experiments with careful evaluation.

Before the sample solution is decided to be the best, two other sample solution were tested. Lysis buffer was replaced by CHAPS-2-mercaptoethanol-Urea for the reason that CHAPS is a better detergent than Nonidet P-40. CHAPS-2-mercaptoethanol-Urea was replaced by CHAPS-DTT-Urea sample solution, because the presence of 2-mercaptoethanol causes an artifact (two parallel horizontal bands on upper part of the gel).

2.5.4.2.2 The first dimension -- IEF

The first dimensional gel -- isoelectric focusing is the most important part of 2DE, and the most difficult part as well. Most of the common problems in 2DE arise from the first dimension. For example, poor resolution of 2DE gel, pH gradient degradation and pH gap in pH gradient are all problems arising during IEF.

Degradation of pH gradient is one of the problems we had. As ampholytes are what is used to form pH gradient, experiments on ampholytes were carried out to find the cause of the degradation. Different concentrations of ampholytes, different ratios of different

ranges of ampholytes, different ranges of pH gradient, and different sources of ampholytes were tested.

pH gradient range of 6 to 8:

When pH gradient range of 6 to 8 was used, the degradation of pH gradient disappeared and a good separation of proteins in IEF gel was achieved. About 800 spots are detected on a gel. The only problem is that spots on the acidic side of the gel are very crowded and the basic side of the gel does not have many spots. The resolution would be increased if the spots are evenly distributed on the gel. I believed that the pH gradient range of 5-7 would give a better distribution of spots.

pH gradient range of 5 to 7:

In the same running conditions, pH gradient of 5-7 is not formed. As running conditions affect pH gradient, a large number of different running conditions have been tested. The result shows that some running conditions produce no pH gradient degradation but give poor resolution. The conditions which produce no pH gradient degradation are those which include a lower temperature, a shorter running time and/or a lower voltage.

Change of electrolytes:

After failing to find out optimum running conditions, different electrolytes were tested. The electrolytes, 0.2% H_2SO_4 and 0.5%

ethanolamide (which were used in Coulthart's study), were replaced by 6mM H_3PO_4 and 20mM NaOH. The effects of different electrolytes were compared. The old electrolytes have a much higher conductivity than the new electrolytes. As a result, the resistance of the gel is increased greatly with the new electrolytes. The increased gel resistance leads to low current and allows much higher voltage to be applied on IEF gel. With the new electrolytes, pH gradient degradation does not occur under running conditions which lead to pH gradient degradation when old electrolytes are used. If the acidic halves of gels run with different electrolytes are compared, it is found that new electrolytes give a higher resolution than old electrolytes, although the basic half of the gel remains poor resolution. The new electrolytes have the potential to produce higher resolution.

Optimum running conditions:

After the new electrolytes have been chosen, optimum running conditions for the new electrolytes were searched for. A number of experiments were done. The experiments are no longer the ones which test a number of running conditions as I did with the old electrolytes. Understanding of the process of isoelectric focusing would help in finding the optimum running condition. The idea of the experiments was to understand the process by which proteins are focused to their pI points.

To visualize the movement of proteins during IEF and to

monitor changes (including degradation) of pH gradient, coloured pI markers were used. As samples are different from markers, the movement of proteins and pH gradient may be different in sample gels from what is observed in marker gels, gels loaded with sample are stained after being run in different conditions. Different patterns of the stained gels would tell the difference of pH gradient of the gels. By the help of the two kinds of visualization, I could observe the movement of proteins during IEF. It is described here.

When electricity is applied, proteins loaded on the cathodic end of a gel move fast towards the anodic end (or acidic end) of the gel in the first few hours, after which they slow down. After a few hours of slow movement, acidic proteins (the proteins on the anodic half of the gel) keep moving forward but at a extremely slow speed (difference can be observed in 3-5 hours). When acidic proteins slow down, basic proteins change direction and move back. In the first couple of hours, they move back fast. Then they slow down.

The acidic proteins form sharp bands very soon. The basic proteins take a long time to form bands. The bands are formed gradually from the middle of the gel to the basic end of the gel. If running voltage and time is not enough, basic proteins will not be focused or basic bands will not be formed. After the basic proteins were focussed, the pH gradient started to degrade. Optimum running conditions were found by noting the point when most basic proteins are focussed and before the pH gradient starts to degrade.

It is very important to mention that running time and voltage

required to focus most basic proteins is dependent on sample amount. The more sample is loaded, the longer time and/or higher voltage is required. If sample is too much, pH gradient degrades before most basic proteins are focussed.

In addition to the experiments which lead to the optimum running condition, some other experiments were also done. High voltage (3000v) was tried to focus more sample. Different ratios of ampholytes were tried. Different crosslinkers were tested. When 3000 voltages were applied, gels were extruded after a few hours of running. When only ampholyte with pH 5-7 is used, the resolution is not better than when the mixture of ampholyte pH 5-7 and pH 3-10 is used. Gels containing 1% pH 5-7 ampholyte and 1% pH 3-10 ampholyte give as good resolution as regular ratio (1.6% pH 5-7 ampholyte, 0.4% pH 3-10 ampholyte). A new crosslinker PDA is tested. PDA makes gels stronger and more flexible than Bis does. PDA does not show better resolution than Bis. In addition, flexibility of IEF gels affects reproducibility of the second dimensional gels. So Bis was not replaced by PDA which is claimed to be a better crosslinker (Hochstrasser, 1988).

2.5.4.2.3 The second dimension -- SDS-PAGE

Uneven distribution of spots on the second dimensional gel was one of the problems associated with the older experimental system. The upper part of the gel is too crowded, whereas the lower part of

the gel is scattered with spots. Running the second dimensional gels for a much longer time has improved spot distribution. When gels were run for 18 to 20 hours, high molecular weight proteins were separated better than when a running time of 5-6 hours is used. A large proportion of high molecular weight proteins which were in upper half of the gel have been moved to the lower half of the gel. None of spots at the bottom of the gel has moved out of the gel.

There is another change which may reduce protein loss during the transfer of IEF gel to the second dimensional gel. In the old system, equilibrium of IEF gel is included. IEF gels are put in equilibrium solution and shaken for 20 minutes before they are transferred to the second dimensional gels. A proportion of proteins especially small proteins may be lost during equilibration. The new experimental protocol has skipped this step, and a transfer solution is used. IEF gels are soaked in 165 μL of transfer solution for a few minutes before they are put on the second dimensional gels. This may be partially why more spots on the bottom part of the gel have been detected with the modified protocol.

2.5.4.2.4 Silver staining

Protein loss during silver staining can also contribute to the scattered spots of the lower half of the gel in the old system. It is possible that loss of small proteins occurs after hours of washing in silver stain, especially when fixation is not complete. I found that the

fixation in the old protocol was not enough. When a gel is placed in a glass tray containing fixation solution, the gel is usually stuck on the bottom of the tray. This prevents a good contact of fixation solution with the gel and, therefore, prevents a good fixation. Poor fixation is evident, when uneven stain of a gel happens. The middle of a gel always shows different colour from the surroundings, when the old protocol is used. In order to make a good fixation, gels are fixed for 2 or 3 days and a piece of nitrocellulose paper is placed between the bottom of the tray and the gel. The better fixation of proteins which may lead to reduced loss of low molecular weight proteins may also contribute to the increased number of spots in the lower part of the gel.

2.5.5 Discussion on two-dimensional gel electrophoresis

2.5.5.1 Two points about SDS-PAGE

There were two other questions about gel electrophoresis that needed to be answered. The first one is why the resistance of a gel keeps increasing during electrophoresis. The second one is why proteins move much faster at the beginning of electrophoresis and then slow down.

When a constant current is applied, the voltage keeps increasing. By the end of electrophoresis, the voltage can reach four times as high as the initial voltage. That means the resistance of the

gel has increased 3 times, as current (I) equals voltage (V) divided by resistance (R) (I = V/R). The answer to this question was found when a gel was run for so long a time that proteins were moving out of the gel. What happened was that as soon as proteins moved out of the gel the voltage started to decrease. The existence of proteins and their even distribution in the gel matrix may have led to the high resistance of the gel. During electrophoresis, the pores of the gel are gradually filled with proteins. So the resistance of the gel increases.

If a constant voltage is applied to a gel, the proteins move very fast at the beginning and then gradually slow down. The reduction of velocity seems to be proportional to the increase in gel resistance. The diminishing velocity of proteins is not explained by the increase of resistance (not resistance in the sense of electronics) in the gel by proteins, because the fast moving proteins do not have any protein molecules in front of them to resist or block them. If there is no increase in viscous drag of the proteins, there should be a decrease in the pulling force. According to the formula, F = (E/D)*(q), where F is the force, E is the potential difference between electrodes (electric field), D is the distance between electrodes, E/D is the field strength, and q is the net charge of molecule, the pulling force (F) should not change with a constant voltage (which is E). Because the distance between electrodes is constant and the net charge of a protein (combined with SDS) does not change very much in buffer. It is very important to realize that the detailed theory of gel electrophoresis is highly complicated and the formula above is a simplified one.

The potential difference E in the formula above is very close to the voltage, when the resistance of the gel is very small. When the gel resistance increases, E decreases; because any amount of resistance would lead to a drop in E, which equals the product of current and the resistance. The understanding is that the increased resistance has led to the decreased potential and therefore to the decrease in the pulling force. That, I think, is the explanation why the velocity of proteins diminishes during electrophoresis.

2.5.5.2 Determination of pH in isoelectric focusing gels

There are three methods which have been used to measure pH gradient in IEF gels. The most popular methods is to cut a gel into segments and diffuse ampholytes and buffers into water and the pH of the solution is then measured. The second method is to use colour pI markers. Different colour bands on an IEF gel indicate different pH values. The third method is the use of electrodes designed to measure pH directly in the gel matrices. All three methods were used in the present study. The first and the second method give a proximate estimate of pH gradient. The third method seems to be a better method.

The measurements gave a rough pH range of the IEF gel from pH 4.5 to pH 7.5. The measurements are not very much precise, because pH is affected by a number of factors. At first, temperature during focusing may be different from the temperature at which the

pH is determined. Secondly, CO_2 in the air may alter the pH of the gels after being extruded. In addition, these methods generally do not account for protein effects or molecular interactions in gels (see discussion in chapter 1 by Dunbar 1987).

2.5.5.3 pH gradient range of IEF gel and proteins out of the range

If the pH gradient of the IEF gel is from pH 4.5 to pH 7.5, there should be some proteins which do not fall into this range and thus they are not picked up by the technique. It is of interest to know how many proteins are out of the range and how many proteins failed to enter the IEF gel. Our experiments give an estimation of about 100 proteins which may not be included by IEF gel. In the experiment, four pH gradient ranges of gels are formed by mixing ampholyte pH 5-7 and ampholyte pH 3-10 in different ratios. The narrowest ranges is pH 5-7 and the broadest range is pH 3-10. The other two range are between them, i.e., one is a slightly broader than pH 5-7 and one is narrower than pH 3-10. When pI markers (pI from 4.6 to 9.6) are loaded on the four gels, the pH gradient range order is shown as expected, i.e., the marker bands cover most of the IEF gel with pH range from 5-7, and the marker bands cover only the middle third of the IEF gel with pH range from 3-10. The interesting thing is what happens when sample (adult flies) is loaded on to the four gels. There is no significant difference in the band pattern of the acidic part (one third) of the IEF gel among the four

gels (which have different pH gradient ranges). As the pattern of bands on IEF gel reflects pH gradient of the gel, similar patterns of the four gels on acidic ends show similar pH gradient of the four gels on acidic end of the gel. The implication here is that pH gradient is altered by the proteins in the sample. Hundreds of species of proteins in the sample are similar to hundreds of small molecules in ampholytes and therefore they may behave similarly as ampholytes do. If the pH gradient of acidic end of the gel is determined by the proteins in the sample, there should not be any protein loss at the acidic end.

There is an observation which confirms the idea that no acidic protein has run out of the IEF gel. When heads of fruit flies are used as sample in IEF gel, two red bands (probably pigment proteins from the eyes of the flies) are present as natural markers. One is a very basic molecule and the other is a very acidic molecule. The acidic band moves to the very front and reach the very end of the gel. When pH range 5-7 gels are compared with pH range 3-10 gels, the red acidic bands were formed on both gels.

The basic side of the IEF gel has a different story. When a sample prepared from adult flies is loaded, basic proteins are not focussed in pH 3-10 gels, so the band pattern can not be compared with that of pH 5-7 gels. When sample prepared from adult fly heads is loaded, the basic marker band mentioned above moves into the gel for about 2 cm and then moves back into the sample solution in which the band remains sharp. In both pH 5-7 and pH 3-10 gels, the

basic marker band is left out in the sample solution. This shows that there are some basic proteins which are not picked up by IEF gel.

It is clear that pH gradient of IEF gel starts right from the surface between the gel and anolyte and goes to the surface between the sample solution and catholyte. As about 1 cm of the pH gradient is sample solution, the proteins which are focussed in this 1 cm are lost.

In order to estimate the number of proteins which are lost in the sample solution region, gels with testis sample and pH range 5-7 is compared with gels with pH 6-8. Roughly, about 50 spots in pH 6-8 gels are not detected in pH 5-7 gels. As there are also some basic protein loss in pH 6-8 gels, the actual number would be higher than 50. Assuming another 50 basic proteins are lost on pH 6-8 gels, the estimated number of lost proteins in pH 5-7 gel is about 100, or about 8-10% of the total observed.

The last line of evidence that supports the loss of basic proteins in 2DE is from stained second dimensional gel. The acidic edges of second dimensional gels do not have vertical smear, where as the basic edges have heavy vertical smears.

The suggestion to minimise basic protein loss is to minimise sample volume.

CHAPTER 3

THE GENETIC BASIS OF HALDANE'S RULE AND THE NATURE OF ASYMMETRIC HYBRID MALE STERILITY AMONG *DROSOPHILA SIMULANS*, *DROSOPHILA MAURITIANA* AND *DROSOPHILA SECHELLIA*

This chapter is a published paper (Zeng, L.-W. and R.S. Singh, 1993 Genetics **134**:251-260). In this paper, genes responsible for hybrid male sterility have been searched for on the Y chromosome and the cytoplasmic factors. The X-Y interaction hypothesis of Haldane's rule has been tested. An asymmetric X-autosome interaction has been shown to be involved.

The Genetic Basis of Haldane's Rule and the Nature of Asymmetric Hybrid Male Sterility Among *Drosophila simulans*, *Drosophila mauritiana* and *Drosophila sechellia*

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ABSTRACT

Haldane's rule (*i.e.*, the preferential hybrid sterility and inviability of heterogametic sex) has been known for 70 years, but its genetic basis, which is crucial to the understanding of the process of species formation, remains unclear. In the present study, we have investigated the genetic basis of hybrid male sterility using *Drosophila simulans*, *Drosophila mauritiana* and *Drosophila sechellia*. An introgression of *D. sechellia Y* chromosome into a fairly homogenous background of *D. simulans* did not show any effect of the introgressed *Y* on male sterility. The substitution of *D. simulans Y* chromosome into *D. sechellia*, and both reciprocal *Y* chromosome substitutions between *D. simulans* and *D. mauritiana* were unsuccessful. Introgressions of cytoplasm between *D. simulans* and *D. mauritiana* (or *D. sechellia*) also did not have any effect on hybrid male sterility. These results rule out the *X-Y* interaction hypothesis as a general explanation of Haldane's rule in this species group and indicate an involvement of an *X*-autosome interaction. Models of symmetrical and asymmetrical *X*-autosome interaction have been developed which explain the *Y* chromosome substitution results and suggest that evolution of interactions between different genetic elements in the early stages of speciation is more likely to be of an asymmetrical nature. The model of asymmetrical *X*-autosome interaction also predicts that different sets of interacting genes may be involved in different pairs of related species and can account for the observation that hybrid male sterility in many partially isolated species is often nonreciprocal or unidirectional.

THE genetic basis of species formation is one of the most challenging problems in evolutionary biology. While the genetic basis of species formation is usually meant to include both the genetic changes that lead to reproductive isolation and changes that lead to species-specific adaptation, the genetic analysis of reproductive isolation between related species has currently become the favored experimental approach to pursue this problem. Seventy years ago, HALDANE (1922) drew attention to the peculiar nature of species hybrid sterility and inviability and pointed out that "when in the F_1 offspring of two different animal races one sex is absent, rare, or sterile, that sex is the heterozygous [heterogametic] sex." This observation has come to be known as Haldane's rule. It is quite general (COYNE and ORR 1989, COYNE, CHARLESWORTH and ORR 1991), although it appears to apply more strongly to hybrid sterility than to viability (WU *et al.* 1992). However, in spite of the generality of Haldane's rule, its genetic basis is still unclear. DOBZHANSKY (1936) pioneered the investigation of the genetic basis of hybrid male sterility in the sibling species of *Drosophila pseudoobscura*. In his studies, a handful morphological markers were used to identify the species origin of chromosomal segments in backcross progenies, and to correlate them with male fertility as measured by testis size. DOBZHANSKY (1937a) concluded that every major chromosome except the *Y* was involved in affecting hybrid male sterility.

Until recently, there has been very little experimental work reported regarding this problem. COYNE (1984), COYNE and KREITMAN (1986), VIGNEAULT and ZOUROS (1986), ORR (1987, 1989), and KHADEMAND and KRIMBAS (1991) following DOBZHANSKY's backcross analysis approach, have studied a number of different *Drosophila* species pairs. The outcome of these and other similar studies have invariably shown that the *X* chromosome plays the largest role in determining hybrid male sterility (COYNE and ORR 1989). The *X* chromosome, of course, does not cause hybrid male sterility by itself; it exerts its effects by interacting with genes from the other species. The genes which interact with heterospecific *X* chromosome can be on autosomes, *Y* chromosome or cytoplasm (in cases where females are heterogametic). Among the three possible two-way interactions involving *X* chromosome, *X*-autosome and *X-Y* interactions are the two most often discussed hypotheses proposed for the genetic basis of Haldane's rule (COYNE and ORR 1989; ZOUROS 1989).

One of the versions of the *X*-autosome interaction

hypothesis is DOBZHANSKY'S (1937a) *X*-autosome imbalance explanation. This explanation is based on the assumption that "many species have a balance of genes in the *X* chromosome and the autosomes peculiar to themselves and different from other species" (DOBZHANSKY 1937b). As female hybrids have a complete homospecific set of autosomes and an *X* chromosome from each parental species, the *X*-autosome balance remains undisturbed. In male hybrids, however, the *X* chromosome corresponding to the paternal set of autosomes is missing, so the genic balance between autosomes and the *X* chromosome is perturbed. Another version of the *X*-autosome interaction theory was suggested by HALDANE (1922) and MULLER (1940, 1942). This explanation is based on complementary interaction of genes on autosomes and major sex chromosome ("*X*" or "*Z*"). Either absence of some sex-linked genes which are necessary complements for those on the autosomes, or expression of some sex-linked deleterious genes (deleterious when in combination with some complementary genes on the alien autosomes) in hybrids of heterogametic sex can lead to sterility or inviability.

The *X-Y* interaction hypothesis, first proposed by HALDANE (1932), explains the preferential heterogametic hybrid sterility and inviability by the genic interactions between *X* and *Y* chromosomes (or "Z" and "W" chromosomes where females are heterogametic) from different species.

COYNE (1985) examined the *X*-autosome imbalance and the *X-Y* interaction hypothesis by using three closely related species *Drosophila simulans*, *Drosophila mauritiana* and *Drosophila sechellia* of the *melanogaster* subgroup (hybridization among the three species produce fertile females and sterile males). His experiments indicated that *X*-autosome imbalance is not involved in this group and that *X-Y* interaction is the most likely explanation for hybrid male sterility in species pair *D. simulans/D. mauritiana*.

To understand the details of interactions among genes on different chromosomes affecting hybrid male sterility, we have carried out a detailed genetic analysis to dissect and map individual loci affecting hybrid male sterility. The search for hybrid male sterility genes was done on autosomes, the *X* chromosome, the *Y* chromosome, and the cytoplasm separately. Here we report results regarding the roles of the *Y* chromosome and the cytoplasm in hybrid male sterility. First, to our surprise, in species pair *D. simulans/D. sechellia* an introgression of the *D. sechellia* *Y* chromosome into the background of *D. simulans* revealed no effect on male sterility. This demonstrates that the *X-Y* interaction is not involved in the sterility of *D. simulans* (♀) × *D. sechellia* (♂) hybrid males. Second, in both species pairs *D. simulans/D. mauritiana* and *D. simulans/D. sechellia*, interspecific cytoplasm introgression has shown that the cytoplasm is also not involved in affecting hybrid male sterility. The exclusion of the *Y* chromosome and the cytoplasm and the consideration of the large effects of the *X* chromosome (COYNE 1984; COYNE and KREITMAN 1986; COYNE and CHARLESWORTH 1986, 1989) make the *X*-autosome interaction the most plausible hypothesis for male sterility in *D. simulans* (♀) × *D. sechellia* (♂) hybrids. Models of *X*-autosome interactions are presented (1) to show when *Y* chromosome substitution between species by the backcross method is or is not possible and (2) to explain the generality of asymmetric (*unidirectional*) reproductive isolation during the early stages of speciation.

MATERIALS AND METHODS

Species stocks: *D. simulans*, *D. mauritiana* and *D. sechellia* stocks were obtained from JEAN DAVID. Another line of *D. sechellia* was obtained from JERRY COYNE. A *D. simulans* line with attached-*X* chromosome was obtained from the National *Drosophila* Species Resource Center (Bowling Green). All crosses and stocks were reared in 8-dram vials containing banana medium. The temperature for the stocks and most crosses were 25°, some crosses were made at 23° as specified.

Species hybridization: Reciprocal crosses between *D. simulans* (denoted as s_i) and *D. mauritiana* (denoted as *m*) were made. The hybrids from crossing s_i (*D. simulans*) females to *m* (*D. mauritiana*) males are denoted as s_im, the reciprocal hybrids as ms_i. In the species pair *D. simulans/D. sechellia*, *D. simulans* females were crossed to *D. sechellia* (denoted as s_e) males, and the hybrids from this cross are denoted as s_is_e. The reciprocal cross is very difficult: only one out of seven *D. simulans* lines used produced a few hybrids (denoted as s_es_i).

***Y* chromosome introgression:** The crossing scheme for introgressing the *D. sechellia Y* chromosome into *D. simulans* background is illustrated in Figure 1. The s_is_e hybrid females were backcrossed to s_e (*D. sechellia*) males, the resultant backcross offspring are denoted as $s_is_es_e$. The $s_is_es_e$ males, which carried the *D. sechellia Y* chromosome, were crossed to s_i (*D. simulans*) females and the offspring produced were denoted as s_e-$s_is_es_es_e$. The s_e-$s_is_es_es_e$ males were backcrossed to s_i females and the progenies produced were denoted as s_i^2-$s_is_es_e$. The s_i^2-$s_is_es_e$ males were backcrossed to s_i females again and this was repeated for three more times. Thus s_i^5-$s_is_es_e$ represents the fifth backcross generation progeny. Similarly, for introgressing *D. simulans Y* chromosome into *D. sechellia*, F_1 hybrid females (s_is_e) were crossed to s_i males and the progenies produced were denoted as $s_is_es_i$. $s_is_es_i$ males, which carried a *D. simulans Y* chromosome, were crossed to s_i females, and the male progenies produced (if fertile) would be backcrossed to s_i females again for a number of generations.

The *D. sechellia Y* chromosome was also introgressed into the background of *D. simulans* by an independent method using *D. simulans* attached-*X* chromosomes as described by JOHNSON and WU (1992).

Classification of hybrid males and measure of male fertility: Hybrid males were classified into four types (1, 2, 3 and 4; see Figure 2) based on their testis development (shape and size) and sperm production. Since mature sperm are stored in seminal vesicles (LINDSLEY and TOKUYASU 1980), the size of a seminal vesicle is a good indicator of the

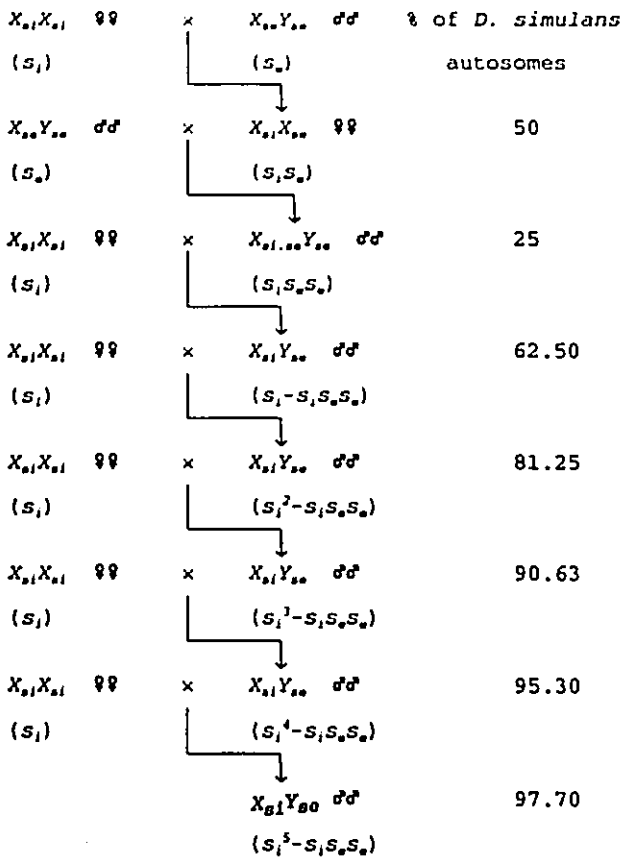

FIGURE 1.—Crossing scheme for *Y* chromosome substitution. Only sex chromosomes are shown in the genotypes of each generation. X_s, X_s and X_{ns} are *D. simulans X* chromosome, *D. sechellia X* chromosome and recombinant *X* chromosome, respectively. Y_s is the *Y* chromosome of *D. sechellia*. The percentages of *D. simulans* autosomes are calculated based on free recombination and random association of chromosomes.

amount of sperm it contains. Dissection showed that "collapsed" seminal vesicles contained no or few sperm; and inflated seminal vesicles contained large amounts of sperm. Regardless of the amount of sperm produced, the sperm were all found to be motile when examined under a compound microscope. The criteria for classification are given in Table 1. Types 1 and 2 males produced no or few sperm and were classified as sterile. Types 3 and 4 males were classified as fertile, as they had normal testes with large amount of sperm (see Table 1).

Male fertility was measured by either evaluating testis development (simply called *testis size method*) or by *mating tests*. By testis size method, we used the percentage of types 3 and 4 males to indirectly measure male fertility. This method is very similar to that used by ZOUROS (1981, 1986), COYNE (1984), and ORR (1987) except that our criterion is based on a combination of both the production of motile sperm and the testis size. In the mating test, individual mature males were mated with normal fertile virgin females for two days, and a male was scored fertile if mated females produced larvae.

In the following, the testis size method was used in all cases except as specified. This method has some advantages over the mating test method. First, examination of large samples is possible by testis size method. Second, males with a few sperm (*e.g.*, 1% of normal) can sometimes inseminate

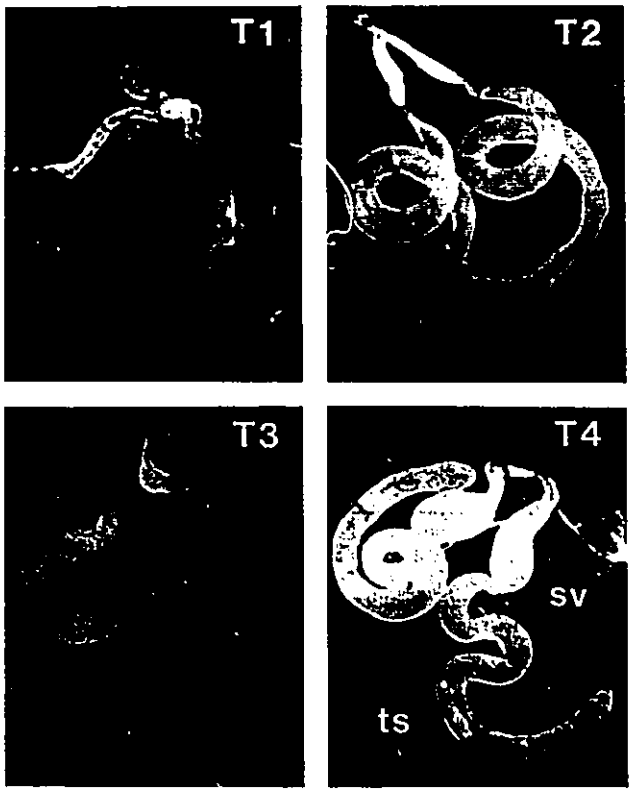

FIGURE 2.—The four types of testes (T1–T4). sv, seminal vesicle; ts, testis; T1, type 1 testes, atrophied testes with small seminal vesicles (no or few sperm); T2, type 2 testes, normal size testes with small seminal vesicles (no or few sperm); T3, type 3 testes, normal size testes with medium size seminal vesicles inflated by lots of sperm (but fewer than normal); T4, type 4 testes, normal size testes with normal amount of sperm.

TABLE 1

Criteria for classification of hybrid male types

Male type	Testis	Seminal vesicle	Sperm
1	Atrophied, smaller than normal size	Very small	None or few
2	Normal	Very small	None or few
3	Normal	Inflated, smaller than normal size	Large quantity (<normal)
4	Normal	Normal size	Large quantity (normal amount)

Hybrid males with two different types of testes were seen in a small proportion (probably due to developmental abnormality). In the present study, if one of a male's testes was normal, it was classified as normal. Flies with mildly atrophied testes but large amounts of sperm were rare and therefore were not included in the classification.

a female (Table 2, a small proportion of type 2 males are fertile) and by the mating test method these semifertile males can not be distinguished from fully fertile males (*i.e.*, males with normal amount of sperm). Third, there are different causes of male sterility; both atrophied testes (*i.e.*, type 1 males) and well developed testes without sperm (*i.e.*, type 2 males) can result in male sterility and these two components of male fertility are controlled by different

TABLE 2

Comparison of fertility/sterility (F/S) by the testis size and direct mating test methods in different backcross males

Male types	$s_i ms_e$		$s_i s_e s_i$		$s_i s_e s_i^{12.8.12}$		$s_i s_e s_i^{12.8.13}$	
	F	S	F	S	F	S	F	S
1	2	37	0	49	0	0	0	1
2	0	87	2	30	6	107	0	158
3	7	0	5	3	4	0	4	2
4	7	1	8	1	94	14	112	6
Total	16	125	15	83	104	121	116	167
3 + 4 (%)	87.5	0.8	86.7	4.8	94.2	11.6	100	4.8
Fertility estimates (%):								
By testis size	10.1		17.3		49.8		43.8	
By mating test	11.3		15.3		46.2		41.0	
G value	0.018		0.173		1.602		0.929	
Probability	>0.8		>0.7		>0.2		>0.3	

Backcross progenies $s_i ms_e$ and $s_i s_e s_i$ were obtained by crossing $s_i m$ and $s_i s_e$ F_1 hybrid females to s_i (*D. simulans*) males respectively; $s_i s_e s_i^{12.8.12}$ and $s_i s_e s_i^{12.8.13}$ are backcross progenies produced by crossing normal *D. simulans* males to females carrying a piece of introgressed *D. sechellia X* chromosome in a *D. simulans* background (L.-W. ZENG and R. S. SINGH, unpublished results). All males tested by mating test were allowed to restore their sperm for 2 days (without females) before being dissected for male (testes) classification.

genes (WU *et al.* 1992; L.-W. ZENG and R. S. SINGH, unpublished results). The testis size method allows us to distinguish and identify the different causes of male sterility. Finally, mating test can be confounded with mating ability of the males being tested.

RESULTS

Male fertility: The two methods of measuring male fertility were compared by scoring hybrid male fertility/sterility in four types of backcross progenies ($s_i ms_i$, $s_i s_e s_i$, $s_i s_e s_i^{12.8.12}$ and $s_i s_e s_i^{12.8.13}$; see Table 2 for explanation) which had a segregation of both fertile and sterile males. The results in Table 2 show that most (from 86.7% to 100%) of the males scored as fertile by the mating tests in all the four backcross generations were types 3 and 4, and most of the males scored as sterile by mating tests were types 1 and 2 with small proportions of types 3 and 4 (from 0.8% to 11.6%). The fertility scores obtained by the two methods were not significantly different (*G* test, Table 2).

The fertility values of parental, hybrid, and various backcross males are shown in Table 3. In pure species (s_i, m, and s_e), over 94% of males were fertile. F_1 hybrid males ($s_i m$, ms_i, and $s_i s_e$) were completely sterile. When F_1 hybrid females were crossed to parental males, the backcross progenies showed different proportions of fertile males.

***Y* chromosome introgression between *D. sechellia* and *D. simulans*:** The *D. sechellia Y* chromosome was successfully introgressed into the background of *D. simulans* by the method shown in Figure 1. However, the introgression of the *D. simulans Y* chromosome into *D. sechellia*, and the attempted introgression of *Y* chromosome between *D. simulans* and *D. mauritiana*, in both directions, were not successful. The results are shown in Table 4.

TABLE 3

Male fertility of *D. simulans* (s_i), *D. mauritiana* (m), *D. sechellia* (s_e), their F_1 hybrids and backcross progenies

Genotypes	Male (testis) types 1	2	3	4	Total	Fertility (%) (type 3 + 4)
s_i	2	34	0	669	705	94.5
m	1	4	0	184	189	97.4
s_e	1	4	0	161	166	97.0
$s_i m$	0	72	0	0	72	0.0
ms_i	1	52	0	0	53	0.0
$s_i s_e$	33	303	0	0	336	0.0
$s_i ms_i$	113	152	14	3	282	6.0
$ms_i s_i$	95	162	0	11	268	4.1
$s_i mm$	234	361	7	7	609	2.3
$ms_i m$	320	322	4	21	669	3.7
$s_i s_e s_i$	165	215	59	58	497	23.6
$s_i s_e s_e$	141	255	13	12	421	5.9

TABLE 4

Fertility of backcross males with heterospecific *X* and *Y* chromosomes

Genotypes	Male types 1	2	3	4	Total	Fertility (%) (type 3 + 4)
s_e-$s_i mm$	0	121	0	0	121	0
s_e-$ms_i m$	9	249	0	0	258	0
m-$ms_i s_i$	1	28	0	0	29	0
m-$s_i ms_i$	—[a]	—	—	—	—	—
s_e-$s_i s_e s_i$	13	23	0	0	36	0
s_i-$s_i s_e s_e$ (23)	3	20	1	6	30	23.3
s_i-$s_i s_e s_e$ (25)	—	—	—	—	33	36.4[b]
s_i^2-$s_i s_e s_e$ (23)	0	7	0	23	30	76.7
s_i^3-$s_i s_e s_e$ (23)	1	12	0	105	118	89.0
s_i^4-$s_i s_e s_e$ (23)	5	6	0	117	128	91.4
s_i^5-$s_i s_e s_e$ (23)	1	1	0	42	44	95.4

[a] No offspring were obtained from cross m ♀♀ × $s_i ms_i$ ♂♂.
[b] Fertility (36.4%) in this case was measured by mating individual males with *D. simulans* females.

The introgression of the *D. sechellia Y* chromosome into *D. simulans* was carried out by repeatedly backcrossing $s_i s_e s_e$ (see Table 3) males to *D. simulans* females for five generations. In the first generation, *sisese* males which had a *Y* chromosome from *D. sechellia* were crossed to *D. simulans* females, and 23.3% to 36.4% (depending on the temperature) of the male offspring (s_i-$s_i s_e s_e$ in Table 4) produced were fertile. The fertile s_i-$s_i s_e s_e$ males (obtained at 23°) were then backcrossed to *D. simulans* females again and this cross was repeated for another four times. All males in the five backcross generations had the complete *D. simulans X* chromosome and an intact *D. sechellia Y* chromosome. The autosomes of the consecutive generations were gradually replaced by that of *D. simulans* and the male fertility increased accordingly. By the

fifth generation, the male fertility had reached normal level (95.4%, s_i^3-$s_iS_eS_e$ in Table 4). The progenies produced by the fifth backcross generation and by the sixth generation in a repeated experiment (data not shown) have been kept by brother-sister mating for 34 generations without any loss of fertility.

Reciprocal introgression by the above method was difficult, because *D. sechellia* females were not receptive to $s_iS_eS_i$ males which had most of their genome from *D. simulans* (3/4 autosomes and 1/2 *X* chromosome). This difficulty of mating is of the same type which usually occurs in crosses involving *D. sechellia* females and *D. simulans* males. However, when a different line of *D. sechellia* (from JERRY COYNE) was used in a repeated experiment, one of three separate crosses between *D. sechellia* females and $s_iS_eS_i$ males produced some offspring (s_e-$s_iS_eS_i$ in Table 4). In the s_e-$s_iS_eS_i$ males, no types 3 or 4 males were found (Table 4). As the s_e-$s_iS_eS_i$ males were sterile, no offspring were produced when s_e-$s_iS_eS_i$ males were further backcrossed to s_e females, and therefore the attempt to introgress the *D. simulans Y* chromosome into *D. sechellia* was stopped.

Y chromosome introgression between *D. mauritiana* and *D. simulans*: To introgress the *Y* chromosome of *D. mauritiana* into *D. simulans*, *D. simulans* females were crossed to the small proportion of fertile s_imm and ms_im (see Table 3) males which carried the *D. mauritiana Y* chromosome. All the male offspring (s_i-s_imm and s_i-ms_im in Table 4) produced from these crosses were sterile. The reciprocal direction of the introgression involved crossing *D. mauritiana* females to ms_is_i and s_ims_i males (see Table 3). The result for crossing *m* females to ms_is_i males was the same–no *m*-ms_is_i fertile males were obtained. Similarly, when *D. mauritiana* females were crossed to s_ims_i males, no offspring were obtained. The complete sterility of s_i-s_imm, s_i-ms_im, and *m*-ms_is_i males (Table 4) thus prevented the transfer of the *Y* chromosome between these two species.

Y chromosome substitution using attached-X chromosomes: The *D. sechellia Y* chromosome was also introgressed into *D. simulans* by using a *D. simulans* stock with attached-*X* chromosomes. F_1 hybrid females from crossing attached-*X D. simulans* females to *D. sechellia* males were crossed to *D. simulans* (standard species stock) males. The male offspring produced from this cross ($s_iS_eS_i$ males), unlike the $s_iS_eS_i$ males in the other method, inherited their *X* chromosome from their fathers (*D. simulans*) and their *Y* from their attached-*X* mothers (*i.e.*, from F_1 hybrid females with attached-*X* chromosomes) which carried a *D. sechellia Y* chromosome. These $s_iS_eS_i$ males with the *D. simulans X* chromosome and the *D. sechellia Y* chromosome showed high fertility when crossed to *D. simulans* females by pair-matings. Twenty one out of 41 pairs produced larvae, and 19 of the 21 pairs produced adult flies which were highly fertile (92.3% on average, $n = 621$). These $s_iS_eS_i$ males were also crossed to *D. simulans* females by mass mating and the male offspring produced were also highly fertile (94.6%, $n = 335$). All the backcross male flies produced by the cross s_i ♀♀ × $s_iS_eS_i$ ♂♂ carried an intact *D. simulans X* chromosome and a *D. sechellia Y* chromosome. The high fertility of these males with heterospecific sex chromosomes shows again that the *D. simulans X* and the *D. sechellia Y* chromosomes are compatible.

The introgression of the *D. simulans Y* chromosome into *D. sechellia* was attempted by crossing $s_iS_eS_i$ (mentioned above) females to *D. sechellia* males and by crossing the male offspring produced from the above cross to *D. sechellia* females repeatedly. The second generation backcross males (produced by cross $s_iS_eS_i$ ♀♀ × s_e ♂♂, and denoted as $s_iS_eS_iS_e$) had an *X* chromosome from *D. sechellia* and a *D. simulans Y* chromosome inherited from the $s_iS_eS_i$ females. If any of the these males ($s_iS_eS_iS_e$ males) were fertile, repeated crosses of these males to *D. sechellia* females would result in the genetic background of the male offspring being gradually replaced by that of *D. sechellia* except the *Y* chromosome which would be from *D. simulans*. However, when the $s_iS_eS_iS_e$ males were mated to *D. sechellia* females, three replicate crosses each involving 20 males and 20 females, no offspring were produced. Dissection of these males also showed 0% ($n = 89$) fertility by the testis size method. The complete sterility of the second generation backcross males made it impossible to introgress the *Y* chromosome of *D. simulans* into *D. sechellia* by using *D. simulans* stock with attached-*X*. The failure of introgressing the *D. simulans Y* chromosome into *D. sechellia* background here is similar to that which occurred by the method using standard stocks. The crosses involved are similar, and the sterile $s_iS_eS_iS_e$ males, which prevented further introgression, had the same chromosome constitution as s_e-$s_iS_eS_i$ males (*D. sechellia X* chromosome, *D. simulans Y* chromosome, 3/8 *D. simulans* autosomes and 5/8 *D. sechellia* autosomes). Nevertheless, it would be interesting to know whether or not this introgression is successful by using an attached-*X D. sechellia* stock if that were available.

Role of cytoplasm in hybrid male sterility: Cytoplasm has long been suspected to have some effect in the asymmetrical hybrid sterility (DOBZHANSKY and STURTEVENT 1935; EHRMAN 1963; DOBZHANSKY 1974) and has been shown to be involved in sterility of *D. pseudoobscura/Drosophila persimilis* hybrid females (ORR 1987) and of *D. pseudoobscura pseudoobscura/D. pseudoobscura bogotana* hybrid males (ORR 1989). The role of the cytoplasm in hybrid male sterility in this group was systematically examined by the recurrent backcross of hybrid females to their

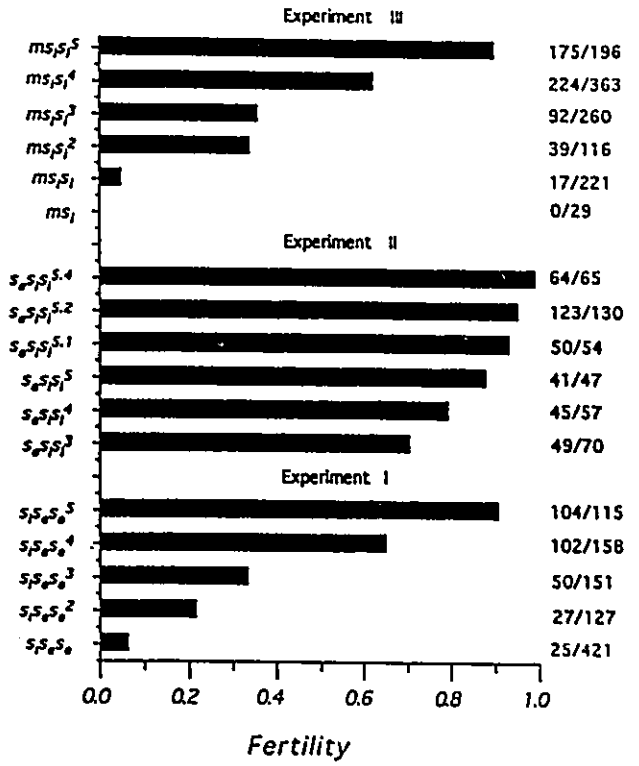

FIGURE 3.—Fertility of backcross males having heterospecific cytoplasm and nuclear genome. $s_iS_es_e^n$ denotes for progenies produced from crossing $s_iS_es_e^{n-1}$ ♀♀ × s_e ♂♂; $ms_is_i^n$ denotes for progenies produced from crossing $ms_is_i^{n-1}$ ♀♀ × s_i ♂♂; and .n indicates progenies produced by nth generation of sib-mating. The fraction at the end of each bar shows number of types 3 and 4 males over number of males scored. Total $n = 2580$.

paternal male parents. During the recurrent backcrosses, the nuclear genome of the backcross progenies is gradually substituted by that of the recurrent parental species, while the cytoplasm remains that from the other species. Both reciprocal directions of cytoplasm introgression in species pairs *D. simulans/D. mauritiana* and *D. simulans/D. sechellia* were performed. Figure 3 shows the results of three experiments for three of the four directions of cytoplasm introgression. In the first experiment, the cytoplasm of *D. sechellia* was substituted by that of *D. simulans* by crossing s_iS_e hybrid females to *D. sechellia* males for five generations. As the backcrosses proceeded the hybrid male fertility steadily increased (experiment I in Figure 3). In the fifth backcross generation, the hybrid males ($s_iS_es_e^5$ in Figure 3) had cytoplasm from *D. simulans* and most of their nuclear genome (more than 97%, calculated based on random association and free recombination of chromosomes) from *D. sechellia*, and these hybrid males (with heterospecific cytoplasm and nuclear genome) have fertility as high as 90.4%. In experiment II, the reciprocal cytoplasm substitution was carried out by crossing s_eS_i hybrid females to *D. simulans* males for five generations. Male fertility of the first two backcross generations was not scored. Male fertility of the following three backcross generations increased to 87.2% ($s_eS_is_i^5$ in Figure 3). The introgression of *D. mauritiana* cytoplasm into *D. simulans* background was carried out in experiment III by the same method. The male fertility of the five backcross generations increased from 4.5% to 89.3% (ms_iS_i *vs.* $ms_iS_i^5$ in Figure 3). The results for introgressing *D. simulans* cytoplasm into the background of *D. mauritiana* are the same (data not shown).

The fertility is retained when the backcross progenies are maintained by brother-sister mating for various numbers of generations. Two independently derived lines with *D. sechellia* cytoplasm and a fairly homogeneous nuclear genome of *D. simulans* (derived from $s_eS_iS_i^5$ in two replicated experiments) have been maintained for 32 generations. Only the first, second and fourth sib-mating generations have been checked for male fertility ($s_eS_iS_i^{5.1}$, $s_eS_iS_i^{5.2}$, and $s_eS_iS_i^{5.4}$ in Figure 3), but the fertility remained high.

The recurrent backcross experiments have shown that substitution of cytoplasm of *D. simulans* by that of *D. sechellia* (or *D. mauritiana*) or vice versa does not affect male fertility. These results show conclusively that cytoplasm is not involved in causing male sterility in *D. simulans* and *D. sechellia* (or *D. mauritiana*) hybrids.

DISCUSSION

Evidence in favor of sex chromosome-autosome interaction: The successful substitutions of *D. simulans Y* chromosome by that of *D. sechellia* demonstrate that the *D. sechellia Y* chromosome is compatible with the rest of the *D. simulans* genome (the *X* chromosome, autosomes and cytoplasm). This shows that the *D. sechellia Y* chromosome is not involved in determining male sterility in *D. simulans/D. sechellia* hybrids. These results rule out the *X-Y* interaction as a general explanation for hybrid male sterility in this species pair because flies with *D. simulans X* chromosome and *D. sechellia Y* chromosome were fully fertile.

Apparently, the unsuccessful substitutions of the *D. simulans Y* by that of *D. sechellia* and between *D. mauritiana* and *D. simulans* can be attributed to the involvement of the *D. simulans* and *D. mauritiana Y* chromosomes which have been shown to carry male sterility genes (COYNE 1985; JOHNSON *et al.* 1992). However, the present study is not conclusive on this point (see below).

The cytoplasm substitution experiments indicate that cytoplasm is compatible with a heterospecific nuclear genome (autosomes and sex chromosomes) in both species pairs *D. simulans/D. mauritiana* and *D. simulans/D. sechellia*. Since both reciprocal cytoplasm substitutions in the two species pairs resulted in normal male fertility, we conclude that cytoplasm, *i.e.*, autonomously replicating cytoplasmic factors, is not

involved in causing hybrid male sterility in the two species pairs.

Hybrid males from crossing *D. simulans* females to *D. sechellia* males carry a haploid set of autosomes from each species, *X* chromosome and cytoplasm from *D. simulans*, and the *Y* chromosome from *D. sechellia*. Possible explanations for the sterility of these hybrid males involve interspecific interactions between (1) *D. simulans* and *D. sechellia* autosomes, (2) *D. simulans X* chromosome and *D. sechellia* autosomes, (3) *D. simulans* cytoplasm and *D. sechellia* autosomes, (4) *D. simulans* autosomes and *D. sechellia Y* chromosome, (5) *D. simulans X* chromosome and *D. sechellia Y* chromosome, (6) *D. simulans* cytoplasm and *D. sechellia Y* chromosome. As neither the *D. simulans* cytoplasm nor the *D. sechellia Y* chromosome is involved, all the above explanations except (1) and (2) can be rejected. In view of the well documented results in this species pair (COYNE and KREITMAN 1986; COYNE and CHARLESWORTH 1989) that sterility factors are located on both autosomes and the *X* chromosome, with those on the *X* chromosome having the largest effects, *X*-autosome interaction must necessarily be involved, although we can not exclude the possibility that the autosome-autosome interaction may also be involved.

X-autosome interaction causing male sterility has been reported in species pairs *D. pseudoobscura/D. persimilis* (DOBZHANSKY 1936,b), *D. pseudoobscura pseudoobscura/D. p. bogatana* (PRAKASH 1972) and *D. hydei/D. neohydei* (SCHÄFER 1978). In the species pair *D. hydei/D. neohydei*, interactions between different autosomes and between the *Y* chromosome and autosomes have also been found (SCHÄFER 1978). A well established case of interactions between autosomes and *Y* chromosome is in the species pair *Drosophila mojavensis* and *Drosophila arizonensis* (VIGNEAULT and ZOUROS 1986; ZOUROS, LOFDAHL and MARTIN 1988). It is possible that interaction between autosomes and sex chromosome(s) is a general explanation of Haldane's rule, probably due to a recessive nature of the interacting genes (for a review, see CHARLESWORTH, COYNE and BARTON 1987) and hemizygosity of sex-linked genes in heterogametic sex. The *X-Y* interaction hypothesis has been suggested, besides HALDANE (1922), by several other workers (CURTIS, LANGLEY and TREWERN 1980; COYNE 1985; ORR 1987); however, convincing evidence supporting this hypothesis is lacking.

Evidence against *X-Y* interaction: In a number of species pairs, it has been shown that *X* and *Y* chromosomes are compatible. For example, there is no incompatibility between the *D. pseudoobscura pseudoobscura X* and the *D. p. bogatana Y* (DOBZHANSKY 1974; ORR 1989), between the *D. mojavensis X* and the *D. arizonensis Y* (VIGNEAULT and ZOUROS 1986), or between the *D. hydei X* and the *D. neohydei Y* (SCHÄFER 1978). However, the F_1 hybrid males with these combinations of interspecific sex chromosomes in all these species pairs are *fertile* although the reciprocal F_1 hybrid males in all these cases except species pair *D. hydei/D. neohydei* are sterile (nonreciprocal hybrid male sterility). Thus the reported compatibility of interspecific *X* and *Y* chromosomes with the same combination as the fertile F_1 hybrid males is not strong evidence against the *X-Y* interaction hypothesis. However, evidence against the involvement of *X-Y* interaction has been obtained from producing highly fertile backcross generation males, which have the same interspecific sex chromosome combinations as the sterile hybrid males in two species pairs, *D. virilis/D. novamexicana* (ORR and COYNE 1989) and *D. pseudoobscura bogatana/D. p. pseudoobscura* (ORR 1989). Both these species pairs show nonreciprocal hybrid male sterility which is *incomplete* [7% fertility in *D. virilis* ♀♀ × *D. novamexicana* ♂♂ hybrids (ORR and COYNE 1989), and 16% male fertility (as measured by sperm motility test) in *D. pseudoobscura bogatana* ♀♀ × *D. p. pseudoobscura* ♂♂ hybrids (ORR 1989)].

Evidence against the *X-Y* interaction reported here was obtained by producing normal fertile flies with the same sex chromosome combination as that of *completely sterile* F_1 hybrid males. Another difference between the present study and previous studies is that in the present study the genetic background was made highly homogeneous with respect to all chromosomes except the *Y* chromosome whose effect was being tested. In the study using standard species stocks, s_i^3-$s_is_is_i$ males had an intact *X* chromosome and about 97.7% of the autosomal genome from *D. simulans*. In the study using attached-*X D. simulans* stock, s_i-$s_is_is_i$ males had an intact *X* chromosome and about 87.5% of the autosomal genome from *D. simulans*.

Related to the *X-Y* interaction hypothesis, a new explanation of Haldane's rule has been proposed which involves *X-Y* meiotic drive (HURST and POMIANKOWSKI 1991; FRANK 1991a,b). This hypothesis has been examined theoretically (COYNE, CHARLESWORTH and ORR 1991) as well as empirically (JOHNSON and WU 1992; Coyne and ORR 1992) and found to be invalid, and apparently is also not supported by our results. No abnormality caused by meiotic drive was observed in the males carrying heterospecific sex chromosomes.

The crossing protocols used in the present study for interspecific *Y* chromosome introgression can be used as a general method to make direct test of the involvement of *X-Y* interaction in hybrid male sterility. These methods are especially important in cases of reciprocal hybrid male sterility, where it is difficult to distinguish between *X*-autosome and *X-Y* interactions by classical backcross analysis because in backcross progenies a full haploid set of autosomes always ac-

companies the *Y* chromosome of the same species (*e.g.*, COYNE and KREITMAN 1986). However, the application of these methods are not restricted to these cases. The method using standard species stocks can be applied to any species pairs which produce fertile F_1 hybrid females and some fertile backcross males. A successful interspecific *Y* chromosome introgression would mean (1) that *X-Y* interaction does not have a significant effect on hybrid male sterility, and (2) that if *X*-autosome (or autosome-autosome) interaction is involved, the interaction is of an asymmetrical nature (discussed below). On the other hand, the method using attached-*X* stocks does not require any interaction to be asymmetrical, but it is restricted to species pairs of which attached-*X* stocks are available. Furthermore when the attached-*X* method is used caution should be taken when very few fertile males are obtained in the early generations of the introgression. A wrong *Y* chromosome can be delivered by female parents in a low frequency due to detachment of the attached-*X* chromosomes. In such cases, it is necessary to verify the introgressed *Y* chromosome by *Y* chromosome specific probing as done by JOHNSON *et al.* (1992).

Models of symmetrical and asymmetrical *X*-autosome interactions: Models of reproductive isolation involving few genes and/or asymmetry have been discussed by many authors (DOBZHANSKY 1937a; MULLER 1940; NEI, MARUYAMA and WU 1983; WU and BECKENBACH 1983; VIGNEAULT and ZOUROS 1986; ZOUROS 1986; ZOUROS, LOFDAHL and MARTIN 1988). Since the symmetrical and asymmetrical *X*-autosome interactions have different consequences for the *Y* chromosome substitution results, we present a detailed symmetrical and an asymmetrical models of *X*-autosome interactions (1) to explain why the failure of *Y* chromosome substitution by our method does not necessarily provide support for the *X-Y* interaction theory, (2) to show that the success of interspecific *Y* chromosome substitution, as here in the case of species pair *D. simulans/D. sechellia*, point to an *asymmetrical nature* of the *X*-autosome gene interaction, and (3) to provide a general explanation for the commonly observed asymmetry (unidirectionality) of hybrid sterility in the early stages of speciation. The symmetrical model assumes that interacting genes in the two species reside symmetrically, *i.e.*, on homologous loci in the two species. The asymmetrical model assumes that interacting genes in the two species reside asymmetrically, *i.e.*, in two different (nonhomologous) loci. As the results in Table 5 show, under the symmetrical model, *Y* chromosome substitution by the backcross substitution method (Figure 1) is not possible as BC2 males are sterile. *Only when the X-autosome interaction is of an asymmetrical nature is it possible to substitute Y of one species by that of another by this method.* Therefore, the successful substitution of *D. simulans Y* by that of *D. sechellia* using this method suggests that the *X*-autosome interaction may be of an asymmetrical type. The same is true if an autosome-autosome interaction is involved. Thus the unsuccessful substitutio is of *D. sechellia Y* chromosome by that of *D. simulans* and between *D. simulans* and *D. mauritiana* do not necessarily show that the *Y* chromosome is involved, because any symmetrical *X*-autosome or autosome-autosome interaction can also prevent the *Y* chromosome substitution. Therefore the test protocol shown in Figure 1 is a one-way test.

The often observed nonreciprocal hybrid male sterility between species can also be accounted for by the asymmetrical model of *X*-autosome interactions. In Table 5, if two loci are involved in the asymmetrical *X*-autosome interaction model, it is apparent that F_1 hybrid males from one direction of hybridization are sterile and those from the reciprocal direction are fertile. An asymmetrical *Y*-autosome interaction model can also provide a similar explanation (VIGNEAULT and ZOUROS 1986; ZOUROS 1986; ZOUROS, LOFDAHL and MARTIN 1988).

Asymmetrical interaction is also expected by the two-locus speciation model of DOBZHANSKY (1937a, pp. 375–376) and MULLER (1940). In this model, reproductive isolation is reached when the ancestral genotype (say, *aabb*) evolves to genotypes *AAbb* and *aaBB* in two separate populations. The incompatibility interaction between alleles *A* and *B* is asymmetrical. The asymmetrical nature of genic interactions between closely related species seems to be a very common pattern and it has been revealed in most species pairs where data regarding sterility interactions are available, *e.g.*, between *Drosophila persimilis* and *D. pseudoobscura* (WU and BECKENBACH 1983), between *D. simulans* and *D. mauritiana* (WU *et al.* 1992), and between *D. arizonensis* and *D. mojavensis* (VIGNEAULT and ZOUROS 1986; ZOUROS, LOFDAHL and MARTIN 1988). These commonly observed asymmetries are expected because the probability of incompatibility arising due to two independent allelic substitutions at the same locus is much lower than that involving substitutions at different loci between species. From this we can further conclude that hybrid sterility in different pairs of species within the same evolutionary group can be expected to involve different sets of interacting genes as indeed appears to be the case in the species subgroup discussed here (WU *et al.* 1992). More information of this issue is crucial for understanding the evolution of hybrid sterility in animal species.

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TABLE 5

Symmetrical and asymmetrical models of *X*-autosome interactions

Parents/Filial generations	Sex	Symmetrical interaction (two loci)[a] Genotypes	Fertile/ Sterile	Asymmetrical interaction[b] (three loci) Genotypes	Fertile/ Sterile
Species 1	♀	$X_1X_1;A_1A_1$	F	$X_1X_1;a_1a_1;B_1B_1$	F
	♂	$X_1Y_1;A_1A_1$	F	$X_1Y_1;a_1a_1;B_1B_1$	F
Species 2	♀	$X_2X_2;A_2A_2$	F	$X_2X_2;A_2A_2;b_2b_2$	F
	♂	$X_2Y_2;A_2A_2$	F	$X_2Y_2;A_2A_2;b_2b_2$	F
F_1 (species 1 ♀ × species 2 ♂)	♀	$X_1X_2;A_1A_2$	F	$X_1X_2;a_1A_2;B_1b_2$	F
	♂	$X_1Y_2;A_1A_2$	S	$X_1Y_2;A_1A_2;B_1b_2$	S
rF_1 (reciprocal)	♀	$X_1X_2;A_1A_2$	F	$X_1X_2;a_1A_2;B_1b_2$	F
	♂	$X_2Y_1;A_1A_2$	S	$X_2Y_1;a_1A_2;B_1b_2$	S
BC1 (F_1♀ × species 2 ♂)	♂	$X_1Y_2;A_1A_2$	S	$X_1Y_2;a_1A_2;B_1b_2$	S
		$X_1Y_2;A_2A_2$	S	$X_1Y_2;a_1A_2;b_2b_2$	S
		$X_2Y_2;A_1A_2$	S	$X_1Y_2;A_2A_2;B_1b_2$	S
		$X_2Y_2;A_2A_2$	F	$X_1Y_2;A_2A_2;b_2b_2$	S
				$X_2Y_2;a_1A_2;B_1b_2$	S
				$X_2Y_2;A_2A_2;B_1b_2$	S
				$X_2Y_2;a_1A_2;b_2b_2$	F
				$X_2Y_2;A_2A_2;b_2b_2$	F
BC2 (BC1 ♂ × species 1 ♀)	♂	$X_1Y_2;A_1A_2$	S	$X_1Y_2;a_1A_2;B_1b_2$	S
				$X_1Y_2;a_1a_1;B_1b_2$	F
BC3 (BC2 ♂ × species 1 ♀)	♂			$X_1Y_2;a_1a_1;B_1B_1$	F
				$X_1Y_2;a_1a_1;B_1b_2$	F

[a] Symmetrical *X*-autosome interaction: A minimum of two loci can be involved in an *X*-autosome interaction, one on the *X* chromosome and one on an autosome. X_1 and X_2 are two alleles of the *X*-linked locus, and A_1, A_2 are two alleles of the autosomal locus in species 1 and 2, respectively. Interaction between X_1 and A_2 or between X_2 and A_1 leads to hybrid male sterility. Therefore, genotypes with X_1 and A_2 or X_2 and A_1 are sterile. Among the four genotypes of BC1 males, only $X_2Y_2;A_2A_2$ (Y_1, Y_2 are the *Y* chromosomes of species 1 and 2 respectively) genotype is fertile. When BC1 fertile males are crossed to females of species 1, the only genotype produced is $X_1Y_2;A_1A_2$ which is the same as F_1 males and are sterile. Thus, further backcrosses can not be performed and a *Y* chromosome transfer between the two species through the present backcross scheme is not possible.

[b] Asymmetrical *X*-autosome interaction: A minimum of two loci are needed to demonstrate this model, and a two-locus model would lead to *nonreciprocal* hybrid male sterility, *i.e.*, one direction of cross produces sterile hybrid males and the reciprocal cross produces fertile hybrid males. To produce sterile hybrid males in both reciprocal crosses under asymmetrical *X*-autosome interaction, three loci are required (as in Table 5). As before X_1/X_2 is an *X*-linked locus, and a_1/A_2, B_2/b_2 are two autosomal loci with capital letters being active alleles and lower case letters being null (or noninteractive) alleles. Suppose active *X*-linked alleles interacting with autosomal active alleles result in hybrid males sterility, then two of the eight genotypes ($X_2Y_2;a_1A_2;b_2b_2$, $X_2Y_2;A_2A_2;b_2b_2$) among BC1 males are fertile and when these fertile males are crossed to females of species 1, one of the two genotypes ($X_1Y_2;a_1a_1;B_1b_2$) is fertile. These fertile males can be further backcrossed to species 1, and both of the two genotypes produced (BC3 in Table 5) are fertile. So the fertile males can be used to make further backcrosses and *Y* chromosome substitution between the two species is possible.

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CHAPTER 4

A COMBINED CLASSICAL GENETIC AND HIGH RESOLUTION TWO-DIMENSIONAL ELECTROPHORETIC APPROACH TO THE ASSESSMENT OF THE NUMBER OF GENES AFFECTING HYBRID MALE STERILITY IN *DROSOPHILA SIMULANS* AND *DROSOPHILA SECHELLIA*

This Chapter consists of a paper (Zeng, L.-W. and R.S. Singh, 1993 *Genetics* **135**:135-147) which addresses a number of issues on hybrid male sterility and Haldane's rule. A new method (2DE) has been introduced, for the first time, to the study of reproductive isolation. This technique combined with classical genetic analysis has been used 1) to assess the number of genes involved in hybrid male sterility; 2) to identify 2D proteins associated with hybrid male sterility, and 3) to test the model of fast evolution of the X chromosome which has been used to explain Haldane's rule and the large effect of the X chromosome.

A Combined Classical Genetic and High Resolution Two-Dimensional Electrophoretic Approach to the Assessment of the Number of Genes Affecting Hybrid Male Sterility in *Drosophila simulans* and *Drosophila sechellia*

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ABSTRACT

We have attempted to estimate the number of genes involved in postzygotic reproductive isolation between two closely related species, *Drosophila simulans* and *Drosophila sechellia*, by a novel approach that involves the use of high resolution two-dimensional gel electrophoresis (2DE) to examine testis proteins in parents, hybrids and fertile and sterile backcross progenies. The important results that have emerged from this study are as follows: (1) about 8% of about 1000 proteins examined showed divergence (presence/absence) between the two species; (2) by tracing individual proteins in parental, hybrid and backcross males, we were able to associate the divergent proteins with different chromosomes and found that most divergent proteins are associated with autosomes and very few with *X* chromosome, *Y* chromosome and cytoplasm; (3) when proteins showing both quantitative and qualitative differences between the two species were examined in F_1 hybrid males, most (97.4%) proteins were expressed at levels between the two parents and no sign of large scale changes in spot density was observed. All the proteins observed in the two parental species were present in F_1 hybrid males except two species-specific proteins that may be encoded (or regulated) by sex chromosomes; (4) when different fertile and sterile backcross male testes were compared, a few *D. sechellia*-specific proteins were identified to be consistently associated with male sterility. These results along with the observation that a large proportion (23.6%) of first generation backcross males were fertile show that hybrid male sterility between *D. simulans* and *D. sechellia* involves a relatively small number of genes. Role of large scale genetic changes due to general genome incompatibility is not supported. The results also suggest that the large effect of *X* chromosome on hybrid male sterility is not due to higher divergence of *X* chromosome than autosomes.

GENETIC theories of species formation, in particular the debate regarding the number and the nature of genes involved, have developed in parallel with the advances in genetics, and consequently the debates on the involvement of "few *vs.* many" genes have gone through several rounds. Classical theories of geographic speciation propose that species differences result from accumulation of many allelic differences of small effects (for review see CHARLESWORTH, LANDE and SLATKIN 1982). MAYR's peripatric model (MAYR 1954, 1963, 1982), an elaborated version of the basic allopatric model, calls for massive reorganization of the gene pool, with extensive allelic substitution, in achieving reproductive isolation. Sympatric theories of speciation, in general, tend to rely on the involvement of few genes of large effects, involving behavior and/or niche segregation, making sympatric speciation possible even in the presence of high gene flow (MAYNARD SMITH 1966; BUSH 1975; TAUBER and TAUBER 1977a, 1977b; TAUBER, TAUBER and NECHOLS 1977). Some recent genetic theories also propose the involvement of relatively few allelic substitutions, but of large effects, to be responsible for the attainment of reproductive isolation (*e.g.*, the transilience model, TEMPLETON 1981). In general, the theories proposing the involvement of few genes in speciation are based on, or meant (1) to make speciation plausible without geographic isolation (*e.g.*, sympatric speciation) (2) to explain or provide a mechanism for rapid speciation (*e.g.*, the transilience model) and (3) to provide support for punctuational evolution (GOULD 1980) by involving mutations with large developmental and/or phenotypic effects.

There have been basically two approaches to estimate the number of genes involved in speciation: the molecular approach and the classical genetic approach. Since we presently lack knowledge to differentiate the genes that affect reproductive isolation *directly* (*i.e.*, genes affecting mating behavior and gametogenesis) from those that affect it *indirectly* (*i.e.*, indirect effects of genes affecting all other aspects of reproductive isolation including ecological divergence), in most molecular studies of speciation, attempts have been made to estimate the total number

of gene differences between related species. As studies of older species pairs would confound the amount of genetic changes that have occurred *during* and *after* speciation, *closely* related species pairs are preferentially chosen for such studies (*e.g.*, see PRAKASH, LEWONTIN and HUBBY 1963; ZOUROS 1973; AYALA 1975; THROCKMORTON 1977; CHOUDHARY and SINGH 1987). This approach was vigorously pursued by the use of gel electrophoresis during 1970s and 1980s (for reviews see LEWONTIN 1974; AYALA 1975; SINGH 1989). The estimates of species divergence between closely related species based on gene-enzymes vary from nearly zero or a few percent between partially isolated species or subspecies to about 10% between sibling species such as *D. melanogaster* and *D. simulans* (CHOUDHARY, COULTHART and SINGH 1992; see SINGH 1989 for a review). But even the estimates between the most closely related species must necessarily be *overestimates* of the actual number of genes involved in speciation, as they contain both relevant and irrelevant changes with respect to speciation.

On the contrary, the classical genetic approaches tend to *underestimate* the number of genes involved in postzygotic reproductive isolation. As heterogametic sex (males in *Drosophila*) sterility is the most common form of postzygotic reproductive isolation between closely related species (Haldane's rule, HALDANE 1922), most studies have focused on the number of genes responsible for hybrid male sterility. The technique used in such studies is backcross genetic analysis of hybrid male sterility between species using morphological markers pioneered by DOBZHANSKY (1936). The outcome of such studies has always been the same: every marked chromosomal region shows some effect on hybrid male sterility. The interpretation of these results has been that polygenes are involved in species hybrid male sterility (DOBZHANSKY 1936, 1974; COYNE 1984; COYNE and KREITMAN 1986; ORR 1987, 1989a; ORR and COYNE 1989; KHADEM and KRIMBAS 1991). A similar conclusion is reached when backcross studies are made with respect to morphological differences (COYNE 1983, 1985; COYNE and KREITMAN 1986; COYNE, RUX and DAVID 1991). However, a limitation of this technique has been that the numbers of morphological markers are limited, and so only a tiny fraction of the genome is represented by the markers. This limitation, and consequently the resulting association of all morphological markers with sterility, provides a basis for the argument that there must be many more genes involved in reproductive isolation than can be detected by the classical genetic approach (COYNE 1984; COYNE and KREITMAN 1986; COYNE and CHARLESWORTH 1989).

Here, for the first time, we used a new combined approach involving classical genetic analysis and two-dimensional gel electrophoresis (2DE) into the molecular study of postzygotic reproductive isolation. In two-dimensional electrophoresis proteins (or polypeptides) are separated based on their intrinsic chemical charges (isoelectric focusing; IEF) in the first dimension and further separated according to molecular size (sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SDS-PAGE) in the second dimension. This technique allows the simultaneous detection of hundreds of proteins, which is unsurpassed by any other technique (HANASH and STRAHLER 1989; GÖRGE 1991; HARRISON and JOSLYN 1991). However, because of the required expertise in achieving adequate reproducibility and truly high resolution, the technique has been used successfully in only a small number of laboratories (HANASH and STRAHLER 1989). In evolutionary genetics, the use of 2DE has been very limited and the reported cases involve estimation of genetic distances and phylogeny (AQUADRO and AVISE 1981; OHNISHI, WATANABE and KIM 1983; OHNISHI, KAWANISHI and WATANABE 1983; GOLDMAN, GIRI and O'BRIEN 1987, 1989; SPICER 1988, 1991; JANCZEWSKI, GOLDMAN, and O'BRIEN 1990; SPICER and FLEMING 1991) or genetic variation (LEIGH BROWN and LANGLEY 1979; COULTHART and SINGH 1988a, 1988b, 1988c). The number of protein spots resolved by most of these studies is in the range of 500–600. With the improved technique in our lab, we are able to detect over 1000 protein spots on a single gel. Application of this improved technique allows us to estimate the number of protein differences of both qualitative (presence/absence) and quantitative (amount of proteins) nature, and thus to discriminate between theories of speciation involving relatively few *vs.* many genes, and those involving structural *vs.* regulatory genes.

Drosophila simulans and *Drosophila sechellia* are two sibling species in the *melanogaster* species subgroup. Crosses between them yield sterile males but fertile females that can be backcrossed to either species to produce sterile and fertile males (Coyne and KREITMAN 1986; ZENG and SINGH 1993). The comparison of 2DE protein profiles in parental, F_1 hybrid and backcross male testes suggests that the number of genes involved in postzygotic reproductive isolation is small. Essentially all proteins detected in both parental species were detected in F_1 hybrids, and most of the divergent proteins (showing both quantitative and qualitative differences) were expressed in F_1 hybrid males at normal levels. A few species-specific proteins were identified to be associated with hybrid male sterility. These results support theories proposing the involvement of a relatively small number of genes in the initial development of postzygotic reproductive isolation and do not support theories that call for the involvement of large-scale polygenic or genomic (ma-

cro-regulatory) changes having pleiotropic effects on reproductive isolation.

MATERIALS AND METHODS

Species, crosses and male sterility measures: *D. simulans* and *D. sechellia* stocks were obtained from Dr. JEAN DAVID. Hybrid offspring produced from crossing *D. simulans* (denoted as s_i) females to *D. sechellia* (denoted as s_e) males were denoted as s_is_e. The s_is_e hybrid females were backcrossed to *D. simulans* males, and the offspring produced were denoted as $s_is_es_i$. All the species stocks and crosses were reared in 8-dram vials containing banana medium at 25°. Hybrid and backcross males were classified into four types as described in ZENG and SINGH (1993). Type 1 males (T_1) have atrophied testes and aspermic seminal vesicles, type 2 males (T_2) have normal testes and collapsed seminal vesicles [containing no (T_{2a}) or a few sperm (T_{2b})], type 3 males (T_3) have normal testes and large amounts of sperm (but less than normal, about half as much as normal) in their seminal vesicles and type 4 males (T_4) have normal testes and normal amounts of sperm. Male fertility was measured by the proportion of type 3 and type 4 males; this measure has been tested and shown to be very reliable (ZENG and SINGH 1993).

Two-dimensional gel electrophoresis (2DE): Two-dimensional electrophoresis was performed as outlined by O'FARRELL (1975) with the modifications of COULTHART (1986), HOCHSTRASSER *et al.* (1988) and ours. There has been considerable amount of effort put in modifying O'FARRELL's (1975) method to apply to *Drosophila* reproductive tract proteins (COULTHART 1986), and the resolution and reproducibility have been significantly improved (COULTHART and SINGH 1988a, 1988b, 1988c). However, our earlier application of this technique did not totally eliminate some of the common problems of 2DE. The first problem was pH gradient degradation at the basic end of the IEF gel. About 2 cm of the basic end of the IEF tube gel were consistently degraded; and, as a result, no spots but a few smears were observed at the basic side of the second dimensional slab gel. The second problem was large spot size and heavy horizontal smears. The third problem was poor separation of high molecular weight proteins (too crowded). Numerous trials and errors involving changes in many experimental conditions have led to a dramatic increase in the resolution and reproducibility of our 2DE method (L.-W. ZENG and R. S. SINGH, unpublished data). The size and shapes of spots have been changed from large with various shapes to small with more uniform (circular) shape. The number of spots has been increased from 500 to over 1000 (with the same tissue) with greater reproducibility (see Figure 1).

Sample preparation: Sample preparation is critical to the resolution of 2DE. There are several possible causes to the heavy smears and big spot size. Too much sample loading causes poor separation in the IEF tube gel and therefore produces horizontal smears and large spots in the second dimensional gel. High sample concentration leads to high molecular weight proteins not being well dissolved. Improper denaturants may result in poor dissolution of proteins in the sample and/or the IEF gel. A number of factors in sample preparation were tested in response to the three possible causes, and the amount of sample loaded was found to have the largest effects on resolution. The sample amount for a gel has been reduced to about one third of what was used in our earlier studies. In addition, three types of sample buffer, lysis buffer (O'FARRELL 1975), CHAPS-2-mercaptoethanol-Urea and CHAPS-DTT-Urea sample buffer (HOCHSTRASSER *et al.* 1988), were tested and the CHAPS-DTT-Urea sample buffer gave the best result. A well-known artifact of 2DE (two parallel horizontal smears in the top part of the gel), which consistently show up when CHAPS-2-mercaptoethanol-Urea buffer is used, disappears when CHAPS-DTT-Urea sample buffer is used.

Young male flies were separated from females and aged for 5–6 days. Seven, 14 or 21 pairs of testes were dissected in Ringer's solution (CHENEY and SHEARN 1983) and placed in 40 μl, 80 μl or 120 μl CHAPS-DTT-Urea sample buffer containing 1% (w/v) DTT, 4% (w/v) Chaps, 9 M urea and 2% ampholyte of pH 3–10 (HOCHSTRASSER *et al.* 1988). The tissues were dissolved in sample solution quickly by a brief vortex and stored at −70° until electrophoresis. Just prior to electrophoresis, samples were alternately thawed and frozen by immersion in liquid nitrogen and a 37° water bath for five freeze/thaw cycles. After the last thaw, samples were centrifuged at 10,000 rpm for 20 min. The supernatants were transferred to new tubes and centrifuged for another 10 min at 14,000 rpm. Thirty μl of supernatant of each sample were loaded for electrophoresis.

The first dimensional gel (IEF): The first dimensional (isoelectric focusing) gel is the most important part of 2DE, and the most difficult part as well. Most of the common problems in 2DE arise from the first dimension; *e.g.*, poor resolution and pH gradient degradation. To maximize the resolution and eliminate the pH gradient degradation, different pH gradient ranges, electrolytes, gel concentration and running conditions (voltage and running time) have been tested. The results of various tests show that pH gradient range from pH 5 to pH 7 give a better separation (even spot distribution) than the range from pH 6 to pH 8. The electrolytes 6 mM H_3PO_4 and 20 mM NaOH have much lower conductivity than 0.2% H_2SO_4 and 0.5% ethanolamide and thus allow low current and much higher voltage to be applied to IEF gel (high voltage is required to focus proteins to their pI points). The running condition that is usually measured by volt-hours (the product of volts and hours) is a critical parameter of 2DE. The optimum value of this parameter (which gives the best resolution and no pH gradient degradation) was found by monitoring the movement of proteins during IEF, the formation and degradation of pH gradient. By using colored pI markers and staining the IEF tube gels after running under different running conditions, we observed that the acidic proteins form sharp bands very quickly and the basic proteins take a long time to form bands. After a certain number of volt-hours (depending on the amount of proteins loaded), the pH gradient starts to degrade. Optimum running conditions were found by noting the point just before the pH gradient starts to degrade and after most basic proteins are focused. It is very important to note that the more the sample is loaded, the more volt-hours are required. If sample is too great, the pH gradient degrades before most basic proteins are focused.

The first dimensional gel contained 2% ampholytes (pH 5–7 and pH 3–10 biolytes mixture in a 4:1 ratio), 1.5% (w/v) CHAPS, 0.5% (v/v) Nonidet P-40, 8.4 M urea and 4.5% acrylamide. The isoelectric focusing was done with the lower reservoir containing 0.06 M phosphoric acid and the upper reservoir containing 0.02 M sodium hydroxide. The gels (16 cm long by 1.5 mm in diameter in glass tubes) were run at room temperature with a constant voltage of 200 V for 2 hr, followed by 500 V for 5 hr, and then 1,000 V for 10 hr. The gels were extruded from the tubes and soaked in 165 μl transfer solution containing 0.07 M Tris-HCl (pH 8.8), 3% SDS and 0.0003% bromphenol blue. The tube gels were then immediately run in the second dimension.

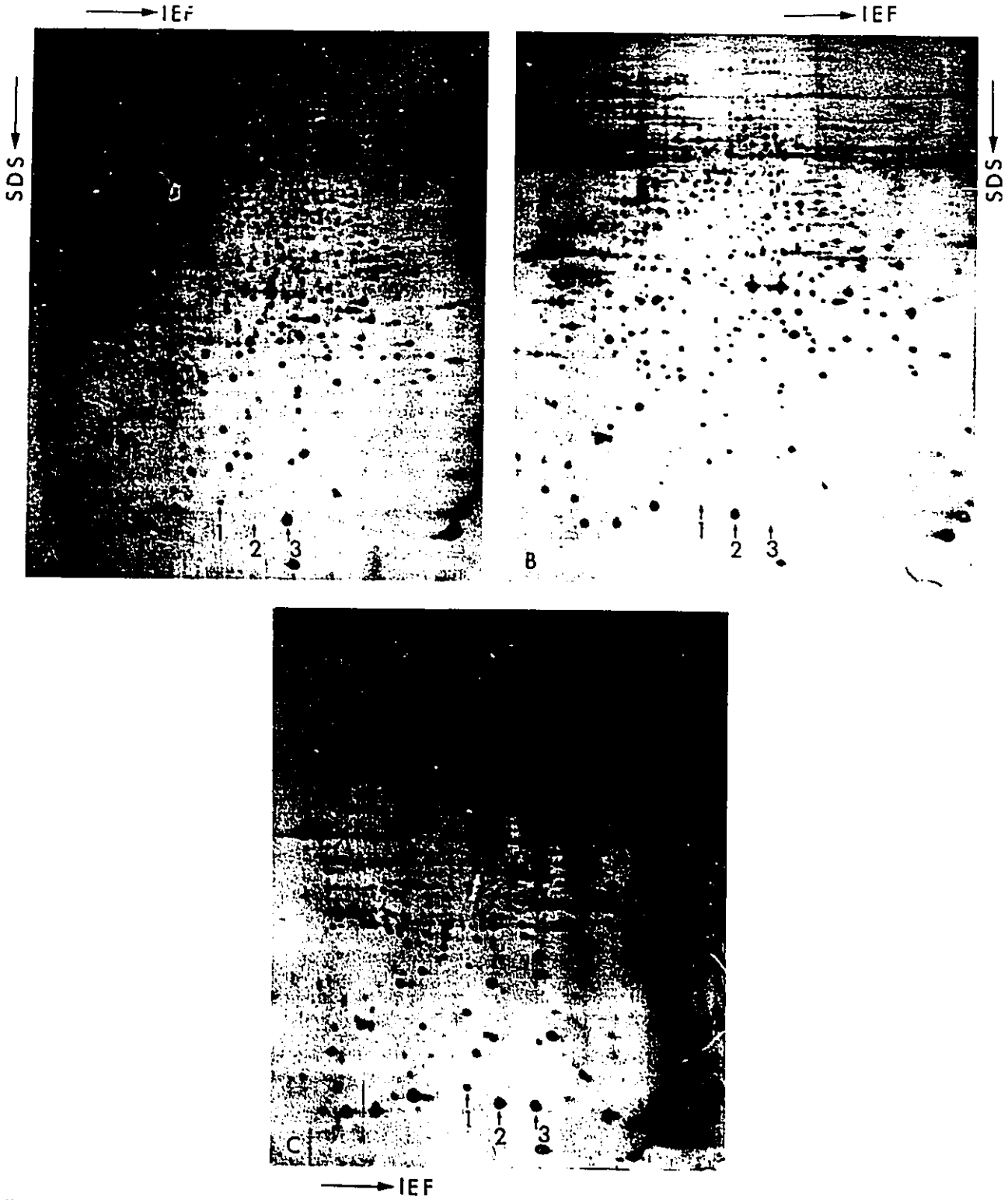

FIGURE 1.—Comparisons of 2D proteins in male testes of *D. simulans* (A), *D. sechellia* (B) and their F_1 (*s,s,*) hybrids (C). Not only all the common proteins but also most of the species-specific proteins are present in F_1 hybrids. For example, *D. simulans*-specific proteins 1 and 3 and *D. sechellia*-specific protein 2 are present in F_1 hybrids.

The second dimensional gel (SDS-PAGE) and silver stain: The second dimension was a 12% acrylamide and SDS free gel of size 20 × 16 × 1.5 mm^3. The gels were run at a constant current of 10 mA per gel for about 18 hr. The prolonged running time improved the separation of high molecular weight proteins. The gels were silver stained by

a method described by COULTHART (1986), which was adapted with some modification from MERRIL, DUNAN and GOLDMAN (1981) and MORRISSEY (1982).

pH gradient range of IEF gel and proteins out of the range: To estimate the number of proteins that were not picked up by the IEF gel, 12 pI marker proteins (pI from 3.5 to 9.6), two red *Drosophila* pigment markers (a basic and an acidic pigment, which were always focused at two extreme ends of an IEF gel) and sample proteins were run on gels with four different ranges of pH gradient (from pH 5–7 to pH 3–10). By comparing the bands of different gels, we learned that (1) sample proteins affected pH gradient, (2) there was no protein loss at the acidic end of the IEF gel and (3) about 100 basic proteins focused in the sample solution were not detected on our 2D gels (L.-W. ZENG and R. S. SINGH, unpublished data).

Gel scoring method: To facilitate comparisons, the gels were run simultaneously using the Protean II 2-D multicell (Bio-Rad) system. Initially we tried to use our 2D analyzing system [a laser scanner and a software (PDQUEST) run on a Sun SPARC station, Protein Databases Inc., New York] to analyze the 2D spots. However, due to the mechanical nature of the computer matching, slight difference in gel shape or size (caused by shrinking or swelling) could lead to systematic mismatch. As hundreds of spots were scored, we found it easier to do a manual match than trying to correct the computer mismatch. We used the computer program to detect the total number of spots on a gel, and the comparison of gels was performed by overlaying gels one on another on a light box. This turned out to be a very efficient method of matching spots. As most spots were identical (overlapping), spot differences between gels were easily identified. Protein spots of both kinds, showing presence/absence or obvious density differences, were scored.

A code was given to each scored spot on a 2D gel to indicate its location and identity. The gel was divided into 6 × 7 (42) 3 cm squares that were numbered from low pI and low molecular weight to high pI and high molecular weight. The first number of a code is the square number. A single gel was taken from each parental species and the F_1 hybrids, and each spot within each square was compared among the three gels. Only those spots that showed obvious different density among the three gels were numbered (the second number of a spot code) and scored. This set of spots were then scored in fertile and sterile backcross males. To score spot density, we used eight values from 0 and 3 (which is the O.D. range detected by the laser scanner) to classify scored spots into eight classes. Each spot was compared to seven reference spots with O.D. values 0.1, 0.5, 1, 1.5, 2, 2.5 and 3 (determined by the computer program). The number 0 indicates absence of a spot and 3 indicates the most dense spots. Spots with density between references were assigned to the nearest class. We have tried to produce eight discrete spot density classes, from what is really a continuous variation in spot density, to provide a rough idea of the magnitude of variation in the quantity of proteins. In addition, ~ and * were used to indicate "faint" and "obvious presence" of a spot, respectively. A list of all the spots scored and their densities in two parental species, F_1 hybrids and sterile and fertile backcross males is available upon request.

RESULTS

Morphology and anatomy of the F_1 hybrid males: The only reliable morphological difference between the two species is in the shape of the male genital arches (COYNE 1983; COYNE and KREITMAN 1986).

TABLE 1

Male fertility estimates based on testis types in *D. simulans*, *D. sechellia*, their F_1 hybrids and backcross progeny

Testis types	*simulans* (s_i)	*sechellia* (s_e)	F_1 (s_is_e)	Backcross ($s_is_es_i$)
T_1	2	1	40	165
T_2	34	4	296	215
T_3	0	0	0	59
T_4	669	161	0	58
Total	705	166	336	497
$T_3 + T_4$ (%)	94.9%	97%	0	23.6%

F_1 hybrid (s_is_e) = *D. simulans* ♀♀ × *D. sechellia* ♂♂, backcross ($s_is_es_i$) = F_1 (s_is_e) ♀♀ × *D. simulans* ♂♂. The four different types of male testes (T_1 to T_4) are described in the MATERIALS AND METHODS.

The genital arches of F_1 hybrid males (*D. simulans* ♀♀ × *D. sechellia* ♂♂) were found to be intermediate between those of the parents (COYNE and KREITMAN 1986). All other morphological characters are either intermediate or the same as those of the parental species.

When hybrid males were placed with fertile hybrid females, no offspring was produced. This is not due to lack of successful mating, as mating was observed. The hybrid male sterility between this species is due to lack of mature sperm and not due to immotile sperm (LACHAISE *et al.* 1986), and this was confirmed by dissecting the reproductive tracts of hybrid males. No sperm were found in the hybrid males' seminal vesicles where mature sperm is stored (LINDSLEY and TOKUYASU 1980).

Where sperm is produced, further examination of testes indicated that most hybrid males had normal well-developed testes (type 2 testes) and some had atrophied testes (type 1 testes) (Table 1). The rest of the reproductive tract (*e.g.*, accessory glands, vas deferens and sperm pumps) appeared normal.

Male fertility: Table 1 shows proportions of the four types of males in the two parental species, F_1 hybrids and backcross progeny. *D. simulans* (s_i) and *D. sechellia* (s_e) had very high frequencies (over 94%) of type 3 and type 4 males. Most of the F_1 hybrid males (s_is_e) were type 2 (88.1%); no type 3 or type 4 males were observed. When hybrid females were backcrossed to *D. simulans* males, the backcross ($s_is_es_i$) males showed a wide distribution of testis types and had a high fertility (23.6%, Table 1).

Protein patterns of parental male testes and species divergence: As hybrid male sterility results from lack of mature sperm, the most relevant tissue—testis—was investigated by using 2DE. Two-dimensional protein profiles of male testes of the two species were compared using a single strain from each species (Figure 1). Initially we started analyzing a number of lines from each species to be able to partition the pairwise line difference (in protein composition) into

TABLE 2

Species specific 2D protein spots and genetic distance between *D. simulans* and *D. sechellia*

	D. simulans	*D. sechellia*	Total	Genetic distance
Total number of spots detected by PDQUEST	1234	1182	2416	0.069
Number of valid spots detected by PDQUEST	1063	983	2046	0.082
Total number of spots detected by eyes	1217	1215	2432	0.069
Number of species specific spots	89	79	168	

The computer program (PDQUEST) detects and evaluates (*e.g.*, faint spots) the total number of spots and then gives the number of valid spots. The genetic distance is calculated by dividing the total number of species specific spots by the total number of spots detected in both species.

two components—true species differences and within species polymorphisms. However, because the overall difference between the two species is very small (COULTHART and SINGH 1988b, also see below), the work reported here was done with a single strain from each species. The total number of spots in each of the two species detected by computer program (PDQUEST) before evaluation is similar to the number obtained from direct counting (about 1200, Table 2). However, the number of valid spots evaluated by the computer program is about 1000 (Table 2). The majority of the protein spots were found to be present in both species. Only a small number (168) of the protein spots have diverged (in terms of presence/absence differences) between the two species. Eighty-nine of the 168 were found only in *D. simulans*, and the remaining 79 spots were found only in *D. sechellia* (Table 2). Based on the total number of species-specific protein spots and the total number of spots detected in the two species, the genetic divergence is estimated to be 7–8% (Table 2).

Protein patterns of F_1 and backcross male testes: When the 2D protein profile of the F_1 (s_is_e) hybrid males was compared with that of the parental species, it was found that most parental proteins were expressed in the hybrids. Not only all the shared spots, but also most of the unshared spots, between the two species were detected in F_1 hybrids (see Figure 1). This was expected as the hybrid males contained one set of genes from each species except the sex linked genes. Protein spots that showed qualitative (presence/absence) or obvious quantitative differences between the two parental species (including all the species-specific spots) or between the parental species and their F_1 hybrids were numbered and compared among the two parental species, the F_1 hybrids, and the sterile and fertile backcross males. The sterile males used in this comparison were $s_is_es_iT_{2a}$, *i.e.*, backcross males with normal shaped testes containing no sperm. The data are summarized in Table 3. There are a total of 231 spots that showed obvious density differences between the two species and only one spot (not shown in Table 3), which showed no obvious density differences between the two species, showed different density in F_1 hybrids. Three gels from each parental species and two gels from the hybrids were used in the comparison. Of the 231 protein spots compared, 63 showed only quantitative differences and all of them were expressed in F_1 hybrids; 168 were species-specific spots and all except two were present in F_1 hybrids (Table 3). One of the two proteins absent from F_1 hybrids was *D. simulans* specific and the other was *D. sechellia* specific, and they may be linked to sex chromosomes (see DISCUSSION below).

TABLE 3

Species-distinguishing protein differences between *D. simulans* (s_i), *D. sechellia* (s_e) and their F_1 hybrids

Protein spot presence in			No. of protein spots
s_i	s_e	F_1	
+	−	+	88
−	+	+	78
−	+	−	1
+	−	−	1
+	+	+	63

F_1 denotes the *D. simulans* ♀♀ × *D. sechellia* ♂♂ hybrids. The 63 protein spots were present in both parental species with different density (quantitative differences). All the other proteins showed qualitative differences between the two species.

TABLE 4

A summary of species distinguishing protein spots with respect to their density patterns in F_1 hybrids

F_1 spot density	Number of spots	Proportion
Higher than the high parent	6	0.026
The same as the high parent	167	0.723
Intermediate between the two parents	42	0.182
The same as the low parent	16	0.069
Lower than the low parent	0	0
Total	231	

Only spots showing species differences in density were compared between the parents and their hybrids.

By comparing the density of the spots between the parents and F_1, it was found that most of the 231 protein spots in F_1 hybrids resembled the parental species with higher density (72.3%, Table 4) and some were intermediates between the two parental species (18.2%, Table 4). Only a small proportion of protein spots resembled the low parent (6.9%, Table 4) or exceeded the high parent (2.6%, Table 4). No protein was expressed at a level lower than the low parent or

was suppressed in the hybrids (Table 4). If the expression of a protein in F_1 hybrids at a level between the two parents can be called normal, most proteins (72.3% + 18.2% + 6.9% = 97.4%) that showed obvious density differences between the two parents were expressed at a normal level. No sign of large-scale disruption of gene expression in F_1 hybrids was observed.

In $s_is_es_i$ backcross males, *all* of the species-specific protein spots, except one *D. sechellia*-specific spot, were present in sterile and/or fertile males (Table 6).

Association of divergent proteins with autosomes, sex chromosomes and the cytoplasmic factors: The 168 species-specific proteins are the total divergent proteins scored between the two species. By tracing these proteins in F_1 and backcross males, it is possible to assign the total divergent proteins to different chromosomes and autonomously propagated cytoplasmic factors. The rationale of the assignment is as follows. The F_1 (s_is_e) hybrid males carry a complete haploid set of autosomes from each species, the *X* chromosome and the cytoplasmic factors from *D. simulans* and the *Y* chromosome from *D. sechellia*. Thus, any species-specific protein encoded (or regulated) by these chromosomes (autosomes of both species, the *X* chromosome and cytoplasmic factors of *D. simulans* and the *Y* of *D. sechellia*) should be present in F_1 hybrids, provided the expression of the protein-coding genes are not suppressed in hybrids, which appears to be the case (see previous paragraphs). Similarly, species-specific proteins encoded (or regulated) by *D. sechellia X* chromosome and cytoplasmic factors and *D. simulans Y* chromosome should be absent from F_1 (s_is_e) hybrid males. The genetic composition of the $s_is_es_i$ backcross males is different from that of F_1 hybrid males. Every $s_is_es_i$ backcross male has a complete haploid set of autosomes, a *Y* chromosome and cytoplasmic factors from *D. simulans*. The other haploid set of autosomes and the *X* chromosome are recombinant mixtures of chromosomal segments or genes, half of which are from *D. sechellia* and are heterogeneous among individuals. The probability of any given *D. sechellia* autosomal or *X*-linked gene to be present or absent in a given backcross offspring is 50%. However, if a randomly pooled sample (*e.g.*, the samples used for 2DE) of backcross offspring is examined, most, if not all, of the *D. sechellia* genes are expected to be present. For instance, the probability of any given *D. sechellia* autosomal or *X*-linked gene to be absent from a sample of 10 backcross offspring is $(1/2)^{10}$, or 10^{-3}. If the sample size is large enough, all the *D. sechellia* autosomal and *X*-linked genes, but not *D. sechellia* Y-linked genes (which are absent from all the backcross males), are expected to be present in $s_is_es_i$ backcross offspring. Knowing the genetic composition of the F_1 and backcross males, we can assign each species-specific protein to different chromosomes (autosomes, *X*, *Y* chromosomes and cytoplasmic factors) based on the pattern of their absence/presence in the F_1 and backcross males.

The association of the 168 species-specific proteins with different chromosomes is shown in Table 5. For *D. simulans*-specific proteins, only the *Y* chromosome-associated proteins can be identified. As 88 of the 89 *D. simulans*-specific proteins were present in F_1 (s_is_e), they can not be on the *Y* chromosome because F_1 (s_is_e) hybrid males do not carry the *D. simulans Y* chromosome. The absence from F_1 hybrid males of the only *D. simulans*-specific protein (protein 28-20) can be explained by its association with the *Y* chromosome. The presence of this protein in backcross males confirms the idea that it may be encoded or regulated by the *D. simulans Y* chromosome, because the *D. simulans Y* chromosome is present in every backcross males. As the *D. simulans* autosomes, *X* chromosome and cytoplasmic factors share the same pattern of presence/absence in F_1 and backcross males, they cannot be identified separately and assigned with different proteins in the present study.

The autosomes, the *X* chromosome, the *Y* chromosome and the cytoplasmic factors of *D. sechellia* have different patterns of presence/absence in F_1 and backcross males, and hence varying proteins can be assigned to different chromosomes individually (Table 5). Of the 79 *D. sechellia*-specific proteins, 78 were present in F_1 hybrid males; so they could be on the autosomes or the *Y* chromosome but they cannot be on the *X* chromosome simply because F_1 (s_is_e) males do not carry *D. sechellia X* chromosome (the reciprocal F_1 males carry the *D. sechellia X* chromosome). If the 78 proteins are all on autosomes, they should be present in pool samples of backcross males. When different backcross males (sterile and fertile) were examined for these proteins, 77 were found to be present and therefore the 77 proteins were assigned to *D. sechellia* autosomes. The one protein (protein 38-3, Table 6) that was absent from all the backcross males examined can be explained by its association with the *D. sechellia Y* chromosome, as the backcross males ($s_is_es_i$) do not carry the *D. sechellia Y* chromosome. However, only backcross males with normal testes were examined; the backcross males with atrophied testes (type 1 males, see Table 1) were not examined. Therefore, the association of this protein with the *Y* chromosome is uncertain. The only protein (protein 33-5, Table 6) that was missing in the F_1 hybrid males was present in backcross males (Table 6), confirming its association with the *X* chromosome. Cytoplasmic factor associated proteins should be absent from both F_1 and backcross males that possess the cytoplasmic factors of *D. simulans*. Of the 79 *D.*

TABLE 5

Association of species-specific proteins with different chromosomes based on their presence/absence in F_1 and backcross males between *D. simulans* and *D. sechellia*

	D. simulans specific proteins (N = 89)				*D. sechellia* specific proteins (N = 79)			
Presence/absence (+/−) in $F_1/s_iS_eS_i$[a]	+/+	+/−	−/+	−/−	+/+	+/−	−/+	−/−
Number of proteins	88	0	1	0	77	1	1	0
Association, or lack thereof (yes/no), between proteins and chromosomes:								
Autosomes	Yes	No	No	No	Yes	No	No	No
X chromosome	Yes	No	No	No	No	No	Yes	No
Y chromosome	No	No	Yes	No	No	Yes	No	No
Cytoplasmic factors	Yes	No	No	No	No	No	No	Yes

[a] Three types of backcross males ($s_iS_eS_iT_{2a}$, $s_iS_eS_iT_{2b}$ and $s_iS_eS_iT_4$, see text for explanation) were used to examine the presence/absence of proteins and the presence of a protein in any of the three types of males was scored as presence in $s_iS_eS_i$ males.

TABLE 6

A summary of *D. sechellia* specific proteins associated with backcross male sterility

	2DE proteins				
Male types	20-2	20-14	21-17	33-5	58-3
D. simulans	0	0	0	0	0
D. sechellia	*	*	*	*	*
s_iS_e F_1	*	*	*	0	*
$s_iS_eS_iT_{2a}$	*	*	*	*	0
$s_iS_eS_iT_{2b}$	*	~	*	0	0
$s_iS_eS_iT_2$	*	~	0	*	0
$s_iS_eS_iT_3$	*	~	*	0	0
$s_iS_eS_iT_4$-1	0	0	0	0	0
$s_iS_eS_iT_4$-2	0	0	0	0	0
$s_iS_eS_iT_4$-3	0	0	0	0	0
Associated with	Normal amount of sperm	Sperm quantity	Normal amount of sperm	Absence of sperm	Testis atrophy (if not *Y*-linked)

The symbols 0, ~ and * denote for "absence," "faint" and "obvious presence," respectively. $s_iS_eS_iT_{2a}$, $s_iS_eS_iT_{2b}$, $s_iS_eS_iT_2$, $s_iS_eS_iT_3$ and $s_iS_eS_iT_4$ denote backcross males without sperm, with a few sperm, a mixture of the previous two, with about half the normal amount of sperm and normal amount of sperm, respectively. $s_iS_eS_iT_4$-1, $s_iS_eS_iT_4$-2 and $s_iS_eS_iT_4$-3 are three different samples of fertile backcross males. A total of 40 *D. sechellia*-specific proteins were examined and only five that showed consistent absence from $s_iS_eS_iT_4$ males are listed here.

sechellia-specific proteins, none was absent from both the F_1 and backcross males.

Although the lack of divergent proteins associated with the *Y* chromosome of both *D. simulans* and *D. sechellia* and the cytoplasmic factors of *D. sechellia* may be due to paucity of proteins encoded by these elements, it is quite clear that the *X* chromosome of *D. sechellia* codes for fewer divergent proteins than expected on the basis of its genome size (about 20% of the total genome). Most of the divergent proteins were associated with autosomes that constitute about 80% of the total genome in these species of *Drosophila*. The reciprocal analysis would give an estimate of divergent proteins associated with the *D. simulans X* chromosome, autosomes and the cytoplasmic factors. However, it is difficult to cross *D. sechellia* females to *D. simulans* males. Only one of seven *D. simulans* lines used in this cross produced a few offspring that were only used to check for presence/absence of the *D. sechellia X* chromosome-associated protein spot. As expected, this spot was present in *D. sechellia* (♀♀) × *D. simulans* (♂♂) hybrid males (data not shown).

Identification of species-specific proteins associated with backcross male sterility: Backcross male progeny is a mixture of males with heterogeneous genetic background (with genes from both parental species). The incompatible interactions involved in backcross males can be complex (Wu and Davis 1993). However, one complete set of autosomes and the *Y* chromosome from one species are uniformly present in every backcross male. In the case of $s_iS_eS_i$ backcross males, a complete set of *D. simulans* autosomes and the *D. simulans Y* chromosome are present in every backcross males. Therefore, any *D. sechellia* gene that is incompatible with *D. simulans* autosomal or *Y*-linked genes (in the background of $s_iS_eS_i$ backcross males) should be consistently absent from fertile backcross males, although it may or may not be present in every sterile backcross male because the sterility of backcross males can be caused by different incompat-

ible genes. Thus, by identifying *D. sechellia*-specific proteins that are consistently absent from fertile backcross males, we can detect *D. sechellia* proteins or genes that are incompatible with *D. simulans* autosomal or *Y*-linked genes. *D. sechellia* autosomal genes that are incompatible with *D. simulans X*-linked genes cannot be detected by this scheme [except when the *X*-autosome interaction is symmetrical (which is not the case in this species pair, ZENG and SINGH 1993)], because the *D. simulans X* chromosome is not present in all backcross males and therefore the *D. sechellia* autosomal genes incompatible with the *D. simulans X* chromosome would not be consistently absent from fertile backcross males.

To identify proteins encoded or regulated by *D. sechellia* genes that are incompatible with *D. simulans* autosomal or *Y*-linked genes, we examined *D. sechellia*-specific proteins in both sterile ($s_iS_eS_iT_{2a}$) and fertile ($s_iS_eS_iT_4$) backcross males. Thirty-six of the 79 *D. sechellia*-specific proteins were present in both sterile and fertile males, 40 were found to be present in sterile and absent from fertile backcross males and three were absent from both sterile and fertile backcross males. We also examined all the 63 common proteins, which showed quantitative difference between the two species, and all the 89 *D. simulans*-specific proteins in sterile and fertile backcross males; and almost all of these proteins (except one common protein) were present in both sterile and fertile backcross males. It is not surprising that so many *D. sechellia*-specific proteins were absent from fertile backcross males, as fertile $s_iS_eS_i$ backcross males must be free of *D. sechellia* incompatible genes and therefore would show absence of all other genes or proteins linked to the incompatible genes.

The incompatible proteins or genes should be consistently absent from fertile backcross males whereas other linked genes would not show consistent absence from fertile backcross males. To check if the 43 *D. sechellia*-specific protein spots were consistently absent from fertile backcross males, detailed examination of these spots were carried out. All except three (which were too faint for unambiguous scoring) of these 43 spots were further examined for presence/absence in two different types of sterile backcross males ($s_iS_eS_iT_{2b}$ and $s_iS_eS_iT_2$, Table 6), one sample of backcross males with about half the amount of sperm as normal ($s_iS_eS_iT_3$) and three independent samples of fertile backcross males ($s_iS_eS_iT_4$, Table 6). Most of the 40 spots scored were present in at least one of the three fertile samples and five were absent from all of the three fertile samples. One of the five proteins (spot 33-5, Table 6), which is associated with the *X* chromosome (see previous section), is strongly associated with *the absence/presence of sperm* in backcross males. It was present in males with no sperm ($s_iS_eS_iT_{2a}$) and absent from males with a few ($s_iS_eS_iT_{2b}$), half ($s_iS_eS_iT_3$) and normal amount of sperm ($s_iS_eS_iT_4$). As the $s_iS_eS_iT_2$ males were a mixture of $s_iS_eS_iT_{2a}$ and $s_iS_eS_iT_{2b}$ males, the protein 33-5 was present in the $s_iS_eS_iT_2$ males. Another protein (protein 20-14, Table 6) is associated with the *amount of sperm* produced by the backcross males. It was present in males with no sperm ($s_iS_eS_iT_{2a}$), faint in males with some sperm ($s_iS_eS_iT_{2b}$, $s_iS_eS_iT_2$ and $s_iS_eS_iT_3$) and it is absent from males with normal amount of sperm ($s_iS_eS_iT_4$). Protein 20-2 is associated with the production of *normal amount of sperm*. It was present in backcross males from the ones with no sperm to the ones with about half the normal amount of sperm. It was consistently absent from males with normal amount of sperm. Protein 21-17 behaved similarly as spot 20-2, except that it was absent from $s_iS_eS_iT_2$ males, and it is associated with the production of *normal amount of sperm*. This association is not affected by the presence of the protein in $s_iS_eS_iT_2$ males, because if more than one sterility gene is involved a given sterility gene is not expected to be present in every sterile backcross males. Proteins 20-14, 20-2 and 21-17 are associated with autosomes (see previous section). Protein 38-3 was absent from all the backcross males examined. As all the backcross males examined have normal shaped testes and males with atrophied testes were not examined, this protein is either associated with the *D. sechellia Y* chromosome (see previous section) or with testis atrophy.

DISCUSSION

Sterility results when two sets of genes from two different species are brought together into hybrid male individuals. As each of the two sets of genes function normally in the parental species, the hybrid male sterility must necessarily be a result of incompatibility of certain genes from the two species. Although this incompatibility may be very complicated, interspecific interactions between genes on the sex chromosomes and autosomes have been shown to be involved, and it is not known if an autosome-autosome interaction is also involved (JOHNSON *et al.* 1992; ZENG and SINGH 1993). The estimation of the number of incompatible or, simply, sterility genes and their genetic characterization has been a major task in the studies of postzygotic reproductive isolation (WU and BECKENBACH 1983; COYNE and CHARLESWORTH 1986, 1989; PANTAZIDIS and ZOUROS 1988; ORR 1989b, 1992; NAVEIRA and FONTDEVILA 1991; COYNE 1992; WU *et al.* 1992; PANTAZIDIZ, GALANOPOULOS and ZOUROS 1993; WU and DAVIS 1993; PEREZ *et al.* 1993).

We have provided data showing that the number of male sterility genes involved in species pair *D. simulans/D. sechellia* is relatively small. The data supporting this conclusion include (1) a low overall genetic

divergence (about 8%) between the two species with little divergence attributable to sex chromosome and cytoplasmic factors based on a sample of over 1000 testis proteins, (2) a normal level expression of most (97.4%) of the divergent proteins in F_1 hybrids, (3) a small number of *D. sechellia*-specific proteins obviously associated with backcross male sterility and (4) a large proportion of fertile males (23.6%) observed in the first generation backcross progeny.

Genetic divergence of sex chromosomes *vs.* autosomes: Data from various sources—morphological and cytological (LACHAISE *et al.* 1988), allozyme (CARIOU 1987; M. CHOUDHARY and R. S. SINGH, unpublished data) and nuclear DNA (COYNE and KREITMAN 1986)—have shown that the genetic distance between *D. simulans* and *D. sechellia* is rather small. By using 2DE, we have sampled more than 1000 polypeptides from testes, which have been shown to be more divergent than other tissues (COULTHART and SINGH 1988a, 1988b, 1988c). This is the largest sample of protein loci used to estimate genetic divergence between any pair of species reported. Our 2DE results show that most protein spots are identical between the two species and only a small proportion (8%) of the protein spots have diverged between the two species.

Not knowing the chromosomal location of proteins, we cannot directly compare the divergence of autosomes *vs.* *X* chromosome between the two species. However, by analyzing the genetic compositions of F_1 and backcross males, we can attribute the divergent proteins to various chromosomes. Assuming that the number of proteins in our sample of 1000 coded by various chromosomes is in proportion to their genome size, the protein-chromosome association method can provide information about the relative divergence of various chromosomes. Following this method in the present study, we can tell if the autosomes and *X* chromosome have diverged equally or unequally. Of the 79 *D. sechellia*-specific proteins, only one was associated with the *X* chromosome (1.3% or 1/79), at most one was associated with the *Y* chromosome and none was associated with cytoplasmic factors. Of the 89 *D. simulans*-specific proteins, just one was associated with the *Y* chromosome. The low protein divergence of the *Y* chromosome and the cytoplasmic factors may be due to relative paucity of proteins encoded by these elements and is consistent with previous findings that the *D. sechellia Y* (JOHNSON *et al.* 1992; ZENG and SINGH 1993) and cytoplasmic factors (ZENG and SINGH 1993) are not involved in hybrid male sterility in this species pair. However, it is interesting that the *X* chromosome-associated protein divergence is far smaller than expected on the basis of its genome size (1.3% observed *vs.* 20% expected). The single *X*-linked *D. sechellia*-specific protein turned out to be strongly associated with presence/absence of sperm in backcross males. In a parallel study involving *D. simulans* and *Drosophila mauritiana*, we found that 20.5% (16/78) of the *D. simulans*-specific proteins and 9.4% (8/85) of the *D. mauritiana*-specific proteins were associated with the *X* chromosome, which are also not larger than expected (L.-W. ZENG and R. S. SINGH, unpublished data). These results show that the *X* chromosome has not diverged more than the autosomes and the large effect of the *X* chromosome on hybrid male sterility (see COYNE and ORR 1989 and references therein) cannot be explained by faster divergence of *X* chromosome as compared with autosomes (CHARLESWORTH, COYNE and BARTON 1987; COYNE and ORR 1989).

The number of sterility genes inferred from backcross data: The proportion of fertile males in backcross progeny can be used as an indication of the number of sterility genes affecting hybrid male fertility (PRAKASH 1972, GUÉNET *et al.* 1990). A large proportion of fertile males observed in the backcross progeny implies that either very few sterility genes are involved in determining hybrid male sterility, or that the genes are clustered on very few chromosomal segments. The high fertility in backcross male progeny obtained in the present study (23.6% in Table 1) and a previous study [36.3% and 26.3% calculated from Table 1 and Table 3, respectively, in COYNE and KREITMAN 1986 (fertility scored by sperm motility)] can be explained by as few as two independent loci each with complete penetrance (the expected fertility is 25% based on independent segregation and random association of the two loci). Although not all incompatible genes between the two species are involved in backcross male sterility (F_1 hybrid male sterility may also not involve all incompatible genes), the ones involved are indicated here to be few.

The number of sterility genes inferred from 2DE: The first indication that a small number of genes are involved in determining hybrid male sterility is the small proportion (8%) of species-specific proteins observed between the two species. This proportion was obtained by comparing a single strain from each species and includes both true species-specific proteins (alternatively fixed alleles in each species) and within species polymorphism. Thus, the proportion of true species-specific proteins should be even smaller. The small proportion of species-specific proteins by itself does not show that the number of sterility genes is small, as in absolute terms a small proportion can still be a large number when a large number of loci are sampled. However, not all divergent proteins can be involved in hybrid sterility. The proportion of divergent proteins involved in hybrid male sterility suggested in this study is small. By tracing the 79 *D. sechellia*-specific proteins in sterile and fertile backcross males, we found most of them were present in

both fertile and sterile males. Only four or five of them were consistently absent from fertile backcross males and therefore were associated with male sterility. Although this number may be lower than the true number (as we could not examine *D. sechellia* autosomal genes incompatible with *D. simulans* X-linked genes, and we did not consider *D. sechellia* autosomal recessive genes incompatible with *D. simulans* genes), it does suggest that most of the *D. sechellia*-specific proteins are probably compatible with *D. simulans* autosomal and Y-linked genes.

It should be kept in mind that the tissue chosen for the present study is the most diverged tissue with respect to protein composition (COULTHART and SINGH 1988a, 1988b, 1988c; THOMAS and SINGH 1992) and obviously directly related to hybrid male sterility. When proteins from other tissues are taken into account, the proportion of divergent proteins has been shown to be much smaller (THOMAS and SINGH 1992). The sterility-associated proteins as a fraction of the total genome would also be smaller as most other tissues are not related to hybrid male sterility.

The second indication of the involvement of only a small number of genes in hybrid male sterility is that not only all shared proteins but also essentially all divergent proteins showing quantitative or qualitative differences between the two species were present in F_1 hybrids and most of these divergent proteins (97.4%) were expressed at a normal level between the two parents. There was no sign of any significant disruption of gene expression (activation of novel genes and/or inactivation of normally expressed genes) in F_1 hybrid males.

Thus, while realizing that estimates of number of genes involved in hybrid male sterility based on 2D genetic divergence would tend to err on the high side and those based on association of 2D proteins to sterility on the low side, our results suggest that genetic changes at a relatively small number of loci affecting gametic processes are sufficient to initiate development of hybrid sterility in the process of speciation. We must differentiate between the number of genes affecting F_1 male sterility and the backcross male sterility. We envision that as species divergence proceeds, other genes, both major and minor, get involved and increase the total number of loci affecting hybrid (F_1 and backcross) male sterility. In this scenario it is easy to see why species (hybrid) sterility is often *asymmetric* (*e.g.*, unidirectional) during the early stages of speciation and becomes *symmetric* (*e.g.*, bidirectional), probably due to cumulative effects of many loci, during the later stages of speciation.

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CHAPTER 5

A GENERAL METHOD FOR THE IDENTIFICATION AND MAPPING OF MAJOR GENES AFFECTING HYBRID MALE STERILITY IN DROSOPHILA

Chapter 5 is a manuscript that has been submitted to *Proceedings of National Academy of Sciences, U.S.A.* for publication. A general method for identifying major hybrid male sterility genes has been described. By using this method, a major hybrid male sterility gene has been identified and mapped to the X chromosome of *D. mauritiana*.

GENETICS

A General Method for the Identification and Mapping of Major Genes Affecting Hybrid Male Sterility in *Drosophila*

(spermatogenesis / hybrid sterility / reproductive isolation / speciation)

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ABSTRACT

The genes responsible for hybrid male sterility are usually identified by introgressing chromosome segments, monitored by visible markers, between closely related species by continuous backcrosses. This commonly used method, however, suffers from two problems. First, it relies on the availability of markers to monitor the introgressed regions and so the portion of the genome examined is limited to the marked regions. Second, the introgressed regions are usually large and it is impossible to tell if the effects of the introgressed regions are due to single (or few) major genes or many minor genes (polygenes). Here we introduce a simple but general method for identifying major genes which is free of these problems. In this method, putative individual hybrid male sterility genes, or very tightly linked gene complexes with large effects, are selectively introgressed from one species into the background of another species by repeated backcrosses. This is done by selectively backcrossing heterozygous (for hybrid male sterility gene or genes) females producing fertile and sterile sons in equal proportions to males of either parental species. With the application of this method, a single Mendelian factor of *D. mauritiana* which produces complete hybrid male sterility (aspermic testes) was identified by 45 generations of backcrossing to *D. simulans*. The sterility factor was mapped at 6.0 ± 0.66 map units from the *forked* locus, at position

62.7 ± 0.66 on the X chromosome. This method can be used with other species pairs that produce unisexual sterility, as no marker gene is required.

INTRODUCTION

Hybrid male sterility is the most common postzygotic reproductive isolating mechanism between closely related animal species and therefore has received special attention in the study of speciation. In animal species with heterogametic males (*e.g.*, mammals and *Drosophila*), it is evident from large numbers of interspecific hybridizations (1) that hybrid male sterility (with fertile hybrid females) is far more common than hybrid inviability. Genetic studies in *Drosophila* have accumulated evidence suggesting the existence of major genes that control hybrid male sterility (2-8).

In an attempt to detect genes responsible for hybrid male sterility, Dobzhansky (9) used morphological markers to identify foreign chromosome segments linked to the markers in backcross progenies, and to examine the effects of the segments on male fertility. The outcome of his and subsequent studies on various groups of *Drosophila* species (10-15) turned out to be virtually the same: every marker used is associated with hybrid male sterility and the largest effects are associated with X-linked markers. The strong association of hybrid sterility with the X-linked markers has led to the postulation that the X chromosome has evolved more rapidly, between different species, than autosomes (16-17). Our recent study in *Drosophila simulans* and *D. sechellia* using two-dimensional electrophoresis (2DE) has shown that the large effect of the X

chromosome is not due to generally higher divergence of the X chromosome than the autosomes (18). This implies that a few major genes may be responsible for the large effect of the X chromosome. If there exists major genes with large effects, we should be able to identify and map them individually. Researchers attempting to identify and map major genes linked to X-linked markers have used an extension of Dobzhansky's backcross analysis approach (9). In this method, the marked chromosome segments of one species are introgressed into the background of another species by continuous backcrosses (2-5, 7, 8, 19-22). The putative hybrid male sterility genes are then mapped by recombination analysis (2-5) or by other genetic and molecular means (7, 8).

However, a general problem of this marker-assisted backcross method is that the introgressed segments are usually very large and it is not known if the segments carry major genes or many polygenes (23). Using introgression of regions marked by three X-linked markers, Coyne and Charlesworth (3) showed three major hybrid male sterility genes linked to the three markers. Using some of the same markers, Naveira (23) showed that the introgressed regions carried polygenes with an additive threshold effect on male fertility.

Another problem of this method is that it is limited by the availability of markers. It can only detect genes closely linked to the markers used; genes which are not closely linked to any marker can not be detected. Not only identification and mapping are difficult in species groups without abundant morphological and molecular

markers (deletion, inversion, asynapsis and DNA markers), but also the detection of sterility genes is biased towards the marked segments of the chromosomes.

Here, we introduce a simple method which is free of these problems. In this method, individual major hybrid male sterility genes, or very tightly linked gene complexes, are introgressed from one species into the background of another species by continuous backcrosses. This is done by selecting females, each generation, which are heterozygous for the sterility gene (and which therefore produce both fertile and sterile sons in equal proportions) and backcrossing them to males of one of the parental species. No marker is required for this introgression, which makes the method one of general use. Once a major gene(s) has been detected it can be mapped and characterized by using conventional genetic and/or molecular techniques (see DISCUSSION).

By applying this method to two closely related species, *Drosophila simulans* and *D. mauritiana*, a major gene has been identified and mapped to the *D. mauritiana* X chromosome. This gene, which functions normally in *D. mauritiana*, causes complete male sterility when placed in the background of *D. simulans*.

MATERIALS AND METHODS

Species stocks, markers and fly culture: *Drosophila simulans* and *D. mauritiana* stocks were obtained from Dr. Jean David. A *D. simulans* strain homozygous for a recessive mutant marker on each of its five major chromosome arms was obtained from Dr. Jerry Coyne. For the purpose of this study, only three markers were used. The three mutant markers used are *forked* (bristle) on the X chromosome (*f*, I-56.7), *net* (wing vains) on the second chromosome (*nt*, II-0), and *ebony* (body color) on the third chromosome (*e*, III-71). All the stocks and crosses were reared in 8-dram vials on banana medium at 24-25° and a 12 hour light/dark regime.

Classification of hybrid males and fertility measure: The method for classifying hybrid males and measuring male fertility is the same as described in Zeng and Singh (24). In F_1 and backcross generations of *D. simulans* and *D. mauritiana*, we found two types of sterile males: males with atrophied testes (type 1 males) and males with normal shaped testes but with no mature sperm in their seminal vesicles (type 2 males). The two types of sterile males appeared in comparable frequencies in the first backcross generation (Table 1). Although both type 1 and type 2 males are sterile, the sterility genes involved are different (L.-W. Zeng and R. S. Singh, unpublished results) and in the present paper a gene responsible for

type 2 male sterility is reported. There were also two types of fertile males both of which had normal testes and large quantity of mature sperm in their seminal vesicles. The difference between these two types of males is that the amount of sperm produced by one type (type 3) is about half of the "normal" amount (type 4). For simplification, in the present study the two types of fertile males are sometimes combined and referred to as type 3/4. Male fertility was measured by the combined proportion of type 3 and type 4 males (or type 3/4). This method has been shown to be very reliable (24). All males were aged for five to six days before being dissected for scoring.

Introgression of hybrid male sterility genes: F_1 hybrid females from crossing *D. simulans* females to *D. mauritiana* males were backcrossed to *D. simulans* males. The progeny produced by the first backcross generation was denoted as BC_1. BC_1 females were individually backcrossed to *D. simulans* males again (one female with one to three males) and thus a number of matriarchal families (BC_1 families) were established. The sons from each family were dissected and their fertility was scored. This measure of fertility of the sons from each family was used as an indication of whether or not the mother of the family carried any major hybrid male sterility gene (from *D. mauritiana*). If the mother carried a major male sterility gene, half of her sons would receive the gene and be sterile.

Similarly, the male sterility gene would be passed to half of her daughters.

The daughters of one of the sterility-gene-carrier-mothers (which produced about 50% sterile males) were individually backcrossed to *D. simulans* males again and a number of second generation backcross (BC_2) families were formed. The sons from each BC_2 families were examined for fertility and the daughters of one of the sterility-gene-carrier-mothers were used to perform the next generation backcross. This backcrossing scheme was continued for 45 generations.

By continuously backcrossing a male-sterility-gene-carrier-female to *D. simulans*, the genetic composition of the resulting backcross offspring was gradually substituted by that of *D. simulans*. The gene or genes from *D. mauritiana* responsible for hybrid male sterility were selectively introgressed into the background of *D. simulans*. In the present backcross method, a given introgression line would carry only one sterility gene (or a tightly linked gene block). All other *D. mauritiana* genes or genome were eliminated by recombination-segregation process. As only one female was chosen in each backcross generation to make the consecutive backcrosses, the progressive reduction of the introgressed region by recombination was very efficient. Independent introgression lines may carry different genes if more than one major gene are involved in sterility.

RESULTS

Male fertility of parental species, F_1 hybrids and first generation backcross progeny: Table 1 shows the proportions of the four types of males in *D. simulans*, *D. mauritiana*, F_1 hybrids and progeny of the first backcross generation. Most of the males in the two parental species were type 4 males and the fertility was very high (> 95%). F_1 hybrid males from crossing *D. simulans* females to *D. mauritiana* males were completely sterile consisting of all type 2 males. When the hybrid females were backcrossed to *D. simulans* males, the male offspring produced were mostly of type 1 and type 2 with a small proportion of type 3 and type 4 (or type 3/4) males (6.0%).

Introgression of a hybrid male sterility gene from *D. mauritiana* into the background of *D. simulans*: By using the introgression scheme described in the MATERIALS AND METHODS, a hybrid male sterility gene with large effect was introgressed from *D. mauritiana* into the background of *D. simulans* by 45 generations of backcrosses. Table 2 shows the family segregation data for the first ten and the last seven backcross generations.

Eight BC_1 (the first backcross generation) females were individually backcrossed to *D. simulans* males and eight families (BC_1 families) were formed. The numbers of the four types of males in each of the eight families are given in Table 2. In most of the BC_1

families, type 1 and type 2 males were still produced, which means that the mothers carried genes responsible for both type 1 and type 2 male sterility. Seven virgin females were collected from one of the eight families (BC_1-family 6, Table 2) to carry out the second generation backcross, and seven BC_2 families were generated. Of the seven BC_2 families, some segregated type 2 and type 3/4 males, some segregated type 1, type 2 and type 3/4 males. The mothers of the families that segregate type 2 and type 3/4 males may carry a gene or genes (in heterozygous state) responsible for type 2 male sterility. The mothers of the families which segregate for type 1, type 2 and type 3/4 males may carry genes responsible for both type 1 and type 2 male sterility.

Three BC_2 families were selected to carry out the third generation of backcross, two (families 2 and 7) of which had a segregation of type 2 and type 3/4 males, and one (family 6) had a segregation of type 1, type 2 and type 3/4 males (Table 2). The data in Table 2 show that the BC_3 families (1, 2, 5, 6, and 7) derived from families 2 and 7 in previous generation were still segregating for type 2 and type 3/4 males and the families (3 and 4) derived from families 6 in previous generation were still segregating for all four types of males. As family 3 produced both types of sterile males, the mother may carry genes responsible for the sterility of both type 1 and type 2 males. So this family was selected to make the fourth generation backcross. However, only type 2 sterile males were produced in BC_4 generation and, for the rest of the 45 generations,

the backcrossing was continued by using families producing type 2 sterile sons (Table 2).

The segregation pattern in the subsequent backcross generations (Table 2) is the same: some families produced no sterile males and some produced about half type 2 (sterile) and half type 4 (fertile) males. This indicates that the mothers of the former families did not carry a sterility gene and the mothers of the latter families carried a gene (or gene complex) for aspermic testes (type 2 males). Assuming one hybrid male sterility (*hms*) gene was introgressed we can denote the *D. mauritiana* allele as hms^{ma}, the homologous *D. simulans* allele as hms^{si}, the sterility-gene-carrier-females as hms^{si}/hms^{ma} (heterozygous) and the non-sterility-gene-carrier-females hms^{si}/hms^{si} (homozygous, the same as pure *D. simulans*).

The introgressed gene behaves as a single Mendelian factor with complete penetrance: From the fourth backcross generation onward, the introgressed *hms* gene responsible for aspermic testes (type 2 males) segregated in a manner of a single major Mendelian factor. The type 2 and type 4 males produced by every heterozygous female in BC_4 or later generation families are roughly in equal proportions (Table 2). A Chi-square test of the combined data of males produced by heterozygous females (from BC_4 on) in Table 3 shows that the segregation ratio of type 2 (hms^{ma}/Y, 348) and type 4 (hms^{si}/Y, 370) males is not significantly different from the 1:1 ratio ($\chi^2_{[1]} = 0.674$, $p > 0.2$, Table 3). The numbers of

homozygous (non-sterility-gene-carriers, hms^{si}/hms^{si}) and heterozygous (sterility-gene-carriers, hms^{si}/hms^{ma}) females also showed a 1:1 ratio (Table 3). From BC_4 generation on (data in Table 2), there were 48 females (or families) which produced type 2 and type 4 males (*i.e.*, heterozygous females) and 37 females (or families) which produced all type 4 males (*i.e.*, homozygous females). The ratio of heterozygous females to homozygous females is not significantly different from the expected ratio of 1:1 ($\chi^2_{[1]} = 1.424$, $p > 0.2$; Table 3).

A segregation ratio of 1:1 for type 2 (sterile) and type 4 (fertile) males produced by heterozygous females indicates that the expression of the introgressed gene has a complete penetrance. The probability that a son receives the introgressed gene from his heterozygous mother is 50%, and the proportion of sterile sons (or type 2 males) is also 50%. The 1:1 ratio of sterile to fertile males produced by heterozygous females shows that the introgressed gene by itself can cause complete male sterility.

Mapping of the male sterility gene: As the introgressed *hms* gene behaves as a single Mendelian factor with complete penetrance in expression, we can use conventional recombination analysis to map this factor. A *D. simulans* strain with three morphological markers on each of the three major chromosomes (f^-; e^-; nt^-) was used to map the gene. Virgin females produced by a heterozygous mother (hms^{si}/hms^{ma}) from the 45th backcross

generation were individually crossed to males of the marker strain. The genotypes ($f^+/f^-;e^+/e^-;nt^+/nt^-;hms^{si}/hms^{ma}$ or $f^+/f^-;e^+/e^-;nt^+/nt^-;hms^{si}/hms^{si}$) of these females were identified by the fertility scores of their sons. The female progenies produced by two of the females with the genotype $f^+/f^-;e^+/e^-;nt^+/nt^-;hms^{si}/hms^{ma}$ were again individually backcrossed to males from the marker strain ($f^-/Y;e^-/e^-;nt^-/nt^-;hms^{si}$), and a number of families were formed. The segregation pattern in these families remained the same: some families produced all fertile males and some produced about half fertile and half sterile males. The males produced by the former families were discarded and the males produced by the latter families (13 families, Table 4), which resemble progeny of a test-cross, were scored for the marker phenotype and fertility. The data shown in Table 4 were used to calculate recombination frequencies between the *hms* gene and each of the three mutant marker genes (Table 5). The recombination frequencies between the sterility gene and two autosomal mutant markers (*net*, *nt*: II-0; *ebony*, *e*: III-71) are close to 50% (Table 5). This means that the introgressed *hms* gene is unlinked to these markers. On the other hand, the recombination frequency between the *hms* gene and the X-linked mutant marker *forked* (*f*: I-56.7) is 6.0%. This shows that the introgressed gene is on the X chromosome located about 6 cM (centimorgans) away from the *forked* locus. Pooling data from 13 families gave a standard error of 0.66 for the distance between *forked* and the introgressed gene (Table 5). The recombination

frequencies of the three pairwise mutant markers are close to 50% as expected, since they are on three separate chromosomes (Table 5).

Viability effect of the *hms* gene: It is of interest to examine if the *hms* gene has any other fitness effect. The segregation ratio of the males produced by heterozygous females is a good indication of the viability effect of the introgressed gene. If the introgressed gene decreases the viability of its carriers, the sterile/fertile male ratio would departure from 1:1 ratio and there would be more fertile males than sterile males. The data presented in Table 2 and summarized in Table 3 fit the 1:1 ratio very well ($\chi^2_{[1]}$ = 0.674, $p > 0.2$) and no segregation bias was observed. This shows that the hybrid male sterility gene does not decrease the viability of the sterile males.

However, the data from the mapping cross showed a different pattern. The segregation ratio of sterile and fertile males was significantly different from 1:1 ($\chi^2_{[1]}$ = 105.38, $p < 0.001$, Table 6). What is more interesting is that there were more sterile males than fertile males. The mutant marker *forked* which is linked to the sterility gene also showed a significant departure from 1:1 ratio ($\chi^2_{[1]}$ = 46.57, $p < 0.001$, Table 6). There were more wild type males than mutant type males. A simple explanation of the data is that the mutant allele (f^-) of the *forked* locus decreases the viability. In agreement with this explanation, one of the two autosomal mutant markers (*net*, *nt*:II-0) also showed a significant departure from 1:1

ratio ($\chi^2_{[1]} = 7.45$, $p < 0.01$, Table 6). There were more wild type males (nt^+/nt^-) than mutant type males (nt^-/nt^-). As this marker is not linked to the introgressed sterility gene, this departure from 1:1 ratio is not due to any introgressed genome from *D. mauritiana* but due to the low viability of the mutant gene and/or other linked genes. If f^- has a lower viability than f^+, the biased segregation ratio of the *hms* locus is expected, because the hms^{ma} is linked to the wild type allele f^+ and the hms^{si} allele is linked to the mutant allele f^- in the mapping cross. However, a larger departure from the 1:1 ratio of the *hms* locus than that of the *forked* locus can not be fully explained by such a linkage. Alternatively, the introgressed gene *i.e.*, the hms^{ma}, may produce a higher viability than hms^{si}. The question is then why the higher viability of the hms^{ma} was not detected in the 45 generations of backcrosses. The difference between the *D. simulans* strain used for the introgression and the multiply marked (five mutant genes) strain may provide an explanation. The hms^{si} allele and/or other linked genes of the marker strain may produce a low viability.

DISCUSSION

A method for detecting major hybrid male sterility genes: The backcross method used in this study along with the selection of females which produce fertile and sterile sons in approximately equal proportions ensures that the selected females carry a gene or genes (from the donor species) which can cause complete male sterility. If a major gene is carried by the female, half of her daughters would inherit the gene and thus the introgression can proceed further. On the other hand, if many unlinked genes each with a minor effect are the basis of the male sterility, these genes would be separated by the processes of recombination and segregation and would then be randomly passed on to different daughters, none or few of which would inherit a full set of polygenes required to produce complete sterility. Since we selected carrier females which produce fertile and sterile sons in approximately equal proportions, the present scheme guarantees detection of any major genes that affect male fertility. Obviously, in the case that several independent major genes are involved, they will be separated by recombination and segregation and only one of them would be kept in a given introgression line by the continuous backcrosses. However, if a tightly linked gene complex is involved which behaves as a single major gene, a very large number of generations of backcrosses would be required to break the linkage.

A second hybrid male sterility gene in the *forked* region: A tight linkage between hybrid male sterility and the marker *forked* was first revealed by Coyne (10) using Dobzhansky's backcross analysis (9). Assuming that the sterility gene was due to a single major gene, Coyne and Charlesworth (2, 3) introgressed the *forked* region from *D. mauritiana* into *D. simulans* by repeated backcrosses and mapped this gene at 1.1 map units from the *forked* locus using a maximum-likelihood method. In an attempt to map this gene at a finer scale, Perez *et al.* (8) introgressed segments of various size around the *forked* region from *D. mauritiana* into the background of *D. simulans*, and mapped a major gene (named *Ods*) within polytene chromosome bands 16D and 16E, or about 1.1 map units proximal to the *forked* locus (calculated from the data in Table 1 in ref. 8), which is the exact position defined by Coyne and Charlesworth (2).

By using the new method described in this paper, we identified another major sterility gene around the *forked* region and mapped it at 6.0 ± 0.66 map units from the *forked* locus. By using a second X-linked marker, we could easily determine on which side of the *forked* locus the gene is located. However, the available information allows us to make this decision without performing another mapping cross. The distal location of our mapping (50.7 ± 0.66) corresponds roughly to the cytological location ranging from band 13F to 14A of the polytene chromosome. The region distal to the *forked* locus covering and exceeding band 13F of the polytene chromosome of *D. mauritiana*, when introgressed into the background of *D. simulans*,

does not cause male sterility (8). Therefore, the *hms* gene must be proximal to the *forked* locus and located at position 62.7 ± 0.66 on the X chromosome.

The different map positions of the *hms* gene (62.7) and *Ods* (57.8) suggests that these genes are not allelic. Perez *et al.* (8) raised the possibility of the existence of a different hybrid male sterility gene proximal to a marker *Beadex* (*Bx*, I-59.4). However, due to lack of markers in the region, they could not investigate this possibility further.

Around the position 62.7 on the X chromosome, many loci have been reported to affect male fertility in *D. melanogaster*. At least five different male sterility mutations have been isolated and mapped to the cytological bands from 18F to 20BC (25, 26). The phenotype of mutant at one locus [*ms*(1)4, I:63, or 18F to 19C] is similar to that of the hybrid male sterility gene identified here. Both the mutant and our sterile males seem to be affected in the later stages of the spermatogenesis (absence of mature sperm). It would be interesting to know if hybrid male sterility genes are divergent copies of normal genes required for male fertility.

The role of major and minor genes in hybrid male sterility: A central question concerning the nature of genes involved in reproductive isolation remains unanswered: *i.e.*, is hybrid sterility or inviability caused by cumulative effect of many minor genes, or by the action of a few major genes, each with a discrete effect? (8). The

involvement of major genes on hybrid inviability is shown by the isolation of mutations that rescue inviable hybrids (27-31). However, for hybrid sterility, the issue remains controversial. Although by using marker-assisted introgression of chromosome segments a number of major genes have been identified and mapped (2-5, 7, 8), the introgressed segments are usually very large and it is difficult to determine if the effects of the introgressed segment are due to mainly major genes or only to a large number of minor genes. It is this problem that has recently led to a renewed controversy on the role of major/minor genes.

The introgression studies with *D. buzzatii* group species (20-22) show that the effects of most introgressed segments are due to large number of genes each with small effect (polygenes). On the other hand, in species pairs *D. simulans/D. mauritiana*, and *D. simulans/D. sechellia*, Coyne and Charlesworth (3) introgressed three marked regions of *D. mauritiana* (or *D. sechellia*) into *D. simulans* and mapped three major genes on the X chromosome. However, contradictory results have been obtained in the same pair of species for the same introgressed regions. Naveira (23) introgressed two of the three regions studied by Coyne and Charlesworth (3) and has shown that the sterility effects associated with these regions are polygenic, and, therefore raised the possibility that most of the previously identified "major genes" (2-5) may be polygenes. While there is no data verifying other identified "major genes", the two major genes identified and mapped in the *forked* region support Coyne and

Charlesworth's (2, 3) conclusion. Their results in species pair *D. simulans* and *D. sechellia* are also verified by a parallel study in our lab (L.-W. Zeng and R. S. Singh, unpublished results). By using the new method in the species pair *D. simulans/D. sechellia*, we have identified two major genes in the *forked* and *yellow-white* regions. The map positions of the two major genes are comparable to those obtained by Coyne and Charlesworth (3).

The data from the present study suggest that both major and minor genes are involved in hybrid male sterility. This is shown by the fact that hybrid male sterility can arise from multiple developmental defects producing atrophied testes (type 1 males) or from failure in sperm production (type 2 males). The disappearance of type 1 males (atrophied testes) in the first few generations of the backcross experiment (Table 2) indicates that this type of sterility may involve polygenes. On the other hand, the sterility of type 2 males (aspermic testes) may involve both major and minor genes. The method described here is meant to detect only major genes. By using this method with other species pairs, we should be able to tell if in these species pairs any major genes are involved.

Mapping and molecular characterization of major hybrid male sterility genes: The hybrid male sterility genes, once identified, can be maintained in different introgression lines by sib-mating or backcrossing using heterozygous females. The phenotype of the genes (male sterility or aspermic testes) is very easily scored.

If any morphological markers are available, the genes can be easily mapped by the conventional recombination method as we did with the *hms* gene. Once the map position of a gene is known, we can search for molecular markers in the mapped region and perform a physical mapping as done by Perez *et al.* (8).

In species pairs where no or few morphological or molecular markers are available, mapping and molecular characterization of the identified genes can also be approached. RAPD (Randomly Amplified Polymorphic DNA) (32, 33) can be used to screen for random primers which amplify polymorphic DNA between sterile (with sterility gene) and fertile (with the normal allele of the locus) males. The polymorphic DNA should be either part of the sterile gene or a tightly linked sequence. In *Drosophila*, it is easy to physically map the polymorphic DNA by *in situ* hybridization. This approach can be used regardless of the availability of markers. Another molecular approach is to use 2DE. Over a thousand testis polypeptides can be detected by 2DE on a single gel (18). By comparing 2DE protein profiles of fertile and sterile males produced by heterozygous females, a protein or proteins associated with the male sterility gene can be identified provided that protein production is affected by the sterility gene and it is detectable by the sensitivity of 2DE. This approach is also not restricted by the availability of markers.

SUMMARY

A simple method for detecting major hybrid male sterility genes has been described. This method, unlike other methods relying on the availability of visible markers, involves selectively introgressing major hybrid male sterility genes from one species into the background of another species by continuous backcrosses. As no marker is required, the application of this method is not restricted to species pairs with available markers; it can be used with any species pair which produce unisexual hybrid sterility (which is the most common form of reproductive isolation in closely related animal species). As this method detects only major genes or genes with large effects, application of this method can help resolve the debate regarding the nature (major *vs.* minor) of genes underlying hybrid male sterility. Once a major gene is identified, conventional genetic and molecular techniques can be used to map and characterize the gene at the molecular level.

Application of this method in species pair *D. simulans*/*D. mauritiana* has led to the identification of a single Mendelian factor in *D. mauritiana* which causes complete male sterility when placed in a background of *D. simulans*. This factor has been mapped at 62.7 ± 0.66 map units on the X chromosome of *D. mauritiana*.

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Table 1

Male fertility of *D. simulans*, *D. mauritiana* , the F_1 hybrids and the first generation backcross (BC_1) progeny.

	Male type					Fertility (%)
	1	2	3	4	Total	(type 3/4)
D. simulans	0	7	0	152	159	95.6
D. mauritiana	1	4	0	184	189	97.4
F_1	0	72	0	0	72	0.0
BC_1	113	152	14	3	282	6.0

The F_1 hybrid males were obtained from crossing *D. simulans* females to *D. mauritiana* males. BC_1 males were obtained by crossing the F_1 hybrid females to *D. simulans* males. Males were classified into four types (type 1 to 4) based on the shape and size of their testes and the amount of sperm produced. Type 1 males had atrophied testes with no or few sperm. Type 2 males had normal shaped testes but with no or few sperm. Both type 3 and type 4 males had normal shaped testes and large quantity of sperm, the difference was that type 4 males had normal amount of sperm and type 3 males had about half as much. The fertility was measured by the combined proportion of type 3 and type 4 males (or type 3/4).

Table 2

Male types and numbers produced in the first ten and the last seven of the 45 generations of backcrosses (BC).

	Family															
Male type	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
	BC_1								BC_2							
1	3	1	2	1	11	1	6	6	0	0	0	2	0	19	0	
2	1	7	7	2	6	6	2	9	1	14	9	11	8	3	12	
3/4	2	4	5	3	3	8	6	2	18	20	7	8	6	6	15	
Total	6	12	14	6	20	15	14	17	19	34	16	21	14	28	27	
	BC_3*								BC_4							
1	0	0	9	1	0	0	0		0	0						
2	9	7	11	10	11	13	0		0	11						
3/4	12	4	4	5	12	9	9		23	19						
Total	21	11	24	16	23	22	9		23	30						
	BC_5								BC_6							
1	0	2	0	0	0	0	0	0	0	0	0					
2	5	3	7	1	8	2	7	3	0	6	2					
4	4	10	4	14	8	8	4	5	11	7	8					
Total	9	15	11	15	16	10	11	8	11	13	10					
	BC_7								BC_8							
2	0	1	3	1	10	0	6	6	2	1	7	1	0	7	4	
4	16	6	3	7	5	12	7	5	11	10	9	16	13	9	9	
Total	16	7	6	8	15	12	13	11	13	11	16	17	13	16	13	

	BC_9								BC_{10}	
2	0	10	1	6	0	0	0	0	0	4
4	5	8	9	6	14	11	6	4	4	5
Total	5	18	10	12	14	11	6	4	4	9

	BC_{39}								BC_{40}				
2	0	5	0	8	0	0	7	0	6	7	4	0	8
4	13	5	8	5	15	11	5	10	5	6	4	12	5
Total	13	10	8	13	15	11	12	10	11	13	8	12	13

	BC_{41}								BC_{42}		
2	5	5	0	3	5	4	5	6	5	0	3
4	7	4	12	6	7	8	12	8	4	9	4
Total	12	9	12	9	12	12	17	14	9	9	7

	BC_{43}								BC_{44}	
2	0	7	4	6	0	0	9	0	6	5
4	8	9	5	5	8	9	4	15	13	13
Total	8	16	9	11	8	9	13	15	19	18

	BC_{45}								BC_{45} (continued)				
2	9	0	11	0	7	13	19	0	16	20	0	15	0
4	8	20	15	18	13	6	15	26	14	12	20	16	27
Total	17	20	26	18	20	19	34	26	30	32	20	31	27

The recurrent parent of the backcrosses was *D. simulans*. The families of BCn generation were formed by individually backcrossing female offspring of n^{th} generation backcross to *D. simulans* males. A sample of sons of each family were dissected for fertility check (male type scoring). The daughters from one of the families which produced about or higher than 50% sterile males were selected for next generation backcross. The numbers of the four types of males in each family at each generation are given in the table. In the first

10 generations, the families selected to propagate next generations are indicated in **bold** face. Type 3/4 are mostly type 4 males with very few type 3 males. From sixth backcross generation on, virtually no type 1 or type 3 males were observed and the columns for male type 1 and 3 are left out.

* In BC_3 families 1-2, 3-4 and 5-7 were derived from families 2, 6 and 7 in BC_2, respectively. In all other backcross generations all families were derived from a single family from the previous generation.

Table 3

Segregation ratio (1:1) of the hybrid male sterility gene (*hms*) in the backcross generations

Genotype	No. of males	No. of females
hms^{si}/hms^{si} (or hms^{si}/Y)	370	37
hms^{si}/hms^{ma} (or hms^{ma}/Y)	348	48
χ^2	0.674	1.424
Probability	> 0.2	> 0.2

The numbers of males and females are pooled data from BC_4 onward in Table 2. Females which produced none, one or two sterile males were considered as homozygous (hms^{si}/hms^{si}, N = 37), and the rest were considered as heterozygous (hms^{si}/hms^{ma}, N = 48). The numbers of fertile and sterile males are the sum of the sterile and fertile males produced by all the 48 heterozygous females.

Table 4 Numbers of males with different genotypes produced in the mapping cross.

Backcross Male Genotype	Family 1	2	3	4	5	6	7	8	9	10	11	12	13	Total
1. $f^+;e^+;nt^+;hms^{ma}$	21	11	8	11	9	10	14	16	15	9	14	15	17	170
2. $f^+;e^+;nt^+;hms^{si}$	1	0	0	0	0	0	0	0	0	0	0	0	1	2
3. $f^+;e^+;nt^-;hms^{ma}$	14	10	8	6	5	19	12	12	29	8	10	16	12	161
4. $f^+;e^+;nt^-;hms^{si}$	0	0	0	0	0	0	0	1	0	0	0	0	0	1
5. $f^+;e^-;nt^+;hms^{ma}$	16	14	15	15	9	17	12	14	23	13	11	12	23	194
6. $f^+;e^-;nt^+;hms^{si}$	0	0	0	0	0	0	0	0	1	0	0	0	0	1
7. $f^+;e^-;nt^-;hms^{ma}$	15	18	11	10	10	10	7	9	27	7	8	7	14	153
8. $f^+;e^-;nt^-;hms^{si}$	1	0	0	0	0	0	0	0	0	0	0	0	0	1
9. $f^-;e^+;nt^+;hms^{ma}$	2	0	2	0	1	2	2	1	1	0	1	1	0	13
10. $f^-;e^+;nt^+;hms^{si}$	10	7	7	7	4	12	11	11	22	4	9	8	9	121
11. $f^-;e^+;nt^-;hms^{ma}$	3	1	0	1	0	3	1	0	4	1	1	0	3	18
12. $f^-;e^+;nt^-;hms^{si}$	11	3	2	1	5	8	6	10	17	3	6	6	10	88
13. $f^-;e^-;nt^+;hms^{ma}$	2	0	0	0	1	3	3	2	2	0	2	2	1	18
14. $f^-;e^-;nt^+;hms^{si}$	9	8	4	6	7	8	9	4	12	3	11	8	6	95
15. $f^-;e^-;nt^-;hms^{ma}$	1	1	2	1	1	0	3	0	2	0	2	0	1	14
16. $f^-;e^-;nt^-;hms^{si}$	6	6	9	3	7	6	12	5	12	3	3	7	7	86
Total	112	79	68	61	59	98	92	85	167	51	78	82	104	1136

Footnote for Table 4:

Males from a *D. simulans* marker strain with the *D. simulans* allele of the *hms* gene (f^-/Y; e^-/e^-; nt^-/nt^-; hms^{si}) were crossed to females heterozygous for the *hms* gene (f^+/f^+; e^+/e^+; nt^+/nt^+; hms^{si}/hms^{ma}). Female offspring produced by the above cross (f^+/f^-; e^+/e^-; nt^+/nt^-, hms^{si}/hms^{ma} and f^+/f^-; e^+/e^-; nt^+/nt^-, hms^{si}/hms^{si}) were individually backcrossed to the males of the marker strain. Only the males produced by the f^+/f^-; e^+/e^-; nt^+/nt^-, hms^{si}/hms^{ma} females were scored for marker phenotypes and fertility. The males produced by females with the genotype f^+/f^-; e^+/e^-; nt^+/nt^-, hms^{si}/hms^{si} were discarded. The numbers of males of each genotype produced by each female (or family) are shown in the table, which are used to calculate the recombination frequencies between the *hms* gene and each of the three marker genes (see Table 5).

Table 5

Recombination frequencies (r) between the hybrid male sterility gene (*hms*) and the markers.

Gene pair	No. of recombinants	Total	r ± S. E. (%)
hms-f	68	1136	6.0 ± 0.66
hms-e	591	1136	52.0 ± 1.18
hms-nt	565	1136	49.7 ± 1.35
f-e	589	1136	51.8 ± 1.44
f-nt	563	1136	49.6 ± 1.28
e-nt	576	1136	50.7 ± 1.27

The r values are weighted means calculated from the data on 13 families presented in Table 4.

Table 6

Tests of the 1:1 segregation ratio of the three markers and the male sterility gene (*hms*^{si}-*hms*^{ma}) in the mapping cross.

Genotypes	Number of males	χ^2	p
e$^+$/*e*$^-$	574	0.13	> 0.5
e$^-$/*e*$^-$	562		
nt$^+$/*nt*$^-$	614	7.45	< 0.01
nt$^-$/*nt*$^-$	522		
f$^+$	683	46.57	< 0.001
f$^-$	453		
hms^{ma}	741	105.38	< 0.001
hms^{si}	395		

The mapping cross is a test cross and the expected segregation ratio of every marker and the male sterility gene is 1:1. However, only one locus showed a 1:1 segregation ratio. The segregation ratios of the other two markers and the male sterility gene are significantly different from 1:1.

CHAPTER 6

GENERAL DISCUSSION

In the previous three Chapters I have presented data which address specific aspects of hybrid male sterility in the three species of *Drosophila melenogaster* subgroup. Here I discuss the implications of these results on a number of general questions regarding hybrid male sterility and Haldane's rule.

6.1 The number of genes responsible for hybrid male sterility

The backcross analyses of Coyne (1984), Coyne and Kreitman (1986) have shown that all the five markers they used (each on a major chromosomal arm) is associated with male sterility in species pairs *D. simulans* / *D. mauritiana* and *D. simulans* / *D. sechellia*. As the X chromosome appears to have the highest concentration of sterility genes (Coyne and Orr 1989), Coyne and Charlesworth (1989) introgessed three regions of the *D. mauritiana* (or *D. sechellia*) X chromosome into *D. simulans* and mapped three sterility genes using a maximum likelihood method. The authors stress that this is the maximum number of genes that could be identified and the actual

number of genes affecting sterility is probably much greater.

Two of the three regions of *D. sechellia* X chromosome have been further studied by Johnson (1992) using molecular marker assisted introgression method. No additional sterility genes were found. Furthermore, a region comprising approximately 1/4 of the X chromosome of *D. sechellia* (from 13F to 18CD on the polytene chromosome) was found not to possess any hybrid sterility factor, when introgressed into the background of *D. simulans* (Johnson 1992). The 2DE data and the genetic analysis data described in Chapter 4 show that the number of genes involved in hybrid male sterility in this species pair is not large, instead, a small number of genes may be involved.

The question concerning the number of genes involved in hybrid sterility is only meaningful if the age of the species pair is considered. There may be more genes causing hybrid sterility in old species pairs than in younger species pairs. We must differentiate between the number of genes initially required for complete hybrid sterility and the total number of hybrid sterility genes that can be measured in the present day species. Hybrid sterility in young species pairs may involve a very small number of genes and may have a fairly simple genetic basis. This is evident from the studies in *Drosophila pseudoobscura pseudoobscura* and *D. p. bogotana* (Orr 1989b and personal communication), in which most chromosomal regions carry no sterility factor and the hybrid male sterility is almost solely due to a small region of the X chromosome.

The simple genetic basis for hybrid inviability has been demonstrated by one of the most remarkable discoveries in the study of reproductive isolation: mutations on single genes can rescue inviable hybrids from crosses between *D. melanogaster* and *D. simulans* (Watanabe 1979; Hutter and Ashburner 1987; Hutter et al. 1990; Sawamura, Taira and Watanabe 1993; Sawamura, Yamamoto and Watanabe 1993; Sawamura and Yamamoto 1993). Although, it remains to be shown if the genetic basis of hybrid inviability in most closely related species pairs is that simple.

6.2 The nature of genic interactions underlying hybrid male sterility

Hybrid sterility and inviability result from epistatic interactions of genes from different species. The nature of such interactions is essential to the understanding of both the development (or origin) of the incompatibility (i.e., hybrid sterility and inviability) and the genetic basis of Haldane's rule. From the review of all reported cases (Chapter 1), it seems that hybrid sterility and inviability can arise from any possible interaction between heterospecific chromosomes. The X-autosome interaction is the most commonly observed form of interaction. Coyne (1985) showed that hybrid male sterility between *Drosophila simulans* and *D. mauritiana* (or *D. sechellia*) was primarily due to an interaction between X and Y chromosomes and such an X-Y interaction was most likely to be the genetic basis of Haldane's rule. In Chapter 3, I demonstrated that *D.*

sechellia Y chromosome is compatible with *D. simulans* X chromosome, and therefore ruling out the X-Y interaction as a general explanation of Haldane's rule. The same result has been obtained independently by Johnson et al. (1992). I have also shown that an X-autosome interaction is involved for hybrid male sterility in this species pair and this X-autosome interaction is of an asymmetrical type.

The asymmetrical nature of incompatible interactions between genes from different species seems to be a very common pattern and it has been revealed in most species pairs where data regarding sterility interactions are available (Wu and Backenbach 1983; Wu et al. 1992; Vigneault and Zouros 1986; Zouros, Lofdahl and Martin 1988). For example, the *se-sh* region of *D. pseudoobscura* X chromosome is compatible with the genetic background of *D. persimilis* whereas the reciprocal introgression results in male sterility (Wu and Backenbach 1983). As discussed in Chapter 3 the asymmetrical interactions (asymmetrical X-autosome interaction or Y-autosome interaction) explain the often observed unidirectional hybrid sterility and inviability (nonreciprocal hybrid sterility and inviability). By extrapolation, the asymmetrical model can also account for the commonly observed unidirectional mating [mating between two species in one direction is easier (or more difficult) than that in the reciprocal direction (Kaneshiro 1976)] in a similar way. The asymmetrical nature of the interactions and the genetic divergence and population processes that lead to it may provide a

key to the understanding of the evolution of reproductive isolation between animal species.

6.3 The nature of genes involved in hybrid male sterility

A central question concerning the nature of genes involved in reproductive isolation remains unanswered: *i.e.*, is hybrid sterility or inviability caused by cumulative effect of many minor genes, or by the action of a few major genes, each with a discrete effect? The involvement of major genes on hybrid inviability is shown by the isolation of mutations that rescue inviable hybrids (Watanabe 1979; Hutter and Ashburner 1987; Hutter, Roote and Ashburner 1990; Sawamura, Taira and Watanabe 1993; Sawamura, Yamamoto and Watanabe 1993; Sawamura and Yamamoto 1993). However, for hybrid sterility, the issue remains controversial. Although by using marker-assisted introgression of chromosome segments, a number of major genes have been identified and mapped (Coyne and Charlesworth 1986, 1989; Pantazidis and Zouros 1988; Orr 1989b; Pantazidis, Galanopoulos and Zouros 1993; Perez, Wu, Johnson and Wu 1993), the introgressed segments are usually large and it is difficult to determine if the effects of the introgressed segments are due to mainly major genes or only to a large number of linked minor genes.

The introgression studies with *D. buzzatii* group species (Naveira and Fontdevila 1986, 1991a, b) show that the effects of most introgressed segments are due to large number of genes each

with small effect (polygenes). On the other hand, in species pairs *D. simulans* / *D. mauritiana*, and *D. simulans* / *D. sechellia*, Coyne and Charlesworth (1989) introgressed three marked regions of *D. mauritiana* (or *D. sechellia*) into *D. simulans* and mapped three major genes on the X chromosome. However, contradictory results have been obtained in the same pair of species for the same introgressed regions. Naveira (1992) introgressed two of the three regions studied by Coyne and Charlesworth (1989). Two regions linked to forked locus and yellow-white loci did not cause sterility when independently introgressed from *D. mauritiana* (or *D. sechellia*) to *D. simulans*, but when introgressed together they caused complete sterility. He concluded that the sterility effects associated with these regions are polygenic, and, therefore raised the possibility that most of the previously identified "major genes" (Coyne and Charlesworth 1986, 1989; Pantazidis and Zouros 1988; Orr 1989b) may be polygenes. Similar introgressions in these three species have been also carried out in Wu's lab (Chung-I Wu, personal communication) and similar results have been obtained.

It is possible the regions around the *forked* locus and the *yellow-white* loci carry both major genes and polygenes and different studies introgressed different subregions which carry different types of genes. Although it is difficult to definitely prove the existence of a major gene, but why all the polygenes causing complete sterility should be clustered in a region of 200 kb in the study of Perez et al. (1993) or in a region 250 kb in the study of Orr (1992). Polygenic mutations causing incompatibility between sibling

species are expected to occur randomly in the genome. The probability of occurrence of a number of incompatible mutations causing complete sterility in a very small region of a chromosome would be very small. The same argument applies to the gene described in Chapter 5. This gene caused complete hybrid male sterility and segregated in a 1:1 ratio for more than 45 generations.

The data presented in Chapter 5 suggest that both major and minor genes are involved in hybrid male sterility. This is shown by the fact that hybrid male sterility can arise from multiple developmental defects producing atrophied testes (type 1 males), failing in sperm production (type 2 males) or affecting the amount of sperm produced (type 3 males). The disappearance of type 1 males (atrophied testes) in the first few generations of the backcross experiment (Table 2, Chapter 5) indicates that this type of sterility may involve polygenes. On the other hand, the sterility of type 2 males (aspermic testes) may involve major genes and possibly minor genes as well. The separation of the two types of male sterility would help resolve the issue of major-minor genes.

The general method for detecting major hybrid male sterility genes described in Chapter 5 can be used in any species pair which produce unisexual hybrid sterility (which is the most common form of reproductive isolation between closely related animal species). It is meant to detect only major genes. By using this method with other species pairs, we should be able to tell if in these species pairs any major genes are involved.

One way to prove that an identified "major" gene is a major gene is to screen for mutants which can restore fertility of males carrying the sterility gene. It can not only prove the major gene, but also it provides information on how the sterility gene causes male sterility and provides clue to molecular isolation of the genes.

6.4 Is the model of fast evolution of X chromosome the correct explanation of Haldane's rule?

The problems associated with the model of fast evolution of X chromosome have been discussed in Chapter 1. The first line of experimental evidence against this model is the data on the divergent 2DE proteins on different chromosomes presented in Chapter 4, which provide a direct test of this model. The results show that the X chromosome has not evolved faster than the autosomes in *Drosophila simulans*, *D. mauritiana* and *D. sechellia*. On the other hand, the number of divergent proteins on the X chromosome is far fewer than expected based on its genome size. This indicates that the X chromosome may evolve slower than autosomes and Haldane's rule is not a result of fast evolution of X chromosome as specified in the model proposed by Charlesworth, Coyne and Barton (1987) and Coyne and Orr (1989).

On the other hand, the slower evolution of X chromosome than autosomes also has a theoretical basis. The major argument for the fast evolution of the X chromosome is that recessive advantageous and underdominant alleles will more often be fixed if they are on the

X chromosome than if they are on the autosomes. However, this argument is weighted by how often these recessive advantageous mutations occur. If recessive advantageous mutations are rare events or most mutations are deleterious or neutral (Ohta 1973), the X chromosome should evolve slower than autosomes because recessive deleterious mutations are more rapidly eliminated when they are X-linked than when they are on autosomes. Autosomal recessive deleterious mutations will be maintained in a higher frequency for a longer period of time than if they are X-linked. These deleterious mutations may change their fitness values when the environment and/or the genetic background changes. Faster elimination of X linked deleterious mutations results in lower polymorphism of X-linked loci, for which there is good evidence (Aquadro 1992, Begun and Aquadro 1992). A compilation of DNA sequences of a number of genes of *Drosophila melanogaster* and *D. simulans* by Begun and Aquadro (1992) shows that the X-linked genes are much less variable than autosomal genes. The nucleotide divergence of these X-linked genes are also slightly smaller than autosomal genes.

6.5 What accounts for Haldane's rule?

Since Jerry Coyne (1985) tested the X-autosome imbalance theory, a variety of hypotheses have been proposed to account for Haldane's rule (reviewed in Chapter 1). However, none of them provides a completely satisfactory explanation (Coyne 1992). Could the failure in finding a satisfactory explanation be because a correct

hypothesis has been rejected? As discussed in Chapter 1, Coyne's (1985) test on sterility is not a good test and a more proper test by Orr (1993) shows that the X-autosome imbalance theory is correct.

Recently, Wu and Davis (1993) reviewed the problems associated with different hypotheses for Haldane's rule, and asserted that hybrid sterility and inviability of Haldane's rule have different genetic bases. They further propose that Haldane's rule is a composite rule and it has no unitary explanation. Orr (1993) also interprets his results that Haldane's rule has multiple causes after demonstrating that the X-autosome imbalance theory works when tested on inviability. However, I find the argument that Haldane's rule is a composite rule as misleading. It does not help in understanding or finding the genetic basis of Haldane's rule. The pattern of the loss of fitness of heterogametic sex applies to both sterility and inviability. This implies that the phenomenon results from a common cause rather than being coincidental.

Male sterility and female sterility may involve different genes, and the actual genetic causes are not expected to be the same. The causes of sterility and inviability may be more different, because the developmental pathways involved are very much different. These differences are intuitive and it is conceptually misleading to use these differences to argue that Haldane's rule has multiple causes. On the other hand, in spite of these differences Haldane's rule still holds for both sterility and inviability, which indicates the existence of a common basis of the rule.

Haldane's rule applies to heterogametic males as well as heterogametic females. The common basis of Haldane's rule must therefore be connected not with sex, but with sex chromosomes. The differences of sex chromosomes between sexes has to be the starting place to explain why heterogametic sex is preferentially affected in hybrids. The first obvious difference is the presence of the Y chromosome (or the W chromosome) in heterogametic sex. Starting from this difference, we have the X-Y interaction hypothesis for which there is little evidence. In addition, as the Y chromosome carries very few genes, the X-Y interaction is not expected to play a central role in underlying Haldane's rule. In Drosophila, Y chromosome is not necessary for viability. The second major difference of sex chromosomes between sexes is the haploidy in heterogametic sex and diploidy in homogametic sex. The consequence of this difference is that both dominant and recessive sex-linked genes are expressed in heterogametic sex and only dominant genes are expressed in homogametic sex. In interspecific hybrids, only sex-linked recessive incompatible genes contribute to Haldane's rule. Sex-linked dominant and autosomal incompatible genes affect both sexes and therefore do not contribute to Haldane's rule. A simple explanation of Haldane's rule is therefore that Haldane's rule results from expression of some recessive sex-linked incompatible genes. In the hybrids of heterogametic sex, the sex-linked incompatible genes are incompatible with genes from alien autosomes. The incompatibility can be sterility, inviability or any other disharmony causing loss of fitness.

This explanation is essentially the same as Muller's (1940, 1942) X-autosome interaction (hemizygosity) hypothesis which has been mixed and "killed" together with Dobzhansky's (1937a) X-autosome imbalance hypothesis in the literature. Muller's hypothesis states that either absence of some sex-linked genes which are necessary complements for those on the autosomes, or expression of some sex-linked deleterious genes in heterogametic sex can lead to sterility or inviability. It is clear that Muller's hypothesis emphasizes the importance of sex-linked recessive incompatible genes in causing hybrid sterility and inviability of heterogametic sex. This hypothesis has largely been ignored in the literature for the reason of being mixed with the X-autosome imbalance. Muller's hypothesis emphasizes the incompatibility at gene level, whereas Dobzhansky's X-autosome imbalance is concerned with incompatibility (imbalance) at the chromosomal level. They are two distinct hypotheses implying very different underlying genetic causes.

One prediction of this explanation is the sex chromosome-autosome interaction which is the most commonly observed type of interaction (see review in Chapter 1). Although other types of interactions (e.g., autosome-autosome interaction) have also been observed, they are not involved in F_1 but in backcross hybrid sterility and inviability. For example, interactions between *Drosophila hydei* fourth chromosome and the third chromosome of *D. neohydei* result in both male and female sterility. The sterility occurs only in backcross progeny as F_1 hybrids between these two species are fertile. Another prediction of this explanation is that the

incompatible genes, i.e., hybrid sterility and inviability genes, are often recessive. Although there are very few well described incompatible genes, the available data do show a recessive nature of incompatible genes. The well studied *SMF* (sperm motility factor) (Vigneult and Zouros 1986; Pantazidis, Galanopoulos and Zouros 1993) is recessive, two copies of the *SMF* are requireed to cause sperm immortility. Another example is the study of Schafer (1978), in which the recessive nature of the autosomal incompatible genes is obvious. The autosomes involved in X-autosome, autosome-autosome and Y-autosome interactions have to be homozygous to produce sterility effects.

This explanation is simple and has virtually no conflict with any experimental data. As a matter of fact, more and more studies are lending support to this hypothesis. The issue would soon be resolved by further studies on hybrid sterility and inviability in different groups of species, because it is a simple testable hypothesis.

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