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 F1 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 F1 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.

G
enetic 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
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; ZOYROS 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; JANCEWSKI, 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, F1 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 F1 hybrids, and most of the divergent proteins (showing both quantitative and qualitative differences) were expressed in F1 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) females to *D. sechellia* (denoted as S) males were denoted as sS. The sS hybrid females were backcrossed to *D. simulans* males, and the offspring were produced as denoted as sSs. All the species stocks and crosses were reared in 8-dram vials containing banana medium at 25°C. Hybrid and backcross males were classified into four types as described in Zeng and Singh (1993). Type 1 males (T1) have atrophied testes and aspermic seminal vesicles, type 2 males (T2) have normal testes and collapsed seminal vesicles (containing no (T2s) or a few sperm (T2z)) type 3 males (T3) 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 (T4) 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 (1988), 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 1988, 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% amylolytic of pH 3–10 (Hochstrasser et al. 1988). The tissues were dissolved in sample solution quickly by a brief vortex 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 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 H₃PO₄ and 20 mM NaOH have much lower conductivity than 0.2% H₂SO₄ and 0.5% ethanalamide and thus allow low current and much higher voltage to be applied to IEF gel (high voltage is required to focus proteins to their pl 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 pl 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% amylolytic (pH 5–7 and pH 3–10 biolyte 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 10,000 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% bromanphenol blue. The tube gels were then immediately run in the second dimension.
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 mm³. 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 Merrill, Dunan and

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 pl marker proteins (pl from
3.5 to 9.5) two red Drosophila pigment markers (a basic
and an acidic pigment, which were always focused at two ex-
treme 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 Protein 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 (overlap-
ing), 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 x 7 (42) 3 cm squares that were numbered from low pl
and low molecular weight to high pl 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
F1 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 to 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 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, F1 hybrids and
sterile and fertile backcross males is available upon request.

RESULTS

Morphology and anatomy of the F1 hybrid males:
The only reliable morphological difference between
the two species is in the shape of the male genital
arches (Coyné 1983; Coyné and Kreitman 1986).

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Male fertility estimates based on testis types in D. simulans, D. sechellia, their F1 hybrids and backcross progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testis types</td>
<td>simulans (s)</td>
</tr>
<tr>
<td>T1</td>
<td>2</td>
</tr>
<tr>
<td>T2</td>
<td>34</td>
</tr>
<tr>
<td>T3</td>
<td>0</td>
</tr>
<tr>
<td>T4</td>
<td>669</td>
</tr>
<tr>
<td>Total</td>
<td>705</td>
</tr>
<tr>
<td>T1 + T4 (%)</td>
<td>94.9%</td>
</tr>
</tbody>
</table>

The genital arches of F1 hybrid males (D. simulans (s) × D. sechellia (sS)) were found to be intermediate
between those of the parents (Coyné 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
atrophyed testes (type 1 testes) (Table 1). The rest of
the reproductive tract (e.g., accessory glands, vas defer-
s and sperm pumps) appeared normal.

Male fertility: Table 1 shows proportions of the
different types of males in the two parental species, F1
hybrids and backcross progeny. D. simulans (s) and D.
sechellia (sS) had very high frequencies (over 94%) of
type 3 and type 4 males. Most of the F1 hybrid males
(ss) were type 2 (88.1%); no type 3 or type 4 males
were observed. When hybrid females were back-
crossed to D. simulans males, the backcross (ssss) males
showed a wide distribution of testis types and had a
high fertility (23.6%, Table 1).

Protein patterns of parental male testes and spe-
cies divergence: As hybrid male sterility results from
lack of mature sperm, the most relevant tissue—
testis—was investigated by using 2DE. Two-dimen-
sional 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
and fertile backcross males. The sterile males used in
but also most of the unshared spots, between the two
pressed in the hybrids. Not only all the shared spots,
detected in the two species, the genetic divergence is
specific protein spots and the total number of spots
hybrids was compared with that of the parental species,
protein spots were found to be present
species were detected in F1 hybrids (see Figure 1).
This was expected as the hybrid males contained one
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 F1 and backcross male testes:
When the 2D protein profile of the F1 (s,s) 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 F1 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 spe-
cies-specific spots) or between the parental species and
their F1 hybrids were numbered and compared among
the two parental species, the F1 hybrids, and the sterile
and fertile backcross males. The sterile males used in

```
<table>
<thead>
<tr>
<th>Total number of spots detected by PDQUEST</th>
<th>D. simulans</th>
<th>D. sechellia</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1234</td>
<td>1182</td>
<td>2416</td>
<td>0.069</td>
</tr>
<tr>
<td>Number of valid spots detected by PDQUEST</td>
<td>1063</td>
<td>983</td>
<td>2046</td>
</tr>
<tr>
<td>Total number of spots detected by PDQUEST</td>
<td>1217</td>
<td>1215</td>
<td>2432</td>
</tr>
<tr>
<td>Number of species specific spots</td>
<td>89</td>
<td>79</td>
<td>168</td>
</tr>
</tbody>
</table>
```

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.

```
<table>
<thead>
<tr>
<th>Protein spot presence in</th>
<th>No. of protein spots</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>88</td>
</tr>
<tr>
<td>-</td>
<td>78</td>
</tr>
<tr>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>+</td>
<td>63</td>
</tr>
</tbody>
</table>
```

F1 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.

### A summary of species distinguishing protein spots with respect to their density patterns in F1 hybrids

```
<table>
<thead>
<tr>
<th>F1 spot density</th>
<th>Number of spots</th>
<th>Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Higher than the high parent</td>
<td>5</td>
<td>0.026</td>
</tr>
<tr>
<td>The same as the high parent</td>
<td>167</td>
<td>0.723</td>
</tr>
<tr>
<td>Intermediate between the two</td>
<td>42</td>
<td>0.182</td>
</tr>
<tr>
<td>parents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>The same as the low parent</td>
<td>16</td>
<td>0.069</td>
</tr>
<tr>
<td>Lower than the low parent</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>231</td>
<td></td>
</tr>
</tbody>
</table>
```

Only spots showing species differences in density were compared between the parents and their hybrids.
was suppressed in the hybrids (Table 4). If the expression of a protein in F1 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 F1 hybrids was observed.

In $s_s$s, 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 F1 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 F1 ($s_s$s) 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) should be present in F1 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 F1 ($s_s$s) hybrid males. The genetic composition of the $s_s$s, backcross males is different from that of F1 hybrid males. Every $s_s$s, 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 $10^{-5}$. 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 $s_s$s, backcross offspring. Knowing the genetic composition of the F1 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 F1 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 F1 ($s_s$s), they cannot be on the Y chromosome because F1 ($s_s$s) hybrid males do not carry the D. simulans Y chromosome. The absence from F1, 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 F1 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 F1 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 F1 hybrid males; so they could be on the autosomes or the Y chromosome but they cannot be on the X chromosome simply because F1 ($s_s$s) males do not carry D. sechellia X chromosome (the reciprocal F1 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 ($s_s$s) 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 F1 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 F1 and backcross males that possess the cytoplasmic factors of D. simulans. Of the 79 D.
sechellia-specific proteins, none was absent from both the F₁ 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 D. simulans backcross males, a complete set of D. simulans autosomes and the D. simulans Y chromosome are present in every backcross male. Therefore, any D. sechellia gene that is incompatible with D. simulans autosomal or Y-linked genes (in the background of D. simulans 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-

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**TABLE 5**

Association of species-specific proteins with different chromosomes based on their presence/absence in F₁ and backcross males between D. simulans and D. sechellia

<table>
<thead>
<tr>
<th>Presence/absence (+/-) in F₁/sechellia*</th>
<th>D. simulans specific proteins (N = 89)</th>
<th>D. sechellia specific proteins (N = 79)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of proteins</td>
<td>+/+</td>
<td>+/-</td>
</tr>
<tr>
<td>88</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Association, or lack thereof (yes/no), between proteins and chromosomes:

- **Autosomes**
  - Yes
  - No
  - No
  - No
  - Yes

- **X chromosome**
  - Yes
  - No
  - No
  - No
  - No

- **Y chromosome**
  - No
  - No
  - Yes
  - No
  - No
  - No

- **Cytoplasmic factors**
  - Yes
  - No
  - No
  - No
  - No

* Three types of backcross males (s,sLs,s,T4, s,sLs,s,T3 and s,sLs,s,T4, 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,sLs,s,T4 males.

**TABLE 6**

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

<table>
<thead>
<tr>
<th>2DE proteins</th>
<th>Male types</th>
<th>20-2</th>
<th>20-14</th>
<th>21-17</th>
<th>33-5</th>
<th>38-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. simulans</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D. sechellia</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>s,sLs,s,T1</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>s,sLs,s,T2</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>0</td>
<td>*</td>
</tr>
<tr>
<td>s,sLs,s,T3</td>
<td>*</td>
<td>*</td>
<td>*</td>
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<td>0</td>
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<tr>
<td>s,sLs,s,T4</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>s,sLs,s,T5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>s,sLs,s,T6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>s,sLs,s,T7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Associated with</th>
<th>Normal amount of sperm</th>
<th>Sperm quantity (if not Y-linked)</th>
<th>Normal amount of sperm</th>
<th>Absence of sperm</th>
<th>Testis atrophy</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. sechellia</td>
<td>Normal amount of sperm</td>
<td>Sperm quantity (if not Y-linked)</td>
<td>Normal amount of sperm</td>
<td>Absence of sperm</td>
<td>Testis atrophy</td>
</tr>
<tr>
<td>D. simulans</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D. sechellia</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>s,sLs,s,T1</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>s,sLs,s,T2</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>s,sLs,s,T3</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>s,sLs,s,T4</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>s,sLs,s,T5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The symbols 0, ~ and * denote for "absence," "faint" and "obvious presence," respectively. s,sLs,s,T4, s,sLs,s,T3 and s,sLs,s,T4 denote backcross males with 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,sLs,s,T4-1, s,sLs,s,T4-2 and s,sLs,s,T4-3 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,sLs,s,T4 males are listed here.
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,sd,T2) and fertile (s,sd,T4) 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 65 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,sd, 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,sd,T2 and s,sd,T2, Table 6), one sample of backcross males with about half the amount of sperm as normal (s,sd,T3) and three independent samples of fertile backcross males (s,sd,T4, 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,sd,T2) and absent from males with a few (s,sd,T2), half (s,sd,T3) and normal amount of sperm (s,sd,T4). As the s,sd,T2 males were a mixture of s,sd,T2 and s,sd,T2 males, the protein 33-5 was present in the s,sd,T2 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,sd,T2), faint in males with some sperm (s,sd,T2, s,sd,T2 and s,sd,T3) and it is absent from males with normal amount of sperm (s,sd,T4). 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,sd,T2 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,sd,T2 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; Pantzidis and Zouros 1988; Orr 1989b, 1992; Naveira and Fontdevila 1991; Coyne 1992; Wu et al. 1992; Pantzidis, 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 F1 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 (Car- Iou 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 F1 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 1999) 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, Guénét 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 (F1 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 genic 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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LITERATURE CITED


