J Mol Evol (1995) 41:1085–1095

JOURNAL OF MOLECULAR EVOLUTION

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

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Received: 30 January 1995 / Accepted: 16 May 1995

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

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

Introduction

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

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

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

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

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

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

Materials and Methods

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

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

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

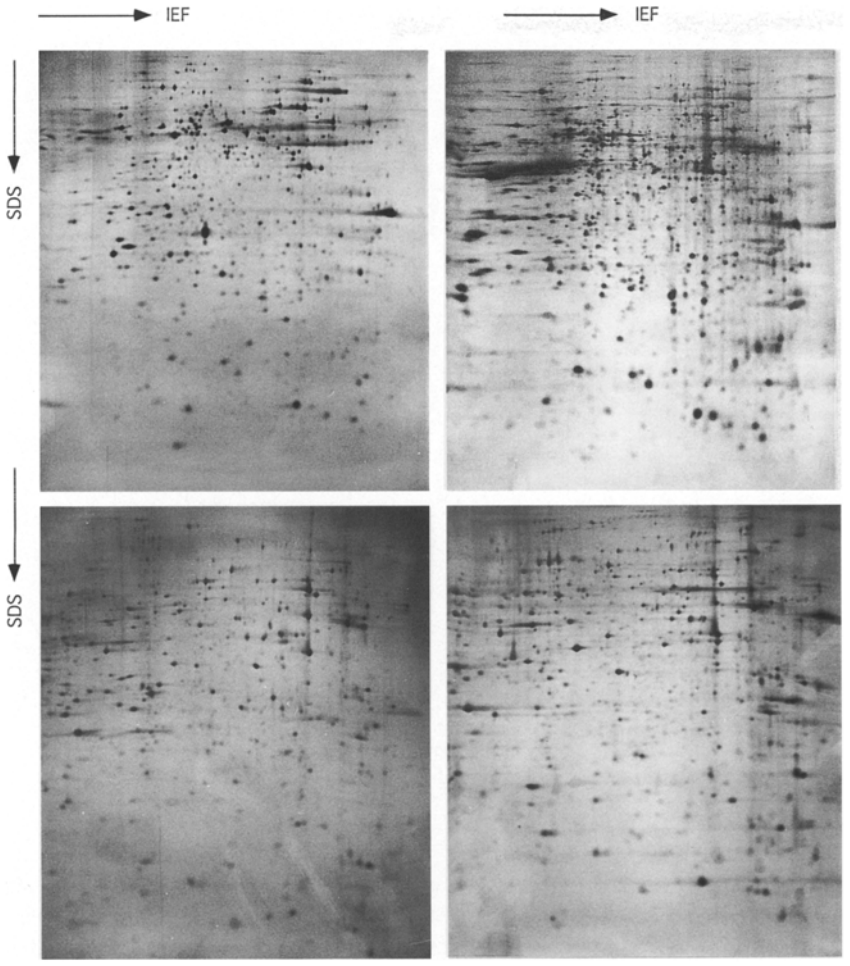

Fig. 1. 2D gels showing protein spots in adult male testes **(upper left),** adult female ovaries **(upper right)** of *Drosophila simulans,* adult malpighian tubules of *Drosophila novamexicana* **(lower left),** and adult brain of *Drosophila americana* **(lower right).** Only well-resolved protein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

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

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

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

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

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

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

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

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

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

Results

High Divergence of Reproductive-Tract Proteins

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

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

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

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

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

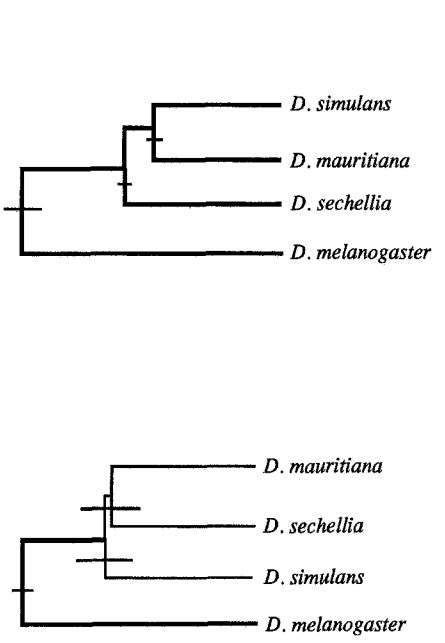

Fig. 2. Phenogram (UPGMA) obtained using the distance values in Table 1. *Thin lines* crossing the branching ones represent the standard deviation associated with the genetic distance estimates. In the case of ovary data, the distance values ($D \pm SD$) among the members of the *simulans* clade overlap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

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

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

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

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

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

[a] Nomenclature as in Table 2

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

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

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

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

[a] NS = nonsignificant results

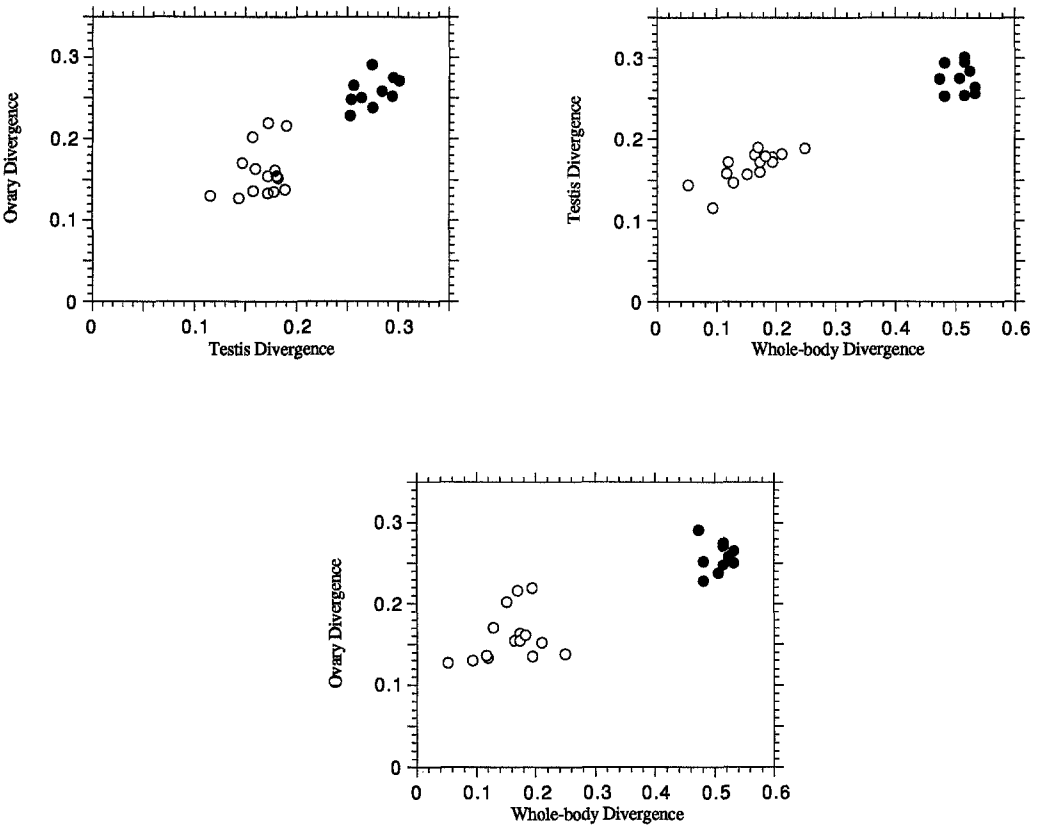

Fig. 3. Correlation between protein divergence ($D = -\ln F$ for testes and ovaries data; $D = -\ln S_{sm}$ for Spicer's whole-body data) in the *virilis* group species estimated from different tissue samples. *Open circles* are data points for species pairs belonging to the same phylad. *Solid circles*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

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

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

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

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

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

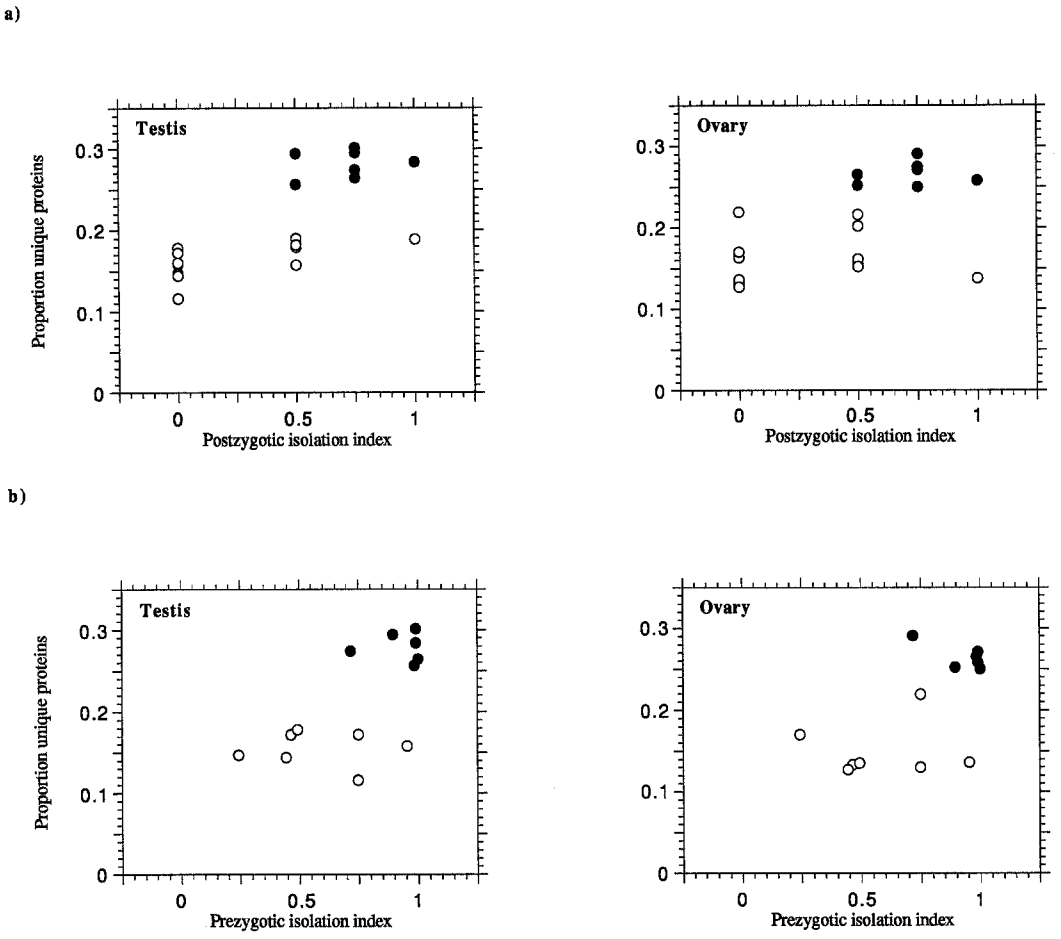

Fig. 4. Correlation between the proportion of unique proteins detected in testis and ovary samples and the level of **(a)** postzygotic reproductive isolation among the *virilis* group species and **(b)** prezygotic reproductive isolation among the *virilis* group species. *Solid and open circles* as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

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

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

Discussion

The Role of Reproductive Tract Proteins in Reproductive Isolation

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

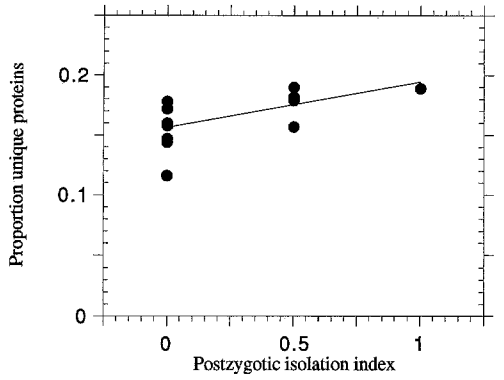

Fig. 5. Correlation between the proportion of unique proteins in testis and postzygotic reproductive isolation among species pairs belonging to the same phylad (either *virilis* or *montana*).

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

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

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

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

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

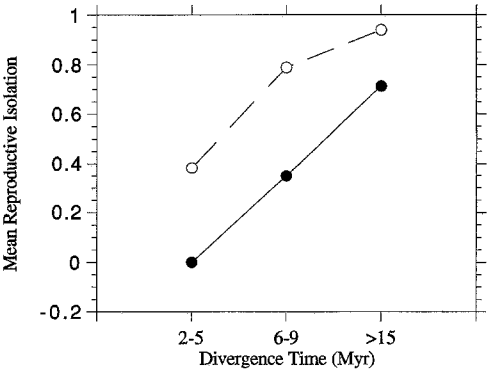

Fig. 6. Mean postzygotic reproductive isolation (*filled circles*) and mean prezygotic reproductive isolation (*open circles*) among species of the *virilis* group plotted against three distinctive species group classes that have diverged for different periods of time.

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

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

Different Rates of Protein Evolution

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

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

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

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

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

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

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

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

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

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

Acknowledgments. This paper constitutes part of the research done by A. Civetta in partial fulfillment of a Ph.D. degree at McMaster University. We would like to thank Dr. Jean David and Dr. Jerry A. Coyne for providing fly stocks. We are grateful to Dr. L.-W. Zeng for technical advice and to Dr. R.A. Morton for comments on the manuscript. This work was supported by a research grant from the Natural Sciences and Engineering Research Council of Canada to R.S.S.

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