GENETIC DIVERGENCE IN SIBLING SPECIES OF *D. melanogaster*

GENETIC DIVERGENCE IN TISSUE AND DEVELOPMENTAL STAGE-SPECIFIC PROTEINS OF SIBLING SPECIES OF *Drosophila melanogaster*

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ABSTRACT

Four sibling species of the Drosophila melanogaster species subgroup (D. melanogaster, D. simulans, D. sechellia and D. mauritiana) were used to study genetic variation at the protein level by improved two-dimensional (2DE) gel electrophoresis. Three of the species, D. simulans, D. sechellia and D. mauritiana, are chromosomally homosequential, but their phylogenetic relationship to each other is a subject of controversy.

Eight tissues representing adult and larval (developmental stage) and reproductive and non-reproductive tissues were analysed for protein variation. The tissues used were as follows: larval testis, brain, haemolymph, wing disc, and adult testis, accessory gland, male and female brain. Close to 400 protein spots were detected per tissue using this sensitive method. Each tissue was compared between species for protein variation. Protein variation was measured on the basis of qualitative differences (presence/ absence) of the protein spots in six pairwise species comparisons and four-way comparisons.

Different levels of protein variation were detected

for the same tissues in different species comparisons and each tissue showed different levels of variation in the same species. Different tissue proteins seemed to evolve independently in different species. But there was no evidence of any trend or pattern to show that either larval or adult patterns are more diverged than the other. The variation between tissues rather than developmental stages appears to be the major determinat of the level of divergence.

The reproductive tract (testis and accessory gland) proteins showed more variation among species comparison than the non-reproductive tissue proteins. Among the four species, D. melanogaster testis proteins, both larval and adult, showed the maximum divergence. From the results it seems that there is a correlation between the level of reproductive tract protein divergence and the degree of reproductive isolation observed among these species. Among the other three closely related species, the levels of divergence of the reproductive tract proteins are similar, but lower than that of D. melanogaster.

The phylogenetic relationship based on 2DE protein divergence showed D. simulans to be closer to D. mauritiana than to D. sechellia.

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INTRODUCTION

The concept of species and the mechanism of speciation are central as well as controversial issues in evolutionary biology. Aristotles' definition of species was typological while Darwin looked at the term species "as one given arbitrarily for convenience sake". The typological concept was also morphological because it was defined on the basis of morphology or "type". Later this was replaced by the biological species concept (Mayr, 1942) also known as the isolation species concept. Since then other species concepts have evolved in order to embrace all important aspects of evolutionary genetic processes. Two other main concepts are the evolutionary and the recognition species concepts (Paterson, 1985). The central criticism of these concepts is that they can be applied only to sexually reproducing organisms (Vrba, 1985). Recently, Templeton (1989) has proposed yet another concept - the cohesion concept. After evaluating the merits and demerits of other definitions he defines species as the most inclusive group of organisms having the potential for genetic and/or demographic exchangeability.

Speciation, by definition, is the process of becoming a species and is taken to represent the formation of the units of evolution (Otte, 1989). Darwin solved the problem of species by showing that species change by "phyletic gradualism" which later came to be recognized as being different from speciation by "cladogenesis" (Singh, 1989). Geographic models of isolation and population differentiation were the proposed mechanisms of speciation before the advent of molecular population biology, except for the theory of macromutational changes involving the whole genome (Goldschmidt, 1940). The lack of knowledge and progress on speciation and the mechanism of speciation are mainly because of the basic problem of studying speciation experimentally and of the doubts as to whether speciation is a separate process independent of adaptation or not (Lewontin, 1974). Study of geographic isolation, adaptation, and quantitative characters are easier than the study of speciation experimentally. So more work has been done on the former and the study of speciation lagged far behind.

1.1 One-Dimensional Electrophoretic Survey of Species Divergence

The new technique of gel electrophoresis was introduced to the study of speciation in a number of different organisms in the early sixties. In this technique the protein products of allelic variants are separated on the basis of charge and visualized by protein or enzyme staining. The widespread application of eletrophoresis revealed more about the nature of genetic variation within and between populations than about the genetic mechanisms of speciation (Hubby and Throckmorton, 1965; Prakash, 1969; Selander, 1969; Ayala and Powell, 1972; Lewontin, 1974). Electrophoretic comparisons of closely related species produced rather similar results and the message was that very little genetic differentiation was needed for speciation (Lewontin 1974; Ayala et al. 1974; Throckmorton 1977). For example, Prakash (1969) found differentiation in only 12.5 % of the loci after comparing 24 loci between Drosophila pseudoobscura and Drosophila persimilis. Kojima et al. (1970) found genetic differentiation at 11.8% of the loci examined between Drosophila melanogaster and Drosophila simulans. Ayala and Powell (1972) studied four sibling species of the Drosophila willistoni group and found that

14%-35% of the loci were differentiated between these species. In all these studies involving sibling species, the apparent absence of alleles which were fixed in one species and lacking in another was quite striking. This lack of genetic differentiation came as a surprise, since Mayer's geographic theory of species formation predicted large amount of genetic differentiation among closely related species. The new revelations of reduced genetic differentiation forced the evolutionary biologists to review their assumptions on species formation. The argument presented was that if fully formed species differed at 15% to 35% of their loci, then a newly formed species would differ at far less than 15% of its loci. The study on Mus musculus musculus and Mus musculus domesticus which are in their second phase of speciation (recontact after geographic isolation) by Selander et al., (1969) showed genetic differentiation at 20% of the loci. The low genetic differentiation among sibling species, sub-species and semi -species supported the theory (Throckmorton and Hubby, 1963) that very little genetic differentiation is required for species formation. The argument was strengthened by pointing out that what little genetic differentiation was observed, included both relevent and irrelevent genetic

changes that have accumulated during and after the speciation event (Throckmorton, 1977).

In order to clarify the argument, many more studies have been done on numerous species over the years. The latest and the largest set of molecular data on genetic differentiation was collected by Singh and Rhomberg (1987) and Choudhary and Singh (1987) on the sibling species of Drosophila melanogaster. This was done to see if the generally small value of genetic divergence among species may have been due to the sampling of a relatively small number of loci. Singh and Rhomberg (1988) have pointed out that over 80% of the studies on genetic variation have sampled less than 30 loci. In order to overcome this drawback, the data were obtained by comparative electrophoretic analyses of 112 structural loci encoding homologous soluble enzymes and abundant soluble proteins in D. melanogaster and D. simulans. An important finding of this study was that the level of genetic variation and species differentiation depended on the types of gene loci sampled. For example, the abundant haemolymph proteins were more polymorphic than the enzymes, and all loci showing complete divergence (7% of the total) were enzymes. In other words none of the abundant protein loci showed

complete species divergence. While 16% of the alleles were unique in D. simulans and 27% in D. melanogaster, most of these alleles tended to be in low frequencies (except of course at the loci that are alternately fixed). In the light of these observations, Singh and Rhomberg (1987) concluded that the enzymes alone do not provide us with a true picture of genic variation, let alone the genetic basis of speciation.

1.2 Two-Dimensional Electrophoretic Survey of Species Divergence

Recently a new and sophisticated technique has been introduced to the study of genic variation in natural populations. Two-dimensional gel electrophoresis (O'Farrell, 1975) combined with silver staining, has tremendously increased the possibility of detecting all proteins of the cells visually. The principle is based on the separation of abundant soluble proteins by a two step process. In the first dimension proteins are separated according to their isoelectric points (pI), and in the second dimension according to their molecular weight (MW). This method gives a very high resolution of protein mixtures because it is very unlikely that two proteins are identical in both pI and MW (Klose, 1983). In order to use this

technique in combination with genetics, it is important that a single charge change can produce a detectable change in protein spot position. In one-dimensional isoelectric focusing, single charge changes are known to produce a significant change in band position (O'Farrell, 1975; Milman et al., 1976; Steinberg et al., 1977; Comings, 1979). In addition to mobility changes, changes in the intensity of protein spots and differences in spot size and shape can also be detected (Klose, 1989; De Vienne et al., 1988). Over the years this tecnique has been improved and modified (Wheeler, 1986; Tindal, 1986; Zazra, 1987) using double labelling, chemical spacers and denaturing agents. The technique of using immobilized pH gradients to prevent cathodic drift and reproducible focusing of the basic proteins has made further improvement (Strahler et al., 1987; Gorg et al., 1987; Hanash et al., 1989). The use of large gels and efficient cooling at the second dimension helped Hochstrasser et al. (1988) to increase the resolution by 1.5-3 fold in spot detection.

The two-dimensional technique has been widely applied in a variety of studies, for example, variation in natural populations (Goldman et al., 1983; Ohnishi et al., 1983), protein polymorphisms in bacteria (Picard et al.,

1987), mutations in human cells (Hanash et al., 1987), total cell proteins in mouse (Klose et al., 1989), molecular phylogeny of the hominoid primates (Goldman et al., 1987) and molecular evolution among some Drosophila species (Spicer, 1988). Two-dimensional gel electrophoresis has also been used to study organ-specific variability and inheritance of maize proteins (Leonardi et al., 1988) and to assess pleiotropic effects of a gene substitution in Peas (Pisum sativum) (Gottlieb et al., 1988).

1.3 An Application of Two-Dimensional Gel Electrophoresis to the species of D. melanogaster Complex

A comparative study of proteins found in reproductive tract tissues and larval wing discs using two-dimensional gel electrophoresis was carried out by Coulthart and Singh (1988c). From these studies, two important observations have emerged. First, the reproductive tract proteins show more divergence between species than non-reproductive tract (larval wing disc) proteins. The overall proportions of completely diverged loci for soluble enzymes studied by 1DE and solubilized proteins studied by 2DE are not very different (about 10% in both cases). The high divergence between species in the

2DE proteins is limited to mostly loci which are monomorphic within species. About 20%-30% of D. melanogaster and D. simulans male-reproductive tract proteins show "presence/absence" type of differences between them, i.e. a protein present in one of the two species lacks a detectable homologous spot in the other species. Differences in protein amounts were also observed among reproductive tract proteins (Coulhart and Singh, 1988). These results raised the question as to whether tissue specific genes and proteins that are expressed during the early stages of development and differentiation may be more conserved than those that are expressed in the later stages. It is important that these results be checked with more tissues and preferably with more species from the D. melanogaster subgroup. The current study was undertaken with this aim in mind.

1.4 The Drosophila melanogaster Species Subgroup

The four species used in this study belong to Drosophila melanogaster species subgroup. This subgroup presently consists of eight species and they are: D. orena, D. erecta, D. yakuba, D. teissieri, D. melanogaster, D. simulans, D. sechellia, and D. mauritiana. The origin of

this subgroup is Afro-tropical. The eight species of the subgroup differ from one another in male genitalia, ecology and patterns of polymorphism of their populations (Lachaise et al., 1988). D. orena and D. erecta are specialists found around mid and south Ivory Coast and are grouped as erecta complex. D. yakuba and D. teissieri are generalists and wide spread on the African mainland and grouped as yakuba complex. The other four species are grouped together as melanogaster complex. D. melanogaster and D. simulans are opportunistic human commensals and are cosmopolitan in their distribution but not always sympatric. D. sechellia and D. mauritiana are insular specialist species. D. sechellia is endemic to Seychelles and D. mauritiana to Mauritius, two islands in the Indian Ocean. In Mauritius, D. mauritiana is broad-niched, opportunistic and a domestic species (David et al., 1987). D. sechellia appears more to be a specialist as it is known to breed only on the fruits of Morinda citrifolia, a plant common in south east Asia but not found in mainland Africa.

The four species of the melanogaster complex are closely related sibling species. By definition sibling species are reproductively isolated species whose morphological differences are cryptic. As mentioned

before, these species can be identified morphologically from each other by the shape of their male genitalia. Even though these four species are closely related, their relation to each other has been a subject of controversy. The relationship of D. melanogaster and D. simulans was not difficult to ascertain since at the time they were the only two species in this complex (Hsu, 1949) and was done on morphological basis. But when new species were discovered which did not conform to the previous definition, it was gradually modified (Bock and Wheeler, 1972; Bock, 1980). It became clear that not a single character but a number of characters should be used to make a correct identification. The recent discovery of D. sechellia (Tsacas and Bachli, 1981) and D. mauritiana (Tsacas and David, 1974) posed the problem of finding their right place in the phylogeny. With the available data on chromosomal, allozymic, mitochondrial DNA, ribosomal DNA, unique DNA sequence and hybridization data, there is general agreement in separating D. melanogaster from the "simulans like" species. The "simulans like" species includes D. simulans, D. sechellia and D. mauritiana. But even after many studies the phylogenetic relationships of D. simulans, D. sechellia and D. mauritiana to each other remains to be controversial.

With the available data and depending on the genetic system used, different phylogenetic trees have emerged. For example, reproductive relationships (Lachaise et al., 1988), cyst length, (Joly, 1987) and mitochondrial DNA variation (Solignac, 1986) show D. simulans closer to D. mauritiana. On the other hand, Adh sequence (Coyne and Krietman, 1986) and some allozyme data (Cariou, 1986) show D. simulans closer to D. sechellia. In the light of all these contradictory evidences the phylogenetic tree A in Figure 1.1 is the one which is most widely accepted (Lachaise et al., 1988). It is hoped that besides, shedding light on the nature of genic differentiation in tissue - and developmental stage - specific proteins, this study may also provide another basis to resolve the phylogenetic relationship of D. simulans, D. sechellia, and D. mauritiana.

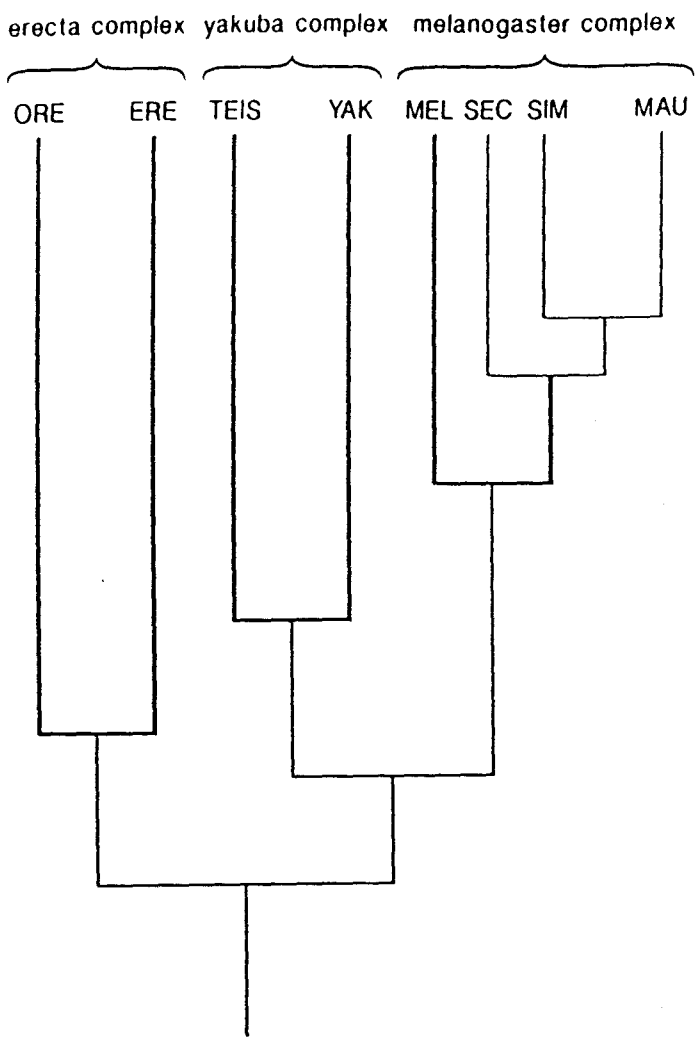


Figure 1.1

Phylogenetic tree A (Lachaise et al., 1989) showing the relationship of the sibling species of D. melanogaster species subgroup.

2

MATERIALS AND METHODS

2.1 Drosophila Stocks

The strains analyzed in the present study are from the four sibling species of Drosophila melanogaster and the information on their origins and sources are as follows:

Species	Strain	Origin	Source
D. melanogaster	Canton Special		Lab strain
D. simulans	Cape Town 1	Cape Town, South Africa (1987)	Dr.J.David
D. mauritiana	LG 24	Mauritius (1987)	Dr.J.David
D. sechellia	Sechellia 12	Mahe, Seychelles	Dr.J.David

2.2 Drosophila Culture

Drosophila strains were maintained as isofemale lines at 22±1°C on banana medium, the composition of which is described in Table 2.1. All the flies were raised in 250 mL glass bottles with 50 mL of banana medium. Efforts were made to keep the culture density below 150-200 individuals per bottle. The adult flies were removed routinely after they had laid eggs for 24 hours to start a new generation.

Table 2.1

Culture Medium (Banana Medium)

Dried brewer's yeast	---60 g
Agar	---20 g
Water	---3.6 L
Banana (medium size)	---2
Sugar	---5 g
Corn syrup	---30 mL
Tegosept soluion (10 g p-hydroxybenzoic acid, methyl ester + 100 mL 95% ethanol)	---36 mL

Boiled agar in 3.6 L of water, added other ingredients (blended) while stirring and boiled again. After the medium was cooled to 45°C, added Tegosept. Stirred well and poured into 250 mL glass bottles.

2.3 Tissue Dissection

2.3.1 Solutions

The sources of chemicals for solutions described in sections 2.3., 2.4 and 2.5 are listed in Table 2.2 along with abbreviations. The recipes are given in terms of weights and volumes required for a final volume of solutions.

The following formula for PIPES - Buffered Ringer's solution is taken directly from Chaney and Shern (1983). The lysis buffer (O'Farrell, 1975) described here is the modified form described by Coulthart (1986).

TABLE 2.2

Sources of Chemicals

Chemical Name	Abbeviation	Source
Acetic Acid (glacial)	---	1
Acrylamide (99.9%)	---	2
Agarose (Type I: Low EEO)	---	5
Ammonium Persulfate	---	3
Biolyte pH 5-7, pH 3-10	---	2
N, N' - Methylene Bisacrylamide	Bis	2
Calcium Chloride (dihydrate)	$CACl_2$ $2H_2O$	3
Citric acid (monohydrate)	---	3
3-[c3-Cholamidopropyl dimethylammonio]	CHAPS	5
1-propanesulfonate Dithiothreitol	DTT	5
Formaldehyde (37% w/v) - Analyzed	---	4
Glucose	---	3
L-lysine (free base)	---	5
Magnesium Sulphate (heptahydrate)	$MgSO_4$ $7H_2O$	
Phosphoric Acid (85% w/v)	H_3PO_4	4
Piperazine-N, N' -bis (2-ethane sulfonic acid)	PIPES	5
Potassium Carbonate (anhydrous)	K_2CO_3	

TABLE 2.2 (Cont'd)

Chemical Name	Abbreviation	Source
Pottassium Chloride	Kcl	3
Silver Nitrate	AgNo3	3
Sodium Carbonate (anhydrous)	Na_2CO_3	3
Sodium Chloride	NaCl	3
Sodium Dodecyl Sulfate	SDS	3
Sodium Hydroxide	NaoH	3
Sucrose	---	3
N, N, N, N'-Tetramethyl Ethylenediamine	TEMED	2
Tris (hydroxymethyl) aminomethane ("sigma 7-9")	Tris	5
Tris (hydroxymethyl) aminomethane	Tris	2
Urea (ultrapure Grade)	---	2

1 - J.T. Baker Chemical Co., Philipsburg, New Jersey

2 - Bio-Rad Laboratories, Richmond, California

3 - BDH Chemicals, Toronto, Ontario

4 - Fisher Scientific Co., Fairlawn, New Jersey

5 - Sigma Chemical Co., St. Louis, Missouri

PIPES - Buffered Ringer's Solution

For 25 ml:

PIPES	--- 665 mg (88 mM)
Glucose	--- 90 mg (20mM)
Sucrose	--- 428 mg (50 mM)
NaCl	--- 80 mg (55 mM)
KCl	--- 75 mg (40 mM)
$MgSo_4 7H_2O$	--- 45 mg (7.3 mM)
0.5 NaOH	--- 5-6 mL
H_2O	--- up to 20 mL

Dissolved all solutes and added

$CaCl_2$ $2H_2O$ (1.02 M)*	--- 1.18 mL (48 mM)
0.5 N NaOH	--- to pH 6.95(25°C)
H_2O	--- to 25 mL

Stored at 4°C.

---

* 1.02 M $CaCl_2$ $2H_2O$ (25 mL):

$CaCl_2$ $2H_2O$	--- 3.76 g
H_2O	--- to 25 mL

Lysis Buffer

For 25 mL:

Urea	--- 14.3 g (9.5 M)
K_2CO_3	--- 17.3 mg (1.5 mM)
L-lysine	--- 12.5 mg (1.5 mM)
DTT	--- 385 mg (100 mM)
H_2O	--- 13.4 mL

Dissolved with gentle warming, dispensed into 0.2 mL aliquots; froze in liquid nitrogen and stored up to 6 months at - 70°C. For use, thawed and added carrier ampholytes to 2% w/v.

2.4 Sample Preparation

For two-dimensional gel electrophoretic analyses of male reproductive tract, accessory gland, adult male and female brain, newly emerged males and females were separated after light anaesthetization with diethyl ether. The males and females were then aged for 4-5 days in 25 x 95 mm glass vials on banana medium before use. To raise larvae for collecting imaginal wing disc (larval wing disc), larval testis, haemolymph, and brain, sexually mature flies were placed in 250 mL culture bottles to lay eggs.

After two hours the flies were cleared and the larvae were allowed to develop for 5-6 days. The third instar larvae were taken from the wall of the culture bottle to make organ tissue samples.

Different organs were dissected with two watchmaker's forceps (A. Dumont et fils, number 5) under cold conditions (adults and larvae were kept on ice). Sharpened tungsten needles were used for tissue cleaning and transfering the tissues. Each larva or adult was dissected in a fresh drop of cold ($4^{o}C$) PIPES -Buffered Ringer's solution. Each tissue was washed before it was collected in a fresh drop of buffer kept on ice.

Lysis buffer for the sample was conveniently prepared in batches of 5 by dividing a thawed 0.02 mL of buffer after adding biolytes(8 uL of 5-7 and 2 uL of 3-10). The 40 uL aliquots in 400 uL polyethylene centrifuge tubes were stored on ice during dissection. The number of tissues varied for different organs, but the volume of lysis buffer was 40 uL for all samples.

From adult male flies ten pairs of testes, ten pairs of accessory glands (with seminal vesicles and anterior ejaculatory duct) and fifteen pairs of brains were dissected, washed and stored on ice until the desired number

was collected. After completion, the 40 uL aliquot was warmed to room temperature and the tissues were quickly transferred to the lysis buffer using a tungsten needle. The suspension was kept at room temperature for one minute, then stirred gently with the plunger of a Hamilton syringe and kept at room temperature for one more minute. Then the lysate was frozen by dipping the tube in liquid nitrogen. The samples were stored at -70^{o}C

Before dissecting different larval organs, the larvae were washed in cold (4^{o}C) PIPES -Buffered Rin solution to remove any trace of medium. Fifteen pairs of brains, twenty pairs of larval testis and twenty pairs of larval wing discs were dissected from washed, mature third instar larvae. After dissection the larval tissues were treated identical to that of adult tissues.

For "haemolymph", nine washed third instar larvae were kept on a slide and the cuticle of each larva was carefully pierced below the anterior spiracle. The haemolymph that oozed out which contained haemocytes and disrupted tissues was collected with a clean Hamilton syringe and was added to the lysis buffer. After keeping at room temperature for two minutes the lysate was frozen and stored at -70^{0C}.

Prior to electrophoresis, the frozen samples were alternately thawed in a 37°C water bath and frozen in liquid nitrogen for a total of five freeze thaw cycles. After the final thaw the samples were centrifuged for eight minutes at 13,000 g in a Beckman micro centrifuge at room temperature. Usually 35 uL of the sample was loaded on the first dimension gel.

2.5 Two-Dimensional Polyacrylamide Gel Electrophoresis

The two-dimensional gel electrophoretic procedures used in the present study are essentially those described by Hochstrasser et al., (1988) with some changes. The primary changes are: a different IEF gel solution (Coulthart, 1986), 2 mm capillary tubes forIEF gels, equilibration buffer (Coulthart, 1986), and agarose overlay method was employed during the transfer of IEF gels to the 2nd dimension resolution gel.

2.5.1 First Dimension Isoelectric Focusing (IEF)

2.5.1.1 Solutions

Gel Solution

For 20 mL:

Urea	--- 11.44 g (9.5 M)
Acrylamide	--- 758 mg (3.7 %)
Bisacrylamide	--- 42.6 mg (0.213 %)
CHAPS	--- 400 mg (2 %)
H_2O	--- 9.5 mL

Dissolved with gentle warming (not above 30°C), filtered, deaerated and added :

Biolyte 3.5 - 10	--- 0.2 mL (0.4% w/v)
Biolyte 5 - 7	--- 0.8 mL (1.6% w/v)

Dissolved, divided into 2.6 mL aliquots, froze in liquid nitrogen and storer at -70°C.

Anolyte

For first dimension gels:

H_3PO_4 (85% w/v)	--- 1.8 mL (6 mM)
H_2O	--- 2.6 mL

Catholyte

10 N NaOH	--- 1 mL(20 mM)
H_2O	--- 500 mL

2.5.1.2 Procedures

To cast four first-dimensional tube gels (2 mm ID, 6 mm OD,180 mm) 2.6 mL of gel solution was thawed at room temperature and stirred thoroughly to dissolve the urea. The capillary tubes were marked at 150 mm from one end. Each capillary was connected to a 1 mL syringe using a small piece of tygon tubing (3/16" ID, 1/4" OD,3/4" in length). Each capillary tube with the syringe connected to the marked end was placed in glass test tubes (12 x75 mm) which were held in a rack. After adding 10.4 uL of 10% freshly prepared ammonium persulfate and 1.56 uL of TEMED, to the thawed gel solution and stirring briefly, 630 uL of the solution was pipetted into each test tube along the inner wall to prevent air bubbles. The capillary tubes were filled by suction to the marked height of 150 mm. After 2-3 hours of polymerisation the capillary tubes were carefully removed after rotating them inside the test tubes. The tubes were pressed against a piece of parafilm to remove the excess acrylamide and the 1 mL syringes were also removed.

The fully polymerised gels were placed in a Hoefer Model GT tube gel apparatus and a drop of anolyte was added to the bottom end of each tube without air bubbles. The tube gels were then placed carefully into the lower reservoir with the anolyte. Each gel was overlaid with 30 uL of catholyte and 35 uL of centrifuged samples were gently layered under the catholyte. After adding 500 mL of catholyte to the upper reservoir, isoelectric focusing was carried out at room temperature with a constant voltage of 200 for 2 hours, 500 volts for 7 hours, followed by 800 volts for 9 hours and a final step of 1000 volts for 2 hours. This change in time and voltage seemed to help in attaining reproducible focusing. For some samples it was necessary to run them at 1000 volts for 4-5 hours to get the basic end of the gel focused. The higher voltage during the last step helped to increase the resolution of the gel. BIO-RAD MODEL 1000/500 power supply with a programmable step mode was used for isoelectric focusing.

2.5.2 Second dimension: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS - PAGE)

2.5.2.1 Solutions

<u>20% SDS Stock</u>

SDS	--- 20 g (20% w/v)
H_2O	--- 90 mL

Dissolved by warming, add H_2O --- to 100 mL

Stored at room temperature in an amber bottle .

<u>10% Ammonium Persulfate</u>

Ammonium Persulfate	--- 100 mg (10% w/v)
H_2O	--- 940 uL

Made fresh for first dimension and second dimension gel solutions.

<u>Acrylamide/Bis Stock Solution</u>

Acrylamide	--- 39.0 g
Bis	--- 1.0 g (40% T/2.5% C)
H_2O	--- to 100 mL

Resolution Gel Buffer Stock Solution

Tris (Bio - Rad)	--- 18.3 g (1.51 M)
1N HCl	--- 20 -22 mL
H_2O	--- 80 mL
Dissolved, added 1 N HCl	--- to pH 8.8 (25°C)
H_2O	--- to 100 mL

Stored in an amber bottle at 4°C.

Cathode Buffer

SDS	--- 0.8 g
Tris	--- 4.8 g
Glycine	--- 23.04 g
H_2O	--- to 800 mL

Made fresh each time.

Anode Buffer

SDS	--- 20 g
Tris	--- 120 g
Glycine	--- 576 g
H_2O	--- to 20 L

Can be used five times if stored at 4°C.

SDS Equilibration Buffer

Resolution gel buffer stock	--- 6.25 mL
Glycerol	--- 1.25 mL (5% w/v)
20% SDS	--- 2.875 mL (2.3% w/v)
DTT	--- 77 mg
0.1% (w/v) Phenol red	--- 1 drop
H_2O	--- to 50 mL

Made fresh every time.

Agarose Solution

Agarose (Sigma Type)	--- 0.3 g (1% w/v)
Resolution gel buffer stock	--- 7.5 mL
20% SDS	--- 0.15 mL
H_2O	--- 23 mL

Boiled to dissolve agarose.

2.5.2.2 Second Dimension Resolution Gel Preparation

The second dimension separations were carried out in the Protean II 2-D Multi cell apparatus from BIO-RAD in which six gels can be run simultaneously. In the present study, four gels were run simultaneously in order to run one sample from each species under the same conditions. The resolution gels measured 160 mm x 20 mm x 1.5 mm thick.

A 12% T/2.5% C resolution gel solution was usually prepared. Four gels were cast on the casting stand using the Protean II sandwich clamps with a single screw mechanism. To prepare 230 mL of gel solution, 57.5 mL of resolution gel buffer stock, 102.6 mL of distilled water and 69 mL of Acrylamide/Bis Stock Solutions were mixed and suction filtered through a Whatman #3 paper into a 250 mL side arm flask and the solution was deaerated for 60-70 minutes.

After breaking the vacuum, the solution was poured into a 500 mL beaker, 736 uL of 10% ammonium persulfate and 138 uL TEMED were added and the solution was stirred briefly. SDS was ommitted from the resolution gel (Hochstrasser et al., 1988). The gel solution was quickly drawn into a 60 mL plastic syringe. A blunted needle with 5mm long polyethylene tubing was placed onto the filled syringe. The needle with the plastic tubing was placed just above the beveled inner glass plate and gel cassettes were quickly filled with gel solution to the top and was immediately overlaid with 0.5 mL of 0.1% SDS solution. All the four gels were poured in the same manner and were allowed to polymerise. After 20 minutes, when a clear interface was visible, the overlay gel solution was poured off and replaced with the resolution gel buffer and the gels

were allowed to polymerise for 4 hours. The lower tank was filled with 18 L running buffer, and a cooler was connected to the cooling core of the lower tank. The cooling core in the lower tank helped to keep the temperature at 8^{o}C at which the gels were being run.

When isoelectric focusing was completed, the gels were removed from the tubes with the aid of a gentle stream of water from a 30 mL plastic syringe fitted with a blunted 22 G needle. After both ends were freed from the tube, another 30 mL syringe fitted with an Eppendorf pipette tip was used to gently extrude the gel from the tube into a plastic weighing boat. The gel was immediately transferred to a 60 mm x 15 mm disposable plastic dish with 12 mL of equilibration buffer. All the gels were removed and transferred in the same manner. Immediately the gel buffer was poured off from the top of the gels and the excess overlay solution was removed using a Kim wipe.

The tube gels were removed from the equilibration buffer and were placed into the Petri dish cover with a few drops of water and the gels were placed around the bottom rim of the Petri dish. Melted agarose solution was added to the top of the resolution gel and using a spatula, the tube gel was slowly pushed from one end while rolling the

petri dish on the beveled edge of the inner glass plate. This way the gel gently slithered into the agarose without trapping air bubbles. The procedure was repeated until all the gels were placed on the resolution gels. Then the gel sandwiches were attached to the central cooling cores. The running buffer was poured into the upper chamber of the gel box and the gels were run at 40 MA constant current for 18 hours with cooling.

2.6 Ultrasensitive Silver Staining of Proteins

The silver staining metod used in the present study was described by Coulthart (1986) which is taken with modifications from Morrissey (1982) and Sammons et al. (1981). The procedure is listed in Table 2.4. The volume of solution per gel was 200 mL. Between each of steps 1-5, a brief wash with water was performed to remove residual solution from the previous step. The entire procedure was carried out with gentle shaking (60-100 revolutions per minute) on a reciprocating platform shaker.

The gels may be stored for long periods of time in 0.75% Na_2 CO_3 with no detectable deterioration, in "Zip-Loc" plastic bags.

TABLE 2.3

Silver Staining Procedure Used in Present Study

Step	Solution	Time	Comments
1. Fix	95% Ethanol/Acetic Acid/H_2O (52.5: 5: 42.5 v/v/v)	Overnight	No shaking
2. Wash	95% Ethanol/H_2O (10.5: 89.5 v/v)	1 hr	Wash with H_2O
3. Wash	Repeat step 2	30 min	
4. Wash	H_2O	30 min	
5. Wash	Repeat step 4		
6. Reduce	5 mg/L DTT	30 min	No Water rinse
7. Silver binding	0.1%(w/v) $AgNO_3$	30 min	
8. Wash	H_2O		
9. Wash	Developer 3%(w/v) Na_2CO_3 + 0.5 mL/L 37% (w/v) formaldehyde		Two quick washes with 150 mL of developer
10. Develop	Developer(step 9)	5-10 min	Shake on rotary shaker
11. Stop	2.3 M Citric acid	5-30 min	7.5 mL per 150 mL developer
12. Store	0.75%(w/v)Na_2CO_3	indefinitely	Gels will yellow if stored in developer

3

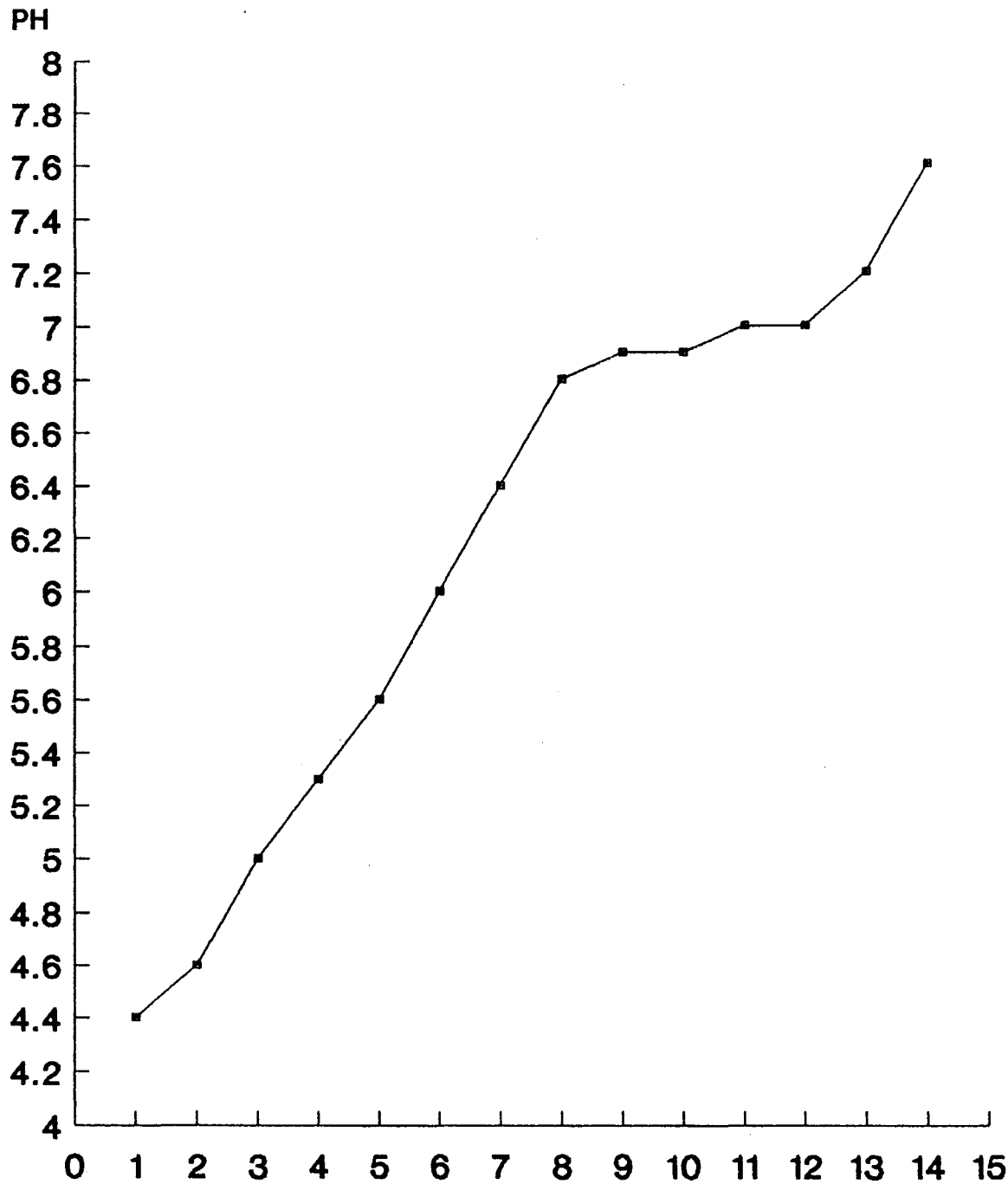
RESULTS

3.1 The Structure of Data Set

The pH gradient obtained during isoelectric focusing is shown in Figure 3.1. With the use of Biolyte ampholines, the pH gradient reached was between 4.6 and 6.8., which was almost near linearity. Not many proteins were focused well beyond the pH 6.8 range. So the few proteins near the basic end of the gel were not scored. Two-dimensional gel electrophoresis was done on eight different tissues from four species of the melanogaster species complex as mentioned in the introduction with details in chapter two. The silver stained gels were stored in zip lock bags to prevent them from drying. Gel scoring was done visually using a light box. Overhead sheets were used to mark the protein spots while comparing two gels. Unlike allozymes where specific stains are used, 2DE method uses general protein stains to detect a large number of proteins. The homology of spots between species are assigned by the relative position and appearance of the protein spots. Once this has been done, allelic variants of homologous protein

Figure 3.1

Graph of pH measued along the length of a first- dimension isoelectric focusing gel after focusing for 13,ooo volt-hours without any protein sample. 1cm sections of the gel were soaked in 1mL of deionized and deaerated water and the pH of the solution was measured.


PH
8
7.8
7.6
7.4
7.2
7
6.8
6.6
6.4
6.2
6
5.8
5.6
5.4
5.2
5
4.8
4.6
4.4
4.2
4
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
GEL LENGTH

can be compared between species. This method seemed quite inadequate and arbitrary to use to compare species. So in order to minimise subjectivity the spots were scored as present or absent. In single line comparison, this method does not create any problem as each line is essentially fixed for a given allele and the number of protein spots estimate the number of loci being compared. In multi line comparison, however, the "presence/ absence" method of scoring each spot would necessarily over estimate the number of loci as what is being scored is the number of alleles and not the number of loci.

All "land mark" - outstanding in size and shape - protein spots were marked first at different areas of the gel while comparing two gels of the same tissue between two species. While scoring, replicate gels from the same tissue was also used to confirm the presence/absence of the proteins. For example, if one protein is present as a faint spot in one gel and is absent in the second gel it was not scored. The common spots - present in both species - were marked with one colour. Unique proteins are those present in one species but absent in the other. Each protein spot

was marked according to its size and shape. Only clearly identifiable protein spots were scored. An effort was made to score approximately the same number of protein spots from all species. Since the number of unique protein spots in each species is different, the total, which is the sum of the common and unique protein spots, is also different. The total number of proteins resolved in each species is also different and this is another reason for the difference in the total numbers in the pairwise species comparisons. The presence of unique proteins may be different because polymorphic alleles may have been fixed in different species, or genes that are present in one species may be absent in others. It may also be due to differences in the time of expression of a gene in different species (Klose et al., 1989).

The photographs of adult brains of D. melanogaster and D. simulans are shown in Figure 3.2 and 3.3 representing the least diverged class of proteins. Close to three hundred proteins of varying intensity are visible in these gels. Some of the major proteins that are unique to each species are marked by arrows. Figure 3.4 and 3.5 are of the larval testis proteins.

Figure 3.2

A gel showing adult male brain proteins of D. melanogaster. Major unique (species-specific) proteins are marked by arrows.

Figure 3.3

A gel of the adult male brain proteins of D. simulans. Major unique proteins are marked by arrows.

D. melanogaster brain

D. simulans brain

Figure 3.4, 3.5

A gel showing larval testis proteins of D. melanogaster and D. simulans respectively. Arrows show some of the unique proteins in both gels.

D. melanogaster larval testis

D.simulans larval testis

The primary data are given in Tables 3.1-3.8. Since the total number of proteins are different in each species the proportion of unique or diverged and common proteins are more relevant than the actual number.

3.2. Analysis of Genetic Divergence

3.2.1. Analysis of Variance

Two-way analysis of variance (Sokal and Rohlf, 1981) was done on the data set in Tables 3.1- 3.8. Since only one strain from each species was studied, we do not have measurements of error among strains within species. Therefore the two values of unique proteins for each species pair were treated as independent values and used for measuring error. Since within-species variation is expected to be lower than between-species variation the error terms used in this study are larger than expected and would result into a conservative test of significance. The results from the analysis of variance are given in Tables 3.9-3.10. The levels of divergence among tissues and species are significantly different and so is the interaction component between tissues and species, suggesting that proteins in different tissues have not diverged at the same rates in all the species. These results are presented in detail below.

TABLE 3.1

Comparison of **larval brain proteins** (common and unique) between four sibling species of *Drosophila melanogaster*

Species* pair	No.of protein spots scored	No. of common spots	No. of Unique spots
mel	359	314 (0.875)	45 (0.125)
sim	344	314 (0.913)	30 (0.087)
mel	356	298 (0.837)	58 (0.163)
sec	354	298 (0.842)	57 (0.158)
mel	358	310 (0.866)	48 (0.134)
mau	351	310 (0.883)	41 (0.117)
sim	344	321 (0.933)	23 (0.067)
sec	351	321 (0.915)	30 (0.085)
sim	352	332 (0.943)	20 (0.057)
mau	353	332 (0.941)	21 (0.059)
sec	357	320 (0.896)	37 (0.104)
mau	351	320 (0.912)	31 (0.088)

*mel *Drosophila melanogaster*
mau *Drosophila mauritiana*
sec *Drosophila sechellia*
sim *Drosophila simulans*

TABLE 3.2

Comparison of **larval wing disc proteins** (common and unique) between four sibling species of *Drosophila melanogaster*

Species pair	No.of Protein spots scored	No.of common spots	No.of unique spots
mel	304	262 (0.862)	42 (0.138)
sim	292	262 (0.897)	30 (0.103)
mel	304	254 (0.836)	50 (0.164)
sec	294	254 (0.864)	40 (0.136)
mel	303	260 (0.858)	43 (0.142)
mau	295	260 (0.881)	35 (0.119)
sim	284	261 (0.884)	33 (0.116)
sec	298	261 (0.842)	47 (0.158)
sim	285	260 (0.884)	33 (0.116)
mau	287	260 (0.878)	35 (0.122)
sec	284	254 (0.873)	36 (0.127)
mau	291	254 (0.852)	43 (0.148)

TABLE 3.3

Comparison of **larval haemolymph proteins** (common and unique) between four sibling species of Drosophila melanogaster

Species pair	No. of protein spots scored	No. of common spots	No. of unique spots
mel	242	168 (0.694)	74 (0.306)
sim	238	168 (0.706)	70 (0.294)
mel	244	159 (0.652)	85 (0.348)
sec	248	159 (0.641)	89 (0.559)
mel	240	172 (0.717)	68 (0.283)
mau	248	172 (0.486)	76 (0.306)
sim	240	199 (0.829)	41 (0.171)
sec	237	199 (0.840)	38 (0.160)
sim	236	207 (0.877)	29 (0.123)
mau	256	207 (0.809)	49 (0.191)
sec	234	181 (0.774)	53 (0.226)
mau	253	181 (0.715)	72 (0.285)

TABLE 3.4

Comparison of **larval testis proteins** (common and unique) between four sibling species of *Drosophila melanogaster*

Species pair	No. of protein spots scored	No. of common proteins	No.of unique proteins
mel	404	312 (0.772)	92 (0.228)
sim	406	312 (0.768)	94 (0.232)
mel	403	280 (0.695)	123 (0.305)
sec	400	280 (0.700)	120 (0.300)
mel	407	271 (0.666)	136 (0.334)
mau	395	271 (0.693)	124 (0.314)
sim	404	350 (0.866)	54 (0.134)
sec	406	350 (0.862)	56 (0.138)
sim	395	346 (0.876)	49 (0.124)
mau	409	346 (0.846)	63 (0.154)
sec	397	346 (0.872)	51 (0.128)
mau	406	346 (0.838)	60 (0.148)

TABLE 3.5

Comparison of **adult female brain proteins** (common and unique) of four sibling species of *Drosophila melanogaster*

Species pair	No. of protein spots scored	No. of Common spots	No. of Unique spots
mel	364	329 (0.904)	35 (0.096)
sim	363	329 (0.906)	34 (0.094)
mel	370	325 (0.878)	45 (0.122)
sec	359	325 (0.905)	34 (0.095)
mel	364	324 (0.890)	40 (0.110)
mau	358	324 (0.905)	34 (0.095)
sim	350	328 (0.937)	22 (0.063)
sec	345	328 (0.951)	17 (0.049)
sim	360	331 (0.919)	29 (0.081)
mau	347	331 (0.954)	16 (0.046)
sec	354	328 (0.927)	26 (0.073)
mau	350	328 (0.937)	22 (0.063)

TABLE 3.6

Comparison of **adult male brain proteins** (common and unique) between four sibling species of Drosophila melanogaster

Species pair	No. of Protein spots scored	No. of common spots	No.unique spots
mel	364	329 (0.904)	35 (0.096)
sim	363	329 (0.906)	34 (0.094)
mel	370	326 (0.881)	44 (0.119)
sec	361	326 (0.903)	35 (0.097)
mel	365	325 (0.890)	40 (0.109)
mau	357	325 (0.907)	32 (0.093)
sim	353	330 (0.935)	23 (0.065)
sec	350	330 (0.943)	17 (0.049)
sim	350	328 (0.937)	22 (0.063)
mau	345	328 (0.951)	17 (0.049)
sec	351	326 (0.929)	25 (0.071)
mau	346	326 (0.942)	20 (0.058)

TABLE 3.7

Comparison of **accessory gland proteins** (common and unique) between four sibling species of Drosophila melanogaster

Species pair	No. of protein spots scored	No. of common spots	No. of unique spots
mel	323	242 (0.749)	81 (0.251)
sim	311	242 (0.778)	69 (0.222)
mel	321	239 (0.745)	82 (0.255)
sec	297	239 (0.805)	58 (0.195)
mel	326	242 (0.742)	84 (0.258)
mau	307	242 (0.788)	65 (0.212)
sim	306	263 (0.859)	43 (0.141)
sec	304	263 (0.865)	41 (0.135)
sim	303	258 (0.851)	45 (0.149)
mau	295	258 (0.875)	37 (0.125)
sec	309	251 (0.812)	58 (0.188)
mau	300	251 (0.837)	49 (0.158)

TABLE 3.8

Comparison of **adults testis proteins** (common and unique) between four sibling species of Drosophila melanogaster

Species pair	No. of Protein spots scored	No. of common spots	No. of unique spots
mel	487	402 (0.826)	85 (0.174)
sim	460	402 (0.874)	58 (0.126)
mel	489	398 (0.814)	91 (0.186)
sec	462	398 (0.861)	64 (0.139)
mel	490	401 (0.818)	89 (0.192)
mau	462	401 (0.868)	61 (0.132)
sim	463	407 (0.879)	56 (0.121)
sec	443	407 (0.904)	36 (0.082)
sim	458	416 (0.902)	42 (0.091)
mau	451	416 (0.918)	35 (0.078)
sec	459	411 (0.895)	48 (0.105)
mau	463	411 (0.888)	52 (0.112)

Table 3.9

Two-way analysis of variance on the proportion of unique proteins in various tissues and species

Source	DF	SS	MS	F-Ratio	Prob
Tissue	7	0.34555	0.04936	123.25	P<0.01
Species pair	5	0.12776	0.0255	63.75	P<0.01
Tissue x species	35	0.06798	0.00194	4.85	P<0.01
Residual	48	0.01922	0.00040		
Total	95	0.56052			

Table 3.10

Analysis of variance on the total number of proteins in various tissues and species

Source	DF	SS	MS	F-Ratio	Prob
Tissue	7	390539.5	55791.4	794.74	P<0.01
Species	5	1840.1	368.0	5.24	P<0.01
Tissue x Species	35	1503.2	42.9	0.61	NS
Error	48	3367.5	70.2		
Total	95	397250.3			

Analysis of variance on the number of unique proteins in various tissues and species

Source	DF	SS	MS	F-Ratio	Prob
Tissue	7	32839.6	4691.4	66.63	P<0.01
Species Pair	5	16495.4	3299.1	46.86	P<0.01
Tissue x Species	35	8056.5	230.2	3.26	P<0.01
Error	48	3379.5	70.4		
Total	95	60771.0			

3.2.2 Variation Among Tissues

For ease of comparison, the proportion of genic divergence for each species pair for various tissues are shown in Tables 3.11-3.16 and as graphs in Figures 3.6-3.9. The tissues are ranked according to the level of divergence as it is in Tables 3.11-3.16. The larval "haemolymph" proteins show more divergence than proteins from any other tissue, a result in agreement with those from one-dimensional analysis of these proteins, even though the haemolymph was without any haemocytes and broken tissues. (Singh and Coulthart, 1982). Larval testis and accessory gland proteins show similar levels of divergence in mel/sim comparison (Figure 3.6), but this is not consistent in all comparisons. For example in mel/sec and mel/mau comparisons, larval testis proteins show more divergence than accessory gland proteins. However, the accessory gland and "haemolymph" proteins show high divergence consistently in all pairwise comparisons.

Adult male and female brain tissues show the same level of divergence in all pairwise species comparisons. Within the resolution and pH range used in this study, no differences between male and female brain proteins of the

Tables 3.11-16

The tables show proportion of common and unique proteins from the pairwise comparison. The tissues are arranged according to the level of divergence in the descending order.

TABLE 3.11

Proportion of common and unique proteins in
D.melanogaster and D.simulans

Tissues/Organs	common proteins observed	unique proteins observed
Larval Haemolymph	0.700	0.300
Larval Testes	0.770	0.230
Accessory Glands	0.764	0.236
Larval Wing Disc	0.880	0.120
Adult Testes	0.842	0.158
Larval Brain	0.894	0.106
Adult Brain (female)	0.905	0.095
Adult Brain (male)	0.905	0.095

TABLE 3.12

Proportion of common and unique proteins in
D.melanogaster and D.sechellia

Tissues/Organs	common proteins observed	unique proteins observed
Larval Haemolymph	0.647	0.353
Larval Testes	0.696	0.304
Accessory Glands	0.775	0.225
Larval Wing Disc	0.850	0.150
Adult Testes	0.838	0.162
Larval Brain	0.840	0.160
Adult Brain (female)	0.892	0.108
Adult Brain (male)	0.892	0.108

TABLE 3.13

Proportion of common and unique Proteins in
D.melanogaster and D.mauritiana

Tissues/Organs	common proteins observed	unique proteins observed
Larval Haemolymph	0.706	0.294
Larval Testes	0.676	0.324
Accessory Glands	0.765	0.235
Larval Wing Disc	0.870	0.130
Adult Testes	0.843	0.157
Larval Brain	0.875	0.125
Adult Brain (female)	0.898	0.102
Adult Brain (male)	0.899	0.101

*Sig. Level

TABLE 3.14

Proportion of common and unique proteins in D.simulans and D.sechellia

Tissues/Organs	common proteins observed	unique proteins observed
Larval Haemolymph	0.835	0.165
Larval Testes	0.864	0.136
Acessory Glands	0.862	0.138
Larval Wing Disc	0.863	0.137
Adult Testes	0.899	0.101
Larval Brain	0.924	0.076
Adult Brain (female)	0.944	0.056
Adult Brain (male)	0.939	0.061

TABLE 3.15

Proportion of common and unique proteins in D.simulans and D.mauritiana

Tissues/Organs	common proteins observed	unique proteins observed
Larval Haemolymph	0.843	0.157
Larval Testes	0.861	0.139
Accessory Glands	0.863	0.137
Larval Wing Disc	0.881	0.119
Adult Testes	0.915	0.085
Larval Brain	0.942	0.058
Adult Brain (female)	0.937	0.063
Adult Brain (male)	0.944	0.056

TABLE 3.16

Proportion of common and unique proteins in
D.sechellia and D.mauritiana

Tissues/Organs	common proteins observed	unique proteins observed
Larval Haemolymph	0.745	0.255
Larval Testes	0.862	0.138
Accessory Glands	0.825	0.175
Larval Wing Disc	0.863	0.137
Adult Testes	0.892	0.108
Larval Brain	0.904	0.096
Adult Brain (female)	0.932	0.068
Adult Brain (male)	0.936	0.064

Figure 3.6

Graphs showing proportion of proteins divergenced in various tissues between D. melanogaster / D.simulans and D. melanogaster / D. sechellia.

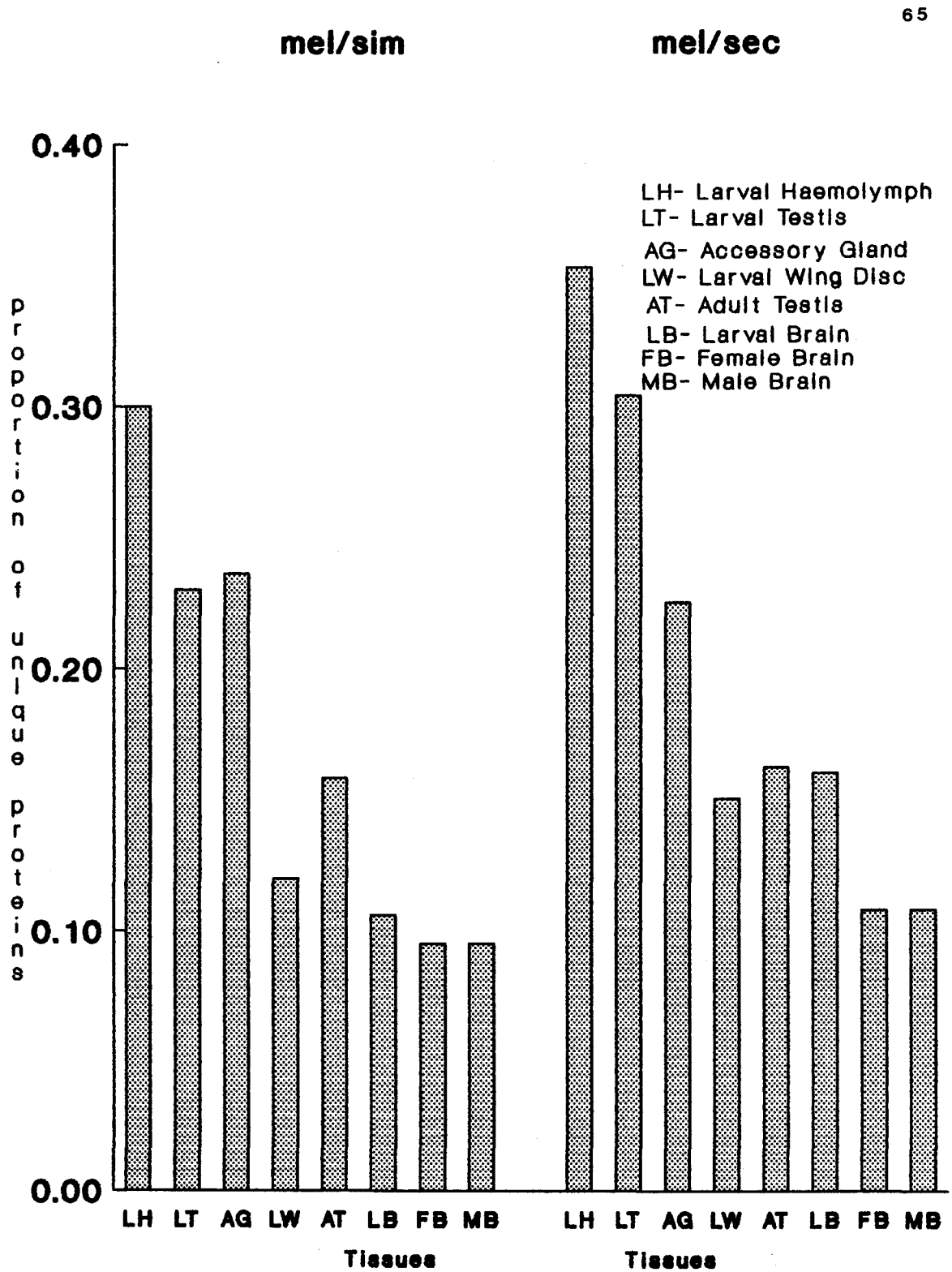

mel/sim
mel/sec
0.40
0.30
0.20
0.10
0.00
proportion of unique proteins
LH- Larval Haemolymph
LT- Larval Testis
AG- Accessory Gland
LW- Larval Wing Disc
AT- Adult Testis
LB- Larval Brain
FB- Female Brain
MB- Male Brain
LH LT AG LW AT LB FB MB
Tissues
LH LT AG LW AT LB FB MB
Tissues

Figure 3.7

Graphs showing the proportion of proteins diverged in various tissues between D. melanogaster / D .mauritiana and D. simulans / D.sechellia.

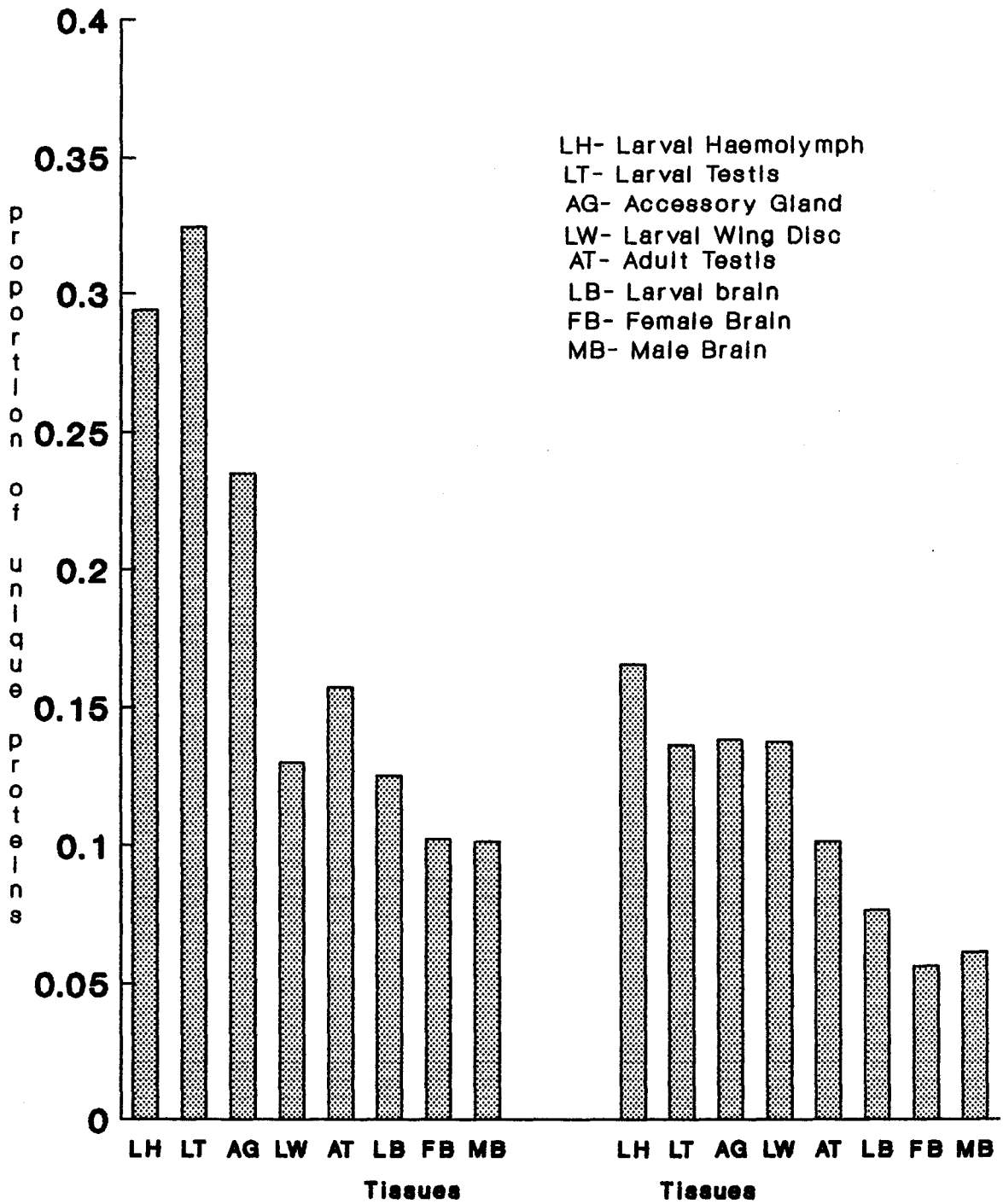

mel/mau
sim/sec
LH- Larval Haemolymph
LT- Larval Testis
AG- Accessory Gland
LW- Larval Wing Disc
AT- Adult Testis
LB- Larval brain
FB- Female Brain
MB- Male Brain
proportion of unique proteins
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
LH LT AG LW AT LB FB MB
Tissues
LH LT AG LW AT LB FB MB
Tissues

Figure 3.8

Graphs showing proportion of proteins diverged in various tissues between D. simulans / D. mauritiana and D. sechellia / D. mauritiana.

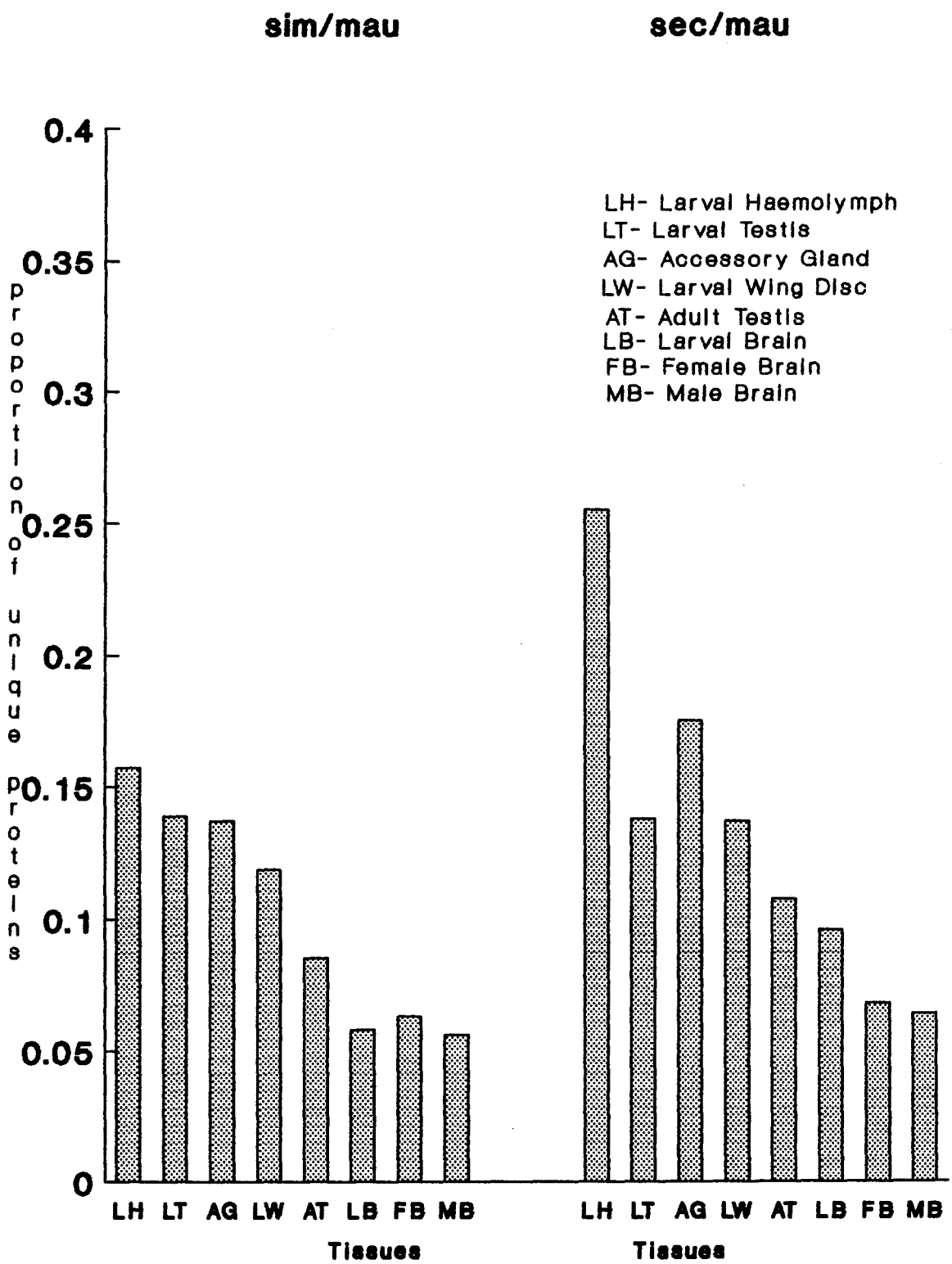
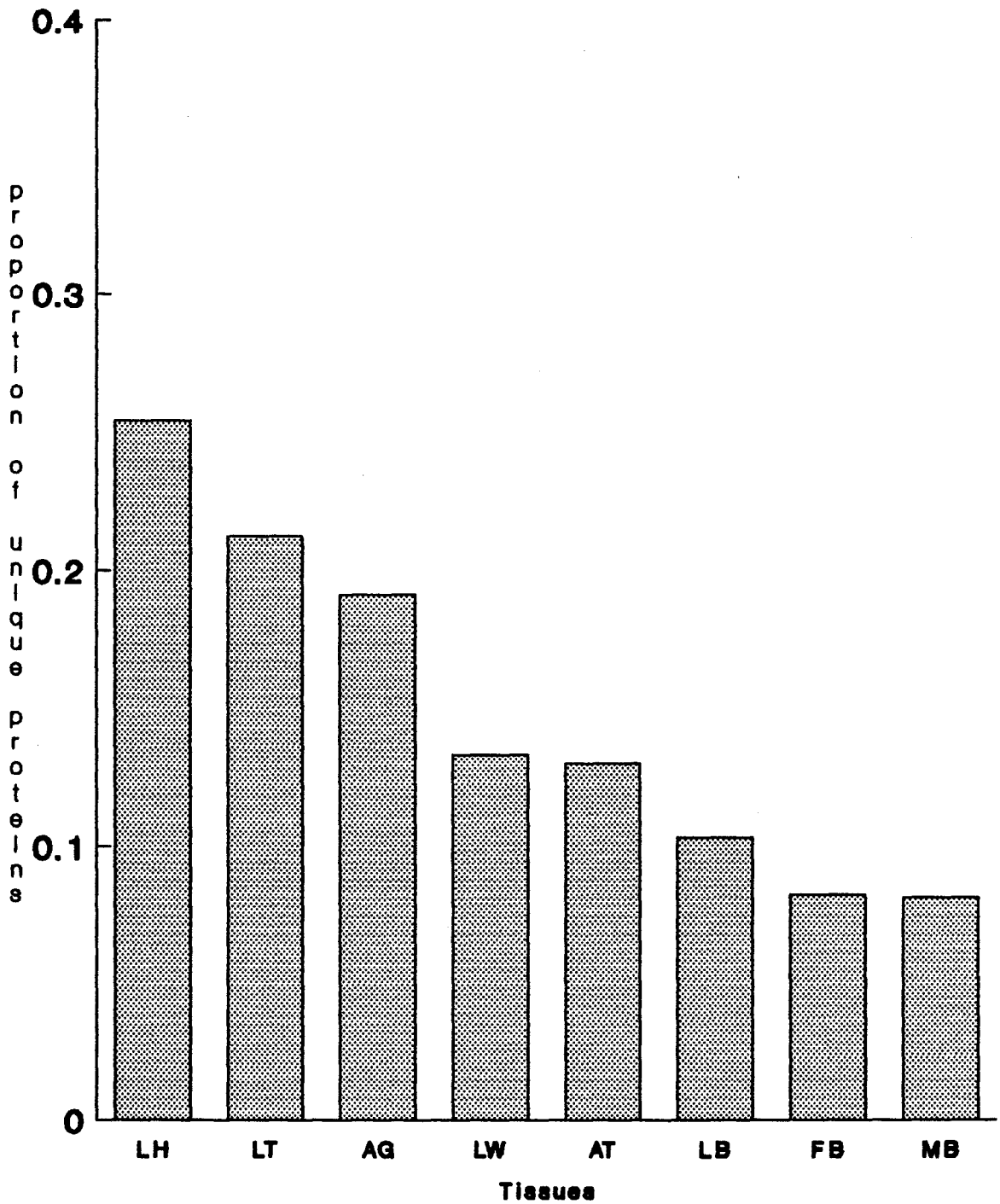

sim/mau
sec/mau
LH- Larval Haemolymph
LT- Larval Testis
AG- Accessory Gland
LW- Larval Wing Disc
AT- Adult Testis
LB- Larval Brain
FB- Female Brain
MB- Male Brain
proportion of unique proteins
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
LH LT AG LW AT LB FB MB
Tissues
LH LT AG LW AT LB FB MB
Tissues

Figure 3.9

Graph showing the mean proportion of protein divergence of (overall four species) in various tissues arranged in decreasing order of the level of divergence.

Mean Proportion of Protein Divergence



same species were observed. This is true for all four species studied. Larval brain proteins also do not show much divergence between species.

As the summary (Figure 3.9) shows there is no trend or pattern to show that either larval or adult proteins are overall (more or less) diverged than the other. In some comparisons, proteins from larval tissues are more diverged than those from the adult tissues. For example, proteins of larval haemolymph and larval testis show more divergence than larval brain and larval wing disc proteins. While adult brains show less divergence than adult testis and accessory gland proteins. Thus, variation between tissues rather than between developmental stages appears to be the major determinant of the level of divergence.

3.2.3 Variation Among Species

The proportion of unique proteins for each tissue in six pair-wise comparisons are shown in Figures 3.10. All comparisons involving D. melanogaster show significantly higher divergence for all tissues except the larval wing disc proteins. The larval wing disc proteins show intermediate level of divergence and all the six values fall within the similar range. Larval haemolymph and larval

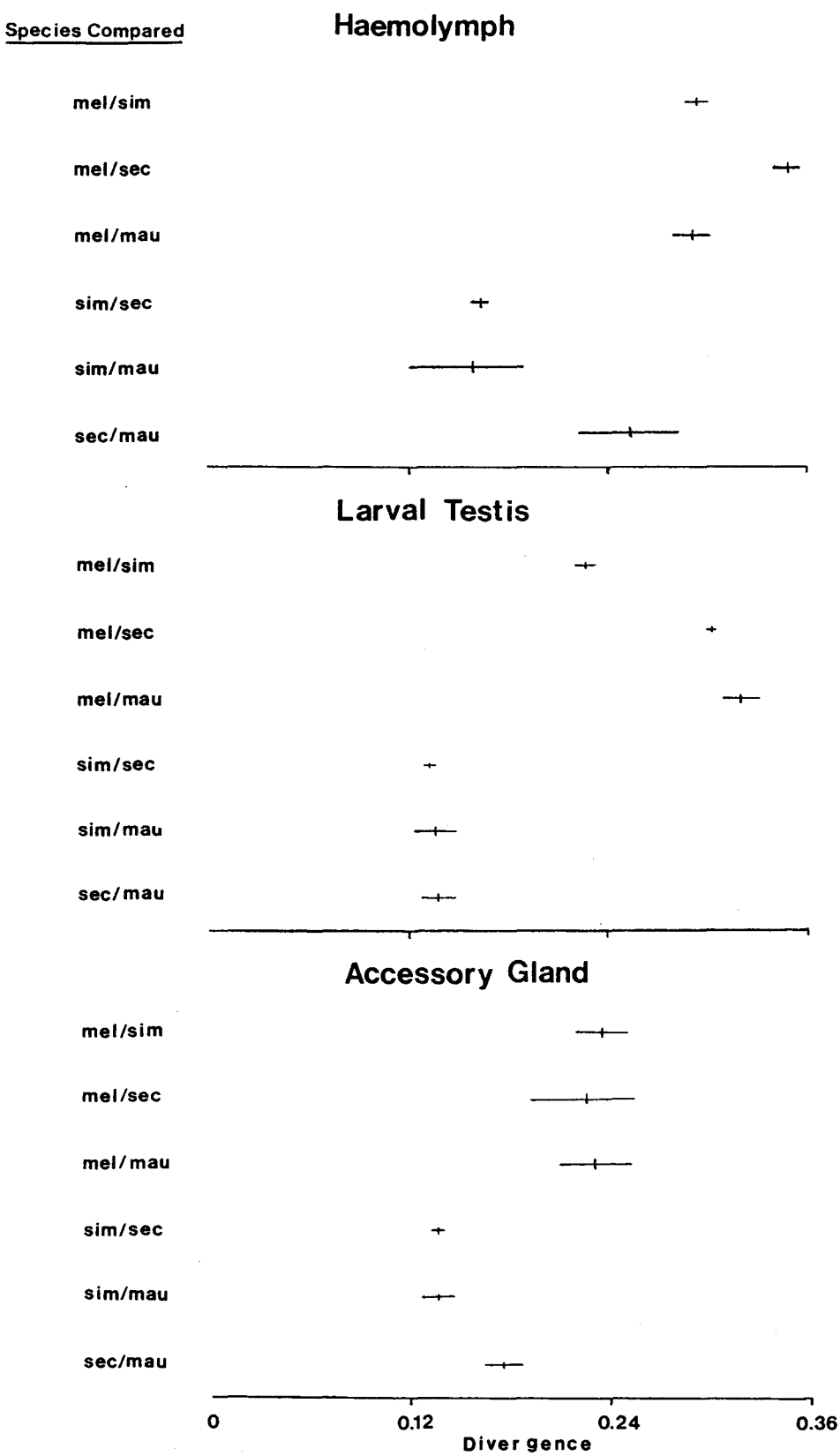
testis proteins show a different picture. D. melanogaster is more diverged than all the other three species which show divergence that fall within the same range. With respect to adult tissues, D. melanogaster shows a higher divergence than the other three species in accessory glands and adult testis proteins, but the level of divergence is much larger for accessory glands than for adult testis. Both male and female adult brain proteins show the lowest level of divergence in all pair-wise comparisons. One common pattern observed is that D. melanogaster has diverged significantly from the other three species except for larval wing disc proteins. All the other three species show a restricted range of divergence in the tissues studied here.

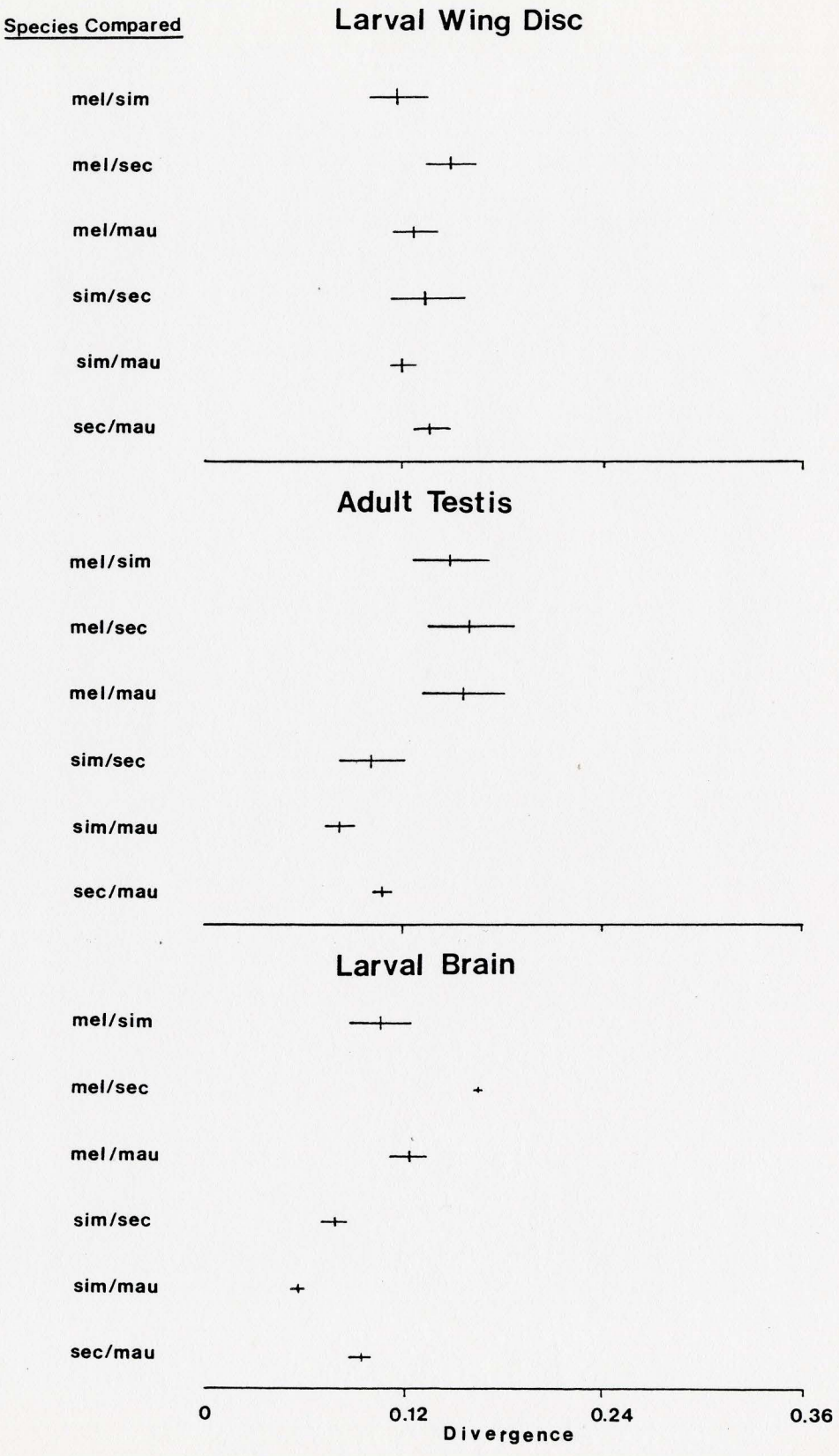
3.2.4 Unique Proteins in Four-Way Species Comparison

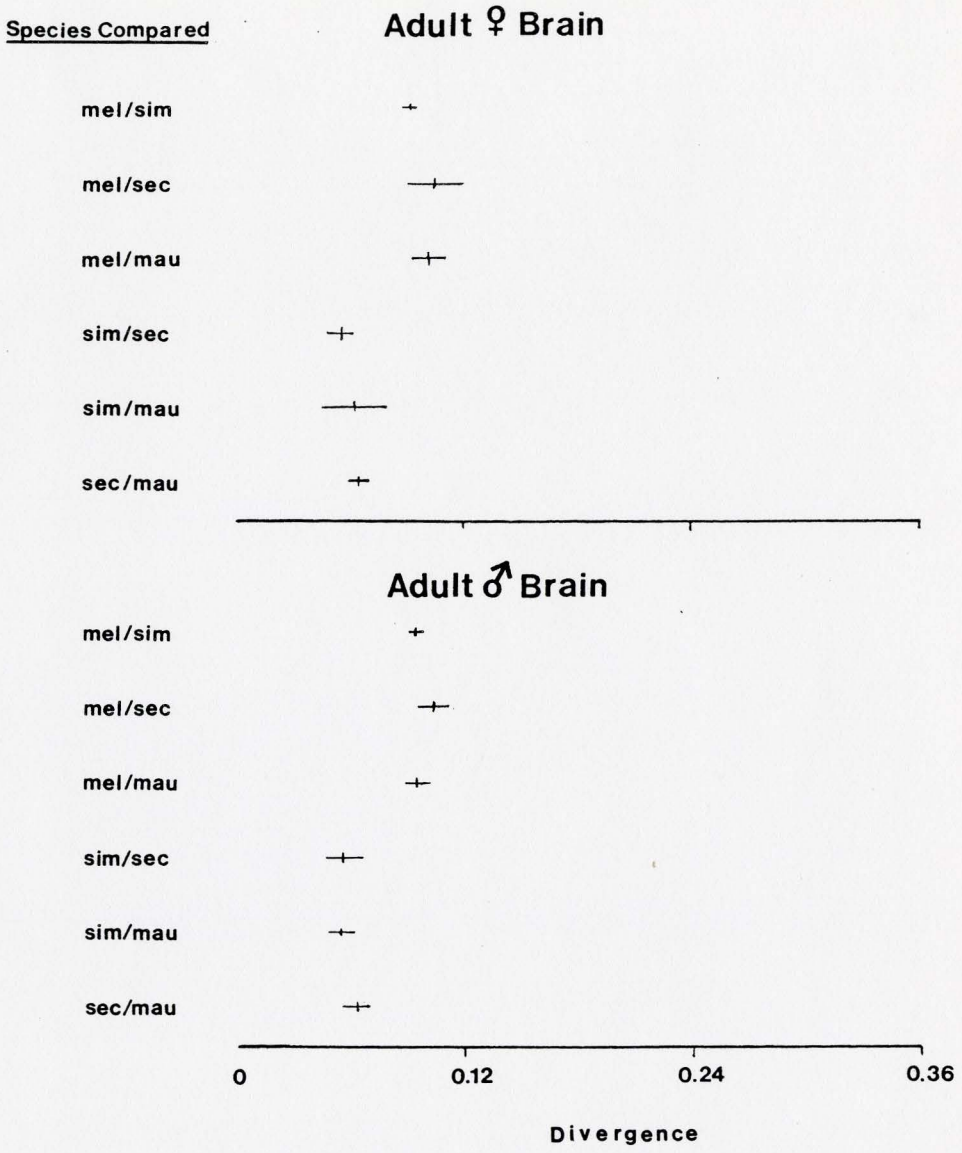
Unique proteins presented in Tables 3.1-3.8 and Figures 3.10 are based on pairwise species comparisons. To see if unique proteins have accumulated at different rates in different species, all four species were compared together. In the data presented in Figures 3.11 unique proteins are defined as those present in one species but absent in the other three. From these graphs it is evident that D. melanogaster has accumulated the largest number of

Figure 3.10 (pages 75, 76, 77)

A graphic display of the level of protein divergence in pairwise species comparison in various tissues. Each bar shows the range of two values for each species and their mean. Note the high divergence of *D. melanogaster*.

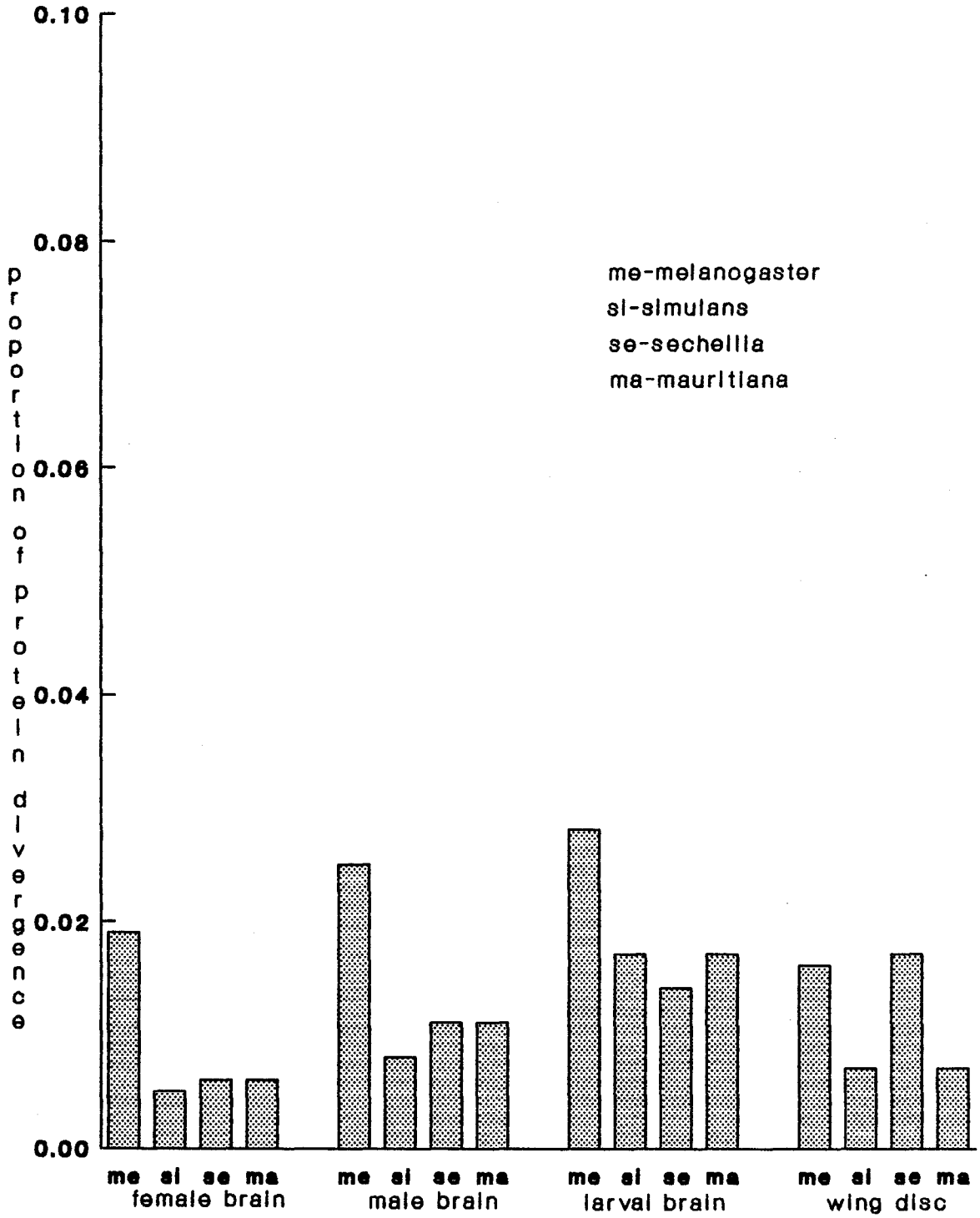

Species Compared
Haemolymph
mel/sim
mel/sec
mel/mau
sim/sec
sim/mau
sec/mau
Larval Testis
mel/sim
mel/sec
mel/mau
sim/sec
sim/mau
sec/mau
Accessory Gland
mel/sim
mel/sec
mel/mau
sim/sec
sim/mau
sec/mau
0
0.12
0.24
0.36
Diver gence

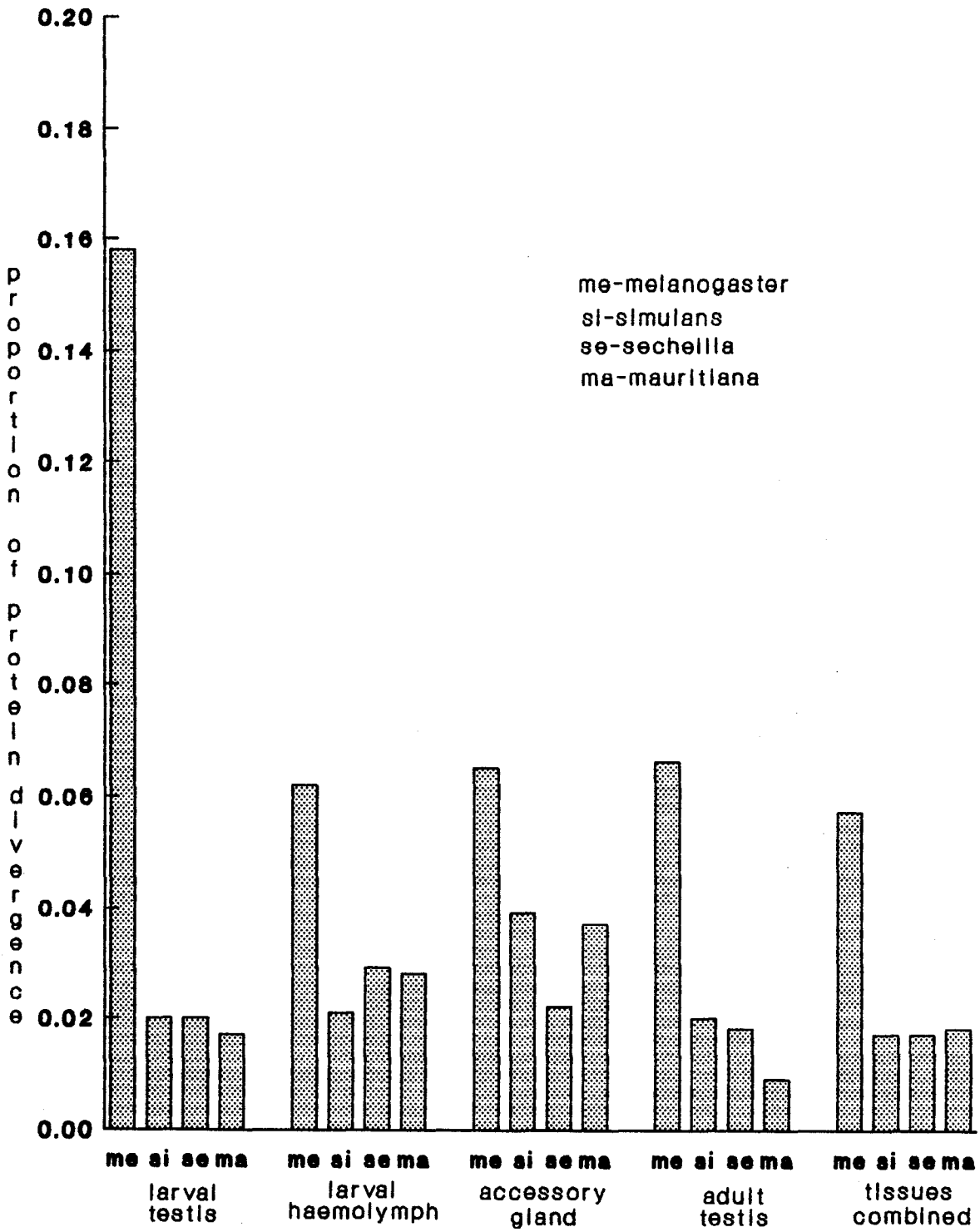

Species Compared
Larval Wing Disc
mel/sim
mel/sec
mel/mau
sim/sec
sim/mau
sec/mau
Adult Testis
mel/sim
mel/sec
mel/mau
sim/sec
sim/mau
sec/mau
Larval Brain
mel/sim
mel/sec
mel/mau
sim/sec
sim/mau
sec/mau
0
0.12
0.24
0.36
Divergence


Species Compared
Adult ♀ Brain
mel/sim
mel/sec
mel/mau
sim/sec
sim/mau
sec/mau
Adult ♂ Brain
mel/sim
mel/sec
mel/mau
sim/sec
sim/mau
sec/mau
0
0.12
0.24
0.36
Divergence

Figures 3.11

Figures showing proportion of proteins in four-way comparisons involving all species. These unique proteins are present in only one species and absent in the other three species.


0.10
0.08
0.06
0.04
0.02
0.00
proportion of protein divergence
me-melanogaster
si-simulans
se-sechellia
ma-mauritiana
me si se ma
female brain
me si se ma
male brain
me si se ma
larval brain
me si se ma
wing disc


0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
proportion of protein divergence
me-melanogaster
si-simulans
se-sechellia
ma-mauritiana
me si se ma
larval testis
me si se ma
larval haemolymph
me si se ma
accessory gland
me si se ma
adult testis
me si se ma
tissues combined

unique proteins in all tissues. Larval testis of D. melanogaster has the largest number of unique proteins followed by adult testis, accesory gland and haemolymph. The other three species show a similar proportion of unique proteins for these three tissues except the accessory gland for which D. mauritiana shows the least number of unique proteins. Both larval and adult testis have accumulated almost the same number of unique proteins. Among the remaining tissues, proteins of adult male and female brains, the larval brain, and wing disc show a similar proportion of unique proteins in all the four species. Klose et al., (1989) also found that in mouse, brain and muscle cell proteins show less variation compared to liver and kidney proteins. It is clear from these results that proteins in different tissues are evolving at different rates within and between species. The tissue and species interaction is significant as shown by the analysis of variance (Tables 3.9-3.10). So, while D. melanogaster, the oldest in this group, is expected to accumulate more unique proteins, it is not true for all the tissues. Besides divergence time, mutation and selection are relevant parameters to be considered, which will be dealt with in the discussion.

3.2.5 Phylogenetic Relationships

As mentioned in the introduction, the phylogenetic relationship of D. simulans, D. sechellia and D. mauritiana is a subject of controversy. The available data on these three species do not unequivocally resolve their phylogenetic relationships. But most of the data (Lachaise et al., 1988) points to the closeness of D. simulans and D. mauritiana. With the use of the proportion of unique proteins as distance matrices (Tables 17- 24) dendrograms were constructed according to Sneath and Sokal (1973) for each tissue separately as well as for all tissues combined (Figures 3.12). Six out of eight tissues (adult male brain, larval brain, larval wing disc, adult testis, accessory gland and haemolymph) show D. mauritiana to be closer to D. simulans than to D. sechellia. The two tissues which show D. simulans to be closer to D. sechellia than to D. mauritiana are adult female brain and larval testis, but the distances in both cases are not significant. It is also significant to note that the larval testis of D.melanogaster shows the highest divergence, while the other three species show more or less the same amount of divergence with regard

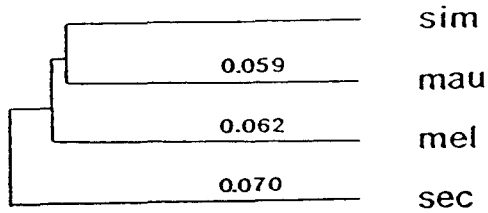
to larval testis proteins. In the case of larval wing disc, D. sechellia is more diverged than all the other three species, but the divergence, again, is not significantly different. The larval wing disc is the only tissue where D. melanogaster has not diverged very much.

While the dendrogram, based on all tissues, does not descriminate among D. simulans, D. mauritiana and D. sechellia, the fact that all except two tissues show D. simulans closer to D. mauritiana supports the previous conclusions made by Lachaise et al., 1984); Singh (1989) that D. mauritiana and D. simulans are much closer to each other than either is to D. sechellia.

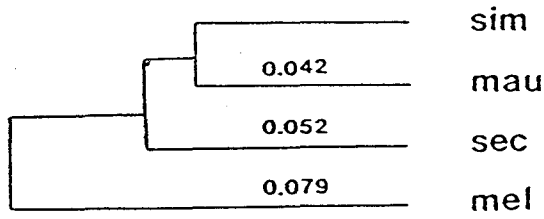
Figure 3.12 (pages 85, 86, 87)

The dendrograms are made using the mean proportion of protein divergence shown in Tables 3.17 - 3.24 given at the end of this chapter according to the UPGMA method (Sneath and Sokal, 1973).

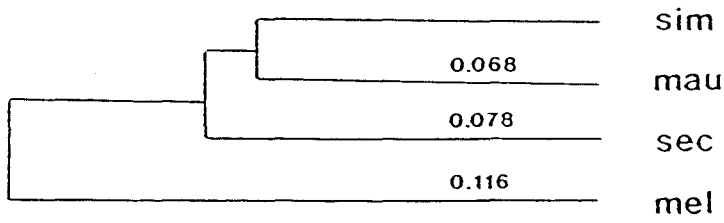
Larval Wing Disc



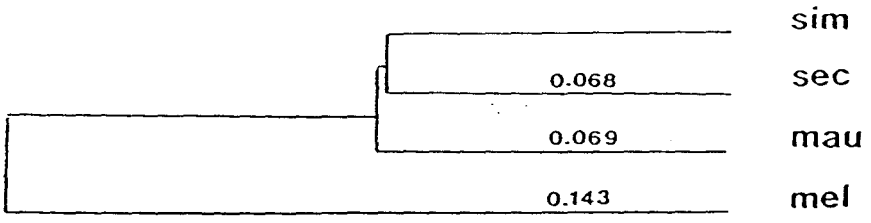
Adult Testis



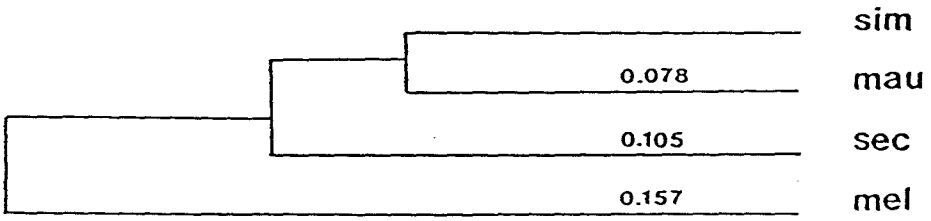
Accessory Gland



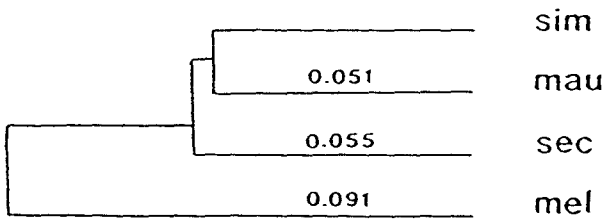
Larval Testis



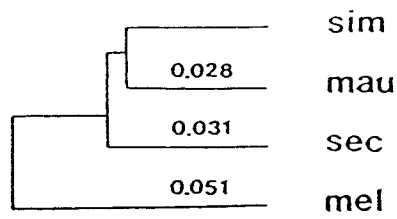
Larval Haemolymph



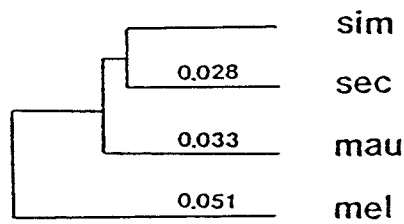
Combined Tissues



Adult ♂ Brain



Adult ♀ Brain



Larval Brain

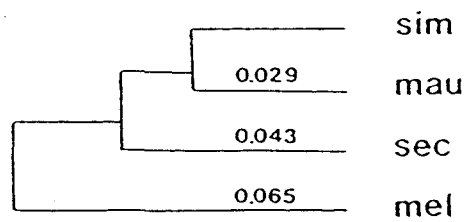


Table 3.17

Mean Proportion of common (upper diagonal) and unique lower diagonal) larval brain proteins of sibling species of Drosophila melanogaster

Species	mel	sim	sech	mau
mel	---	0.894	0.840	0.875
sim	0.106	---	0.924	0.942
sec	0.160	0.076	---	0.904
mau	0.125	0.058	0.096	---

TABLE 3.18

Mean Proportion of common (upper diagonal) and unique (lower diagonal) larval wing proteins of four sibling species of Drosophila melanogaster

Species	mel	sim	sech	mau
mel	---	0.880	0.850	0.870
sim	0.120	---	0.863	0.881
sec	0.150	0.137	---	0.863
mau	0.130	0.119	0.137	---

TABLE 3.19

Mean proportion of common (upper diagonal) and unique (lower diagonal) larval haemolymph proteins of four sibling species of D.melanogaster

Species	mel	sim	sec	mau
mel	---	0.700	0.647	0.706
sim	0.300	---	0.835	0.843
sec	0.353	0.165	---	0.745
mau	0.294	0.157	0.255	---

TABLE 3.20

Mean Proportion of common (upper diagonal) and unique lower diagonal) larval gonad proteins of four sibling species of Drosophila melanogaster

Species	mel	sim	sech	mau
mel	---	0.770	0.696	0.676
sim	0.230	---	0.864	0.861
sec	0.304	0.136	---	0.862
mau	0.324	0.139	0.138	---

TABLE 3.21

Mean Proportion of common (upper diagonal) and unique lower diagonal) adult female brain proteins of four sibling species of Drosophila melanogaster

Species	mel	sim	sech	mau
mel	---	0.905	0.892	0.898
sim	0.095	---	0.944	0.937
sec	0.108	0.056	---	0.932
mau	0.102	0.063	0.068	---

Table 3.22

Mean proportion of common (upper diagonal) and unique (lower diagonal) adult male brain proteins of four sibling species of D. melanogaster

Species	mel	sim	sech	mau
mel	---	0.905	0.892	0.899
sim	0.095	---	0.939	0.944
sec	0.108	0.061	---	0.936
mau	0.101	0.056	0.064	---

TABLE 3.23

Mean proportion of common (upper diagonal) and unique (lower diagonal) accessory gland proteins of four sibling species of Drosophila melanogaster

Species	mel	sim	sech	mau
mel	---	0.764	0.775	0.765
sim	0.236	---	0.862	0.863
sec	0.225	0.138	---	0.824
mau	0.235	0.137	0.176	---

TABLE 3.24

Mean Proportion of common (upper diagonal) and unique lower diagonal) adult testes proteins of four sibling species of Drosophila melanogaster

Species	mel	sim	sec	mau
mel	---	0.842	0.838	0.843
sim	0.158	----	0.899	0.915
sec	0.162	0.101	---	0.892
mau	0.157	0.085	0.108	---

TABLE 3.25

Mean Proportion of common (upper diagonal) and unique (lower diagonal) proteins of all tissues combined

Species	mel	sim	sec	mau
mel	---	0.833	0.804	0.817
sim	0.167	---	0.891	0.898
sec	0.196	0.109	---	0.887
mau	0.183	0.102	0.113	---

4

DISCUSSION

4.1 Sensitivity of Two-Dimensional Gel Electrophoresis to Detect Variation

The study of genetic variation within and between species is a first step in understanding the evolutionary process of speciation. The main goal of this study was to understand the nature of genetic variation at the protein level by two-dimensional gel electrophoresis in reproductive and non-reproductive tissues of Drosophila. Almost all currently existing data on genetic variation among species have been obtained by one-dimensional native gel electrophoresis. Relatively few investigators have used two-dimensional gel electrophoresis to study genetic variation. Since two-dimensional electrophoresis is a relatively new and less utilized technique, it is important to consider some technical and methodological aspects of this technique before discussing the significance of the results obtained from the present study.

As pointed out earlier, 2DE proteins are extracted from samples using solubilising agents and separated under fully denaturing conditions (O'Farrell, 1975).

The denatured polypeptides are separated on the basis of charge in the first dimension and on the basis of size in the second dimension. It is this independence of charge and size in the separation that makes it such a powerful analytical tool. Another advantage is that potentially all the proteins in a cell or tissue can be visualized in a single gel (Klose, 1982). The use of silver stain (Merril et al., 1981), flourography (Bonner and Laskey, 1974) and autoradiography have been used to increase the potential of 2DE still further. However, some doubts regarding its sensitivity have been raised by several investigators. Early investigators (Leigh, Brown and Langley, 1979; Racine and Langley, 1980; Aquadro et al., 1982; Ohnishi et al., 1982) found that heterozygosity values obtained by 2DE were two-to-five fold lower than those obtained by 1DE. This is evident from the 1DE and 2DE data presented in Table 4.1. The number of loci scored in 1DE and 2DE are quite different and so it makes the comparison rather difficult. But if the mean heterozygosity values in Table 4.1 can be taken at their face values the 2DE estimates are consistently two-to-five-fold lower than the 1DE estimates in all cases except one. The exception is the study of Rosenblum et al., (1984)

TABLE 4.1

Proportion of loci polymorphic (P), average heterozygosity (H) and number of loci (N)[a] studied by 1DE for enzymes and by 2DE for proteins

Species		P	H	N		References
Drosophila melanogaster	1DE	0.420	0.102	117	9 population	1
	2DE	0.110	0.040	54	1 population	2
		0.080	0.024	244	2 population	1
Drosophila simulans	1DE	0.417	0.063	24	2 population	3
		0.288	0.094	114	5 population	1
	2DE	0	0	70	1 population	3
		0.085	0.029	271	2 population	
Mus musculus	1DE	0.20	0.084	92	2 population	4
	2DE	0.042	0.020	72		5
Homo sapiens	1DE	0.231	0.063	104		6
	2DE	0.101	0.020	168	fibroblasts	10
		0.102	0.024	186	lymphoblasts	10
		0.175	0.056	40	serum	10
		0	0	83	kidney	9
		0.087	0.031	46	erythrocytes	11
		0.250	0.062	20	plasma	12

a For 2DE surveys, number of loci is taken as equal to number of polypeptides scored

References - (1) Choudhary et al., (1988); (2) Leigh Brown and Langley (1979); (3) Ohnishi et al., (1982); (4) Rice et al. (1980); (5) Racine and Langley (1980); (6) Harris (1980); (7) Mc Conkey et al., (1979); (8) Walton et al., (1979); (9) Smith et al., (1980); (10) Goldman and Merril (1983); (11) Rosenblum et al., (1984); (12) Rosenblum et al., (1983).

who estimated variability of plasma proteins and obtained a high heterozygosity value close to that of 1DE (Table 4.1). On the other hand, no genic variation was detected in human kidney (Smith et al., 1980) or in an early study of D. simulans (Ohnishi et al., 1982).

Two main reasons have been proposed for the low level of polymorphism detected by 2DE. One is that the polypeptides accessible to analysis by 2DE come from a different class of proteins such that a higher proportion of gene loci are either monomorphic or have less heterozygosity (Jones, 1980). For example while 1DE is usually limited to soluble proteins, membrane proteins and organelle components are also visualized on 2DE gels. The general thinking is that these proteins may interact with each other and the interaction may impose constraints on the tolerance of variation in these proteins (Wanner et al., 1982). The second argument is that 2DE is not as efficient as 1DE in detecting variation (McLellan et al., 1983). In the 2DE method a uniform set of conditions apply to all polypeptides while in 1DE, gel conditions most appropriate to the resolution of the variants at a particular locus are used (Asakawa et al., 1988).

Furthermore 2DE may not be detecting the same variants, or only a subset of the variants detected by 1DE.

The most direct way to compare the sensitivity of 2DE with 1DE would be to do cross calibrations using known variants of abundant polymorphic proteins. McLellan (1983) used five haemoglobin variants of known sequence to study the sensitivity of 2DE method. McLellan could not distinguish the five proteins of known sequences by 2DE, but those variants formed four mobility classes in 1DE (Ramshaw, Coyne and Lewontin, 1979). McLellan (1983) also compared nine -GPDH variants from six different Drosophila species. The mobility classes of these -GPDH variants were known from previous study (Coyne et al., 1979). McLellan could see only four out of the nine classes of -GPDH on 2DE while seven were seen on 1DE starch gels (Coyne et al., 1979). In another study by McLellan and Inouye (1986) fourteen myoglobin proteins of known sequence were examined by isoelectric focusing with and without urea. Fourteen sequence variants formed six distinct mobility classes on gels without urea and three classes on those with urea. So they came to the conclusion that under denaturing conditions isoelectric focusing performed poorly and that

only unit charge differences could be detected by 2DE. The level of genetic variation as well as the number of polypeptides resolved by 2DE is partly dependent upon the technical procedures used (Damervalet al., 1986). Many researchers are using this technique and in turn are showing that the resolving power of 2DE can be improved by using technical improvements as in the case of any new technique. New improvements such as, the use of zwitterionic detergents (Klose et al., 1984; Damerval et al., 1986; Hochstrasser et al., 1988), flourography instead of autoradiogrphy (Klose and Zeindl, 1984 and doublelabelling (Wheeler, 1986) have helped to visualize more proteins in a gel. Use of flourography by Klose and Ziendl (1984) enabled them to visualize 96% of the proteins from epithelial-like human carcinoma cell lines. Increased pore size of the IEF gels (Spicer, 1988) and the size of the second dimension gels (Young, 1984; Hochstrasser et al., 1988) also helped to improve the resolution of the proteins. Wanner et al., (1982) resolved sixteen out of seventeen known allelic variants using 2DE by changing the pH gradient. Damerval et al., (1986) using the same two unrelated wheat lines, but by improving extraction, electrophoretic and silver staining

procedures increased the 2DE estimate of genetic polymorphism from 6.7% to 15.2%. But the ratios of qualitative (differences in mobility) to quantitative variation (differences in protein amounts) were similar (1.4 versus 1.5) in the two studies. In a recent study Spicer (1988) has re-examined the five whale myoglobin protein variants studied by McLellan et al., (1984). Spicer also could not separate the five myoglobins using three different denaturing isoelectric focusing conditions. But by using denaturing nonequilibrium pH gradient electrophoresis in the first dimension, all five whale myoglobin proteins were identified. At the second dimension also, all the myoglobin proteins were separated, but the differences did not correspond to their molecular weights. This led Spicer (1988) to conclude that 2DE can be more sensitive to differences in primary protein structure and not just to unit charge differences as previous studies have shown.

4.2 Genic Variation in Tissue-Specific Proteins

The level of genic variation detected by 2DE appears to be very much dependent on the type of tissue sampled. This should not be surprising as unlike the majority of the enzymes sampled by 1DE which tend to occur in most cell and

tissue types, a significant proportion of 2DE proteins appear to be tissue-specific. These proteins may be susceptible to varying levels of mutation rate and/or natural selection. A clear example of variation in tissue specific proteins, besides the present study, is shown in Table 4.2. Rosenblum et al. (1983) and Asakawa et al. (1985) studied plasma proteins by 2DE and Neel (1978) studied serum and erythrocyte enzymes by 1DE in the same populations of Japanese, Caucasians and Amerindians. In these 2DE studies the same polypeptides were scored for heterozygosity. Besides 1DE and 2DE comparisons, heterozygosity among three ethnic groups using two different staining methods (silver and coomasie blue) are also available in this data set. These data on serum and erythrocyte enzymes and plasma proteins (Asakawa et al., 1985; Neel, 1978) can also be compared with erythrocyte and lymphocyte proteins shown in Table 4.1. The 2DE estimates of genic variation in plasma proteins are comparable to 1DE estimates of enzymes but are higher than the 2DE estimates of erythrocytes, lymphocytes, kidney and fibroblast proteins. This suggests that the lower estimate of genic variation detected by 2DE cannot be all due to its lower

TABLE 4.2

Comparison of heterozygosity in human plasma proteins and erythrocyte enzymes

Population & Technique	P	H	N	References
Amerindian				
2DE (plasma)				
silver stain	0.18	0.045	11	Asakawa et al., (1985)
coomasie stain	0.33	0.032	12	Asakawa et al., (1985)
1DE (serum & erythrocyte enzymes)		0.054	28	Neel et al., (1978)
Caucasian				
2DE (plasma)				
silver stain	0.27	0.080	11	Asakawa et al., (1985)
coomasie stain	0.58	0.087	12	Neel et al., (1984)
1DE (serum & erythrocyte enzymes)		0.078	28	Neel et al., (1978)
Japanese				
2DE (plasma)				
silver stain	0.18	0.057	11	Asakawa et al., (1985)
coomasie stain	0.05	0.040	12	Asakawa et al., (1985)
1DE (serum & erythrocyte enzymes)		0.077	28	Neel et al., (1978)

sensitivity. The differences may be due to varying levels of neutral mutation, natural selection and functional constraints expressed by tissue-specific proteins.

In the present study we sampled various tissues of presumably different functions and of two different developmental stages (larva and adult). Individual tissues must contain some tissue-specific proteins besides general proteins or enzymes that are present in all tissues. Like the results reported by Klose (1989) on different mouse tissues and by Leonardi et al., (1987) on maize tissues both qualitative (presence/absence) and quantitative (differences in amount) variation were found in the present study, although in the present analysis we are only concerned with qualitative differences.

The two adult reproductive tissues - accessory gland and testis - show high divergence but low heterozygosity (Coulthart and Singh, 1988a). Chen (1984) has also reported that major protein components of the accessory gland in each species differ in isoelectric points as well as in their molecular weights even though the ultrastructure of accessory gland in different species appear very similar. 2DE analysis have shown that accessory gland proteins in

Drosophila are highly species specific (Stumm-Zollinger and Chen, 1984).

The eight tissues studied here showed different levels of divergence in each species. For example, adult as well as larval testis proteins of D. melanogaster are more diverged than any of the other three species. This is not surprising considering the relationship of D. melanogaster to the other three species. D. melanogaster being the oldest among the four species shows a high level of reproductive isolation with some hybrid inviability and complete hybrid male sterility. Thus the high divergence observed in the adult and larval testis of D. melanogaster is in line with its high reproductive isolation, uniparental progeny and hybrid sterility. The apparent absence of the same level of divergence in all the non-reproductive tissues examined in this study may indicate a preferential genetic involvement of male reproductive tract proteins in species formation. But this conclusion must remain tentative until more in-depth studies have been done.

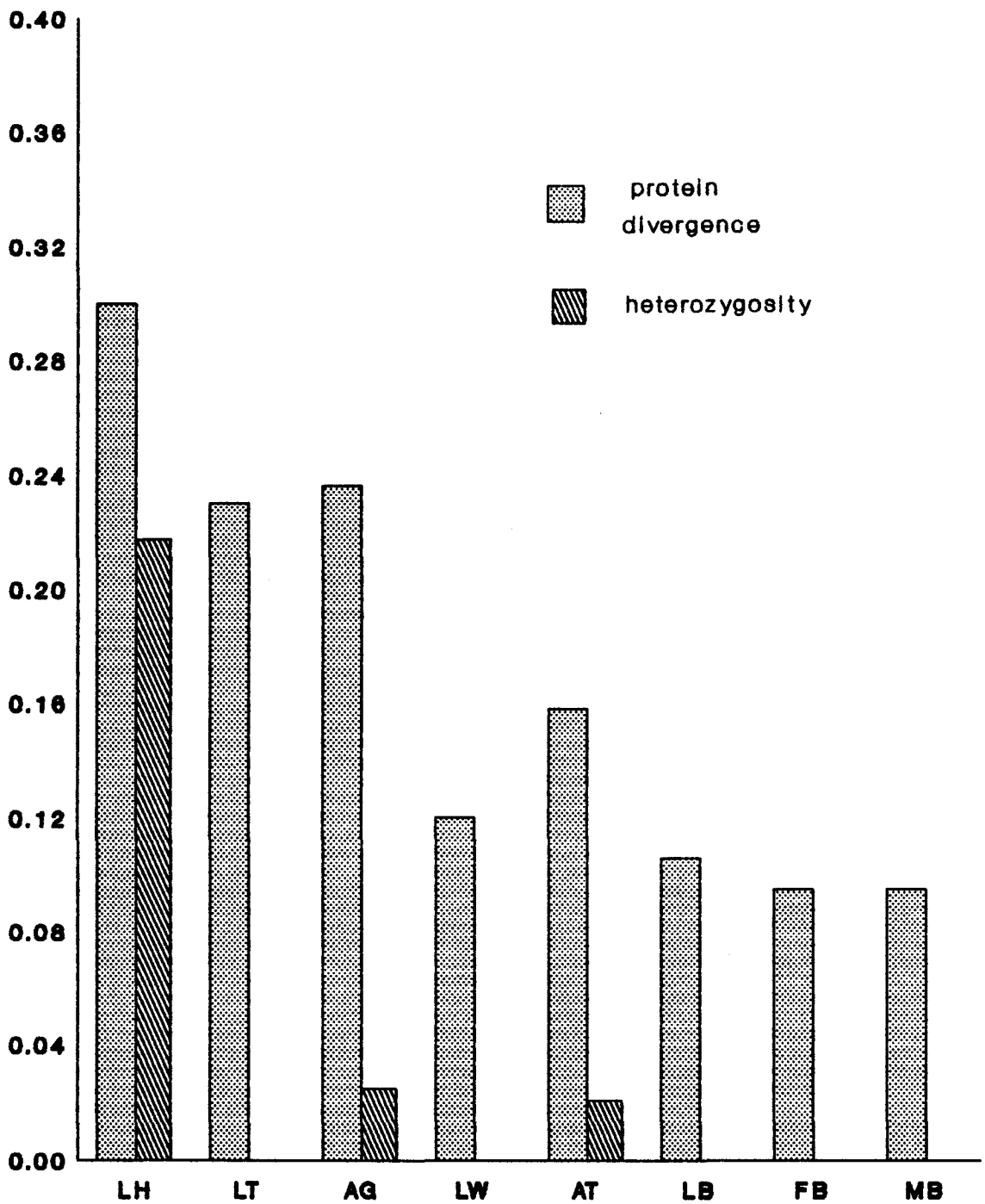
4.3 Relationship Between Heterozygosity and Genetic Distance

In most genetic studies of species comparisons Nei's genetic distance (D) is used as a measure of genetic

divergence. The calculation of D requires gene frequency data on homologous gene loci between the species or taxa being compared. In the present study the data are based only on single line from each species. Therefore the divergence statistics used here is not D and is based on the proportion of protein spots diverged between two species (Materials and Methods). Heterozygosity data are available from our laboratory on three of these tissues : larval haemolymph proteins by 1DE (Singh and Coulthart, 1982) and adult testis and accessory gland proteins by 2DE (Coulthart and Singh, 1988a). The estimates of heterozygosity in D. melanogaster and D. simulans are plotted against the 2DE protein divergence data obtained from these species (Figure 4.1). The haemolymph proteins show high heterozygosity and high divergence. Since the "haemolymph" used in this study contained other organelle like haemocytes and disrupted tissue components (Rizki, 1978), the observed values do not reflect the divergence of true haemolymph proteins. Like fibronopeptides in humans high heterozygosity and high divergence of "haemolymph" proteins may mean that these proteins are under less functional constraints (Kimura, 1989).

Figure 4.1

Relationship between genic hterozygosity and proportion of genic distance (D) as calculated in this study. The genic heterozygosity data is from Singh and Coulthart (1988). The divergence data is the mean proportion of unique proteins between D. melanogaster and D. simulans as shown in Figure 3.6 (page 65).


0.40
0.36
0.32
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0.00
protein divergence
heterozygosity
LH
LT
AG
LW
AT
LB
FB
MB

The testis and accessory gland proteins, due to their involvement in reproduction and possibly in reproductive isolation, may also be under functional constraints. Their low heterozygosity suggests that these proteins may remain relatively monomorphic within species (by purifying selection) but favourable mutations get incorporated rather rapidly between species. At this point a detailed study of both heterozygosity and genetic divergence involving species from different groups of Drosophila would be most valuable.

4.4 Two-Dimensional Gel Electrophoresis and Genetic Divergence Between Species

Few investigators have used 1DE allozyme data and 2DE protein data to compare genetic divergence among different species (Ohnishi et al., 1983; Goldman et al., 1987; (Goldman and Giri, 1989). The levels of genetic divergence obtained by these two methods are different and the 2DE estimates are usually lower. Ohnishi et al., (1983) used 1DE and 2DE data to compare genetic distances among eight species of D. virilis group, six species of D. melanogaster subgroup, and four species of D. auraria complex. The genetic distance values obtained by

2DE were generally lower than those obtained from 1DE in all comparisons, but the phylogenetic relationships obtained from the 2DE data were topologically similar to those from the 1DE data. For example the phylogenetic relationship obtained for D. melanogaster subgroup using 2DE data was similar to that obtained by other investigators using cytological data (Lemeunier and Ashburner, 1976); mtDNA (Barnes et al., 1978) and rDNA (Tartof, 1979). Goldman et al., (1987) used 2DE data from fibroblast cells to calculate genetic distances among the hominoid primates. In this case also the phylogenetic reltionship was consistent with those generated by other molecular procedures, and the data supported the human-chimpanzee-gorilla trichotomy in favour of a more recent association of humans and chimpanzees. In the latter study the phylogenetic relationships obtained by 1DE allozymes and 2DE proteins were similar, even though the 2DE distance values were generally lower than the 1DE values. The lower 2DE estimates of D, which are clearly based on a much larger number of proteins than the 1DE estimates, suggests that because of functional constraints and purifying selection estimates of chronological divergence time from protein data would be grossly underestimated.

4.5 Phylogenetic Relationships of Species in D.melanogaster Complex

The phylogenetic relationship of D. simulans, D. mauritiana and D. sechellia is an unsettled matter. The data gathered from different sources show various proposed relationships and are presented in Table 4.3. A variety of molecular, morphological and behavioural traits have been used to infer their relationships. Phylogeny based on morphological and behavioural data may be more ambiguous because of the possible involvement of selection on these characters. The C-banding (Lemeunier, 1984) is the only characteristic that showed an unambiguous D. sechellia- D. mauritiana grouping. In the Adh sequence comparison (Coyne and Krietman, 1986) D. mauritiana lines showed more divergence than the average divergence of D. simulans and D. sechellia and because of this the latter two species appeared very close to each other. The authors themselves have admitted that it was difficult to reach an unambiguous conclusion with only one sequence.

In the case of scDNA (Caccone et al., 1988), D. mauritiana and D. sechellia turned out to be a monophyletic group branching from D. simulans. Even though

TABLE 4.3

Comparison of phylogenetic relationship among the three sibling species of D. melanogaster based on various traits. "+" refers closer phylogenetic relationship between two of the three species compared

Traits	si-ma	si-se	se-ma	Reference
Molecular traits				
C-banding			+	1
Adh sequence		+		2
Allozyme data		+		3
ScnDNA			+	4
mtDNA	+			4
mtDNA	+			5
2DE proteins	+			6
2DE proteins	+			7
Morphological traits				
Asymmetrical mating	+			6
Cyst length	+			8
Number of sex comb		+		9
Testis colour		+		9
Genital shape	+			9
Cross fertility		+		9
Cross fertility	+			10

References: (1) Lemeunier and Ashburner (1984); (2) Coyne and Krietman (1986) (3) Cariou (1987); (4) Caccone et al., (1988); (5) Solignac et al., (1986) (6) Lee and Watanabe (1987); (7) Coulthart and Singh (1988); (8) Joly (1987); (9) Coyne and Krietman (1986); (10) David et al., (1984).

the authors studied four D. mauritiana lines to get an unambiguous relationship, the divergence was not significant. The mtDNA data (Caccone et al., 1988; Solignac et al., 1986) showed that D. mauritiana is closer to D. simulans than D. sechellia. Allozyme data (Cariou, 1987) based on 33 loci grouped D. mauritiana and D. sechellia together, but the divergence is again not significant.

2DE data from previous studies (Lee and Watanabe, 1987; Coulthart and Singh, 1988c) showed D. mauritiana to be closer to D. simulans than to D. sechellia. The present data based on eight tissues also confirm this relationship. The three tissues showing most divergence (haemolymph, accessory gland and adult testis) and the three tissues showing least divergence (adult male brain, larval brain, and wing disc) showed D. mauritiana closer to D. simulans than to D. sechellia. (Figure 3.15). In the remaining two tissues the divergence is not significant. The combined data from all tissues also grouped D. mauritiana with D.simulans. This is an important result since it is based on a number of tissues showing different divergence rates within and between species. If all the available data is

considered along with the present data, the most plausible grouping is D. mauritiana and D. simulans together. More allozyme and DNA data may help to reaffirm these results.

4.6 Conclusion

The main findings of this study can be summarised as follows:

1. Different tissues of the same species show dissimilar levels of divergence suggesting that proteins of each tissue are evolving at different rates. There is no obvious trend or pattern to suggest that either the larval or adult tissue proteins are more diverged than the other.
2. Adult testis proteins and accessory gland proteins show high divergence consistantly in all pairwise and four-way comparisons. Larval testis proteins of D. melanogaster also show high divergence, suggesting that the differences are probably related to function rather than to developmental stage.
3. D. melanogaster, the oldest among the four species shows the highest level of divergence and a high level of reproductive isolation. Among the other three species the levels of divergence of the reproductive tract proteins are similar but lower than those of D. melanogaster.

The high divergence of reproductive tract proteins observed in the most reproductively isolated of these species, (D.melanogaster) suggests a correlation between reproductive isolation and divergence of reproductive tract proteins.

4. Phylogenetic relationships based on different tissue proteins show D. mauritiana to be closer to D. simulans than to D.sechellia.

4.7 Future Prospects

The three sibling species studied here are chromosomally homosequential. All possible crosses yield fertile females and sterile males except for one cross involving D. simulans male and D. sechellia female which is not successful. In all successful crosses hybrid males are sterile, but with well developed testes. This limited study has shown that proteins of male reproductive tissues from both larval as well as adult stages have diverged among these species. It will be worth while to extend the study to female reproductive tissues to see if those proteins also show high divergence. Since hybrid females are fertile, one may be tempted to predict that female reproductive tissues would show less divergence.

All the four sibling species studied here show post-mating reproductive barriers. Besides extending this

study to other members of the D. melanogaster subgroup it will be interesting to involve species from other Drosophila species groups which show only pre-mating isolating barriers. If the high reproductive-tract-protein divergence is related to reproductive isolation and speciation, then one may predict that species pairs showing only pre-mating isolation, (i.e., producing fertile hybrids) would show less divergence in their reproductive tract proteins. Species from the Mulleri and Hawaiian Drosophila groups will be ideal for this study.

Adult testis proteins showed less divergence than larval testis proteins in D. melanogaster; in the other three species, D. simulans, D. sechellia and D. mauritiana, larval testis showed lower divergence and all the values fell in the same range. It will be worth while to undertake a developmental study of larval testis proteins in D. melanogaster since it is possible to distinguish male and female larvae from the first instar (Demerec, 1965), and so the study will be quite feasible. The developmental profile of reproductive tract proteins with species pairs showing pre-mating and post-mating barriers would show whether the possible genetic causes of hybrid male sterility can be correlated to genetic divergence of reproductive tract

proteins. This study will show whether the hybrid male sterility is due to pleotropic effect of overall genetic divergence in the genome, or due to few specific genes that are critically involved in spermatogenesis.

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