VARIATION AND EVOLUTION IN PROTEINS OF THE *Drosophila* MALE REPRODUCTIVE TRACT

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ABSTRACT

Improved two-dimensional gel electrophoresis (2DE) and silver staining were applied to Drosophila male reproductive tract proteins. Genic variation was scored for about 300 polypeptides in 20 isofemale lines each of Drosophila melanogaster and D. simulans. Approximately 10% of loci were polymorphic within each species, with average heterozygosity in the range: 2-3%. These estimates are significantly lower (2-5 fold) than analogous ones from one-dimensional gel electrophoresis (1DE) of soluble enzymes in the same Drosophila populations. This confirms earlier reports of low variability in 2DE proteins from Drosophila, Homo and Mus. It is argued that the technical improvements applied, as well as other considerations, indicate that the variability differences are not artifacts of electrophoretic technique. 2DE was also used to compare male reproductive tract proteins between D. melanogaster and D. simulans, D. simulans and D. mauritiana, and D. simulans and D. sechellia. About 10% of loci were apparently fixed for different alleles between D. melanogaster and D. simulans. About 25% of polypeptides within D. melanogaster or D. simulans lacked a detectably homologous spot in D. simulans or D. melanogaster, respectively. Many of these unpaired spots may represent

large (>10-fold) changes in polypeptide expression. Male reproductive tract proteins may be evolving faster than other proteins in these four species, as judged by 2DE comparisons of imaginal disc proteins and 2DE proteins of whole-body extracts. Finally, polypeptides localized to glandular tissues of the male reproductive tract in D. simulans were on average more highly polymorphic than polypeptides expressed in both testis and glandular tissues or only in testes. Glandular polypeptides of D. simulans were also more highly diverged from those of the other three species, compared to polypeptides expressed in testes. These increases occurred along with decreases for polypeptides co-expressed in testes and glands. These patterns were weaker in D. melanogaster. The results are discussed with reference to theories of balanced genetic structure in populations, and shifts in such balance during species formation.

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GENERAL INTRODUCTION

The purpose of this chapter is to place the research undertaken for this thesis into the context of a broad biological problem of central importance to our understanding of evolution. The first section of the chapter contains a general statement of this problem. Precisely because the problem is such a broad one, it will next be clarified by briefly tracing certain aspects of its history of formulation and reformulation in the nineteenth and twentieth centuries. Then, an interpretation of its current status will be offered, emphasizing the unique role that data on macromolecular structure have had in this most recent formulation. Finally, with this background laid, the rationale for the current research will be explained.

Population Genetics, Species Divergence, and the Mechanism of Evolution

In Darwin's (1859) original formulation of a mechanistic theory of organic evolution by means of natural selection, and in later articulations of the basic structure of the theory (Monod, 1971; Lewontin, 1970; Maynard Smith, 1975; Dawkins, 1976), evolution is understood as the outcome of interaction between two essential components of biological systems- heritable variation in populations of organisms, and

constraints imposed on that variation by the environments which the organisms encounter. As described in numerous recent accounts (e.g. Provine, 1971; Lewontin, 1974; Wills, 1980; Mayr, 1982a; Turner, 1983; Bowler, 1984; Clark, 1984), the post-Darwinian period has been one of extension and refinement in our understanding of the operation and true significance of this interactive mechanism in evolution. The period has progressed through several historical phases, each characterized by spirited and sometimes ardent debate.

Interestingly, the boundary dates of these phases were each correlated with major advances in genetics. Moreover, interwoven with the historical phases are two genetic themes that have lent organization and purpose to theoretical and experimental research in the field. Both of these themes have been reformulated repeatedly, in accord with the methods, data and concepts prevailing in genetics at the time. Thus, the role of genetics in evolutionary biology has been a crucial one. This has been largely by virtue of its success in illuminating the rules governing the behaviour of the "heritable variation" component of Darwin's evolutionary mechanism, but also to some extent by providing data and concepts to help understand the nature of biological divergence resulting from the evolutionary process.

The first of the two recurrent genetic themes has been embodied in the diverse models which have been tacitly assumed or explicitly articulated to describe the

genetic structure of populations. Among the many possible distinguishing features which could be used to classify these various models, most important are their different assumptions regarding the amount and character of genetic variation present in natural populations of organisms, and also the assumed relative importance and exact mode of interaction of natural selection and chance in the maintenance and organization of this genetic variation.

The second genetic theme incorporates an even more diverse array of ideas, concerning the nature of genetic differences between species. Schools of thought have tended to form here along lines analogous to those seen in discussions of population structure; disagreements have related to the numbers and kinds of gene substitutions that underlie species differentiation, and to the relative roles of natural selection and chance in "driving" the various phases of genetic divergence between populations in the process of evolving into species.

The view is taken here, in keeping with that expressed by Mayr (1982b), that together these two themes represent, in condensed form, many if not most of the fundamental outstanding questions about the mechanism of organic evolution. This helps to explain the amount of attention and effort they have commanded in the past. It also implies that further efforts to understand population variation and species divergence on the genetic level, and especially to

grasp the connections between these two sets of phenomena, will probably contribute greatly to a deeper understanding of the entire evolutionary process.

This last point - that advances in understanding the connections between intraspecific variation and interspecific divergence will be of unique value - is difficult to overemphasize. Population variation and species divergence have not always explicitly been considered together, although it is clear that a viewpoint favoured in one of these two areas will tend to affect that favoured in the other area. For example, if one believes that most of the genetic polymorphism in natural populations is neutral with respect to the fitness of organisms, then much of evolution may be viewed as dependent on stochastic processes (Kimura, 1983). On the other hand, postulation of widespread selectively-maintained polymorphism predisposes one towards thinking of evolutionary change as driven by adaptive forces (Wills, 1980). However, at this point the basis for making either logically or empirically justified leaps from statements about population variation to statements about the genetics of species differences is even slimmer than is our knowledge of either of the two separately. The experimental approach taken in the present research was, therefore, designed to yield relevant data both on within-species genetic variation and on species divergence. As will be seen, the data

obtained suggest that much remains to be learned about the connection between these two sets of phenomena.

Historical Overview

The Mechanism of Heredity and the Emergence of the Mendelian Population. The primary concern for Darwin's theory after the publication of The Origin of Species was the demonstration of the possibility that natural selection could operate as a mechanism for evolution. Perhaps the most fundamental deficiency in the theory at the time, as recognized by Darwin himself, was the lack of a well-articulated theory of the mechanism of heredity. Thus in 1867 the Scottish engineer Fleeming Jenkin criticized the idea of natural selection as fundamentally unsound because, he maintained, the blending nature of inheritance in a randomly breeding sexual population would rapidly dilute any adaptively significant variation, before natural selection could have an effect on the population's characteristics. But the rediscovery, around 1900, of Mendel's work demonstrating the particulate (non-blending) nature of hereditary determinants provided a basis for the eventual obviation of objections like Jenkin's, and marked the end of the first major historical phase of the post-Darwinian era.

The second phase, extending between 1900 and 1918, saw the establishment of three important conclusions regarding the operation of the Mendelian mechanism. The

first of these was the mathematical demonstration by Hardy and by Weinberg that a randomly breeding diploid sexual population would maintain an absolute stability of allele frequencies ("Hardy-Weinberg equilibrium") in the absence of perturbing influences (such as differential reproduction of the various genotypes). The second was that phenotypically continuous variation in a population actually has a basis in normal Mendelian inheritance, assuming only that alleles of individually small effect are segregating at many loci. And thirdly, it was repeatedly shown that artificial selection (and thus by implication natural selection) could dramatically shift the population mean of a continuously varying phenotypic trait - often well beyond the original range exhibited by the parent population.

These advances had several effects on thinking about the mechanism of evolution. One was that the demise of theories of blending inheritance was finalized. But even more important were the new concepts of the nature of genetic variation in populations under a Mendelian system of inheritance. Not only was allelic variation capable of being stored in such a population as a simple consequence of the mechanism of inheritance, but this type of variation was apparently present in abundance for most phenotypic traits in most organisms, and could respond readily to changing selection pressures. Thus, simplistic ideas of population structure espoused, for example, by William Bateson and Hugo

de Vries, to the effect that populations were genetically essentially uniform and that natural selection required new mutations of large and specific phenotypic effect in order that new species could evolve, became untenable. A concept of the Mendelian population as a complex, dynamic entity with high evolutionary potential emerged: the way was thus paved for the construction of a mathematical theory of population genetics and evolution (Fisher, 1930; Wright, 1931; Haldane, 1932).

The Modern Synthesis and a Shift in Focus. The third phase, occupying roughly the period between 1920 and 1950, was stimulated by the growing body of work elucidating the nature of the gene as a physiological entity that was extremely stable but nevertheless had an enormously broad and subtle potential for mutation (Muller, 1927). These genes were now known to be physically ensconced in a stable linear order within a specific cellular structure, the chromosome, which was at the same time capable of extensive recombination between homologous genetic sites (Morgan et al., 1915; Darlington, 1932). And in terms of their mechanisms of action, genes were also shown to be somehow in control of specific, individual biochemical (enzymatic) functions of the cell (Beadle and Tatum, 1941). This period culminated in what J. S. Huxley (1942) termed "The Modern Synthesis", referring to the fusion of Mendelian genetics with the now mathematically -formulated theory of natural selection and

with systematics and paleontology. The first basic proposition of this synthesis was that gene mutations and their recombinations provide the essential "raw material" upon which natural selection acts to cause evolution within populations. Subsidiary roles were assigned to gene flow patterns (migration), population size fluctuations (random genetic drift) and deviations from random mating (inbreeding); the function of each of these factors was to influence the actual distribution of genotypes available in populations for natural selection to act upon. The second basic proposition was that no additional genetic mechanisms beyond those operative within populations - only greater numbers of generations - are required in the process of evolutionary divergence between populations, species, and higher taxonomic groupings.

Considering the essentially universal acceptance of the validity of the Modern Synthesis, it may at first sight be surprising that major controversies remain over the nature of genetic structure in populations and genetic differences between species, and over the role played by natural selection in shaping variation and driving evolution. But such is indeed the case, and in fact, the most difficult issues only became clearly focused after the most basic and general questions - those about the workings of the hereditary mechanism, the existence of genetic variation in populations, the potential of natural selection to effect

allele substitutions, and the general outlines of the process of evolutionary divergence - had been adequately answered in the Modern Synthesis. The origins and the difficulty of the issues that grew out of the Synthesis can be traced to two basic sources. First, it became important not to be content simply with descriptions of possible evolutionary mechanisms, but to attempt to make decisions as to what the actual mechanisms have been. Thus, neither theory nor extrapolation from laboratory experiments were any longer in themselves adequate means for testing the hypotheses which were being proposed: collection and careful interpretation of relevant data from natural populations and species were required. And second, these more realistic hypotheses could not be framed in terms of clear-cut alternatives. The correct answers to the questions being asked were not expected to be categorical statements, but rather, quantitative, conditional and qualified ones - How important is natural selection, in comparison to other factors, in driving interspecies divergence? Under what conditions? In which groups of species? For what proportion and which types of gene loci? and so on. Evolutionary genetics since the Modern Synthesis has to a large extent been preoccupied with meeting this dual challenge, on both theoretical and experimental levels.

Parallel with and complementary to this post-Synthesis sophistication in formulation of the evolutionary questions, moreover, came the methodological and conceptual

sophistication of molecular genetics. In keeping with the general historical pattern, but on an unprecedented scale, the post-1950 advances in genetics have profoundly altered the character of discussion in the field of evolutionary biology. It is probably safe to say that in principle, essentially any gene can now be analyzed for population variation and evolutionary divergence. However, the challenge will come in the interpretation of this data in terms of precisely and shrewdly formulated mechanistic hypotheses.

The Classical and Balance Hypotheses: The Mendelian Population as a Balanced System. In an introductory essay to the 1955 Cold Spring Harbor Symposium on "Population Genetics: The Nature and Causes of Genetic Variability in Populations", Dobzhansky (1955) formulated what he considered to be one of the most basic questions of population genetics, which was - What is the genetic structure of a Mendelian population that is at or near its "adaptive norm"? By "adaptive norm", Dobzhansky meant "array of related genotypes consonant with the demands of the environment". At that time, according to Dobzhansky, two basic points of view on this question were being espoused by different groups of geneticists; he termed these the "classical" and "balance" hypotheses.

The classical hypothesis, Dobzhansky maintained, pictured a well-adapted population to be essentially homozygous, with a variety of relatively rare variant alleles present: deleterious recessives in the process of selective elimination; selectively neutral or nearly neutral alleles undergoing primarily random changes in frequency and frequently being lost thereby; an occasional selectively balanced pair or set of alleles (maintained polymorphic as a result of either spatial or temporal fluctuation in sign of their relative fitnesses); and (especially rare) advantageous mutations in the process of replacing the "wild-type" alleles at their respective loci. This view was articulately propounded by H. J. Muller (e.g. Muller, 1949), but is derived directly from a mathematical theory of natural selection constructed by R. A. Fisher (Fisher, 1930). In Fisher's theory, evolution takes place by selective allele substitution in large, randomly-mating populations. The substituted alleles have individually small effects on organismal fitness and act additively (i.e. have minimal interaction with the genetic background); they are accumulated one by one over long periods of time. Thus, in the "classical" view, populations have a relatively simple genetic structure, being maintained mostly homozygous by a "purifying" action of natural selection. Evolution of a biologically meaningful sort proceeds slowly and gradually by an orderly process of serial substitution of favourable

mutant alleles at many loci; species divergence thus could be viewed as an almost inevitable by-product of the continual improvement of adaptation within populations, requiring only the occasional occurrence of extrinsically imposed barriers to gene flow between populations in order to initiate the "fission" process (Fisher, 1930: Chapter 6).

Sewall Wright (see review in Wright, 1977: Chapter 12) was highly critical of Fisher's theory on the grounds of its being based on oversimplification both of the ways in which genes act to determine phenotypes, and of the ways in which natural populations are genetically structured. Wright constructed his own "shifting balance" theory of the genetic mechanism of evolution, including several elements which Fisher's theory did not: large amounts of selectively nonequivalent allelic variation in populations; strong gene interaction and pleiotropy in the genetic determination of organismal fitness; spatially subdivided, locally inbred population structure; and variable and potentially high rates of evolution that depended significantly on chance factors (e.g. random genetic drift, migration, recombination, environmental change) for their specific magnitudes at any given time (Wright, 1977, Chapter 13).

Wright's view of population structure and evolution is profoundly different from that of Fisher and Muller. It visualizes the gene pool of a species as an <u>internally balanced system</u> of variability. The domain of the gene pool

is a multidimensional, many-peaked surface of fitness values that vary in relation to gene frequencies (Wright's "adaptive landscape"); this is quite distinct from Fisher's model of the gene pool where there is a single globally optimal genotype at any given time, and therefore much less opportunity for the balanced storage of variation. At any given time, in Wright's model, various subpopulations of a species tend to occupy different local fitness peaks in the adaptive landscape, thus permitting much genetic diversity to be maintained in the species as a whole. The shape of Wright's adaptive landscape is determined by constraints, thresholds and other "system properties" expressed by the gene pool as it interacts with the organized physiological and developmental systems of the organism and also with the environment. As a balanced system, the gene pool can respond in very diverse ways to both deterministic and random forces. These responses may involve stable and unstable states, multiple homogeneous or heterogeneous equilibria, variable and sometimes rapid rates of transition from one state to another, and disproportion between the immediate and ultimate magnitudes of the effects of perturbations.

The "balance hypothesis" of population structure described by Dobzhansky in his 1955 paper holds much in common with Wright's view. The central distinguishing feature of Dobzhansky's model is the importance that it assigns to heterosis (selection in favour of heterozygotes)

as an internal balancing mechanism for maintaining genetic variation in a Mendelian population, even in the immediate neighbourhood of its adaptive norm. The balance hypothesis thus predicts that allelic polymorphism will prove to be the rule, rather than the exception, for gene loci as they are analyzed in natural populations. The same general viewpoint was put forth by Mather (1953) with his concept of "relational balance" in polygenic systems, and by Lerner (1954), with his emphasis on the importance of intermediate fitness optima and on the phenotypic intermediacy of heterozygotes with respect to most metric traits. Thus, while preserving Wright's approach to the gene pool as an integrated system, the "balance school" of population structure placed heterosis and other forms of balancing selection in a key position among the factors underlying the basic structure of the system.

The balance hypothesis circumvented major shortcomings inherent in the Fisher-Muller model of population structure and evolution. Some of these shortcomings had already been perceived by Wright - lack of biological realism, extreme slowness and the consequent requirement for constancy of small selective differentials over long periods, and poor explanation of the evolution of complex adaptations controlled by many interacting genes. The point most heayily stressed by Dobzhansky, however, was something he saw as a paradoxical logical consequence of a

simplistic view of the operation of natural selection: How could such a process, with its inexorable restriction of the range of variability in gene pools *via* the systematic elimination of all but the fittest genotypes, be compatible with the continued evolutionary flexibility of the lineage? Obviously, the paradox disappears if natural selection, while still optimizing fitness (*adaptedness*) on the phenotypic level nevertheless favours heterogeneity (which was equated with *adaptability*) on the genetic level. But perhaps most revealing of the fundamental attitude of the balance school was the fact that they postulated the widespread existence of systems of genetic variability that not only *responded* to natural selection by changing their composition, but actually owed the very *existence* and *structure* of their variability to natural selection. The variation in a population was not simply seen as "noise" resulting from the random effects of mutation; it could, in one sense, almost be considered an adaptive characteristic in itself - an adaptation the function of which was to promote evolutionary flexibility.

One important consequence of this way of thinking was the reduction of the role of chance to an even smaller one than that envisioned in the classical model (in the form of mutation to favourable alleles), and the ascription of even more power to natural selection as the overwhelmingly dominant factor in evolution. Change was pictured as a process of reorganization of the genetic system, which was

otherwise held in a state of quasi-equilibrium by a combination of its own internal balance mechanisms and the constraining effects of the environment. Chance was involved in the form of environmental change, recombinational assembly of new genotypes, and, in Wright's model at least, random fluctuations in gene frequencies. But because of the abundance of variation assumed in the system concept of the gene pool, the evolutionary potential of populations was considered to be only rarely limited for very long by lack of the appropriate genetic variation for adaptation.

Probably the most far-reaching influence of the balance model was based on its attempt at greater biological realism, even if this was bought at the price of considerably increased theoretical complexity. This realism and complexity helped to precipitate the major shift in focus mentioned earlier - away from construction of simplified scenarios describing the operation of individual mechanistic processes in evolution and towards empirical testing of the closeness of fit of the theoretical models to actual populations and species. This shift in focus was to point the way towards the next and most recent major phase in the development of evolutionary genetics.

Selection versus Neutrality: The Consequences of Having Molecular Data. As revealingly sketched by Lewontin (1974), the early empirical methodology used to study genic variation in natural populations, because of inherent factors of

imprecision and bias, was profoundly inadequate to the actual task of measurement. It was only in 1966 that molecular methods (protein electrophoresis) capable of relatively precise and unbiased, if indirect, measurement of diversity in a sample of allelic DNA sequences were first consciously applied to problems of population genetics, and high-quality genetic variation data began to flow in large quantities. The immediately surprising result was the large amount of allelic polymorphism present in almost all species investigated (Lewontin, 1974; see also Table 3.1). Since the mere existence of large amounts of genetic variation was sufficient ground for abandonment of the classical hypothesis as it was laid out by Dobzhansky, an initial, "naive" conclusion that the balance model had been vindicated or at least strengthened might have seemed justified. But as was quickly pointed out by Kimura (1968), such a conclusion was not justified. Kimura demonstrated elegantly that the mere fact of high variation was not enough to show that balancing selection was operative, since very high levels of variation could theoretically be present in populations simply by virtue of its irrelevance to organismal fitness (i.e. its selective neutrality) combined with the random genetic drift behaviour expected of such neutral variation if population size was even occasionally drastically reduced. Thus began the "selection versus neutrality" controversy over the

importance of natural selection in the maintenance of extensive molecular polymorphism in populations.

Lewontin (1974) labels the neutral mutation - random drift theory of Kimura the "neoclassical" theory, pointing out its similarity to Fisher's theory in terms of its emphasis on the rarity of balancing selection; we could add to this the feature of reinstatement of initially rare mutations to an important evolutionary role, whether they be neutral and fixed by drift or advantageous and fixed by selection. But as Wills (1980) suggests, the similarity may go deeper than this. For both the classical and neutral models of population structure are very simple models, both in terms of the general homozygosity (at least functionally speaking) postulated for most loci affecting fitness in natural populations, and in terms of the small number of uniformly acting causal factors underlying allelic variation where it does exist. Almost completely absent are the complex and dynamic interactions and system properties ascribed to the gene pool by Wright and by the balance school. This simplicity appears again at the level of the predictions made by the neutral theory for the rate and mechanism of evolution above the species level (Kimura, 1968). Here, Kimura adduced two observations: the apparent constancy over time ("clock-like" behaviour) of amino acid substitution rates for most proteins in most lineages, and the apparent functional irrelevance of most substitutions

that have occurred. He concluded that chance fixation of neutral mutations in occasional "population bottlenecks" would explain the observed evolutionary pattern, and indeed that most evolution at the molecular level may be of precisely this type. The mere fact of its simplicity does not, of course, validate or invalidate the neutral theory. But it is not very difficult to see why initial reaction against it was so strong, especially for those biologists who had been trained in the tradition of Wright and Dobzhansky.

The selection-neutrality controversy is not likely to be resolved in the near future. Neither theory can be proven: to do so for the neutral theory would require some means of conclusively demonstrating the effective absence of selection pressures on the alleles at a presumably enormous number of polymorphic loci. And it is only slightly less difficult to imagine a conclusive demonstration of the validity of the balance model. To actually show how natural selection operates to maintain polymorphism at even one locus is also an enormous task, fraught with practical and theoretical difficulties (Lewontin, 1974; Wills, 1980); and the balance theory calls for the majority of polymorphisms in the genome to be maintained in this way. However, probably the most important thing to keep in mind is that, as Lewontin (1974) aptly expressed it:

> "...the question was not simply, How much genetic variation is there? nor even, How much genetic variation in fitness is there? but rather, How much genetic variation is there that can be the basis of

> adaptive evolution? To answer that question it is not sufficient to measure genetic variation, which we can now do, nor to measure the present variation in fitness associated with that variation, which we have not done but which may be possible with a proper reorientation of theory. We require, further, that we be able to assess the potentiality for adaptive evolution in genetic variation that may currently be nonadaptive. But such an assessment will depend on an understanding of the relation between gene and organism that far transcends any present knowledge of development, physiology and behaviour. In fact, it demands the answer to every other question that now lies open in biology."

Stated slightly differently, this means that even if we were able to develop an accurate, quantitative understanding of the distribution of causal factors that currently maintain genetic polymorphism in populations, only one part of the answer would have been found, since we would then need to assess the relevance of the variation found within species to that seen between species. And the difficulty in accomplishing this is not only because of the temporal inaccessibility of most of the important intermediate evolutionary stages between extant species. In spite of the exquisite sensitivity of our molecular analytical techniques, we are profoundly ignorant of the ways in which the tolerance ranges and limits built into the internally balanced organization of individual organisms are or are not translated into internal balance at the level of evolving populations and species. The selection-neutrality controversy has helped us to glimpse the immensity of the

research program that lay latent in the structure of Darwin's theory.

Two Views of Species Divergence. The theme of the genetic structure of natural populations is more easily traced through its historical development than is the theme of genetic divergence between species, so this is where the present account has been focused up to now, treating evolutionary mechanisms as relatively straightforward extrapolations from the population models as indeed they tended to be by their original authors. This is by no means the only approach which has been taken, however, and in fact many evolutionary biologists with training in taxonomy and systematics, rather than attempting to understand the evolutionary potential of populations by studying the genetic variation they contain, have chosen to infer genetic mechanisms of divergence on the basis of extensive comparative work with extant species in actively evolving groups. No attempt will be made here to describe in detail the historical development of ideas on the genetic basis of species differences. Instead, an illustration will be given to show that the issues in this area are closely analogous to those that arise in discussions of population structure.

Probably the most energetic proponent of this "top-down" approach has been Ernst Mayr, who proposed and has continued to defend (Mayr 1954, 1963, 1970, 1982b) a genetic theory of species formation that he termed the "peripatric

theory". Mayr was impressed, at an early stage in his research on the systematics of birds of Indonesia, by the frequency with which phenotypic divergence accompanied the colonization of small islands by founder populations. This seemed to occur even in the absence of obvious differences between the environment of the island population and that of the ancestral population. He was thus led to propose that in small, peripherally isolated populations of normally outbred animals, forced inbreeding and random drift of gene frequencies so altered the shape of the adaptive landscape (in the sense of Wright) and/or the population's location within the adaptive landscape, that formerly maladaptive gene combinations could occur, increase in frequency, and eventually be fixed in the new population. Furthermore, Mayr postulated that an extensive web of physiologically and developmentally coadapted gene interactions usually exists between the specific alleles present at most of the different loci in the genome, and that these interactions serve as a cohesive influence normally preventing genetic divergence between populations of a species. Thus, evolutionary change of the kind he proposed, even though initially occurring at only a few loci, could trigger a massive adaptive reorganization of the gene pool. This, he said, would often amount to a "genetic revolution", with allele substitution taking place at a majority of loci in the genome. The revolutionized genomes of the peripheral isolates, at some

point during the divergence process, would as a secondary consequence become reproductively incompatible with the ancestral genome, and a new species would thus be formed.

Mayr's theory clearly belongs to the "gene-pool-as-a-balanced-system" tradition of Wright and Dobzhansky. It predicts that populations at a stage of divergence advanced enough to give reproductive isolation (i.e. biological species) should share few alleles at homologous loci, with loci that are monomorphic within species being alternately fixed for different alleles between species, and intraspecifically polymorphic loci showing strongly or completely non-overlapping sets of segregating alleles. It also suggests that the amount of genetic difference seen between two different populations will be a function primarily of the total number of genetic revolutions (speciation events) undergone since the lineages represented by the populations originally began to diverge. Thus, below the level of species there should be little or no genetic differentiation between populations, and where it is seen it should affect only a minuscule proportion of loci and/or should only involve quantitative shifts in allele frequencies. The initiation and the complete achievement of reproductive isolation should both be associated with large changes in the composition of the gene pool. At the time it was proposed, the main alternative to Mayr's theory of the genetics of speciation was that described along with the

Fisher-Muller model of population structure, where species differences accumulate steadily at the genetic level as a result of allele substitutions driven by natural selection. (Mayr referred to this approach as the "beanbag" school of population genetics). If this is the way that evolution proceeds, then gene differences between populations should not be correlated primarily with how many times reproductive isolation has arisen in their ancestral lineages since their initial divergence, but with the time elapsed since that divergence. Also, this second model predicts that the amount of genetic difference between reproductively isolated species need not be very large, and in fact may involve only a few loci.

Interestingly, early results from the application of protein electrophoresis to the question of species differences (reviewed by Lewontin, 1974; Ayala, 1975; Avise, 1976) were used to refute the genetic revolution model of Mayr, with the primary rationale being the absence of clear evidence for the kinds of massive and complete allele substitution during speciation which are predicted by Mayr's theory. This result appeared to weaken the concept of the gene pool as a highly integrated system, or at least to suggest that such system-like properties where they do exist are not very relevant to speciation. In this case the critical backlash came, in one sense, from the opposite direction as did the neutralist objections to the initial

interpretations of the data on genic variation within populations; that is, from authors who were proposing that species formation did tend to involve substantial reorganization of the organism (and putatively the genome). For example, Eldredge and Gould (1972) argued that Mayr's peripatric speciation model was actually the one most consistent with an observable pattern in the fossil record of rapid change followed by extended stasis ("punctuated equilibria"). And Wilson, Maxson and Sarich (1974) suggested that extensive changes in genetic regulation systems frequently accompany the evolution of mammalian species, whereas, by contrast, evolution of protein sequences proceeds in a clocklike fashion as argued by Kimura. Controversy continues unabated over the importance of the two modes of genetic change during speciation (see for example Carson and Templeton, 1984; Barton and Charlesworth, 1984). Therefore, molecular data have had a net effect on current thinking about species divergence mechanisms quite analogous to their effect in the population structure debate: the issue of whether the variation analyzed is relevant to the questions asked has been raised and sharpened, but is still very much an open one.

Purpose of the Present Research

The progress of genetics since 1900 has strongly influenced the various contemporaneous models of evolutionary

mechanisms which have been formulated, by supplying the terms in which the models have been framed as well as the methods available for genetic analysis of population variation and species divergence. But perhaps most important has been the tendency for the "state of the art" in genetics to influence perceptions and opinions as to what specific problems of genetic analysis incorporate, at any given time, the best combination of amenability to solution and importance for the Darwinian theory of evolution. Hence the shifts from elucidation of the mechanism of inheritance before 1920, to theoretical elaboration of the population consequences of these mechanisms between 1920 and 1950, to empirical testing of realistic theories in the period since 1950, with much emphasis on molecular analysis since 1966.

Another interesting observation that emerges from the preceding historical overview is that two distinct approaches, using what are ostensibly quite different starting points (populations and species) for the empirical study of the Darwinian mechanism, have both led to formulation of the same fundamental question: How are the effects of organization at the level of the organismic system felt in terms of processes occurring at the level of the gene pool?

The current preoccupations of experimental population genetics and evolutionary theory display a great deal of continuity with these historical trends. The selection -

neutrality controversy developed following an attempt to test the balance theory of population structure, and the original molecular data was based directly on the knowledge from molecular genetics that genic DNA encodes, in a colinear fashion, the primary structure of polypeptides. It was also assumed (quite reasonably) that the type of genetic variation which is reflected in polypeptide sequence variation in a "representative sample" of proteins from the genome provided a valid means to approach the study of genetic variation in populations. Soluble enzymes, for operational reasons as well as genetic ones (i.e. the "one gene - one enzyme" principle) were assumed to be representative proteins. Species comparisons using protein electrophoresis are based on the same genetic principles and assumptions; the questions asked here were also derived from the hypotheses of earlier workers (especially Mayr) who were concerned with the behaviour of gene pools as systems in evolution.

Another aspect of the parallelism between the molecular approach to populations and that to species, as suggested above, is that they both caused attention to be directed to the question of how relevant the variation being studied was to the questions being asked. The main contribution of the neutral theory may have been its demonstration of just how radically the variation we observe at any given point in a population's history may be disconnected with any sort of adaptive process. There are,

however, at least three additional ways in which "the variation being studied" may be understood as being relevant or irrelevant. The first way is by defining the variation as the structural changes detectable in proteins by electrophoresis. As discussed in more detail in Chapter 3, not all amino acid changes result in a change in electrophoretic mobility under the experimental conditions commonly employed to measure genetic variation. If the proportion of undetected variation is large, the result could be serious underestimation or distortion of the actual amount and pattern of structural gene variation. The second sense of "variation being studied" refers to the sampling universe to which the set of structural loci being analyzed actually belongs. It is interesting to note that the molecular data to which the current controversy is directed have come mostly by sampling a set of about 100 specific proteins (in total), with all but the best-studied organisms having been analyzed for 30 or fewer of these. It is, therefore, crucial to ask the question: How representative are these loci of the total set of structural loci in the genome of a species? It will be argued in Chapter 5 that this question becomes especially important in the analysis of genetic differences between species. And thirdly, we must eventually understand the relative significance of variation and evolution in the different "sectors" of genomic DNA other than that encoding the primary structure of proteins. The assumption that

variation in genic (protein-encoding) DNA commonly underlies adaptation and evolution has facilitated the collection of much data, but no general empirical basis exists at present for assessing the relative importance of coding and non-coding DNA in Darwinian evolution.

It was with the goal of addressing the question of relevant variation, most directly with respect to the universe of structural loci sampled by electrophoresis, that the experimental approach taken in this thesis was developed. The significance of the results derives partly from the author's successful optimization of recently developed techniques for the simultaneous high-resolution electrophoretic separation of large numbers of polypeptides from crude tissue extracts, and for ultrasensitive metal-stain detection of the separated polypeptides in the electrophoresed mixture. In addition, methods were developed for the preparation of protein samples from specific sets of Drosophila organs (male reproductive tracts) which are very likely of unique importance in the speciation process in this genus of flies. Thus, the search for relevant variation has been both broadened to include a larger number of loci, and narrowed to include only those proteins which are found in a specific, evolutionarily interesting part of the organism.

The aspects of data relevance concerned with sensitivity of electrophoretic detection of polypeptide sequence variation, and with variation and evolution in non-

coding DNA, were addressed only indirectly in this study. However, the optimization of technique had the consequence that one important source of insensitivity to allelic variation, especially acute with the new electrophoretic technique used and a hindrance in the interpretation of many earlier results, was virtually eliminated. The basis for this contention is discussed in detail in Chapter 3. Also, the fact that many of the differences found between species in the present study (see Chapter 5) may be most readily explained by genetic changes that are independent of polypeptide sequence changes suggests that evolutionary changes involving nongenic DNA may be accessible to at least partial analysis with the techniques employed.

However, it is the first-mentioned sense of the term "relevant variation" - i.e. that which refers to the relationship between the variation we can study in populations and that which we can study between species - that perhaps deserves the most attention. Not only is this the aspect of the problem least likely to yield to technical advances alone: it is also the aspect most inextricably involved with future development of the Darwinian theory of evolution. It is for this reason that the experimental approach developed here placed almost equal emphasis on the analysis of both variation within species and divergence between species.

2

MATERIALS AND METHODS

2.1 *DROSOPHILA* STOCKS

The strains analyzed to provide the data of Chapters 3-5 are listed in Table 2.1, with information on their origins and the sources from which they were obtained.

2.2 FLY CULTURE

Drosophila lines were maintained under ambient atmospheric humidity, with a diurnal photic cycle of 12 hr. light and 12 hr. dark, at 22 ± 1° C. The compositions of the two culture media used are described in Table 2.2. *D. mauritiana* and *D. sechellia* were raised on culture medium 2 only. For population surveys of genic variation in male reproductive tract proteins in isofemale lines of *D. melanogaster* and *D. simulans*, cultures were raised in 25 x 95 mm glass vials on medium 1. Samples of male reproductive tracts from flies of these two species grown on medium 2 were also analyzed. The gel patterns for flies raised on these two culture media showed no qualitative differences in the spots studied. Larvae for collection of imaginal discs were reared in 250 mL glass bottles on medium 2. An effort was made to keep larval density below 50 individuals per vial and 100 individuals per bottle.

TABLE 2.1

Lines of *Drosophila* used in this study.

Line(s)	Type/Species	Origin: Place	Origin: Time	Source
France (10 lines)	isofemale/ *D. melanogaster*	Villeurbanne, France (46.1°N)	1978	Dr. J. David, Laboratoire De Biologie et Génétique Evolutives, C.N.R.S., Gif-sur-Yvette, France
Benin (10 lines)	isofemale/ *D. melanogaster*	Benin, West Africa (6.3°N)	1978	J. David
South France (10 lines)	isofemale/ *D. simulans*	Porquerolles, France (43.0°N)	1983	J. David
Brazzaville (10 lines)	isofemale/ *D. simulans*	Brazzaville, Congo (4.3°S)	1983	J. David
Dmr 1	mass culture/ *D. mauritiana*	Mauritius	1973	J. David
Dsch 1	mass culture/ *D. sechellia*	Seychelles	1982	Dr. J. A. Coyne, Dept. of Zoology, University of Maryland, College Park, Maryland, U.S.A.

TABLE 2.2

Composition of culture media

Medium 1

Yellow cornmeal	... 67 g
Dried brewer's yeast	... 30 g
Agar	... 10 g
Malt powder	... 31 g
Corn syrup	... 50 mL
Tegosept solution (10 g p-hydroxybenzoic acid, methyl ester + 100 mL 95% ethanol)	... 24 mL
Water	... 1 L

Stir thoroughly to suspend solids; boil; cool to 70°C; add Tegosept; pour

Medium 2

Dried brewer's yeast	... 60 g
Agar	... 20 g
Water	... 3.6 L
Bananas	... 1.5 (medium size)
Malt powder	... 1.5 T
Corn syrup	... 2 T
Tegosept solution (see Medium 1)	... 36 mL
H_2O	... 80 mL

Stir yeast, agar and 3.6L water to suspend; boil; add other ingredients (blended); simmer 10 min; cool to 43°C; add Tegosept; cool to 41°C; pour

2.3 LINE SAMPLING

For two-dimensional electrophoretic analyses of male reproductive tract proteins, 2-3 days were allowed to elapse after the onset of eclosion in a culture, and males were collected after light anaesthetization with diethyl ether. These males were then aged in groups of approximately 20, in vials containing medium 1 and a moistened piece of Kimwipe tissue, for 3-5 days before dissection.

To rear larvae for imaginal disc dissection, adult flies were placed in a culture bottle and maintained until approximately 100 eggs were visible on the surface of the medium. The flies were then cleared and the larvae allowed to develop for 5 days. At this point, third-instar larvae were collected from the wall of the culture bottle. Only larvae whose movement had begun to slow markedly in preparation for pupariation were selected. This has the dual advantages of facilitating larval capture and of ensuring the presence of large imaginal discs in a well-standardized stage of development for sample preparation.

2.4 TISSUE DISSECTION AND SAMPLE PREPARATION

2.4.1. Solutions

The sources of chemicals for solutions described in sections 2.4, 2.5 and 2.6 are listed in Table 2.3 along with appropriate abbreviations employed. Where the composition of solutions is described below, recipes are given in terms of weights and volumes required for a given final volume of solution. Final compositions in terms of molarities and percentages by weight or volume are listed in parentheses.

The following formulation for PIPES - buffered Ringer's solution is taken directly from Cheney and Shearn (1983). The Lysis Buffer described is modified from that described by O'Farrell (1975). The inclusion of 5 mM K_2CO_3 follows a suggestion of Horst et al. (1980); the intended purposes are to inhibit proteolysis and to aid protein solubilization by raising pH to approximately 10.3. L-lysine is included to scavenge isocyanate formed in urea solutions. DTT was substituted for 2 -mercaptoethanol primarily for the convenience of its much less noxious odour.

TABLE 2.3

Sources of Chemicals[a]

Chemical Name	Abbreviation	Source[b]
Acetic Acid (glacial)	--	1
Acrylamide (>99.9%)-for IEF Gel Solution (section 2.5.1.1)	--	2
Acrylamide ("Electran" Grade) - for SDS-PAGE Monomer Stock (section 2.5.2.1)	--	3
Agarose (Type I: Low EEO)	--	8
Amberlite MB-1 Ion Exchange Resin	MB-1	8
Ammonium Persulfate	--	3
Ampholines pH 5-7 pH 3.5-10	--	6
Biolytes pH 7-9	--	2
N, N' - Methylene Bisacrylamide	Bis	4
Calcium Chloride (dihydrate)	$CaCl_2 \cdot 2H_2O$	3
Citric acid (monohydrate)	--	3
Diallyltartardiamide	DATD	2
Dithiothreitol	DTT	8
Ethanolamine	--	5
Formaldehyde (37% w/v) - Analyzed	--	5
Glucose	--	3
Glycine	--	8
L - Lysine (free base)	--	8
Magnesium Sulfate (heptahydrate)	$MgSO_4 \cdot 7\ H_2O$	3
Nonidet P-40	NP-40	4
Phosphoric Acid (85% w/v)	H_3PO_4	5
Piperazine - N, N'- bis (2-ethane sulfonic acid)	PIPES	8
Potassium Carbonate (anhydrous)	K_2CO_3	3

TABLE 2.3 (CONT'D)

Chemical Name	Abbreviation	Source
Potassium Chloride	KCl	3
Silver Nitrate	$AgNO_3$	3
Sodium Carbonate (anhydrous)	Na_2CO_3	3
Sodium Chloride	NaCl	3
Sodium Dodecyl Sulfate	SDS	3
Sodium Hydroxide	NaOH	3
Sucrose	—	3
Sulfuric Acid (concentrated)	H_2SO_4(conc)	1
N, N, N', N' -Tetramethyl Ethylenediamine	TEMED	2
Tris (hydroxymethyl) aminomethane ("Sigma 7-9") - for SDS-PAGE Anode Buffer (see section 2.5.2.1)	Tris	8
Tris (hydroxymethyl) aminomethane (Electrophoresis Purity - for SDS-PAGE Gel and Cathode Buffers) (see Section 2.5.2.1)	Tris	2
Urea (Ultrapure Grade)	—	7

[a] Unless otherwise specified, all chemicals used were of reagent grade.

[b] 1 - J. T. Baker Chemical Co., Phillipsburg, New Jersey

2 - Bio-Rad Laboratories, Richmond, California

3 - BDH Chemicals, Toronto, Ontario

4 - Bethesda Research Laboratories, Rockville, Maryland

5 - Fisher Scientific Co., Fairlawn, New Jersey

6 - LKB Produkter, Bromma, Sweden

7 - Schwarz-Mann Inc., Spring Valley, New York

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

PIPES - Buffered Ringer's Solution

For 25 mL:

PIPES	...665 mg (88 mM)
Glucose	...90 mg (20 mM)
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 N NaOH	...5-6 mL
H_2O	...to approx. 20 mL

Dissolve all solutes; add

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

Store at 4°C for no more than 1 month.

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

$CaCl_2 \cdot 2H_2O$	...3.76 g
H_2O	...to dissolve; to 25 mL

Lysis Buffer

For 25 mL:

Urea	...14.3 g (9.5 M)
K_2CO_3	...17.3 mg (5 mM)
L-lysine	...12.5 mg (1.5 mM)
DTT	...385 mg (100 mM)
H_2O	...13.4 mL

Dissolve with gentle warming; add

NP-40 ...0.75 mL (3% v/v)

Swirl to dissolve NP-40; dispense into 0.5 mL aliquots; freeze in liquid nitrogen and store up to 6 months at -70°C. For use, thaw and add carrier ampholytes to 2% w/v.

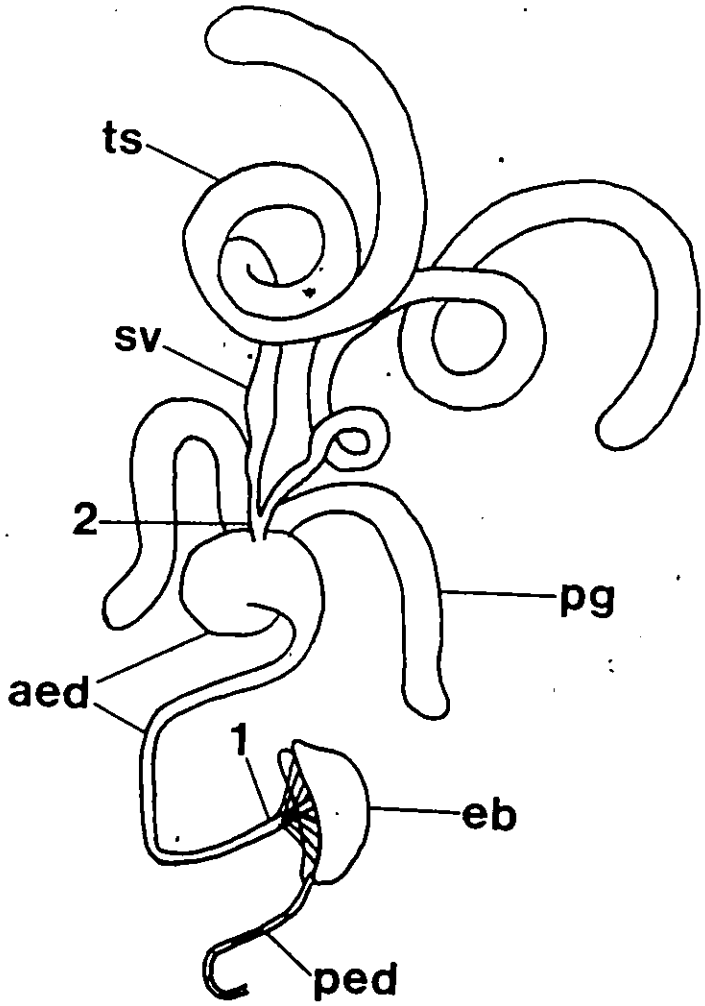
2.4.2. Procedures

Male flies to be dissected were etherized for approximately 5 minutes. The reproductive tracts (Figure 2.1) of 10 individual flies were dissected by hand with two pairs of watchmaker's forceps (A. Dumont et fils, number 4) which had been sharpened on a fine whetstone. Sharpened tungsten needles were employed for tissue cleaning and transfer after the abdomens had been opened with the forceps. Each fly was dissected in a fresh drop of PIPES-Buffered Ringer's at room temperature, and the organs pooled and stored in a fresh aliquot of PIPES-Buffered Ringer's at room temperature until dissection was completed (about 20 minutes). The ejaculatory bulb was discarded from each tract, leaving testes, seminal vesicles, paragonial glands and anterior ejaculatory duct to be included either separately or together in the lysate for electrophoresis (see Figure 2.1).

Upon completion of dissection, an aliquot of Lysis Buffer (40 µL per 10 sets of organs in a 400 µL plastic sample tube) was warmed to room temperature. The aliquots

FIGURE 2.1

Diagram of male reproductive tract of *Drosophila melanogaster*. The anatomy is almost identical between species of the *melanogaster* subgroup. **ts:** testis, **sv:** seminal vesicle, **pg:** paragonial gland, **aed:** anterior ejaculatory duct (glandular), **ped:** posterior ejaculatory duct, **eb:** ejaculatory bulb. For "whole-tract" preparations, all tissue *anterior* to the ejaculatory bulb was included, after severing the aed at point 1. For separate preparations of "testes" and "glandular tissues", testes and seminal vesicles were severed from paragonia and ejaculatory duct at point 2.


ts
sv
2
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are conveniently prepared in batches of 12 by dividing a thawed 0.5 mL aliquot of Lysis Buffer after adding carrier ampholytes (usually Bio-Lytes 7-9) to 2% (w/v). Note that the stock solutions of ampholytes as supplied by the manufacturers are 40% (w/v). The 40 µL aliquots may then be stored on ice for up to 8 hours while dissection proceeds. The organs were transferred quickly to the Lysis Buffer on the slightly curved tip of a tungsten needle. The suspension was incubated at room temperature for 1 minute, then further disrupted by gentle stirring with the flamed-shut tip of a Pasteur pipet. After one more minute of incubation at room temperature, the lysate was frozen by dipping the tip of the sample tube into liquid nitrogen. The samples may be stored at -70°C for several months with no detectable effect on the protein pattern obtained by gel electrophoresis.

Third-instar imaginal wing discs were dissected by hand from larvae staged as described in Section 2.3, after brief washing of the larvae in distilled water. Individual larvae were processed separately, each in a fresh drop of PIPES - Buffered Ringer's until 20 discs had been collected and pooled in a second aliquot of PIPES - Buffered Ringer's; the discs were then transferred to 40 µL of Lysis Buffer with a tungsten needle. Subsequent processing of the lysates was identical to that described for male reproductive tract lysates, except that mechanical disruption with the Pasteur pipet tip was unnecessary.

Just prior to electrophoresis, samples were alternately thawed and frozen by immersion in liquid nitrogen and a 37°C water bath, for a total of 5 freeze/thaw cycles. After the final thaw, the samples were centrifuged for 10 minutes in a Beckman microfuge. All but the bottom 3-5 µL of the lysate could then be used for electrophoresis. Usually all of this "supernate" volume was applied to the first-dimension gel.

2.5 TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS

The two-dimensional electrophoresis procedures employed are essentially those described by O'Farrell (1975) and O'Farrell et al. (1977) but they have been modified and simplified. The primary modifications are: the substitution of DATD for bisacrylamide as gel cross-linker in the first dimension, the substitution of H_2SO_4 and ethanolamine for H_3PO_4 and NaOH as first-dimension terminal electrolytes (Zurfluh and Guilfoyle, 1982), and application of sample at the anodal rather than the cathodal end of the first-dimension gel. The simplifications include: frozen storage of first-dimension gel solution, omission of the water-overlay and lysis-buffer overlay steps during tube gel polymerization, shortening of the SDS equilibration step from 2 hr. to 30 min., and elimination of stepped voltage schedules during isoelectric focusing and prefocusing.

2.5.1. First Dimension: Isoelectric Focusing (IEF) or Nonequilibrium pH Gel Electrophoresis (NEPHGE)

2.5.1.1. Solutions

Gel Solution

For 50 mL:

Urea	...28.6 g (9.5 M)
Acrylamide	...2.125 g
DATD	...375 mg. (5.0% T/15%C)
H_2O	...22.0 mL

Dissolve with gentle warming.

Deionize by stirring with 2.5 g Amberlite MB-1 ionic exchange resin (20 min.), while deaerating.

Filter; add

Ampholines pH 3.5-10	...0.5 mL (0.4% w/v)
Ampholines pH 5-7	...2.0 mL (1.6% w/v)

or

Ampholines pH 3.5-10	...0.5 mL (0.4% w/v)
Ampholines pH 4-6.	...2.0 mL (1.6% w/v)

or

Ampholines pH 3.5-10	...2.5 mL (2.0% w/v)

Deaerate further (20 min.); add

NP-40	...1.5 mL (3% v/v)

Swirl to dissolve; divide into 3 mL aliquots; freeze in liquid nitrogen and store at -70°C up to 6 months.

Gel Overlay

Lysis Buffer (see Section 2.4.1)	...0.5 mL
Ampholines pH 3.5-10	...5 µL (0.4% w/v)
Ampholines pH 5-7	...20 µL (1.6% w/v)

Invert tube to mix thoroughly.

Sample Overlay

Add 0.1 volumes H_2O to Gel Overlay and mix thoroughly.

Anolyte

For all first-dimension gels:

Ethanolamine	...15 mL (0.5% v/v)
H_2O	...to 3 L

Catholyte

For pH 5-7 IEF and pH 3.5-10 NEPHGE gels:

H_2SO_4	...2 mL (0.2% v/v)
H_2O	...to 1 L

For pH 4-6 IEF gels:

H_3PO_4 (85% w/v)	...2 mL
H_2O	...to 1 L

2.5.1.2 Procedures

To cast 7 first-dimension cylindrical tube gels (11 cm. x 2 mm.), 3 mL of gel solution was thawed at room temperature and stirred thoroughly to re-dissolve the urea. Polymerization was then initiated by the addition of 1.8 µL TEMED and 12 µL freshly prepared ammonium persulfate solution (10% w/v). After stirring briefly, the mixture was poured into the gel tubes (135 mm. long x 2 mm. i.d. x 7 mm. o.d.), which were sealed at the bottom with Parafilm and a rubber cap. A cannula needle attached to a 3 mL disposable plastic syringe was useful to accomplish this. Care was taken to insure that air bubbles were not trapped in the tubes. The tops of the tubes were then sealed with Parafilm and polymerization allowed to proceed for 2 hours.

The fully set gels were placed in a Hoefer Model GT vertical tube gel apparatus. The rubber caps and Parafilm were gently removed from the bottoms of the tubes, and a drop of catholyte applied to the end of each gel with a Pasteur pipet, taking care to remove any floating bubbles. After assembling the chamber with catholyte in the lower reservoir, for isoelectric focusing (IEF) separations the gels were then each overlaid with 30 µL Gel Overlay and the tubes filled with anolyte. The upper reservoir of the apparatus was filled with 500 mL of anolyte, and the gels prefocused (with anode at the top and cathode at the bottom) for 1 hr at 250 V.

At the completion of prefocusing, the anolyte was poured off and the gel overlay solution removed from two tubes with a gentle stream of fresh anolyte from a 3 mL plastic syringe fitted with a 27G needle and a short piece of polyethylene tubing. The two gels were then overlaid immediately with 30 µL of Sample Overlay. This removal/overlay procedure was repeated, two tubes at a time, for the remaining gels. Up to 38 µL of sample was then layered under the Sample Overlay. After adding 500 mL of fresh anolyte to the upper reservoir, isoelectric focusing was carried out at 400 V for 16-20 hrs. (6400-8000 V-hr.), with the same polarity as used for prefocusing.

For non-equilibrium (NEPHGE) separations in the first dimension (O'Farrell et al. 1977), the prefocusing step was omitted, electrophoresis carried out for only 1800-2400 Volt-hours, and the tube gels and Sample Overlay contained only Ampholines 3.5-10 (see section 2.5.1.1); otherwise the procedure was identical to that described above for IEF gels.

Finally, when using the Pharmacia EPS 500/400 power supply for first-dimension separations, it was necessary to connect a 0.5 megohm load resistor in parallel with the electrophoresis tank across the second pair of power outlets. This is because the current across the gels drops during the separation, usually to a total value less than the 7 µA threshold that activates the automatic safety shut-off mechanism built into this power supply. Connection of the

load resistor avoids such premature abortion of the experiment.

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

2.5.2.1 Solutions

20% SDS Stock

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

Dissolve with warming; add

H_2O	...to 100 mL

Store at room temperature in an amber bottle.

10% Ammonium Persulfate

Ammonium persulfate	...1.0 g (10% w/v)
H_2O	...to dissolve
H_2O	...to 10 mL

Make fresh for first-dimension gels; store 1 day (4°C) for use in resolution and stacking gels of second dimension.

SDS-PAGE Monomer Stock

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

Store at 4°C in an amber bottle.

Resolution Gel Buffer Stock

Tris (BioRad)	...18.3 g (1.51 M)
1N HCl	...20 - 22 mL
H_2O	...to 80 mL
Dissolve; add	
1N HCl	...to pH 8.8 (25^oC)
H_2O	...to 100 mL

Store at 4^oC in an amber bottle.

Stacking Gel Buffer Stock

Tris (BioRad)	...5.8 g
1N HCl	...48 mL (0.48 N)
H_2O	...to 80 mL
Dissolve; add	
2M Tris (BioRad)	...to pH 6.8 (25^oC)
H_2O	...to 100 mL

Store at 4^oC in an amber bottle.

Cathode Buffer

Glycine	...28.8 g (192 mM)
Tris (BioRad)	... 6.0 g (25 mM)
H_2O	...to 2 L
20% (w/v) SDS	...10 mL (0.1% w/v)

Make fresh every time.

Anode Buffer

Tris (Sigma)	...121.14 g (62.5 mM)
HCl (conc.)	...75 mL (50 mM)

H_2O	...to 16 L
SDS	...16 g

Re-use up to 3 times; store at 4°C between uses.

SDS Equilibration Buffer

Stacking Gel Buffer Stock	...12.5 mL
Glycerol	... 2.5 mL (5% v/v)
20% (w/v) SDS	...5.75 mL (2.3% w/v)
DTT	...154 mg (20mM)
0.1% (w/v) Phenol Red	...2 drops
H_2O	...to 100 mL

Make fresh every time; may be stored overnight (4°C).

Agarose Gel Solution

Agarose (Sigma Type I)	... 0.3 g (1% w/v)
Stacking Gel Buffer Stock	... 7.5 mL
20% (w/v) SDS	... 0.15 mL (0.1% w/v)
H_2O	... 23.0 mL

Make fresh every time.

2.5.2.2 Procedures

Gel Casting. The second dimension separations were carried out in a Hoefer SE 700 Multiple Vertical Slab Gel Electrophoresis unit, in which 6 slab gels may be run simultaneously. The resolution gel slabs measured 14 cm wide x 15 cm long x 0.75 mm thick, and the stacking gel layer was

1 cm deep (excluding agarose gel solution used to embed the first-dimension gel atop the slab). A 12.0%T/2.5%C resolution gel solution was routinely prepared for the second dimension of electrophoresis in a 50 mL batch for every three slab gels. Two such 50 mL batches were prepared and poured separately for every set of 6 gels cast, so as to allow adequate working time during the pre-gelation phase of polymerization. To prepare 50 mL of gel solution, 12.5 mL of Resolution Gel Buffer Stock, 22.25 mL distilled water and 15.0 mL SDS-PAGE Monomer Stock were mixed and suction-filtered through Whatman #1 paper into a 125 mL sidearm flask, and the solution deaerated for 30-60 min. by aspiration on the domestic water supply.

After breaking the vacuum, the solution was poured into a beaker, 0.25 mL of 20% SDS Stock, 30 µL TEMED and 160 µL 10% (w/v) ammonium persulfate were added, and the solution stirred briefly. The gel solution was drawn into a 30 mL plastic syringe lacking a needle, and then a blunted 22G needle was placed on the filled syringe. The needle could then be inserted between the glass plates of the gel cassettes and the cassettes filled to within 0.75 cm. of the top. When three cassettes had been filled in this way, the resolution gel solution was overlaid immediately with a 0.1% (w/v) solution of SDS. SDS and catalysts were then mixed into the second 50 mL batch of gel solution and the pouring

and overlayering processes repeated for the second set of three slab gels.

After a sharp gel interface became visible (about 15 min.), the overlay solution was poured off and replaced with resolution gel buffer containing 0.1% (w/v) SDS. The cassettes were covered with a sheet of plastic film to reduce evaporation, and the gels were allowed to polymerize overnight (about 16 hr.).

Stacking gel solution (4.75%T/2.5%C) was prepared in a single 30 mL batch for 6 gels. Distilled water (18.6 mL), Stacking Gel Buffer Stock (7.5 mL) and SDS-PAGE Monomer Stock (3.6 mL) were mixed and deaerated in a 50 mL sidearm flask for 30 minutes. 20%(w/v) SDS (0.15 mL), 30 µL TEMED and 100 µL 10%(w/v) ammonium persulfate were added and stirred in briefly. The overlay buffer was poured off the resolution gel slabs, and the surface rinsed quickly with stacking gel solution, using a Pasteur pipet. The stacking gels (all six) could then be poured to a height such that the outer ends of the menisci formed at the edges of the spacers on the sides of the cassettes just barely reached the top edges of the glass plates. This allowed overlayering with a minimum volume of 0.1% SDS solution, so that after stacking gel polymerization only 2-3 mm. at the top of the cassette remained to be filled with agarose gel solution.

When stacking gel polymerization had proceeded for 30 min. after the appearance of a sharp gel surface, the

cassettes were detached and the plain rubber strips replaced with the slotted gaskets. After blotting excess overlay solution from the surfaces of the stacking gels with a tissue, the tanks could be reassembled and placed in the anode buffer chamber containing Anode Buffer pre-warmed to room temperature. Final assembly of the gel tanks should take place only just prior to completion of SDS equilibration of the first-dimension gels (described below), so as to minimize dehydration of the upper surfaces of the stacking gels.

First-Dimension Gel Removal and SDS Equilibration. Upon completion of the first-dimension separation, the glass tubes containing the gels were cooled on ice for 10 min. The gels were removed with the aid of a gentle stream of water from a 30 mL plastic syringe fitted with a blunted 22G needle. If necessary, after the gel was thus freed from the tube wall, gentle air pressure was applied through a short piece of silicone rubber tubing fitted over one end of the gel tube. By using a peristaltic motion on the rubber tubing with thumb and forefinger, the gel could be coaxed out of the tube into a plastic weighing boat without risk of breakage. Gels were transferred to plastic culture tubes on ice, where they were held until the removal procedure was completed (about 20 min.). For gels to be run immediately in the second dimension, 6-7 mL of SDS Equilibration Buffer was added to each tube. Gel and buffer were transferred by pouring into a

60 x 15 mm disposable plastic petri dish, covered, and shaken on a rotary platform shaker at 20 cycles per minute for 30 minutes at room temperature.

For gels to be stored frozen and run on a separate day in the second dimension (as for NEPHGE gels), 1 mL of SDS Equilibration Buffer was added to each gel in its plastic tube. The tubes were capped, and after ensuring that the gels were immersed in the buffer, the gels and buffer were frozen by dipping the end of the tube into liquid nitrogen for about 10 seconds. For storage of a few days or less, the frozen gels were kept at -20°C. For longer periods of storage, -70°C is recommended. Thawing was carried out by incubating the tubes in a water bath at 35°C. Equilibration was then completed as described above for non-frozen gels, after adding 5-6 mL of SDS Equilibration Buffer.

Transfer of First-Dimension Gels to Slabs. When the SDS equilibration step was completed, the first-dimension gels were each transferred to a small amount of distilled water in a fresh petri dish, shaken briefly by hand, and the water carefully removed with a Pasteur pipet. The gels then tend to cling around the outer rim of the floor of the Petri dish. They could be transferred using a spatula and a slow rolling motion of the dish onto a strip of Parafilm folded lengthwise. The gels were easily carried on the Parafilm and placed beside the gel-holding troughs of the upper slab cell reservoirs.

Freshly melted Agarose Gel Solution was added through the gasket slot to the top of one slab gel, using a Pasteur pipet. Extreme care must be taken to avoid entrapment of bubbles beneath the gasket at this point. If the temperature of the agarose solution is close to 100°C when pipetted, this difficulty is rarely encountered. Using a spatula, the tube gel was then pushed immediately off the edge of the Parafilm into the molten agarose and aligned exactly over the gasket slot. This application process was then repeated one gel at a time for the other five gels, and the agarose was allowed to set for 5-10 minutes. Cathode Buffer (600-700 mL) was added to each upper reservoir, and electrophoresis carried out at 12 mA constant current per gel. The separation was continued until the tracking dye had exited from the lower end of the gel and the trailing edge of the SDS stack (about 1 cm. behind the tracking dye) had migrated to within 0.5 cm. of the end of the gel. Total electrophoresis time in the second dimension was about 7 hr.

2.6 ULTRASENSITIVE SILVER STAINING OF PROTEINS

The method used is that developed by Merril et al. (1981), with modifications taken from Morrissey (1982) and Sammons et al. (1981). The sequence of steps is listed in Table 2.4. Volumes of solution per gel are 150 mL for every step except the silver binding step, for which a 100 mL volume was used. Between each of steps 1-5 and its following

TABLE 2.4

Silver Stain Used in this Study

Step	Solution	Time	Comments
1. Fix	95% Ethanol/ Acetic Acid/H_2O (52.5:5:42.5 v/v/v)	Overnight (16 hr.)	No shaking May be fixed longer (e.g. 40 hr.)
2. Wash	95% Ethanol/H_2O (10.5:89.5 v/v)	1 hr	Avoid gel tearing by freeing corners from tray with H_2O stream and floating by hand shaking Correct gel curling
3. Wash	Repeat Step 2		May be reduced to 30 min.
4. Wash	H_2O	30 min	
5. Wash	Repeat Step 4		
6. Reduce	5 mg/L DTT	30 min.	No water rinse following this step
7. Silver binding	0.1% (w/v) $AgNO_3$	30 min.	
8. Wash	H_2O	10 sec.	Float gel in tray with hand shaking
9. Wash	Developer 3%(w/v)Na_2CO_3 +0.5 mL/L 37% (w/v) formaldehyde	10 sec.	Float gel in tray with hand shaking Minimal finger pressure on gel when draining
10. Wash	Repeat Step 9		
11. Develop	Developer (see Step 9)	5-10min.	Shake on rotary platform shaker (40-60 cycles/min) Gel will float free in 1-2 min.
12. Stop	2.3M Citric acid	5-30 min.	7.5 mL per 150 mL developer
13. Store	0.75%(w/v)Na_2CO_3	in-definitely	Gels will yellow quickly if stored in neutralized developer

step, as well as between steps 12 and 13, a brief rinse with water was performed to remove residual solution from the previous step. Unless otherwise mentioned, the entire procedure was carried out with gentle shaking (60-100 excursions per minute) on a reciprocating platform shaker. For gels of the size used here, 2.0 L Pyrex trays (Corning number 222) were ideal. The trays should be kept tightly covered with plastic film throughout, except during the development and stop processes.

Two precautions should be observed to avoid mechanical damage to the gel. First, only minimal pressure with gloved fingers should be applied to the gel to hold it in the tray when inverting the tray to drain solutions. Gloves should be changed frequently. Second, at several points in the procedure the gel has a tendency to stick to the glass tray. This is especially true after steps 1 and 9. Gel tearing may be avoided by following the suggestions made in Table 2.4.

Finally, the gels may be stored for long periods (e.g. 6 months) in 0.75% Na_2CO_3 with no detectable deterioration, in "Zip-Loc" plastic food storage bags (Dow Chemical Corp.). The only problem in storage is gel dehydration, which can be reversed at any time by rehydrating the gel inside the bag.

2.7 CLEANING OF GLASSWARE

Immediately following a first- or second- dimension electrophoretic separation and gel removal, glass tubes or plates were soaked overnight in a solution of warm tap water and laboratory detergent (Sparkleen - Fisher Scientific). The tubes were then rinsed thoroughly and cleaned further by soaking for at least 24 hours in concentrated nitric acid. After thorough rinsing of tubes or plates in warm tap water and then distilled water, they were then air-dried and used for gel casting. To reduce artifactual streaking caused by interaction of dust particles and silver stain reagents in the slab gel, the plates were given a final distilled-water rinse immediately before slab gel casting and were dried manually with Kimwipes.

INTRODUCTION

Diverse Estimates of the Amount of Genic Variation in Animal Genomes

Over the past two decades, most of the molecular data on genetic variation in natural populations have been acquired through the application of techniques for protein separation by gel electrophoresis. The most common procedure employs a one-dimensional separation, under non-denaturing conditions, of "soluble" proteins extracted from biological samples in aqueous buffers of low ionic strength and moderate pH. The preservation of tertiary and quaternary protein structure under such conditions permits post-electrophoretic assay of gels for specific enzymes present in very small amounts. Moreover, the system is highly sensitive to the slight differences in the physical properties of proteins that result from amino acid substitutions in their constituent polypeptides; many of these substitutions are expressed as differences in electrophoretic mobility (Ramshaw, Coyne and Lewontin, 1979). Genetic variation in protein structure can, therefore, be detected as enzyme mobility variation ("allozyme variation") between genotypes in population samples, and can be assigned with confidence to specific loci encoding the amino acid sequences of the

polypeptides (Harris, 1966; Hubby and Lewontin, 1966; Lewontin and Hubby, 1966).

In well-studied organisms, the accumulation of such one-dimensional electrophoresis (1DE) data has formed a basis for estimation of fundamental parameters related to the average amount of genic variation in the genome as a whole. The two most widely used indices of the extent of genic variation in a sample of loci are the fraction of loci found to be polymorphic (P) and the index of heterozygosity ($\bar{H}$). A locus is usually defined as polymorphic if the frequency of the most common allele is less than .99. $\bar{H}$ is really a composite index of allelic "diversity" (in terms of number of alleles and their evenness of frequency) at an average locus (Nei, 1975). H is conveniently calculated for a single locus from the frequencies of the various alleles observed in a sample, using the formula: $H = 1 - \Sigma p_i^2$, where the p_i are the allele frequencies. These single-locus H values are then averaged arithmetically to give $\bar{H}$. $\bar{H}$ is interpretable either as the fraction of the population expected to be heterozygous at a randomly chosen locus, or as the fraction of loci expected to be heterozygous in a randomly chosen individual, assuming Hardy-Weinberg proportions for diploid genotypes. Estimates of P and $\bar{H}$ by 1DE in five well-studied animal species are shown in Table 3.1, in the lines labelled "1D".

TABLE 3.1

Estimates of proportion of loci polymorphic (P), average heterozygosity per locus ($\bar{H}$), and number of loci studied (N)[a], in four animal species for enzymes studied by one-dimensional (1D) and proteins studied by two-dimensional (2D) gel electrophoresis.

Species		P	$\bar{H}$	N	Comments	References[b]
Homo sapiens	1D	.231	.063	104	-	5
	2D	0	0	83	kidney	6
		.102	.024	186	lymphocytes	9
		.087	.031	46	erythrocytes	10
		.25	.062	20	plasma	8
Drosophila melanogaster	1D	.575	.158	81	15 populations	11
	2D	.11	.04	54	1 population	1
Drosophila simulans	1D	.417	.063	24	2 populations	7
	2D	0	0	70	1 population	7
Mus musculus	1D	.24	.094	46	Population 1	3
		.17	.073	46	Population 2	3
	2D	.042	.02	72	-	2
Felis catus	1D	.22	.07	55	-	4

[a] For 2D surveys, number of loci is taken as equal to number of polypeptides scored

[b] References - (1) Leigh Brown and Langley (1979); (2) Racine and Langley 1980; (3) Rice et al. 1980; (4) O'Brien 1980; (5) Harris 1980; (6) Smith et al. 1980; (7) Ohnishi et al. 1982; (8) Rosenblum et al. 1983; (9) Goldman and Merril 1983; (10) Rosenblum et al. (1984); (11) R. S. Singh (unpublished data)

One of the most immediately obvious aspects of these genic variation estimates obtained by 1DE is their general similarity among distantly-related species that differ widely in their ecologies, life histories and population structures. The tendency to higher 1DE values of P and $\bar{H}$ for Drosophila is consistent with a general tendency to higher levels of variation in invertebrate versus vertebrate species (Powell, 1975; Selander, 1976). The apparently lower average level of 1DE variation in Drosophila simulans as compared to its close relative, D. melanogaster, has also been confirmed by repeated observations. We will return to this point later in this chapter. But the most general aspect of the data, and the one that initiated the neutrality-selection debate which was discussed in the General Introduction (e.g. Kimura and Ohta, 1971; Lewontin, 1974; Nei, 1975), is the unexpectedly large amount of polymorphism. It is worth noting at this point that 1DE under a single set of conditions commonly does not reveal all amino acid substitutions in soluble enzymes (Bernstein, Throckmorton and Hubby, 1973; Singh, Lewontin and Felton, 1976; Coyne, Felton and Lewontin, 1978; Singh, 1979; Buchanan and Johnson, 1983; reviewed by Coyne, 1982). Neel (1984) has used estimates of the extent of electrophoretically "cryptic" variation in enzymes to suggest that the true index of heterozygosity in man could be close to 15%. Singh (1979) actually observed an increase in average heterozygosity from 33% to 42% for 10 polymorphic

loci in Drosophila pseudoobscura, when electrophoretic conditions were altered to reveal cryptic variation. These results suggest that average levels of genic polymorphism in most animal genomes may even be considerably higher than indicated by the 1DE estimates in Table 3.1.

A question that arises naturally about such extrapolations is to what extent the average genic variation in soluble enzymes reflects the true level of genic variation for the genome as a whole. This question is supported by at least two different considerations. First, even large enzyme surveys such as those listed in Table 3.1 only include on the order of 0.1-1 percent of the total number of structural genes expressed by the respective genomes (Levy and McCarthy, 1975; Hastie and Bishop, 1976; Holland et al., 1980). And second, although the small number of genes being studied need not cause difficulty if the sample of loci can be assumed to be "representative", the validity of this assumption is very difficult to ascertain. Gillespie and Langley (1974) go so far as to suggest that the representation of different types of loci in typical 1DE surveys is very unbalanced and thus misleading. They maintain that estimates by 1DE of amounts of polymorphism in the genome are strongly biased upwards by a tendency to oversample certain highly polymorphic enzyme loci.

An interesting perspective on this question has been provided by the recent application of a more sophisticated

technique of protein electrophoresis to the study of genic variation in natural populations. In this technique (O'Farrell, 1975), proteins are first extracted from tissue samples in the presence of solubilizing agents, and then electrophoretically fractionated in two dimensions under fully denaturing conditions (see Section 2.4). The first dimension (isoelectric focusing - IEF) separates polypeptides on the basis of charge, and the second (sodium dodecyl sulfate electrophoresis - SDS-PAGE) on the basis of size. The high degree of independence of charge and size for a given polypeptide permits extremely high electrophoretic resolution of the different polypeptides in a complex mixture. This means that general protein zone detection techniques (based on dyes, metals or radioisotopes) can be used without sacrificing the clarity of resolution required to score variation in protein mobility. Also, it has been demonstrated that some allelic variation in protein structure is readily detected in this system, at least in the first dimension (O'Farrell, 1975; Milman et al., 1976; Steinberg et al., 1977; Zechel, 1977; Comings, 1979). These features indicate that the technique has the potential not only to increase greatly the number of structural gene loci accessible to population-genetic study, but also perhaps to provide data on a more "balanced" sample from the range of structural and functional classes of proteins encoded by the genome.

This potential was soon recognized by various workers, and by 1980 two-dimensional gel electrophoresis (2DE) had been applied to Drosophila, Homo and Mus in an effort to expand the samples of loci studied for genic variation. The results of these various studies were quite consistent, and quite interesting (Table 3.1). Estimates of the proportion of loci polymorphic and of average heterozygosity per locus are reduced two- to fivefold when the results of 2DE are compared to those of 1DE. In two of the studies (human kidney and Drosophila simulans), no genic variation was detected in samples of 83 and 70 polypeptides, respectively. Furthermore, the difference between the vertebrates and Drosophila seems to be greatly lessened, if not to disappear. A notable exception was the 2DE analysis of variation in 20 human plasma polypeptides by Rosenblum et al. (1983); the possible significance of this deviation will be discussed further in Chapter 4.

The discrepancy between the results of 1DE and 2DE appears to extend to estimates of genic divergence between species. Aquadro and Avise (1981) determined genetic distances between 8 rodent taxa that formed a series of increasingly distant phylogenetic relationships. They found that genetic distance estimates by 1DE and 2DE were perfectly rank-correlated with each other when Peromyscus maniculatus from a Georgia population was compared pairwise with each of the 7 other taxa. However, the distance estimate for a given

pair of taxa by 2DE was markedly lower in every case (i.e. at every level of relatedness) than the distance estimate for the same two taxa by 1DE, suggesting that proteins analyzed by 2DE evolve at a uniformly slower rate than those analyzed by 1DE.

McConkey (1982) provides very striking evidence that the proteins assayed by 2DE are highly constrained in structure during evolution. He found that, when 370 HeLa cell polypeptides and 373 Chinese hamster ovary cell polypeptides were compared rigorously on the same 2D gel by co-electrophoresis and double-label autoradiography, the electrophoretic mobilities of 220 of the polypeptides (about 59%) were identical between the two species. This result is strongly discordant with expectation, based on the estimated divergence time for the two phylads to which man and rodents belong and on general rates of protein sequence evolution; nor can it be explained by lack of resolving power of the technique (McConkey, 1982).

Diverse Interpretations

These results seem consistent with the suggestion that the 1DE data have been providing a non-representative view of the extent of genic polymorphism in natural populations of animals. That is, if the potential of 2DE as outlined above was actually achieved in the studies listed in Table 3.1, then perhaps genic polymorphism is limited to a small minority of loci in the genome, with most loci

represented by a single naturally-occurring allele within species or even throughout much larger taxonomic groupings. But before such a conclusion can be reached, certain methodological questions must be addressed. These questions are of two types. The first concerns the possibility that 2DE, regardless of its apparent potential, in practice detects substantially less of the total genic variation than does 1DE, and that this explains much of the discrepancy between the results of the two techniques. The second has to do with whether 2DE as it has been used in genic variation surveys actually does sample a set of structural loci more representative of the genome than does 1DE.

Detection of Variation by 2DE. As discussed by various authors (e.g. Edwards and Hopkinson, 1980; Aquadro and Avise, 1981; Singh and Coulthart, 1982), 2DE might conceivably suffer from a lack of sensitivity (relative to 1DE) in the detection of allelic variation in protein structure. One physical consideration is that isoelectric focusing under denaturing conditions (the first dimension of the O'Farrell technique, and the one where allelic variation is assumed to be most readily visible) clearly would not be sensitive to variants that depended for their detection on the full preservation of tertiary or quaternary protein structure. What is not clear, in most cases, is to what extent the

detection of genic variation by 1DE depends on these levels of protein structure, although Ramshaw et al. (1979) were able to demonstrate that some "charge-equivalent" or even chemically equivalent amino acid substitutions in different parts of human hemoglobin chains were separable from each other by 1DE. Also, as pointed out by Ramshaw et al. (1979; see also references therein), there is empirical evidence that separation of variant proteins by differences in their isoelectric points may be intrinsically less readily accomplished than non-equilibrium separation of the proteins on the basis of their different rates of electrophoretic migration at a pH displaced from their isoelectric points.

Direct attempts to resolve known 1DE variants by 2DE have given varying results. For instance, McLellan, Ames and Nikaido (1983) were able to distinguish by 2DE only 4 of a set of 9 known alpha - glycerophosphate dehydrogenase variants from different Drosophila species, while 7 of these 9 variants could be resolved simultaneously by 1DE in starch gels at pH 8.6. When the authors assayed five human beta-hemoglobin variants known to differ by single amino acid changes from normal HbA, four of them were resolved under a single set of 1DE conditions and no variation was detected by 2DE. In contrast, however, Wanner, Neel and Meisler (1982) were able to resolve 13 of 17 1DE variants in 5 human polypeptides by "standard" 2DE. Interestingly, by also using an altered first-dimension pH gradient these workers were

able to distinguish a combined total of 16 of the 17 variants under the two sets of 2DE conditions. Neel et al. (1983) admit that 2DE may in some cases be less sensitive to allelic variation in polypeptide structure than is 1DE. However, they conclude that the results of direct tests do not support the hypothesis that an overall difference in sensitivity explains the contrasting estimates of genic variation obtained by the two techniques.

Another type of data bearing on the physical capability of 2DE to resolve allelic protein variants is that reported by deJong, Zweers and Cohen (1978), Noel, Nikaido and Ames (1979), Wilson, Tarr and Kelley (1983), and Vlasuk, Inouye and Inouye (1984). These studies all demonstrate that certain single amino acid substitutions can markedly alter the electrophoretic mobility of a polypeptide when it is complexed with SDS. In 8 of 9 such substitutions examined by these authors, no changes in the intrinsic charges of the polypeptides were involved. However, in 8 of the 9 cases the substitution did involve a change in hydrophobicity of the amino acid side chain. This suggests that certain substitutions not detectable by "charge-separation" techniques like IEF or 1DE may be detectable in the second dimension of 2DE. A final note of interest is that Levin et al. (1984) observed that two isoforms of rat cytochrome P-450, known from complete sequence data to have no differences in net charge or chain length (although they differed in

sequence), were nonetheless separable in both dimensions of O'Farrell gels. Clearly, the task of accurately estimating sensitivity factors for cross-calibration of the physical abilities of 1DE and 2DE to detect allelic protein variants will require more time, and data from several different sources.

A second possible source of technical bias originates not in the electrophoretic separation itself but in the mind of the researcher as primary data is acquired and interpreted. The procedure for detection of variation in 2DE is usually one of visual comparison of spot positions on two different gels, with reference to a two-dimensional array ("constellation") of neighbouring spots. This contrasts with the usual procedure in 1DE, where the test samples being scored can be compared on the same gel, either inter se or with a standard. This means that the observer's confidence in the gel-to-gel reproducibility of 2DE constellations will strongly influence his/her ability to score a spot difference as a putative genetic variant, against an inevitable background of nongenetic fluctuation in the behaviour (position, intensity, etc.) of a spot and its reference constellation. Given the physical nature of the O'Farrell separation technique, it is likely that a given observer will be (rightly) more conservative in scoring genetic variation with 2DE than with 1DE (the data of Aquadro and Avise (1981) contain some interesting examples of substantive differences

between observers in their estimates of spot differences between the same 2D gels). The increased effort involved in 2DE relative to 1DE also renders more difficult any rigorous estimation of the actual gel-to-gel reproducibility being achieved in a specific laboratory, at a specific time, by a specific worker.

Conservatism in gel scoring may arise in at least two other ways. First, the usefulness of reference constellations is directly proportional to the number of spots per unit area in the gel pattern. With the less sensitive general protein detection techniques (e.g. Coomassie Blue staining), the number of spots available for close reference may be quite limited. The more spots visualized on a gel, the less serious this factor will be. And second, to measure genic variation by electrophoresis, protein mobility shifts must be organized into a set of Mendelian models, each with a pair (or set) of segregating alleles. This may be difficult if many protein moieties with a similar appearance are present in a small area of gel, especially when more than one of a set of allelic variants is present on a gel simultaneously. This is frequently the case when heterozygous individuals or several pooled individuals from a segregating population are analyzed. Thus, the more information available to identify the genetic homology of a variant, the more successful the effort of genetic interpretation will be. It should be clear that this task is

usually much more straightforward in 1DE than in 2DE, with a resulting possibility than genic variation can be documented more thoroughly by 1DE.

Finally, the question of fitting a genetic model to the protein data arises at another level, when an estimate is made of the total number of structural gene loci being scored for genic variation. As noted in Table 3.1, each spot on a 2D gel is usually assumed to represent the polypeptide product of a separate locus; yet the capacity of 1DE, IEF and SDS-PAGE to detect post-translational "microheterogeneity" resulting from *in vivo* or *in vitro* modification of a single polypeptide species is well known. Thus a potential exists for underestimation of *average* levels of variation per sampled locus. This potential increases with the number of invariant bands or spots in a gel pattern, and is presumably also greater when *two* dimensions of separation are both capable of detecting microheterogeneity. There is also a dearth of genetic data for the unidentified, poorly-studied majority of the proteins that appear on 2D gels. To summarize: in comparison to 1DE, 2DE may yield not only artificially smaller numerators (number of polymorphic loci, or their summed heterozygosities) but also artificially larger denominators (total number of loci sampled) in the fractions used to calculate average levels of genic polymorphism.

Composition of the Genomic Sample. Do the protein loci which have been surveyed by 2DE actually comprise a set more representative of the overall level of genic polymorphism in the genome than those loci surveyed by 1DE? Or are they simply a different subset, perhaps much less polymorphic than soluble enzymes but no more (or perhaps less) representative of the whole? It might appear that the consistency of the reduced variation estimates obtained with 2DE using diverse sets of proteins is in itself an argument in favour of representativeness. It is not immediately obvious what unrepresentative features might be held in common by radiolabeled polypeptides of human lymphocytes and Coomassie-stained polypeptides of whole Drosophila (see Table 3.1).

However, it has been pointed out repeatedly that 2DE is biased, in terms of sensitivity of detection and feasibility of analysis, towards the more abundantly represented polypeptide species in the biological sample. McConkey (1982) proposes that a majority of the abundant polypeptides appearing on 2D gels are probably synthesized by cells in large, "stoichiometric" amounts to carry out structural roles in highly precise, complex and diverse molecular interactions (e.g. in cytoskeletons, ribosomes, membranes, chromatin, etc.). He argues that this may explain the low levels of variation and low rates of evolution of these polypeptides, since the precision, complexity and

diversity of molecular interactions in which a polypeptide is involved are probably correlated directly with the likelihood and intensity of negative selection experienced by mutant forms of that polypeptide.

The same type of "functional constraint" argument may apply to polypeptides which are expressed in a wide range of cell types, as the diversity of molecular interactions experienced by broadly distributed polypeptides would presumably be greater than that experienced by those with expression localized to a particular cell type. Clearly also, a correlation may exist between a polypeptide's having a structural role and its having a broad tissue distribution, so that the most abundant polypeptides in histologically heterogeneous samples may be strongly constrained in both of these ways. In 2DE studies of Drosophila proteins (Leigh Brown and Langley, 1979; Ohnishi et al., 1982), where whole-body homogenates are electrophoresed, the relative abundance of a polypeptide in the sample should be a function not only of its level of expression within the particular cell and tissue types included but also of the breadth of its distribution between these cell and tissue types. Thus the sample of polypeptides studied by 2DE may represent a very highly constrained group of loci, extremely invariant on the scale spanned by the loci in the genome. They may be "unrepresentative" to the extent that loci coding for polypeptides (e.g. enzyme subunits) present in smaller,

"catalytic" quantities within cells and perhaps expressed in fewer cell types constitute a preponderant fraction of the loci in the genome.

It is probably not possible at present to arrive at a convincing description of what types and proportions of structural loci a representative genome sample might contain. A suggestion as to how this question might be profitably approached will be made in the Discussion section of this chapter. For the present, however, it seems premature to conclude that the loci analyzed for genic variation by 2DE are anything more than a different and (to date) a larger sample of loci than those analyzed by 1DE.

The Present Work in the Context of Estimates of Genic Variation in the Drosophila Genome

In this study, 2DE surveys of protein variation within Drosophila melanogaster and within Drosophila simulans were carried out in order to assess the relationship between genic polymorphism within these two species and genic differences between them. However, the results of these within-species surveys are also of interest in the context of the preceding discussion of the apparent difference in overall level of genic variation between the proteins studied by 1DE and those studied by 2DE, and its possible significance for the question of relevant variation which was posed in the General Introduction.

The original data contributions presented in this chapter are of several types. First of all, technical improvements, based on work published since the first Drosophila 2DE studies were done, have been applied to the material analyzed. The use of modified sample preparation and electrophoresis techniques, combined with "ultrasensitive" silver staining of the separated polypeptides, have reduced the effects of technical bias in the detection and scoring of genic variation by 2DE. Second, the number of loci surveyed has been increased three- to fourfold over previous 2DE work with Drosophila populations: approximately 300 polypeptides are represented from each species. Third, two populations - one temperate and one tropical - were sampled from each species, as opposed to the single temperate populations from each species which were studied by Leigh Brown and Langley (1979) and Ohnishi et al. (1982). Fourth, extensive 1DE data are available for each of these four populations, so that rigorous comparisons can be carried out between the variation estimates obtained with the different sets of proteins analyzed by the different electrophoresis techniques. Finally, this appears to be the first 2DE survey of genic polymorphism in Drosophila proteins from a specific set of tissues - those of the adult male reproductive tract (see Section 2.4). The results support two basic conclusions. The first is that different sets of proteins sampled from the genome have different distributions

of variability. This conclusion re-emphasizes the necessity for caution in extrapolating from a small sample of loci to the entire genome. The second is that our impression of the structure of genic variation in populations may have to be revised to accomodate a larger class of loci which are maintained essentially monomorphic through the removal of allelic variation by natural selection.

RESULTS

General Considerations

Electrophoretic Resolution and Stain Sensitivity.

Photographs of silver-stained 2D polypeptide patterns, resulting from IEF (pH5-7)/SDS-PAGE separations of whole - reproductive - tract homogenates from Drosophila simulans and Drosophila melanogaster, are shown in Figures 3.1 and 3.2. Well over 400 polypeptide spots are visualized clearly with whole-tract preparations of either species, at the level of loading (10 tracts per gel) routinely employed in this study.

As is evident by inspection of the spot patterns, polypeptides of very different relative abundance levels were detected by the silver stain. Dilution experiments suggested that these relative abundances were spread over at least two orders of magnitude, although interpretive caution must be exercised here, since different silver-stained polypeptides may exhibit stain-density vs. protein-density functions with very different slopes (Merril et al., 1982). For additional perspective, it can be noted that Coomassie Brilliant Blue R250 staining of identical gels detected at most 20 spots, with some very prominent silver-stained spots (e.g. D. melanogaster spots 23 and 24) barely visible. This stain sensitivity, in combination with the analysis of only a subset of Drosophila tissues, may aid in the sampling of a

FIGURE 3.1

Photograph of a two-dimensional electrophoretic gel, on which polypeptides from 10 male reproductive tracts of Drosophila simulans were separated and stained with silver. Directions of migration in the first (IEF) and second (SDS) dimensions are shown. The mean pH of each of ten 1-cm. sections of the first-dimension gel is indicated at the top of the figure. Landmark spots are numbered (some were omitted for the sake of clarity - see Appendix 1 for a complete map of the spot pattern). Polymorphic spots are all numbered, and indicated by an underline.

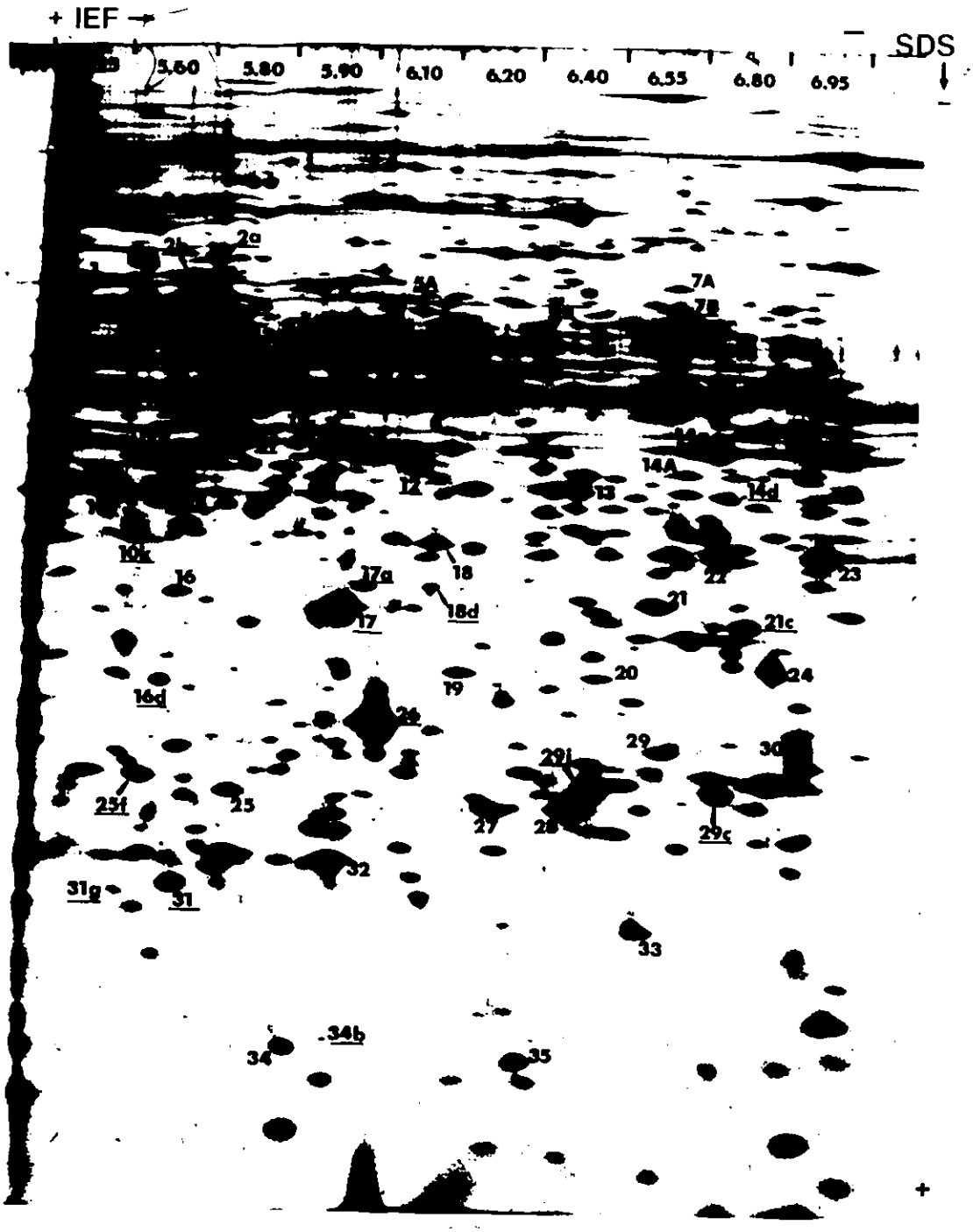

+ IEF →
SDS
5.60
5.80
5.90
6.10
6.20
6.40
6.55
6.80
6.95
2a
7A
7B
14A
14d
16k
16
17a
18
18d
17
21
21c
16d
19
20
24
26
29
29l
30
25f
25
27
28
29c
32
31g
31
33
34b
34
35

FIGURE 3.2

Photograph of a two-dimensional electrophoretic gel, on which polypeptides from 10 male reproductive tracts of Drosophila melanogaster were separated and stained with silver. Directions of migration in the first (IEF) and second (SDS) dimensions are the same as those shown in Figure 3.1, as is the pH gradient in the first dimension. Landmark spots are numbered (some were omitted for the sake of clarity - see Appendix 1 for a complete map of the spot pattern). Polymorphic spots are all numbered, and indicated by an underline.

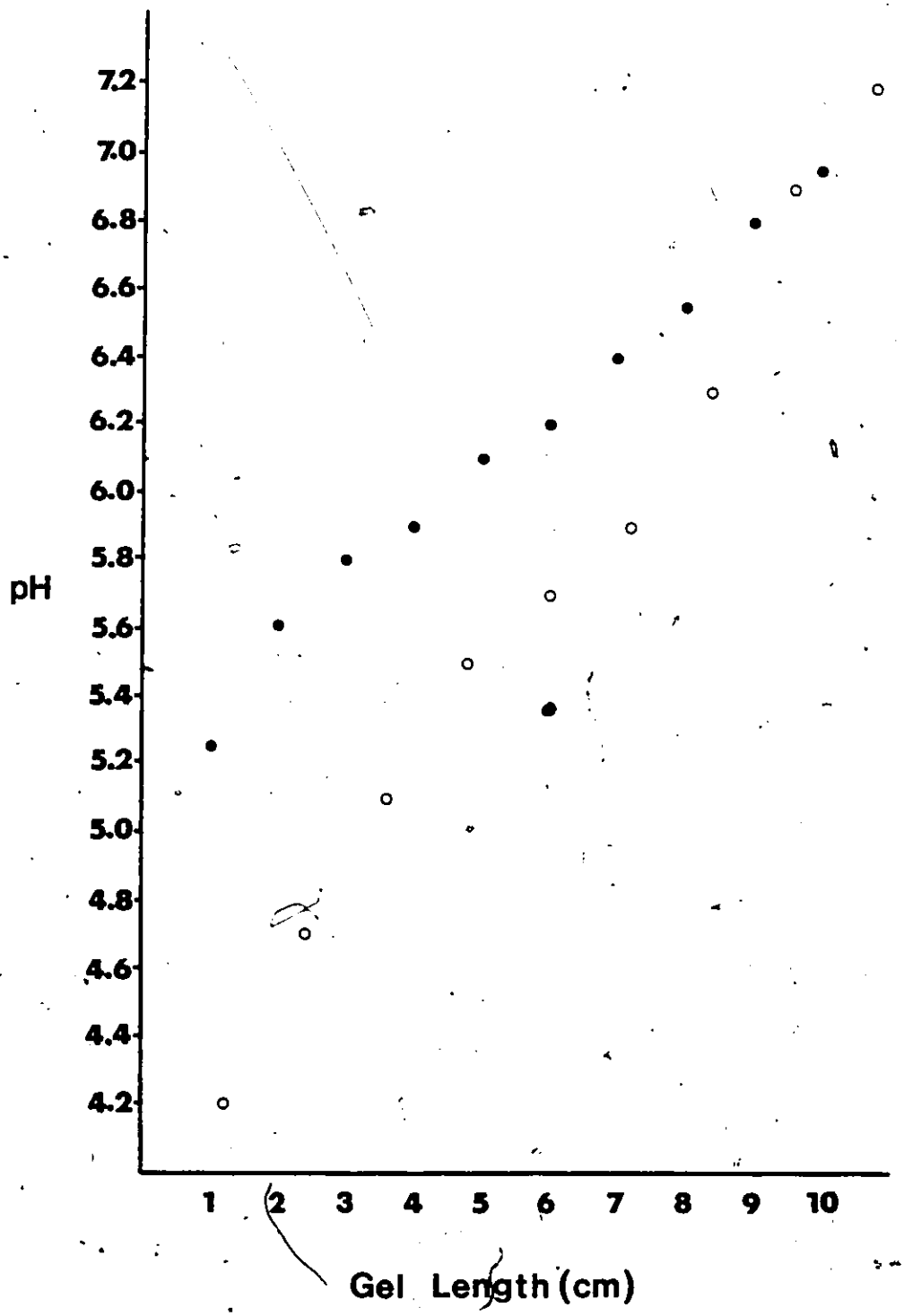

1
2g
3a
4A 4B
4
3c
31
32

set of gene loci less biased towards superabundantly and/or ubiquitously expressed polypeptides. This does not mean that there is no significant bias, in the present system, towards detection and analysis of the more abundant polypeptides on the gel. Many of the relatively minor spots, although reproducible, were too small and featureless to be good material for rigorous scoring of polymorphic alleles. These minor spots were, however, very useful for reference purposes in scoring variation of the more abundant polypeptides.

The pH gradient obtained in the first-dimension gel is shown numerically at the top of Fig. 3.1 and depicted graphically in Fig. 3.3. Excluding the terminal sections of the IEF gel (the pH of the acidic end section is included in Fig. 3.3, but not the alkaline end section), the gradient closely approximated linearity between pH 5.4 and pH 6.95, over a 9 cm. distance. For comparison, the "standard" pH gradient (O'Farrell, 1975) employed by Leigh Brown and Langley (1979, their Fig. 1) for 2DE study of genic variation in D. melanogaster, is also plotted in Fig. 3.3. Average slopes of the two gradients (in pH units/cm) were calculated to be 0.18 and 0.30, respectively, for the total interval of overlap between the two gradients, and 0.17 and 0.31, respectively, for the portion of the gradient showing approximate linearity in the present study. Since detection of allelic variation is presumably more sensitive on shallower pH gradients, the present system should be, for

FIGURE 3.3

Plot of pH (•) measured along the length of a first-dimension isoelectric focusing gel, after the completion of focusing (6800 Volt-hours). No protein sample was applied to the gel. pH was measured by soaking 1-cm. sections of the gel overnight in 1 mL of deaerated distilled water, and then measuring the pH of the solution with a microelectrode. The pH gradient used by Leigh Brown and Langley (1979) is also plotted (o).


7.2
7.0
6.8
6.6
6.4
6.2
6.0
5.8
5.6
5.4
5.2
5.0
4.8
4.6
4.4
4.2
pH
1
2
3
4
5
6
7
8
9
10
Gel Length (cm)

this purpose, an improvement over O'Farrell's original formulation (at least for the pH interval between 5 and 7). It is also worth noting that by far the largest concentration of polypeptides on an O'Farrell -type pH gradient run by the author (gels not shown) focused within the pH 5-7 interval.

An attempt to precisely calibrate the second dimension of the present gel system for the molecular weights of the separated polypeptides, using standard proteins intended for use with Coomassie Blue staining, was not successful. However, it is estimated that polypeptides of M_r > 10 Kd are clearly resolved in the gel. In addition, spots 10 in Figs. 3.1 and 3.2 appear to represent actin (M_r = 40 Kd).

The reproducibility of the details of the 2D spot pattern with this system appears to be excellent, as judged from more than 200 gels run up to 8 months apart on over 50 lines in 6 species of Drosophila. The primary non-genetic sources of variation appear to be related to (i) culture conditions: crowded larval cultures produced smaller adults, with consequent reduction in size of reproductive organs and their protein content, and (ii) time of isoelectric focusing: 6400 - 7000 Volt-hours appeared optimal, whereas beyond 8000 Volt-hours the basic end of the pH gradient began to decay. The silver staining procedure of Table 2.4 gave extremely reproducible results when followed even within fairly broad procedural limits, and is not considered to be an important

source of non-genetic variation in spot patterns.

Thus, the technical improvements applied by the author to the original two-dimensional electrophoresis scheme described by O'Farrell (1975) have resulted in high-resolution, extremely reproducible separation and visualization of more than 400 polypeptides from Drosophila male reproductive tracts. This power and reliability cannot help but reduce the tendency towards conservatism in gel scoring on the part of the researcher.

Appearance of Spots. One of the most useful general features of the 2D system used here is the diversity of appearance of the polypeptide spots when gels were stained according to Table 2.4. Most striking was the variety of colours of the stained spots. This effect, which requires for full development the use of ethanol in gel fixation (Sammons et al., 1983), appears to depend largely on the amino acid composition of the polypeptide (Nielsen and Brown, 1984). Spots were stained black, white, pink, orange, red, yellow, green, and various shades of gray and brown. Colour can be used in combination with other features such as shape, size and texture (sharpness of outline, density and fineness of grain) to recognize a definite and reproducible "morphology" for a polypeptide spot. The ability to define such spot morphologies has been a valuable aid during the course of this study, in the assignment of homology between polypeptides occupying different gel positions both within

and between species. Many of the distinctive visible features of spots on gels stained as outlined in Table 2.4 are simply not available when polypeptides are visualized by Coomassie Blue staining or by autoradiography. Thus, the use of silver staining has reduced further the importance of interpretive obstacles in the genetic analysis of spot variation, by greatly reducing uncertainties concerning the homologies of variable spots.

Spot Nomenclature. The system adopted was organized around "landmark" spots or spot groups, which were designated as such because of their ease of recognition when scanning the pattern visually and were chosen to be distributed as evenly as possible over the area of the gel. Single landmark spots (see Figures 3.1 and 3.2) were assigned a number (e.g. 10); members of a landmark grouping were given an upper-case letter as well (e.g. 4A, 4B, 4C). Where it seemed clear that the multiplicity of a set of prominent spots chosen as a landmark was actually the result of microheterogeneity rather than the presence of several separate gene products, the grouping was simply named as a single landmark spot (e.g. 12, 17). In the interests of simplifying comparisons between D. simulans and D. melanogaster, an effort was made to assign the same number to at least some of the clearly homologous landmark spots in the two species, although compromise was sometimes necessary between this desirable feature and that

of choosing the best landmarks for use within species.

After landmarks were named, the remaining spots were named in neighbouring groups around the landmarks, by attaching letters to the number of the landmark (see Appendix 1 for the spot maps generated for D. simulans and D. melanogaster). It should be borne in mind that this system is not only arbitrary but also temporary, and will eventually be replaced by a more meaningful system of names conveying information about polypeptide functions.

Detection and Scoring of Genic Variation

Measurement of genic polymorphism in Drosophila simulans and Drosophila melanogaster was based on whole-tract separations of the type shown in Figs. 3.1 and 3.2, for 10 isofemale lines from each of two populations within each species. One population of each species was from a temperate European region (France), and the other from a tropical African region (Benin and The Congo), as listed in Table 2.1. Gel comparisons were facilitated by choosing one gel (usually the one with best resolution) as a standard from a set of 10 gels selected as suitable for scoring. The other gels were then compared side by side visually, one at a time, to this standard. A transparent plastic overhead projector sheet laid over a gel being compared to the standard was useful to mark spot differences with the aid of coloured pens. Later, the complete set of these "score sheets" could be used to simplify the recognition of recurring variant spots as

putative genic polymorphisms.

Variant spots were paired with an allelic spot on the scored and/or the standard gel. The "spot morphology" criteria listed above were very useful here, as were assumptions (i) that the members of an allelic pair of spots were likely to differ in the charge dimension, with or without a shift in the size dimension, (ii) that spot shifts were likely to be small, so that a process of elimination could be applied to a limited set of nearby candidates for homology, and (iii) that presence of a variant should be accompanied by a visible change in its allelic spot, i.e. a reduction in staining intensity (for segregating lines) or occasionally (for homozygous lines) a disappearance. Optimally, both of these phenotypes - segregating variant and homozygous variant - should be observed for each variant allele. However, in practice, with small samples of lines such as were analyzed here, observation of segregating - variant phenotypes sometimes had to suffice. Only variant spots which could be paired with their homologs according to these criteria were accorded status as genic polymorphisms. Examples of variation at such polymorphic loci are shown in Fig. 3.4 and Fig. 3.5. Not all of the discrete, recurring line-to-line variation could be fitted precisely to such a Mendelian model. For instance, in D. simulans 13 such "non-scorable" variable spots were seen (listed in Table A4.1, Appendix 4). Some of these spots clearly showed homozygous

FIGURE 3.4

Examples of electrophoretic variants observed in Drosophila simulans reproductive tract proteins. (A) 17^2 homozygous line. (B) Line segregating for $17^2/17^3$. (C) Triallelic line, segregating for $17^1/17^2/17^3$. Note that two "isoforms" of each locus 17 protein exist in the lateral (pH) dimension.

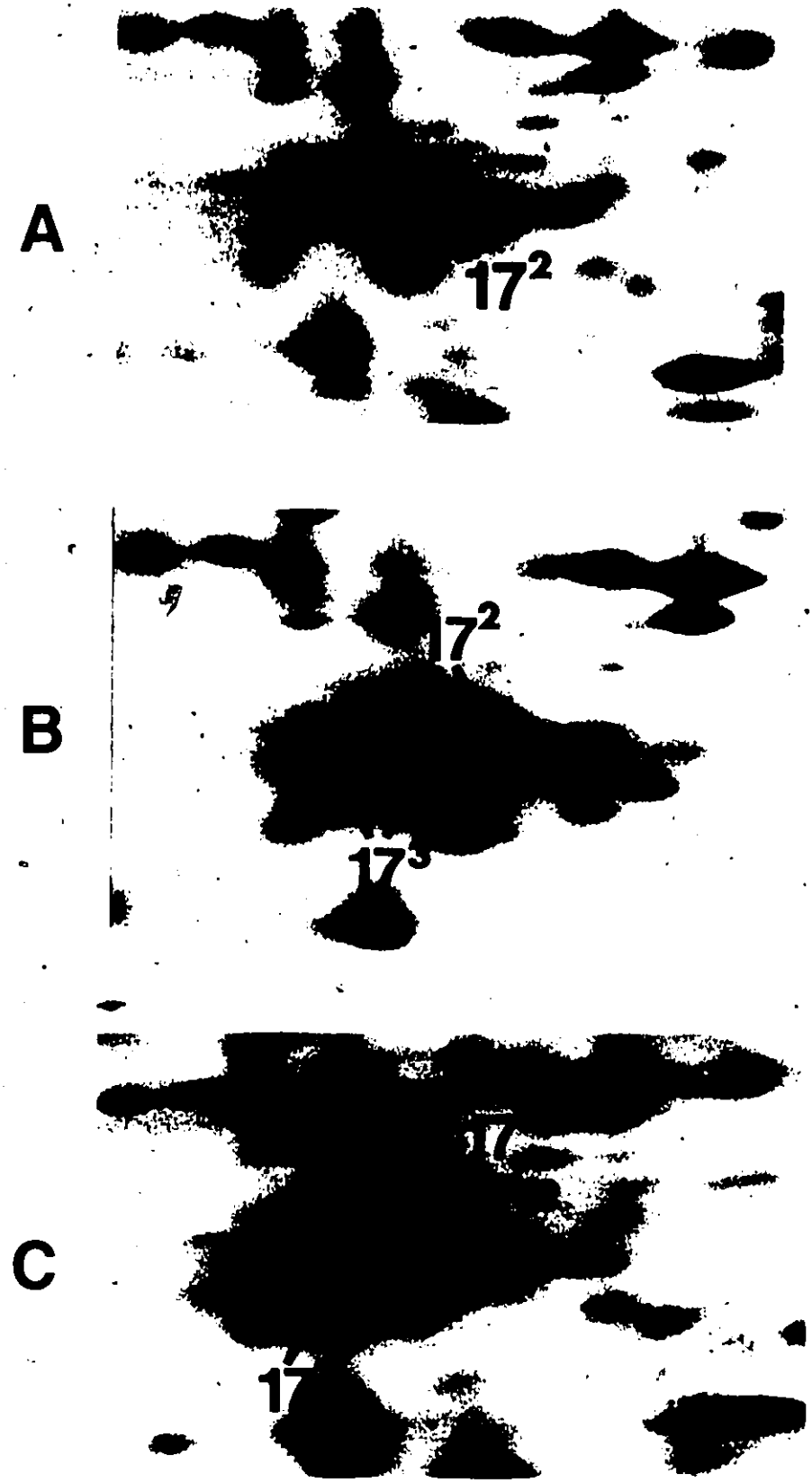
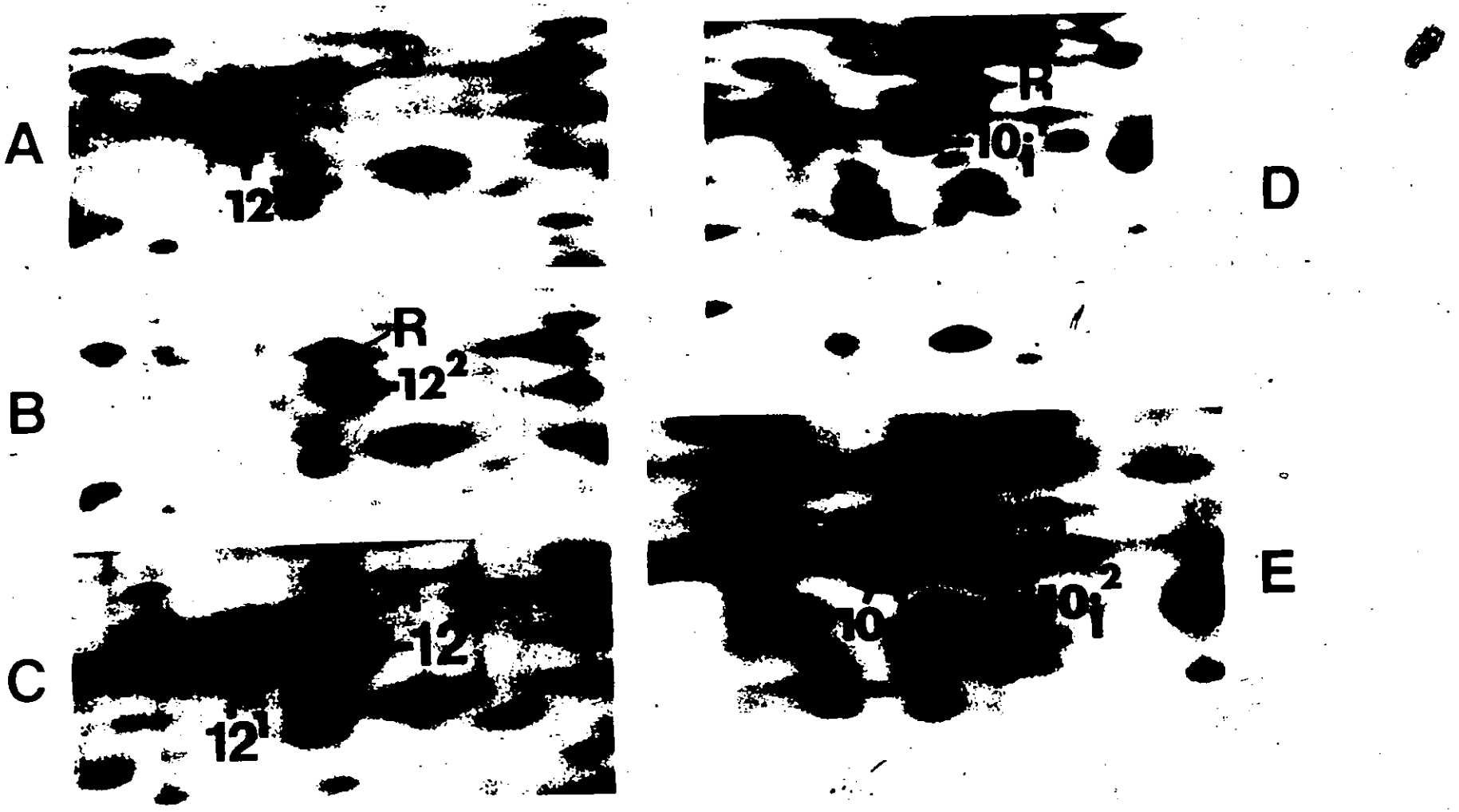

A
17²
B
7²
17³
C
17

FIGURE 3.5

Examples of electrophoretic variants observed in Drosophila simulans reproductive tract proteins. (A) Line homozygous for 12^1. (B) Line homozygous for 12^2. (C) Line segregating for $12^1/12^2$. (D) Line homozygous for $10j^1$. (E) Line segregating for $10j^1/10j^2$. Spots labelled "R" are indicated for reference.


A
12
B
R
12²
C
12
12¹
D
R
10¹
E
10
10¹²

and heterozygous phenotypes, but their peripheral location near the boundaries of the gel pattern prevented exact determination of phenotype for every line analyzed. Others showed no apparent heterozygotes, although spot shifts were seen. However, in only two isofemale lines in the entire study was a variant spot detected which could not be paired with any homologous spot on the scored or the standard gel. Thus, the requirement that the variation in spots scored as polymorphic be codified in terms of a locus with countable, segregating structural alleles implies that some truly polymorphic loci will be excluded simply because they could not be analyzed precisely. But almost none of this bias appeared to be caused, in the course of the current work, by inability to identify allelic homologies. The putative allelic relationships which were assigned could be verified further at any time by controlled crosses, although these were not performed here.

To compute the allele frequencies for a polymorphic locus in a population sample, each isofemale line, descended from the progeny of a single inseminated wild-caught female, was assumed to represent a sample of two alleles from the original gene pool. This is because, over time as the lines are cultured in the laboratory, the original sample of at least four alleles per line (two from the wild-caught female plus a minimum of two paternal alleles) is inevitably reduced in size by random genetic

drift within lines. Nevertheless, many of the lines maintained in this laboratory still segregate for known allozyme variants even after many years of such culture; thus an assumption of two alleles per line rather than just one seemed reasonable a priori. The only deviation from this rule of computation occurred with occasional lines of D. simulans that were still segregating for three alleles, presumably as a result of their relatively recent origin (see Table 2.1). In these cases, only the visibly triallelic lines were treated as such, and the allele frequencies calculated with respect to this slightly larger total sample of alleles for that locus. The sample size taken for each of the four populations was of a size (20 genomes) expected to be capable of detecting variant alleles present at a frequency of .05 or greater in the original population.

Summary of Genic Variation in Drosophila simulans and Drosophila melanogaster

The polymorphic spots identified in these two species are shown in Figures 3.1 and 3.2, designated by an underlined number. Twenty-seven such loci were identified in D. simulans and twenty-seven in D. melanogaster. The gel positions of the alleles identified are indicated in Figures 3.6 and 3.7. These alleles are numbered beginning with the most acidic and highest - M_r forms and with decrease in M_r taking precedence over increase in pI when naming the next

FIGURE 3.6

Pictorial summary of variant alleles of Drosophila simulans which are listed and numbered in Table 3.1. Positions of variants not present in the line analyzed on this gel are related to the allele present by a dot and a connecting line. Identities of the polymorphic loci can be obtained by referring to Figure 3.1. Note that spots 31 and 31g were found to co-segregate, and are thus considered to be coded by a single locus.

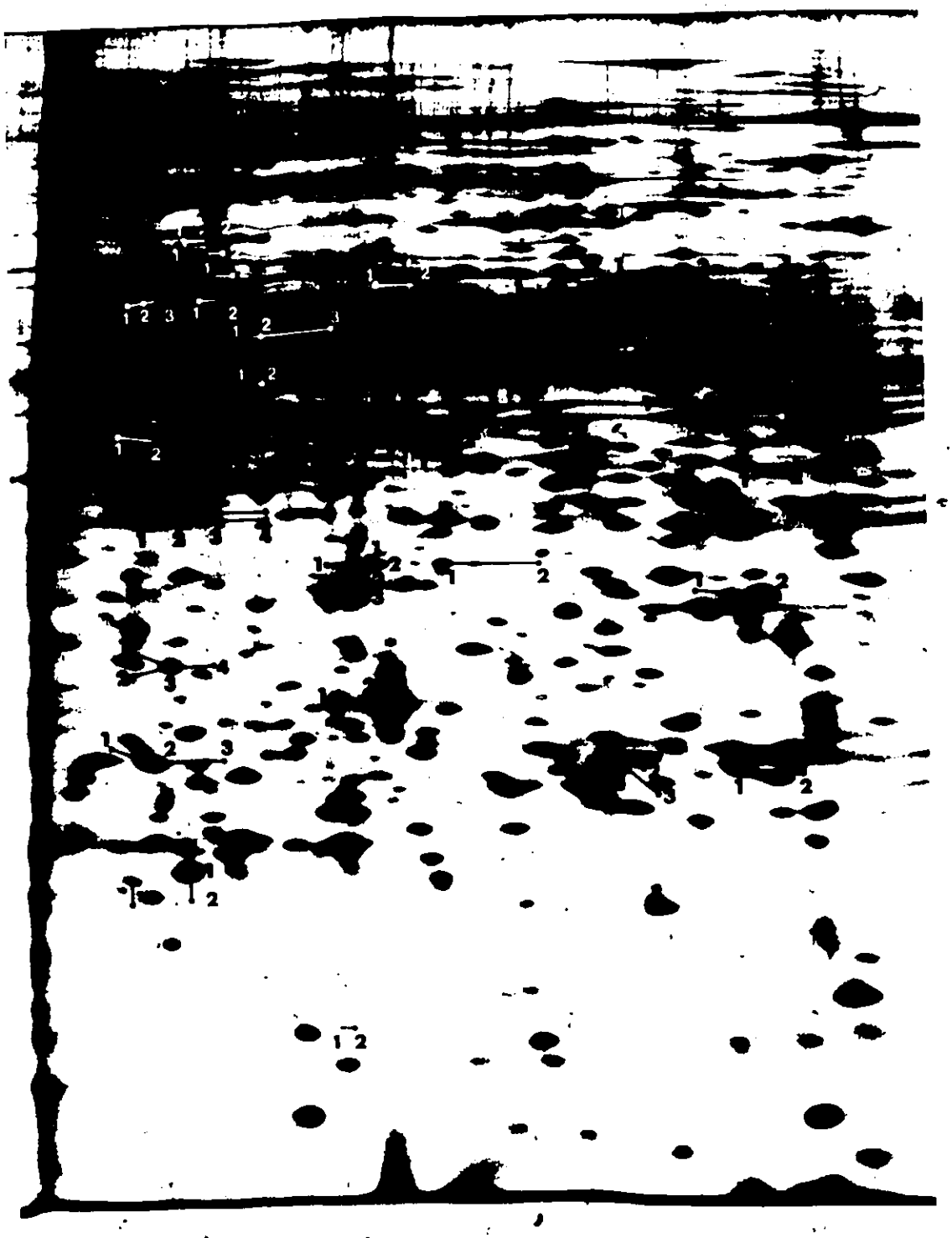


FIGURE 3.7

Pictorial summary for variant alleles of Drosophila melanogaster. Refer to Table 3.2 and Figure 3.2 for numerical data and identities of polymorphic loci, respectively. Positions of variants not present in the line analyzed on this gel are related to the allele present by a dot and a connecting line.

allele in a series. Polymorphic spots appeared in all regions of the gels, and considerable variety was encountered with respect to number of alleles, direction of shift, and presence or absence of co-ordinated shifts of multiple spots. Some indication can be gained of the relative importance of charge shifts and size shifts, in terms of their contributions to the overall variation, if all pairwise differences in spot positions at the polymorphic loci in each species are classified according to whether a charge shift only, a size shift only, or a charge-plus-size shift differentiates the pair of alleles. When these proportions were calculated for D. simulans, out of a total of 62 pairwise allele comparisons, 32 (51.6%) involved only a charge shift, 25 (40.3%) involved shifts in both charge and size, and 5 (8.1%) involved a size shift only. In D. melanogaster, a total of 35 such allele pairs was divided into 25 (71.4%) with only a charge shift, 9 (25.7%) with a size shift and a charge shift, and 1 (2.9%) with only a size shift. Summaries of allele frequencies, sample sizes, and expected heterozygosities (calculated as $1-\Sigma p_i^2$, where p_i = the frequency of the i^{th} allele) are given in Tables 3.2 and 3.3. Missing entries in these tables (e.g. locus 19e in the France population of D. melanogaster) represent cases where it was not possible to score allele frequencies with precision on the gels available. The allelic constitutions

TABLE 3.2

Allele frequencies, expected heterozygosities, and numbers of genomes sampled for 27 polymorphic loci analyzed by two-dimensional gel electrophoresis in a temperate (S. France) and a tropical (Brazzaville) population of Drosophila simulans.

Locus	Allele	Allele Frequency/ Expected Heterozygosity (H)[a] S. France	Brazzaville	Mean[b]	Genomes Sampled[a]
2a	1	.15	.10	.125	40
	2	.85	.90	.875	
	H	.255	.180	.218	
2c	1	1.00	.95	.975	40
	2	–	.05	.025	
	H	0	.095	.048	
2e	1	.35	.10	.225	42
	2	.65	.90	.775	
	H	.455	.180	.318	
2f	1	–	.455	.228	42
	2	.10	.136	.118	
	3	.90	.409	.655	
	H	.180	.609	.395	
3a	1	.40	.409	.400	42
	2	.60	.364	.482	
	3	–	.227	.114	
	H	.480	.533	.507	
3e	1	.80	.30	.550	40
	2	.20	.70	.450	
	H	.320	.420	.370	
3f	1	–	.095	.048	41
	2	.55	.333	.442	
	3	.40	.571	.486	
	4	.05	–	.025	
	H	.535	.553	.544	
5A	1	.60	.95	.775	40
	2	.40	–	.200	
	3	–	.05	.025	
	H	.480	.095	.288	
7B	1	–	.05	.025	40
	2	1.00	.95	.975	
	H	0	.095	.048	

Table 3.2 (Cont'd.)

Frequency/
Expected Heterozygosity (H)

Locus	Allele	S. France	Brazzaville	Mean	Genomes Sampled
10e	1	.20	.70	.450	40
	2	.80	.30	.550	
	H	.320	.420	.370	
10j	1	.95	.67	.810	41
	2	.05	.29	.045	
	3	-	.04	.145	
	H	.095	.465	.280	
10k	1	.60	.375	.488	44
	2	.40	.292	.346	
	3	-	.208	.104	
	4	-	.125	.063	
	H	.480	.715	.598	
11k	1	.45	.55	.500	40
	2	.55	.45	.500	
	H	.495	.495	.495	
12	1	.80	.85	.825	40
	2	.20	.15	.175	
	H	.320	.255	.288	
14a	1	.238	-	.119	41
	2	.714	.50	.607	
	3	.048	.50	.274	
	H	.431	.500	.466	
14d	1	.80		.800	20
	2	.20	-	.200	
	H	.320		.320	
16d	1	.10	-	.050	40
	2	.05	-	.025	
	3	.85	.95	.900	
	4	-	.05	.025	
	H	.265	.095	.180	
17	1	.048	.10	.074	41
	2	.429	.90	.665	
	3	.524	-	.262	
	H	.542	.180	.361	

Table 3.2 (Cont'd.)

Locus	Allele	Frequency/ Expected Heterozygosity (H) S. France	Brazzaville	Mean	Genomes Sampled
17a	1	.40	-	.200	40
	2	.60	1.00	.800	
	H	.480	0	.240	
18d	1	.40		.400	20
	2	.60	-c	.600	
	H	.480		.480	
21c	1	.05	-	.025	40
	2	.80	.60	.700	
	3	.15	.40	.275	
	H	.335	.480	.408	
25f	1	.25	-	.125	40
	2	.75	.95	.850	
	3	-	.05	.025	
	H	.375	.095	.235	
26	1	-	.05	.025	40
	2	1.00	.95	.975	
	H	0	.095	.048	
29c	1	.85	.778	.814	38
	2	.15	.222	.186	
	H	.255	.346	.301	
29i	1	1.00	.55	.775	40
	2	-	.15	.075	
	3	-	.30	.150	
	H	0	.585	.293	
31/31g[d]	1	.40	-	.200	40
	2	.60	.60	.600	
	3	-	.40	.200	
	H	.480	.480	.480	
34b	1	.90	1.00	.950	40
	2	.10	-	.050	
	H	.180	0	.090	

[a] See text for computation methods.
[b] Arithmetic mean (unweighted) over two populations.
[c] Not scored.
[d] Spots 31 and 31g show coordinate mobility shifts.

TABLE 3.3

Allele frequencies, expected heterozygosities, and numbers of genomes sampled for 27 polymorphic protein loci analyzed by two-dimensional gel electrophoresis in a temperate (France) and a tropical (Benin) population of Drosophila melanogaster.

		Allele Frequency/ Expected Heterozygosity (H)			
Locus	**Allele**	**France**	**Benin**	**Mean**	**Genomes Sampled**
2g	1	.55	.20	.375	40
	2	.45	.80	.625	
	H	.495	.320	.408	
3a	1	.389	.50	.444	38
	2	.611	.50	.556	
	3[a]	-	-	-	
	H	.475	.500	.488	
3c	1	-	.15	.150	40
	2	1.00	.85	.850	
	H	0	.255	.128	
3e	1	.75	.15	.450	40
	2	.25	.85	.550	
	H	.375	.255	.315	
3f	1	.20	.50	.350	40
	2	.80	.50	.650	
	H	.320	.500	.410	
5i	1	[b]	.50	.500	20
	2		.50	.500	
	H		.500	.500	
7C	1	-	.10	.050	40
	2	1.00	.90	.950	
	H	0	.180	.090	
7b	1	1.00	.20	.600	40
	2	-	.80	.400	
	H	0	.320	.320	
7c	1	.555	.55	.553	38
	2	.445	.45	.447	
	H	.494	.495	.495	
9j	1	-	.20	.100	38
	2	.111	.80	.456	
	3	.889	-	.444	
	H	.197	.320	.259	

Table 3.3 (Cont'd.)

Allele Frequency/
Expected Heterozygosity (H)

Locus	Allele	France	Benin	Mean	Genomes Sampled
10d	1	.50	.55	.525	40
	2	.50	.45	.475	
	H	.500	.495	.498	
10'	1	.95	.75	.850	40
	2	.05	.25	.150	
	H	.095	.375	.235	
10'h	1	-	.05	.025	40
	2	1.00	.95	.975	
	H	0	.095	.048	
11d	1	.30	.40	.350	40
	2	.70	.60	.650	
	H	.420	.480	.450	
15b	1	1.00	.95	.975	40
	2	-	.05	.025	
	H	0	.095	.048	
17	1	1.00	.75	.875	40
	2	-	.25	.125	
	H	0	.375	.188	
18c	1	.05	.15	.100	40
	2	.05	-	.025	
	3	.90	.85	.875	
	H	.185	.255	.220	
19d	1	.65	.95	.800	40
	2	.45	.05	.200	
	H	.455	.095	.275	
19e	1		.20	.200	20
	2	-b	.50	.500	
	3		.30	.300	
	H		.620	.620	
19l	1	-	.05	.025	40
	2	-	.20	.100	
	3	1.00	.75	.875	
	H	0	.395	.198	

Table 3.3 (Cont'd.)

Allele Frequency/
Expected Heterozygosity (H)

Locus	Allele	France	Benin	Mean	Genomes Sampled
22a	1	1.00	.95	.975	40
	2	-	.05	.025	
	H	0	.095	.048	
23e	1	-	.05	.025	40
	2	1.00	.95	.975	
	H	0	.095	.048	
24	1	1.00	.95	.975	40
	2	-	.05	.025	
	H	0	.095	.048	
24b	1	[b]	.80	.800	20
	2		.20	.200	
	H		.320	.320	
25	1	.95	1.00	.975	40
	2	.05	-	.025	
	H	.095	0	.095	
28a[a]	1	-	-	-	40
	2	-	.05	.025	
	3	1.00	.95	.975	
	H	0	.095	.048	
28b	1	-	.10	.05	40
	2	1.00	.90	.95	
	H	0	.180	.090	

[a] Alleles $3a^3$ and $28a^1$, although found segregating in other sources of D. melanogaster, were not found in the France or Benin material analyzed.

[b] Not scored.

of the individual isofemale lines analyzed are listed in Appendix 2.

Although the specific values obtained for the allele frequencies in Tables 3.2 and 3.3 cannot be taken as definitive, because of the small number of genomes sampled, certain general features of the frequency data warrant comment. First, there is a high degree of genetic similarity between the two populations of each species. In D. simulans, 17 of the 25 polymorphic loci which were scored in both populations (68%) shared the same leading allele in each population. However, exactly the same number of loci (17 of 25) were segregating for an apparently population-specific allele or alleles in either the South France or the Brazzaville sample; some of these population-specific alleles were present in fairly high frequencies (Table 3.2). The pattern in D. melanogaster showed 17 of 24 loci (70.8%) sharing the same leading allele in both populations, and 15 of 24 loci (62.5%) segregating for "population-specific" alleles. These latter alleles, however, tended to be present at low frequencies in this species (Table 3.3), and therefore may only appear to be population-specific because of inadequate sampling of the population that seemed to lack them.

Some of the polymorphic loci listed in Table 3.2 and 3.3 are homologous between D. simulans and D. melanogaster. These loci will be discussed more fully in Chapter 5, in the

context of a thorough analysis of divergence between the male reproductive tract proteins of these two species.

Table 3.4 contains summary statistics (percentages of polymorphic loci and average expected heterozygosities per locus) not only for the proteins studied by 2DE here but also, for purposes of comparison, in samples of loci analyzed by 1DE in the same two species. The 1DE data are for soluble enzymes (Singh, unpublished data; Singh and Choudhary, unpublished data) and "abundant soluble proteins" visualized on Coomassie Blue - stained gels (Singh and Coulthart, 1982); these data were obtained from the same four populations of D. simulans and D. melanogaster as well as from a more inclusive set of populations intended to represent each species on a broader geographic basis. This approach, by reducing the possible effects of between-population divergence and sampling variation, enhances the rigour of comparisons made between different sets of loci with respect to their variability, even though global perspective on variability within a species may be sacrificed. The "maximum" total numbers of loci listed in Table 3.4 for the 2DE study represent the total numbers of reproducible, prominent spots on the gels in each species (see maps in Appendix 1). The "minimum" total numbers represent this larger total minus a fraction comprising peripheral spots that, especially by their proximity to the lateral edges of the spot pattern, could have harboured segregating variation

TABLE 3.4

Number of loci analyzed, mean heterozygosity per locus, and percentage of loci polymorphic for various sets of proteins studied by one-dimensional gel electrophoresis (1DE) and two-dimensional gel electrophoresis (2DE) in *Drosophila simulans* and *Drosophila melanogaster*. The 1DE values for soluble enzymes and larval proteins of *D. simulans* are from Singh and Choudhary (unpublished data), for *D. melanogaster* soluble enzymes from Singh (unpublished data), and for *D. melanogaster* abundant soluble proteins from Singh and Coulthart (1982).

Protein Set/Population(s)	Number of Loci	Mean Heterozygosity Polymorphic Loci ($\bar{H}_p$)	Mean Heterozygosity All Loci ($\bar{H}$)	Percentage of Loci Polymorphic (P)
I. Soluble Enzymes (1DE)				
D. simulans				
S. France	39	.381	.059	15.4
Brazzaville	39	.348	.089	25.6
Overall[a]	39	.222	.063	28.2
D. melanogaster				
France	80	.314	.129	41.3
Benin	80	.306	.134	43.8
Overall[a]	80	.274	.158	57.5
II. Abundant Soluble Proteins (1DE)				
D. simulans (larval)				
S. France	11	.425	.193	45.5
Brazzaville	11	.390	.177	45.5
Overall	11	.343	.187	54.5
D. melanogaster (Larval-Homologous)[b]				
France	11	.283	.128	45.5
Benin	11	.329	.149	45.5
Overall	11	.255	.140	54.5
D. melanogaster (Larval/Adult)				
France	20	.203	.081	40.0
Benin	20	.163	.090	55.0
Overall	20	.151	.098	65.0

Table 3.4 (Cont'd.)

Protein Set/Population(s)	Number of Loci	Mean Heterozygosity: Polymorphic Loci	Mean Heterozygosity: All Loci	Percentage of Loci Polymorphic
III. Male Reproductive Tract Proteins (2DE)				
D. simulans				
S. France	250[c]	.363	.034	9.2
	295		.029	7.8
Brazzaville	250	.373	.032	9.2
	295		.027	7.8
Mean	250	.368	.033	10.8
	295		.028	9.2
D. melanogaster				
France	243[c]	.311	.015	4.9
	307		.012	3.9
Benin	243	.299	.032	10.7
	307		.025	8.5
Mean	243	.305	.024	11.1
	307		.018	8.8

[a] "Overall" refers to pooled data of S. France, Tunisia, Congo (Brazzaville) and South Africa populations of D. simulans, or to 15 populations (worldwide) of D. melanogaster (see Singh and Coulthart, 1982).

[b] Larval proteins of D. melanogaster homologous to 11 larval proteins surveyed in D. simulans.

[c] Two total numbers of loci refer to minimum and maximum estimates (see text).

that was undetectable because it fell beyond the spot pattern boundary. Although the contribution of hidden variation in most such spots to overall heterozygosity would probably have been slight (since the "normal" allele was consistently seen), their contribution to the overall percentage of polymorphic loci might have been substantial if they could have been scored accurately.

The summary statistics in Table 3.4 were compared among themselves in various ways. To facilitate these comparisons, the values in Table 3.4 were "standardized" by expressing them as ratios, in the following ways: (i) for a given 1DE protein set in relation to the 2DE set analyzed in the same population(s) (Table 3.5), (ii) for a given protein set in D. simulans in relation to the same protein set in D. melanogaster (Table 3.6), and (iii) for a protein set of a temperate population in relation to the same protein set in the conspecific tropical population (Table 3.7). The remainder of the Results section will be concerned with the patterns that emerge from these comparisons.

First, when all loci from each protein set are included, the percentages of polymorphic loci and average heterozygosities per locus are much lower with the 2DE set than with any of the 1DE sets. Both D. simulans and D. melanogaster 2DE proteins include polymorphic loci at about a 10% frequency, and overall mean heterozygosities per locus are in the vicinity of 2-3% (Table 3.4). These values agree

TABLE 3.5

The 1DE data of Table 3.4 were expressed as ratios of the values obtained for the same population or set of populations by two-dimensional gel electrophoresis. Comparisons are thus carried out between values within a single line of the table.

Protein Set/Population(s)	Mean Heterozygosity: All Loci ($\bar{H}$)	Mean Heterozygosity: Polymorphic Loci ($\bar{H}_p$)	Percentages of Loci Polymorphic (P)
I. Soluble Enzymes (1DE)			
D. simulans			
S. France	1.73	1.05	1.67
Brazzaville	2.78	0.93	2.78
Overall	1.91	0.60	2.61
D. melanogaster			
France	8.60	1.01	8.43
Benin	4.18	1.02	4.09
Overall	6.58	0.90	5.18
II. Abundant Soluble Proteins (1DE)			
A. Larval			
D. simulans			
S. France	5.68	1.17	4.95
Brazzaville	5.53	1.05	4.95
Overall	5.67	0.93	5.05
D. melanogaster			
France	8.53	0.91	9.29
Benin	4.66	1.10	4.25
Overall	5.83	0.84	4.91
B. Larval + Adult			
D. melanogaster			
France	5.40	0.65	8.16
Benin	2.81	0.55	5.14
Overall	4.08	0.50	5.86

quite closely with those obtained in earlier 2DE studies with D. melanogaster, but show an interesting contrast with the previously reported complete lack of variation in the 2DE proteins of D. simulans (see Table 3.1). Within D. simulans, the order of protein sets from the most frequently polymorphic (or most heterozygous) to the least is (Table 3.5): abundant larval proteins > soluble enzymes > 2DE proteins; within D. melanogaster the order is: soluble enzymes > abundant larval proteins > abundant larval + adult proteins > 2DE proteins. Note that the unusually high standardized values for the 1DE sets in the France population of D. melanogaster (Table 3.5) are traceable to lower variation in the 2DE set, rather than higher variation in the 1DE sets (see Table 3.4). Thus, regardless of the technical refinements and differences in the 2DE procedure employed by the author, the estimates of genic variation obtained for these proteins remain quite low in comparison to those derived from 1DE.

Second, if only the polymorphic loci within each set are considered (Table 3.5, column 2), most of the mean-heterozygosity differences between protein sets disappear. The most striking exceptions to this effect are the larval and adult abundant soluble proteins of D. melanogaster, the polymorphic loci of which are about half as heterozygous on average as the polymorphic 2DE loci; however, average heterozygosity at polymorphic enzyme loci for the more

inclusive set of populations in D. simulans is also lower than that for polymorphic 2DE proteins. D. simulans larval proteins, when polymorphic, appear to be more heterozygous than the average for that species (Table 3.4). This pattern is not consistent with the idea that a global lack of sensitivity to allelic variation explains the low variability estimates obtained by 2DE. If sensitivity played a significant part here, one would expect the polymorphic loci themselves also to have a lower mean heterozygosity with 2DE than with 1DE. But the main source of the differences between the estimates from soluble enzymes and 2DE proteins appears to be the preponderance (ca. 90%) of apparent monomorphism among the 2DE proteins. We will return to this point presently.

Third, it appears that the least polymorphic set, aside from 2DE proteins, is that comprised by the enzyme loci of D. simulans. A fourth and connected point is that of all three protein sets which have been sampled both in D. simulans and in D. melanogaster, soluble enzymes show the largest difference in variability between the two species (Table 3.6). D. simulans enzymes are about half as likely to be polymorphic and about 40% as heterozygous overall, as are D. melanogaster enzymes. The mean heterozygosity of the polymorphic enzyme loci of D. simulans is also lower than that of D. melanogaster. This pattern contrasts with that in the other two protein sets which were compared between

TABLE 3.6

The D. simulans data of Table 3.4 were expressed as ratios of the corresponding values for D. melanogaster. Comparisons were carried out between temperate populations (S. France/France), between tropical populations (Brazzaville/Benin), and between mean (for H) or total (for P) values of the more inclusive sets of populations sampled from each species (see footnote [a] to Table 3.4).

Protein Set/Population(s)	Mean Heterozygosity: All Loci ($\bar{H}$)	Mean Heterozygosity: Polymorphic Loci ($\bar{H}_p$)	Percentages of Loci Polymorphic (P)
I. Soluble Enzymes (1DE)			
Temperate	0.46	1.21	0.37
Tropical	0.66	1.14	0.58
Mean/Total	0.56	1.17	0.50
Overall	0.40	0.81	0.49
II. Abundant Soluble Larval Proteins (1DE)			
Temperate	1.51	1.50	1.00
Tropical	1.19	1.19	1.00
Mean/Total	1.34	1.33	1.00
Overall	1.34	1.35	1.00
III. Male Reproductive Tract Proteins (2DE)			
Temperate	2.27	1.17	1.88
Tropical	1.00	1.25	0.86
Mean/Total	1.38	1.21	0.97

species, with respect to both of which D. simulans is, if anything, substantially more heterozygous than D. melanogaster. The possible significance of this pattern of species differences will be taken up in more detail in the Discussion.

The fifth point concerns the standardized data in Table 3.7, with reference to which comparisons can be carried out between populations within each species. The only large interpopulation differences in variability were those seen for soluble enzymes in D. simulans and for 2DE proteins in D. melanogaster. In each case, the temperate population showed considerably less variation than its conspecific Afrotropical counterpart, but this difference was essentially absent when only the average heterozygosities of the polymorphic loci were considered.

This result demonstrates that, if different protein sets were used to assess population-variability differentiation within each of these two species, and these patterns of variability differentiation were then themselves compared between species, quite different impressions could be gained from the different protein sets. For example, with respect to soluble enzymes D. simulans appears to be much less variable (heterozygosity and percentage polymorphism) in the temperate (South France) population than in the tropical (Brazzaville) population, whereas D. melanogaster does not show such a difference (Table 3.7). However, although the

TABLE 3.7

The temperate population data of Table 3.4 were expressed as ratios of corresponding data for the tropical population of the same species.

Protein Set/Population(s)	Mean Heterozygosity: All Loci ($\bar{H}$)	Mean Heterozygosity: Polymorphic Loci ($\bar{H}_p$)	Percentage of Loci Polymorphic (P)
I. Soluble Enzymes (1DE)			
D. simulans	0.66	1.09	0.60
D. melanogaster	0.96	1.03	0.94
II. Abundant Soluble Proteins (1DE)			
A. Larval			
D. simulans	1.09	1.09	1.00
D. melanogaster	0.86	0.86	1.00
B. Larval + Adult			
D. melanogaster	0.89	1.25	0.73
III. Male Reproductive Tract Proteins (2DE)			
D. simulans	1.06	0.97	1.00
D. melanogaster	0.47	1.04	0.46

ratios for male reproductive tract proteins measured by 2DE also show such a difference between species, the difference is in exactly the opposite direction (Table 3.7).

In summary, various electrophoretic techniques, which exploit different physical, chemical and biochemical features of proteins for their separation and visualization, can be used to study genic variation in largely nonoverlapping sets of loci. The data presented in this chapter very clearly illustrate what is now becoming established as a general pattern: when these different sets of loci are analyzed, diverse estimates of average heterozygosity and the proportion of loci polymorphic in natural populations result. The different techniques also yield distinctive patterns of differences in overall variability between species and between populations within a species. The analysis and discussion of this and other patterns of heterogeneity between sets of loci in the genome, and discussion of their possible significance, will constitute most of the rest of this Chapter, and most of Chapter 4.

Where such heterogeneity has been identified, the data also suggest that it can be characterized further as being primarily traceable to differences in the pattern of variation either "between loci" or "within loci". This distinction can perhaps be seen most clearly when, for a given comparison of the type listed in Tables 3.5 - 3.7, we compare the standardized value of total mean heterozygosity

("$\bar{H}$") to the standardized values of mean heterozygosity for the polymorphic loci ("$\bar{H}_P$") and of percentage of loci polymorphic (P).

The "between-locus" type of pattern is exemplified by many of the differences seen between 1DE and 2DE estimates of heterozygosity (Table 3.5). The standardized values of P and $\bar{H}$ for soluble enzymes in this table are, for many of the individual comparisons, almost identical to each other although not equal to one, and the standardized values of $\bar{H}_P$ are very close to unity. This is what is expected when diverse estimates of total heterozygosity for different sets of loci are primarily ascribable to differences in the frequencies of monomorphic loci. In contrast, a "within-locus" type of pattern may be seen with the species difference for larval proteins (Table 3.6). Values of P are identical between the two species (standardized values all = 1.00), while the standardized values of $\bar{H}$ and $\bar{H}_P$ are identical to each other for each of the interspecies comparisons made (temperate-temperate, tropical-tropical, etc.), but not equal to one. This suggests that there is no overall difference in the incidence of monomorphic loci in the sets compared; the differences in $\bar{H}$ are caused by differences in the levels of variation within the subset of polymorphic loci. As it turns out, in this small sample of larval protein loci most of the difference in $\bar{H}$ between species is traceable to one highly polymorphic locus (locus

15) in D. simulans. However, this does not detract from the essential point: that the frequencies of monomorphic abundant larval protein loci in D. simulans and D. melanogaster are the same, and the difference in $\bar{H}$ is a within-locus effect.

Finally, although less common, "mixed" cases are also seen, for example when abundant soluble proteins (larval + adult) of D. melanogaster are compared to 2DE proteins of the same species (Table 3.5). In this case, although the abundant soluble proteins are much more heterozygous and polymorphic with respect to the whole sample of loci than are 2DE proteins, the average heterozygosity of the polymorphic abundant soluble protein loci is substantially lower than that of the polymorphic 2DE proteins. An examination of Table 1 of Singh and Coulthart (1982) shows that this result is connected with the high incidence, among abundant soluble protein loci, of weakly or sporadically polymorphic loci. Thus, both between- and within-locus effects appear to be operative here.

DISCUSSION

Adequacy of the Data. The salient result presented in this chapter is that, despite significant differences, improvements and expansion in methodology and biological materials in comparison to earlier 2DE studies, the protein loci sampled by 2DE in the present study were still found to be significantly less genetically variable than a sample of loci studied by 1DE in the same four populations of Drosophila simulans and D. melanogaster. In both species, 2DE yielded figures of about 10% of male reproductive tract protein loci polymorphic, and 2 - 3% average heterozygosity, for samples of between 250 and 300 loci. This contrasts with the 1DE figures for enzyme loci, i.e. 15 - 25% polymorphism and 6 - 9% heterozygosity for D. simulans, and 41 - 44% polymorphism and about 13% heterozygosity for D. melanogaster.

The two electrophoretic methods also give different results when the data are used for comparisons of mean heterozygosity between the two species; in contrast with much earlier 1DE data, D. simulans appeared to be at least as genically variable as D. melanogaster with respect to male reproductive organ proteins analyzed by 2DE. Discrepancies between the results of 1DE and 2DE were also suggested in terms of latitudinal differences in variability between

populations within each of the two species. The heterogeneity in the results of the different methods could be traced, in most instances, either to disparities in the frequencies of polymorphic loci in the entire set, or to differences in the heterozygosities of the polymorphic loci themselves. These two patterns were loosely termed the "between-locus" and "within-locus" patterns, respectively. The former pattern typified the differences between the results of 1DE and 2DE as applied to the same population, especially when the loci examined by 1DE were those coding for enzymes.

As argued earlier, these results probably cannot be explained simply by a lower physical sensitivity of 2DE to allelic variation in protein structure. Standing against such explanation are both the results of cross-calibration experiments with known variants, and the fact that the polymorphic loci in 2DE tend on average to be as heterozygous as the polymorphic loci in 1DE (Table 3.5), and frequently exhibit multiple alleles (Tables 3.2, 3.3).

More data would be helpful in adding strength to this conclusion. In the terminology used by Ramshaw et al. (1979), both "forward" and "backward" calibration experiments are needed. The "backward" type of experiment, exemplified by the work of McLellan et al. and of Neel's group which was discussed earlier, depends on the availability of previously characterized protein variants. In the "forward" type of

experiment, proteins are sampled from populations without prior knowledge of their allelic variability, either with an aim simply to compare the results obtained for the same or similar sets of proteins with different electrophoretic techniques (e.g. Singh and Coulthart, 1982) or as an attempt to exhaustively determine the amount of protein sequence variation present (Singh et al., 1976).

The latter attempt is usually made, with 1DE separations, by varying electrophoretic conditions or by applying heat denaturation treatments, measurements of enzyme activity, etc. to the sampled alleles in an effort to split electrophoretically homogeneous classes of alleles into subtypes (Coyne, 1982; Neel, 1984). With 2DE, the equilibrium nature of the first-dimension separations most commonly used, and the denaturing conditions to which the polypeptides are subjected, tend to make an exactly analogous approach rather cumbersome at best. However, one possibility for a systematic attack is suggested by methodology described by Fey et al. (1983). These researchers demonstrate the feasibility of peptide mapping of polypeptides excised from two-dimensional gels, after partial proteolytic cleavage of the polypeptides into oligopeptides. Ayala (1982) has shown that electrophoretically cryptic allelic variation in alcohol dehyrogenase of Drosophila melanogaster can be detected by such peptide mapping. These techniques could probably be applied towards the generation of "baseline" data on the

physical sensitivity of 2DE to allelic variation, without depending on the availability of previously characterized variants.

Even if 2DE does prove on further careful examination to be physically less sensitive than 1DE to allelic variation in protein structure, the effects of uncovering electrophoretically hidden variation on the distribution of polymorphism between loci studied by 1DE (summarized by Coyne, 1982) suggest that most of the monomorphic 2DE loci should remain monomorphic even after closer examination. This is because the 1DE studies of cryptic variation via "forward" experiments have yielded a strong positive correlation between the number of alleles detected at a locus under a single "standard" set of conditions and the number of additional alleles revealed by altering the conditions (see Coyne, 1982: Figure 2). Moreover, there is a very marked tendency for loci that are initially apparently monomorphic or diallelic to harbour no cryptic variation. Combined, the results of "backward" experiments with 2DE and "forward" experiments with 1DE strongly suggest that much of the monomorphism seen with 2DE will prove to be an accurate reflection of the underlying genetic situation.

The question of population sampling should also be addressed. The time and labour entailed in preparation, execution and data acquisition with 2DE necessarily restricted the number of independent isofemale lines which

could be analyzed. The sample of lines (about 10 per population, for each of 2 populations per species) was carefully chosen to strike a good compromise between a manageable sample size and truly adequate sampling within populations and species. Nevertheless, it is likely that substantial sampling error is present in the estimates of allele frequencies listed in Tables 3.2 and 3.3. Care was taken, therefore, not to base conclusions too heavily on the allele frequencies as such. Heterozygosity estimates based on the formula: $H = 1 - \Sigma\ p_i^2$, because of their relative insensitivity to the (squared) frequencies of rare alleles, are robust to small sample sizes. And where topics such as the similarity of allelic composition at various loci between populations and species are discussed (see below), the analysis is qualitative and is treated as preliminary. The estimates obtained for percentage of loci polymorphic (P) are probably lower than those which would be expected from larger samples of lines and populations, but this bias was minimized by estimating P from the pooled data (N = about 40 genomes) of both populations in each species. Future work, especially that directed towards characterizing allele frequencies within populations, and patterns of differentiation in allele frequencies between populations, should be based on larger samples of lines. However, since the major conclusions made here are not very dependent on the exact estimation of these frequencies, and because the results are in many respects

quite similar to those from previously published work, this is not considered to be a major deficiency in the present interpretation of the data.

A last consideration of the effects of methodology on data adequacy regards the composition of the sample of loci. As mentioned earlier, it is likely to remain very difficult to define exactly what is meant by a sample of loci that represents the general average level of genic variation in the genome.

The only working alternative to attempting to frame such a definition would seem to be the gradual accumulation of results from a large number of loci that together will represent such a large fraction of the genome that representativeness cannot help but be improved. With the technical improvements in resolution, reproducibility and sensitivity of detection of both radioactive and nonradioactive proteins which have recently been made in 2DE (see for example, Celis and Bravo (1983); Dunn and Burghes 1983a, 1983b; Klose, 1983) it can now be applied to the analysis of genic variation in protein samples from many tissues and stages of Drosophila. Results of separations of over 200 imaginal disc proteins will be described in Chapter 5. Other candidates for such analysis are larval brain and salivary glands, whole embryos, and female reproductive tissues. Since estimates of the total number of structural genes transcribed into messenger RNA in Drosophila fall in

the range of 5,000 - 10,000 (Levy and McCarthy, 1975; Hough-Evans et al., 1980; Hall, Mason and Spierer, 1983; Spierer et al., 1983), this suggests that if 2DE were applied to the measurement of genic variation in 1,000 different polypeptides then 10 - 20% of the structural genes of the genome would have been included in the sample - an increase of at least an order of magnitude over that currently possible using 1DE of soluble enzymes.

Implications for Models of Population Structure. As explained in the General Introduction, data describing genetic variation in populations is open in several ways to the question of its relevance to Darwinian theory. The data contained in this chapter are being discussed from this perspective. The question of electrophoretic sensitivity has already been discussed, as has the representativeness of the sample of proteins for the structural loci of the genome as a whole. Now we shall turn to some less straightforward aspects of the problem. It will not be possible to conclusively prove or eliminate specific hypotheses, but as we shall see, the data can be used to suggest that some interpretations are more probably true than others, and to help formulate some thought-provoking questions.

The most prominent feature of the data, as stated earlier, is the high proportion (about 90%) of loci that are monomorphic. Two basic types of model can be used to explain this observation. Monomorphism could be the result of

drastic reductions in population size ("bottlenecks"): there is no allelic variation because the individuals being sampled are all related to a small number of ancestral individuals in the relatively recent past, and there has not been enough time for variant alleles to reappear *via* mutation and to reach high frequencies by drift and/or selection. Alternatively, the loci might be *maintained* monomorphic by natural selection acting in a "purifying" mode against mutant alleles, all of such mutations occurring in the recent past having had a deleterious effect on the fitness of the individuals that carried them.

There is no completely reliable way to know that population bottlenecks have not occurred in the recent ancestry of the lines sampled in this study. However, the tropical regions of Africa (represented by the Brazzaville population of *D. simulans* and the Benin population of *D. melanogaster*) can be considered to represent the portions of these species' ranges that are most likely to have evolved to an "equilibrium" level of genetic variation (Tsacas, Lachaise and David, 1981). It seems, therefore, that purifying selection has been important, if not necessarily the only factor, in the monomorphism characteristic of 2DE proteins of the male reproductive tract in these two species.

The selective maintenance of large numbers of structural genes in the monomorphic condition raises fundamental questions for both the neutral-mutation/random

drift model and the balancing selection model of population structure. Since both theories are fundamentally theories of the origin and maintenance of variation and of the evolutionary importance of this variation, their relevance to the population structure and evolution of protein-coding DNA sequences diminishes in proportion to the relative rarity of polymorphic proteins in the genome. If a conclusion is desired as to whether the gene pool behaves as an internally balanced system, it is important to have some confidence that the class of genetic elements analyzed for evidence of such integration is in fact the appropriate one. It has been suggested, for instance, by Wallace and Kass (1974) that heterosis based on structural rearrangements in regions of nontranslated DNA that function to control gene activity is common. For this reason, if for no other, it will be very important to eventually obtain accurate estimates of the proportion of monomorphic protein-structural loci in eukaryotic genomes. This will require not only a greatly expanded sample of loci, which should be obtainable with currently available 2DE techniques, but also expanded population samples and a careful search for electrophoretically cryptic variation.

But regardless of whether the 90% monomorphism figure holds true for the genome, the much more reliable observation of heterogeneity of variation levels between different major groups of loci can help decide between alternative

explanations for levels and patterns of genic variation. For instance, the systematically lower estimates of protein variation in the same biological material by 2DE as compared to 1DE in almost every case examined so far suggest quite strongly (to the extent that purely technical factors can be discounted) that purifying selection is more important with 2DE proteins than with the soluble enzymes and other proteins analyzed by 1DE. It is interesting also that this putatively greater purifying selection on the majority of 2DE proteins appears to coexist with a subclass of 2DE proteins that are as highly polymorphic as are polymorphic enzyme loci. This "between-locus" effect in turn coexists with "within-locus" differences in average heterozygosity between abundant soluble protein loci, on one hand, and 2DE proteins or allozymes, on the other; these patterns tend to reinforce further the notion that the nonspecific effects of population history cannot totally explain the variation.

Another example of the same type can be seen when variation data from different types of proteins are used for parallel estimates of differences in average genic variability between species. D. simulans and D. melanogaster are both distributed worldwide over tropical and temperate regions, and the two species co-occur extensively (David and Tsacas, 1981). Yet, with respect to various genetic elements, such as chromosome inversions (Ashburner and Lemeunier, 1976), middle repetitive DNA (Dowsett and Young,

1982), structural genes coding for Coomassie-stained 2DE proteins of whole-body homogenates (Ohnishi et al., 1982) and allozymes (Hyytia et al., 1985; R. S. Singh and M. S. Choudhary, unpublished data), mitochondrial DNA (Hale and Singh, 1985) and genes underlying quantitative variation in phenotypic traits (Hyytia, et al., 1985), D. simulans has been shown either to be monomorphic while D. melanogaster is polymorphic, or to be significantly less polymorphic than D. melanogaster.

These observations could again be explained in two basic ways (excluding a generalized difference in mutation rates between the two species, which constitutes a real possibility) - i.e. as the result of a difference in the action of natural selection on similar types of genetic elements in the two species, or as a result of a difference in their population histories. More specifically, it is possible that of the two species D. simulans has tended to employ a genetic "strategy" that relies on the broad adaptability of a "general purpose genotype" (Baker, 1965), or actually experiences a coarse "environmental grain" (Levins, 1968), while D. melanogaster has responded to new habitats by evolving genetic polymorphisms to deal with variation in the environment, possibly because it experiences a finer grain in its environment. Alternatively, it could be that in the geologically recent expansion of these two species (which probably involved colonization of temperate

regions by migrants from ancestral Afrotropical populations), D. simulans has experienced more serious "bottleneck" effects in its gene pool because of severe reductions in population size associated with colonization.

The measurements of genic variation by 2DE reported herein argue against an interpretation solely in terms of population bottlenecks in D. simulans. Although the allozyme data from the four populations studied by 2DE conform to the expectation of lower variation in D. simulans (Table 3.6), the 2DE data from the same populations argue that D. simulans is, if anything, more heterozygous than D. melanogaster. Population bottlenecks should tend to reduce variation at all loci in the genome, and different samples of loci should reflect this nonspecific effect to the same average extent. Therefore, such a difference in behaviour between different sets of loci in the same populations suggests, in a way analogous to the rejection of a null hypothesis and the consequent reinforcement of an alternative hypothesis via a statistical test, that natural selection has played an important role in the origin and/or maintenance of the species differences in variability where they do occur. Further support for this conclusion comes from comparison of the temperate and tropical populations of D. simulans and D. melanogaster (Table 3.7). Although the temperate (South France) population of D. simulans is less heterozygous for allozyme loci than its conspecific Afrotropical counterpart,

the two other protein sets do not show such a difference. In addition, D. melanogaster 2DE proteins are less polymorphic in the temperate (France) population than they are in the Afrotropical (Benin) population of that species, whereas allozymes are not.

Summary. The application of high-resolution two-dimensional gel electrophoresis of proteins to population genetics is in its earliest stages, despite the existence, since 1975, of published methodologies. One reason for this is perhaps that the techniques, in comparison to the older one-dimensional "native" protein methods, appear formidably complex, time-consuming and even unreliable. But in recent years we have witnessed a rapid evolution of 2DE, from a rather esoteric tool reserved primarily for the trained biochemist into a generally applicable, relatively simple, and highly sensitive approach to the investigation of genetic variation. Some of the more recent developments in procedures and equipment in this field were exploited in this study, with interesting results. It is expected that the protocols described in Chapter 2, because of their simplicity and reliability will find significant further application and undergo much improvement in future population genetics studies.

Another reason why 2DE has been underexploited may be that the interest of the genetic data it can yield has not been fully appreciated. The mere adventitious introduction,

into a scientific field, of techniques developed in other fields to answer different sorts of questions may seem to enjoy a low probability of having a significant effect on the progress of the recipient field. But by now it seems clear that, by virtue of its providing a generalized perspective of unparalleled breadth on the patterns of genic variation in the genome, or simply by sampling a different class of genes, 2DE has already led us to question some of the basic assumptions that had seemed safe to make about the relevance of the older allozyme data to the task of understanding the genetic structure of populations. One of these assumptions was that genic variation is present at a large fraction of structural loci in animal genomes (about 50% in Drosophila). As discussed above, the frequency of polymorphic loci in at least some major groups of Drosophila proteins may be as low as 10%, with average heterozygosities correspondingly reduced to 3-4% compared to alloyzme values in the range 10-15% for many species. And the marked, systematic heterogeneity in variation levels of different sets of proteins in the same populations suggests that single-factor explanations of variation such as those offered by the neutral theory are sometimes not adequate to explain the total pattern of variation.

INTRODUCTION

Genic Variation in Different Classes of Proteins. Soon after measurements of genic variation in soluble enzymes began to accumulate, it became apparent that the incidence of polymorphic loci and their average heterozygosities in a species were correlated with membership of the enzymes themselves in certain structural and functional classes (Gillespie and Kojima, 1968). Several aspects of enzyme structure and function have been suggested as major determinants in this between-locus pattern of variation: internally generated (single) *versus* externally encountered (multiple) substrates (Gillespie and Kojima, 1968); direct involvement in glucose catabolism *versus* function in other pathways (Kojima, Gillespie and Tobari, 1970); regulatory function in metabolic feedback loops *versus* lack of such function (Johnson, 1974); complexity of subunit aggregation (Harris, Hopkinson and Edwards, 1977); and subunit size (Koehn and Eanes, 1978).

Problems of Interpretation. Such relationships strongly suggest that natural selection frequently acts directly on the allelic variation in the structure of the proteins. However, beyond such basic statements interpretation of these

"structure/function correlations" is very difficult. From a neutralist point of view, the tendency for a certain class of structural genes to exhibit more polymorphism than another reflects a higher rate of mutation to selectively neutral alleles. This in turn might reflect a higher proportion of amino acid residues that are nonessential to biochemical function, a higher likelihood that a change in protein function has no effect on the physiological and developmental processes in which the genes are involved, or even involvement of the genes in phenotypic traits having no major effects on fitness. From a selectionist point of view, some form of balancing selection, such as heterozygote superiority or superiority of different homozygotes under different conditions, is assumed to underlie genetic polymorphism in general. The structure/function correlation is then explained by postulating a tendency for certain types of molecules to have structures or functions that make them especially good candidates for the action of balancing selection on mutant alleles. As with neutral alleles, these balancing effects can potentially be mediated at any of several different levels between gene and overall organismal fitness.

The central concept in a neutralist explanation of structure/function correlations is <u>functional constraint</u> (Kimura and Ohta, 1971). An effort is made to identify characteristics of a gene or its product that correlate with

the breadth of organismic tolerance to mutational changes in the gene, and thus with the likelihood of neutral polymorphism at the locus. In an analogous way, devising a selectionist explanation for a correlation is essentially a matter of finding molecular indices that reveal the propensity of polymorphism at particular types of loci to engender an increase in fitness at the individual and/or population level, in comparison to individuals and/or populations that are homozygous at those loci. A complicating factor here is that several fundamentally different types of basic functional mechanisms can be envisaged for balancing selection, and these are by no means mutually exclusive for a given organism (Johnson, 1976). This tends to make the choice of a single structural or functional index on a selectionist hypothesis rather difficult.

One of the problems encountered in applying either of these explanatory frameworks to actual variation data is the simple lack of the data necessary to classify many loci according to whatever index of functional constraint or likelihood of balancing selection has been chosen. This has seriously limited the numbers of soluble-enzyme loci with which correlation analysis can be carried out, and the validity of even positive results must often be questioned on this basis alone. One approach which has been taken to offset this difficulty involves the pooling or repeated

testing of the data from homologous loci of different species (Kojima, Gillespie and Tobari, 1970; Johnson, 1974; Ward, 1977, 1978; Koehn and Eanes, 1978). But these tactics could easily obscure correlations that are present in some species but absent or weaker in others. Conversely, the demonstration of correlations using pooled data may indicate the general existence of a relationship of the sort hypothesized, but indicates nothing specific about the causation of variation within the systems of populations that constitute the individual species.

Another difficulty is the ever-present possibility of spurious correlations caused by some "hidden factor" that is itself correlated with the index explicitly used for locus classification. This problem is exacerbated by the repeated use of relatively small, often homologous samples of soluble enzymes to represent a class of loci in ostensibly independent tests of a correlation hypothesis in different organisms. It would be desirable, in many cases, to be able to test a supposed correlation found with one set of loci by sampling nonhomologous loci showing a similar structural or functional heterogeneity. This would help in defining the actual basis for the correlation, as well as suggesting specific mechanistic hypotheses which can be probed experimentally on a single-locus basis (e.g. Di Michele and Powers, 1982; Koehn, Zera and Hall, 1983; Watt, Carter and Blower, 1985).

The third and most fundamental type of difficulty is concerned with partial overlap between the predictions made from neutralist and selectionist points of view. The problem is especially acute if one considers the set of amino acid codons that can mutate to give rise to balanced polymorphisms to be a subset of those codons that can mutate to give non-deleterious alleles (Fitch, 1972). This would mean that amino acid sites subject to lower functional contraint would be more likely to develop both selected and neutral polymorphisms; correlation of variability with indices of functional constraint could then be used to support either neutralist or selectionist hypotheses. The interpretive ambiguity could be resolved, in principle, if the index used for classification of loci into more- and less-variable groups is one that defines the between-locus distribution of the "privileged" subset of codons that are able or likely to respond to balancing selection, independently of their membership in the weakly constrained set of codons. This type of independent index has been sought (Gillespie and Kojima, 1968; Gillespie and Langley, 1974; Johnson, 1974), but has not been completely successful, partly because of the inherent difficulty in identifying such indices and partly because of lack of the data needed to apply the index.

2DE in the Study of Structure/Function Correlations.

Although many structural and functional data are no easier to obtain for the proteins separated by 2DE than they are for

the soluble enzymes studied by IDE, certain advantages do arise from the use of this technique in studies of correlations between genic variation and protein structure/function. First, large numbers of loci are accessible to genic variation analyses (see Chapter 3). Second, data on certain interesting biochemical and biological features of the proteins are often obtainable directly from the gels. Prominent among these features is the distribution of a protein between, for instance, different prepared cellular subfractions or different organs (Klose and Feller, 1981; Klose, 1982). And third, the combination of the above two characteristics, especially with some means to identify specific proteins on gels from different preparations, should sometimes readily permit the independent testing of postulated correlations using nonhomologous sets of loci from the same species.

The focus of this chapter is an analysis of correlations between aspects of tissue distribution of proteins within the male reproductive tracts of Drosophila simulans and Drosophila melanogaster, and the amounts of genic variation observed in those proteins within each species. Two types of correlation are examined. First, the general question is asked: Is the distribution of a protein's expression between different male reproductive tract tissues correlated with its level of genic variation? And second, an independent test is carried out on a correlation suggested by

the data of Singh and Coulthart (1982) describing genic variation in abundant soluble proteins of D. melanogaster and D. pseudoobscura. In these earlier samples of loci studied by IDE, those proteins expressed in the larval hemolymph were markedly more polymorphic and heterozygous, as a group, than were proteins characterized by detectability in the tissues of the larval carcass or the adult fly but not in the larval hemolymph. One statistical hypothesis which could be advanced on the basis of this observation is that proteins which occur and function in extracellular fluids (such as the larval hemolymph) have a greater tendency to be polymorphic than do proteins that carry out their functions primarily within cells.

However, several other hypotheses could be advanced to describe the correlation, one of these based not on any specific structural or functional feature shared by extracellular proteins in general but, rather, on the paralogous relationship between at least four of the five highly polymorphic hemolymph protein loci via recent gene duplication events in D. melanogaster (Roberts and Evans-Roberts, 1979). This genetic relationship, together with the fact that naturally-occurring "null" alleles were found at two of the four loci (Singh and Coulthart, 1982), suggests that some degree of functional redundancy may exist between these four loci, and may help to explain their high level of variability. Thus, it was deemed important to test the

pattern proposed, as truly being based on extracellular versus intracellular function, by analyzing a separate set of loci that were not homologous but did include some proteins that function in an extracellular environment.

Such criteria are fulfilled by many of the proteins which are synthesized by the accessory glandular tissues (paragonia and anterior ejaculatory duct - see Figure 2.1) of the Drosophila male reproductive tract. These tissues apparently function solely to provide accessory substances which are transferred in the ejaculate, along with spermatozoa, to the female during copulation. The transferred accessory substances comprise a variety of high- and low-molecular weight compounds, which are thought to perform functions ranging from sperm cell activation and maintenance to pheromonal control of female re-mating receptivity (Leopold, 1976). Most of the substances, including the proteins, which are secreted by these glands in D. simulans and D. melanogaster are of unknown function. In fact, this study includes what is, to the author's knowledge, the first descriptive 2DE analysis of the proteins of the male accessory sex glands in any insect; analogous low-resolution studies have been carried out using one-dimensional electrophoresis (Chen, 1976; von Wyl, 1976). Two notable exceptions to the general lack of knowledge of function of the secreted proteins are Esterase-6, which catalyzes the hydrolysis of a putative female antiaphrodisiac

precursor (Mane, Tompkins and Richmond, 1983), and glucose oxidase, which may play a role in antisepsis of sperm stored by the female after copulation (Cavener, 1980). This lack of information on the specifics of function does not prevent the study of male sex gland proteins as extracellular proteins per se, in order to test the correlation postulated above.

RESULTS

Classification of Male Reproductive Tract Polypeptides by Tissue Distribution. Three tissue sample types were prepared from adult male reproductive tracts of *Drosophila simulans* and *D. melanogaster*: (i) "whole tracts", including testes, seminal vesicles (containing mature sperm), paragonial glands and anterior (glandular) ejaculatory duct; (ii) "testes", including testes and seminal vesicles; and (iii) "glands", including paragonial glands and anterior ejaculatory duct (see Figure 2.1). In each sample type, the tissues from 10 flies were used to prepare the homogenate; standardization of amount of protein applied per gel was on this basis only. To improve control over physiological and developmental variation in protein expression, the testis and gland fractions were prepared from a single set of 10 reproductive tracts; these tracts were taken from flies raised in the same culture vial and collected at the same time as were the 10 flies used to prepare the whole-tract samples. The three samples were then electrophoresed simultaneously on three separate gels, and the stained gels were compared.

Polypeptides were classified as detectable: (i) in both testis and gland fractions ("common" spots - Class 1), (ii) only in the testis fraction ("testis-specific" spots - Class 2), (iii) only in the gland fraction ("gland-specific"

spots - Class 3), or (iv) in both testis and gland fractions but predominantly in one or the other ("testis-elevated" and "gland-elevated" spots - Classes 4 and 5). The detection of a polypeptide in a tissue sample obviously depends not only qualitatively on the expression of the gene in the tissue but also quantitatively on polypeptide abundance and on stain sensitivity. For this reason, some of the minor spots (22 spots in D. simulans and 57 spots in D. melanogaster), even though they were judged suitable for scoring genic variation, were not assigned to a tissue distribution class, since incorrect classification as a "tissue-specific" spot was more likely with such minor polypeptide species (Note, however, that if a minor spot is seen clearly to occur in both of two tissue fractions, no problem is encountered in classifying it as a "common" spot). Moreover, the distinction between quantitative ("-elevated") differentiation of expression between tissues and a non-differentiated ("common") pattern must be somewhat subjective. But in this particular study, consistency of application of the classification scheme was improved by its being carried out by one worker. Also, after examining a large number of spots, it became clear that the distinction between common spots and tissue-elevated spots was more natural than might seem likely a priori, with the expression pattern of the majority of spots falling clearly into one of these two types. Finally, it should be noted that the protein classification index used here is probably

freer from such definitional and operational imprecisions than are the indices used in some other structure/function correlation studies. One advantage is the ease of preparing testes and glandular tissues in almost completely separate fractions. It could also be argued that a classification based ultimately, as this one is, on a single readily observable biochemical parameter - protein quantity - is more objective, precise, realistic and verifiable than, for example, a classification based on "substrate variability" or "regulatory function" of enzymes. The spot classifications arrived at for D. simulans and D. melanogaster are shown in diagrammatic form in Appendix 3.

The division of the loci into these four classes allows a first attempt at comparing levels of polymorphism between sets of loci having relatively broad tissue distribution (Class 1, with or without Class 4 or 5) and sets of loci having relatively narrow tissue distribution (Classes 2 and 3, with or without Class 4 or 5). In addition, most of the polypeptides that appear gland-specific in this analysis are likely to be secretory products. This is especially likely since amounts of protein in the total gland fraction and in individual Class 1 spots of the gland fraction were as a rule noticeably lower than they were in the testis fraction. Given this generalized bias, it seems that the apparently gland-specific proteins identified are those which are superabundantly expressed in glandular tissue, which is

expected of secreted proteins (see, for example, spots 3a, 3f, 12, 14a 17 and 29i in Figure 3.1). More rigorous verification of the secreted status of these gland-specific polypeptides would be desirable for future work, especially for investigations of their sexual function. This could perhaps be done by collecting and analyzing secretions directly, by depleting secretions through repeated mating of the males, by monitoring transfer of the proteins to females, or by comparing more tissues in order to verify further the gland-specific nature of the expression pattern. For the present, however, a high degree of correlation between gland-specific abundant expression of a polypeptide (in relation to testes) and its functioning as a secreted product is assumed, and used as a basis for testing the hypothesis that extracellular proteins are more polymorphic than intracellular proteins.

Genic Variation in Different Tissue Distribution Classes.

Tables 4.1 - 4.4 summarize estimates of the various parameters of genic variation for the four distribution classes of loci which were delineated, with Tables 4.1 and 4.3 dealing only with the averages for polymorphic loci and Tables 4.2 and 4.4 with the averages over all loci which could be classified. The "gland-elevated" class was absent among the polymorphic loci in *D. simulans* and rare (one locus) in *D. melanogaster*; this single locus was excluded from the analysis in *D. melanogaster*.

TABLE 4.1

Mean heterozygosity ($\bar{H}$), mean number of alleles per locus ($\bar{N}_a$) and number of loci with 2, 3 and 4 alleles, for polymorphic male reproductive tract proteins of Drosophila simulans classified as to tissue distribution between "testis" and "gland" fractions of the reproductive tract. See Figure 2.1 for description of fractionation method.

Tissue Distribution Class	$\bar{H}$ S. France	$\bar{H}$ Brazzaville	$\bar{N}_a$	Alleles Per Locus 2	3	4
1. Common	.380 (5)[a]	.206 (5)	2.33	4	2	-
2. Testis-specific	.306 (7)	.381 (9)	2.11	6	3	-
3. Gland-specific	.375 (7)	.474 (7)	3.00	2	4	2
4. Testis-elevated	.351 (4)	.095 (2)	2.75	2	1	1
Total/Mean	.351 (23)	.346 (23)	2.52	14	10	3

[a]Numbers in parentheses are numbers of polymorphic loci in each tissue class in each population.

TABLE 4.2

Number of loci, percentage of loci polymorphic, and overall mean heterozygosity per locus ($\bar{H}$) for four tissue distribution classes of proteins in the male reproductive tract of Drosophila simulans.

Tissue Distribution Class	Number of Loci	Percentage of Loci Polymorphic	$\bar{H}$ S. France	$\bar{H}$ Brazzaville
1. Common	131	4.6	.015	.008
2. Testis-specific	87	10.3	.025	.039
3. Gland-specific	25	32.0	.105	.133
4. Testis-elevated	28	14.3	.050	.007
Total/Mean	273	[illegible]	.030	.029

TABLE 4.3

Mean heterozygosity ($\bar{H}$), mean number of alleles per locus ($\bar{N}_a$) and number of loci with 2, 3 and 4 alleles, for polymorphic male reproductive tract proteins of Drosophila melanogaster classified as to tissue distribution between "testis" and "gland" fractions of the reproductive tract. See Figure 2.1 for description of fractionation method.

Tissue Distribution Class	$\bar{H}$ France	$\bar{H}$ Benin	$\bar{N}_a$	Alleles Per Locus 2	3	4
1. Common	.494 (1)[a]	.223 (8)	2.13	7	1	-
2. Testis-specific	.308 (6)	.287 (9)	2.44	5	4	-
3. Gland-specific	.357 (3)	.382 (6)	2.17	5	1	-
4. Testis-elevated	.320 (1)	.418 (3)	2.00	3	-	-
Total/Mean	.342 (12)	.303 (27)	2.22	20	6	-

[a]Numbers in parentheses are numbers of loci in each tissue class in each population.

TABLE 4.4

Number of loci, percentage of loci polymorphic, and overall mean heterozygosity per locus ($\bar{H}$) for four tissue distribution classes of proteins in the male reproductive tract of Drosophila melanogaster.

Tissue Distribution Class	Number of Loci	Percentage of Loci Polymorphic	$\bar{H}$ France	$\bar{H}$ Benin
1. Common	110	7.3	.004	.016
2. Testis-specific	74	12.2	.025	.035
3. Gland-specific	26	23.1	.041	.088
4. Testis-elevated	34	8.8	.009	.037
Total/Mean	248	10.5	.017	.031

Informal inspection of the frequencies of polymorphic loci in the various tissue classes in D. simulans (Table 4.2) suggests that Class 1 loci are less likely to be polymorphic (Percent polymorphism ≅ 5%) than other classes, and Class 3 loci much more likely to be polymorphic (P = 32%). These potential associations and others were tested for statistical significance (Table 4.5). Expected numbers of polymorphic loci in each tissue distribution class were calculated from the representation of the class in the overall sample of loci. These expected numbers were then tested for goodness of fit (Chi-square test) to the observed distribution of numbers of polymorphic loci over tissue classes. The procedure therefore, tests for deviations from randomness of the proportions in which a preset total number of polymorphic loci (i.e. the total number of polymorphic loci observed) "fall" into the variously-sized tissue-distribution classes. Several tests were performed, with the tissue distribution classes taken singly and in various combinations (Table 4.5).

In D. simulans, the overall deviation from expectation was highly significant ($X^2_3 = 16.89$; $P < .005$). The largest deviations of individual classes from expectation were the underrepresentation of Class 1 loci and the overrepresentation of Class 3 loci in the polymorphic subset; for these classes taken singly against the rest of the loci both X^2_1 values were highly significant (Table 4.5, comparisons A and C). These results suggest that there may be a

TABLE 4.5

Observed and expected numbers of polymorphic loci in each of four tissue distribution classes of proteins in the male reproductive tract of *Drosophila simulans*. Chi-square tests of goodness of fit of observed and expected numbers are shown in the bottom half of the table..

Tissue Distribution Class	Number of Polymorphic Loci	
	Observed	Expected[a]
1. Common	6	12.96
2. Testis-specific	9	8.60
3. Gland-specific	8	2.47
4. Testis-elevated	4	2.77

Comparison	Chi-square	d.f.	p[b]
Overall	16.89	3	***
A. 1/2 + 3 + 4	7.62	1	**
B. 2/1 + 3 + 4	0.22	1	n.s.
C. 3/1 + 2 + 4	13.75	1	***
D. 2 + 3/1 + 4	5.44	1	*

[a] Expected values were calculated from proportions of loci in each tissue class in the total sample (see Table 4.2).

[b] n.s. - not significant at .05 level (one-tailed test)
* - significant at .05 level or lower
** - significant at .01 level or lower
*** - significant at .001 level or lower

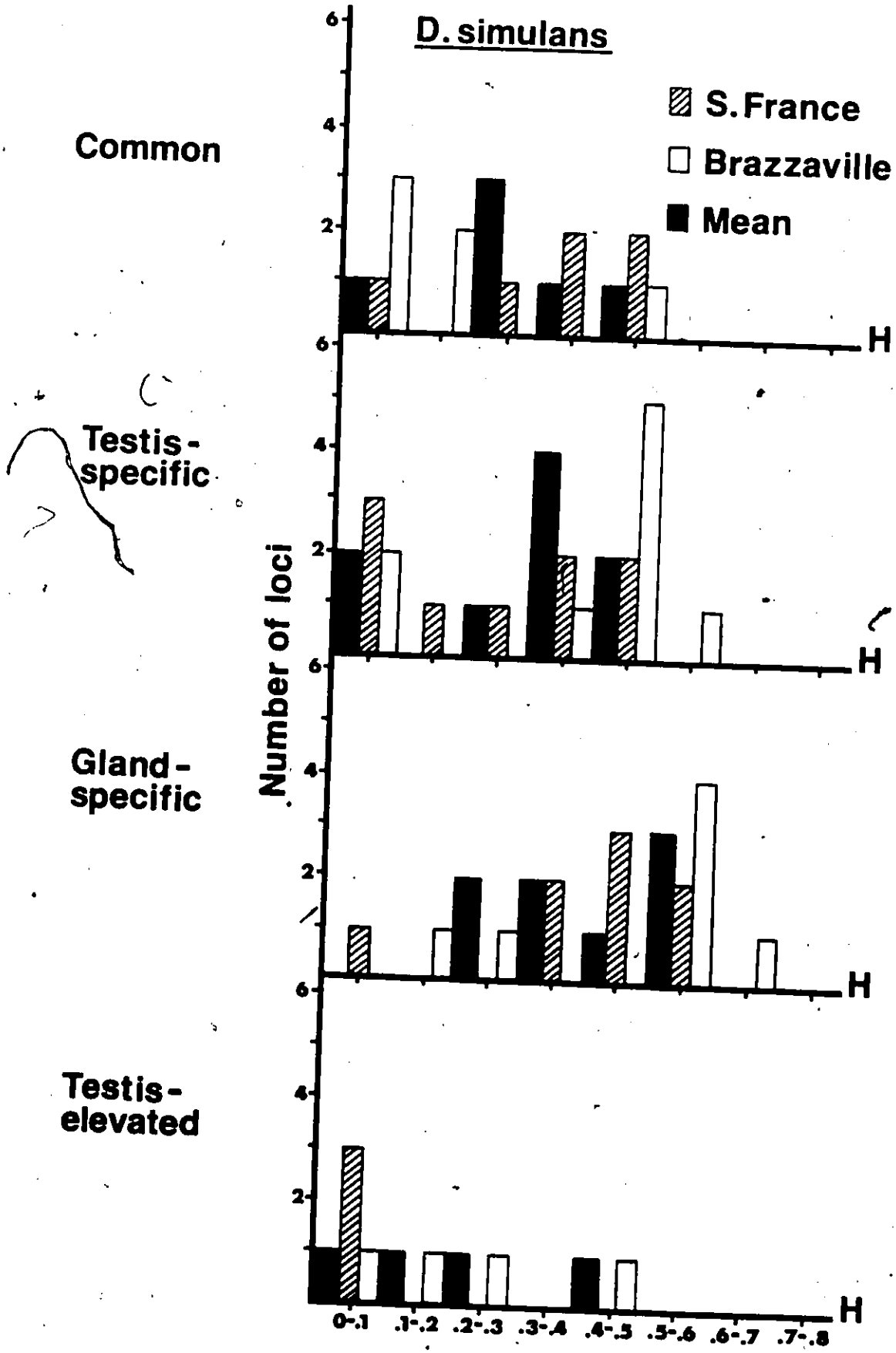
constraining effect of broad tissue distribution, on whether or not a structural locus is polymorphic in D. simulans. A high frequency of polymorphic loci among those coding for secreted proteins, a pattern suggested previously by independent data, (Singh and Coulthart, 1982) is also confirmed by the present data (Table 4.5).

The tendency towards lower variation in Class 1 proteins and higher variation in Class 3 proteins is also reflected in the mean numbers of alleles per locus in D. simulans. The mean for each class deviates from the overall mean number of alleles in the direction expected from the differences in the frequencies of polymorphic loci in the different tissue classes (Table 4.1).

Tests for association between tissue distribution and heterozygosity values of polymorphic loci were also carried out. The mean heterozygosity values for each class can be found for D. simulans in Table 4.1, and the heterozygosity distributions are plotted in Figure 4.1. Informal inspection of this data suggests that in the Brazzaville population there is a tendency for polymorphic Class 1 loci to be less heterozygous and for polymorphic Class 2 and 3 loci to be more heterozygous than the overall average. In addition, there is an apparent tendency for the most heterozygous loci to be testis-specific or gland-specific in their expression. Nonparametric Mann-Whitney U-tests (Sokal and Rohlf, 1981) were carried out to test for significant differences between

FIGURE 4.1

Distributions of heterozygosity values for loci coding for 2DE proteins of male reproductive tracts of *Drosophila simulans*, with loci classified according to their distribution of expression between testis and gland fractions of the reproductive tract. Heterozygosities measured within the Afrotropical (Brazzaville) and temperate (S. France) populations are plotted separately, as well as the arithmetic means between the two populations. Note, therefore, that for each interval of heterozygosity values (H) in the histograms, *three* values for "number of loci" are plotted. Empty classes are frequent.


D. simulans
S. France
Brazzaville
Mean
Common
Testis-specific
Gland-specific
Testis-elevated
Number of loci
H
0-.1
.1-.2
.2-.3
.3-.4
.4-.5
.5-.6
.6-.7
.7-.8

the rather widely dispersed distributions of heterozygosities in the four tissue classes; the comparisons made and the results of these are listed in Table 4.6. Of all the comparisons made, the only significant differences between heterozygosity distributions occurred in connection with the data from the Brazzaville (Afrotropical) population. These differences are reflected both in the U_s values for that population alone and in the U_s values obtained when the Brazzaville data was averaged with the South France (temperate) population data. Thus, the polymorphic "tissue-specific" proteins (Class 2 plus Class 3: comparison D) and the gland-specific proteins (Class 3: comparison F) were apparently sampled from a population of loci with a higher-tending distribution of heterozygosities than the population of loci from which the polymorphic proteins expressed in both testes and glands were drawn.

These results suggest that some tissue-specific loci are more variable than broadly-expressed loci and support the hypothesis that secreted proteins are more variable than non-secreted proteins. However, it should be noted that in no case was the testis-specific class, taken alone, significantly more variable than the class which was expressed in both tissues. This suggests that it is the Class 3 (gland-specific) proteins that constitute the subset responsible for most of the upward deviations from the mean level of variation in the "tissue-specific" class, while the

TABLE 4.6

Pairwise tests for different distributions of heterozygosity values at polymorphic loci belonging to different tissue distribution classes in the male reproductive tract of *Drosophila simulans*. U_S = Mann-Whitney U-test statistic.

	U_S			P^a		
Comparison	S. France	Brazzaville	Mean	S. France	Brazzaville	Mean
A. 1/2 + 3 + 4	73.5	85.0	-	n.s.	n.s.	-
B. 2/1 + 3 + 4	110.5	74.5	-	n.s.	n.s.	-
C. 3/1 + 2 + 4	104.5	84.5	-	n.s.	n.s.	-
D. 2 + 3/1 + 4	96.5	126.5	125.5	n.s.	**	*
E. 2/1 + 4	57.5	57.5	-	n.s.	n.s.	-
F. 3/1 + 4	51.5	60.0	68.0	n.s.	**	*
G. 2/1	34.0	35.5	-	n.s.	n.s.	-
H. 3/1	31.5	38.0	-	n.s.	n.s.	-

[a] n.s. - not significant at .05 level (two-tailed test)
* - significant at .05 level or lower
** - significant at .01 level or lower

Class 1 proteins are the source of most of the downward deviations. In addition, the presence of these correlations in the Afrotropical population in D. simulans and their apparent absence in the temperate conspecific population is intriguing and should be further confirmed by more extensive data from other populations.

Similar analyses of the D. melanogaster data were carried out. The results can be found in Tables 4.3, 4.4, 4.7 and 4.8 and in Figure 4.2. The overall frequency of polymorphic loci in this species (P ≅ 10%) was again intermediate between the frequency in Class 1 proteins (P ≅ 7%) and the frequency in Class 3 proteins (P ≅ 23%), as can be seen in Table 4.4. The only comparisons between classes that gave a significant deviation of the Chi-square value from expectation for this parameter, however, were those comparisons involving Class 3 loci alone versus other classes (Table 4.7). The differences between classes with respect to mean numbers of alleles per locus were also much weaker in D. melanogaster than in D. simulans, with only the testis-specific loci showing a possible tendency towards a higher value. Finally, as seen in Figure 4.2 and Table 4.8, there is no statistically significant tendency for any tissue-distribution class of polymorphic loci to be more heterozygous than another in D. melanogaster, despite the apparently higher mean heterozygosity for gland-specific

FIGURE 4.2

Distributions of heterozygosity values for loci coding for 2DE proteins of male reproductive tracts of Drosophila melanogaster, with loci classified according to their distribution of expression between testis and gland fractions of the reproductive tract. Heterozygosities measured within the Afrotropical (Benin) and temperate (France) populations are plotted separately, as well as the arithmetic means between the two populations.

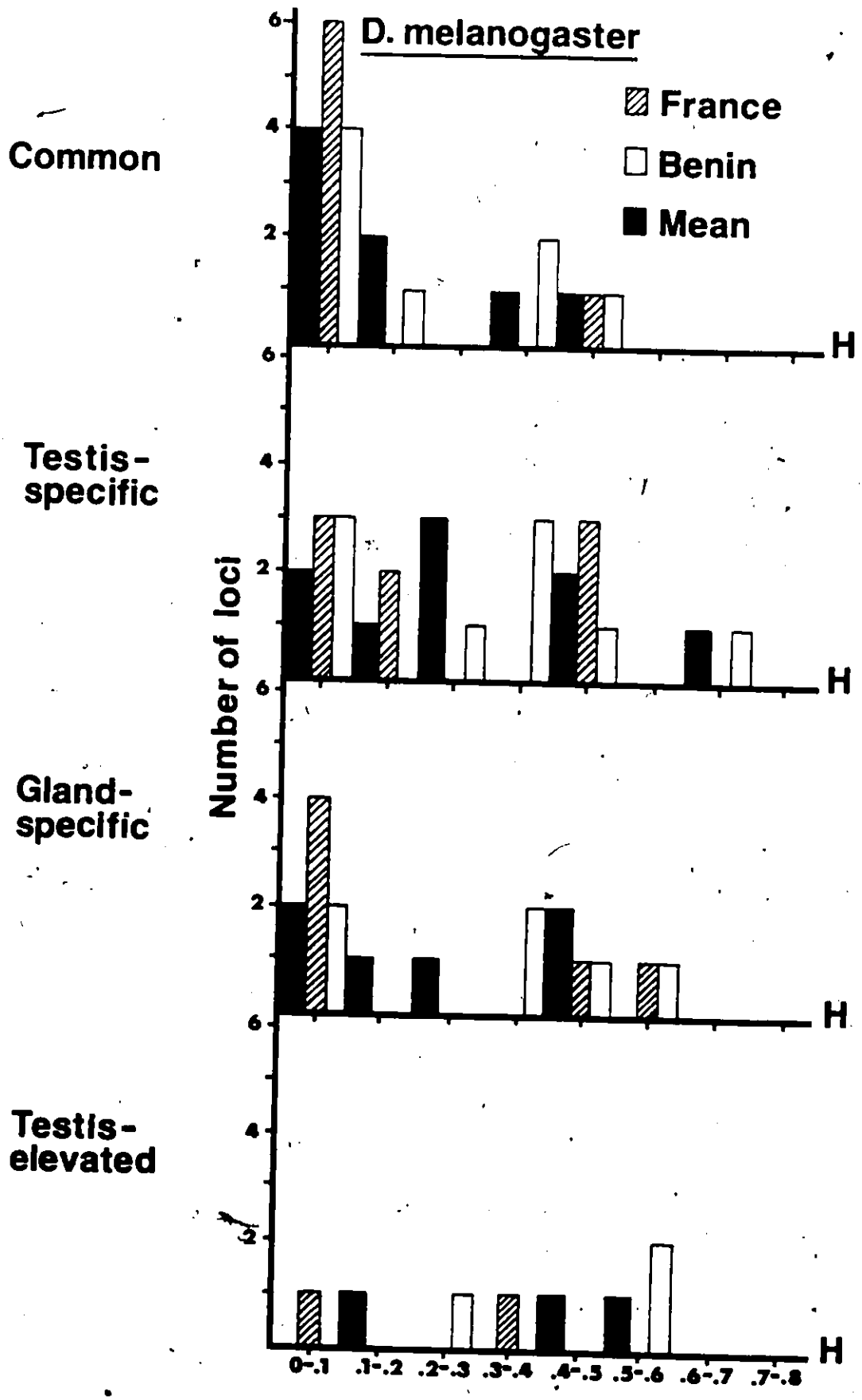

D. melanogaster
France
Benin
Mean
Common
Testis-specific
Gland-specific
Testis-elevated
Number of loci
H
0-.1
.1-.2
.2-.3
.3-.4
.4-.5
.5-.6
.6-.7
.7-.8

TABLE 4.7

Observed and expected numbers of polymorphic loci in each of four tissue distribution classes of proteins in the male reproductive tract of *Drosophila melanogaster*. Chi-square tests of goodness of fit of observed and expected numbers are shown in the bottom half of the table.

Tissue Distribution Class	Number of Polymorphic Loci	
	Observed	Expected[a]
1. Common	8	11.53
2. Testis-specific	9	7.76
3. Gland-specific	6	2.73
4. Testis-elevated	3	3.56

Comparison	Chi-square	d.f.	P[b]
Overall	5.70	3	n.s.
A. 1/2 + 3 + 4	2.61	1	n.s.
B. 2/1 + 3 + 4	0.78	1	n.s.
C. 3/1 + 2 + 4	4.70	1	*
D. 2 + 3/1 + 4	3.48	1	n.s.

[a]Expected values were calculated from proportions of loci in each tissue class in the total sample (see Table 4.2).

[b]n.s. - not significant at .05 level (one-tailed test)
* - significant at .05 level or lower

TABLE 4.8

Pairwise tests for different distribution of heterozygosity values at polymorphic loci belonging to different tissue distribution classes in the male reproductive tract of Drosophila melanogaster. U_s = Mann-Whitney U-test statistic.

	U_s			p^a		
Comparison	**S. France**	**Benin**	**Mean**	**S. France**	**Benin**	**Mean**
A. 1/2 + 3 + 4	79.5	95.5	-	n.s.	n.s.	-
B. 2/1 + 3 + 4	91.5	90.5	-	n.s.	n.s.	-
C. 3/1 + 2 + 4	55.0	67.0	-	n.s.	n.s.	-
D. 2 + 3/1 + 4	87.0	87.0	-	n.s.	n.s.	-
E. 2/1 + 4	52.5	49.5	-	n.s.	n.s.	-
F. 3/1 + 4	37.5	37.5	-	n.s.	n.s.	-
G. 2/1	42.5	38.0	-	n.s.	n.s.	-
H. 3/1	28.0	32.5	-	n.s.	n.s.	-

[a] n.s. - not significant at .05 level (two-tailed test)

polymorphic loci in the Afrotropical (Benin) population (Table 4.3).

In summary then, it appears that some support is lent to both of the hypotheses put forward in the Introduction to this chapter, by the genic variation data in D. simulans and D. melanogaster. In D. simulans, proteins co-expressed in the testis and the gland fractions of the male reproductive tract are less likely to be polymorphic, and when they are polymorphic they are more likely to be diallelic (versus triallelic or tetrallelic), than are the polymorphic loci with a more differentiated pattern of expression between testes and glands. Also, proteins expressed in the glandular tissues and not detectable in the testes show the opposite tendency. That is, they are more likely to be polymorphic, and when they are polymorphic they have a tendency to be segregating for more alleles and to be more heterozygous than loci with testis-specific or undifferentiated patterns of expression. However, the correlation of tissue distribution with heterozygosity of polymorphic loci, when tested in D. simulans for statistical significance in a temperate and a tropical population separately, could only be detected in the latter population. These correlations were weaker or absent in the material of D. melanogaster which was analyzed. The only statistically significant pattern observed in this species was a higher likelihood for gland-specific loci to be polymorphic than for loci with other tissue distributions.

DISCUSSION

Study of correlations between variability of genes and structure or function of the genes or their products is an attempt to demonstrate and to understand the relevance of the genetic variation in populations to natural selection and adaptation. The promise of such an approach was that it might yield conclusions without the necessity of being in possession of detailed information on the ecology or demographic history of the populations in which the variation was found segregating. However, formidable conceptual and practical obstacles have so far prevented achievement of this objective. The correlation analyses and discussion in this chapter are intended as contributions to the clarification and eventual remediation of some of these problems.

The most prominent correlation found in the present analysis was that between apparent functioning of proteins as components of extracellular fluids, and comparatively high values for certain measures of genic variability, i.e. percentage polymorphism among loci, heterozygosity per locus, and number of alleles per locus. The effect was most noticeable in D. simulans, especially in an Afrotropical (Congo) population, but was also statistically significant in D. melanogaster, with the Afrotropical (Benin) population of this species also being the one most strongly affected. As

well as its confirming a pattern suggested by 1DE for Drosophila larval hemolymph proteins (Singh and Coulthart, 1982), this result agrees with similar 1DE data on extracellular proteins (those of blood serum) from primate species in comparison to erythrocyte enzymes from the same species (King and Wilson, 1975; Palmour et al., 1980). 2DE estimates of variation in human plasma polypeptides are also considerably higher than those for polypeptides of erythrocyte lysate (see Table 3.1), and Juneja et al. (1981) report greater variation in 2DE analyses of plasma proteins of the domestic dog than in 1DE of erythrocyte enzymes in this species.

The main significance of the present finding is its demonstration of one use of 2DE in the study of structure/function correlations. In some cases, especially when the structural or functional data of interest can be obtained directly from the gels, as was done here, the technique will be helpful in the rapid identification and genetic analysis of large, independent samples of structural genes chosen in order to test the basis for a supposed correlation. Although more data and different types of experiments will be required to establish the reasons why extracellular proteins appear to be more polymorphic, we are now at least able to place more confidence in the non-spurious nature of the correlation.

With regard to the other type of correlation found - that between breadth of tissue distribution of a protein and its level of variation - the results were more tentative. Despite the fact that in both species the largest deviations from expected frequencies of polymorphic loci besides those found for gland-specific proteins were those seen with testis/gland-common proteins (Tables 4.5, 4.7), the differences from expectation were not statistically significant in D. melanogaster. The lack of significance may partly be a consequence of the fact that distribution between only two organs (accessory glands and testes) is a narrow basis on which to assess general breadth of tissue distribution, so that a real correlation may have been partly obscured by inadequate characterization of tissue distribution. Also, although truly testis-specific proteins are found in Drosophila (Kemphues et al., 1979; Cavener, 1980), Ingman-Baker and Candido (1980) note that all of the sperm polypeptides they studied were also synthesized by other (somatic) cell types. This suggests that many of the "testis-specific" abundant proteins identified in the present study, if they are actually components of sperm, are not truly specific to the testes. This, too, would tend to decrease the relevance of the tissue distribution breadth classification used here. 2DE experiments run on isolated sperm, and on other somatic tissues, should help to clarify this correlation, if it does exist.

It is appropriate to ask, at this point, exactly what pattern of correlation might be expected to exist between genetic variation and breadth of tissue distribution of a gene product. As mentioned in Chapter 3, an argument can be made that broad tissue distribution of a protein should be associated with a high level of functional constraint on its amino acid sequence, and thus a low level of variation. But one important type of balancing selection involves heterosis which is mediated by a putatively greater average physiological and developmental homeostasis, over a tandem series of life cycle stages, enjoyed by functionally intermediate heterozygotes (Gillespie and Langley, 1974; Mitton and Grant, 1984). This mechanism predicts higher levels of selectively balanced polymorphism in broadly distributed proteins. Negative results in correlation analyses are usually quite difficult to interpret. On the other hand, positive results on a neutralist "functional constraint" hypothesis can usually be explained equally well on the basis of opportunity for balancing selection, and independent indices of the tendency to undergo balancing selection are usually difficult to define and/or to apply. Therefore, a single operationally applicable index (i.e. breadth of tissue distribution) that makes a prediction of positive results under both neutrality and selection, but indicates opposite directions for the correlations, may have an advantage of interpretability over other

structure/function indices.

Regardless of whether the structure/function correlation approach is ultimately successful in discriminating between selective and neutral mechanisms for maintenance of genetic variation in populations, the approach should prove to be of great value on a purely descriptive basis. For instance, certain major classes of gene products, such as enzymes of mammalian brain and erythrocytes (Wade Cohen et al., 1973), proteins of Drosophila ribosomes (Berger and Weber, 1974), and proteins of mouse brain and liver membrane (Klose and Feller, 1981), appear to contain much less genic variation, on average, than do the general samples of structural loci which have been used to characterize overall levels of genic variation for the respective species' genomes. At the other end of the spectrum are genes like the K and D loci of the mouse H-2 histocompatibility complex, which have a minimum of 200 alleles each (Klein, 1978). Such findings are very helpful in demonstrating the range of genic variability levels which can be supported by populations. They may also, in combination with estimates of the relative sizes of the different gene classes represented, eventually provide a basis for more rational extrapolation from the results of variation studies on small samples of loci to predictions about variation in the genome.

INTRODUCTION

The melanogaster Species Subgroup. Drosophila simulans and Drosophila melanogaster are placed taxonomically, within the genus Drosophila, in a subgroup of eight sibling species (the melanogaster subgroup), which in turn belongs to a set of eleven subgroups that comprise a total of 141 known species and together make up the melanogaster species group (Lemeunier et al., 1984). All of the melanogaster subgroup species are endemic to Afrotropical regions, except D. simulans and D. melanogaster, which are cosmopolitan.

The term "sibling species" was introduced by Mayr (1942) to describe "morphologically similar or identical populations that are reproductively isolated". They are very common in the genus Drosophila (Patterson and Stone, 1952), where they are both a challenge to the taxonomist and a rich source of material for the geneticist with an interest in the early stages of species formation and divergence. The eight sibling species of the melanogaster subgroup are almost identical with respect to external morphology: the only reliable taxonomic character for species identification is the shape of the male genital process, located on the ninth abdominal tergite. However, many comparative studies of, for

instance, polytene chromosome banding patterns (Lemeunier and Ashburner, 1976, 1984), electrophoretic mobilities both of enzymes separated by 1DE (Eisses, van Dijk and van Delden, 1979; Gonzalez et al., 1982) and of proteins of whole-body homogenates separated by 2DE (Ohnishi et al., 1983), sequence similarity in repetitive (Barnes, Webb and Dover, 1978) and single-copy (Bodmer and Ashburner, 1984) DNA, and behavioural traits (Bos and Boerema, 1981), have helped to clarify patterns of relationship between the species of the subgroup. The results of these studies agree quite closely in dividing the subgroup into two sets of four species each - D. simulans, D. melanogaster, D. mauritiana and D. sechellia on one hand, and D. teissieri, D. yakuba, D. orena and D. erecta on the other. The former set of four is referred to informally as the melanogaster species complex, and constitutes the material investigated in the present comparative study of proteins of the Drosophila male reproductive tract.

Within this species complex, the above-cited comparative studies are unanimous in placing D. mauritiana and D. sechellia, which are endemic, respectively, to the island of Mauritius and to the Seychelles Islands (both in the Indian Ocean), much closer to D. simulans than to D. melanogaster. Most clearly revealing among the strictly comparative data is the fact that D. simulans, D. mauritiana and D. sechellia are mutually homosequential with respect to

polytene chromosome banding pattern, whereas D. melanogaster is distinguishable from these three species by one major and several minor paracentric inversions (Lemeunier and Ashburner, 1976; 1984). It is not unreasonable to suggest that D. mauritiana and D. sechellia originated in connection with island colonization events by D. simulans, although it is difficult to disciminate conclusively against competing phylogenetic hypotheses at present (Lemeunier and Ashburner, 1984).

Male Reproductive Function in Drosophila Speciation. The conclusions about species relationships reached by comparative studies within the melanogaster species complex are supported by sexual hybridization studies. Of particular relevance to the current study are viability and fertility effects seen in F_1 hybrids between the species (David et al., 1974; Roberts, 1985). Male and female hybrids between D. simulans and D. melanogaster are both completely sterile. When the cross is made with D. melanogaster as female parent, only female adult progeny are produced; in the opposite direction of crossing, mostly or exclusively male progeny survive. In contrast, hybrids of both sexes between D. simulans and either D. mauritiana or D. sechellia are viable, and females are highly fertile, although the males are sterile and lack motile sperm. The hybrid females in these latter two cases can be backcrossed to males of either

parental species, whereupon some male fertility is regained in the B_1 progeny (David et al., 1974; Coyne, 1984).

Aside from the confirmation of species relationships offered by patterns of hybrid male sterility, such patterns illustrate an interesting aspect of species divergence in the genus Drosophila. This is that, very frequently, F_1 hybrids between species in this genus are completely sterile, although viable. Moreover, as summarized in a recent review by Bock (1985), in 141 of 142 cases where both hybrid sexes are viable but one is sterile, it is the male which is affected. Thus in many cases, as with D. simulans crossed to D. mauritiana or D. sechellia, the only gross abnormality seen in the F_1 is male sterility. This, of course, does not argue conclusively in favour of a causal role for hybrid male sterility in the initiation or even the consolidation of species formation in Drosophila. But it suggests strongly that the phenomenon is very often one of the earliest correlates of cladogenesis within the genus. Deeper understanding of genetic differences between Drosophila species with respect to male reproductive function should, therefore, at least help to understand some of the early events in species divergence, and may prove to be directly useful in understanding the process of establishment of the species per se.

Molecular Comparisons Between Drosophila Sibling Species.

Early molecular studies of genetic differences between

Drosophila sibling or incipient species relied heavily on 1DE analysis of electrophoretic mobility differences in enzymes and other soluble proteins. The functions of these enzymes and proteins were either completely unknown or bore no known relationship to male reproductive function or, for that matter, to any other portion of the phenotype of probable or possible importance in the process of species formation (Hubby and Throckmorton, 1967; Prakash, 1969, 1972; Ayala and Powell, 1972; Kojima, Gillespie and Tobari, 1970; Ayala et al., 1974). Furthermore, because of practical limitations, the samples of loci were small in relation to the total size of the genome: even the extensive analysis of the Drosophila willistoni species complex carried out by Ayala and co-workers (Ayala et al., 1974) included a total of only 36 loci. Also, electrophoretically cryptic variation usually was not taken into account. Thus, several questions about the adequacy of electrophoretic data on proteins, exactly analogous to those addressed in Chapter 3 in the context of population variation, also arise in molecular studies of species divergence.

One of these problems has been examined experimentally. Sequential electrophoresis and other supplementary techniques designed to uncover allelic variation not detected under standard 1DE conditions have been applied to comparisons of 6 loci between the sibling species Drosophila pseudoobscura and D. persimilis

(summarized by Coyne, 1982). They have also been applied to comparisons of 16 loci between populations of D. pseudoobscura from the main body of this species' range in North and Central America and from an isolated population in Bogota, Colombia that is considered to be in the first stages of species differentiation from D. pseudoobscura (Coyne, 1982; Singh, 1983). At 1 of the 6 loci in the former comparison and at 7 of the 16 loci in the latter comparison, substantial numbers of new species-specific alleles were discovered within electrophoretic classes previously thought to be equivalent between species. The data of Singh (1983) include 3 cases of loci that previously appeared nearly identical in their allele distributions between main-body and Bogota populations of D. pseudoobscura, but on closer examination proved to have essentially disjunct allele distributions. The disclosure of such hidden divergence suggests that previous estimates of the extent of genic differentiation, at least of some loci, between incipient or sibling species of Drosophila may be too conservative even with respect to the loci which have been studied by IDE.

In contrast with the substantial body of information on electrophoretically cryptic variation as it relates to IDE enzyme comparisons between closely related Drosophila species, there is a notable lack of experimental perspective on the other aspects of data adequacy. Moreover, the problem of representativeness may in some ways be more acute in the

study of species formation than it is in the analysis of population structure. In the latter situation, the presupposition that a "sample" of loci taken from the genome is relevant to the question is perhaps more readily justified - all the more so if large amounts of variation are actually found in the limited sample. But in attempting to understand the genetics of species formation, the key questions will ultimately be more closely concerned with exactly which genes were involved in the formation of particular species.

Mayr originally formulated his "genetic revolution" model of species formation to entail allelic substitutions at most of the loci in the genome, and workers who have set out to test Mayr's theory have taken this extreme version as the one to be tested (Hubby and Throckmorton, 1967; Lewontin, 1974, Chapter 4). Mayr's extreme formulation may have been part of an attempt to lend generality to his model, by integrating the origin of reproductive isolation, via pleiotropic effects, with general processes of adaptive change in populations. But there exists at present no theoretical basis for estimation of the magnitude of observed genetic change that would be required to confirm or reject Mayr's theory, or any variant of it that involves "reorganization" of the gene pool during species formation. It seems entirely possible that, although genetic revolutions do take place, they involve only a relatively small number of loci that are "tightly" coadapted to each other, leaving most

of the rest of the genome unchanged. We thus require data on species differentiation in larger samples of loci.

The need for larger samples of loci in molecular species comparisons is paralleled by a need for appropriate samples of loci. Again: when population structure is under study, there is usually little a priori reason to consider one set of loci as relevant to adaptation and another set as irrelevant. But when comparing two species, we are really examining the results of two sets of processes: those that led to the initial establishment of the species as two reproductively independent population systems, and those that were involved either concurrently or subsequently in divergence between the species, but occurred independently of the establishment of reproductive isolation. So if our aim is to come to grips with the genetic events underlying species formation, we must attempt to exclude as many irrelevant loci as possible from the analysis, and/or to include as many potentially relevant ones as possible. To do this, information on the mechanisms of reproductive isolation presently operative between the extant species must be available, as well as some means of precise (i.e. molecular) genetic analysis of the isolation mechanism.

Furthermore, we must not be limited, in trying to analyze the genetic differences that underlie species differentiation, to any restricted subset of the different kinds of alterations in DNA that might have a critical

phenotypic effect in the species and/or their hybrids. Changes in the DNA encoding the amino acid sequences of proteins are just such a restricted subset: it has been established for some time that DNA changes outside of such coding regions can have large quantitative effects on the expression of nearby structural genes in eukaryotes (Chovnick et al., 1976). Ultimately, the analysis of genetic elements that do not encode a protein product must be extended even further than this, to include DNA sequences that can exert phenotypic effects without having direct effects on protein expression, for example through their effects on an RNA product, or even in the absence of any encoded product.

Finally, in analogy with the interpretive difficulties encountered immediately following the discovery by LDE of extensive genic variation in populations, it will not be enough simply to describe the amount and pattern of genetic divergence between species, however "large" or "appropriate" the sample of loci. We will eventually be obliged to demonstrate the connections between the patterns observed at the genetic level and particular phenomena at the phenotypic level; in this case, reproductive isolation.

Two-Dimensional Gel Electrophoresis and Genetic Divergence Between Drosophila Species. As demonstrated in Chapter 3, one of the clearest advantages offered by 2DE is the much larger number of structural genes accessible to analysis, in

comparison to 1DE of enzymes and other soluble proteins. This advantage has been applied, in the present study, to carry out expanded molecular comparisons between the 4 sibling species of the melanogaster complex, using proteins of the male reproductive tract. The direct examination of male reproductive tract tissues has been made possible by virtue of a second technical advantage of the methodology used - i.e. sensitivity of the protein zone detection technique. In comparisons between D. melanogaster and D. simulans, and between D. simulans and the 2 island endemics which are closely related to it (D. mauritiana and D. sechellia), 2DE data on genic variation in these proteins within one or both of the compared species is also available. This permits an assessment of the pattern of interspecies divergence in the context of existing intraspecies polymorphism. Yet a third technical advantage available with the present techniques is that silver stains, which interact with the polypeptides themselves in zone visualization, give a more easily interpreted picture of relative amounts of a polypeptide in two different samples than do the histochemical activity stains generally used in 1DE. Thus, certain types of alterations in gene expression should be at least preliminarily detectable in silver-stained gels, even though the ascription of particular cases of changes in protein quantity to specific changes in gene expression will always require further work.

The most radical difference between the data presented in this chapter and that from previous molecular comparisons of sibling species of Drosophila is derived from the choice of material (male reproductive tract) used in sample preparation. As noted above, genetic changes that affect reproductive function in hybrid males are a very common early feature of species divergence in Drosophila, and by their very nature may contribute strongly to the initial establishment of reproductive isolation between populations. This is not to say that the electrophoretic analysis of male reproductive tract proteins in these species is necessarily capable of revealing the crucial genetic differences. The analysis has limited scope and sensitivity, and the sibling species of the melanogaster complex seem to have been diverging for millions of years already (Easteal and Oakeshott, 1985), making them less-than-ideal material for study of the earliest stages of species differentiation. But the approach taken here is representative of the kind of high-resolution analysis of carefully chosen sets of genes that will be necessary in order to eventually understand the genetic changes underlying species formation in Drosophila.

RESULTS

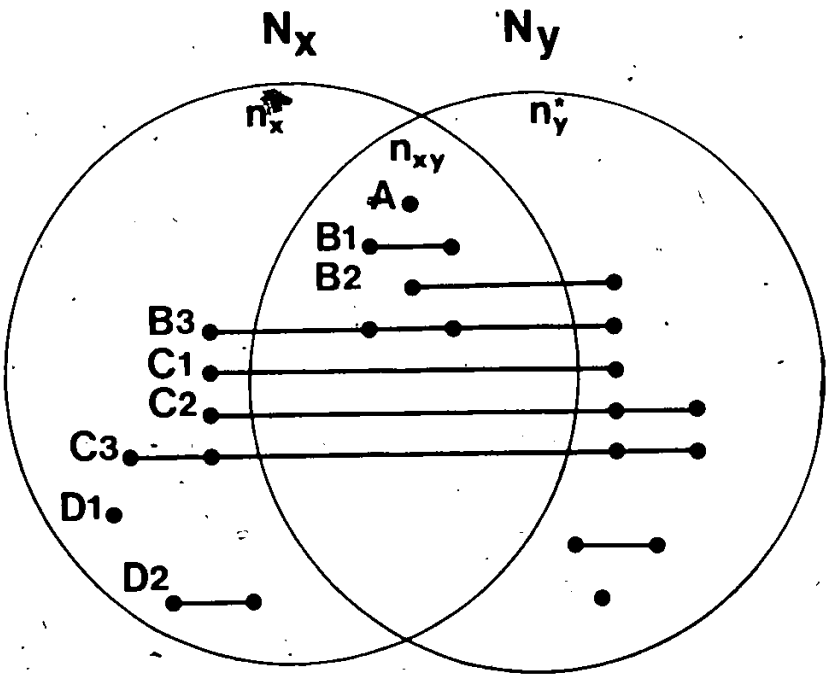
Approaches to Pairwise Comparison of Different Species by 2DE. Figure 5.1 is a diagrammatic representation of the different empirical results possible when the polypeptides of homologous tissue samples from two different species are separated by 2DE and the spot patterns compared. Class A consists of invariant, shared polypeptides. Class B contains polymorphic polypeptides, with subclasses B1-B3 to take account of different degrees of polymorphism (i.e. one or both species) and either partial or complete overlap in allele distribution. Each of these subclasses actually includes a wide range of possibilities for total numbers of alleles involved, and Figure 5.1 only contains three simple examples to represent the qualitative distinctions between subclasses. Class C includes all identifiably homologous pairs of polypeptides with no allelic overlap between species, and is also divided into three subclasses. Class D accommodates those polypeptides of each species that have no recognizable Homolog in the other species. Cases of Class D differences might be attributable to any of several distinct types of evolutionary change. Large shifts in electrophoretic mobility (especially in the first dimension) could lead to exclusion of the homologous polypeptide of one of the two species from the resolution "window" of pI and M_r used in the experiment. Or, large differences in polypeptide expression might render the polypeptide undetectable or

FIGURE 5.1

Venn-diagrammatic representation of possible divergence relationships between proteins resolved by 2DE from homologous tissues of two different species. Classes A-D are explained in the text and summarized in Table 5.1. N_x and N_y are the total protein sets in species x and y, for which appropriate data on polymorphism and homology are available. n_{xy} is the total number of spots shared between the two species. n_x^* and n_y^* are the total numbers of unshared spots in species x and y, respectively.

TABLE 5.1

Summary of criteria for definition of protein divergence classes A-D, with subclasses.



Divergence Class	Allele Overlap	Species Polymorphic	Homolog Identified
A	Complete	Neither	Yes
B1	Complete	Both	Yes
B2	Partial	One	Yes
B3	Partial	Both	Yes
C1	None	Neither	Yes
C2	None	One	Yes
C3	None	Both	Yes
D1	?	No	No
D2	?	Yes	No

unrecognizable in one of the two species. Alterations in chemical composition could also potentially change the silver staining properties of a polypeptide so radically that it would not be recognized as homologous. Finally, it is conceivable that the gene encoding a polypeptide has been lost, silenced or even acquired (for example, by gene duplication) in one of the two species, so that a Class D spot could represent a species-specific genetic function.

To undertake a spot classification of this type, data on within-species polymorphism and a basis for discerning interspecies spot homologies are needed. Since the visual appearances of the spots on these gels were highly diverse and yet reproducible within a species, this appearance could be used to pair many spots that migrated to dissimilar gel positions in two different species. Here, essentially the same criteria were used as were applied to the scoring of allelic variation within species (see Results, Chapter 3). In addition, tissue distribution information (see Results, Chapter 4) was very useful: 17 electrophoretically divergent spots in D. simulans and 14 in D. melanogaster which could not be confidently paired between these two species on the basis of appearance alone were pairable when tissue distribution was taken into account. It was interesting that in only one case among the electrophoretically divergent spots was the putative homology based on spot appearance not in agreement with that based on tissue distribution. This

attests to the general veracity of spot appearance alone as a tool for recognition of homology, at least between closely related species.

It is clear that in any species comparison where comprehensive spot classification is attempted, many spots that appear reproducibly on the gels will be excluded from the analysis because of lack of data on polymorphism and/or homology. The sets N_x and N_y are, therefore, necessarily subsets of the total number of spots appearing on the gels. For excluded spots with identifiable homologs (Classes A-C), this restriction of the working sets of polypeptides presents no obvious problem of bias. For Class D spots, the attempt to find a homolog was not restricted to the N_x or N_y subset, but included all spots from the other species; thus, the absence of an identifiable homolog is not a simple artifact of the restricted membership of $N_x + N_y$.

When reliable data on polymorphism or tissue distribution are lacking for some or all of the polypeptides in one or both species, useful comparative information can still be obtained from these polypeptides even though discrimination between certain of the spot classes of Figure 5.1 is impossible. This consideration arose, for instance, in comparisons involving D. mauritiana and D. sechellia; for these two species only single isofemale lines were available. Also, in all four species many minor spots not suitable for the rigorous analysis of polymorphism or tissue distribution

could at least be assigned a shared versus unshared or pairable versus non-pairable status between a single pair of lines representing two species. Data from comparisons such as these were conveniently summarized in terms of a similarity measure suggested by Aquadro and Avise (1981): $F = 2n_{xy}/N_x + N_y$, where n_{xy}, N_x and N_y are as defined previously (see Figure 5.1), and in terms of another statistic, $F_p = a/n_x^* + n_y^*$, where n_x^* and n_y^* are as defined in Figure 5.1, and a is the number of spots not shared but capable of being paired homologously between two gels. F may be referred to as the "shared fraction" (of the total number of spots scored), and F_p as the "pairable fraction" (of the total number of unshared spots). Note that 1-F and $1-F_p$ can both be calculated and used as measures of interspecies divergence of different types. These statistics are most useful when a single measure of interspecies similarity or divergence is required, as it is when degrees of divergence in different sets of polypeptides are compared, or when phylogenetic relationships are being assessed.

When incomplete data are used in comparisons, a definable range of possibilities exists for alternative classifications of a spot or spot pair, were additional data on polymorphism or homology available. For instance, in comparisons between D. simulans and D. mauritiana, much polymorphism information was available for the former species but not for the latter. So if a spot which is known to be

polymorphic in D. simulans has a homolog in D. mauritiana with an electrophoretic mobility not found for any D. simulans allele, we can provisionally label the spot as a member of Class C2', to indicate its tentative status as a case of allelic non-overlap between homologous proteins of the two species. More information might lead to its being reclassified as Class C3, or even as Class B, but never as Class A and almost never as Class D. The same type of consideration can be applied to the "F" indices defined above. When only one line in each of two species is sampled, the membership of n_{xy} (Class A') is made up of spots which would only be reclassified as A or B if more information were available. Similarly, the pairable members of n_x^* plus n_y^* (together: Class C') could only be from Class B or Class C. In general, the values of n_{xy} and $(n_x^* + n_y^*)$ obtained in single-line comparisons for any two species will vary reciprocally over a range equal to the number of Class B loci, with the exact shape of the sampling distribution being strongly dependent on the allele frequency distributions at the Class B loci in the two species.

Interspecies Divergence Classification of Total Reproductive Tract Proteins. The classification of male reproductive tract proteins in a comparison between D. simulans and D. melanogaster is summarized in Table 5.2. Of the total maximum numbers of spots found suitable for scoring allelic

TABLE 5.2

Numbers and proportions of male reproductive tract proteins of *Drosophila simulans* and *D. melanogaster* falling into each divergence class as defined in Figure 5.1 and Table 5.1.

Divergence Class	*D. simulans*		*D. melanogaster*	
	Number	Proportion	Number	Proportion
A	114	.530	114	.528
B1	0	0	0	0
2	16	.074	16	.074
3	6	.028	6	.028
C1	23	.107	23	.106
2	2	.009	2	.009
3	2	.009	2	.009
D1	48	.223	47	.218
2	8	.037	10	.046
Total	215		216	

polymorphism within D. simulans (295) and D. melanogaster (307), 67 in D. simulans and 79 in D. melanogaster were paired homologously with minor spots not included within the scorable spot sets of the other species. In addition, 11 spots in D. simulans and 2 more in D. melanogaster were of the "nonscorably polymorphic" type (see Results, Chapter 3), even though pairable between species. Since exact placement of such spot pairs in one of Classes B or C was difficult, they were excluded from this phase of the analysis, leaving $N_{simulans} = 215$ and $N_{melanogaster} = 216$.

The largest class in each species (Table 5.2) was Class A, comprising about 53% of D. simulans or D. melanogaster spots. The next largest class was Class D, with 26% representation in D. simulans and 26.4% in D. melanogaster. Some of these Class D spots will probably be reclassified eventually as Class A, B or C when more information becomes available on their homology relationships. However, as mentioned above, the use of spot appearance and tissue distribution together still failed to help identify homologs for these Class D spots. Moreover, pH 4-6 IEF/SDS-PAGE and pH 3.5-10 NEPHGE/SDS-PAGE separations were carried out (gels not shown). These failed to reveal any cases of simple charge-shift differences between D. simulans and D. melanogaster that might have led to exclusion of one species' spot from the pH range, 5.25-6.95. A third

point is that many of the Class D spots of both species were still plainly visible and identifiable by appearance in gels run with 10-fold dilutions of the 10-tract protein loads routinely electrophoresed. Such considerations suggest that most of the Class D species-specific spots are the results of large (≥ 10-fold) quantitative differences, and perhaps even qualitative differences between D. simulans and D. melanogaster in the expression of individual polypeptides in male reproductive tracts.

The next largest class of proteins (10.6%-10.7%) was Class C1, representing pairable spots apparently monomorphic for different alleles in the two species. This figure is very close to the analogous figure for enzymes which have been analyzed by IDE in both of these species (R. S. Singh and M. S. Choudhary, unpublished data): of 70 enzyme loci examined, 7 (10%) are apparently fixed for alternate alleles in D. simulans and D. melanogaster.

It is striking that Classes C and D together make up 38.6% of the total number of D. simulans spots included in the Table 5.2 total; the corresponding figure for D. melanogaster is 38.9%. Finally, about 10% of polypeptides in this comparison fell into Class B, with Class B2 being about twice as frequent as Class B3. No case of completely overlapping polymorphism (Class B1) was identified.

In comparing D. simulans to D. mauritiana or D. sechellia, a slightly different classification scheme was

required, as explained above. In Table 5.3, Class A' is defined as the set of spots, monomorphic in D. simulans, for which the corresponding spot in the single D. mauritiana line analyzed had an identical electrophoretic mobility. Class B' spots were those that were polymorphic in D. simulans and for which the D. mauritiana form was the same as one of those found segregating in D. simulans. In Class C1' are included spots monomorphic in D. simulans, for which the D. mauritiana homolog had a mobility different from that of D. simulans. Class C2' contains cases of non-overlap where the D. simulans spot is polymorphic. Class D spots could be treated in the same way as they are when polymorphism data is available for both species, except of course that no distinction can be made between D1 and D2 loci in D. mauritiana. As with the D. simulans/D. melanogaster comparison, homologous pairing between species took information on both spot appearance and tissue distribution into account. Nonscorably polymorphic D. simulans spots not belonging to Class D (of which there were 10 in this comparison) were again excluded from the analysis. The total sets for each species are here somewhat larger than they were in the D. simulans/D. melanogaster comparison. This is because the 67 D. simulans spots that were excluded from the latter analysis owing to lack of data for the D. melanogaster homolog were included in the D. simulans/D. mauritiana comparison.

TABLE 5.3

Numbers and proportions of total male reproductive tract proteins of D. simulans and D. mauritiana falling into different divergence classes. **Class A'**: monomorphic in D. simulans, same allele in D. mauritiana. **Class B'**: polymorphic in D. simulans, D. mauritiana allele found within D. simulans. **Class C1'**: monomorphic in D. simulans, different allele in D. mauritiana. **Class C2'**: polymorphic in D. simulans, D. mauritiana allele not found in D. simulans **Class D**: As per Figure 5.1, Table 5.1.

Divergence Class	D. simulans		D. mauritiana	
	Number	Proportion	Number	Proportion
A'	235	.789	235	.800
B'	23	.077	23	.078
C1'	16	.054	16	.054
C2'	4	.013	4	.014
D	20	.067	16	.054
Total	298		294	

TABLE 5.4

As per Table 5.3, for D. simulans and D. sechellia.

Divergence Class	D. simulans		D. sechellia	
	Number	Proportion	Number	Proportion
A'	222	.742	222	.831
B'	19	.064	19	.071
C1'	6	.020	6	.022
C2'	4	.013	4	.015
D	48	.161	16	.060
Total	299		267	

As with D. simulans and D. melanogaster, the most common finding was nondivergence between the polypeptides of D. simulans and D. mauritiana, with about 80% of spots falling into Class A'. The next most frequent classes were D and B' (6.7% and 7.7% respectively) in D. simulans, and B' (7.8%) in D. mauritiana. Slightly less frequent were Classes D and C1' in D. mauritiana, and C1' in D. simulans, with 5.4%. Together, Classes C' and D accounted for 13.4% of D. simulans spots and 12.2% of D. mauritiana spots.

A similar analysis was carried out for D. simulans and D. sechellia (Table 5.4), with similar results. However, a conspicuous difference is apparent between the contents of Table 5.4 and those of Table 5.3: D. simulans Class D spots number 48 when this species is compared to D. sechellia, whereas only 20 D. simulans spots were classified as this type when compared to D. mauritiana. This was in spite of the fact that the other divergent classes of polypeptides (C1', C2' and D) did not occur in greater numbers in D. sechellia than in D. mauritiana.

If this difference between the results of D. simulans/D. sechellia and D. simulans/D. mauritiana comparisons is a result of greater structural divergence in D. sechellia polypeptides from those of D. simulans, leading to greater difficulty in the identification of homologous interspecific spot pairs, then some of the 48 D. simulans spots placed in Class D with respect to D. sechellia may

eventually be reclassified, for instance as Class C. But if this is the case the relative degree of divergence should not be lessened; rather, it would simply be seen as structural rather than quantitative.

A more seriously misleading interpretive ambiguity could possibly arise in connection with the fact that the total protein loads applied to gels, in samples containing 10 reproductive tracts from these three species, were not exactly equivalent; the differences were in the order: D. simulans > D. mauritiana > D. sechellia. This overall intensity reduction could conceivably "generate" more Class D spots in D. simulans when compared to D. sechellia than when compared to D. mauritiana, because of their having fallen below a threshold of identifiability or even detectability in D. sechellia.

That this is not the case is argued by the observation that, on examination of 39 individual D. simulans spots that were Class D with respect to D. sechellia but not with respect to D. mauritiana, at least 28 of these (including 10 landmark spots) clearly contained too much protein for the simple reduction in overall protein loading to account for detectability in D. mauritiana but not in D. sechellia. This was especially clear since many Class A' spots, common to all three species and of approximately the same intensity as individual D. simulans Class D spots, were available to coarsely "calibrate" the effects of the overall

reductions in loading. Experiments in which protein loads were carefully standardized or systematically varied would answer this question more rigorously. But in the interim it can be tentatively concluded that reduction in level or abolition of expression may characterize the evolutionary divergence of many more of the individual male reproductive tract polypeptides of D. sechellia than of D. mauritiana, when each of these two species is compared to D. simulans. In fact, the number of Class D spots in D. simulans is about the same whether it is compared to D. sechellia or to D. melanogaster (Tables 5.2, 5.4) despite the fact that several other types of genetic divergence between D. simulans and D. melanogaster have progressed to a significantly greater extent than they have between D. simulans and D. sechellia.

Interspecies Divergence in Different Sets of Polypeptides. Considering those polypeptides for which distribution between male reproductive tract tissues is known (see Chapter 4), it is possible to cross-classify individual polypeptides of a species with respect to divergence class (in relation to a specified second species) and tissue distribution class. Tables 5.5, 5.7, 5.9 and 5.11 contain the results of such cross-classification of D. simulans and D. melanogaster polypeptides, on the basis of mutual comparisons between these two species and of comparisons between D. simulans and either D. mauritiana or D. sechellia.

TABLE 5.5

Observed and expected numbers of *D. simulans* male reproductive tract polypeptides, arranged in a two-way classification by: (i) observed tissue distribution in *D. simulans* (see Chapter 4, Results), and (ii) observed divergence class with respect to *D. melanogaster*.

Tissue Distribution Class	Observed/ Expected	Divergence Class A	B	C	D	Total
1. Common	Obs.	71	8	10	15	104
	Exp.	55.24	10.82	13.31	24.65	
2. Testis-specific	Obs.	28	10	11	16	65
	Exp.	34.51	6.76	8.32	15.40	
3. Gland-specific	Obs.	2	3	6	12	23
	Exp.	12.21	2.39	2.94	5.45	
4. Testis-elevated	Obs.	11	1	0	7	19
	Exp.	10.08	1.97	2.43	4.50	
Total		112	22	27	50	211

TABLE 5.6

Results of Chi-square tests of independence of tissue distribution and divergence, and of goodness of fit between proportions within single rows and columns and total proportions, for the data in Table 5.5.

Comparison	Chi-square	d.f.	p[a]
Overall	37.62	9	***
Rows within Column:			
A	14.35	3	***
B	2.92	3	n.s.
C	7.29	3	n.s.
D	13.06	3	**
Columns within Row:			
1	9.83	3	*
2	3.66	3	n.s.
3	19.75	3	***
4	4.38	3	n.s.

[a] n.s. - not significant at .05 level (one-tailed test)
* - significant at .05 level or lower
** - significant at .01 level or lower
*** - significant at .005 level or lower

TABLE 5.7

Observed and expected numbers of *D. melanogaster* male reproductive tract polypeptides, arranged in a two-way classification by: (i) observed tissue distribution in *D. melanogaster* (see Chapter 4), and (ii) observed divergence class with respect to *D. simulans*.

Tissue Distribution Class	Observed/ Expected	Divergence Class A	B	C	D	Total
1. Common	Obs.	60	7	10	22	99
	Exp.	52.47	10.89	13.31	22.28	
2. Testis-specific	Obs.	22	8	9	11	50
	Exp.	26.50	5.50	6.75	11.25	
3. Gland-specific	Obs.	7	4	6	6	23
	Exp.	12.19	2.53	3.11	5.18	
4. Testis-elevated	Obs.	17	3	2	6	28
	Exp.	14.84	3.08	3.78	6.30	
Total		106	22	27	45	200

TABLE 5.8

Results of Chi-square tests of independence of tissue distribution and divergence, and of goodness of fit between proportions within single rows and columns and total proportions, for the data in Table 5.7.

Comparison	Chi-square	d.f.	p[a]
Overall	11.87	9	n.s.
Rows within Column:			
A	4.36	3	n.s.
B	3.38	3	n.s.
C	5.13	3	n.s.
D	Ø.15	3	n.s.
Columns within Row:			
1	3.32	3	n.s.
2	2.66	3	n.s.
3	5.88	3	n.s.
4	1.16	3	n.s.

[a] n.s. - not significant at .Ø5 level (one-tailed test)

TABLE 5.9

Observed and expected numbers of D. simulans male reproductive tract polypeptides, arranged in a two-way classification by: (i) observed tissue distribution in D. simulans (see Chapter 4, Results), and (ii) observed divergence class with respect to D. mauritiana.

Tissue Distribution Class	Observed/ Expected	Divergence Class A'	B'	C'	D	Total
1. Common	Obs.	113	7	7	4	131
	Exp.	102.74	9.17	9.57	9.57	
2. Testis-specific	Obs.	65	9	5	8	87
	Exp.	68.28	6.10	6.36	6.36	
3. Gland-specific	Obs.	12	11	7	7	27
	Exp.	21.19	1.89	1.97	1.97	
4. Testis-elevated	Obs.	24	2	1	1	28
	Exp.	22.05	1.97	2.05	2.05	
Total		214	19	20	20	273

TABLE 5.10

Results of Chi-square tests of independence of tissue distribution and divergence, and of goodness of fit between proportions within single rows and columns and total proportions, for the data in Table 5.9.

Comparison	Chi-square	d.f.	p[a]
Overall	39.25	9	***
Rows within Column:			
A'	5.34	3	n.s.
B'	2.51	3	n.s.
C'	14.36	3	***
D	17.04	3	***
Columns within Row:			
1	5.46	3	n.s.
2	2.45	3	n.s.
3	30.09	3	***
4	1.25	3	n.s.

[a] n.s. - not significant at .05 level (one-tailed test)
*** - significant at .005 level or lower

TABLE 5.11

Observed and expected numbers of D. simulans male reproductive tract polypeptides arranged in a two-way classification by: (i) observed tissue distribution in D. simulans (see Chapter 4, Results), and (ii) observed divergence class with respect to D. sechellia.

Tissue Distribution Class	Observed/ Expected	Divergence Class A'	B'	C'	D	Total
1. Common	Obs.	107	5	2	17	131
	Exp.	95.00	10.09	4.32	21.62	
2. Testis-specific	Obs.	61	8	4	14	87
	Exp.	63.14	6.71	2.87	14.37	
3. Gland-specific	Obs.	8	4	2	13	27
	Exp.	19.59	2.08	0.89	4.46	
4. Testis-elevated	Obs.	22	4	1	1	28
	Exp.	20.39	2.17	0.93	4.64	
Total		198	21	9	45	273

TABLE 5.12

Results of Chi-square tests of independence of tissue distribution and divergence, and of goodness of fit between proportions within single rows and columns and total proportions, for the data in Table 5.11.

Comparison	Chi-square	d.f.	p[a]
Overall	38.06	9	***
Rows within Column:			
A'	8.59	3	*
B'	6.13	3	n.s.
C'	3.13	3	***
D	20.21	3	***
Columns within Row:			
1	6.33	3	n.s.
2	0.77	3	n.s.
3	26.41	3	***
4	4.55	3	n.s.

[a] n.s. - not significant at .05 level (one-tailed test)
* - significant at .05 level or lower
*** - significant at .005 level or lower

Once the data are in this form, they can be analyzed in a 4 x 4 contingency table, and the overall independence of tissue distribution and interspecies divergence can be assessed using a Chi-square test with 9 degrees of freedom. In addition, each row or column of the 4 x 4 table can be tested separately for goodness of fit to the proportions of the grand total represented by the marginal column or row totals, respectively. These Chi-square goodness-of-fit tests each have 3 degrees of freedom. The results of the Chi-square tests are presented in Tables 5.6, 5.8, 5.10 and 5.12.

In each of the three comparisons in which D. simulans polypeptides were cross-classified, a highly significant deviation from independence was detected (Tables 5.6, 5.10 and 5.12). The largest deviations from expected proportions occurred within rows 1 and 3 of the 4 x 4 table, reflecting the overrepresentation of testis/gland-common polypeptides belonging to divergence class A or A', and/or overrepresentation of gland-specific polypeptides in divergence class C' or D. In contrast, when D. melanogaster polypeptides were cross-classified with respect to tissue distribution and divergence status in the comparison with D. simulans, no statistically significant deviations from independence of the two classification criteria or from random proportions within individual rows or columns were detected (Table 5.8). Note, however (Table 5.7), that the deviations of observed from expected numbers in this species

include some of the same types of deviations as are found in D. simulans.

For additional perspective on interspecies divergence in different sets of polypeptides, a few 2DE comparisons were carried out for polypeptides extracted from imaginal wing discs of late third-instar larvae of D. simulans, D. melanogaster and D. sechellia (wing disc samples from D. mauritiana were not analyzed). The results of these comparisons, summarized by means of F and F_p statistics (defined above), are presented in Table 5.13. Also included are F and F_p values for comparisons of male reproductive tract proteins between single randomly chosen isofemale lines from each of the same three species, plus D. mauritiana. The number of spots compared in wing disc samples was approximately 250 in each species; in reproductive tract samples the number was about 400. In order to make the results from imaginal discs and reproductive tracts more comparable, both major and minor spots were included in N_x and N_y for both sample types. Also, spot pairing between species was carried out on the basis of electrophoretic behaviour and spot appearance only: tissue distribution was not employed here for pairing of reproductive tract proteins. Since these single-line comparisons were actually done before the data on reproductive tract tissue distribution were applied to the task of interspecies spot pairing, the unconscious

TABLE 5.13

Values of F (shared fraction of total spots), F_p (pairable fraction of unshared spots), and $F + (1-F)F_p$ (homologous fraction of total spots), obtained in comparisons of single randomly-chosen isofemale lines of D. simulans, D. melanogaster, D. mauritiana and D. sechellia, by 2DE of polypeptides in male reproductive tracts and third-instar imaginal wing discs.

Species Compared	Wing Discs			Reproductive Tracts		
	F	F_p	$F + (1-F)F_p$	F	F_p	$F + (1-F)F_p$
D. simulans/	.833	.65	.942	.639	.28	.740
D. melanogaster		.55[a]	.925		.27	.736
D. simulans/	–	–	–	.848	.42	.912
D. mauritiana					.44	.915
D. simulans/	.856	.68	.954	.791	.31	.856
D. sechellia		.68	.954		.39	.873
D. melanogaster/	–	–	–	.636	.24	.723
D. mauritiana					.35	.763
D. melanogaster/	.700	.51	.853	.664	.37	.788
D. sechellia		.56	.914		.45	.815
D. mauritiana/	–	–	–	.781	.43	.875
D. sechellia					.33	.853

[a] F_p values refer to species listed on that line of the table, in column 1.

utilization of tissue distribution information in the single-line comparisons was minimized. Moreover, if this tissue distribution information did affect the F_p values with reproductive tract protein comparisons, the effect would be to raise these values above what would be obtained in the absence of the information. This biases the results, if anything, in a conservative direction in relation to the conclusions drawn.

A prominent feature of the data in Table 5.13 is that the imaginal disc values of F and F_p are higher in every case than are the corresponding values for reproductive tracts. Thus, of the polypeptides studied in imaginal discs, fewer appeared in only one of the two species being compared; and of the spots that appeared thus to be species-unique, a higher fraction could be matched with an homologous spot in the other species, than could be so matched among reproductive tract polypeptides. If it is borne in mind that the total range of sampling variation in F, due to random inclusion of structural alleles at Class B loci in the pair of lines selected for comparison, will be no greater than the percentage of Class B loci within the total sample of loci, then these F-value differences between imaginal discs and reproductive tracts become more significant. For instance, Class B loci occur at a frequency of about 10% between D. simulans and D. melanogaster (Table 5.2); the difference between the two F values for this species comparison in Table

5.13 is about 20%. Also, most of the polypeptides detected in samples of 20 imaginal discs were much less abundant than those detected and scored in the 10-reproductive-tract samples, so that spot pairing should theoretically have been less efficient, for technical reasons, with imaginal discs. If the pairable fraction is added to the shared fraction for each comparison as shown in the columns marked: $F + (1-F)F_p$, it can be seen that the proportion of spots that appear identical, or differ at most by changes in electrophoretic behaviour between species, is uniformly and markedly higher with imaginal discs than with reproductive tract polypeptides. Although such two-gel species comparisons were carried out for isolated testis preparations only between D. simulans and D. melanogaster, the results here agreed closely with those obtained with whole reproductive tracts. The value of F here was .653; F_p was .25 for D. simulans and .23 for D. melanogaster; and $F + (1-F)F_p$ was .740 for D. simulans and .733 for D. melanogaster.

A second item of note is that the species relationships outlined in the Introduction to this chapter are not contradicted by the data of Table 5.13. D. simulans, D. mauritiana and D. sechellia show the highest F values inter se, with values for D. simulans x D. melanogaster somewhat lower and those for D. melanogaster x D. mauritiana and D. melanogaster x D. sechellia as low as this or lower still. Interestingly, the samples of D. mauritiana and D.

sechellia reproductive tract polypeptides appear no more similar to each other than either does to the corresponding sample from D. simulans; this is consistent with the former two species having diverged independently from D. simulans.

DISCUSSION

Lewontin (1974) reviewed the results of some early 1DE comparative studies of enzyme loci between Drosophila sibling species. Referring to the data from one such comparison by Prakash (1969) between D. pseudoobscura and D. persimilis (a readily hybridizable pair of sibling species), he notes:

> "If there are 'species-distinguishing' genes as indeed we suppose there must be, since these species are ecologically differentiated and reproductively isolated, they have not been picked up in a random sample of 24 loci. Thus, even if such species-differentiating genes are large in absolute number, they must be a small fraction of the whole genome, almost surely less that 10 percent of it. An alternative is that there are no such species-distinguishing genes but that the difference between species lies in the accumulation of quantitative differences in allelic frequencies, as in the case of the esterase-5 locus. This latter hypothesis is not particularly attractive because it assumes that species differences simply represent very low probabilities of total genetic identity between individuals. Yet with the degree of polymorphism within species that has been revealed, the probability of genetic identity within a species is already essentially zero. For example, using only the 20 most polymorphic genes known at present in man, the probability of genetic identity between two Englishmen is already less than 10^{-6}. It seems more reasonable to suppose that D. persimilis and D. pseudoobscura do indeed differ completely at certain loci, like those found by Dobzhansky in his study of sterility in their hybrids, but that only a special part of the genome is involved, while most of the genome remains undifferentiated."

After discussing the results of two more 1DE sibling-species comparisons, that between D. simulans and D. melanogaster for 17 loci by Kojima, Gillespie and Tobari (1970) and for 39 loci between four species of the D. willistoni complex (Ayala

and Powell, 1972), Lewontin observes that "The feature held in common is the general absence of alleles that are fixed in one species and lacking in another." And then, in what is one of the stronger statements made about the nature of genetic differentiation between species, he concludes that "... the overwhelming preponderance of genetic differences between closely related species is latent in the polymorphisms existing within species."

In light of the discussion and data presented in the preceding portions of this thesis, it is perhaps legitimate to ask the question whether evidence of the type to which Lewontin refers still constitutes overwhelming support for such a conclusion.

Species-Diagnostic Structural Divergence in Male Reproductive Tract Proteins. Ayala and Powell (1972) defined a "diagnostic locus" in an electrophoretic comparison between two species as one having allele frequency distributions such that overlap between the diploid genotype frequency distributions in the two species is .01 or lower. In extreme examples of this type, the locus will be fixed for alternate alleles in each of two species, or the locus may be polymorphic in one or both species with no allelic overlap between species. These cases correspond to Class C spots in this study, and are the ones cited by Lewontin as being absent from the small "random" samples of enzyme loci studied by IDE.

In each of the three comparisons summarized in Tables 5.2-5.4, Class C or C' loci were detected in substantial numbers: they constituted 12.5% of the spots analyzed in D. simulans and D. melanogaster. The largest subclass within Class C was that wherein both or at least one species was apparently monomorphic (i.e. Class C1 or C1'). Thus, a sizeable fraction of structural loci, although presently apparently monomorphic within species, nevertheless seem capable of undergoing complete evolutionary differentiation between species.

This finding, if borne out by more extensive population sampling within species, is clearly inconsistent with the notion that no species-specific genes exist between closely related species of Drosophila. But in attempting to interpret the possible significance of such differences, it was noted that the ratio of the number of C1 loci found in the D. simulans/D. melanogaster comparison to the number of C1' loci found in the D. simulans/D. mauritiana comparison (23/16 = 1.44: Tables 5.2, 5.3) is in good agreement with the ratio of divergence times estimated for these two species pairs by Easteal and Oakeshott (1985) from DNA sequence data (range 1.37 - 1.55 for 5 different divergent DNA segments; mean = 1.46). Also, in a large-scale (70 loci) IDE study of enzymes in D. simulans and D. melanogaster (R. S. Singh and M. S. Choudhary, unpublished data), as mentioned earlier, 7 of the loci (10%) showed alternate fixation for different

alleles. This percentage is very close to the percentage of Class C1 loci seen in the present 2DE comparison between these two species. These considerations are consistent with the notion that allele substitutions at monomorphic structural loci occurred throughout the genome at a relatively constant rate over the time since species formation, as a result of independent phyletic evolution within each of the lineages. The relatively large absolute numbers of such substitutions detected in the 2DE sample of male reproductive tract proteins may be more a function of the increased sample of loci than of any special involvement of these proteins in the genetic determination of critical species differences. What is brought out clearly by these data is that no necessary connection exists between the polymorphism observed at a locus at an arbitrarily chosen time in the history of a pair of species, and the ability of the locus to undergo divergence between the species.

The possible genetic mechanisms underlying the divergence are many; different processes may even occur simultaneously at different loci and not all of them necessarily involved allele substitutions occurring in the period since divergence. Natural selection or random genetic drift may well have both been involved, and there is nothing in the current data that could help to date the actual occurrences of genic divergence in relation to the events of species formation. But the existence of allelically fixed

diagnostic loci between closely related species has been clearly demonstrated, and forces us to admit the possibility that they have played a role in the critical events of species formation. Knowledge of the specific functions of the C1 and C1' loci will be very useful; as Tables 5.5-5.12 show, they occurred in all tissue distribution classes among the reproductive tract polypeptides sampled.

Possible Non-Structural Divergence. Ayala and Powell (1972) listed one locus (esterase-5) as "diagnostic" for D. tropicalis, in comparison to its three sibling species in the willistoni group, because of the fact that D. tropicalis lacked any detectable esterase activity traceable to this locus. Cases such as this have generally been reported merely as interesting or even distracting peripheral observations, in the context of more easily codified electrophoretic mobility divergence for enzymes detectable in all of the species compared. In the present study, however, cases like that of esterase-5 in D. tropicalis occurred at such high frequency that it seemed appropriate to draw some useful, if tentative, conclusions about their significance.

Class D spots, for which no interspecifically homologous spot could be identified, occurred at a frequency of about 25% in each of the two species in the D. simulans/D. melanogaster comparison, and at somewhat lower but still considerable frequencies in the other two comparisons (Tables 5.2-5.4). These Class D differences between species are

unlikely to be artifacts of inconstant culture conditions or mating history: these variables were controlled fairly rigidly, and the 2DE spot patterns were extremely reproducible within species. The involvement of some as-yet-unidentified difference in nutritional or other environmental requirements between species, which could lead to reproducible yet physiologically "distorted" protein expression profiles in one or more of the four species, cannot be ruled out. However, no abnormalities in development time or vigour of either larvae or adults were observed.

Gross anatomy of the reproductive tissues in the four species studied is extremely similar. This suggests (but of course does not conclusively demonstrate) that histological differences between species involving, for example, simply different proportions of the same cell types, are not a likely possibility. Selective protein loss during sample preparation would seemingly have to be very complete and selective indeed to explain the results, in view of the within-species reproducibility of spot pattern and the evenly scattered occurrence of Class D spots over the gel area.

Probably the most difficult source of artifact to rule out is the possible failure to pair homologous spots between species, when in fact the homolog is present but unrecognizable, or simply has migrated off the gel. Preliminary experiments with extended first-dimension pH

gradients failed to confirm the latter possibility. However, these altered separations, although quite successful, were not optimized, and this aspect should be pursued further. It was noted earlier that auxiliary information (i.e. tissue distribution) can help significantly to reduce uncertainties in the assignment of spot homologies by appearance alone. It may therefore be expected that more rigorous criteria of homology assessment, such as one-dimensional peptide mapping (Fey et al., 1983), will eventually reduce the number of Class D spots. But it should be remembered that (i) most Class D spots were apparently monomorphic within D. simulans and D. melanogaster (Table 5.2); this suggests that if the divergence class status of these spots is altered, they will become Class C1, and thus the overall degree of spot divergence will be unreduced, (ii) although it is not immediately clear what an alteration of appearance and/or tissue distribution of a particular spot to the extent that it is unrecognizable between species means in molecular or cellular terms, no indication of this kind of alteration was observed within species, and (iii) it seems unlikely that all Class D spots can be homologously paired between species with a member of the set of silver-stained polypeptides studied; a certain proportion will probably prove to represent bona fide alterations in levels of gene and/or protein expression.

Taken together, these three considerations imply that detailed investigation of the actual physical, biochemical

and genetic bases of the Class D spots should prove very interesting, whatever the results may be. If Class C1 is greatly enlarged by the successful homologous pairing of many Class D spots, this would suggest that male reproductive tract polypeptides do in fact evolve structurally more rapidly than do the enzymes usually studied by IDE. Judging from the numbers of Class D spots observed, up to a threefold difference in the rate of structural evolution could prove to be involved. If the situation is that greater amounts or different types of structural changes tend to accumulate in individual polypeptides during species divergence than characteristically are found in the form of polymorphisms segregating within species populations, this, too, would lead us to question the general validity of statements like those quoted from Lewontin, to the effect that species differences are of a genetic type which can be found, latent, in the variation occurring within species. Or if quantitative changes in gene or protein expression are the most frequent type of genetic difference found between species, then perhaps a whole new analytical approach is required in the study of species formation at the molecular level. The results of these more detailed analyses should be especially interesting in the case of the D. simulans/D. sechellia pair, where 17% of the D. simulans spots were placed in Class D, in spite of the extremely close relationship between the two species according to other criteria.

Patterns of Species Divergence in Different 2DE Protein Sets. When the interspecies divergence data were examined for possible correlations with distributions of polypeptide expression in different male reproductive tract tissues, some significant correlations were found. The strongest correlations among reproductive tract proteins were seen in D. simulans, where Class C' and D differences were disproportionately represented in gland-specific polypeptides, and Class A or A' in testis/gland-common polypeptides. Thus, on this basis, no statistically significant positive deviations from expected numbers of strongly divergent loci were detected for the polypeptides expressed in testes.

However, when the basis for comparison was altered, some evidence for higher rates of evolution in male reproductive tract proteins was obtained. When male reproductive tract proteins were compared not among themselves but with proteins expressed in third-instar imaginal wing discs, in every species comparison a greater degree of interspecies divergence was detected with the former set of proteins than with the latter. And within the divergent subset of imaginal disc polypeptides, a significantly larger fraction could be paired with putative homologs between species, in spite of the expectation that technical factors (i.e. protein loading) would tend to create a bias in the opposite direction.

This result could be taken to mean that male reproductive tract polypeptides evolve at a more rapid rate and/or in a different molecular "mode" (i.e. quantitative changes in expression vs. structural changes) in comparison to most other polypeptides. Or it might mean that the polypeptides expressed in late third-instar imaginal discs are unusually constrained in their evolution. Support for the latter hypothesis could be drawn, conceptually, from the consideration of the relatively undifferentiated state of imaginal discs at this stage of larval development, suggesting that the genetic functions expressed in them may be "general" cellular functions and thus perhaps highly constrained ones (see Introduction to Chapter 3). On the empirical side, it was found by Greenberg and Adler (1982) that, of a total of over 500 silver-stained spots detected in 2DE separations of imaginal disc polypeptides, none showed reproducible qualitative differences between different disc types (haltere, wing, leg, eye/antenna). This seems consistent with the idea that the imaginal disc polypeptides detected in the present study have generalized cellular functions, perhaps as components of cell structure or enzymes of intermediary metabolism.

Interestingly, Ohnishi, Kawanishi and Watanabe (1983), in single-line comparisons of proteins in whole-body homogenates separated by 2DE and stained with Coomassie Blue, obtained F values of .811 for D. simulans/D. melanogaster,

.955 for D. simulans/D. mauritiana, and .803 for D. melanogaster/D. mauritiana. These values are much closer to those for imaginal discs than they are to those for male reproductive tracts (see Table 5.13). Measurements like these for other major groups of proteins, by standardized 2DE techniques, will be of extreme interest.

This discussion illustrates some of the interpretive problems associated with the structure/function correlation approach, outlined in Chapter 4, of which the data analysis in this chapter is essentially a variant. Even if the alternative hypotheses and their predictions are relatively well-defined; even if data are not seriously limited in quantity; and even if independent tests of the same hypotheses are possible, the results must still be interpreted cautiously, especially with respect to statistical methodology and to potentially misleading features of the actual sets of loci analyzed for correlations. In future work involving the correlation studies approach to analysis of genetic differences between species, careful attention must be paid to these difficulties. It should also be kept in mind that even successful correlation studies only provide a preliminary statistical perspective in the search for effects that many ultimately be mediated at the level of single loci. The statistical analyses should therefore be followed by detailed functional analyses of single loci of putative adaptive and/or evolutionary significance.

Mention was made earlier of the purely descriptive value of data on variation in different sets of proteins, in terms of understanding the range of possibilities that occur in nature. The same consideration applies to data on interspecies divergence in different sets of proteins, regardless of their possible connection with crucial aspects of species divergence such as reproductive isolation. The unusually rapid evolution of gland-specific polypeptides suggested by this study may be representative of a general tendency towards rapid evolution in functions accessory to the primary male sexual functions of spermatogenesis and fertilization. The unique value of exterior male genitalia as taxonomic characters in Drosophila, already mentioned, is consistent with this possible tendency. Comparative studies of the co-occurrence in the genus Drosophila of adult-male-limited expression of glucose dehydrogenase activity and expanded (presumably glandular) anterior ejaculatory ducts (Cavener, 1985) as well as the distribution within the melanogaster subgroup of male-specific elevation of esterase-6 activity in the anterior ejaculatory duct (Morton and Singh, 1985), also attest to the evolutionary potential of accessory male sexual functions, in this case on a taxonomic scale larger than is represented by the melanogaster species complex. Comparative 2DE studies of accessory gland proteins between species of Drosophila may uncover numerous examples of differences in developmental

patterns of protein expression between closely related species. In this event, some of these may provide favourable material to go beyond statistical description of divergence patterns in different groups of loci, to detailed genetic and molecular analysis of the mechanistic basis of evolutionary changes in gene expression.

6

GENERAL SUMMARY AND CONCLUSIONS

Chapter 1 began with an outline description of some fruitful interactions that took place between Mendelian genetics and evolutionary theory during the first half of this century. The interaction produced a diverse but largely untested array of theoretical genetic models of population structure and species formation, generally grouped under the rubric of "The Modern Synthesis". Largely inspired by the work of Dobzhansky and his colleagues, evolutionary geneticists next began to make serious efforts to test the validity of certain aspects of the theory. The initially dominant questions concerned the hypothetical internally balanced nature of naturally-occurring gene pools, and the degree to which alteration of such putative internal balances might be involved in species formation.

The late 1960's saw the first systematic application of relatively simple biochemical techniques (one-dimensional enzyme electrophoresis - 1DE) and genetic knowledge (the "one gene-one polypeptide" principle) borrowed from molecular genetics, to the direct measurement of genic diversity within populations and between species. It was originally hoped that such data would permit definitive testing of theory.

However, the sufficiency of the data as a basis on which to test the theory was questioned, on several different grounds: (i) the imperfect sensitivity of 1DE to allelic variation in protein structure; (ii) the small and perhaps unrepresentative set of structural loci accessible to study by 1DE; (iii) the lack of information, in most 1DE analyses, on allelic variation in nontranslated DNA; and (iv) the relative ease with which the large amounts of genic variation detected by 1DE can be explained equally well on the basis of either the presence or the complete absence of balancing mechanisms at the variable loci in question. This last-mentioned point formed the basis for the so-called "selection-neutrality" debate.

The major contribution of this thesis lies in its delineation of an approach to the collection of genetic data that may be better suited to the task of evaluating the theory. This has been done in two main ways. First, more sophisticated biochemical techniques (two-dimensional protein electrophoresis - "2DE", and ultrasensitive silver staining of proteins) have been applied, and improved to increase the precision with which large numbers of polypeptides can be analyzed for genic variation. And second, attention has been restricted to a set of organs (the Drosophila male reproductive tract) that undergo evolutionary change in interesting and possibly crucial ways in this genus of flies. These two methodological changes have had the effect of

simultaneously broadening scope and sharpening focus in the attempt to understand the adaptive and evolutionary significance of genetic variation in populations and genetic divergence between species.

Two main sets of measurements were carried out using this methodology. The first was a set of estimates of amounts of genic variation in four natural populations of Drosophila simulans and Drosophila melanogaster. Even with substantial improvements and differences in techniques and material over earlier 2DE studies carried out by other workers with these two species, the results of those earlier studies were confirmed. About 10% of loci were found to be polymorphic, and average expected levels of heterozygosity were in the neighbourhood of 2-3% - a 2-6 fold reduction compared to estimates obtained by one-dimensional native electrophoresis (1DE) of enzymes in the same four populations of flies.

The second set of measurements was a set of estimates of the amounts and types of divergence between species of the Drosophila melanogaster species complex, again with respect to male reproductive tract proteins examined by 2DE. One significant finding here was the frequent occurrence of apparent "fixation" of different alleles at homologous loci between species (about 10% of loci between D. simulans and D. melanogaster, 6-7% of loci between D. simulans and D. mauritiana, and 2-3% of loci between D. simulans and D.

sechellia). Another finding was the high frequency of male reproductive tract protein differences that were probably the result of large changes in level of protein expression, as distinct from changes in electrophoretic behaviour of the protein. The frequency of this type of species difference among the proteins analyzed was about 27% between D. simulans and D. melanogaster, 5-7% between D. simulans and D. mauritiana, and 16 and 6% for D. simulans and D. sechellia, respectively. The third significant finding was that the total degree of interspecies divergence is significantly higher for male reproductive tract proteins than for wing imaginal disc proteins separated and detected by the same 2DE and silver stain technique, for the species pairs: D. simulans/D. melanogaster, D. simulans/D. sechellia and D. melanogaster/D. sechellia.

The genic variation measurements mentioned above support two tentative conclusions about the genetic structure of populations. Along with the growing body of 2DE data on genic variation in other organisms, these measurements strengthen the observed pattern of lower variation in the proteins examined by 2DE than in soluble enzymes examined by 1DE in the same organisms. The more diverse the sets of proteins which are analyzed by 2DE and give such results, the stronger the case will be that 1DE of soluble enzymes generally overestimates the amount of genic variation in the genome as a whole. The next major research tasks in this

area will be: to establish definitive cross-calibrations for the relative sensitivity of 1DE and 2DE to allelic variation in protein structure; to expand the sample of structural loci so as to improve the representation of the genome; and to study variation in the genes coding for sets of proteins with distinctive structural or functional properties, in order to discover any systematic differences in variability that might exist between such defined sets of loci.

If it should turn out that the 2DE estimates accurately reflect the overall level of genic variation in animal genomes, this may tend to change our present conception of the Mendelian population from one where genic polymorphism is common (or perhaps the rule) to one where it is relatively rare (or perhaps even the exception). If 90% of structural loci are monomorphic within a species, it becomes more difficult to maintain an unqualified conviction of the widespread importance of either balancing selection or neutral mutation and drift in shaping the genetic structure of populations. A result such as this would highlight the value of studying, for example: variation in nongenic DNA, and the question of how many variable loci are necessary to maintain adaptive flexibility.

The second conclusion supported by the 2DE genic variation data is that patterns in the amount of genic variability between populations or between species may not be the same for different sets of loci in the genomes being

examined. This was found to be the case for differences between temperate and tropical populations within species, and between D. simulans and D. melanogaster, when the 1DE data were examined in parallel with the 2DE data from the same material. Results of this kind provide evidence that historical factors alone, affecting genic variation through population size fluctuations and thus expected to affect all loci in the genome simultaneously, cannot explain the total pattern of variation.

The results from the 2DE species comparisons also suggest that fresh thinking is in order. It seems clear that alternate fixation for different structural alleles at a locus can occur between fairly closely related sibling species like Drosophila simulans and D. melanogaster. Such alternate fixation seems to occur only at a minority of loci and may depend primarily on time elapsed since species divergence. However, its existence attests to the possibility that allele substitutions may sometimes occur relatively rapidly in evolution, with an initially rare allele at an essentially monomorphic locus passing through only a transient phase of polymorphism that involves no intermediate "balanced" phase. Also, it does not seem necessary yet to entertain the "recombinational" hypothesis of species divergence that was mentioned earlier as a serious alternative to the presence of fixed genic differences.

Finally, when the pattern of polypeptide expression is taken into account, either separately or together with structural divergence, male reproductive tract proteins appear to evolve more rapidly, as a group, than do some other major groups of proteins in the melanogaster species complex. The effect of this observation may be to re-open the question of the role of large shifts in "genetic balance" during species formation, on a more concrete basis. As with investigation of the adaptive significance of genic variation, the most difficult task will be that of demonstrating the biological relevance of the observed patterns of interspecies divergence. Careful choice of species, thorough quantitative molecular analysis of a large number of gene products in a serious search for species differences of potential importance in hybrid male sterility, and extension of that analysis from the parent species to the hybrids themselves, may eventually lead to a precise genetic understanding of an important aspect of reproductive isolation in Drosophila. The present study has yielded promising preliminary evidence that we may be closer to identifying that "special part of the genome" postulated by Lewontin to underlie the formation of new species. Until this kind of analysis has been accomplished, theories of evolution based on the changing genetic structure of Mendelian populations will remain untested abstractions.

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APPENDIX 1

FIGURES A1.1, A1.2 (INSIDE BACK COVER)

Diagrammatic representations of reproducible, major-spots of D. simulans and D. melanogaster male reproductive tract polypeptides, separated by 2DE and stained with silver. Landmark spots are identified by boldface numbers; others by a number and lower-case letter.

FIGURE A1.1

D. simulans

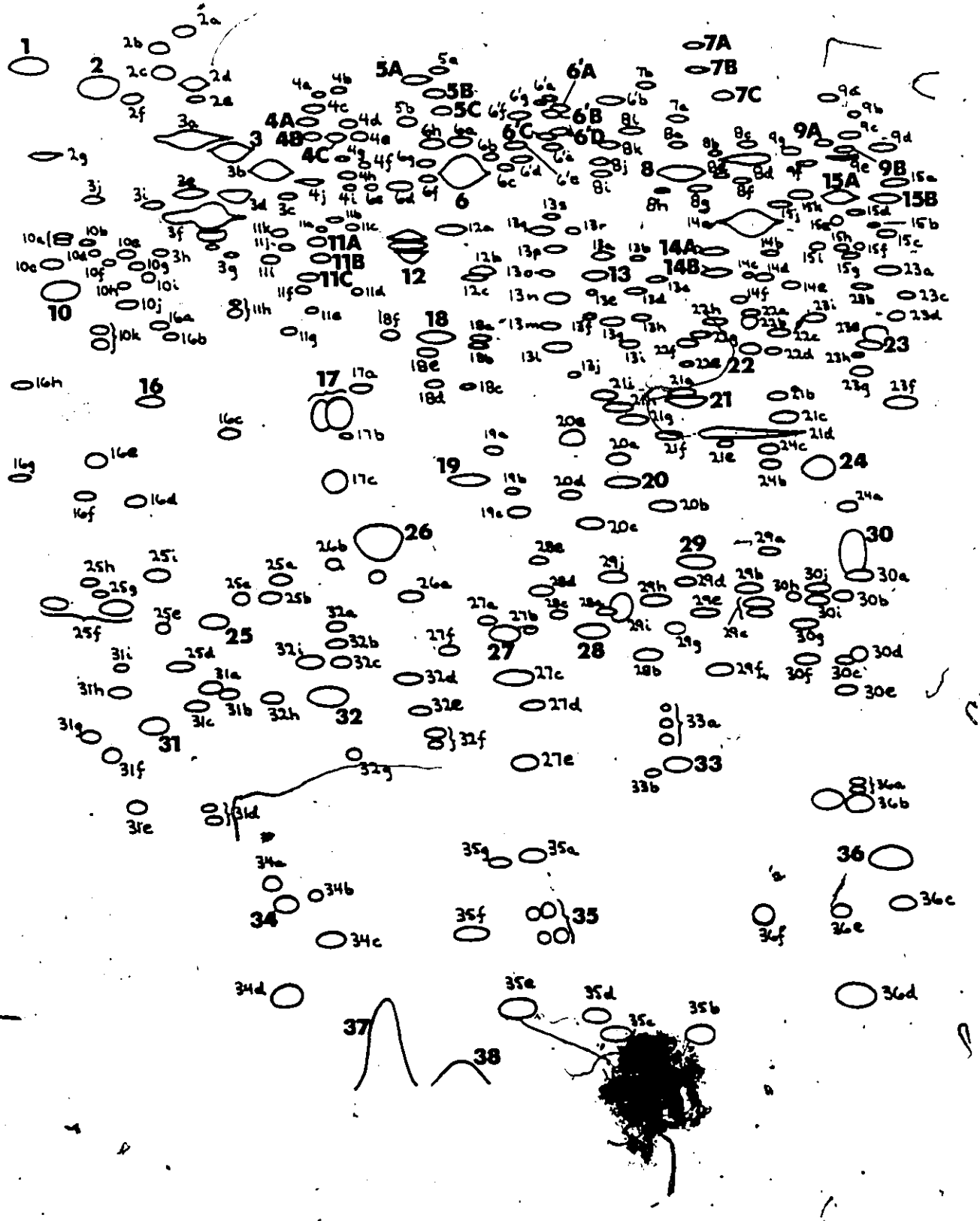
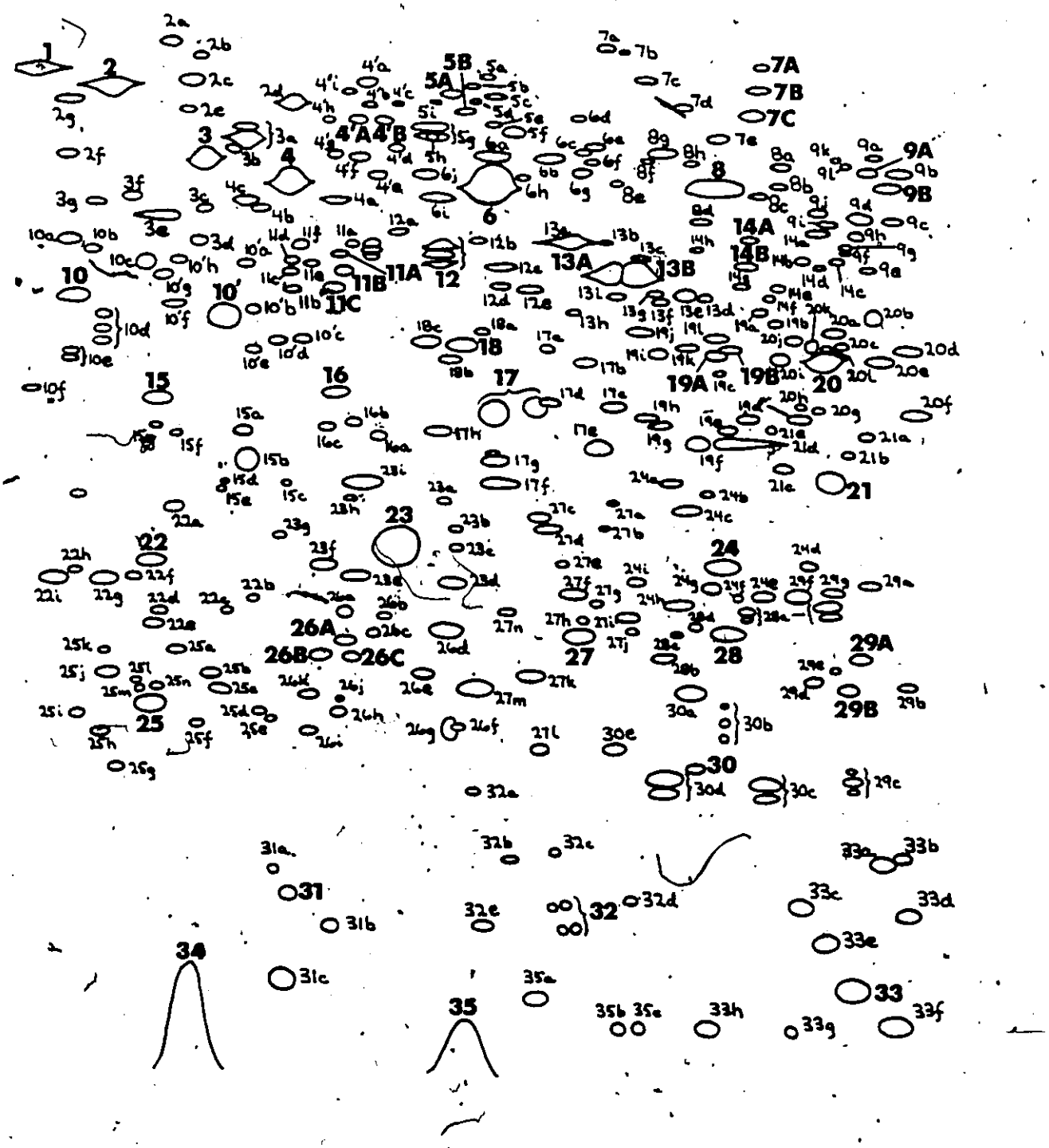


FIGURE A1.2

D.melanogaster



APPENDIX 2

TABLE A2.1

Structural allelic compositions of individual isofemale lines of *Drosophila simulans* from a temperate (South France) and a tropical (Brazzaville) population, with respect to 27 polymorphic male reproductive tract protein loci.

South France

		Isofemale Line									
Locus	**Allele**	**1**	**3**	**5**	**13**	**14**	**15**	**16**	**20**	**21**	**27**
2a	1			+			+				+
	2	+	+	+	+	+	+	+	+	+	+
2c	1	+	+	+	+	+	+	+	+	+	+
	2										
2e	1	+			+	+	+		+	+	+
	2	+	+	+	+	+	+	+	+	+	+
2f	1										
	2		+								+
	3	+	+	+	+	+	+	+	+	+	+
3a	1		+	+		+	+	+		+	+
	2	+	+	+	+	+	+	+	+		+
	3										
3e	1	+	+	+	+	+	+	+	+	+	
	2							+		+	+
3f	1										
	2	+	+	+	+		+	+	+	+	+
	3	+	+	+		+	+	+		+	+
	4					+					
5A	1	+	+	+	+	+	+	+	+	+	+
	2	+	+	+	+	+		+	+	+	
	3										
7B	1										
	2	+	+	+	+	+	+	+	+	+	+
10e	1		+		+	+					+
	2	+	+	+	+	+	+	+	+	+	+
10j	1	+	+	+	+	+	+	+	+	+	+
	2						+				
	3										

APPENDIX 2 (CONT'D.)

TABLE A2.1, SOUTH FRANCE (CONT'D.)

		Isofemale Line									
Locus	Allele	1	3	5	13	14	15	16	20	21	27
10k	1	+	+	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+		+		+
	3										
	4										
11k	1	+		+	+		+	+		+	+
	2	+	+	+	+	+	+		+		+
12	1	+	+	+	+	+	+	+	+	+	+
	2			+	+				+	+	
14a	1		+	+		+	+				+
	2	+	+	+	+	+	+	+	+	+	+
	3						+				
14d	1	+		+	+	+	+	+	+	+	
	2		+							+	+
16d	1							+			+
	2									+	
	3	+	+	+	+	+	+	+	+	+	+
	4										
17	1			+							
	2	+	+	+	+	+		+	+	+	
	3	+	+	+	+	+	+	+	+		+
17a	1		+	+	+		+		+	+	+
	2	+	+	+	+	+		+		+	+
18d	1	+			+		+	+	+		+
	2	+	+	+		+	+	+	+	+	
21c	1						+				
	2	+	+	+	+	+	+	+	+	+	+
	3	+	+					+			
25f	1		+	+	+					+	
	2	+	+	+		+	+	+	+	+	+
	3										
26	1										
	2	+	+	+	+	+	+	+	+	+	+
29c	1	+	+	+	+	+	+	+	+	+	+
	2							+		+	

APPENDIX 2 (CONT'D.)

TABLE A2.1, SOUTH FRANCE (CONT'D.)

		Isofemale Line									
Locus	Allele	1	3	5	13	14	15	16	20	21	27
29i	1	+	+	+	+	+	+	+	+	+	+
	2										
	3										
31/31g	1	+		+	+		+			+	
	2		+	+	+	+		+	+	+	+
	3										
34b	1	+	+	+	+	+	+	+	+	+	+
	2					+		+			

Brazzaville

Isofemale Line

Locus	Allele	10	27	28	31	33	34	36	40	42	47
2a	1									+	+
	2	+	+	+	+	+	+	+	+	+	+
2c	1	+	+	+	+	+	+	+	+	+	+
	2		+								
2e	1			+	+						
	2	+	+		+	+	+	+	+	+	+
2f	1	+	+	+	+	+	+	+	+	+	+
	2				+		+				+
	3	+	+	+	+	+		+	+	+	+
3a	1	+	+	+	+			+	+	+	
	2	+			+	+	+	+		+	
	3	+	+							+	+
3e	1	+					+	+	+	+	+
	2	+	+	+	+	+	+	+	+	+	+
3f	1			+							+
	2	+	+	+			+	+	+	+	
	3	+	+	+	+	+	+	+	+	+	+
	4										
5A	1	+	+	+	+	+	+	+	+	+	+
	2										
	3										+
7B	1				+						
	2	+	+	+	+	+	+	+	+	+	+
10e	1	+	+			+	+	+	+	+	+
	2			+	+	+		+			
10j	1	+	+	+	+	+	+	+	+	+	+
	2		+	+	+		+	+			+
	3							+			

APPENDIX 2 (CONT'D.)

TABLE A2.1, BRAZZAVILLE (CONT'D.)

		Isofemale Line									
Locus	Allele	10	27	28	31	33	34	36	40	42	47
10k	1	+	+	+			+	+	+	+	+
	2	+		+	+	+		+	+		+
	3	+	+			+		+			+
	4				+				+	+	
11k	1	+			+	+	+	+	+	+	+
	2		+	+			+	+	+	+	+
12	1	+	+	+	+	+		+	+	+	+
	2		+				+				
14a	1	+	?	+	+		+		+	+	
	2				+	+		+	+		+
	3										
14d	1	not scored									
	2										
16d	1										
	2										
	3	+	+	+	+	+	+	+	+	+	+
	4				+						
17	1			+							+
	2	+	+	+	+	+	+	+	+	+	+
	3										
17a	1										
	2	+	+	+	+	+	+	+	+	+	+
18d	1	not scored									
	2										
21c	1										
	2	+	+		+	+		+	+		+
	3			+			+		+	+	+
25f	1										
	2	+	+	+	+	+	+	+	+	+	+
	3						+				
26	1									+	
	2	+	+	+	+	+	+	+	+	+	+
29c	1	+	+	+	+	?	+	+	+	+	+
	2	+	+		+				+		

APPENDIX 2 (CONT'D.)

TABLE A2.1, BRAZZAVILLE (CONT'D.)

Isofemale Line

Locus	Allele	10	27	28	31	33	34	36	40	42	47
29i	1		+	+	+	+	+	+	+	+	+
	2				+					+	+
	3	+	+			+		+	+		
31/31g	1										
	2	+	+	+	+	+	+		+	+	+
	3	+	+		+	+	+	+			+
34b	1	+	+	+	+	+	+	+	+	+	+
	2										

APPENDIX 2 (CONT'D.)

TABLE A2.2

Structural allelic compositions of individual isofemale lines of Drosophila melanogaster from a temperate (France) and a tropical (Benin) population, with respect to 27 polymorphic male reproductive tract protein loci.

France

		Isofemale Line									
Locus	Allele	3-1	5-2	8-1	10-1	11-2	14-2	16-2	17-2	18-2	19-2
2g	1	+	+	+			+	+	+		+
	2		+		+	+		+		+	+
3a	1	+			+			+	?	+	+
	2	+	+	+		+	+	+			+
	3										
3c	1										
	2	+	+	+	+	+	+	+	+	+	+
3e	1	+	+	+	+	+	+	+	+	+	+
	2	+	+	+				+	+		
3f	1	+								+	
	2		+	+	+	+	+	+	+		+
5i	1	not scored									
	2										
7C	1										
	2	+	+	+	+	+	+	+	+	+	+
7b	1	+	+	+	+	+	+	+	+	+	+
	2										
7c	1		+	?	+		+	+	+		+
	2	+				+	+	+		+	
9j	1			?							
	2								+		
	3	+	+		+	+	+	+		+	+
10d	1	+	+		+	+	+		+	+	
	2			+	+	+	+	+	+		+
10'	1	+	+	+	+	+	+	+	+	+	+
	2							+			
10'h	1										
	2	+	+	+	+	+	+	+	+	+	+

APPENDIX 2 (CONT'D.)

TABLE A2.2 FRANCE (CONT'D.)

		Isofemale Line									
Locus	Allele	3-1	5-2	8-1	10-1	11-2	14-2	16-2	17-2	18-2	19-2
11d	1	+				+				+	
	2		+	+	+		+	+	+		+
15b	1	+	+	+	+	+	+	+	+	+	+
	2										
17	1	+	+	+	+	+	+	+	+	+	+
	2										
18c	1	+									
	2			+							
	3	+	+	+	+	+	+	+	+	+	+
19d	1	+			+	+		+	+	+	+
	2		+	+			+		+		
19e	1	not scored									
	2										
	3										
191	1										
	2										
	3	+	+	+	+	+	+	+	+	+	+
22a	1	+	+	+	+	+	+	+	+	+	+
	2										
23e	1										
	2	+	+	+	+	+	+	+	+	+	+
24	1	+	+	+	+	+	+	+	+	+	+
	2										
24b	1	not scored									
	2										
25	1	+	+	+	+	+	+	+	+	+	+
	2							+			
28a	1										
	2										
	3	+	+	+	+	+	+	+	+	+	+
28b	1										
	2	+	+	+	+	+	+	+	+	+	+

Benin

Locus	Allele	Isofemale Line 1C	4EA	6CA	9A	10C	11A	18C	23C	24C	25C
2g	1		+	+	+		+				
	2	+	+	+	+	+	+	+	+	+	+
3a	1	+		+		+	+	+	+	+	+
	2	+	+	+	+		+		+	+	+
	3										
3c	1				+						+
	2	+	+	+		+	+	+	+	+	+
3e	1	+						+		+	
	2	+	+	+	+	+	+	+	+	+	+
3f	1		+	+	+		+	+	+	+	
	2	+		+		+	+	+	+		+
5i	1	+	+	+			+	+			+
	2	+			+	+	+		+	+	
7C	1					+	+				
	2	+	+	+	+	+	+	+	+	+	+
7b	1		+			+			+		
	2	+	+	+	+		+	+	+	+	+
7c	1	+	+	+	+	+			+		
	2					+	+	+		+	+
9j	1						+		+		
	2	+	+	+	+	+		+		+	
	3										+
10d	1	+	+	+	+	+	+	+	+		
	2	+		+	+			+	+	+	+
10'	1	+	+	+	+	+	+	+		+	+
	2						+		+	+	+
10'h	1		+								
	2	+	+	+	+	+	+	+	+	+	+

APPENDIX 2 (CONT'D.)

TABLE A2.2 BENIN (CONT'D.)

Locus	Allele	Isofemale Line 1C	4EA	6CA	9A	10C	11A	18C	23C	24C	25C
11d	1		+	+	+			+	+	+	
	2	+		+		+	+	+	+	+	+
15b	1	+	+	+	+	+	+	+	+	+	+
	2				+						
17	1	+	+	+		+	+	+	+	+	+
	2	+			+		+			+	
18c	1			+				+			
	2										
	3	+	+		+	+	+	+	+	+	+
19d	1	+	+	+	+	+	+	+	+	+	+
	2									+	
19e	1		+						+	+	
	2	+			+	+	+			+	+
	3			+		+		+	+		
191	1									+	
	2						+		+	+	
	3	+	+	+	+	+	+	+			+
22a	1	+	+	+	+	+	+	+	+	+	+
	2			+							
23e	1		+								
	2	+	+	+	+	+	+	+	+	+	+
24	1	+	+	+	+	+	+	+	+	+	+
	2							+			
24b	1	+	+	+	+	+	+	+	+	+	+
	2	+	+	+			+				
25	1	+	+	+	+	+	+	+	+	+	+
	2										
28a	1										
	2				+						
	3	+	+	+	+	+	+	+	+	+	+
28b	1	+	+								
	2	+	+	+	+	+	+	+	+	+	+

APPENDIX 3

FIGURES A3.1, A3.2 (INSIDE BACK COVER)

Diagrammatic summary of tissue distributions of the spots illustrated in Figures A1.1 and A1.2, within the male reproductive tracts of *D. simulans* and *D. melanogaster*. Not all spots could be assigned a distribution pattern; these are left unshaded.

FIGURE A3.1

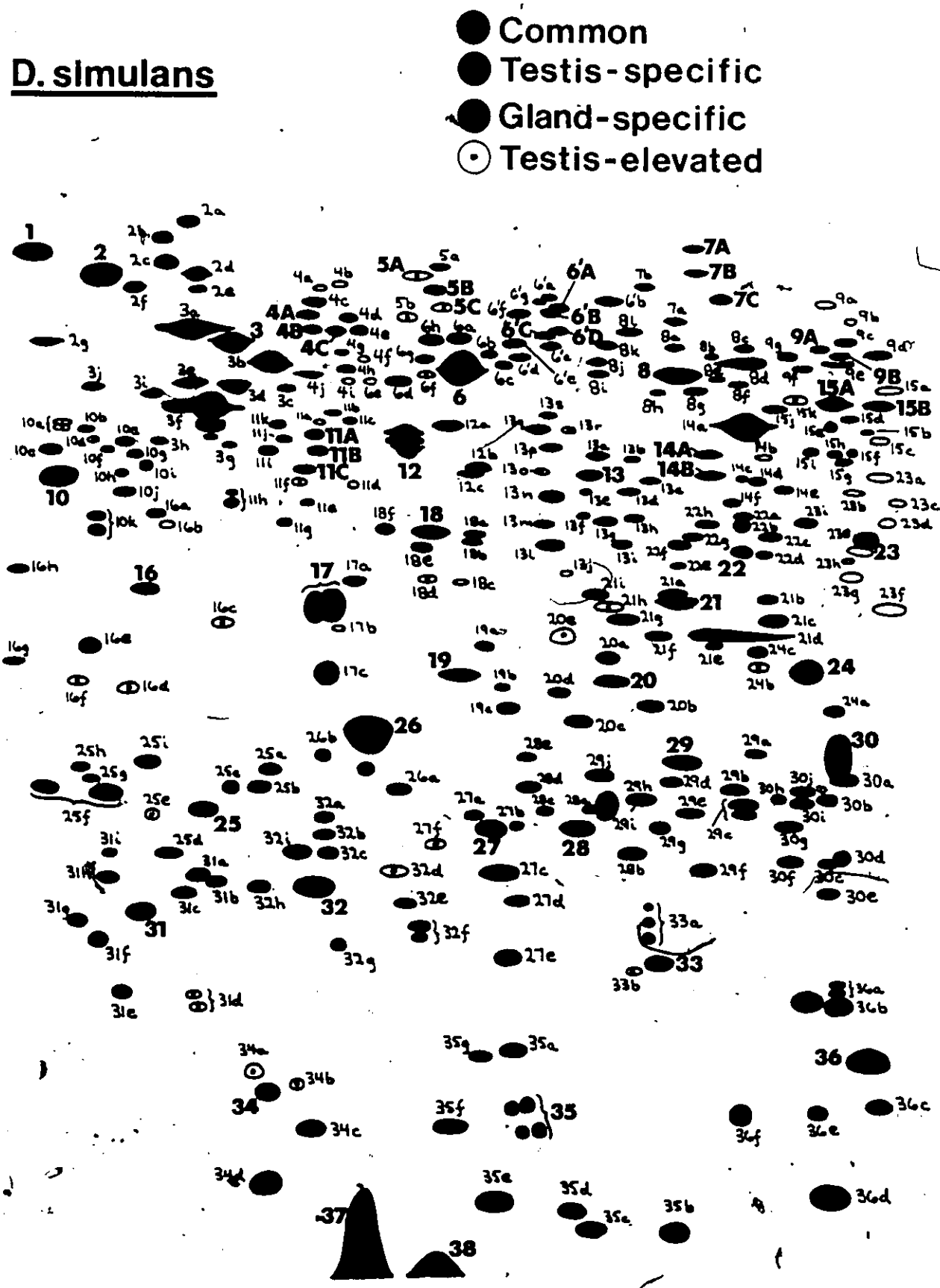
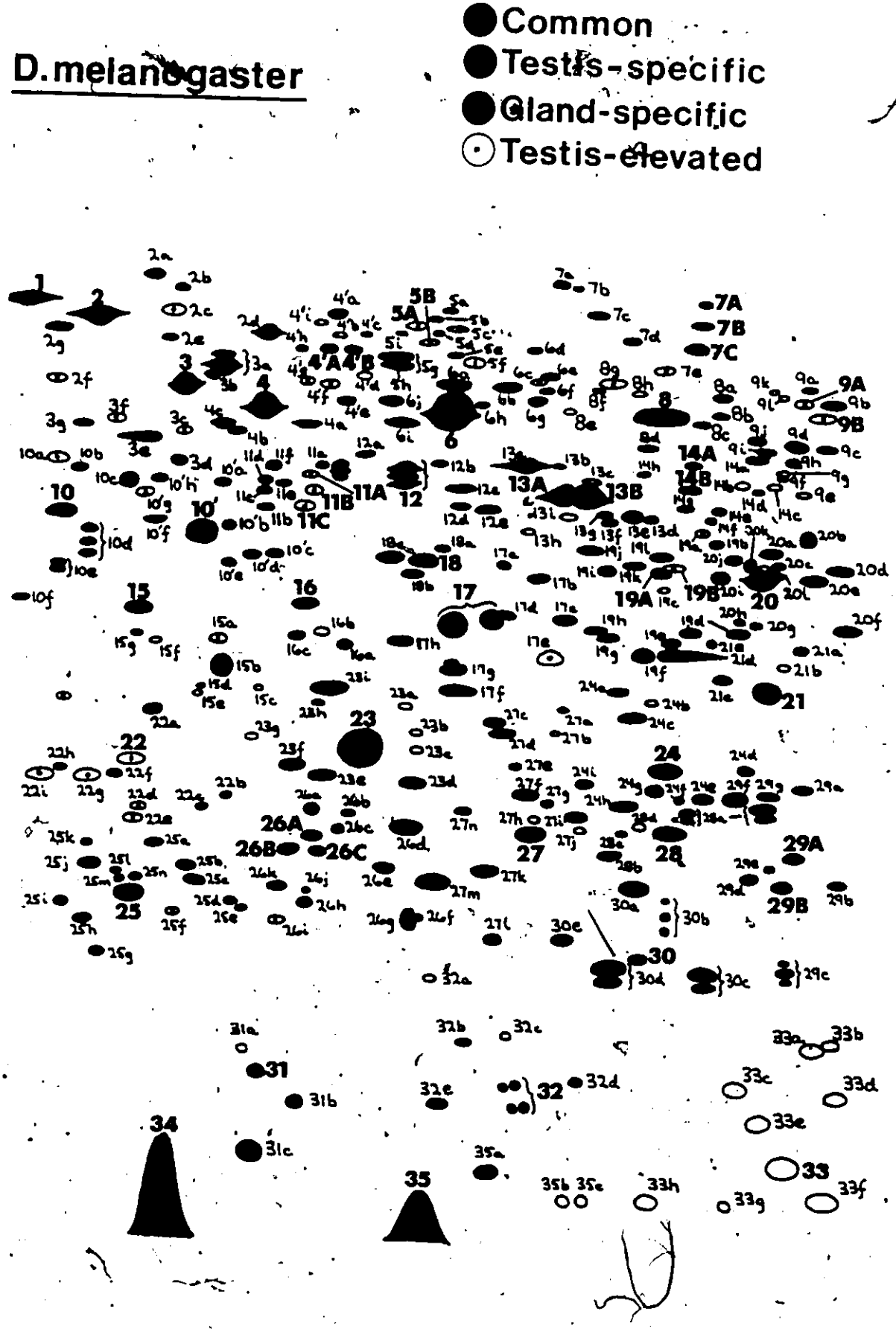

D. simulans
Common
Testis-specific
Gland-specific
Testis-elevated

FIGURE A3.2


D.melanogaster
Common
Testis-specific
Gland-specific
Testis-elevated

APPENDIX 4

TABLE A4.1

Classification of individual male reproductive tract polypeptides of Drosophila simulans into divergence classes, with respect to male reproductive tract polypeptides of D. melanogaster. For classes A-C, the putatively homologous spot in D. melanogaster is also listed. In each case, the D. simulans spot number precedes the slash mark: "/", and the corresponding D. melanogaster spot number follows it. The members of D. simulans landmark groupings occur sequentially within the table, so that it has been necessary to list the number of the landmark grouping only once. For definitions of divergence classes, see Figure 5.1 and Table 5.1, as well as adjacent portions of the text in Chapter 5. For spot map locations and photographic illustration of individual spots, see Appendix 1 and Figures 3.1 and 3.2.

CLASS A

1/1

2/2

3b/4
d/4b
j/3g

4B/4'g
C/4'f
j/4a

5B/5B
a/5b
b/5h

6/6
a/6a
d/6i

6'C/6c
D/6e
c/6f
e/6b
g/6d

7a/7e

8/8
c/8a
e/8c
h/8d
l/8g

9A/9l
B/9A
c/9a
d/9b

10/10
a/10a
g/10'g
i/10'c

11A/11A
B/11B
C/11C
g/10'c
h/10'b
i/11c
j/11e

12b/12d

13d/13f
g/19j
i/19i
l/17b
r/13b

14B/14g
c/14e

15e/9h
g/9e
i/14c
k/9j

16/15
c/15a
h/10f

18/18
e/18b

19c/27c

20b/27c
e/17e

21d/21d
f/19f
g/19g
h/19h

22/20i
c/20k
e/19c
g/19B

23/20d
f/20f

24/21
b/21c

25c/22b
d/25a
e/22d
i/22

27a/27n
c/27k
e/27l

28a/27i
c/27h
d/27f
e/27e

29b/24e
d/24g
e/28
j/24i

30a/29a
d/29A
e/29B
j/29g

31a/25b
e/25g
f/25h
i/25k

32a/26a
b/26A
c/26C
d/26e
f/26g
h/26k
i/26B

33/30

34/31
a/31a
c/31b
d/31c

35/32
a/32c
e/35a
f/32e
g/32b

38/35

CLASS B

B1

None

B2

2a/2a
c/2c
e/2e

3h/10'h

5A/5A

7B/7B
c/7C
b/7d

10e/10c
j/10'f

11k/11f

12/12

17a/16

26/23

28b/28b

29/24

B3

2f/2g

3a/3a
e/3e

10k/10d

25f/22g

31,31g/25,25i

CLASS C

C1

2d/2d

3/3

6b/6g

6'f/5f

7A/7A

11b/12a
c/11a

12a/13a

15A/9d
B/9c

18a/17a

19/23i

20,20a/17f,17g

21i/17c

22h/19k

27,28/26d,27.

30c/29b
f/29d
h/24f

33a/30b

36a/29c

37/34

C2

3i/3c

15j/9j

C3

17/17

29c/28a

CLASS D

3f

4A
c
d
f
i

5C

6e

6'A
B
a
b
d

8a
b
j

9g

10b
c
h

11d

13
f
h
m
n
o
p
s

14a
d
f

18d

16e
d

19a

20d

21
a
c

22a

23b
c
i

24c

25
b
g

26a
b

29f
g
i

30g

32

34b

TABLE A4.2

Class D male reproductive tract polypeptides of Drosophila melanogaster, classed as such with respect to male reproductive tract polypeptides of D. simulans. For definitions of divergence classes, see Figure 5.1 and Table 5.1, as well as adjacent portions of the text in Chapter 5. For spot map locations and photographic illustrations of individual spots, see Appendix 1 and Figure 3.2.

3f

4'c
d
e
e

5c
d
e
g
i

6h

7c

8b
d

10'
a

9g
f

11b
d

12e

13e
g

14a
b
f

16a

17d
h

19a
b
d
e
l

20
g
j

21a

22a
e
f

23a
b
d
f

24a
b

25c
d
e
f

26i
j

27m
b

28c

30a

TABLE A4.3

Classification of individual male reproductive tract polypeptides of *Drosophila simulans* into divergence classes, with respect to male reproductive tract polypeptides of *D. mauritiana*. All *D. simulans* spots identified in Figure A1.1 (see Appendix 1) and not listed here belong to Class A', except spots 12c, 14A, 17b, 17c, 20c, 20d, 22f, 23e, 29h and 31h, which were nonscorably polymorphic in *D. simulans*. For definitions of divergence classes, see text in Chapter 5-Results. For spot map locations and photographic illustration of individual spots, see Appendix 1 and Figure 3.1

CLASS B'

2a
b
c
e
f
3e
5A
7B
10e
j
k
11k
12
16d
17a
18d
21c
25f
26
29c
31,31g

CLASS C'

C1'

2d
3d
13
n
15A
20e
22h
c
25
h
i
27d
29e
31c
36a
37

C2'

3a
f
14d
17

CLASS D

4A
c
6b
6'a
b
8a
b
c
13d
14a
f
16e
18f
22g
24c
26b
29i
30
32h
35g

TABLE A4.4

Classificaiton of individual male reproductive tract polypeptides of Drosophila simulans into divergence classes B', C' and D, with respect to male reproductive tract polypeptides of D. sechellia. All D. simulans spots identified in Figure A1.1 (see Appendix 1) and not listed here belong to Class A', except spots 12c, 17b, 17c, 20c, 20d, 22f, 23e, 29h and 31h, which were nonscorably polymorphic in D. simulans. For definitions of divergence classes, see text in Chapter 5 - Results. For spot map locations and photographic illustration of individual spots, see Appendix 1 and Figure 3.1.

CLASS B'

2a
b
c
e
f

3a
e

5A

10e
j
k

11k

12

16d

18d

21c

26

29c

34b

CLASS C'

C1'

2d

5C

13s

15a

27

36a

C2'

7B

17

25f

31,31g

CLASS D

3f

4A
a
c
d

6b
c

6'A
B
a
b

8b
c
g

9A
c

10c

11c

13d
n

14A
a
d

15c
f
h
j

16
e

17a

18f

21
a
i

23b

25a
b
c
g

28c

29f
i

30
f

31b
c

32
h