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DROSOPHILA SYSTEMATICS AND BIOCHEMICAL EVOLUTION

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INTRODUCTION

The pendulum swings back and forth, and then back again, and the species problem seems always to be with us. This certainly is not an area of biology about which we dare be complacent, though some would have it so, and a few of those concerned with *Drosophila* are not least among them. Fortunately the field advances whether we will or no, and recent work with *Drosophila* does contribute its share to the eventual decline of controversy over speciation and its mechanisms. These contributions derive most directly from studies of protein evolution, and this review concentrates on that. It does not deal with protein variation within populations, which has been much reviewed recently (35, 52, 62). Some topics, particularly rates of molecular evolution and biochemical systematics, are commented upon when the occasion permits.

MAJOR INVESTIGATIONS

Early Protein Studies

These focused on the potentialities of biochemical approaches for shedding light on evolutionary problems. They emphasized the possibilities for measuring evolutionary change by tracing the pathways of molecular evolution and for analyzing the speciation process. In the latter case they sought direct evidence for the extensive reorganization of gene pools so much emphasized by Mayr (44) and questioned by others (74). The earliest studies were expected, as it were, to explore the routes to answers rather than to arrive at the answers themselves. In spite of that they produced suprisingly potent data.

THE VIRILIS GROUP The first of these pathfinders was a small study of soluble proteins from one strain each of nine members of the virilis group (32). These could not be related to specific gene loci, and even the number of loci contributing to the

data could be estimated only crudely. Nonetheless, the distribution of proteins of identical mobilities among the species could be determined, and since a chromosome phylogeny was available for these species a partial recontruction of ancestral gene pools could be, and was, attempted. The resulting picture was consistent with established evolutionary theory, but not necessarily with the theories of established evolutionists. Genetic change during evolution from the group ancestor had been gradual, with some gain and some loss of ancestral alleles. Changes in some lineages were more extreme than the changes in others, and the members of one cytological phylad diverged less from the group ancestor than did those of the other. The members of the less conservative phylad showed greater differences among themselves than did the members of the less conservative phylad (a mean similarity of 41% compared to 72%). The average species diverged from its immediate ancestor in about 25% of its proteins (range, 16–41%), and the gene pool of the average species comprised 60% of alleles from the group ancester, 25% of alleles from the ancestor of its cytological phylad, and 15% of alleles unique to itself. The subspecies, *D. a. americana* and *D. a. texana,* appeared to have diverged in the same manner and to the same degree as had full species. The phenetic relationships of these species based on their protein similarites bore only a rough resemblance to the established chromosome phylogeny, although Nei (48, 50) thought otherwise. By no means definitive at the time, these data did suggest the following working hypotheses: (*a*) Biochemical evolution does not occur at a constant rate, either for individual speciation events or averaged over several events; (*b*) there is no evidence for a quantitative difference between speciation and subspeciation; and (*c*) protein "phylogenies" are not necessarily good genealogies.

With respect to item *b,* two matters need to be clarified. First, the "divergence" between species measured in this study, and most others since, confounds at least two types of genetic change: that "necessary" for speciation, and that involved in evolution independent of speciation. Thus, when we look at species differences, we are not concerned with averages. When we ask how much change can be attributed to speciation we look only for minimum difference. This will be a single observation for any particular study. When we have found this minimum we still must treat it as a maximum estimate of the change we are trying to assess, because it incorporates both relevant and irrelevant genetic changes. Hence, in reporting results of the various studies I emphasize the observed minimum divergence. The observation in the study of the virilis group that both species and subspecies diverged in the same manner and to the same degree raised immediate questions. The chief one was, of course, whether speciation necessarily involved a reorganization of the gene pool at all. Might speciation instead involve only a few loci that have special effects on the biology of populations. Might it involve changes only in control systems (70)?

THE SIBLING SPECIES STUDY The study of general proteins provided challenging data, but it was desirable that these questions be reappraised using methods that permitted more accurate identification of the genetic systems involved. The utility of assay systems for specific enzymes (31) for this kind of work was demonstrated next (33).

Seven assays were used, six for specific enzymes and one for hemolymph proteins. Sibling species representing six species groups and three subgenera were selected, and a nonsibling near relative was chosen to match each pair. Nine triads, a sibling pair and one near relative, were used. Siblings on the average shared proteins of identical mobility 50% of the time (range, 22–85%), while a sibling and a morphologically distinct member of the same group shared proteins of identical mobility only 18% of the time. The approximately 15% minimum divergence compared surprisingly closely with the approximately 16% minimum divergence noted in the virilis group. Even when the apparent defects of this study were duly acknowledged (small sample size, failure to detect cryptic alleles, complications arising from polymorphism, etc), the parallel was too strong to ignore. There seemed to be no sharp difference between intraspecific and interspecific differentiation. It was concluded that an extensive reorganization of gene pools did not appear to be required for speciation, although it was uncertain just how slight the reorganization could be.

The Bandwagon Era

THE OBSCURA GROUP In other laboratories similar work had been going on briskly. The first detailed comparison of closely related species was between *D. pseudoobscura* and *D. persimilis* (53, 55), long studied by Dobzhansky and his coworkers (23) and, after *D. melanogaster* and *D. simulans,* probably the most widely known sibling species pair. The comparison was based on one population of *D. persimilis* and on several of *D. pseudoobscura.* The study was later expanded. Summarizing the evidence to 1974, Lewontin (43) noted that there was slight or no gene frequency differentiation at 88% of the loci, clear gene frequency differentiation at 8%, and something approaching differentiation at 4% of the loci. He concluded that they probably differed completely at certain loci involving just a special part of the genome and that most of the genome was not differentiated.

Until recently the known range of *D. pseudoobscura* was from the Rocky Mountains to the Pacific Ocean and from British Columbia to Guatemala. Then a population was discovered in the Andes near Bogotá, Colombia (27). When analyzed cytologically and genetically it was discovered to differ from the rest of the species, and it is regarded as rather old, probably resulting from passive transport thousands of years ago (21). In one direction crosses between it and the North American populations produce fertile female and sterile male hybrids (54). When compared electrophoretically with other populations it differs from them by about 20% and has been described as a distinct subspecies, *D. p. bogotana* (4). A 20% difference between this and the nominate subspecies contrasts with the 12% difference between *D. p. pseudoobscura* and *D. persimilis.* When full species differ by less than subspecies the importance of giving weight only to minimum differences is underscored.

Lakovaara and his colleagues have used genetic distance from protein study to evaluate evolutionary relationships among 22 species of the obscura group (41, 42). Enzymes at 21 loci were checked, but only the most common allele at each locus was used in calculating genetic distance. Values ranged from about 14% to about

65%. The greatest similarity, 65%, between *D. pseudoobscura* and *D. persimilis,* was rather different from the approximately 88% observed when populations were compared more thoroughly (43). A dendrogram was included and some of the difficulties in equating this with genealogy were considered.

D. athabasca is one of the North American members of the affinis subgroup of the obscura group, and it is made up of a western and two eastern forms sometimes referred to as semispecies (46). The "western" and the "eastern A" forms are sympatric through an area east and north of Minnesota, but they do not overlap outside this region. Strongly isolated ethologically, with only 10% probability of the gene flow between them, there is limited and equivocal evidence from chromosomes for gene flow even though hybrids produced in the laboratory are fully fertile. Protein evidence indicates that 4 of 17 loci (24%) show evidence of significant differentiation between the two forms. Among populations within forms similarities range from 0.93 to 0.99, with an average of 0.97 (Nei's index), and between the two forms the similarity is 0.90. Rogers' index (61) and Nei's index (49) have been used most commonly for comparisons of this type, and both can be equated roughly with percentages, for present purposes at least, without serious distortion. When allozymes are checked for progeny of wild caught females from areas where both forms were collected together, there is no indication of gene flow between the "western" and "eastern" forms. It was decided that these forms are most probably species, and since the minimum divergences between local populations (7%) and between eastern and western forms (10%) were so nearly the same, speciation required very little gene pool reorganization (34).

THE MELANOGASTER GROUP The best known pair of siblings is *D. melanogaster* and *D. simulans.* Of seventeen loci studied (40), none was fixed for alternative alleles, although one was nearly so, and there was clear gene frequency differentiation at 11% of the loci. The authors made no comment on the significance of these data for the species problem, but the pattern is very similar to that seen for *D. pseudoobscura* and *D. persimilis.*

The bipectinata complex is found mainly in southeast Asia and nearby areas, and its protein variation has been reported by Yang et al (78). They observed allelic distributions at 23 loci over 194 isofemale lines from 30 localities. Four species were involved, two of which were composed of two subspecies each. The subspecies are completely interfertile. Crosses between species usually produce some F_1s, at least in one of the reciprocal crosses, and hybrid males are sterile. Highest similarities, about 92%, were seen between one of the pairs of subspecies, followed closely by the most similar species pair, *D. bipectinata* and *D. malerkotliana,* at 91%. This is, accordingly, another case where speciation and subspeciation show no quantitative differences. Chromosomally the four species share no intraspecific inversions but they have accumulated an average of 9.3 fixed interspecific inversions, and the authors suggest that speciation in this complex was more closely associated with the accumulation of inversion differences than with genetic changes. Not surprisingly, they see no evidence that an extensive reorganization of the gene pool is necessary for speciation. The bipectinata complex provides another case of two species, inter-

crossable in the laboratory, maintaining themselves as separate species in nature. They are separated by ten fixed inversions and no shared inversions, and when sympatric neither inversions nor proteins are found intermingled.

THE NASUTA COMPLEX Samples of three species collected from Australia, New Guinea, Malaysia, Hawaii, and points between were assayed for eight enzymes. Two of the species, *D. albomicans* and *D. sulfurigaster,* can be regarded as siblings. *D. pallidifrons* is an only slightly more distant relative. Three subspecies of *D. sulfurigaster* were included. The results, while not presented quantitatively, indicated that the differences between species were few and in close agreement with degrees of relationship established by cytological and hybridization studies. *D. pallidifrons* was most different, diverging from the others at about 40% of its loci. *D. albomicans* differed from its sibling, *D. sulfurigaster,* at about 25% of its loci, and all strains of all three subspecies of *D. sulfurigaster* were indistinguishable. Thus, species were clearly distinct and subspecies were not (37).

THE MESOPHRAGMATICAS In 1971 a study of six species of the mesophragmatica group was completed. These are primarily Andean in their distribution, from Colombia to Chile. Proteins at 24 loci were assayed, and a cytological phylogeny was available for the group. Using this it was possible to reconstruct the ancestral gene pool in a manner similar to that used for the virilis group. It could then be inferred that 11 out of 24 loci had been polymorphic in the ancestral gene pool as compared, for example, with 12 out of 24 polymorphic in one of the extant species, *D. pavani.* When similarities were calculated using Rogers' index, *D. brncici* and *D. gascici* were closest (0.77); next closest were *D. mesophragmatica* and *D. gasici* (0.67); while a sibling pair, *D. gaucha* and *D. pavani* came in at 0.63. The minimum divergence here, about 30%, is somewhat larger than observed elsewhere. The authors conclude that changes at a large number of loci are not required for speciation, and they suggest that changes in genetic regulatory mechanisms and polygenic systems are the critical factors in the origin of species. The observed genetic variation showed a close relationship to the morphological and cytological variations between species and phylogenetic relationships paralleled the postulated cytotaxonomic relationships. Thus, isozyme variations can be useful diagnostic aids for taxonomy (47).

THE HAWAIIAN DROSOPHILA A new perspective on the species problem was added with the emergence of investigations of the Hawaiian Drosophilidae. Recent reviews describe the evolutionary biology of these forms and note their diversity and the paucity of sibling species among them (14, 17). Among other unusual features are pairs or groups of species for which no detectible differences are observed in the banding sequences of any of the polytene chromosomes. For continental forms, and even for sibling species, this is rarely the case. Such species are referred to as "homosequential" (15).

An early study of population variation included a small comparison between the very similar *D. mimica* and *D. kambysellisi.* The results were not quantified and

the proteins were described only as being very similar (59). In a much larger study the allozymes of about 80 of the Hawaiian species were reported (60). These represented a wide cross section of Hawaiian groups, including most of the major types. Homosequential species were compared in lieu of siblings, and divergence was measured as the proportion of the gene pool that was unique between species. It ranged from 26% to 80% for homosequential species, with a mean divergence of 55% and a variance of 133. A random sample of an equal number of species pairs gave a range of 44% to 87%, a mean divergence of 66% and a variance of 15. Thus, randomly chosen pairs are less similar (34% compared to 45%) than are homosequential species, homosequentials are somewhat less similar (45% compared to 50%) than siblings, and random pairs of Hawaiian species are considerably more similar (34% compared to 18%) than the average nonsiblings reported elsewhere (33). The minimum divergence (26%) is comparable to that seen in the nasutas, the mesophragmaticas, and so on. When substitutions between homologous loci were calculated the homosequential species gave a value of 35%. This contrasts sharply with the value of 12% for complete or near complete substitution between *D. pseudoobuscura* and *D. persimilis,* and of 11% for *D. melanogaster* and *D. simulans* (43). The authors conclude that the large range and high variance of the homosequential sample emphasize the essential lack of parallel between gene sequence stability and genic similarity, and that speciation among Hawaiian *Drosophila* is not accompanied by a major reorganization of the gene pool.

Johnson and his collaborators provide a detailed look at a more compact group of Hawaiian species (36). This is the planitibia subgroup of the Hawaiian picture wing *Drosophila.* These are among the world's largest *Drosophila,* most of them are single island endemics, and usually each is restricted to a single Hawaiian volcano. A cytological phylogeny is available for them, although the direction of evolution within it cannot be specified unambiguously. For sixteen species the similarities ranged from 0.11 to 0.99, with a mean of 0.44 (Roger's index). These values do not differ greatly from those of the sibling species study (33), except that the minimum divergence is much less, here approaching zero. Out of 120 comparisons possible between 16 species, 4 show a divergence of less than 10%, 11 show a divergence of less than 20% and 18 (15% of the comparisons) show a divergence of less than 30%. The authors conclude that the four cases of unusually high similarity, from 0.96 to 0.99, probably resulted from allopatric speciation occurring through fragmentation of a widely distributed population by the appearance of strong isolating barriers (e.g. the rise of sea level to separate formerly continuous land masses, disruption of host ranges by climatic change, etc). They argue that one would expect minimal divergence in that case unless the isolates were subjected to strong differential or directional selection. They regard the most dissimilar species as most likely to have been founded by a few individuals, through interisland colonization for the most part, and hence to have undergone a "flush" and "genetic revolution" as postulated by Carson (10–12). Regardless of how differences are explained, these are the first cases where almost no difference is seen between full species.

Comparing the pattern of genetic relationships shown by the allozyme data with the cytological phylogeny, only a rough congruence is seen. Some subclusters agree, but for the most part the patterns in one set are a scrambled version of those in the other. It is true that in neither case can the direction of evolution be unequivocally specified, more nearly so for the chromosomes than for the enzymes, but the disagreements are not such that they can be reconciled by any simple reorientation of a diagram. The authors conclude that the rate of divergence between species varies greatly depending on the kind of speciation event involved, and similarity values have small phylogentic significance. Whether or not this interpretation is correct, the conflict between allozyme and chromosome results casts doubt on the reliability of protein data for phylogenetic research.

D. setosimentum and *D. ochrobasis* are members of the adiastola subgroup of the picture wing group and are near sibling species, with males distinct but living females not easily separable. They are widely distributed in wet highland forests on the island of Hawaii, show altitudinal and breeding site differences, and both are found together at some localities. Chromosomes and allozymes show significant differences between species and between some of the local populations within species. There is evidence for the differentiation of a low altitude ecotype, and at one locality (Mawae) recent hybridization has been postulated to account for unusual variation observed there (13). At one area (Kahuku Ranch) natural hybridization was detected, with 4 unusual specimens of 180 examined (2.2%), one an F_1 and three backcross hybrids. So it is possible that gene exchange can occur between these species. The gene pools, however, give no evidence of merging. The species pair is regarded as representing a stage that may be rather common for allopatric speciation in general, that is, where two separated populations come back into contact after nearly completing "integration" of "new" gene pools. Reciprocal gene flow is thought to be insignificant in this case because natural selection works against inferior F_2 and backcross combinations (16).

A quantitative assessment of the differentiation, both for chromosomes and for allozymes, has been made (13, 18). *D. setosimentum* could be divided into a set of six highland populations, the "main body" of the species, and one Kona population; *D. ochrobasis* could be divided into two southern populations, the "main body" of the species, and one population from Ohu isolated at the north end of the island. Comparisons within *D. setosimentum* showed similarities of 0.98 (Nei's index) within the main body of the species and 0.92 with the Kona population. Within *D. ochrobasis* the similarity was 0.98 within the main body of the species of 0.87 with the isolated Ohu population. Comparisons between species, however, show that while the main bodies of the two are rather distinct (0.79), the isolated Ohu population is very similar (0.98) to the main body of *D. setosimentum.* Chromosome comparisons give somewhat different results, with a mean intraspecific similarity of 0.96, compared to 0.94 for the allozymes; and for interspecific comparisons a mean value of 0.69 is seen with chromosomes, compared to 0.90 for allozymes. The allozymes are much less precise in distinguishing these species than the chromosomes. This is the reverse of the case for homosequential species, where 100%

similarity of chromosomes contrasts with only 20–74% similarity for allozymes. The authors conclude that for these species, as for *D. silvestris* and *D. heteroneura* (36), speciation has occurred with only a small amount of allozymic reorganization. They suggest this is a result of the recency of speciational events, and that speciation may involve regulatory genes which usually are not revealed by electrophoretic data.

THE WILLISTONI GROUP The biology of these species is reviewed by Dobzhansky and Powell (24). Those of greatest interest here, *D. willistoni* and its siblings, including the semispecies of *D. paulistorum* (26), comprise one of three major clusters among the 25 species that make up the group (73). There are six siblings, three of which consist of a pair of subspecies each and one of which consists of six semispecies. The siblings are nearly indistinguishable morphologically and their distributions overlap broadly. In many areas three or four siblings are collected together, but the range of *D. willistoni,* from southern Florida and central Mexico to northern Argentina, is the greatest of the six. Sexual isolation between these species is quite strong and only occasional sterile hybrids are produced in laboratory crosses. Some fertile hybrids have been reported (76, 77), but they do not seem to appear regularly or generally (25). The subspecies show no sexual isolation in the laboratory, but at least one of the reciprocal crosses between them produces sterile males, and they are largely allopatric (5, 6).

The semispecies of *D. paulistorum* are not distinguishable morphologically (51). With few exceptions, when crossed in the laboratory they produce fertile female and sterile male progenies. A range from little to almost complete sexual isolation is exhibited, depending on the cross (9, 22). They thus show incomplete reproductive isolation from each other. Transitional strains also exist which partially bridge the reproductive gaps, at least in theory, especially between the Centroamerican and the Andean-Brazilian semispecies. In many cases two or three semispecies exist sympatrically without losing their identity. The area of highest sympatry, northern South America, is also the area of highest sympatry for the sibling species themselves (65).

The first protein comparison appeared in 1970 with a report of genetic differentiation at 14 loci among four of the siblings (3). Other reports over the years have filled out the story, and a complete report is given by Ayala and his collaborators (7). More limited discussions are also available (1, 2, 24). Dealing with averages, and using Nei's index as a comparator, it is shown that a graded series of similarities exists among these forms, from local populations to nonsibling species. The local populations range in similarity from 0.91 for transitional strains of *D. paulistorum* to 0.99 for local populations of *D. willistoni,* with a mean for local populations of 0.97. Between subspecies the values were 0.81 and 0.78 with a mean of 0.80, and for semispecies the mean was 0.80 for similarities ranging from 0.81 to 0.96. For sibling species the range was from 0.28 to 0.80, and the mean was 0.56. The nonsibling comparison was made between the siblings and *D. nebulosa* and gave a mean similarity of 0.35. The minimum divergence between species, approximately 20%, is in the usual range for continental species, and this range is overlapped by that for subspecies, which is also about 20%, and for semispecies, which ranges from

about 5% to 20%. These data are in nice accord with other results, and they extend the series first described in the sibling species study (33) to the level of the local population. The series of means now stands: local populations, 0.97; subspecies, 0.80; semispecies, 0.80; sibling species, 0.56 (compared to 42% for the sibling species study); nonsibling near relatives, 0.35 (compared to 20% for the sibling species study); and from the sibling species study only, somewhat less than 6% for distantly related species.

The unique thing about this study is, of course, the inclusion of two different subdivisions between local populations and species. The authors regard subspecies and semispecies as different evolutionary levels, using the ranking: geographical populations, subspecies, semispecies, sibling species, morphologically distinct species. Subspecies are regarded as the "first" stage of species formation, representing a type of allopatric differentiation that might give rise to species upon sympatry, according to the model of geographic speciation. Because of their often nearly complete reproductive isolation, the semispecies of *D. paulistorum* are regarded by them as representative of the "second" state of speciation, when reproductive isolation is being completed. Since substantially the same amount of divergence exists between subspecies and between semispecies, on the average, it is concluded that a considerable amount of genetic differentiation occurs during the first stage of speciation but that little is needed during the second stage. In fact, however, the minimum divergence, the operational value for evaluating these results, is only about 5%, and that is for semispecies. The minimum value for subspecies, about 20%, is much higher. Hence, the conclusion of a first stage of considerable divergence, and a second stage of little divergence, is not really supported by these data.

No chromosome phylogeny is available for these species. The chromosomes have been repatterned extensively, with *D. paulistorum,* as the extreme example, exhibiting at least 85 rearrangements of its own (38, 39). Ayala and his coworkers provide a dendrogram constructed from the protein data following one of the "maximum parsimony" approaches (29), and hence having the limitations that result from such procedures. The tree is, nonetheless, a very fair approximation of that derived by Spassky et al (65) from morphology, behavior, and so on. An interesting relationship is observed for the pairs of subspecies, each of which consists of one widespread form and one of narrower range. In each case, the more widely distributed subspecies (the main body of the species, as it were) is more similar to the other siblings than is the narrowly distributed form. It is speculated that the less conservative subspecies is an offshoot of the main body of the species; it has adapted to new habitats and hence has diverged most. Or, they suggest, the less conservative subspecies may have originated through colonization and so may give evidence of a "founder effect" (44) or a "population flush" (10). However that may be, the evidence is thought to indicate an inconstancy of the rate of genetic change among different evolutionary lineages.

THE MULLERI SUBGROUP Two species pairs have been investigated closely: *D. aldrichi* and *D. mulleri,* and *D. mojavensis* and *D. arizonensis* (79). *D. mojavensis* is divided into two races, and one of these is further subdivided into two subraces.

The biological relationships are somewhat parallel to those among the siblings and semispecies of the willistoni group, and the closeness of the pairs can be appreciated by the fact that *D. mojavensis* was originally described as a subspecies of *D. mulleri.* Crosses between *D. aldrichi* and *D. mulleri* produce either sterile hybrids or none at all. *D. arizonensis* and *D. mojavensis* are fully interfertile. Nevertheless they remain cytologically separate entities, and in the cytological phylogeny the members of pairs are phylogenetically closer to each other than to members of the other species pair (75). The two races of *D. mojavensis* reflect both chromosomal and morphological differences, they are allopatric, and they are completely interfertile. The subraces of *D. mojavensis* are allopatric, one in Arizona and Sonora, the other in Baja California and the islands of the Gulf of California, and they differ in the species of host cactus they exploit. The Arizona and Sonora populations of *D. mojavensis* are sympatric with *D. arizonensis.* The strains of *D. aldrichi* and *D. mulleri* were from Texas, where the two species are sympatric. Similarity means for these forms provide another nicely graded series: populations, 99% subraces, 97%; races, 88%; sibling species, 84%; and nonsibling species, 70%. The values for siblings and nonsiblings are considerably higher than those from the willistoni group, which were about 56% and 35% respectively. The minimum difference, about 12% for *D. aldrichi* and *D. mulleri,* is in the usual range. It is also less than the 20% minimum difference between *D. arizonensis* and *D. mojavensis,* even though this pair shows much weaker post-mating isolating mechanisms, being fully fertile in crosses, than *D. aldrichi* and *D. mulleri* which produce sterile hybrids or none at all. This is a good example of the failure of reproductive divergence to serve as an index of evolutionary divergence. The author concludes that: (*a*) the hybrid sterility or breakdown observed in species crosses may be the result of changes in a rather small proportion of the genome; (*b*) degree of reproductive isolation is a less reliable index of the genetic change involved during early evolutionary divergence than is the degree of ecological differentiation; and (*c*) while there is a regular accumulation of genic difference as speciation procedes, most genetic change appears only after genetic isolation is insured.

A rather different type of study has been carried out by Richardson and his collaborators (56–58). Stressing that single electromorphs need not represent single alleles, electrophoretic mobilities were analyzed and compared by analysis of variance. A distance measure based on average electrophoretic mobilities was defined for investigating evolutionary relationships. Seven assay systems were employed with species of the mulleri complex whose cytological relationships are specified (75). Variation among species was greater than within species and that among sibling species clusters was greater than that within clusters. Grouping on the basis of average relative mobility yielded results similar to, but not identical with, those given by the chromosomes. The authors conclude that such clusterings can be taxonomically useful in a gross way but that uncritical use of mobility can lead to difficulty (57); modes of evolution are usually different among different loci (56); and the taxonomic diversity of larval substrates and the electrophoretic diversity of *Drosophila* populations using them appear to be associated (58).

THE VIRILIS GROUP AGAIN The virilis group originated most probably in warm temperate Asia somewhat more than 15 million years ago. It subsequently split into two groups, as is indicated by the chromosome phylogeny. The descendents of one, the virilis phylad, are associated so far as is known with willow in riparian communities of the temperate zone, the probable ancestral habitat; those of the other group, the montana phylad, are associated with aspen and alder near lakes and streams and on mountainsides at higher altitudes, higher latitudes, or both. At present the group has a Holarctic distribution, the new world having been colonized by at least one population from each of the phylads, apparently shortly after their founding (73). Thus, by comparing phylads we can check relative rates of evolution, and from biogeographical evidence we can make "ball park" estimates of absolute rates of biochemical evolution.

The first update on proteins came in 1972 with a report of assays covering a wide cross section of laboratory strains from nine species and dealing with 11 loci (71). Genetic changes during speciation averaged 12% in one phylad and 27% in the other, and these amounts were in the same range as those seen for intraspecific differentiation among the same populations. The average speciation event involved a reduction of genetic variability, new species budding off, as it were, from stem populations that retained the reservoirs of genetic variability. The phylads evolved at different rates. The one that exploited novel ecological opportunity evolved at approximately twice the rate of the one that remained in the ancestral habitat (willows). There was a heterogeneous pattern of change at the individual loci, with different loci changing at different rates in different lineages and parts of lineages. It was concluded that: (*a*) there was no evidence for an extensive reorganization of the gene pool during speciation, (*b*) there is no constant rate of biochemical evolution, (*c*) there is evidence for the maintenance of polymorphism through natural selection since polymorphisms had persisted at about 25% of the loci since the founding of the group (72).

The most recent version of these data has not yet been published, although there is some prospect of that (L. H. Throckmorton and J. L. Hubby, in preparation). The number of strains assayed has been increased five-fold, covering large new samples from many new localities for almost every one of the eleven species available. The results substantially agree with and enlarge upon previous ones. The gene pool of the average species consists 62% of ancestral alleles, 31% of phylad specific alleles, and 7% of alleles unique to itself. This compares favorably with the 60%, 25%, and 15% values from the general protein study (32). The proportion of unique alleles per species ranges from 2.2% to 14.4%, giving by that criterion a minimum divergence of about 2%. The similarity between the closest species, 80% for *D. montana* and *D. lacicola,* approximates that of 82% for the most different of local populations, so by that criterion also, interspecific and intraspecific differentiation do not differ markedly.

Having added 1.3 compared to 2.2 alleles per million years of evolution, the ecologically conservative (willow) phylad changed at 60% the rate of species evolving in new habitats and over diverse terrain. The average speciation event still

involved a reduction in genetic variation, diverging populations gaining 0.8 times as many alleles and losing 2.4 times as many as the stem population. There was a more than five-fold difference in evolutionary rates among the different loci, with some loci even evolving more rapidly in the conservative phylad, although the total gene pool of those species was changing more slowly. Polymorphism was perpetuated at 56% of the loci from the time of founding of the group (for more than 15 million years), and only partial agreement was obtained between the protein phenogram and the cytological phylogeny. The protein data did partition the group into the same two phylads as did the chromosome data, but the details within the phylads differed greatly.

The obvious and by now tedious conclusions follow: (*a*) There is no evidence for an extensive reorganization of the gene pool during speciation. (*b*) There is no evidence for a usable molecular clock operating in these proteins. The rate of molecular evolution is apparently related most closely to the opportunity open to the evolving population. (*c*) Most speciation events in the group involve a reduction of genetic variability. (*d*) The dendrogram based on "genetic distance" is not a good phylogeny.

Still More Genetic Variability

REFORMATION AND ENLIGHTMENT Our first paper on protein differences introduced the topic of cryptic variation; that is, of genetic change not disclosed by electrophoresis of proteins, and specifically of alterations of proteins that did not result in a net charge change for the molecule (32). The topic turned out to have much in common with the weather. Lots of people talked about it, but no one did anything about it. On the basis of early evidence it seemed that about two thirds of the actual variation might be disclosed by electrophoresis. As time passed the estimated fraction grew smaller. The consequences of crypic variation for comparative studies became more serious.

The problem was finally explored by adding a discriminating technique to the standard procedure for screening alleles. Heat denaturation was used with the *xanthine dehydrogenase* (*XDH*) locus of the virilis group. Almost twice as many alleles were discovered, both within populations and within species, and across the group as a whole the number of alleles increased from 11 to 32. On the basis of small samples, geographically close populations were found to have three of four electromorphs in common but only one of nine "thermoelectromorphs". The average similarity between species dropped to 35% of the value based on electromorphs alone (8).

Suddenly the outlook was bleak if not actually grim. The specter of still more genetic variation had always been with us. Some had wished to ignore it. Now it turned out to be more material than had been expected. It was necessary to take stock at this turn of events.

TWELVE YEARS OF WORK DOWN THE DRAIN? The news was received with astonishment, some resentment, and some disbelief; but additional studies verified

the phenomenon. At the *octanol dehydrogenase* (*ODH*) locus in the virilis group heat denaturation disclosed 2.6 times as many alleles as before observed, and similarities between species dropped accordingly. The major electromorph of this study was one that was shared by all species of the group and usually it was the predominant allele, having a frequency of 85% or higher in eight of the ten species investigated. After heat sensitivity studies there was still one predominant allele with this mobility, but its importance was much reduced. It was present in 17 of 34 populations investigated, and still present in all species. Samples were too small to tell much about frequencies within species (63).

Alleles at the *esterase-6* locus in *D. melanogaster* increased in number from four to seven when heat denaturation was used (19). In this case no allele was unique to any one of the six populations but the genetic similarity between populations decreased. One allele remained most common in different populations, but two different alleles were now second most important in some of them. The sharp differentiation seen for heat sensitive alleles between populations for *XDH* in the virilis group was not seen here.

The *esterase-5* locus was reinvestigated in gels of different concentrations and with different buffer systems. Six different alleles were revealed within the most frequent class and three in the second most frequent one, to make this a highly heterogeneous system (45). Another study of *D. pseudoobscura* employed four different electrophoretic conditions and a heat sensitivity test on *XDH*. Thirty-seven alleles were revealed where only six had been observed before, and *D. p. bogotana* was shown to be different from *D. p. pseudoobscura* at this locus where before they had been thought to share the same most common allele (64). A detailed comparison was also made between *D. p. pseudoobscura* and *D. persimilis* for cryptic variation at the *XDH* locus (20). Sequential electrophoresis was used with different gel concentrations and different gel buffers. Five alleles became twenty-three. Most were unique or nearly so to the populations where they were seen, but the same allele predominated in all populations. Interpopulation genetic similarities (Nei's index) that had ranged from 0.966 to 0.995 for electromorphs now ranged from 0.858 to 0.956, a drop of about 10%. The number of alleles shared at this locus between *D. p. pseudoobscura* and *D. persimilis* dropped from 25% to 6%, and the similarity value fell from 0.55 to 0.03. The most common alleles in the two species are quite different. The author concludes that previous interspecific comparisons now bear reinvestigation. Until now the evidence indicated that marked genetic change need not accompany the appearance of reproductive isolation, but that conclusion may have been premature.

SPECIES AND SPECIATION

The species controversy, in its most recent incarnation, materialized in the early 1960s. In part it was born of reaction to the extreme views of Mayr (44) and particularly to his opinions regarding the cohesion of the gene pool and the universality of the geographic mode of speciation. According to his view, speciation

entailed an extensive reorganization of the gene pool, and the only situation in which a gene pool can be completely reconstituted genetically, while all of its elements remain well integrated and coadapted, is geographic isolation.

As I pointed out at the time (74), the evidence supporting this position was meager and equivocal, and it by no means required the conclusions that Mayr insisted upon. Legitimate alternatives existed. His view of speciation was in fact a set of hypotheses resting upon yet other unproven hypotheses. Scientists should not accept opinions uncritically, as many of our contemporaries were doing, but rather should try to show where the truth lies. Biochemical methods were available with which to launch a direct assault upon the problem. After these methods had been exploited to the full we would be nearer to an appreciation of the facts.

At least some of these methods have now been in use for over a decade, and not all of the conclusions from them have been made obsolete by the most recently perceived variability. The genetics of the speciation process remains unsettled, nevertheless. The conclusion of Coyne (20) that previous interspecific comparisons must be reinvestigated is quite proper. Until a thorough reinvestigation is completed we will not *know* whether the situation at the *XDH* locus in *D. pseudoobscura* and *D. persimilis* exposes a general pattern or just a quirk in the data. The heterogeneities among recent results are such as to indicate the latter, but we cannot be certain. The data from the *XDH* locus in the virilis group indicated as much intraspecific divergence as interspecific divergence, suggesting that new information might only shift the mean similarity downward without changing the general pattern. And it also suggested that much of the total variability might be unseen by natural selection. That is, it seemed to have the random distribution that might be expected for neutral alleles or for a mixture of selected and neutral alleles (8). But when Cochrane sought randomness in the distribution of heat sensitive alleles in *D. melanogaster* —randomness of the sort suggested by the data from the virilis group—he did not find it (19).

The data from the *ODH* locus in the virilis group are also pertinent. There one electromorph, common and at high frequency in all species of the group, was discovered to consist of six alleles, one of which was present in all species and apparently predominant in at least one species from each of the phylads. Again, the samples of individual species were small, but with one allele distributed throughout the group it does not seem that speciation necessarily or often completely replaced alleles at this locus. This contrasts with Coyne's data from the obscura group. So, for the present, no common pattern is yet evident, and there is much to indicate that earlier work was not badly misleading.

As documented in the preceeding pages, investigators have been nearly unanimous in agreeing that there is no evidence for extensive reorganization of gene pools during speciation. The basis for that conclusion is easy to see. The concept elicits a vision of speciation that entails adaptive changes at many loci, with these changes precipitating others until a swelling cascade of revision overtakes most loci—a molecular domino effect that leaves little sacred and less untouched. But when the gene pools were assayed, in many different species from all parts of the world, no

eivdence of such a cataclysm appeared. True, some siblings may differ at well over 30% of their loci, and that sounds like a lot. But the consequences of high levels of heterozygosity, the observed high levels of intraspecific differentiation in some cases, and the extremely low levels of interspecific differentiation in others, place this in perspective. Against the yardstick of infraspecific differentiation, speciation does not stand out as a special process. And a very strange situation must pertain if it is to appear otherwise when the data from cryptic variation are in. The new variants would have to be predominantly disposed so as to sharpen the differences between species and to minimize those within them, which would imply a peculiar and positive relation between the speciation process and cryptic alleles. Adaptation would then involve chiefly electromorphs while speciation would involve chiefly the cryptic variants within electromorphs. Such a case seems very improbable. Why should we expect extensive reorganization of gene pools to be visible only at the level of variability within electromorphs?

Therefore, while recent work raises uncertainties concerning the details of population genetics and the details of genetic differences between species, it does not negate the conclusions reached to date. These cannot be discarded until strong positive evidence is mustered against them. And while the probability of discovering such evidence is not negligible, it is small. We can expect to discover the dynamics of populations in even more discriminating detail—especially the relationships of selected and nonselected alleles. And we can expect still to be left with no evidence for an extensive reorganization of gene pools and with every indication that changes at a few loci are sufficient for speciation.

MOLECULAR CLOCKS

In principle the discovery of extensive additional variation, variation unassessed in the major studies to date, casts doubt on the reliability of conclusions about molecular clocks and rates of molecular evolution. The doubt is of the same sort raised with respect to the genetics of speciation, and its significance must be questioned on the same grounds. Let us use the data from the virilis group as a case in point. It will be recalled that the two phylads evolved at different rates. The one remaining in its ancestral habitat added fewer alleles than did the one evolving in new environments. If the "within electromorph" alleles are to change this evidence to allow the molecular clock to keep accurate time, they must do it by very nicely countering the differences recorded to date. Most cryptic variants must be added to the conservative phylad's gene pools. But why should populations evolving within ancestral habitats evolve cheifly through "within electromorph" variation while other populations do not? Present evidence increases the uncertainty regarding the accuracy of some estimations of evolutionary rates, but it does not invalidate the general conclusions derived from them. Data from Hawaiian *Drosophila* (36), the willistoni group (7), and the virilis group (32, 71, 72) indicate an inconstancy of evolutionary rates. (The most decisive data are from the virilis group.) New evidence must be abundant and very one-sided if this view is to be changed.

BIOCHEMICAL SYSTEMATICS

The problems of systematics are so diverse that some selection must be made among them. It happens that, aside from speciation, phylogeny is the problem that attracts the interest of most investigators employing protein analysis. Since it most interests me also (66–69), and since protein phylogenies are being promoted quite ostentatiously now, I emphasize phylogeny here. The appropriate questions are, "Can adequate phylogenies be obtained from protein data using present analytical methods?" And, "Why or why not?"

Phylogeny means many things to many people, and I use it in the sense of "genealogy," to refer to the sequence of origin of different taxa during the course of evolution. In studies of the proteins of *Drosophila* two general procedures have been employed to produce "phylogenies." One uses distance measures of some sort combined with one or another of the conventional phenetic clustering methods. The other uses one of the so called "maximum parsimony" methods. The first of these is unsound because, among other things, its validity would depend on the accuracy of molecular clocks. Since molecular clocks do not operate accurately, methods relying on them are unsound. Occasionally, e.g. (6), it is asserted that even though there is some heterogeneity of evolutionary rate, over the long term the average is constant, and hence usable. But even if rates were constant over the long term, so long as they are heterogeneous over the short term they would be unusable for constructing phylogenies. It is a question of what one can and cannot do with averages. One cannot, on the basis of the average height of the United States citizen, predict the height of the next person to enter a room. Neither can one, on the basis of an average evolutionary rate, assert the position, or the length, of any particular internode on a dendrogram. The different sections of a dendrogram are as much individual observations as are the heights of individuals, and they cannot be specified by an average.

The "maximum parsimony" methods are equally unsound, partly because many of them use the inference of constant evolutionary rates as an expedient for "rooting" their trees (30), but mostly because, in fact, they are not parsimonious at all. These undertake to produce trees of minimum length, and they try to arrange the different branches in such a way that the fewest possible character state changes occur over the entire tree or some sections of it (29). The difficulty here, of course, is that evolutionary theory has never proposed that evolution proceeds by a minimum number of steps. At most it requires that changes from one generation to the next be by small steps—and not necessarily by the smallest possible steps. There is nothing whatever in evolutionary theory to suggest that a collection of species should have evolved one from the other, or from annectant ancestors, over the shortest possible pathway. These methods, far from being maximally parsimonious, are completely nonparsimonious. They, and the genetic distance methods, force sets of data to conform to preset bias, very much in the tradition of the Biogenetic Law and Orthogenesis. It is not surprising that while some *Drosophila* studies (32, 36, 57, 71, 72) find protein "phylogenies" incompatible with available information, some (47) find that they match rather well. Protein "phylogenies" may or may not

approximate the real genealogy for the species. But they are only as sound as the analyses that produce them. And since there is no guarantee that they must match genealogy, or even that there is good reason to think they will, they have only limited usefulness.

CONCLUSION

Three topics have been dealt with, two with dispatch and one more thoroughly. The *Drosophila* literature on the genetics of speciation, including the most recent disclosures of unanticipated variation, provides no evidence for an extensive reorganization of gene pools during speciation. Hence speciation mechanisms involving only minimal genetic change are not precluded on those grounds. This evidence also contradicts widespread faith in molecular clocks and in the usual analytic methods for deriving "phylogenies" from proteins.

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