Perspectives

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Twenty-Five Years Ago in GENETICS: Electrophoresis in the Development of Evolutionary Genetics: Milestone or Millstone?

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WHEN I entered TH. DOBZHANSKY's laboratory as a graduate student in 1951, the problematic of population genetics was the description and explanation of genetic variation within and between populations. That remains its problematic 40 years later in 1991. What has changed is our ability to characterize variation at the genic and nucleotide level and, linked to the ability to give detailed descriptions of variation, the development of a theory of population genetics that takes into account the full implication of historical ancestries in real populations.

In the 1950s and before, observations of genetic variation were confined to two sorts of data. On the one hand, some morphological variation was a consequence of the segregation of alleles at single loci in classical Mendelian fashion and these could be studied either by direct observation of phenotypes in nature or, if there was complete dominance of one allele, by test crosses in species that could be bred. The blood group and hemoglobin polymorphisms in humans (BOYD 1950; ALLISON 1955), shell markings in snails (LAMOTTE 1951; CAIN and SHEPPARD 1954), wing patterns in Lepidoptera (FORD 1953), and rare recessive mutations in Drosophila were the materials of such studies. Although they provided individual model systems for the study of evolution in action, it was not clear how general a picture of genetic variation they represented. Moreover, it was notoriously difficult to establish differential fitness for different genotypes and up to the present no convincing selective story has been given either for snail shells or human blood groups.

In contrast, it was possible to measure large fitness differences between genotypes when whole chromosomes were the unit of observation. There were extensive studies by DOBZHANSKY and his school of inversion polymorphism in Drosophila (summarized in LEWONTIN *et al.* 1981). But the largest single form of data was on the fitness distributions of whole chromosome homozygotes and heterozygotes in Drosophila using some variant of MULLER's *ClB* technique [see LEWONTIN (1974) for a summary]. There was universal agreement that genomes totally homozygous for one or more chromosomes were on the average lower in viability and fecundity than were random heterozygotes. The problem was that the observations could not be interpreted at the gene level. Was the inbreeding effect the consequence of a few nearly recessive deleterious alleles carried by each genome as a consequence of the constant rain of mutations, or was it the consequence of homozygosity at very large numbers of loci that were normally heterozygous, held in heterozygous state in natural populations by some form of balancing selection?

This same problem was at issue in corn breeding. The entire hybrid corn industry depended on the fact that *some* inbred lines, when crossed, produced hybrids with higher yields than the open-pollinated population from which the lines were drawn. Was this because heterozygotes at individual loci gave higher yields than either homozygote (overdominance) or was it simply the effect of covering the effect of partly dominant deleterious genes? If the former, then the inbred-hybrid method was optimal for producing high-yielding corn. If the latter, then a program of selection would be best. Whether in studies of natural populations or in agricultural genetics, the problems could not be solved because no method existed for identifying genotypes at individual segregating loci unless allelic differences at single loci led to clearly distinguishable phenotype classes. So it was impossible

This is emphatically not meant to be a review of the immense literature on the subject, but a commentary on salient points. Thus, the literature actually cited is spotty, unsystematic, and in some ways unrepresentative of the vast corpus of knowledge in the field.

to describe the genetic variation for the genome as a whole in populations, nor to make inferences about the effects of single allelic substitutions. Yet, these were the very data that population genetic theory demanded for making causal explanations.

Needless to say, nothing could be asserted about the genetic differences *between* species either. Under the circumstances, it is not surprising that evolutionary geneticists were divided into opposing schools with more or less uncompromising views of the truth. DOBZHANSKY and his followers belonged to what he called the "balance" school (DOBZHANSKY 1955), holding that every individual in a sexually reproducing population was heterozygous at most or all of its loci. DOBZHANSKY's opponents were derogatorily called by him the "classical" school, whose most influential spokesman was H. J. MULLER, a school that believed nearly all loci to be essentially homozygous, with rare deleterious mutations segregating to produce a "genetic load" (MULLER 1950). Population genetics seemed doomed to a perpetual struggle between alternative interpretations of great masses of inevitably ambiguous data.

All the while, molecular and biochemical genetics was developing a picture of the relationship between genes and proteins that could provide a way out. Putting aside redundant nucleotide substitution at silent sites, a point mutation in a coding sequence would result in an amino acid substitution in a coded protein and, in principle at least, that substitution could be detected unambiguously by the analysis of the protein. In practice, however, the application of this knowledge to population genetics seemed hopeless. No on could seriously propose the amino acid sequencing, by the laborious chemical methods available, of even one protein from hundreds of single individuals sampled from a population, nor would single individuals provide nearly enough purified protein for the analysis. What was required was some technique that would be sensitive enough to amino acid sequence variation to detect that variation in single individuals as small as Drosophila and be applicable to large population samples with reasonable effort. In 1966 two laboratories, one in Chicago and one in London, independently published experimental results that apparently solved the problem (HARRIS 1966; HUBBY and LEWONTIN 1966; LEWONTIN and HUBBY 1966), initiating 20 years of intensive investigation of protein variation in natural populations by hundreds of laboratories. (The latter two papers were published in GENETICS 25 years ago this month.)

The method introduced by HARRIS, HUBBY and LEWONTIN was gel electrophoresis of proteins. It was already well known that single enzyme species could be visualized in unpurified extracts from single individuals by cytochemical staining, and that single amino acid substitutions could change the pI of a protein sufficiently that it would move at a detectably different rate in a charge field. As a consequence, electrophoretic variation in proteins was already known to exist. A survey of the literature by SHAW (1965) found 16 different enzymes in 20 species of organisms from flagellates to mammals for which evidence of electrophoretic variation existed. It remained only to adapt this method to large-scale surveys of individual genomes from natural populations and to demonstrate that any electrophoretic differences observed did indeed mendelize.

The first results were startling. Of 18 proteins (loci) surveyed by LEWONTIN and HUBBY in five natural populations of *Drosophila pseudoobscura*, an average of 30% were polymorphic (*i.e.*, had more than one allele present at a frequency greater than 1%) within populations and the average heterozygosity was 11.5%. For the human population studied by HARRIS, the comparable values were 30% polymorphic and 9.9% heterozygosity. The extraordinarily high genetic variation seemed, on the face of it, to support definitively the views of DOBZHANSKY and WALLACE on the ubiquity of genetic variation segregating within species.

The publication of these results in 1966 had an immediate effect on experimental population genetics and research on species comparisons. Here was a technique that could be learned easily by any moderately competent person, that was relatively cheap as compared with most physiological and biochemical methods, that gave instant gratification by revealing before one's eyes the heritable variation in unambiguously scoreable characters, and most important, could be applied to *any* organism whether or not the organism could be genetically manipulated, artificially crossed, or even cultivated in the laboratory or greenhouse. It is little wonder that there was a virtual explosion of electrophoretic investigations. A comprehensive literature search made by NEVO, BEILES and BEN-SHLOMO (1984) 18 years after the first experiments were published, found studies of intraspecific variation in 1111 species, with an average of 23 loci and 200 individuals per species examined. The range of organisms that have been studied to date includes bacteria, fungi, vascular and nonvascular plants, many phyla of invertebrates, especially insects and mollusks, and vertebrates from fish to humans. While this immense collective body of work is sometimes derisively referred to as the "find 'em and grind 'em" school of population genetics, it established a general fact about genetic variation that could not have been otherwise determined. A typical species population for most organisms is polymorphic for about 1/3 of its loci that code for enzymes and other soluble proteins, and an average individual is heterozygous (or, for haploid

organisms, has a probability of nonidentity with another individual) at about 10% of its loci. Of course, there are differences among species. A few, like the cheetah, are virtually completely monomorphic. Fossorial animals as different as moles and mole crickets have very low variation. Vertebrates are somewhat less variable than invertebrates, probably because of their generally smaller population sizes, with average polymorphisms of about 25% and heterozygosity of 7%, but the difference from invertebrates is not large.

In addition to the purely population genetic applications, gel electrophoresis became a widely used tool for species comparisons. Morphological differences between species involve unknown numbers of genes and, to some extent, are not genetic but a consequence of different developmental environments. Electrophoretic phenotypes ("electromorphs") are discrete differences, almost certain to be a consequence of single gene differences, and are immune to developmental variation. As a result, a widespread and often uncritical use was made of electromorphic characters for systematics. It is not always appreciated that two indistinguishable electromorphs may result from two different amino acid sequences so that false convergences will appear in phylogenies, and that the order of mobility of electrophoretic variants from fast to slow does not correspond to an ordering of successive amino acid substitutions, so that there is no rationale for ordering character states in an evolutionary sequence, as there may be for morphological characters. When phylogenetic reconstruction based on electromorphs disagrees with a phylogeny based on morphology, there is no *a priori* reason for preferring one or the other. On the other hand, electrophoresis has been a powerful device for discriminating populations whose specific or subspecific status is in doubt and for detecting hybrid zones between differentiated populations. The boundaries between geographically contiguous species can be found and complex patterns of related species biogeography can be resolved, as for example in Mus (SELANDER, HUNT and YANG 1969).

From the publication of the first results of electrophoretic surveys of variation in 1966, the problem of the explanation of the variation became primary. Is the large amount of standing genetic variation in populations a consequence of some form of variation-preserving natural selection, such as overdominance or frequency dependent selection, or is the variation simply what one would expect from the random accumulation of selectively neutral mutations reaching intermediate frequencies by genetic drift in finite populations accompanied by some small migration between populations? If the former were the case, then the protein variation seen is the stuff of adaptive evolution and is the proper object of genetic studies of evolution by natural selection. If, on the other hand, the latter is true, then the observed variation is more or less irrelevant to adaptive evolution, at least in the ecological conditions prevailing at present. Then, to the extent that the protein variation is the actual precursor of species differentiation, it would simply be a stage in nonselective neutral divergence between species. The struggle between these two views of genetic variation was evident from the beginning of electrophoretic studies. LEWONTIN and HUBBY, already in 1966, pointed out the immense genetic load that would exist in a population with 10% heterozygosity if it were maintained by simple overdominant selection, even very weak selection. Various more complex selective schemes were immediately proposed to meet the difficulty (KING 1967; MILKMAN 1967; SVED, REED and BODMER 1967). On the other hand, a theory of selectively neutral evolution of protein differences between species was proposed by KIMURA (1968) and KING and JUKES (1969), and it was KIMURA's view that electrophoretic polymorphism was simply a stage in this neutral evolution of species differences (KIMURA and OHTA 1971). Thus, the old struggle between those who saw natural selection as the preserver of variation and those who saw it as essentially a purifying process, was transferred to the domain of electrophoretic polymorphism. Although no one could now deny that there was indeed a great deal of genetic variation in natural populations, the assumption that this variation was unselected made the observations perfectly compatible with a view that when selection *did* occur, it was purifying in nature.

The question was, would the immense body of information from electrophoresis resolve the issue? The electrophoretic variation provided two categories of data that could be brought to bear: *static* data and *functional* data. By static data, I mean the observed frequency distributions of electromorphic variants within and between populations. By functional data, I mean observations on physiological and fitness differences among electromorphs, including correlations between electromorph frequencies and ecological variables.

The simplest form of static data is the gross proportion of loci polymorphic and the average heterozygosity. While heterozygosity *might* have turned out to be so low or so high as to exclude one or another hypothesis, it turns out, in fact, to be just in the range that makes the interpretation totally ambiguous. See LEWONTIN (1974) for a detailed discussion of this problem.

It might have been, however, that heterozygosity was grossly overestimated or underestimated because of artifacts of the technique. On the one hand, the loci surveyed were those coding for soluble proteins (mostly enzymes) or enzymes that could easily be freed from their association with subcellular particles. In-

soluble structural proteins like lens, muscle, and membrane proteins might be highly monomorphic. Unfortunately, the methods available to study these require their denaturation and charge saturation so that only size variation could be observed electrophoretically. To the present, we do not know how variable in amino acid sequence such proteins are. On the other hand, gel electrophoresis, depending upon charge changes as it does, might not have been able to detect all amino acid substitutions. Only about 1/4 of all random code changes that lead to an amino acid substitution results in a change from one charge class to another. So, loci found to be monomorphic might easily have been polymorphic, and a serious underestimate of genetic variation would have resulted. As pointed out by JOHNSON (1974), the usual conditions of electrophoresis were just those that provide the least sensitivity to charge differences. To investigate this question, SINGH, LEWONTIN and FELTON (1976) developed the system of sequential gel electrophoresis that uses various pH values and buffer systems to detect hidden electrophoretic variation, and RAMSHAW, COYNE and LEWONTIN (1979) used the sequential system to calibrate electrophoresis on a sample of proteins with known amino acid substitutions. The result of these experiments were that sequential electrophoresis could detect about 85% of all amino acid substitutions at different positions in the polypeptide chain. Application of the method to a variety of proteins (COYNE and FELTON 1977; KEITH 1983; KEITH *et al.* 1985) gave a clear result. Loci that had been revealed as polymorphic originally by electrophoresis increased in their observed heterozygosity by the discovery of new alleles and in some cases the increase of the number of alleles was dramatic (from 8 to 27 in xanthine dehydrogenase). But loci previously classified as monomorphic remained monomorphic. The result is that the estimate of average polymorphism did not change, and the average heterozygosity over all loci increased only slightly. The data remained ambiguous.

A second consequence of the introduction of sequential electrophoresis was that a few extremely rich data sets became available so that more sophisticated tests of neutrality or selection could be applied to the static frequency distribution of alleles within and between populations. Tests for the operation of selection like those of EWENS (1972) and WATTERSON (1977) are most powerful when applied to multiple allelic loci. When these tests were applied to the extremely polymorphic loci studied by KEITH, loci that had identical frequency distributions in two populations separated by 300 miles, again the results were ambiguous. Even the richest available static data set on electrophoretic variability lacks the statistical power to discriminate unambiguously between selection and neutrality in large populations with a small amount of migration.

When we turn to functional data, the situation is not much better. The earliest attempts to find fitness differences between electromorphs by population cage selection experiments seemed to show very large fitness effects (*e.g.*, BERGER 1971). But these effects turned out to be the result of linked fitness modifiers and when large samples of independently derived electromorphs were tested using replicated large populations in laboratory culture, differences in fitness were found to be extremely small or nondetectable (YAMAZAKI 1971; ARNASON 1982) as might have been expected. There has been considerable success in demonstrating enzyme kinetic differences and differences in total enzyme activity for numerous polymorphic enzymes as, for example, in a variety of human polymorphic enzymes (see review in HARRIS 1980), lactate dehydrogenase in fish (POWERS, DIMICHELE and PLACE 1983) and a variety of enzymes in Drosophila (LAURIE-AHLBERG *et al.* 1982). But it has been rather more difficult to relate these differences consistently to fitness differences, and especially when exogenously provided substrates like alcohol or starch are involved, there is great sensitivity of fitness estimates to the exact conditions of the experiment. At this point, the only case of convincing fitness differences in nature is for alcohol dehydrogenase in *D. melanogaster* where a combination of consistent altitudinal and latitudinal clines, laboratory selection experiments, and kinetic data come together. Good evidence of some selection on electromorphs is the observation of strong linkage disequilibrium between loci within inversions and of these loci with the inversion karyotype, maintained over very long evolutionary time (PRAKASH and LEWONTIN 1968). However, no such disequilibria are observed in the absence of the extreme recombination suppression created by inversions, so epistatic fitness interactions between loci cannot be large.

Attempts to understand protein polymorphisms by studies of comparative heterozygosity are suggestive but not compelling. So, enzymes in the glycolytic pathway in Drosophila are less variable than other enzymes (KOJIMA, GILLESPIE and TOBARI 1970) and weak correlations between the degree of heterozygosity of a species and aspects of its ecology have been found for various environmental factors (NEVO, BEILES and BEN-SHLOMO 1984). Because loci differ markedly in their heterozygosity, the standard error of average heterozygosity for a species is very large and results are very sensitive to the sample of loci studied. On the order of 100 loci per species would be needed before ecological correlations would be convincing, although there are extreme cases, like the absence of heterozygosity in fossorial mammals and

insects. Attempts to predict heterozygosity from evolutionary plasticity have proved disappointing. Thus the morphologically conservative horseshoe crab, Limulus, often thought of as a phylogenetic "relic," is no less heterozygous than the mouse (SELANDER *et al.* 1970). If Limulus is morphologically conservative in its evolution, it is not a general lack of genetic variation that is the cause.

The question raised in the subtitle of this commentary is whether electrophoresis has been a milestone or a millstone in the development of evolutionary genetics. It has been a milestone, literally, because it marked the first stage in a new path of evolutionary genetics, a path that was so ostentatiously announced in the title of HUBBY and LEWONTIN's paper, "A molecular approach to the study of genetic heterozygosity in natural populations." Molecular biology and evolutionary biology are in constant danger of diverging totally, both in the problems with which they are concerned, that is, the "how" as against the "why," and as scientific communities ignorant and disdainful of each other's methods and concepts. The introduction of electrophoresis in evolutionary studies went some way toward impeding that separation and led naturally to an important second stage, the introduction of DNA sequence studies into population genetics.

Electrophoresis was also a milestone in that it provided for the first time the possibility of including virtually any organism in the study of evolutionary variation on the basis of a common denominator across species. It thus broke the monopoly of a few genetically manipulable forms like Drosophila, mouse and corn as subjects for general genetic and evolutionary studies. As a consequence, it has been possible, by the collective work of large numbers of investigators, to characterize the genetic potential for evolutionary change for organisms in general.

The immense outpouring of data on genetic variation has also been a millstone around our necks. Its first effect was a considerable depauperization of the diversity of empirical work in evolutionary genetics. Within a few years experiments on fitness variation in natural and laboratory populations, selection experiments on morphological and physiological traits, studies of developmental regulation and flexibility in an evolutionary context, work on chromosomal variation, studies of segregation distortion in natural populations–all of the rich diversity of evolutionary genetic investigation–nearly disappeared from the literature of our subject as one investigator after another discovered the joys of electrophoresis. But the problems raised by those earlier studies have not been solved. They have only disappeared from our collective consciousness. Nor are they likely to reappear now, as the ever more seductive offspring of electrophoresis, DNA sequencing, becomes the mode.

The second reason that electrophoresis is a fardel that we bear is that the result it has generated is so rich and so general, yet not, in itself, rich enough to solve the riddle of its own existence. So, ironically, the methods introduced to break the old impasse of evolutionary genetics has created a new and more frustrating impasse precisely because the data are so tantalizingly clear-cut and universal.

Those of us who now study DNA sequence variation believe that at this level we will resolve the problems generated by electrophoretic studies and that finally, because the structure of the observation of DNA sequences is qualitatively different from observations of amino acid variation, that the ambiguities will disappear. But that is another story, and anyway it is somewhat reminiscent of one that I remember telling before, about 25 years ago.

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