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# Hubby and Lewontin on Protein Variation in Natural Populations: When Molecular Genetics Came to the Rescue of Population Genetics

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**ABSTRACT** The 1966 *GENETICS* papers by John Hubby and Richard Lewontin were a landmark in the study of genome-wide levels of variability. They used the technique of gel electrophoresis of enzymes and proteins to study variation in natural populations of *Drosophila pseudoobscura*, at a set of loci that had been chosen purely for technical convenience, without prior knowledge of their levels of variability. Together with the independent study of human populations by Harry Harris, this seminal study provided the first relatively unbiased picture of the extent of genetic variability in protein sequences within populations, revealing that many genes had surprisingly high levels of diversity. These papers stimulated a large research program that found similarly high electrophoretic variability in many different species and led to statistical tools for interpreting the data in terms of population genetics processes such as genetic drift, balancing and purifying selection, and the effects of selection on linked variants. The current use of whole-genome sequences in studies of variation is the direct descendant of this pioneering work.

**KEYWORDS** Hubby; Lewontin; electrophoresis; heterozygosity; molecular variation

THE back-to-back papers of Hubby and Lewontin (1966) and Lewontin and Hubby (1966) on genetic variability in *Drosophila pseudoobscura* represent a landmark in the study of variation in natural populations. The authors introduced the concept of studying variability across the genome, unbiased by prior knowledge of variability at the loci in question. Their experimental technique was gel electrophoresis of enzymes and soluble proteins, which detects most charge change variants. Electrophoresis had already been used to study within-species variation at specific loci (Hubby 1963; Shaw 1965). It had also provided a tool for studying genetic divergence between species (Hubby and Throckmorton 1965), which continued to be employed through the 1980s (*e.g.*, Coyne and Orr 1989). Our main focus in this *Perspectives* is on unbiased studies of variation within species.

Hubby and Lewontin (1966) and Lewontin and Hubby (1966), together with the slightly earlier paper by Harris (1966) on human electrophoretic variability, initiated the modern era of the study of natural genetic variation at the molecular level. While Harris explicitly recognized the need for unbiased surveys of enzyme variability, his work was more concerned with problems in human genetics than general questions in evolutionary genetics, and has thus been somewhat less influential. As Lewontin (1974, 1991) eloquently explained, his work with Jack Hubby was motivated by the impasse that had been reached by the use of classical and quantitative genetics methods for studying genetic variability in nature. There was evidence for abundant genetic variation in quantitative traits, as well for "concealed variability" revealed by inbreeding experiments, but the numbers of genes involved, the frequencies of allelic variants at the underlying loci, and the sizes of their effects on the traits in question, were all unknown. A few examples were known of single-gene inheritance of polymorphisms for visible traits in natural populations, and chromosomal polymorphisms had been studied in *Drosophila* and a few other species, as

well as a handful of biochemical polymorphisms such as human blood groups. But the 1950s debate between the "classical" and "balance" views of variability remained unresolved.

The classical view was that the typical state of a gene in a population was a functional wild-type allele, with deleterious mutant alleles present at low frequencies (Muller 1950). In contrast, the balance hypothesis proposed that many genes might have two or more alleles maintained at intermediate frequencies in populations by balancing selection (Dobzhansky 1955). Without a way to measure genetic variability at the level of individual genes, strong tests of these hypotheses could not be carried out.

## The Problem and Its Solution

Hubby and Lewontin (1966) began their paper with a lucid outline of the problem and the requirements for solving it, a summary that could hardly be bettered today:

> A cornerstone of the theory of evolution by gradual change is that the rate of evolution is absolutely limited by the amount of genetic variation in the evolving population. Fisher's Fundamental Theorem of Natural Selection" (1930) is a mathematical statement of this generalization, but even without mathematics it is clear that genetic change caused by natural selection presupposes genetic differences already existing, on which natural selection can operate. In a sense, a description of the genetic variation in a population is the fundamental datum of evolutionary studies; and it is necessary to explain the origin and maintenance of this variation and to predict its evolutionary consequences. It is not surprising, then, that a major effort of genetics in the last 50 years has been to characterize the amounts and kinds of genetic variation existing in natural or laboratory populations of various organisms.

> The reason for our present lack of knowledge about the amount of heterozygosity per locus in a population is that no technique has been available capable of giving a straightforward and unambiguous answer even under ideal experimental conditions. Any technique that is to give the kind of clear information we need must satisfy all of the following criteria: (1) Phenotypic differences caused by allelic substitution at single loci must be detectable in single individuals. (2) Allelic substitutions at one locus must be distinguishable from substitutions at other loci. (3) A substantial portion of (ideally, all) allelic substitutions must be distinguishable from each other. (4) Loci studied must be an unbiased sample of the genome with respect to physiological effects and degree of variation. Requirements 1 and 2 really amount to the condition that the phenotypes used have a simple Mendelian inheritance without important environmental variation. Requirements 3 and 4 come from the need to make statements about variation in the genome as a whole from a necessarily restricted sample.

Their paper reasoned that studies of variation in electrophoretic mobility of enzymes and proteins provided a way to fully meet all these requirements except the third (see below), making use of the one-to-one relation between gene and polypeptide that had recently been established by stunning advances in molecular genetics. They wrote:

> The phenotypic differences are detectable in single individuals. Allelic substitutions at different loci are distinguishable from each other because the simple genetics of each difference can be investigated as for any phenotypic character. As it turns out, all the electrophoretic differences to be described turn out to be single Mendelizing entities. This fact is most important because it frees the method of any a priori assumptions about gene action. Moreover, it allows us, as a first order of approximation, to equate a protein without any detectable variation to a gene without detectable variation. That is, we can count up the number of loci in our sample that show no variation, as well as the number that do have alternative alleles. This is the cornerstone of the method for it then allows us to estimate the proportion of all loci that show variation in populations. The third requirement, that a substantial portion of all possible changes is detectable, is only met in part ... Finally, the enzymes and high concentration proteins used in the study have been chosen without reference to their known variability in the population, but only because assay techniques exist for them.

The first of the two papers described the technical details of their study of genetic differences among strains derived from wild-caught *D. pseudoobscura*. This was the focal species in Theodosius Dobzhansky's (Lewontin's Ph.D. advisor) monumental series of 43 papers *The Genetics of Natural Populations* (mostly published in *GENETICS*), which included important studies of inversion polymorphisms. Hubby and Lewontin first verified the single locus, codominant inheritance of electrophoretic differences in this species. Studying 43 strains derived from nature, they found allelic variation at 9 of 18 loci (including both enzyme loci and loci controlling abundant larval proteins.

An important contribution of the second paper was the introduction of summary statistics for measuring variability, notably the heterozygosity ($H$), a statistic that has become standard in analyses of molecular variability, when applied to variability per nucleotide site rather than per gene (Nei 1987). The proportion of polymorphic loci ($P$) was also introduced. This used the arbitrary criterion that a locus should show more than a single allele among the strains or individuals studied, and is little used today, although it is related to the estimator of nucleotide site diversity based on the number of segregating sites in a sample (Watterson 1975).

$H$ makes use of the estimated allele frequencies at a locus to determine the overall chance that two randomly sampled alleles at a locus are different, by calculating the mean frequency of heterozygotes per locus under the assumption of random mating. With random mating, $H$ for a set of loci is equivalent to the expected fraction of these loci that are heterozygous within an individual. Especially when dealing with nonrandomly mating populations, however, one can avoid confusion by referring to $H$ as the "expected heterozygosity," "allelic diversity," or simply "diversity" (Nei 1973, 1987). These estimates were applied to five different *D. pseudoobscura* populations; the $P$ and $H$ values were similar for each population, with a mean $P$ of 0.3 and a mean $H$ of 0.12. Later, more extensive surveys of electrophoretic variability

in *D. pseudoobscura* did not substantially change these estimates (Lewontin 1974).

The Discussion section of the paper opened with an analysis of the potential biases in the method. The most important problem is that only a fraction of changes to protein sequences are detectable by electrophoresis (so that requirement three was not fully satisfied), implying that diversity was likely to be underestimated. The paper ended with a consideration of the relative roles of selection, mutation, and genetic drift in explaining the polymorphisms, with a tentative leaning toward a role for a selective advantage to heterozygotes, at least at some of the loci. The question of the causes of natural molecular variation has been the target of much subsequent research.

## Refinements to Electrophoretic Approaches

For molecular population genetics data to be useful in testing *any* theory about the forces controlling natural variation, they need to satisfy the third requirement just mentioned— the approach used *must detect most allelic differences*. This was by no means guaranteed in the protein electrophoresis era. For over a decade, most workers used a set of constant, lab-specific electrophoretic conditions to assay variation: for a given protein, a buffer of a single pH and chemical composition was used, with gels of a fixed starch or acrylamide concentration. The next advance was to try more varied experimental conditions, including different gel pH values to alter protein charge and shape, varying gel concentrations to detect conformational differences between proteins with the same charge, and heat treatments of organismal extracts. This *sequential electrophoresis* procedure was expected to distinguish otherwise identical variants, including sequence changes that affected the likelihood of protein denaturation.

Lewontin's lab indeed found "hidden" electrophoretic variants by such methods—often many of them. At least in *Drosophila*, loci that were already variable under the single "standard condition" consistently proved even more variable when multiple conditions were employed—with up to sixfold more alleles—whereas loci that appeared to be invariant ("monomorphic") within a species under a single condition remained so under sequential electrophoresis (Singh *et al.* 1976; Coyne *et al.* 1978). But this was not true for protein differences *among* species: quite often, species or subspecies that initially appeared to be either monomorphic for the same variant, or to have similar polymorphism levels, were shown to have different alleles (Coyne 1976; Coyne and Felton 1977; Coyne *et al.* 1979), revealing that differences between taxa had been severely underestimated. We now know that this reflects the far greater number of amino acid differences that distinguish homologous proteins from different species, compared with differences within a species. DNA sequences are, of course, now the main source of data for the study of interspecific differences.

What proportion of all alleles was missed by the "standard" electrophoretic procedure? To estimate this, one needs to apply the procedure to proteins of known sequence. This test was done by Ramshaw *et al.* (1979), using variants of human hemoglobin that had been detected because they either caused disease or were picked up in routine screening. While substitutions that involved a change in net protein charge were often detectable under the standard conditions, sequential electrophoresis found more than twice as many variants, including ones that did not differ in net charge. When combined with heat denaturation, it could even distinguish nearly 80% of cases where sites at different places in the same human hemoglobin protein varied for the same two amino acids (*e.g.*, lysine *vs.* asparagine). Very careful work could thus potentially detect most amino acid variants in this well-studied protein.

This laborious procedure has not been replicated with other proteins, but the overall conclusion that variants are more abundant than detected by standard electrophoresis probably applies to other soluble proteins, of the type that were mostly used in electrophoretic studies of genetic diversity. In contrast, the use of 2D gel electrophoresis to study variation in nonsoluble "structural" proteins showed much less variability, suggesting that the results from soluble proteins were not necessarily representative of the whole genome (Leigh Brown and Langley 1979b; Smith *et al.* 1980). This revealed a further technical limitation to standard electrophoresis, and a violation of Hubby and Lewontin's fourth criterion, since it suggested that soluble proteins are not necessarily representative with respect to their level of variability. In the early 1980s, however, restriction mapping and DNA sequencing of cloned fragments of the genome became possible, allowing studies of variation in the DNA itself. The first population survey using direct sequencing was performed by Martin Kreitman in the Lewontin lab (Kreitman 1983). Protein electrophoresis therefore became largely obsolete in evolutionary genetics, except as an inexpensive source of genetic markers for genetic mapping and for studies of inbreeding and population subdivision.

## Influence on Later Research in Population Genetics

The initial discovery that molecular variants were readily available, and distributed throughout the genome, opened the way for many advances in genetics and population genetics. It triggered an explosion of "find 'em and grind 'em" studies of variability in natural populations of numerous different species, from bacteria to humans, which showed that the levels of variability originally found in *Drosophila* and humans were not unusual (Lewontin 1974, 1985, 1991). These studies mostly used enzymes, and the term "allozyme," introduced by Prakash *et al.* (1969), became widely used to denote electrophoretically polymorphic enzyme loci.

Efforts to detect selection soon showed that selection on allozyme variants was usually too weak to be directly

detectable, for example from allele frequency changes in experimental populations, except in very sensitive bacterial chemostat experiments (Dykhuizen 1990). However, allozyme work, such as studies of alcohol dehydrogenase in *D. melanogaster*, sometimes revealed repeatable clinal patterns in allele frequencies, suggesting the action of selection at some loci (Oakeshott *et al.* 1982; Eanes 1999). Occasionally, convincing evidence of selectively caused shifts in allele frequencies in laboratory populations was obtained, as well as for selective differences among allozyme genotypes in natural populations (reviewed by Gillespie 1991, Eanes 1999, and Watt and Dean 2000). Conflicting views on whether electrophoretic variation predominantly resulted from classical processes of mutation, drift, and purifying selection *vs.* balance processes of active maintenance of variability by selection were advocated by Kimura (1983) and Gillespie (1991), respectively, using essentially the same data.

The inadequacy of evidence from surveys of electrophoretic variation to resolve the problem of the causes of variation had already been noted by Lewontin (1974) in this famous passage (p. 189):

> For many years, population genetics was an immensely rich and powerful theory with virtually no suitable facts on which to operate..…. Quite suddenly the situation has changed … and facts in profusion have been poured into the hopper of this theory machine. And from the other end has issued nothing. It is not that the machinery does not work, for a great clashing of gears is audible, if not deafening, but it somehow cannot transform into a finished product the great volume of raw material that has been provided. The entire relationship between the theory and the facts needs to be reconsidered.

Despite the difficulties in using protein electrophoretic data to infer the processes responsible for controlling levels of genetic variation, these variants provided biologists with useful tools based on Wright's $F_{ST}$ statistic (Wright 1951) to estimate the partitioning of diversity within and between populations (Lewontin 1972; Nei 1973, 1987). This represented a major breakthrough compared to what had previously been possible. [It is interesting to note that Lewontin's 1972 conclusion (Lewontin 1972) that most human molecular genetic diversity occurs within rather than between populations was verified by later studies at the DNA level, *e.g.*, Barbujani *et al.* 1997]. Such analyses, initially ignoring the possibility that selection might affect frequencies of allozyme variants, revealed interesting and important patterns of diversity that are still being discussed today, such as the remarkable constancy of levels of allozyme polymorphism across species with very different population sizes (Lewontin 1974). This observation stimulated the development of the first model of the genetic hitchhiking of neutral variants by favorable mutations (Maynard Smith and Haigh 1974). In addition, electrophoretic markers also made it possible to use $F_{ST}$ to detect outlier loci that might indicate the action of selection (Lewontin and Krakauer 1973). This approach has become a major way in which DNA sequence

variants can be used to detect loci involved in adaptive changes (*e.g.*, Foll *et al.* 2014).

Early allozyme surveys also stimulated the development of sophisticated statistical methods based on the full information in a sample of individuals from a population, exemplified by Ewens' sampling distribution (Ewens 1972). This eventually led to the powerful coalescent process model (Kingman 1982), widely used today in analyses of genome-wide variability (Wakeley 2008). They also stimulated discussions of the problem of the genetic load that would be caused by large numbers of loci maintained polymorphic by selection (Sved *et al.* 1967; Wallace 1968; Franklin and Lewontin 1970; Lewontin 1974), which contributed to the development of the neutral theory of molecular evolution and variation (Kimura 1968, 1983; King and Jukes 1969). The high level of genome-wide variation at both coding and noncoding sequences revealed by modern genomics has revived interest in this question (Kondrashov 1995; Lesecque *et al.* 2012; Charlesworth 2013).

In addition, allozyme data provided genetic markers that, for the first time, allowed mating systems to be inferred from genotype frequencies in natural populations or from progeny raised from parents living in the wild (Brown and Allard 1970; Ritland and Jain 1981). Results accumulated from such studies revealed a strong tendency for diversity to be lower in inbreeding than in outcrossing populations, associated with much higher subdivision in inbreeders as measured by $F_{ST}$ (Hamrick and Godt 1990). The mating system is the strongest factor affecting patterns of genetic diversity in flowering plants (Charlesworth and Wright 2001).

These examples illustrate how the population genetics concepts that have become central to present-day analyses of DNA sequence variation were often prompted by earlier allozyme studies of natural populations.

## Classical *vs.* Balance Models of Variability

Dick Lewontin's own *Perspectives* on the 1966 papers (Lewontin 1991) was written at the beginning of the era when DNA sequencing was becoming easy enough for population geneticists to use in studies of variation at multiple loci. He was optimistic that many of the difficulties associated with the use of allozymes would be resolved when extensive DNA sequence data became available. Was this optimism justified by the subsequent quarter of a century of work? The answer is that we now understand a lot more about the forces acting on genetic variability than in 1966 or 1991, but there is still much to learn, especially about the extent of balancing selection across the genome and the importance of selection on noncoding sequences (see Charlesworth 2010 for a brief overview).

In this context, it is useful to note that, from early on, it was evident that only a minority of electrophoretic loci had variants segregating at intermediate frequencies, so that there is a contribution to *H* from loci with low frequency alleles as well from loci with alleles at intermediate frequencies. *H* values

were also found to differ systematically among different classes of loci, *e.g.*, the classification of enzymes into group I (enzymes with single substrates, low $H$) and group II (multiple substrates, high $H$) (Gillespie and Kojima 1968; Gillespie 1991), and the positive correlation between the molecular weight of polypeptides and $H$ (Leigh Brown and Langley 1979a).

A survey of *D. melanogaster* based on a large number of genes showed a bimodal distribution of $H$ values for individual loci (see figure 5 in Singh and Rhomberg 1987). This suggested that the low diversity loci have alleles maintained by the balance between drift, mutation, and purifying selection, while those with high diversity values are candidates for the action of balancing selection or local adaptation. Subsequent analyses of DNA sequence variability at enzyme loci have supported this interpretation (Eanes 1999), implying that electrophoretic variation reflects a mixture of the classical and balance mechanisms.

We now know from genome-wide surveys of DNA sequence variability that most nonsynonymous variants in coding sequences are present at low frequencies within populations (*e.g.*, Boyko *et al.* 2008 and Keightley *et al.* 2016). Only a few of them appear to be maintained at intermediate frequencies by possible balancing selection within populations, or geographically varying selection pressures. The large mean size of a coding sequence (around 500 codons in eukaryotes) implies that a very low level of variability per nonsynonymous site can result in a high probability that a randomly sampled allele of a gene includes an alteration in the corresponding polypeptide sequence. For example, with a mean frequency of a nonsynonymous variant at a given nucleotide site in the population of $0.7 \times 10^{-3}$, the value for an African *D. melanogaster* population (Campos *et al.* 2014), a sequence of 1000 nonsynonymous nucleotide sites has an expected number of amino acid variants of 0.7 and a probability of $1 - \exp(-0.7) = 0.5$ of carrying at least one such variant. Inferences from population genomic data suggest that the vast majority of new nonsynonymous mutations in both humans (Boyko *et al.* 2008) and *Drosophila* (Kousathanas and Keightley 2013) are subject to sufficiently strong purifying selection that Muller's mutation–selection model (Muller 1950) should apply to them. This classical mechanism, supplemented by the random drift of nearly neutral nonsynonymous mutations, is thus sufficient to explain most low-frequency variation in protein sequences among individuals within a population. Nonetheless, population genomic data also provide clear evidence for the operation of balancing selection at a small minority of loci (Charlesworth 2006; Gao *et al.* 2015).

## Conclusions

Five decades on, the small but revolutionary window that Hubby, Harris, and Lewontin opened on genomic variation has become a vast panorama. The fundamental questions that they addressed remain central, and their basic approach is still relevant for further progress toward answering them, although the amount of data on within-population variation and the sophistication of methods of analysis have both increased enormously.

The near completeness of the information provided by modern genomics tools is driving new experimental designs and stimulating the development of ever-more sophisticated tools for statistical analyses of population genetics data. In particular, combined analyses of genomic patterns of between-species divergence and within-population variation allow the contributions of selective and neutral processes to evolutionary change to be evaluated with increasing precision (Boyko *et al.* 2008; Kousathanas and Keightley 2013). Functional genomics is "annotating" genomes with rich mechanistic information that continues to be incorporated into evolutionary genomic studies. Genomic data sets are currently providing huge amounts of information, especially on humans, that allow inferences of populations' demographic histories to be made, revealing our "out of Africa" history, and introgression from Neanderthals. Genome-wide association studies for analyzing complex traits and genetic diseases, are now possible in our own species, and powerful new tools for detecting the footprints of recent natural selection in both wild and domesticated populations of plants and animals are available (Haasl and Payseur 2016), building on the early insight of Maynard Smith and Haigh (1974) that the spread of an advantageous mutation event causes a "selective sweep," leaving the associated region of the genome with low diversity for many generations afterward.

Both the creators and observers of historic first steps, like those made by Hubby, Harris, and Lewontin, often recognize them as important advances. The initial success and subsequent expansion of our understanding of genetic variation that rests on the Hubby/Lewontin/Harris approach depended heavily on their clear thinking about the implications of the profound advances of genetics that had occurred in the five decades that preceded their paper, as the quotations at the beginning of this article show. But no-one in 1966 foresaw the scope of the research that was to follow.

We end by asking what today's genomic sequencing results tell us about the accuracy of the estimates of levels of genome-wide variability made by Lewontin and Hubby (1966) and Harris (1966)—a question that has apparently not been asked previously. Thanks to studies by Y.-C. Lee and S. Schaeffer (personal communication), the overall amount of charge change variation in proteins in natural populations of *D. melanogaster* and *D. pseudoobscura* is now known with high accuracy. Lewontin and Hubby (1966) estimated an $H$ value of 0.12 for charge change electrophoretic variants in *D. pseudoobscura*. Allozyme surveys of *D. melanogaster* quickly followed, yielding a similar value. Based on his DNA sequencing survey of 15 *D. pseudoobscura* 3rd chromosomes (20% of the genome, 2669 genes), S. Schaeffer estimated $H$ for such variants to be 0.237; Y.-C. Lee's estimate for *D. melanogaster*, from the full sequences of many randomly sampled genomes, is 0.252. The precision of the estimates

from the early allozyme studies was thus surprisingly good, despite the small numbers of genes that could be studied, as well as the insensitivity of standard electrophoretic methods (see above). This is the ultimate confirmation of the work using the sequential electrophoresis of a single human protein (Ramshaw *et al.* 1979), which suggested that the initial results were likely to be widely applicable. Like many other significant scientific advances, the seminal initial studies not only introduced a new perspective on the study of natural genetic variation, but also introduced a rigorous and reliable way of quantifying levels of variability.

## Acknowledgments

## Literature Cited

Barbujani, G., A. Magagni, E. Minch, and L. L. Cavalli-Sforza, 1997   An apportionment of human genetic diversity. Proc. Natl. Acad. Sci. USA 94: 4516–4519.

Boyko, A., S. H. Williamson, A. R. Indap, J. D. Degenhardt, R. D. Hernandez *et al.*, 2008   Assessing the evolutionary impact of amino-acid mutations in the human genome. PLoS Genet. 5: e1000083.

Brown, A. H. D., and R. W. Allard, 1970   Estimation of the mating system in open-pollinated maize populations using isozyme polymorphisms. Genetics 66: 133–145.

Campos, J. L., D. L. Halligan, P. R. Haddrill, and B. Charlesworth, 2014   The relationship between recombination rate and patterns of molecular evolution and variation in *Drosophila melanogaster*. Mol. Biol. Evol. 31: 1010–1028.

Charlesworth, B., 2010   Molecular population genomics: a short history. Genet. Res. 92: 397–411.

Charlesworth, B., 2013   Why we are not dead one hundred times over. Evolution 67: 3354–3361.

Charlesworth, D., 2006   Balancing selection and its effects on sequences in nearby genome regions. PLoS Genet. 2: e2.

Charlesworth, D., and S. I. Wright, 2001   Breeding systems and genome evolution. Curr. Opin. Genet. Dev. 11: 685–690.

Coyne, J. A., 1976   Lack of genic similarity between two sibling species of Drosophila as revealed by varied techniques. Genetics 84: 593–607.

Coyne, J. A., and A. A. Felton, 1977   Genetic heterogeneity of two alcohol dehydrogenase loci in *Drosophila pseudoobscura* and *Drosophila persimilis*. Genetics 87: 285–304.

Coyne, J. A., and H. A. Orr, 1989   Patterns of speciation in *Drosophila*. Evolution 43: 362–381.

Coyne, J. A., A. A. Felton, and R. C. Lewontin, 1978   Extent of genetic variation at a highly polymorphic esterase locus in *Drosophila pseudoobscura*. Proc. Natl. Acad. Sci. USA 75: 5090–5093.

Coyne, J. A., W. F. Eanes, J. A. M. Ramshaw, and R. K. Koehn, 1979   Electrophoretic heterogeneity of alpha-glycerophosphate dehydrogenase in many species of *Drosophila*. Syst. Zool. 28: 164–175.

Dobzhansky, T., 1955   A review of some fundamental concepts and problems of population genetics. Cold Spring Harb. Symp. Quant. Biol. 20: 1–15.

Dykhuizen, D. E., 1990   Experimental studies of natural selection in bacteria. Annu. Rev. Ecol. Syst. 21: 373–398.

Eanes, W. F., 1999   Analysis of selection on enzyme polymorphisms. Annu. Rev. Ecol. Syst. 30: 301–326.

Ewens, W. J., 1972   The sampling theory of selectively neutral alleles. Theor. Popul. Biol. 3: 87–112.

Foll, M., O. E. Gaggiotti, J. T. Daub, A. Vatsiou, and L. Excoffier, 2014   Widespread signals of convergent adaptation to high altitude in Asia and America. Am. J. Hum. Genet. 95: 394–407.

Franklin, I. R., and R. C. Lewontin, 1970   Is the gene the unit of selection? Genetics 65: 701–734.

Gao, Z., M. Przeworski, and G. Sella, 2015   Footprints of ancient-balanced polymorphisms in genetic variation: data from closely related species. Evolution 69: 431–466.

Gillespie, J. H., 1991   *The Causes of Molecular Evolution*. Oxford University Press, Oxford, UK.

Gillespie, J. H., and K. Kojima, 1968   The degree of polymorphism in enzymes involved in energy production compared to that in nonspecific enzymes in two *Drosophila ananassae* populations. Proc. Natl. Acad. Sci. USA 61: 582–585.

Haasl, R. J., and B. A. Payseur, 2016   Fifteen years of genomewide scans for selection: trends, lessons and unaddressed genetic sources of complication. Mol. Ecol. 25: 5–23.

Hamrick, J. L., and M. J. W. Godt, 1990   Allozyme diversity in plant species, pp. 43-63 in *Plant Population Genetics, Breeding and Genetic Resources*, edited by A. H. D. Brown, M. T. Clegg, A. L. Kahler, and B. S. Weir. Sinauer Associated, Sunderland, MA.

Harris, H., 1966   Enzyme polymorphisms in man. Proc. R. Soc. Lond. B Biol. Sci. 164: 298–310.

Hubby, J. L., 1963   Protein differences in Drosophila. I. *Drosophila melanogaster*. Genetics 48: 871–879.

Hubby, J. L., and L. H. Throckmorton, 1965   Protein differences in Drosophila. II. Comparative species genetics and evolutionary problems. Genetics 52: 203–215.

Hubby, J. L., and R. C. Lewontin, 1966   A molecular approach to the study of genic heterozygosity in natural populations. I. The number of alleles at different loci in *Drosophila pseudoobscura*. Genetics 54: 577–594.

Keightley, P. D., J. L. Campos, T. R. Booker, and B. Charlesworth, 2016   Inferring the site frequency spectrum of derived variants to quantify adaptive molecular evolution in protein-coding genes of *Drosophila melanogaster*. Genetics 202: 975–984.

Kimura, M., 1968   Evolutionary rate at the molecular level. Nature 217: 624–626.

Kimura, M., 1983   *The Neutral Theory of Molecular Evolution*. Cambridge University Press, Cambridge, UK.

King, J. L., and T. H. Jukes, 1969   Non-Darwinian evolution. Science 164: 788–798.

Kingman, J. F. C., 1982   On the genealogy of large populations. J. Appl. Probab. A19: 27–43.

Kondrashov, A. S., 1995   Contamination of the genome by very slightly deleterious mutations: why have we not died 100 times over? J. Theor. Biol. 175: 583–594.

Kousathanas, A., and P. D. Keightley, 2013   A comparison of models to infer the distribution of fitness effects of new mutations. Genetics 193: 1197–1208.

Kreitman, M., 1983   Nucleotide polymorphism at the alcohol dehydrogenase locus of *Drosophila melanogaster*. Nature 304: 412–417.

Leigh Brown, A. J., and C. H. Langley, 1979a   Correlation between heterozygosity and subunit molecular weight. Nature 277: 649–651.

Leigh Brown, A. J., and C. H. Langley, 1979b   Reevaluation of level of genic heterozygosity in natural populations of *Drosophila melanogaster* by two-dimensional electrophoresis. Proc. Natl. Acad. Sci. USA 76: 2381–2384.

Lesecque, Y., P. D. Keightley, and A. Eyre-Walker, 2012   A resolution of the mutation load paradox in humans. Genetics 191: 1321–1330.

Lewontin, R. C., 1972   The apportionment of human diversity. Evol. Biol. 6: 381–398.

Lewontin, R. C., 1974   *The Genetic Basis of Evolutionary Change.* Columbia University Press, New York.

Lewontin, R. C., 1985   Population genetics. Annu. Rev. Genet. 19: 81–102.

Lewontin, R. C., 1991   Twenty-five years ago in Genetics: electrophoresis in the development of evolutionary genetics: millstone or milestone? Genetics 128: 657–662.

Lewontin, R. C., and J. L. Hubby, 1966   A molecular approach to the study of genic heterozygosity in natural populations. II. Amount of variation and degree of heterozygosity in *Drosophila pseudoobscura*. Genetics 54: 595–609.

Lewontin, R. C., and J. Krakauer, 1973   Distribution of gene frequency as a test of neutrality. Genetics 74: 175–195.

Maynard Smith, J., and J. Haigh, 1974   The hitch-hiking effect of a favourable gene. Genet. Res. 23: 23–35.

Muller, H. J., 1950   Our load of mutations. Am. J. Hum. Genet. 2: 111–176.

Nei, M., 1973   Analysis of gene diversity in subdivided populations. Proc. Natl. Acad. Sci. USA 70: 3321–3323.

Nei, M., 1987   *Molecular Evolutionary Genetics.* Columbia University Press, New York.

Oakeshott, J. G., J. B. Gibson, P. R. Anderson, W. R. Knibb, and G. K. Chambers, 1982   Alcohol dehydrogenase and glycerol-3-phosphate dehydrogenase clines in *Drosophila melanogaster* on different continents. Evolution 36: 86–96.

Prakash, S., R. C. Lewontin, and J. L. Hubby, 1969   A molecular approach to the study of genic heterozygosity in natural populations. V. Patterns of genic variation in central, marginal and isolated populations. Genetics 61: 841–858.

Ramshaw, J. A. M., J. A. Coyne, and R. C. Lewontin, 1979   The sensitivity of gel electrophoresis as a detector of genetic variation. Genetics 93: 1019–1037.

Ritland, K., and S. Jain, 1981   A model for the estimation of outrcossing rate and gene-frequencies using N independent loci. Heredity 47: 35–52.

Shaw, C. R., 1965   Electrophoretic variation in enzymes. Science 149: 936–943.

Singh, R. S., and L. R. Rhomberg, 1987   A comprehensive study of genic variation in natural populations of *Drosophila melanogaster*. II. Estimates of heterozygosity and patterns of geographic differentiation. Genetics 117: 255–271.

Singh, R. S., R. C. Lewontin, and A. A. Felton, 1976   Genic heterogeneity within electrophoretic "alleles" of xanthine dehydrogenase in *Drosophila pseudoobscura*. Genetics 84: 609–629.

Smith, S. C., R. R. Racine, and C. H. Langley, 1980   Lack of variation in the abundant proteins of the human kidney. Genetics 96: 967–974.

Sved, J. A., T. E. Reed, and W. F. Bodmer, 1967   The number of balanced polymorphisms that can be maintained in a population. Genetics 55: 469–481.

Wakeley, J., 2008   *Coalescent Theory: An Introduction.* Roberts and Company, Greenwood Village, CO.

Wallace, B., 1968   Polymorphism, population size, and genetic load, pp. 87–108 in *Population Biology and Evolution*, edited by R. C. Lewontin. Syracuse University Press, Syracuse, NY.

Watt, W. B., and A. M. Dean, 2000   Molecular-functional studies of adpative genetic variation in prokaryotes and eukaryotes. Annu. Rev. Genet. 34: 593–622.

Watterson, G. A., 1975   On the number of segregating sites in genetical models without recombination. Theor. Popul. Biol. 7: 256–276.

Wright, S., 1951   The genetical structure of populations. Ann. Eugen. 15: 323–354.

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