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Molecular variability and hypothesis testing

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In the last decade, the field of evolutionary biology has been revitalized with discoveries made using techniques borrowed from molecular biology. Extensive stores of protein polymorphism characterize nearly all natural populations. Evolutionists have become polarized in their views on the maintenance of molecular variation; some subscribe to the proposition that molecular variation is important in adaptation and hence under the control of natural selection, while others believe most variation is neutral with respect to fitness. The debate is not yet settled, but the new observations have already greatly affected our concepts about individual uniqueness. The controversy per se is of philosophical interest because it demonstrates, contrary to common opinion, that powerful scientific methodologies such as strong inference can often be applied to problems in evolutionary theory.

Scientific knowledge accumulates through critical tests of hypotheses against observations gathered with the intent of falsifying those hypotheses.[1]

In the Baconian tradition, the base of an inductive tree consists of objectively gathered observations, upon which alternative explanatory hypotheses may be erected and tested. The stimulus for major advances in a scientific field often results from a novel observation or set of data, sometimes generated by the development and application of new measuring or monitoring techniques. Science proceeds most efficiently when procedures of strong inference are rigorously applied to the problem of explaining such a new set of observations.[2]

The steps involved in conditional inductive logic, or strong inference, are as follows:

(1) alternative hypotheses are devised to explain the problem;

(2) *crucial* experiments or other empirical tests are designed with the intent of falsifying one or another of the hypotheses;

(3) the experiments or tests are carried out, and false hypotheses (those inconsistent with results of the test) are discarded;

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(4) steps (1) to (3) are repeated, by generating and testing hypotheses consistent with the refined possibilities which remain.

Conclusions in science are thus exclusions; hypotheses whose logical implications lack congruence with results of relevant experiments and empirical observations, are rejected.

About 12 years ago, improvements in electrophoretic techniques allowed evolutionists for the first time to objectively quantify levels of genetic variability in natural populations. The results were conclusive and astounding: the genomes of individuals and the gene pools of populations are characterized by tremendous stores of genetic variability–far more than had been predicted according to certain models of population genetics. This exciting discovery generated a controversy, which is now entering its second decade, about the causal processes responsible for maintaining all this genetic variation. At its best, the controversy has contained brilliant formulations of alternative hypotheses, and elegant attempts to test these hypotheses. At its worst, the controversy has generated uncritical data and as much heat as light. The subject of molecular variability in natural populations is of interest both for its profound implications for evolutionary processes, and as an example of the manner in which science proceeds ideally and in practice.

Basis of the controversy: levels of genetic variability

The amount of genetic variation in a population determines its evolutionary potential. The effectiveness of natural selection is dependent upon stores of genetic variability originally derived through mutation, recombination, and gene flow. The Fundamental Theorem of Natural Selection[3] expresses the concept as follows: 'The rate of increase in fitness of a population at any time is equal to its genetic variance in fitness at that time.' Given the signal significance of genetic variation, it may seem ironic that until very recently, evolutionists could not quantify this parameter, even to a first approximation.

In order to determine the proportion of genes in a population which are polymorphic, genes must be sampled which average no more or less variable than the remainder of the genome: the sample of genes must be unbiased with respect to level of variability. Yet classical Mendelian techniques can only determine the presence of a gene by observing the segregational behaviour of its different alleles in progeny of specific crosses. Genes which are monomorphic cannot even be detected.

A conceptual breakthrough occurred in 1941 with the recognition that genes encode proteins.[4] Any method which can distinguish structural properties of proteins encoded by a gene may provide indirect evidence about the gene itself. One such method, electrophoresis, had been discovered in 1937 by Tiselius, and has become a method of choice today for study of many evolutionary problems. Electrophoretic techniques separate enzymes and other proteins in an electric field, primarily according to their net electric charge. Differences in charges of proteins are

attributable to differences in the nucleotides of the codons which encode them. Proteins which differ in electrophoretic mobility are coded for by different genes or by different alleles if they are the product of a single gene. Importantly, proteins encoded by a particular gene and sharing an electrophoretic mobility may also be observed, and we may infer that they are encoded by identical alleles.* By selecting for study proteins encoded by a number of genetic loci, estimates may be made of the proportion of genes which are variable within a population, and the proportion of genes containing different alleles in different populations.

Table 1. Typical estimates of genetic variability in natural populations

Organism	No. of loci	Mean percentage genes heterozygous per individual
Krill (*Euphasia*)	36	5·8
Horseshoe crab (*Limulus*)	25	9·7
Killer clam (*Tridacna*)	30	20·2
Crickets (*Gryllus*)	20	14·5
Fruit flies (*Drosophila*)	11–33	14·5
Sunfish (*Lepomis*)	18	5·9
Lizards (*Uta*, *Anolis*, *Sceloporus*)	15–29	5·8
Sparrow (*Zonotrichia*)	15	5·9
Mice (*Peromyscus polionotus*)	32	4·8
Deer (*Odocoileus*)	22	12·9
Man (*Homo*)	70	6·7

In the early 1960s two laboratories independently undertook to determine heterozygosity levels† by conducting large-scale electrophoretic surveys of many randomly chosen proteins. The results greatly surprised many people. Of eighteen genetic loci in the fruit fly, *Drosophila pseudoobscura*, 30 per cent had electrophoretic variants and an average individual was heterozygous at 11·5 per cent of its loci.[5,6] And of ten genetic loci in man, 30 per cent were polymorphic, and average individual heterozygosity was 9·9 per cent.[7] Similar high levels of genetic variability have now been documented in a very wide array of organisms, ranging from killer clams, horseshoe crabs, and crickets, to mice, deer, and man. Typical results are summarized in Table 1.

One might have thought that these exciting observations would lead to a rapid resolution of several outstanding problems in evolutionary theory. Such has not been the case. In order to understand why, we must first examine two schools of thought concerning levels of genetic variability, and how their proponents' views have adapted to the new observations on molecular variability.

* This inference is not always correct since electrophoretic techniques are not sensitive enough to pick up all protein differences. Electrophoresis *underestimates* the level of genic variability in populations.

† Heterozygosity is here defined as the mean proportion of loci heterozygous per individual.

Classical, balance, and neoclassical theories of genic variability

The classical theory of genome structure envisions a strictly purifying role of natural selection; selection functions to 'weed out' the continual influx of mutations, the vast majority of which are detrimental to the success of their bearers in leaving offspring. Of course, a few mutations may occasionally increase an organism's fitness, and these new alleles will spread through the population. Thus evolutionary change by natural selection is by no means denied. A logical consequence of this theory, and in fact the cornerstone of the classical model of population structure, is that most genes in the population gene pool will be fixed or monomorphic for the allele conferring highest fitness. Most of what little genetic variation is present results from rare adaptive mutations, recently introduced into the population and presently on their way to fixation. The classical view has received much theoretical support, primarily from the concept of the cost of natural selection or genetic load.

The term 'genetic load' was introduced in 1950 by H. J. Muller in an attempt to convince the medical profession and public of the serious health consequences of increased mutation rates. Muller was convinced of the deleterious nature of most mutations, and recognized that they would tend to be eliminated by natural selection.[8] But natural selection of any form involves a cost—the price is paid in reduced population fitness, and in genetic deaths. According to Muller,[8] Kimura & Crow,[9] and others, genetic load meant that strict limits would be imposed on the amount of genetic variability; populations necessarily must be monomorphic at nearly all loci.

The balance theory of genome structure took a different view of natural selection. Dobzhansky and his colleagues proclaimed that natural selection frequently acted to increase and preserve genetic variability in populations. A typical population was thought to be polymorphic at a relatively large proportion of loci. A number of modes of balancing natural selection have been recognized as potentially capable of maintaining genetic variation: (1) heterosis, in which the heterozygote is superior in fitness to either homozygote; (2) frequency dependence, in which an allele is favoured when rare but selected against when common; (3) diversifying selection, in which selection operates in different directions in different sexes, or in different stages of the life cycle. Diversifying selection acting in different habitats may also maintain genetic variation between populations, or, when coupled with gene flow, increase genetic variation within populations. Early work on phenotypic and viability characters suggested that much genetic polymorphism was present in populations, but the evidence was inconclusive. The recent discoveries of high molecular variability in natural populations have shown the classical view to be fallacious, and have triumphantly vindicated the balance view of population structure. The classical view has been firmly put to rest—or has it?

By an interesting shift of reasoning, the former followers of the classical theory have regrouped and drafted a challenge to the balance theory which has shaken it to its very roots. The intellectual heirs to the classical school, the neo-classicists, cannot and do not deny that high levels of genetic variability are normally present in natural populations. Rather, they deny that natural selection plays a key role in maintaining the variability. They accept the conclusions of the balance school, but not its premises. In a sense, this challenge to the balance theory is far more serious than the former challenge of the classical school.

The neoclassicists, or neutralists, hold that most of the recently discovered genic variability in populations is neutral with respect to fitness. That is, it makes no difference to an individual's fitness whether that individual is homozygous or heterozygous at a given locus; the alleles are adaptively equivalent. They go further to argue that the discovery of high variability was inevitable once the use of high resolution, sophisticated techniques such as electrophoresis became practical. What difference can it possibly make to an organism whether it has, for example, a lysine or a glycine in position 47 of its lactate dehydrogenase molecules? Nonetheless, this difference might well be electrophoretically detectable.

According to the neutralists, most genetic variation is maintained by a balance between the influx of variability through recurrent mutation and migration, and its loss through the process of chance sampling through generations (genetic drift). A very elegant and elaborate theory has been developed which predicts for any particular population the amount of genetic variability and the rates of gene frequency change through time, given the relevant parameters of mutation rate, migration rate (where applicable), and population size. Conspicuously absent from the calculations, of course, are selection coefficients (measures of fitness of different genotypes), since the alleles are assumed to be neutral. The challenge from the neutralists' prediction cuts to the very heart of the balance school. As Lewontin puts it, 'The balance school sees the maintenance of variation within populations and adaptive evolution as manifestations of the same selective forces, and therefore it regards adaptive evolution as immanent in the population variation at all times. Because the alleles that are segregating in a population are maintained in equilibrium by natural selection, they are the very alleles that will form the basis of adaptive phyletic change.'[10]

A theory or hypothesis is scientific only if there are, at least in principal, relevant observations which could falsify it. Any hypothesis which is so general or vague that it could readily explain any conceivable state of affairs does not belong within the realm of science. In other words, a hypothesis must be testable by relevant observations and experiments if it is to have scientific value or empirical content. Popper has shown that the empirical content of a hypothesis is measured by the number of its potential falsifiers.[1] The neutrality theory makes a number of predictions which can be directly tested by relevant observations and experiments. As such, the neoclassicists have made a very significant contribution to

science, whether or not their views are ultimately vindicated. In the following section, I will provide examples of some predictions made by neutrality theory, and how these predictions have been tested by observations and experiments with natural populations.

Testing neutrality hypotheses

Neutrality theory is able to make specific predictions based largely on mutation rates and effective population size. Since these parameters, in particular population sizes, are likely to be very different for different kinds of organisms, it is best to critically test neutrality predictions separately for each species or group of closely related species. One of the most complete sets of data currently available has been gathered by Francisco Ayala and coworkers from the *Drosophila willistoni* complex of fruitflies. The following arguments are based on this work.[11] Arguments, similar in principle, could be cited for other organisms as well. Readers are advised that the neutralist hypotheses and their tests are presented below in only the barest outline; the primary purpose is to exemplify scientific methodology in the field. For more complete presentations see Kimura & Ohta[12] and Lewontin.[10]

The number, n_e, of electrophoretically detectable *neutral* alleles in a population at equilibrium is predicted to be

$$n_e = (8\,N\mu + 1)^{1/2} \qquad (1)$$

where N is the effective size of the population, and μ is the per locus per generation mutation rate to neutral alleles. Also, in a sexually reproducing, random mating population

$$n_e = 1/(1 - \bar{H}) \qquad (2)$$

where $\bar{H}$ is the average heterozygosity.

The fruit fly *Drosophila willistoni* is distributed throughout most of Central America and northern South America. A common result reported by Ayala is that allele frequencies at polymorphic loci are often very similar in different populations, even when the populations are as far apart as southern Brazil and Costa Rica, for example. Neutrality theory could explain this observation if it is assumed that sufficient migration occurs between populations to insure allele frequency homogeneity. In this case the entire species would behave as a single population. The size of this population is immense. In many localities, hundreds or thousands of flies may be collected with just a few sweeps of a net over banana bait. A conservative estimate of the population size for the species is 10^9.

More than twenty-five randomly chosen genetic loci have been electrophoretically examined in *Drosophila willistoni.* Mean heterozygosity (the mean proportion of loci heterozygous per individual) is $\bar{H} = 0{\cdot}179$. Substituting this value into (2) yields $n_e = 1{\cdot}22$, the observed effective number of electrophoretically detectable alleles. If these alleles are neutral, substituting the values of $n_e = 1{\cdot}22$ and

$N = 10^9$ into (1) yields $\mu = 6{\cdot}1 \times 10^{-11}$. This value for the neutral mutation rate is much lower than typical estimates which place $\mu \geqslant 10^{-7}$. Clearly, the observations on *D. willistoni* are not in line with predictions of neutrality. To look at it differently, assume again that $N = 10^9$ (a conservative estimate), and that μ does indeed equal 10^{-7} (also conservative). Then predicted n_e equals 28·3, signigicantly greater than the observed n_e which equals 1·22.

Other evidence on the pattern of allelic variation within species appears incompatible with neutrality theory. Although for most genes, allele frequencies are very similar throughout the range of *D. willistoni*, for a few genes they are very different. Neutralists predict that different populations, not connected by gene flow, should exhibit different alleles and in different frequencies, due to stochastic allele frequency changes through generations. In order to assume homogeneity in neutral allele frequencies between populations, gene flow must be sufficient, or else not enough time has elapsed since the populations separated from a common ancestor for chance to have had a significant influence. However, genetic drift and migration affect all loci simultaneously. That is, if enough time has elapsed for some neutral loci to diverge in allele frequency, enough time has elapsed for some neutral loci as well. And if migration between populations is sufficient to maintain allelic similarity at some neutral loci, all neutral loci should be similar. Such is apparently not the case. Note that although all of the loci examined in *D. willistoni* cannot be neutral, we cannot yet say which ones are selected. It could be that geographic homogeneity in allele frequency represents a response to natural selection; or it could be that genes with geographically heterogeneous allele frequencies are selected; or it could be that all genes are selected.

Many tests of the neutrality hypothesis, similar in outline to those presented in the two preceding paragraphs and utilizing the observed distributions of genetic variability within and between species, may be cited. Some people are unsatisfied by these tests. They point out that the neutrality hypothesis cannot be falsified until we know much more about the true effective size of populations, true mutation rates, and true levels of variability within populations. And in any event, the results do not exclude the possibility that many loci are neutral. Other tests have been conducted which are not subject to these criticisms, and represent especially good examples of strong inference.

Natural populations of *Drosophila equinoxialis* and *D. tropicalis*, other members of the *willistoni* group of flies, are nearly monomorphic for different alleles at the gene encoding malate dehydrogenase. *D. equinoxialis* is almost monomorphic for allele '95', and *tropicalis* is nearly fixed for allele '86'. Experimental cage populations were set up in which allele frequencies were artificially perturbed from their frequencies in nature. On the assumption that the alleles were neutral, the prediction was made that the allele frequencies should remain at their perturbed levels, since the experimental population sizes were large and chance sampling errors would be unlikely to significantly alter allele frequencies in a few generations. On

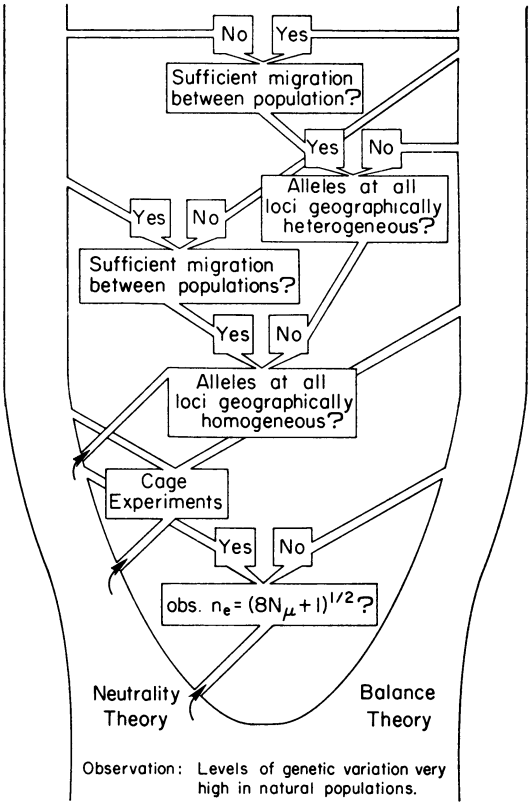

Fig. 1. Conditional inductive tree, with examples of hypotheses and decision points, based upon the new observation of high levels of molecular variability in natural populations.

the other hand, if the fitness of an allele was a function of the physiological or genetic background in which it acts, allele frequency changes should be directional. After about ten generations, allele 95 significantly increased in frequency in the *equinoxialis* populations, and allele 86 significantly increased in the populations of *tropicalis*.[13] These results cannot be explained by neutrality theory.*

The neutralists have contributed significantly to science by formulating important predictions which are eminently testable. A great deal of valuable research has been stimulated. In most of this research, neutrality predictions have constituted the null hypotheses, that is, the hypotheses to be tested. Critics of the balance theory sometimes argue that some form of natural selection could account

* The possibility that selection was acting at the levels of chromosomal inversions rather than at the malate dehydrogenase locus itself was not eliminated.

for any conceivable state of affairs in nature; the concept of natural selection cannot be falsified and therefore is not a scientific hypothesis. This argument is fallacious. Natural selection does indeed have very powerful explanatory power, and can frequently account for alternative observations. But in any particular set of evolutionary circumstances, natural selection can only account for one possible outcome. Nonetheless, an examination of Fig. 1 correctly indicates that neutrality predictions have usually generated the hypotheses which have been subject to falsification. In the future, it will be especially valuable to 'fill out the branches' of the balance theory. Knowing that at least some loci are selected is not enough. Testable predictions based upon alternative modes of natural selection in specific evolutionary circumstances must be formulated and tested before we can safely proclaim that we understand the significance of the recently discovered generic viability.

Molecular variation and individual uniqueness

Near the close of his treatise on *The Origin of Species*, Charles Darwin wrote that through the study of evolution by natural selection, 'much light will be thrown on the origin of man and his history'. Evolutionary thought has since led to the recognition of the close ties which bind man to other biological species, and to his evolutionary past. It has also led to the recognition of the extraordinary evolutionary uniqueness of man. The recent discoveries of molecular variation also have profound implications for man's concept of himself. I will mention just one of these.

Recent estimates based on forty or more loci demonstrate that roughly 40 per cent of man's genes are polymorphic. Using a conservative estimate of 10,000 genetic loci in the genome, this would mean that 4000 genes in mankind are polymorphic. Let us assume, for sake of argument, that this estimate is for some reason inflated by more than an order of magnitude so that only 200 loci are polymorphic. Let us further assume that each gene has only two alleles (this is extremely conservative–many loci in fact are known to have several alleles). Rules of Mendelian heredity show that the possible number of human genotypes is then 3^{200}, or roughly 10,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000, 000,000,000,000,000,000,000,000,000,000,000,000,000,000,000. And this is a very gross underestimate! The total number of people estimated to be alive in the year 2000 is 6,270,000,000. The total number of people who have ever lived on earth will be roughly 12,688,000,000 by then. Thus the potential number of different human genotypes vastly exceeds the number of people that have ever inhabited the earth. With the exception of identical twins, no person is likely to be genetically identical to any other human presently living, to any human who has lived in the past, or to any human who will ever live in the future! Studies in molecular variability provide a biological basis for the recognition of human individuality and uniqueness.

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