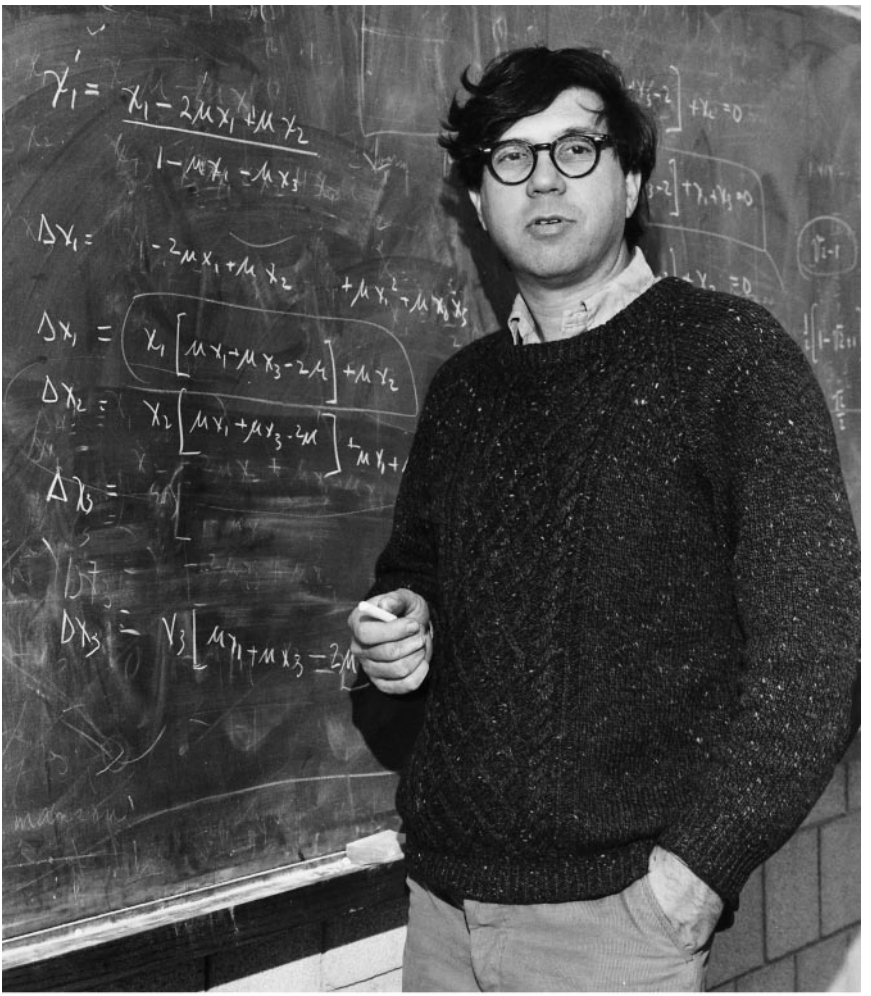

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DIRECTIONS IN EVOLUTIONARY BIOLOGY

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■ **Abstract** In order to understand both the past and future directions of research in evolutionary biology we need to begin by understanding in what way these programs of research differ from the model of most scientific work. The study of evolutionary processes and, in particular, the genetics of the evolutionary process must confront special difficulties in both the conceptual and the methodological aspects of research. On the conceptual side, unlike for molecular, cellular, and developmental biology, there is no basic mechanism that evolutionists are attempting to elucidate. There is no single cause of the evolutionary change in the properties of members of a species. Natural selection may be involved but so are random events, patterns of migration and interbreeding, mutational events, and horizontal transfer of genes across species boundaries. The change in each character of each species is a consequence of a particular mixture of these causal pathways.

CONTENTS

THE GENERAL SHAPE OF EVOLUTIONARY RESEARCH

In order to understand both the past and future directions of research in evolutionary biology we need to begin by understanding in what way these programs of research differ from the model of most scientific work. The study of evolutionary processes and, in particular, the genetics of the evolutionary process must confront special difficulties in both the conceptual and the methodological aspects of research. On the conceptual side, unlike for molecular, cellular, and developmental biology, there is no basic mechanism that evolutionists are attempting to elucidate. There is no single cause of the evolutionary change in the properties

of members of a species. Natural selection may be involved but so are random events, patterns of migration and interbreeding, mutational events, and horizontal transfer of genes across species boundaries. The change in each character of each species is a consequence of a particular mixture of these causal pathways. One of the most detailed and convincing studies of selection in natural populations is the 30-year project of P.R. and B.R. Grant on two species of Darwin's finches, *Geospiza fortis* and *G. scandens*. While they demonstrated changes between generations in bill length, bill shape, and body size that were predictable from their measurements of reproductive fitness, they report that "Natural selection . . . varied from unidirectional to oscillating, episodic to gradual." Moreover, changes over the entire 30-year study were unpredictable from fitness differences and were the consequence of repeated unforeseen hybridizations between the species. Their conclusion after this massive study is that "Continuous, long-term studies are needed to detect and interpret rare but important events and non-uniform evolutionary change" (8). Nor is this only a property of intraspecific change in characters. Different kinds of genetic and environmental events lead to speciation in different cases, and there is no single cause of species extinction. Not even basic cellular processes are exempt from this contingency. That the genetic code is not universal, but differs between nuclear and mitochondrial genomes and among different mitochondrial genomes is a consequence of multiple independent evolutionary changes in mitochondria from an intracellular symbiont. The very relationship between DNA sequence and protein that is described by the standard dogma for most organisms differs in some protists that edit the transcribed RNA by inserting large numbers of U's into the immature message to make a final set of translatable codons. Of course the DNA code and the machinery of transcription and translation are *nearly* universal so the program of cellular biology can generally be carried out by a judicious choice of an experimental system that will represent the great mass of organisms. But for evolutionary biology both the similarities between organisms that are a consequence of their common descent and the differences between them that have occurred in their evolution are the objects of inquiry. For molecular, cellular, and developmental biology differences between cases are an annoying complication. For the evolutionist, differences are what evolution is all about and are expected, whereas persistent similarities across organisms need to be explained.

Second, evolutionary biology is particularly plagued by the discrepancy between the problematic posed by its agreed-upon subject matter and methods that are available to answer the questions posed. This has not been simply a matter of waiting until an appropriate methodology is developed, although there are indeed such cases, but for many problems the difficulty lies in the relationship between processes that have occurred in the past and the evidence about those processes that can only be recovered from presently existing organisms, with a little help from fossils. In the first place, the fossil record is so sparse that no quasi-continuous process of change and diversification can be followed as a detailed dynamic process. Second, the fossil record provides evidence primarily of morphological change,

and nothing can be reconstructed about underlying genetic change and only rarely about physiological and biochemical changes. Nor can living species be used, except under exceptional circumstances, to follow evolutionary changes now occurring. Processes of change and divergence of species are usually extremely slow compared not only to the lifetime of an investigator but of science as an institution. This low speed is, in turn, a consequence of the weakness of most evolutionary forces most of the time. It is now generally agreed that selection differences in nature for most of the alleles segregating at most loci are likely to be of the order of 10^{-3} or less (although the evidence for the intensity of selection, as is discussed below, is indirect). Random changes in allelic composition consequent on extreme fluctuations in population size between generations are not great since, except for very rare alleles, even a population size of 100 is sufficient to keep allelic frequencies within a few percent of their values in the previous generations. Moreover, a small amount of migration between populations, as little as one or two individuals per generation, is sufficient to prevent random divergence among populations. The consequence of the weakness of selective and random forces is that the processes of evolution in living species cannot, except very rarely, be followed as a *dynamic* process in time. Instead, the evolutionary biologist must depend on *static* data, observations of patterns of variation within and between species, to infer the dynamic processes that could not be directly observed.

To infer dynamic processes from static differences within and between species requires a combination of antecedent theoretical developments. In order to estimate the rates and directions of heritable change between species over time, as well as to identify conserved features, it must be possible to reconstruct the chains of common ancestry that connect living species. That is, evolutionary explanation depends upon systematics. The problem is that to reconstruct patterns of ancestry we have only the observed similarities and differences between organisms as data, and we must impose on the data a theory of how character states succeed each other in evolution, a theory that we are trying to construct and verify in the first place by using the inferred relationships. This apparent circularity is not fatal, however, although it leads to ambiguities. The most widely used modern systematic practice depends upon the assumption that a change from character state A in one species to character state B in a descendant species occurs once and once only in the evolutionary process and that this process is irreversible so that B never returns to A. In this scheme, there are no independently derived parallel evolutionary changes, nor convergences from a variety of states to a single one. So, if two species share a character state different from other species, it is because they are more closely related to each other through a recent common ancestor than they are to other species. Given this principle of parsimony, a scheme of common ancestry for all the species is derived that uses all the characters that have been observed. This scheme always contains a number of internal contradictions, so-called homoplasies, inferred character changes that contradict the basic assumptions, but the scheme with the fewest such contradictions is taken as the correct one. Sometimes the choice is easy because one scheme has many fewer homoplasies than its closest competitor, but not

infrequently there a several equally or nearly equally parsimonious phylogenies. Given a very well supported and unproblematic phylogeny, the evolutionist can then use the conservations and changes in the characters to study evolutionary phenomena. The homoplasies become particularly revealing because they represent states that have been repeatedly independently derived and so provide evidence of selective or mutational constraints or of hybridization or horizontal genetic transfer between species. For example, Wells (25) studied the evolution of the glycerol-3-phosphate dehydrogenase locus in the genus *Drosophila* by sequencing the gene in a number of species. The phylogeny of *Drosophila* was well supported by the standard parsimony analysis so that he was able to infer the direction of amino acid change for a number of independent evolutionary trajectories. What he discovered was that for four amino acid positions there had been repeated transitions back and forth among several alternatives, providing strong evidence for the existence of a small subspace of amino acid replacements that were presumably roughly functionally equivalent and of high fitness as contrasted with other possible substitutions at the same positions that apparently were selected against.

Within the general field of evolutionary studies, there is a divergence of view about the purpose of research in systematics. For the systematist, the revelation of the true ancestral relationships among organisms is a goal in itself, because a valid part of evolutionary inquiry is the description of what has actually happened in the history of life. The systematist recognizes that inferences about phylogenies are likely to be inexact to some degree and, moreover, that there is no possible independent verification of the details of a phylogeny outside of the methods of systematics itself. So, if there are a number of phylogenies that are more or less equally supported, the systematist accepts the imperfection of knowledge as the closest one can come to the truth. Indeed, this uncertainty is central to one of the methods of phylogenetic reconstruction, the use of maximum likelihood statistics, whose aim is specifically to *estimate* the correct phylogeny, with all the uncertainty that the statistical notion of estimation involves. While not denying that a revelation of the history of species has an independent interest, evolutionary geneticists and those concerned with the dynamical process of evolutionary change see this essentially historical study as only of instrumental value. For the biologist interested in the dynamical processes of evolution, the reconstruction of the correct phyletic relationships among organisms, the bare description of who is related to whom and how closely, is of no interest in itself but only as a prerequisite to making inferences about processes leading to differentiation and stasis. But the uncertainty about the correctness of a phylogeny may have severe consequences when the "correct" phylogeny is needed to make inferences about dynamic processes. If the phylogeny of *Drosophila* species were not so well supported, it would not have been possible to demonstrate the independent substitutions back and forth between a few amino acids in the evolution of the glycerol-3-phosphate dehydrogenase protein. This apparent shifting back and forth might have been taken as strong evidence that the phylogeny was in error. There is a great deal at stake for the reconstruction of evolutionary processes in having a correct phylogeny.

The other methodological apparatus for reconstructing past events from present organisms is meant to estimate the actual magnitude of forces of natural selection and random events that have led to genetic differences between closely related species or to genetic differences between populations of the same species. It involves a mixture of the mathematical theory of population genetics and statistical theory. It begins with a complex mathematical apparatus that is designed to carry the state of a population forward in time from some initial condition. It predicts rates of genetic change from an initial state and possible equilibria that will result from selection, mutation, migration, and recombination. This must be a stochastic, rather than a deterministic, theory to account for random changes that result from genetic drift in finite populations, so that the form of the prediction is not a unique state at future time, but a probability distribution of states. This is the stochastic theory of population genetics originally produced by Wright (26) and further elaborated by Kimura & Ohta (12).

Second, a probabilistic theory is needed that can reverse the deductions of the first theory and infer backwards in time from a particular observed state at present what the most likely dynamical forces were that have led to the actual present situation. But a difficulty arises here. A dynamical theory that predicts the present state generally requires that we know not only the nature and magnitude of the forces that have operated, but also the initial condition and how long the process has been in operation. That means that if we wish to use a backward inference from the present state to estimate the forces that have operated, we would need to know the initial condition and how long the process has been going on as well as assuming that the forces have not changed during the process. But this is precisely what we cannot know. Either we assume that we know the forces, in which case we can make probability statements about the initial conditions, or else we assume that we know the initial conditions, in which case we can make estimates of the forces that have led to the present. We cannot do both. There is one solution to this dilemma. If the evolutionary process has gone on for a sufficiently long time with no changes in the forces, then there is an equilibrium probability distribution of the present states, the so-called steady-state distribution, that is reached irrespective of the original state of the population. So, if we can observe many genetic variations all of which can be assumed to be the result of the same forces, then the distribution of those variations can be used to estimate those forces. The most important development in recent evolutionary theory has been the creation and application of *coalescent theory*, which allows inferences about forces and times since common ancestry from observations of current genetical variation within and between populations on the assumption that the variation is at the stochastic steady state (22).

Third, we require a set of statistical procedures that can test the agreement between the static observations and various hypotheses about the strength of the different forces, especially whether the observations indicate the operation of natural selection as opposed to purely random drift events [for example, (3, 7, 11, 17)]. These mathematical and statistical tools are now the standard methodology for

detecting and estimating the selective forces involved in molecular evolution of DNA and protein sequences. As is immediately obvious, this methodology involves many assumptions that we will further examine in the discussion of some specific directions in current research.

The integral importance of formal, mathematical, and statistical structures in the reconstruction of evolutionary relationships and in the testing of hypotheses about evolutionary forces marks out much of the research program of evolutionary biology from most other biological inquiry. Tools and methods are, of course, integral to all research, and a new methodology or machine may take over and remake the problematic of a field, as the mechanization of DNA sequencing has done in molecular genetics. Despite their dramatic effect on research, the development of the methods themselves is not generally a major preoccupation of research in the field. The invention of the PCR machine and automated DNA sequencer had a revolutionizing effect on genetic research, but the engineering of those devices was not a major preoccupation of genetical research. In the case of evolutionary biology and genetics, however, the development of formal conceptual tools has consistently been an important field of research throughout the history of modern evolutionary studies. Only recently, with the use of statistical methods for the localization of quantitative trait loci, has research on mathematical and statistical methods come to play a major role in a biological field outside of evolution.

Both the detailed study of particular natural historical cases of observed dynamical changes and the use of static data to infer unobservable dynamical forces have dealt with a small number of specific examples of general phenomena: How are the changes in bill and body size in Darwin's finches to be explained by the observed reproductive behavior of the finches? Is there evidence that amino acid replacements in alcohol dehydrogenase that occurred in the evolutionary divergence of two species of Drosophila were the result of natural selection (11)?

There are two other directions of evolutionary research that are concerned with other levels of generality. One uses high volume methods of data acquisition to look for some generalities and regularities in the characteristics of organisms, usually their genomes, that are relevant to evolutionary processes. The possibility of evolution is constrained by the amount of genetic variation within species. Evolutionary and population geneticists, conscious of the central position that genetic variation plays in the evolutionary process, struggled for 35 years to assess the amount of genetic variation in natural populations, but the methods available to them were inadequate for the gene-by-gene characterization of genetic variation. Then, with the advent of protein gel electrophoresis in the 1960s and routine DNA sequencing in the 1980s, the description of protein and DNA variation in large numbers of different species from all branches of the living world became possible. The generality that emerged is that a very large amount of protein and DNA variation exists in the genomes of nearly all species. Moreover, the differences in variation among species, among genes, and across different functional regions of DNA have revealed general features of evolution at the gene level. For example, it is now clear that the unequal use of alternative codons for amino acids is

universal and also shows both phylogenetic patterns and amino acid and protein class specific biases that transcend phylogenetic boundaries. It is also clear that, with the exception of recently evolved pseudogenes, there is no class of DNA including introns, synonymous codon positions, and downstream flanking DNA sequences that are not subject to some selective constraint in their variation (20). Protein gel electrophoresis provides a striking example of how the introduction of a simple, easily acquired technique can completely dominate and alter the entire direction of research in a field. Before the mid-1960s experimental evolutionary genetics was an extremely heterogeneous field of research, encompassing, among other subjects, studies of the effect of selection on quantitative characters, research on developmental canalization, on species hybridization, on the sensitivity of fitnesses of genotypes to environmental variation, on the evolutionary dynamics of chromosomal rearrangements, and segregation abnormalities in natural populations. The introduction of protein gel electrophoresis as a tool to investigate the standing variation within and between species almost totally depauperized evolutionary genetics for 20 years. The immense diversity of research directions was replaced by a massive program of grinding up every species that lay at hand and visualizing their proteins by gel electrophoresis. The rest of the program of evolutionary genetic research became marginalized or totally inactive, and it has yet to recover its diversity. It remains to be seen what generalities will emerge from the vast amount of DNA sequence that now is being produced by various genome projects. Although these unquestionably will show some general evolutionary patterns, as did protein gel electrophoresis, it is not clear whether the program of evolutionary research will be enriched or impoverished.

The other direction of investigation, in many ways the most interesting, is the discovery and exploration of specific phenomena and relationships that illustrate the complexity and diversity of evolutionary processes. These include, for example, the discovery of RNA editing; of the remarkable conservation of the genetic basis of certain developmental pathways in animals; of the relative ease, in some cases, with which evolutionary novelties may arise; and in others, of the very constrained pathways of successive change in mutational space that may allow the passage from one functional state to another. While none of these are general phenomena of evolution, their importance is that they are examples of the diversity of evolutionary processes and products.

SPECIFIC RESEARCH DIRECTIONS

Case Studies in Nature: Ecological Genetics

During the entire history of evolutionary research, there have been research programs that have concentrated on a single polymorphism or observed change in heritable characters of a species in nature, with the aim of explaining the maintenance of the polymorphism or the rapid evolutionary change that has occurred. The *locus classicus* of such studies was the attempt to explain the dramatic

increase in the frequency of the melanic form of the peppered moth in Britain during the late nineteenth and early twentieth centuries. The textbook explanation of this evolutionary event is the following story of natural selection involving protection of the moths against bird predators. Light-colored moths resting on tree trunks on which there were patches of grayish lichens were cryptically colored so that bird predators could not see them. The increase in air pollution caused by industrialization resulted in the failure of lichens to grow so that light-colored moths now stood out against the dark lichen-less tree trunks while the dark form of the moth was now cryptically colored and protected. This story was bolstered by field experiments in which noncryptically colored moths were actually observed to be eaten by birds. Unfortunately for the neatness of the story, the actual rate of bird predation appears to be negligible because moths spend rather little time resting on a tree. The field experiments, it is now known, involved tethering the moths to keep them in place. Moreover, the caterpillars of the genetically melanic forms have a higher survival rate although no melanin has yet been formed.

Despite its unsatisfactory result, the moth case has inspired a number of more successful case studies, such as the long-term study of *Geospiza* in the Galapagos in which short-term changes in morphology were successfully explained by differences in reproductive success of individuals of different types, while long-term changes involved occasional hybridizations between species (8). This case, and two other older ones, illustrate the general features of such research and raise the issue of its purpose in the general program of evolutionary studies. Lamotte (14) studied the polymorphism of shell color and banding in a large number of French populations of the snail, *Cepaea nemoralis*. Different local populations differ in the frequency of shells with or without bands. The advantages of this system for a case study are that the presence or absence of bands is determined by a single allelic difference that is easily scored without disturbing the animals, that snails can be captured, marked, and released for migration studies and estimates of population size, that snails have low dispersal rates, that deposits of shells of dead snails leave a record of previous populations, and most important, that such shells can be scored as broken or intact, differentiating those that have been preyed upon by thrushes from those which have not. Lamotte then estimated migration rates, effective population sizes, and searched for a correlation between predation by thrushes and presence of bands in local populations of different vegetational cover as a way of detecting natural selection on banding. He found no such correlation, but this was his only measure of natural selection. He then showed that there was a relation between the physical distance between colonies and the difference in allele frequency between them, a relation that fell off rapidly with distance, as was to be expected from the low measured migration rates of the snails. Finally, he fit the distribution of the allele frequencies among colonies to a stochastic steady-state distribution without natural selection, but with genetic drift and migration. Thus he found negative, although by no means compelling, evidence against selection and positive evidence for the importance of genetic drift and migration. The other illustrative study is that of Christiansen & Frydenberg (4) on an esterase polymorphism in the fish, *Zoarces*. This fish was chosen because it is live-bearing

so that offspring broods could be matched to their mothers. By genotyping (using electrophoresis) of mothers and their offspring, the genotype of fathers could also be inferred, and all three pieces of genotypic information could be used to estimate male, female and pair-mating probabilities, fertilities in both sexes, and probabilities of survivorship of genotypes both within broods and between generations in both sexes. This unusually complete set of reproductive information was then used to estimate selection. Despite a sample size of over 1100 females and their broods, no selection was detected.

The examples of the finches, snails, and fish have in common that the model systems were carefully chosen to have biological characteristics that made them particularly suited to the estimation of important parameters of an evolutionary process. Such model systems are very rare. It would not be possible, for example, to carry out such studies in any small mammal or any species of Drosophila. Moreover, they are extremely labor intensive. One of the studies found evidence of selection and two did not. One found evidence of random genetic drift and one of interspecific hybridization. There is no question of making such studies over and over again for many different organisms and many different genes or phenotypes. They neither allow us to make generalizations about forces nor do they uncover new and unexpected evolutionary phenomena. Certainly they do not test any general hypothesis. Rather, they are illustrative of how the forces of evolutionary change may operate in a particular case. There also seems to be some influence of prior theoretical commitment in the choice of the system and the design of the study. While the finch study might have failed to find a correlation between reproductive properties of the birds and the short-term evolution in morphology, its design would not have allowed conclusions about genetic drift affecting the distribution of properties among populations. In contrast, the snail study would not have allowed a complete study of components of natural selection, but was optimally designed to detect genetic drift. The *Zoarces* study, on the other hand, was a purely methodological one with no prior commitment to the size of selective forces, but was rather an exploration of the power of an optimally designed experiment to detect natural selection if it is occurring.

Although well-designed and gratifying in their results, the question then arises as to the role that model natural historical case studies play in evolutionary studies in general. They seem to be entirely illustrative and supportive, to show that a theoretical system of explanation can, in fact, be cashed out in a real case in nature, making the abstract concrete. That is an important goal for a natural science, but the value of yet further examples is unclear. How many finches and snails do we need to convince ourselves that natural selection and genetic drift really do occur?

DETECTING SELECTION FORMALLY

Attempts to measure selection in natural systems by means of natural historical observations of different types is close to Darwin's concrete view of natural selection as the consequence of the differential fit of organisms to their environments leading to differential reproductive success. But one can eliminate the physiological,

behavioral, ecological, and morphological underpinnings of selection, i.e., the *biology* of natural selection, and ask simply whether there is evidence that changes in the characters of species must have been biased for or against some types as opposed to others, so that observed differences cannot be accounted for by purely random events. There is no way to answer this question for physiological, morphological, or behavioral characteristics. We do not know the environments of past organisms in any detail and changes in organisms induce changes in their relations to other species and the physical world. Thus, all claims that such changes have been a consequence of natural selection can only be invented stories with no means of verification or falsification. On the other hand, amino acid and nucleotide substitutions in evolution constitute a very large and relatively homogeneous class of changes of known genetic status so that the question can be asked without recourse to natural historical explanations. This question has been a major preoccupation of evolutionary studies since King & Jukes (13) first called attention to the fact that amino acid substitutions in proteins appeared to occur at a constant rate across long periods of evolutionary time. The struggle over a "neutralist" versus "selectionist" view of protein evolution in the broad sense is no longer an active issue in evolutionary studies. If we include in the neutral theory that selection coefficients on amino acid substitutions may be non-zero but of the same order or less than the reciprocal of effective population sizes, then the broad picture of amino acid substitutions over hundreds of millions of years is one of "quasi-neutrality."

The present direction of evolutionary research on this question focuses on much shorter time intervals, on the transitions between closely related species, and the variation within species. The questions are: How much does selection constrain the amino acid and nucleotide variation within species and how important has selection been in driving amino acid substitutions in species divergence? Because it is impossible to observe the dynamics of selection at the amino acid and nucleotide level either within or between species, the techniques of analysis of static data discussed earlier must be used. Attempts to carry out such static data analysis when only amino acid substitution data were available failed because statistical tests were ambiguous in their interpretation (16). Since the availability of complete DNA sequences of genes, however, it has been possible to use the contrast between nucleotide substitutions in different functional parts of the sequence to ask questions about selective constraints and selectively driven substitutions. The data on selective constraints on amino acid variation within species are copious and unambiguous, and no longer an active subject of research. If we assume, as a first approximation, that synonymous subsitutions in codons are selectively unconstrained, then the nucleotide polymorphism within a species will be same for synonymous and replacement substitutions only if replacement substitutions are also selectively neutral. But a large accumulation of sequence data has shown that there is always a large deficiency of amino acid replacement polymorphisms as compared with synonymous variations. In *Drosophila pseudoobscura* this may range from a complete absence of amino acid polymorphisms in a gene with a 5% synonymous polymorphism rate [alcohol dehydrogenase (23)] to the retention of

about 15% of the amino acid variation that is expected from the synonymous rate [xanthine dehydrogenase (21)]. Most mutations leading to amino acid changes are deleterious. An unsolved puzzle is how selection can be so discriminating as to eliminate every amino acid replacement in the alcohol dehydrogenase protein that is over 20% leucine, isoleucine, and valine. Can it really be that every leucine-valine substitution matters significantly to the physiology of this organism in nature when it can, in fact, survive under general laboratory conditions of culture with the gene for this alcohol dehydrogenase completely inactivated?

Using the contrast between standing variation in synonymous nucleotide positions and replacement positions, it is also possible to ask whether there is evidence that amino acid substitutions between species have been driven by selection. These make use of the statistical tests based on the mathematical apparatus described in the introductory section, involving data on synonymous and replacement substitutions within and between species. These have been applied to a number of cases where population samples of sequences are available and the results have varied. For genes in *Drosophila* a number of cases of selectively driven amino acid replacements between species have been inferred, but different statistical tests have been used in different cases, and there may be some reporting bias since the failure to find evidence for selection is less interesting. While each gene is a separate case study, unlike for natural historical investigation large numbers of different gene sequences can be determined with modern automated techniques so that an overall picture of how much selection is involved in protein sequence evolution now becomes possible. Because of the large amount of data accumulating on sequence differences, it will be possible to make generalizations over genes and species similar to those made in the past about the extent of genetic diversity based on gel-electrophoresis and DNA sequencing and to observe patterns of differences in selective substitution between classes of genes and species with different biology. The only study so far that uses the same theoretical procedure on a large number of genes is that of Bustamante et al. (3), which found that of 34 *Drosophila* genes, 32 gave estimates of positive selection of the substitution, of which 10 were deemed statistically significant, whereas among 12 *Arabidopsis* genes, 10 gave estimates of some selection against the successful substitutions, of which 6 were statistically significant. The authors ascribe this difference between organisms to the smaller effective population size in *Arabadopsis*, which is inbred. The theoretical issue that remains to be resolved before it is worthwhile pursuing this direction further is the adequacy of the statistical procedures for turning static data into dynamic inferences. All the tests so far used make assumptions about the amount of recombination between sites, about the constancy of effective population size during the evolutionary history of the species, and most important of all, assume that the populations are in stochastic steady state. That is, they assume that all traces of initial conditions have disappeared and that no serious disturbances of population structure have occurred since the divergence of the species. But we know that real populations are constantly being demographically disturbed and that episodes of migration, foundation of new populations, and population mixing are a common

feature of population life histories. It is particularly ironic that two species of *Drosophila*, *D. melanogaster* and *D. simulans*, which are human commensals in North America, and a weed, *Arabidopsis*, have been chosen for the application of tests that assume the irrelevance of recent history. Before we can be secure about the results of these studies of selection, we need to know far more about the operating characteristics of the inferential methods when the assumption of steady state is not met.

WHOLESALE SEQUENCING OF GENOMES

The directions of research in any science are a consequence of two interacting forces. First, questions are generated by the phenomena that are the subject matter of the science. What is the chemical nature of the gene? What are the genetic differences that lie at the basis of speciation? How important is natural selection or random drift in driving evolutionary change? But many questions cannot be answered without the development of new experimental techniques, and until those techniques have been introduced the field is filled with reasonable but untestable speculations. The attempts to understand the chemical nature of the gene in the second quarter of the twentieth century relied on the mutational effects of chemicals and radiation, but these observations proved to be inadequate, and the solution had to await the development of crystallographic analysis of macromolecules. Nor could the organization of the genome be understood until methods of DNA sequencing became a routine part of laboratory practice. Once such methods become available, they become a powerful force in directing future directions in the science. Scientists do what they know how to do. As P.B. Medawar put it, science is "the art of the soluble." Any biologist with some money can now sequence vast amounts of DNA, so a major direction of research in evolution will undoubtedly consist in asking, "What will I find out by sequencing the entire genome of . . . ?" There are, however, some problems in evolutionary biology that are already part of the program of study and that will benefit immensely from comparative studies of genomes.

First, comparative genomics is a powerful systematic tool that can resolve problems in phylogenetic reconstruction, which, in turn, will make inferences that use phylogenies more secure. It is already clear that chromosomal and genic rearrangements including inversions, translocations, insertions, deletions, and duplications are an extremely common feature of evolution of the genome. Sequence studies allow the determination of the location of such events down to the nucleotide position, and it is extremely unlikely that the same insertion or rearrangement will recur independently at exactly the same sequence positions in different species or that an insertion, for example, will be excised perfectly at a later time without leaving a trace. For such genomic events, then, the first rule of parsimonious phylogenetic construction holds true, and this provides the possibility of secure phylogenies on which evolutionary influences can be built. Nor is it necessary to sequence the entire genome of species of interest, but only sufficient stretches to include a

good sample of syntenies. In the long run this may be the most widespread use of large-scale sequencing in evolutionary studies.

Second, complete genome sequences will be used simply as gene finders. For studies of the evolution of complex phenotypic and physiological traits, the first step in the analysis is to localize regions of the genome that appear to contribute to their variation. This involves the standard techniques of quantitative trait locus identification that associate phenotypic variation with chromosomal regions marked by segregating genetic variants. The next step requires an identification of candidate genes in these regions, genes whose variation can explain the variation of phenotype on the basis of molecular and developmental mechanisms. An important case for evolutionary research at present and in the foreseeable future is the elucidation of the genetic basis of the barriers to gene exchange between closely related species, barriers that include both interference with interspecific mating or fertilization and inviability and infertility of hybrids if they are formed. For example, in *Drosophila* species, gene differences leading to hybrid inviability are located more or less equally on all the chromosomes, but genetic changes causing hybrid sterility are more concentrated on the X chromosome (5). Ultimately, it will be necessary to identify the genes and understand how their reading by the cell in hybrids causes developmental and physiological abnormalities that, in turn, lead to inviability or infertility. The genetic and physiological bases of these mechanisms will vary considerably from one group of organisms to another so that no general mechanism will emerge. Instead, the elucidation of this fundamental feature of evolution will require gene localization and analysis from a variety of forms and the result will be a menu of different explanations.

Third, genome sequencing allows the detection of hidden homologies. The immense diversity of living organisms is based on the divergence and amplification of genomes derived from the earliest life. The mapping between genomic changes and functional and developmental changes is an extremely complex one so that all trace of the common evolutionary origin of diverse phenotypic characters is often lost when we study only the outcome of development. There are parallelisms, convergences, divergences, and novelties at the phenotypic level that make the facile textbook distinction between homology and analogy almost empty. It used to be said that the wings of bats and the wings of birds were homologous, but that the wings of birds and the wings of insects were only analogous because they were based on utterly different developmental processes with different genetic bases. The *Hox* gene complex has changed all that. By matching DNA sequences between species over an immense range of organisms, it is now possible to discover the common origin and trace the evolution of features of organisms irrespective of the degree of their apparent similarity or difference at any phenotypic level. Of course, all trace of common origin may disappear in particular cases as sequence divergence becomes greater, but if enough intermediate forms are sequenced, it will usually be possible to trace their evolution.

The possibility of following DNA and amino acid sequence evolution makes it possible to detect patterns of conservation at other levels. The amino acid

differences between yeast and vertebrate lysozymes are sufficient to obscure their homology in the absence of intermediate forms, but the conservation of their three-dimensional structure, despite wholesale amino acid substitution, shows that proper lysozyme function can be achieved by a large array of amino acid compositions, provided that these compositions allow the conservation of a particular three-dimensional structure.

The unification of processes in development of very different organisms that is made possible by the study of sequence similarity is perhaps not so surprising given that the early steps in development of different organisms involve similar serial segmentation patterns. But it is also possible to follow gene evolution through sequence space even when there are radical changes in molecular function. The G-protein–coupled receptor proteins are a group of molecules that include such diverse functions as wing development in *Drosophila*, bone morphogenesis in vertebrates, and animal visual pigment systems. Yet, using sequence information, it has been possible to make a complete phylogeny of this immensely diverse set of molecules (19).

EVOLUTION IN BASIC GENETIC MECHANISMS

The general outline of how genes are transcribed and translated is now well understood, and a great deal of the detailed structural biology of the molecular processes is already known. An examination of the details in a variety of organisms, however, has revealed certain constraints and variations on the basic theme that are evidence of the evolutionary plasticity of this most basic of cellular processes. For example, the genetic code is not universal, but differs between mitochondrial and nuclear DNA and from one mitochondrial genome to another. The difference between nuclear and mitochondrial codes might be expected from the origin of mitochondria from invasion by a symbiont, but even if that symbiont differed in its nuclear code from the current nuclear code the current diversity of mitochondria must be the result of subsequent evolution of the mitochondrial codon set. It is not clear how this ancient set of events can be subject to further experiment or analysis. There are, however, other aspects of variation in the coding system that are part of the project of ongoing research.

Synonymous positions in codons are not unconstrained, as shown by the unequal use of alternative codons (codon usage biases) that are present for all proteins in all species but which differ from protein to protein and species to species. There are some generalities. For example, codon usages of highly transcribed genes are more biased than the average (24) but there are many regularities of codon bias that remain to be understood. Using the direction of species origins from the phylogeny of *Drosophila*, Akashi (1) asked how many codon changes in the divergence of species had occurred from highly used codons to less used ones and the reverse, thus enabling him to estimate the selection intensity in the evolution of codon bias. This is a question that remains part of the active problematic of evolutionary genetics and is accessible to experiment. For example, the Adh protein in *Drosophila* species

has 12 or 13 isoleucines but virtually never uses AUA in any species. By in vitro mutagenesis and insertion of a mutated Adh gene into an Adh null line, it would be possible to replace allowed isoleucine codons by the prohibited AUA and measure the fitness decrease and effect on Adh production caused by increasing numbers of such replacements.

It is clear from studies of the variability in polymorphism and species divergence across different regions within a gene that there are patterns of nucleotide conservation within introns and within flanking regions that are without known function. Introns do sometimes contain enhancer elements that need to be conserved, and there are interactions between 5′ and 3′ ends of RNA sequences that may be important in constraining the evolution of gene sequences. Moreover, the possibility of selective constraints on RNA raises the issue of how much of amino acid conservation in proteins is a consequence of the selective effects at the protein level, as we usually assume, and how much is purely the consequence of structural constraints on messenger RNA. If, say, a certain 6-nucleotide stretch in the message is conserved because the three-dimensional structure of the molecule influences its lifetime or its rate of transcription or translation, then that conservation will appear as a conservation of two amino acids. The question of selection of structural properties of messenger RNA is yet to be investigated.

The most remarkable variation in the coding mechanism is the existence of RNA editing in certain flagellates and ciliates. In one form of editing, the DNA sequence is compressed, leaving out T-A pairs so that the template DNA sequence does not correspond to the final sequence of amino acids. The RNA is then edited to add the missing U's, according to separately coded information in the genome, to producing the mature message (15). In another form of editing, the DNA sequence occurs in segments whose order on the chromosome is scrambled. The production of the message unscrambles this order and assembles it in the correct reading order (6). While protozoa have been the organisms in which these editing mechanisms have so far been found, we cannot assume that they are restricted to that group or that the array of bizarre variations that have evolved has been exhausted.

NOVELTIES AND PHENOTYPE-GENOTYPE MAPS

A number of experiments and observations in evolution call attention to a variety of phenomena that arise from the peculiarities of the relation between phenotypes and genotypes. These phenomena have appeared in a disparate group of experiments held together only by the fact that they are all concerned, in one way or another, with the origin of novel morphologies and physiologies. Precisely because they are novelties they are unpredictable and one cannot design a long-term research program to create them, yet each case has something important to teach us about the nature of the evolutionary process.

Hall (9) set about to select *Escherichia coli* that could use a novel carbon source, lactobionate, for its energy, instead of the usual lactose. For this purpose he used a gene, *Ebg* (extra beta galactosidase) that had a low efficiency for cleaving

the galactosidic bond of lactose and could be dispensed with in normal lactose metabolism. Using a mutagen, he finally succeeded in accumulating mutations of *Ebg* that would allow growth on lactobionate, but the evolutionary path to this state was not direct. He was not able to select directly for the new substrate. First, he had to select for increased activity on lactose, followed by a second stage of selection for an intermediate substrate lactulose, and then finally from a strain that could ferment lactulose, he successfully selected strains to grow on lactobionate. Moreover, at each stage of selection there were several strains that acquired the same biochemical phenotype but only some that could be further selected to the next stage. For example, two strains that fermented lactulose did so as a consequence of different mutations, only one of which could be further mutated to a lactobionate fermenter. This result illustrates that the pathway through the space of genotypes from one phenotypic state to another is complex, rather like a maze with many dead ends. Only a restricted subset of all the pathways that lead to the first adaptation are open to the next so that evolution of a novelty may be very difficult to achieve. This suggests one reason for the apparent conservatism of intermediary metabolism.

In contrast, a biochemical novelty may arise by a single very small molecular change. Newcomb et al. (18) found that the acquisition of organo-phosphate herbicide resistance in the blowfly, *Lucilia cuprina*, is a consequence of a single amino acid substitution in the active site of a carboxylesterase that abolished the esterase activity and converted the enzyme to an organophosphatase. Moreover, that qualitative change in specificity was a consequence of a small change in the angle at which the new residue was held in the folded molecule so that a water molecule could now be bound at the active site, a water molecule that could participate in the attack on the phosphate bond of the organophosphate substrate. That this change was not an extraordinary event was shown by the discovery of a second, different amino acid substitution that had the same effect. So, small genetic changes may lead to qualitatively novel adaptive properties.

The last phenomenon, which is likely to be more common, and which can be the object of planned experimental search, concerns the basis for widespread phenotypic conservation of a feature. In *Drosophila*, although there is considerable variation size between species and some variation within species, wing shape is conserved. That is, there are correlations between various wing measurements such that those correlations are characteristic of the variation among individuals within a species, and also between species. Haynes (10) studied a pair of wing measurements that are negatively correlated among individuals within *D. melanogaster* and also among all species of the genus. The assumption originally made was that this represented a developmental constraint that could not be broken. In 15 generations of artificial selection in *D. melanogaster*, she succeeded in reversing the correlation. The same phenomenon was demonstrated for anterior and posterior eyespots on the wings of the butterfly *Bicyclus anynana* by Beldade et al. (2). A strong positive correlation in the size of anterior and posterior eyespot size and other serially repeated features is the rule in butterflies and has been assumed to be a consequence of basic developmental mechanisms of anterio-posterior differentiation. The experiment reversed the correlation within 11 generations of

selection. In both cases, despite the universality of the correlations in nature, there was enough genetic variation in growth relations within a population to allow a selective reversal of the pattern within a few generations. It is part of the theoretical commitment of "evo-devo," the study of the evolution of development and the influence of developmental pathways on evolution, that shape is greatly constrained by basic developmental relations resulting from cell-to-cell signaling and gradients in gene transcription that are more or less fixed across a wide range of organisms. That may indeed be true for some features of development, but it is also clear that the observed constancy of some feature is not in itself a demonstration of such genetically determined invariance. At least for wings in flies and moths, we must assume that natural selection is playing a stabilizing role in preventing evolutionary change in these organisms that is already possible with the genetic variability that they possess.

CONCLUSION

What is already known about evolution shows us that there are no universal rules and even what appear to be regularities have many informative exceptions. Evolution is a loose and complex process, the result of a number of interacting, individually weak forces with many alternative outcomes, and at all times contingent on previous history. The best answer to any question about evolution is the lawyer's answer to any general question about the law: "It depends on the jurisdiction." That is why the program of evolutionary investigation never comes to an end—and, so often, never to a conclusion.

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LITERATURE CITED

1. Akashi H. 1995. Inferring weak selection from patterns of polymorphism and divergence at "silent" sites in *Drosophila* DNA. *Genetics* 139:1067–76
2. Beldade P, Koops K, Brakefield PM. 2002. Developmental constraints versus flexibility in morphological evolution. *Nature* 416:844–47
3. Bustamante CD, Nielsen R, Sawyer SA, Olsen KM, Purugganan MD, et al. 2002. The cost of inbreeding in *Arabidopsis*. *Nature* 416:531–34
4. Christiansen FB, Frydenberg O. 1973. Selection component analysis of natural polymorphisms using population samples including mother-child combinations. *Theor. Pop. Biol.* 4:425–45
5. Coyne JA, Orr HA. 1998. Evolutionary genetics of speciation. *Philos. Trans. R. Soc. London Ser. B* 353:287–305
6. Curtis E, Landweber LF. 1999. The evolution of gene scrambling in ciliate micronuclear genes. *Ann. NY Acad. Sci.* 870:349–50
7. Fu Y-X, Li W-H. 1993. Statistical test of neutrality of mutations. *Genetics* 133:639–709
8. Grant PR, Grant BR. 2002. Unpredictable evolution in a 30-year study of Darwin's finches. *Science* 296:707–11
9. Hall B. 1978. Experimental evolution of a new enzymatic function. II. Evolution of multiple functions for *EBG* enzyme in *E. coli*. *Genetics* 89:453–65

10. Haynes A. 1989. *On developmental constraints in the* Drosophila *wing*. PhD thesis. Harvard Univ., Cambridge. 115 pp.
11. Hudson RR, Kreitman MK, Aguade M. 1987. A test of neutral molecular evolution based on nucleotide data. *Genetics* 116:153–59
12. Kimura M, Ohta T. 1971. *Theoretical Aspects of Population Genetics*. Princeton, NJ: Princeton Univ. Press
13. King JL, Jukes TH. 1969. Non-Darwinian evolution: random fixation of selectively neutral mutations. *Science* 164:788–98
14. Lamotte M. 1951. Recherches sur la structure génétique des populations naturelles de *Cepaea nemoralis* L. *Bull. Biol. Fr. Belg.* 35(Suppl.):1–238
15. Landweber LF. 1992. The evolution of RNA editing in kinetoplastid protozoa. *BioSystems* 28:41–45
16. Lewontin RC. 1974. *The Genetic Basis of Evolutionary Change*. New York: Columbia Univ. Press
17. McDonald JH, Kreitman M. 1991. Adaptive protein evolution at the *Adh* locus in *Drosophila*. *Nature* 351:652–54
18. Newcomb RD, Campbell PM, Ollis DL, Cheah E, Russell RJ, et al. 1997. A single amino acid substitution converts a carboxylesterase to an organophosporus hydrolase and confers insecticide resistance on a blowfly. *Proc. Natl. Acad. Sci. USA* 94:7464–68
19. Rice K. 1997. *The origin, evolution and classification of G-protein-coupled receptors*. PhD thesis. Harvard Univ., Cambridge. 224 pp.
20. Richter B, Long M, Lewontin RC, Nitasaka E. 1997. Nucleotide variation and conservation at the *Dpp* locus, a gene controlling early development in *Drosophila*. *Genetics* 145:311–23
21. Riley MA, Hallas ME, Lewontin RC. 1989. Distinguishing the forces controlling genetic variation at the *Xdh* locus in *Drosophila pseudoobscura*. *Genetics* 123:359–69
22. Sawyer SA, Hartl D. 1992. Population genetics of polymorphism and divergence. *Genetics* 132:1161–76
23. Schaeffer SW, Miller EL. 1992. Molecular population genetics of an electrophoretically monomorphic protein in the alcohol dehydrogenase region of *Drosophila pseudoobscura*. *Genetics* 132:163–78
24. Sharp PM. 1989. Evolution at "silent" sites in DNA. In *Evolution and Animal Breeding: Reviews in Molecular and Quantitative Approaches in Honour of Alan Robertson*, ed. WG Hill, TFC Mackay. Wallingford, UK: CAB Int.
25. Wells RS. 1996. Excessive homoplasy in an evolutionarily constrained protein. *Proc. R. Soc. London Ser. B* 263:393–400
26. Wright S. 1931. Evolution in Mendelian populations. *Genetics* 16:97–159