

THE APPLICATION OF ALLOZYME EVIDENCE TO PROBLEMS IN THE EVOLUTION OF MORPHOLOGY

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ABSTRACT

Living species often offer the only means of inferring the phylogenetic history of particular groups. Since such inferences are usually based on morphology, it is difficult to use them to test ideas concerning rates of adaptive morphological evolution. Allozyme data offer a partial escape from this dilemma. Plethodontid salamanders have been well studied morphologically and electrophoretically. Examples are given of a number of ways in which allozyme data have contributed importantly to an understanding of morphological evolution in the group. When used in the context of a broad-based, systematic approach to particular lineages, allozyme data greatly enrich analytical possibilities. Specific areas in which allozyme data have proven of value include identification of cryptic species, determination of the evolutionary status of allopatric populations, dynamics of species interactions in hybrid zones, and sequence and relative time of divergence of related taxa in relation to morphological evolution. Despite their utility, allozyme data may provide a false sense of security, and some problems are discussed.

INTRODUCTION

The past fifteen years have witnessed an explosion of information describing genic differentiation in natural populations of plants and animals. Initially, evolutionary biologists saw starch-gel electrophoresis as a means of measuring genic variation in natural populations in conjunction with studies in theoretical population genetics (see review by Lewontin 1974). A succeeding phase was the detailed investigation of patterns of geographic variation in allozymes within species, and the comparison of congeneric and more distantly related species (see review by Ayala 1975). The volume of information available is now great, but within any given major taxon coverage is far from uniform.

In this paper I concentrate on one aspect of the utilization of allozymic information for evolutionary studies--the application of such information to investigations of the evolution of morphology within the context of research programs in systematic biology.

MORPHOLOGICAL AND ALLOZYMIC EVOLUTION

Much attention has been given to the degree to which morphological and allozymic evolution proceed in a concordant manner. One approach is to concentrate on cladograms and classification, and to investigate the degree to which trees or classifications of the same organisms based on

different data sets postulate the same groupings (taxonomic congruence, Mckevich 1978). Several methods exist for generating classifications from morphological and molecular data and methods exist for comparing such classifications (summarized by Mckevich 1978; Prager & Wilson 1978; Throckmorton 1978). As data sets grow and as the number of entities increases, the number of possible trees also increases. Many nearly equivalently acceptable trees (using some predetermined measure of goodness of fit) may occur (Felsenstein 1978). The degree of taxonomic congruence of data sets based on allozymes and morphology offers new opportunities for analyzing the phylogenetic history of adaptive morphological evolution. A prior assumption is made that allozymic evolution is mainly a time-dependent phenomenon. Once data are available, this assumption can be tested by constructing dendrograms using algorithms such as that of Fitch and Margoliash (1967). Analysis of the data, then, requires no assumptions concerning molecular evolutionary clocks. By comparing groups obtained from the two data sets, it is possible to determine where in the different trees there have been changes in rates of molecular or morphological evolution. The expectation from many empirical results is that trees based on molecular data are more internally consistent than are trees based on morphology (for example, see three data sets analyzed by Mckevich 1978), and as a general rule, the tree based on molecular data is an appropriate point of departure for comparative studies

(Wilson et al. 1977).

A second approach is to examine the relative rates of morphological and allozymic evolution on a somewhat more ad hoc basis, looking at particular species pairs or small groups of closely related species. The best known of these studies is that dealing with human-chimpanzee relationships (King & Wilson 1975; Bruce & Ayala 1979). These two species differ substantially in morphology but allozyme differences are similar to those commonly recorded among congeneric species of other mammals. The interpretation is that while molecular evolution has proceeded at more or less "normal" rates, morphological evolution has been very rapid. Many workers have now found instances in which relatively great morphological differentiation has been accompanied by little or no allozymic differentiation (for example, in studies of teleost fishes by Avise et al. 1975; Sage & Selander 1975; Kornfield & Koehn 1975). Conversely, species that are essentially identical in morphology have been found to be extremely differentiated genically (Highton 1979; Larson & Highton 1978; Yanev 1978, 1980). The most direct conclusion is that rates of morphological evolution vary greatly from group to group as evolutionary biologists have long known (e.g., Simpson 1944). Among vertebrates, many groups of teleost fishes (e.g., Avise & Smith 1977; Avise et al. 1977), birds (e.g., Avise et al. 1980), and mammals evolve rapidly in adaptively significant morphological features while amphibians and, to lesser extents, reptiles (Adest 1977; Bezy et al. 1977; Wyles & Gorman 1980), evolve relatively slowly.

In this paper I focus particularly on salamanders of the family Plethodontidae. I do so because I believe that in order to use allozyme data effectively to gain an understanding of morphological evolution it is necessary to study specific lineages from a phylogenetic perspective. Plethodontid salamanders have been well studied morphologically, and now a great amount of information on allozymes and other molecular aspects of their evolution has become available.

THE USE OF ALLOZYME DATA IN MORPHOLOGICAL AND SYSTEMATIC STUDIES OF PLETHODONTID SALAMANDERS

I will illustrate some applications of allozyme data by specific examples. The examples are grouped into four categories, all of which involve to some extent the two general approaches outlined above. The categories are organized in the form of specific problems faced by systematic bio-

logists, and include: 1) identification of cryptic species; 2) the systematics of allopatric populations which are similar in morphology; 3) dynamics of interactions in hybrid zones; and 4) sequence and relative time of divergence of related taxa in relation to the evolution of morphology. In all of these examples I make the assumption that the major problem to be understood is the nature of adaptive morphological evolution.

Identification of Cryptic Species

In recent years we have become aware of the fact that many species in nature are so similar in morphology that we have mistakenly considered them to be conspecific. One of the most practical applications of allozyme techniques has been the separation of sympatric, cryptic species. A striking example is the work of Yanev (1978, 1980) on the slender salamanders, *Ambystoma*, in central coastal California. Formerly a single species, *A. attenuatus*, was thought to be continuously distributed from extreme southwestern Oregon along the coast to southern California, with a disjunct group of populations in the Sierra Nevada (Hendrickson 1954). Brame and Murray (1968) used morphological evidence to segregate a group of coastal populations south of Monterey Bay as a distinct species (which they named *A. relictus*), but they continued to recognize *A. attenuatus* as being continuously distributed for nearly the entire length of the state. In the Monterey Bay area it was more interior in relation to the parapatrically distributed *A. relictus*. Using allozyme data, Yanev found *A. attenuatus* in this region was a complex of three parapatrically distributed species exhibiting a complex pattern of species replacement (fig. 1). The central of these three species was found to be a semispecies of *A. relictus*. The two semispecies meet and interact in a narrow zone, but they cannot be distinguished morphologically. They are distinct genetically, with an average genetic distance (all genetic distances in this paper are those of Nei [1972] unless otherwise noted) of .45, a substantial difference. Away from the coast, in the inner coast range, morphologically similar species are arranged in a north to south sequence. In the north, *A. attenuatus* (in the now restricted sense of Yanev 1980) is replaced on its southern borders by the innermost of the two semispecies. The genetic distance between them is about 1.5. In turn, this semispecies is replaced to the south by *A. nigriventris*; these species are separated by a genetic distance of about 1.0. The genetic distance between *A. nigriventris* and *A. attenuatus* is about 1.5. The areas of

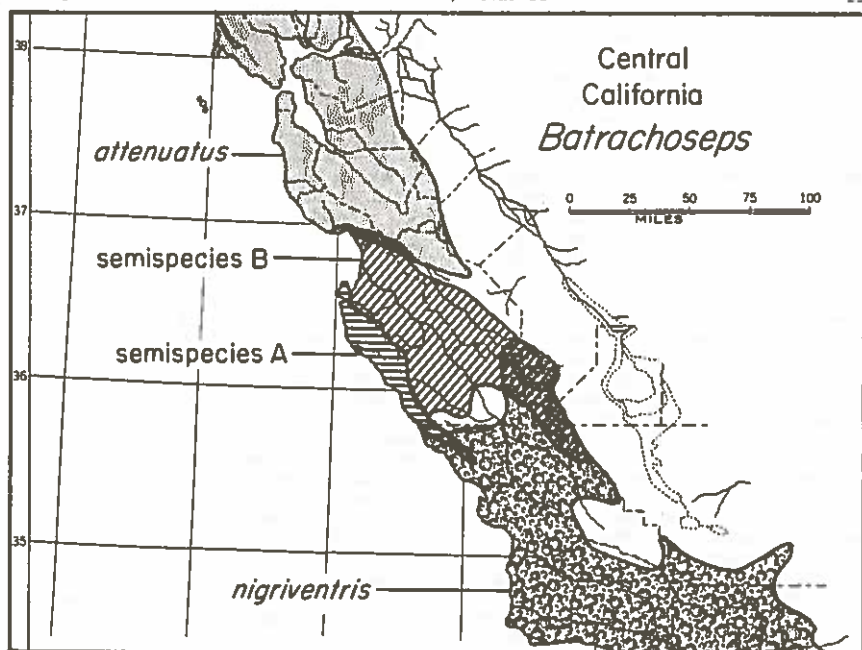


Fig. 1. Distribution of three species of plethodontid salamanders of the genus *Batrachoseps* in central coastal California. The two semispecies were recently assigned to the species *B. pacificus* by Yanev (1980), but earlier authors have assigned populations of semispecies A to *B. relictus* and semispecies B to *B. attenuatus*.

replacement are characterized by narrow zones of sympatry, but no evidence of hybridization has been found. These three species cannot be separated on morphological grounds, even when powerful multivariate morphometric techniques such as discriminant function analysis are used. However, once the three species are sorted on the basis of allozyme data, subtle morphometric and osteological differences can be detected, even though these do not aid in identifying the species.

An even more extreme instance of genetic differentiation without morphological differentiation involves the *Plethodon welleri* species group of salamanders in the eastern United States. A group of populations in the southeastern part of the range of *P. dorsalis* was found to have an average genetic distance of approximately 2.0 from supposedly conspecific populations to the north and west (Larson & Highton 1978). In contrast, the average genetic distance among the southeastern populations, some of which are isolated geographically, is only about .08. Subsequently, Highton (1979) discovered one

locality in Jefferson County, Alabama, where the two species are microsympatric, and described the southeastern population as a new species, *P. websteri*. Highton notes that although the two species share less than 20% of the alleles discovered at 26 electrophoretically detectable loci, no morphological differences in standard taxonomic characters used to distinguish taxa in the genus have been detected. No morphometric analysis has been performed on these species, but there is little question concerning their similar morphology.

In several instances allozyme information has facilitated increased sophistication of morphological analysis. This has been especially true of the plethodontid salamanders of the New World tropics, a speciose group that includes over one-half (about 150) of the living species of salamanders. Tropical species frequently are differentiated by subtle morphological traits that usually have not been used to distinguish North American species. There has been some question as to whether there really are large numbers of species in the tropics (Dunn [1926] recognized only 31).

On Cerro San Felipe, Oaxaca, Mexico, relatively large salamanders of the genus *Pseudoeurycea* occur. Taylor (1941) recognized two species, *P. smithi* and *P. unguidentis*, but samples were small and the rather subtle differences between the two were not documented. On the basis of extensive field work and study of large numbers of additional specimens, Bogert (1967) recognized but one species. Lynch and associates (1977) performed an electrophoretic analysis, and followed this with a morphometric analysis, based on microsympatric samples. The genetic distance of $D = .61$ is relatively large for morphologically similar species. Discriminant function analysis and regression analysis performed on eight morphometric variables revealed highly significant differences between the samples, once they had been properly sorted.

Hanken (1980) examined Mexican salamanders of the genus *Thorius* in detail, using a combination of electrophoretic and morphological approaches. These are the smallest salamanders in the world, and perhaps the smallest tetrapods (sexual maturity is attained in some males at a body size of about 15 mm). The size of these animals hindered detailed analysis in the past, and there were questions concerning the number of valid species. As in the *Batrachoseps* and *Pseudoeurycea* examples, once the species of *Thorius* were separated on the basis of allozyme information, Hanken was able to document morphological differences between them. The genetic differences between these supposedly slightly differentiated species were high, frequently $D = 1.0$.

It remains to be seen if other instances of species thought to be morphologically similar but well differentiated genetically are morphologically identical or not. Workers often have relied only on "standard" taxonomic characters, and have not performed detailed morphometric and osteological analyses. For example, the members of the plethodontid genus *Desmognathus* have been considered to be confusingly similar in morphology, and there is little question but that they are. No detailed morphological study of the group has been completed. Recently Tilley and colleagues (1978) reported the discovery of two similar kinds of salamanders in the southern Appalachian Mountains of North Carolina. At some localities the two were readily separable on the basis of complex coloration differences. At other localities it was apparent that the total range of color variation was great, but two groups could not be distinguished. Allozymic differences proved to be great, with $D = .59$ between *D. ochrophaeus* and the mor-

phologically similar species *D. imitator*. The authors state: "Unfortunately, the taxonomic arrangements suggested by electrophoretic data and color patterns do not coincide". They go on to note that a major disadvantage of electrophoretic data in their study is that the taxonomic units they generate are otherwise indistinguishable. Detailed morphometric studies of these species, which are apparently very similar in morphology, are badly needed.

The dangers of relying on "standard" morphological characters in salamander taxonomy are demonstrated in another example. Brodie (1970) described a new species of plethodontid salamander from west-central Oregon. *Plethodon gordonii* was said to be distinct from its sympatric associate, *P. dunni*, in coloration and morphology. Feder et al. (1978) showed that the two were virtually identical in allozymes ($D = .001$). Re-evaluation of the morphological data demonstrated that the sole difference was the color pattern, an apparent polymorphism. A similar color polymorphism in *P. cinereus* has been shown to be the result of a relatively simple genetic trait (Highton 1959, 1975).

The most intensive study of the relation between geographic variation in standard taxonomic (morphological) characters and allozymes is that of Highton (1977), which focused on *Plethodon cinereus* of the Del-Mar-Va Peninsula east of Chesapeake Bay in eastern United States. Two "standard" taxonomic characters, one coloration polymorphism (striped versus unstriped color phases) and one morphological polymorphism (number of trunk vertebrae) were compared with five variable electrophoretic loci. There was little concordance in the patterns of variation in the two morphological characters (177 localities), but there was a high level of concordance among the five loci (36 localities). On this basis, Highton argued that allozymes offered more insight into the past history of groups than did the morphological characters used. It is unlikely that any insight into the evolution of the restricted kinds of morphological features previously used in the classification of species of *Plethodon* will come directly from the study of allozymes.

In summary, allozymic information is proving to be an invaluable, nearly essential tool in detecting cryptic species and sorting color morphs in salamanders. This is an essential first step for any analysis of morphologic evolution in taxa. There are many reasons to believe that there are far more species of plethodontid salamanders

than have been recognized. Despite the occasional instance of invalid species, the dominant theme has been detection of unrecognized, cryptic species. Work completed but yet unpublished and in progress (especially by Guttman, Hanken, Highton, Tilley, Wake and Yanev) will provide additional documentation of this conclusion.

Allopatric Populations Similar in Morphology

The allozyme revolution has posed new problems for those interested in adaptive evolution, especially those concerned with the evolution of morphology. Nowhere is this more apparent than in the venerable problem of how to deal with allopatric populations of morphologically similar animals. While allozymic information adds an important new dimension to the problem, no solution is provided. Some specific examples illustrate the new problem.

The red-backed salamander, *Plethodon cinereus*, has been intensively studied by Highton and his co-workers over a number of years, and it is one of the best known vertebrates in terms of geographic patterns of variation. The species is continuously distributed from North Carolina, eastern Illinois and Minnesota northeasterly through southeastern Canada, and east to the Atlantic seaboard. To the south and west from this area of continuous distribution are five isolated geographic units that differ either in coloration, numbers of trunk vertebrae, or both, from the nearest populations in the area of continuous distribution. Highton and Webster (1976) performed an extensive electrophoretic analysis throughout the geographic range of the species, including the five isolates. Differentiation within the area of continuous distribution was low ($D = .11$, range 0-.24), and comparable levels were found among the five southern isolates ($D = .07$, range 0-.19). However, genetic distance between these units averaged .42 (range .31-.67). For four of the 24 electrophoretic loci examined no alleles were shared by the two units, and there were strong differences at two additional loci. Highton and Webster noted that there was no empirical basis for assignment of species status to the unit composed of the five isolates. They attributed the similarity within the taxa they recognized to common ancestry, and argued that evolution of reproductive isolation often accompanied genic divergence of the magnitude they measured. Plethodontid salamanders seem to speciate according to the classical allopatric model (Mayr 1963), and implicit in the argument of Highton and Webster is the notion that genic divergence as measured by

allozyme comparisons builds as a function of time since separation. The longer the period of separation, the greater the likelihood that accumulated genetic differences will be sufficiently great that the units would behave as biological species if they re-established contact. For these reasons, and on the basis of some biogeographic considerations, Highton and Webster assigned the five isolates to a single species, *P. serratus*. The five isolates share no unique morphological characteristics.

Guttman and co-workers (1978) examined another allopatric pattern in *Plethodon*. In this case, an isolated group of populations in southern North Carolina had been described as a distinct species, *P. longicrus*, based on its morphological distinctness (in such features as limb length) from its closest relative, *P. yonahlossee*, located to the north and east (Adler & Dennis 1962). Only three of 21 loci were polymorphic, and there are no significant differences between the two supposed species. The greatest genetic difference recorded between populations assigned to the two species was less than .10. They attributed some of the morphological differentiation to clinal geographic variation, and demonstrated that the remaining morphological differences were less significant than had been thought originally. They reduced *P. longicrus* to the synonymy of *P. yonahlossee*.

At present, for salamanders these examples represent the limits of application of allozymic evidence to problems of allopatry. Morphologically similar, but distinct, species of sympatric *Plethodon* have genetic distances as low as .24 (*P. cinereus* and *P. shenandoah*--Highton & Larson 1979), but some allopatric units that are differentiated only on the basis of minor morphological and genetic differences are recognized as distinct species. An example are the closely related allopatric species *P. punctatus* and *P. wehrlei* of eastern United States, which are similar in morphology (Highton 1972) and in allozymes ($D = .18$; Highton & Larson 1979). Highton and Larson recognized several morphologically distinct, geographically isolated populations that had subspecific status as separate species. They reasoned that such a decision was required for purposes of "taxonomic consistency", since these populations had minimal genetic distances from other populations greater than those between several sympatric species pairs that never hybridize in nature.

Highton (pers. comm.) has elaborated his position for me. Taxonomists must

make judgements concerning the status of most allopatric populations that are only a little better than "educated guesses". In the past, judgements usually were based on degree of morphological divergence. For several reasons, degree of morphological divergence can be misleading. Character displacement (a controversial phenomenon that is difficult to demonstrate but is generally accepted as being of considerable significance) increases the morphological divergence of sympatric species known to be isolated reproductively, and this leads to a bias when comparing allopatric populations. Further, as I demonstrate in this paper, morphological evolution is frequently not concordant with genetic evolution. Highton argues that buildup of genetic distance is a better predictor of whether two populations will interbreed upon recontact than is morphology, and he is probably correct. But this is all relative, and I am not convinced that buildup of genetic distance is very useful in establishing taxonomic status, except in extreme cases.

Whether one uses a biological or an evolutionary species concept, there is little ground for a priori determination of species status. If we consider biological species concepts for the moment, there is no information in salamanders concerning the relationship, if any, between rate of allozymic evolution and rate of evolution of reproductive isolation. As genetic distance between allopatric populations increases, the likelihood that reproductive isolating mechanisms have evolved by chance alone also increases. Whether this likelihood is increased when buildup in genetic distance is also accompanied by morphological differentiation is simply a matter of speculation. Perhaps chance-based isolating mechanisms evolve strictly as a function of time rather than as a function of morphological differentiation, but when morphological differentiation is a result of adaptive responses one might expect isolating mechanisms to evolve as a result of pleiotropy or other factors affecting the whole organism (see Mayr 1963; Templeton 1980). There seems to be no theoretical basis for the argument of "taxonomic consistency", which appears to be no more than a rule of thumb.

In fact, application of the taxonomic consistency rule might lead to chaos. We know that morphologically distinct species of *Drosophila* have genetic distances as low as .063 (Sene & Carson 1977), and such levels are characteristically found between populations of the same species in the genus (Ayala 1975). Morphologically distinct species of birds frequently are only slightly differentiated in allozymes. Bar-

rowclough and Corbin (1978) found genetic distances between species of wood warblers to average only .10, and between genera of Parulidae to be only .18. The Veery (*Catharus fuscenscens*) and Swainson's thrush (*C. ustulatus*) have a genetic distance of only .01 (Avisé et al. 1980). Six sympatric species of Galapagos ground finches (*Geospiza*) cannot be distinguished electrophoretically (Yang & Patton 1981). It seems inevitable that sympatric species of salamanders will be found with low genetic distances, and Highton and Larson (1979) have already reported values as low as .26 (*Plethodon shenandoah* and *P. cinereus*). Instances are known of allopatric populations of salamanders only slightly differentiated in morphology having genetic distances greater than .2 to .3 (Yanév 1978; Yanév & Wake 1981; Larson & Highton 1978; Hedgecock & Ayala 1974; Larson 1980), and examples will multiply with time (work in progress by Highton, Wake, Yanév, Yang and others).

Many examples of the difficulties encountered with allopatric populations have been discovered among the tropical salamanders. The plethodons of eastern United States form a nearly continuous "carpet" throughout the region, and one has a chance of an occasional "sympatry test". In contrast, tropical species often have extreme elevational limits (Wake & Lynch 1976), and may be separated from related populations by significant spatial gaps. Salamanders associated with *Pseudoeurycea leprosa* on the southern part of the Mexican Plateau are today mainly limited to elevations in excess of 2500 m. Wake and his colleagues (in prep.) studied seven populations of *P. leprosa* and compared them with single populations each of the related species, *P. robertsi* and *P. altamontana*, from the same area. One population of *P. leprosa* is sympatric with *P. altamontana*, and there is no hybridization so we know that these two represent distinct species. They are similar in morphology, but can be separated on rather subtle morphological characteristics (*P. altamontana* is somewhat more robust, with a broader head and longer limbs). One additional population (called Villa Victoria for reference) had been assigned previously to *P. robertsi* but we felt that it was not very similar to that species, and considered it to be simply one more population of *P. leprosa*. *Pseudoeurycea robertsi* has been recognized as a distinct species by all previous workers, and it is rather distinct in morphology from other populations included in our study.

Some populations of *Pseudoeurycea leprosa* are close to others with genetic distances as low as .002. However, there is

a buildup of genetic distance with geographic distance and two remote populations have a genetic distance of .54 from each other. The species as a whole is distinct from both *P. robertsi* and *P. alta-montana* (genetic distance of greater than 1.5 to both), but to our surprise the distance to our Villa Victoria population is even greater (1.8). Further, the genetic distances between the Villa Victoria population and both *P. robertsi* and *P. alta-montana* are greater than 1.0. Conversely, these two latter, morphologically distinct but allopatric species are relatively close genetically ($D = .15$). Perhaps the Villa Victoria population should be described as a distinct species simply because the genetic distances to potential relatives are so extreme.

One cannot as yet conduct hybridization tests between populations of plethodontid salamanders, and even if we could the results would be of dubious value. The allopatric populations problem is destined to plague us forever, and it is likely to be especially acute in plethodontid salamanders, where the allopatric mode of speciation seem to dominate. As a general rule of thumb I would argue that allopatric populations should not be given species status on grounds of allozyme differentiation alone in the absence of significant morphological differentiation.

Dynamics of Interactions in Hybrid Zones

Details of allozymic interactions in hybrid zones have been studied in only four instances to date: *Plethodon ouachitae*-*P. fourchensis* in Arkansas (Duncan & Highton 1979), *P. jordani*-*P. glutinosus* complex in North Carolina (Peabody 1978), *Desmognathus fuscus*-*D. ochrophaeus* in Ohio and Pennsylvania (Karlin & Guttman 1981), and *Bolitoglossa franklini*-*B. resplendens* in Guatemala (Wake et al. 1980). Of these the latter two sets involve the most genetically distinct groups ($D = .46$ and $.48$, respectively) and they are the most distinct morphologically as well. The *P. jordani*-*P. glutinosus* situation is under intense study by Highton currently, and I will discuss it no further here.

All hybrid zones so far studied have been detected first on morphological grounds (mainly coloration). Duncan and Highton (1979) did not attempt to analyze coloration in detail in the hybrid zone, but simply noted that the hybrid populations were intermediate, with individuals in these populations showing a great deal of variation.

An attempt was made to compare electrophoretic phenotypes and morphological phen-

otypes directly in the *Bolitoglossa* study (Wake et al. 1980). Separate electrophoretic and morphological hybrid indices were established, and the results compared. While hybrid populations were more variable in coloration, good numbers of parental morphotypes were found in the main hybrid population. There were strong heterozygote deficiencies for the three electrophoretic loci that showed fixed differences between parental populations. Several suspected F₁ individuals were found using electromorphic data, but only one of these was assigned a hybrid morphological score. There was poor concordance between the two indices. The species with the narrowest ecological niche, *B. franklini*, has the most resilient morphological phenotype; it accepts a large dose of *B. resplendens* alleles before any outward sign of hybridization is detected. In contrast, *B. resplendens* has a labile morphological phenotype and resembles *B. franklini* when only slight evidence of hybridization can be found from allozyme analysis. Hybridization here may be a relatively recent phenomenon, but in the case reported by Duncan and Highton (1979) no heterozygote deficiencies are reported and there seems to be a smooth, but abrupt, transition from one species to the other over a distance of less than 10 km, and perhaps much less. The *Bolitoglossa* situation appears to represent a kind of tension zone, in which a species better suited to ecological disturbance is introgressing into and possibly replacing a more narrowly adapted one. In the *Desmognathus* case hybridization appears to be rare with introgression occurring in both directions. The *Plethodon* case seems to be a simpler situation resulting from secondary contact of incompletely differentiated units.

Mode of speciation is a major factor in assessing the systematic implications of hybridization. Templeton (1980) has recently contrasted modes of speciation which he terms transilience and divergence. The former are associated with small populations and founder effect, and there is little evidence that it has been important in the evolution of plethodontids. Plethodontids appear to speciate divergently, either adaptively (establishment of an extrinsic isolating factor, followed by independent microevolution and establishment of isolating mechanisms) or clinally (the isolation by distance model). Transilient speciation frequently involves hybridization as a part of the speciation process, and Hall (1977) has presented arguments that stable hybrid zones get established that serve not as foci of introgression or species breakdown, but as "sinks". In contrast, when hybridization occurs following re-establishment of units that have

speciated divergently, stable hybrid zones are not expected. Hybridization is likely to be ephemeral, or it is likely to lead to such profound changes as replacement of one species by another (as in the *Bolitoglossa* example) or such extensive mutual introgression as to blur the distinction between the units. When hybridization occurs between species thought, on independent grounds, to have speciated divergently, care should be taken to assure either that the hybridization is limited, or that there is no extensive mutual introgression before recognizing the units as distinct species. Should these conditions not be met, and in the absence of morphological or other phenotypic differences, such situations are probably best treated as instances of secondary intergradation within a single species.

Sequence and Relative Time of Divergence of Related Taxa in Relation to the Evolution of Morphology

There now exist a number of methods to estimate the phylogenetic history of groups of organisms. Here I will simply demonstrate some of the ways in which these methods can be combined to provide insight into the evolution of morphology. The general approaches used for discrete morphological characters are those of Hennig (1966). The allozymic and other molecular approaches to be used were recently reviewed by Throckmorton (1978) and some general methods applicable both to allozymic and morphometric data are considered by Mickleitch (1978) and Larson (1980).

A first step is the generation of phylogenetic hypotheses from one set of data, and the second is the evaluation of those hypotheses by comparison with another, or several, sets of data. Those individuals who work with *Drosophila* are fortunate to have phylogenetic hypotheses well worked out from chromosome inversion distributions. Most of the rest of us have had to use morphology alone for the generation of phylogenetic hypotheses. When it is the evolution of morphology, or the adaptive history, of groups that we wish to discuss, the argument quickly becomes circular, or, at a minimum, self-serving. Allozyme information offers the possibility of generating phylogenetic hypothesis independent of morphology. If we can find yet another way of testing the phylogenetic hypothesis based on allozymes, we then have a method for examining the adaptive history of groups.

One approach is illustrated in figure 2. This is an analysis by Wake and associates (1978) of correlated variation in discrete allozymes, using the same logic

as that of Throckmorton (1978) for a method he called covariation analysis. This is a method whereby ancestral and derived alleles are identified, and the derived ones used to develop a phylogenetic hypothesis. Any allele that occurs in all species of a taxon is ancestral. In addition, any allele that is found in a related outgroup and one or more of the species is considered to be ancestral. This is an academic issue as far as *Hydromantes* is concerned, for no other salamanders are sufficiently closely related to constitute an appropriate outgroup. In any large data set, conflicts immediately arise, and a variety of phylogenetic hypotheses can be generated rather easily. In general, the greater the number of alleles and the greater the number of entities, the greater will be the number of potential phylogenetic hypotheses. In many instances it will be impossible to generate one hypothesis that is definitively the best, in the sense that it is robustly the most parsimonious. In order to achieve this status a hypothesis should require several fewer evolutionary events than competing trees, and should be relatively immune to change with the addition of more loci or the uncovering of undetected variation by more sophisticated technique.

The genus *Hydromantes* includes five species, three of which occur in California. The other two are the only plethodontid salamanders in the Old World and they occur in extreme southeastern France and Italy, including the island of Sardinia. The lineage incorporating the American species and the lineages associated with the two European species each display from six to nine unique, fixed alleles (fig. 2). The question is whether the European species form a distinct clade, or if, instead, *H. genei* from Sardinia is more closely related to the American species than to *H. italicus* from the European mainland? Each of these hypotheses is supported by data from three alleles. The three joining *H. genei* and *H. italicus* are fixed in both species. The three joining *H. genei* and the American species are not fixed in all populations investigated, and one is absent in some populations of the American species.

We tested the hypotheses by using the independent molecular method of micro-complement fixation, an immunological technique using antisera prepared to albumin (in the present instance). The mean reciprocal immunological distance (ID) between *Hydromantes genei* and the American species is 44.3 ± 7.3 , and the one-way ID from the American species to *H. italicus* is fairly close to this (49; the comparable one-way ID to *H. genei* is 48). In contrast, the

HYDROMANTES

	American Species			European Species	
	<i>brunus</i>	<i>shastae</i>	<i>platycephalus</i>	<i>genei</i>	<i>italicus</i>
1.	x	x	x	(x)	(x)
2.	x	x	x	(x)	(x)
3.				x	x
4.	x	x	x		
5.	x	x	x	x	
6.	x	x	x	x	
7.	x	x	x		x
8.	x	x	x		
9.	x	x	x		
10.	x	x	x		
11.	x	x	x		

Fig. 2. Analysis of correlated variation in eleven allozymes in the plethodontid salamander genus *Hydromantes*, based on Wake et al. (1978). Distribution of allozymic electromorphs among species is indicated. For allozymes 1 and 2, different electromorphs of the same allozyme are fixed in the American and European species, respectively.

one-way ID from *H. genei* and *H. italicus* is only 15 (Maxson & Wake, unpubl. data). The hypothesis that *H. genei* is more closely related to the American species of *Hydromantes* than to *H. italicus* is rejected.

In this instance the available morphological data are in concordance with the picture developed from molecular data. The American species share a peculiar muscular arrangement in their tail base, associated with use of the tail as a locomotory appendage (Serra & Stefani 1974), and the European and American species each share unique arrangements of the anterior cranial elements of the skull (Wake 1966).

Hydromantes illustrates an instance in which cladistic analysis of allozymes and morphology corresponds both with data on albumin evolution (essentially phenetic at this point) and biogeography, giving a reasonably well-rounded picture of the phylogenetic history of the group.

Recently another group of five species has been analyzed. The genus *Aneides* includes three species from the west coast of North America, one from the mountains of New Mexico, and one from the southern Appalachian region of eastern United States. Its close relative, *Plethodon*, also occurs in eastern North America, restricted portions of the Rocky Mountains, and along the west coast from northern California to southern British Columbia. These genera have been well studied from

a number of perspectives, including morphology, allozymes, and micro-complement fixation, and details are published elsewhere (Larson et al. 1981).

The five species of *Aneides* are surprisingly remote from one another in allozymes, and all two-way genetic distances approximate or exceed 1.0. The species were so remote that covariance analysis of allozymes was of little value.

Immunologically, *Aneides* is closer to the western assemblage of *Plethodon* than the latter is to the eastern assemblage of *Plethodon*. Larson et al. (1981) suggest that *Plethodon* is a paraphyletic taxon.

Curiously, the species of *Plethodon* are all similar in details of osteology, as well as being generally similar in external morphology and ecology. They are woodland salamanders of terrestrial habitats. In contrast, *Aneides* is sharply distinct from *Plethodon* in many aspects of osteology, and its species all have arboreal tendencies; some of the species are strongly arboreal.

The major morphological features distinguishing *Aneides* from *Plethodon* are specializations associated with the jaws and feeding, and another series of specializations associated with the appendages and locomotion. At the base of each of these series of specializations is a single "key innovation": for feeding mechanisms, the fusion of the premaxillary bones and for locomotion the rearrangement of the carpus and tarsus. The premaxillary fusion is the first of a series of strengthening specializations that are associated with enlarged, specialized dentation. Carpal and tarsal rearrangements are associated with redistribution of forces in the hands and feet leading to the evolution of grasping capabilities and climbing. Both innovations involve relatively simple developmental changes, and could have occurred quickly. Although simple developmentally, the co-occurrence of these unrelated features had profound adaptive significance for these salamanders.

The combined molecular data strongly suggest that *Aneides* is relatively old, but not nearly so old as *Plethodon*. *Aneides*, as a genus, is between 24 and 38 myr old, and *Plethodon* is between 48 and 64 myr old (see Larson et al. 1981; Maxson et al. 1979; Maxson & Maxson 1979; Wilson et al. 1977, for rationale and discussion of timing). Only during a relatively brief period (about 2.3 myr, using the immunological data, and about 9 myr, using the allozyme data) was the com-

mon *Aneides* lineage involved in adaptive morphological evolution. The two key innovations appeared nearly simultaneously (relative to the age of the genera) and this has been followed by a period of gradual adaptation in the sublineages leading to the five extant species. While the allozymic and immunological data are of little value in analyzing the phylogenetic history of the species of *Aneides*, cladistic analysis of osteological data generated an apparently robust hypothesis of interspecific relationships that is not refuted by the molecular data.

Sometimes genetic differentiation is inadequate to permit covariance analysis of allozymes, but we can still use the genetic distances to provide some information on sequence of divergence if we handle the data carefully (and, of course, keep in mind Throckmorton's (1978) warning about the use of genetic distances for time estimates). The Fitch and Margoliash (1967) method of construction of phylogenetic trees is used with a matrix of Rogers' (1972) genetic distance (a metric) for this purpose. In one recent example, Larson (1980) studied geographic variation in allozymes in *Aneides flavipunctatus* in California. Coastal populations of this salamander resemble one another in coloration and proportions, and Lynch (1981) has suggested that this similarity is the result of paedomorphosis. There are two implicit hypotheses. Either the morphologically similar populations are close phyletic relatives, the standard explanation in the absence of evidence to the contrary, or they have evolved their relatively complex morphologies in parallel as a result of a single developmental phenomenon that has many correlated morphological manifestations. Phylogenetic analysis of genetic distances suggested that the latter hypothesis is the more appropriate. Apparently the complex of morphological features attributed to paedomorphosis evolved at least twice within the species.

The morphological characters studied in *Aneides flavipunctatus* relate directly to protective coloration and feeding (Lynch 1974, 1981). Frequently general external morphological features of salamanders are thought to be directly related to ecological adaptation. However, we now have evidence that at least in some groups apparently adaptive morphologies might be very ancient. The eastern assemblage of *Plethodon* has long been informally divided into "large" and "small" groups (e.g., Highton 1962). Many workers have suggested that the primitive morphology for *Plethodon* is that found in the large eastern species, but Highton has argued that the small,

slender eastern species, which closely resemble some species in the western United States, probably preserved the primitive morphology. Molecular evidence is now strong that the eastern small species do not form a natural group. The *P. welleri* group is closer to the eastern large *Plethodon* than to the other eastern small *Plethodon* on immunological grounds (Maxson et al. 1979), and on the basis of allozyme evidence as well (Highton & Larson 1979). The genetic distances are great and thus somewhat suspect for this kind of use. The great genetic distance (nearly 2.0) between *P. websteri* and the essentially identical, parapatrically distributed *P. dorsalis* (Larson & Highton 1978) argues strongly for the primitiveness of their external morphology.

A final example in which a supposedly derived morphology can be demonstrated to be relatively ancient is the case described by Yanev (1978, 1980) in *Batrachoseps*. The very slender, elongate species of the genus were long thought to be conspecific; we now know that they are separated by relatively enormous genetic distances on the order of 1.5. Individual slender species are much more closely related to some large, robust, and presumably primitive species (for example, the slender *B. nigriventris* and the robust *B. sinatus*) than they are to other slender species (*B. attenuatus*). Marlow and associates (1979) have suggested that the robust species of *Batrachoseps* might be derived from slender, relatively paedomorphic ancestors similar to present-day *B. attenuatus* and *B. nigriventris* through a process involving reactivation of developmental programs "turned off" in the past.

As our knowledge of full lineages becomes more complete I expect that we will find other instances in which we have been deceived by our ideas concerning what is a recent and what is an ancient morphology. Frequently we think in terms of specialized forms representing recent adaptation to present conditions, but it may well be that species can be highly specialized in morphology and yet have sufficient general adaptation to persist almost unchanged for tens of millions of years. If taxonomists persist in current trends to eliminate paraphyletic taxa, we will be in for some great changes in taxonomy at the generic level, for taxonomists of the past frequently united species on the basis of appearance alone. The danger will be greatest for some of the groups that have retained generalized, but distinctive morphology (as in *Plethodon*), but have sublineages which have given rise to highly specialized, derivative lineages with entirely different adaptations (e.g., *Aneides*).

In the Amphibia, but also in other phyla in which the phenomenon has been less thoroughly studied, the relation between ontogeny and phylogeny (e.g., Alberch et al. 1979) can be of extremely great importance. The possibility that morphologically specialized lineages can give rise to forms that have the more generalized morphology of their ancestors will only be detected through the application of molecular techniques, and of these the most generally useful involves analysis of allozyme data.

CONCLUSIONS

Historically the evolution of morphology has been analyzed through the use of morphology alone. This has involved a great deal of subjectivity in most instances, for tests of morphological hypotheses have been difficult or impossible. Electrophoretic techniques have become an important new component in morphological studies. When integrated in a systematic program of investigation, traditional and molecular methods greatly increase the potential for hypothesis generation and testing.

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