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Speciation and Evolution of Marine Fishes Studied by the Electrophoretic Analysis of Proteins[1]

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ABSTRACT: Electrophoretic analysis of proteins can be utilized to clarify the taxonomic status of species as well as the evolutionary interrelationships of populations, species, and higher taxa. Electrophoretic data for over 50 gene loci in the bonefish *Albula "vulpes"* (Albulidae) demonstrate the existence of two discrete species in Hawaii and throughout the Indo-West Pacific. Similar studies of lizardfishes (Synodontidae) in the genera *Synodus* and *Saurida* reveal that several unreported and/or undescribed species occur in the Hawaiian Islands. Both of these studies emphasize the power of electrophoresis in distinguishing morphologically cryptic species. The interrelationships of species and genera of lizardfishes and of goatfishes (Mullidae) were investigated by using values of genetic distance derived from protein similarities and differences. These comparisons and the analysis of the two bonefish species, provide additional examples of the basic independence of the rates of biochemical and morphological evolution.

Published electrophoretic investigations of fish speciation and evolution are reviewed and several guidelines for future applications of the technique are proposed. The importance of sympatric samples, the use of large numbers of gene loci, and the conservative interpretation of genetic distance values are emphasized. The utility of electrophoretic data for (*a*) identifying species (especially juvenile, larval, and embryonic stages, or isolated animal products such as fillets); (*b*) identifying F_1 interspecific hybrids; and (*c*) estimating absolute and relative divergence times between taxa are discussed. Finally, the combined use of electrophoretic data from fresh specimens together with multivariate morphometric analyses of both the fresh specimens and preserved museum type specimens is recommended as a robust approach for sorting out nomenclatural problems.

FOR GONOCHORISTIC, SEXUALLY REPRODUCING organisms such as most fishes, the species concept is based upon the reproductive isolation of groups of true-breeding populations from other such groups. In practice, species are nearly always distinguished and described on the basis of anatomical differences. It is reasonable to expect that nearly all currently recognized species should be morphologically distinct from one another, given this practice.

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However, anatomical differentiation is neither a necessary nor a sufficient basis for the recognition of separate species. The literature is filled with examples of species that exhibit dramatic anatomical polymorphisms yet are conspecific and with examples of morphologically cryptic species complexes that are, in fact, independent genetic units (Borden et al. 1977, Gould, Woodruff, and Martin 1975, Grassle and Grassle 1976, Salmon et al. 1979).

An alternative criterion for the recognition of distinct species—that of actual reproductive isolation—seems obvious given the above definition of species. However, this criterion is weakened by the numerous examples of occasional interbreeding between well-accepted species. Indeed, such interspecific hybridization under laboratory and/or natural field conditions is well documented for fishes (Schwartz 1972, 1981) and other groups of animals.

The technique of gel electrophoresis of proteins provides a powerful, although indirect, test of the validity of presumed species. Because this technique allows the measurement of genetic relatedness among individuals (due to the codominant expression of most alleles at protein-coding loci), it can serve as a means for determining the genetic uniqueness of any set of organisms (i.e., identifying distinct species). The approach is particularly robust in cases of true sympatry (in space and time). In such cases, genetically differentiated species are easily recognized when fixed allelic differences are detected. Populations that are sympatric and characterized by fixed allelic differences have clearly evolved effective means of reproductive isolation. Such populations must therefore be considered true biological species. On the other hand, observations of genetic uniformity, either in terms of similar allele frequency distributions among samples or especially in terms of invariant loci identical in all individuals, are consistent with, but do not definitively establish, the conspecific nature of populations (but see Graves and Rosenblatt 1980, Manooch et al. 1976, Sage and Selander 1975, Turner and Grosse 1980).

In cases of allopatry (in space or time), the above distinctions become blurred due to the potentially confounding effects of geographic or temporal differences in the allelic composition of organisms. Geographically, this may take the form of apparent clines in allele frequency or, given discontinuous sampling in space or time, may even appear as apparent fixed allelic differences among samples (Aspinwall 1974, Powers and Place 1978). Extreme care must therefore be exercised in interpreting such data for allopatric samples, because reproductive isolation resulting from spatial or temporal allopatry may not have any biological basis. It is, at best, very difficult to determine whether allopatric populations would or could freely interbreed if contact were restored under natural conditions.

Besides providing a robust measure of the reproductive relationships of sympatric populations, the electrophoretic approach yields additional benefits. One is the unambiguous identification of interspecific F_1 hybrids between two species having multiple fixed allelic differences. This is a direct result of the codominant expression of alleles characteristic of protein-coding loci and is a clear improvement over the use of morphological criteria which are generally less powerful due to the quantitative (blending) inheritance of most anatomical characteristics. A second benefit of the biochemical analysis of species derives from the molecular clock hypothesis (Maxson and Wilson 1974, Nei 1971), which assumes that proteins evolve at relatively constant rates. Thus, by appropriate calibration, it is possible to estimate the approximate time of divergence of any two species (or higher taxa) based on values of genetic distance derived from electrophoretic studies.

This paper describes our electrophoretic investigations of three groups of inshore marine fishes and provides some general guidelines concerning the application of electrophoresis to studies of speciation and evolution.

MATERIALS AND METHODS

Fish specimens were obtained by field capture or by purchase from commercial sources. All animals were stored frozen at $-20°C$ until used. Methods of sample preparation, gel

electrophoresis (vertical starch and polyacrylamide), and enzyme histochemical staining have been described elsewhere (Shaklee and Tamaru 1981, Shaklee, Champion, and Whitt 1974, Shaklee, Kepes, and Whitt 1973). Each enzyme system was analyzed using only one gel and buffer system. Calculations of genetic distance follow Nei (1978). Dendrograms based on estimates of genetic distance were constructed using the unweighted pair-group method with arithmetic means (UPGMA) of Sokal and Sneath (1963).

RESULTS AND DISCUSSION

Bonefishes (Genus Albula)

The genus *Albula* exhibits a pantropical distribution, being found in all warm seas except the Mediterranean. Although it has a synonomy of well over 20 distinct species names, the common bonefish has been regarded by ichthyologists as a single species, *Albula vulpes*, for the last 60 yr. Significant amounts of quantitative variation both within and among localities have been noted for several morphological characters (Greenwood 1977, Nelson 1976, Shaklee and Tamaru 1981, unpublished), yet no one has seriously questioned the integrity and validity of the species.

Our electrophoretic analysis of enzyme and general protein characteristics of bonefish (primarily from Hawaii and the Indo-West Pacific) has revealed the existence of two distinct species throughout this region (Shaklee and Tamaru 1981, unpublished). We first analyzed nearly 200 individual bonefish collected in the Hawaiian Islands. Levels of within-species genetic variability were low for both species ($\bar{P}_{0.95} < 0.05$; $\bar{H} \leq 0.005$). However, between-species genetic differentiation was marked with essentially fixed allelic differences at 58 of the 84 presumed genetic loci examined. These data yield estimates of genetic similarity (I) and distance ($D = -\ln I$) of 0.3 and 1.1, respectively. This substantial amount of genetic differentiation between samples, which were sympatric in both space and time, is a clear indication of reproductive isolation and therefore establishes the existence of two distinct species of bonefishes in Hawaii. We observed no evidence of interspecific hybridization between the two species.

Because the bulk of this divergence resulted from fixed allelic differences, the estimated genetic similarity and genetic distance values were nearly identical whether the estimate was based on data for over 100 fish of each species or the first specimen of each species examined (cf. Shaklee and Tamaru 1981). These results are similar to those predicted by Nei (1978) and demonstrated empirically by Gorman and Renzi (1979), and indicate the robustness of the estimate in the face of small sample sizes when a large number of gene loci are surveyed and the species are characterized by low levels of within-species genetic variation. The magnitude of genetic differentiation separating the two bonefish species is unexpectedly large when compared with published values of interspecific comparisons for other congeneric fish groups (average $I = 0.75$; $D = 0.30$; see below). This amount of genetic divergence is consistent with an estimated evolutionary age of the two species of 20–30 million yr based on the molecular clock hypothesis (Gorman and Kim 1977, Nei 1972, Vawter, Rosenblatt, and Gorman 1980, Wilson, Carlson, and White 1977). This result is even more surprising given the small amount of anatomical differentiation between these morphologically cryptic species.

In the case of the bonefish, we were able to determine the valid scientific names of the two species by employing both electrophoretic data for fresh specimens and multivariate morphometric data for fresh and for preserved type (museum) specimens (Shaklee and Tamaru 1981). Similar nomenclatural applications, although with somewhat different results, have been reported for other organisms (Gould et al. 1975, Richardson and Sharman 1976).

We have extended this study to bonefishes from 11 other localities in the Indo-West Pacific. Typical results are shown in Figure 1. Bonefishes from throughout the Indo-West Pacific exhibited the same two characteristic biochemical phenotypes found in Hawaii. Ten of the 52 presumed loci screened (Table 1)

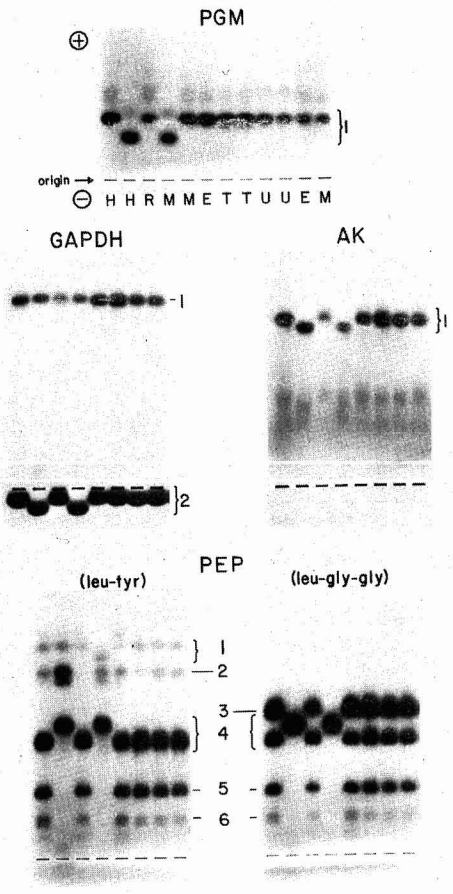

FIGURE 1. Isozyme patterns of Indo-West Pacific bonefishes. Vertical starch gel electrophoresis as described in Shaklee and Tamaru 1981. Isozymes are indicated by numbers and are scored as separate gene products. The enzymes are PGM = phosphoglucomutase, GAPDH = glyceraldehyde phosphate dehydrogenase, AK = adenylate kinase, and PEP = peptidase. The order of samples is the same on all gels and, as indicated at the top for PGM, is (starting from the left): two specimens from Hawaii (=H), one specimen from the Red Sea (=R), two specimens from the Marquesas Islands (=M), one specimen from Enewetak (=E), two specimens from Tarawa (=T), two specimens from the Tuamotu Islands (=U), another specimen from Enewetak (=E), and another specimen from the Marquesas Islands (=M).

were monomorphic and identical in the two bonefish species. Twenty-four loci were essentially invariant in each species but exhibited different alleles in the two species. Each of the remaining loci exhibited genetic variation in at least one of the two bonefishes but only 6 of these had alleles in common in the two species. These data indicate that, throughout their ranges, the two species of bonefishes have fixed allelic differences at over half of the gene loci surveyed, resulting in an overall genetic similarity I of 0.25 and an average genetic distance D of 1.4. It is notable that the fixed allelic differences and large genetic distance values between the two species throughout their respective ranges were also seen in the two localities (Hawaii and the Marquesas Islands) where sympatric samples of both species were compared.

Goatfishes (*Family* Mullidae)

There are about 55 recognized species of goatfishes in six genera worldwide. In this family, species are generally recognized by a combination of differences in meristic and/or morphometric anatomical characters, and differences of coloration and/or pigmentation patterns.

We have electrophoretically analyzed nine species of Hawaiian goatfishes in order to study the interrelationships of species and genera in this family. Twenty-six presumed gene loci were screened in each species (Table 1). Genetic variability in these fishes was low ($\bar{P}_{0.95} = 0.064$; $\bar{H} = 0.021$). Three loci were monomorphic and apparently identical in all nine species examined. Pairwise between-species values for genetic similarity and distance are presented in Table 2. These ranged from a value of $D = 0.11$ ($I = 0.90$) for the two most similar species (*Parupeneus bifasciatus* and *P. chryserydros*) to a value of $D = 1.85$ ($I = 0.16$) for the two most divergent species (*Upeneus taeniopterus* and *P. chryserydros*). Although the estimated genetic distance between *P. bifasciatus* and *P. chryserydros* was relatively small, there can be little doubt that these represent valid species since the sympatric samples examined in this study exhibited fixed allelic differences at both the Gpi-B and Cat loci as well as large differences in allele frequencies at the Pgdh and Iddh loci. No individuals with F_1 hybrid phenotypes were observed.

A dendrogram illustrating the inferred ge-

TABLE 1

NUMBER OF PROTEIN LOCI EXAMINED

ENZYME (E.C. NUMBER)	FISH GROUP		
	BONEFISHES	GOATFISHES	LIZARDFISHES
Adenosine deaminase (3.5.4.4)	1	1	1
Adenylate kinase (2.7.4.3)	2	—	—
Alcohol dehydrogenase (1.1.1.1)	1	1	—
Aspartate aminotransferase (2.6.1.1)	3	2	2
Catalase (1.11.1.6)	—	1	—
Creatine kinase (2.7.3.2)	2	2	2
Esterase (3.1.1.–)	6	—	5
Glucose-6-phosphate dehydrogenase (1.1.1.49)	1	—	—
Glucosephosphate isomerase (5.3.1.9)	2	2	2
Glutamate dehydrogenase (1.4.1.2)	—	1	—
Glyceraldehyde phosphate dehydrogenase (1.2.1.12)	2	1	1
Glycerol-3-phosphate dehydrogenase (1.1.1.8)	2	1	1
L-Iditol dehydrogenase (1.1.1.14)	1	1	1
Isocitrate dehydrogenase ($NADP^+$) (1.1.1.42)	3	2	1
Lactate dehydrogenase (1.1.1.27)	2	3	3
Malate dehydrogenase (1.1.1.37)	3	2	3
Mannosephosphate isomerase (5.3.1.8)	1	—	—
Peptidase (3.4.11.–)	6	2	1
Phosphoglucomutase (2.7.5.1)	1	1	1
Phosphogluconate dehydrogenase (1.1.1.44)	1	1	—
Purine nucleoside phosphorylase (2.4.2.1)	1	—	—
Superoxide dismutase (1.15.1.1)	2	1	1
Triosephosphate isomerase (5.3.1.1)	1	—	—
Xanthine dehydrogenase (1.2.1.37)	1	1	—
General muscle proteins	3	—	4
General brain proteins	4	—	—
Totals	52	26	29

netic interrelationships of the species and genera of goatfishes studied is shown in Figure 2. As would have been predicted by the formal taxonomy, the three genera were more distinct from each other than were any of the species within a genus. More precise interpretations of the interrelationships of species within a genus would be hazardous due to the large standard errors associated with such estimates of genetic distance.

Although the general patterns of species relationships suggested by the dendrogram in Figure 2 are consistent with patterns of morphological similarities within the family, one instructive exception (involving two species of *Mulloidichthys* and two species of *Parupeneus*) is worth noting. *Parupeneus bifasciatus* and *P. chryserydros* are biochemically the most similar pair of species examined, having an estimated genetic distance of only 0.11. Yet these two species are readily distinguished by significant differences in several morphological characteristics, including body coloration, barbel length, head and eye size, and the numbers of pectoral fin rays and gill rakers (Gosline and Brock 1960, Lachner 1960). On the other hand, *M. vanicolensis* and *M. flavolineatus* have an estimated genetic distance of 0.34, yet, with the exception of subtle pigmentation differences when alive, these two species fail to exhibit substantial morphological differentiation (Gosline and Brock 1960). The former situation is an example in which two perfectly good species show relatively little biochemical differentiation, emphasizing that widespread electrophoretic differences are not a necessary correlate of speciation (see also Avise, Smith, and Ayala 1975, Kirkpatrick and Selander 1979, Ryman, Allendorf, and Stahl 1979, Turner 1974).

TABLE 2

ESTIMATES OF GENETIC SIMILARITY, *I* (Nei 1978), ABOVE THE DIAGONAL AND GENETIC DISTANCE, $D = -\ln I$, BELOW THE DIAGONAL FOR NINE SPECIES OF GOATFISHES

	U. taeniopterus	*M. flavolineatus*	*M. pflugeri*	*M. vanicolensis*	*P. bifasciatus*	*P. chryserydros*	*P. multifasciatus*	*P. pleurostigma*	*P. porphyreus*
Upeneus taeniopterus ($\bar{N} = 23$)	—	0.39	0.31	0.47	0.20	0.16	0.19	0.28	0.19
Mulloidichthys flavolineatus ($\bar{N} = 26$)	0.93	—	0.48	0.71	0.41	0.35	0.37	0.44	0.29
M. pflugeri ($\bar{N} = 16$)	1.17	0.74	—	0.54	0.28	0.28	0.20	0.27	0.24
M. vanicolensis ($\bar{N} = 25$)	0.76	0.34	0.61	—	0.32	0.35	0.31	0.45	0.27
Parupeneus bifasciatus ($\bar{N} = 9$)	1.63	0.89	1.29	1.13	—	0.90	0.74	0.74	0.66
P. chryserydros ($\bar{N} = 21$)	1.85	1.04	1.29	1.05	0.11	—	0.73	0.71	0.69
P. multifasciatus ($\bar{N} = 15$)	1.64	1.00	1.63	1.18	0.30	0.32	—	0.70	0.58
P. pleurostigma ($\bar{N} = 14$)	1.29	0.82	1.31	0.79	0.30	0.34	0.36	—	0.56
P. porphyreus ($\bar{N} = 26$)	1.67	1.23	1.43	1.44	0.41	0.37	0.54	0.58	—

NOTE: $\bar{N}$ = average number of individuals screened per locus.

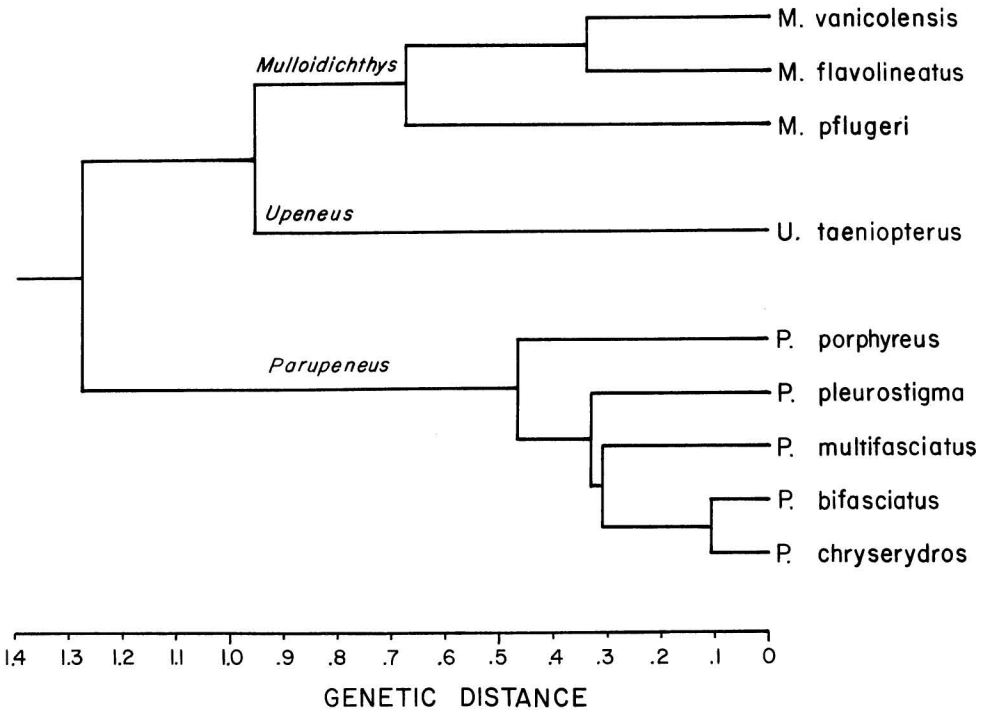

FIGURE 2. Dendrogram (UPGMA, Sokal and Sneath 1963) of genetic distance (Nei 1978) based on all possible pairwise comparisons of the nine species of goatfishes analyzed.

The common occurrence of fixed allelic differences between species makes it possible to construct biochemical keys for the identification of the taxa involved (Avise 1974, Buth 1980). When numerous fixed differences occur, several alternative keys involving different enzyme systems can be formulated. The associated redundancy in these generally independent characters greatly increases the power of this method for species identification. One such key, based on commonly studied enzymes, is presented for the Hawaiian goatfishes in Table 3.

Lizardfishes (*Family* Synodontidae)

This family presently consists of over 40 species in about four genera. Four of the six species commonly recognized in Hawaiian waters are in the genus *Synodus*, with one species each in *Saurida* and *Trachinocephalus* (Gosline and Brock 1960). The four species of *Synodus* are generally distinguished on the basis of small meristic differences (e.g., numbers of transverse scale rows, lateral-line scales, and dorsal fin rays) and/or pigmentation differences.

Our electrophoretic analysis of Hawaiian lizardfishes, using 29 presumed gene loci (Table 1), has revealed the existence of several morphologically cryptic and previously unrecognized species in this family. Two previously unrecognized species of *Saurida*, one of which had never before been described (Waples 1981), and one species of *Synodus* (*S. englemani*), not reported by Gosline and Brock (1960) to occur in Hawaii, were discovered as a result of this electrophoretic screening. Each of these "new" species was initially recognized because it possessed several fixed allelic differences that gave it a unique biochemical phenotype (distinct from that of any other known Hawaiian lizardfish species). Figure 3 illustrates such species-

TABLE 3

BIOCHEMICAL KEY TO HAWAIIAN GOATFISHES

1.	a.	Aspartate aminotransferase (cytosolic) same 2
	b.	Aspartate aminotransferase (cytosolic) slower 5
2.	a.	L-Iditol dehydrogenase same 3
	b.	L-Iditol dehydrogenase slower ..... *Parupeneus chryserydros*
3.	a.	Phosphogluconate dehydrogenase same or faster 4
	b.	Phosphogluconate dehydrogenase slower ..... *Parupeneus multifasciatus*
4.	a.	Malate dehydrogenase (mitochondrial) same ..... *Parupeneus pleurostigma*
	b.	Malate dehydrogenase (mitochondrial) faster ..... *Parupeneus bifasciatus*
5.	a.	Isocitrate dehydrogenase (mitochondrial) faster 6
	b.	Isocitrate dehydrogenase (mitochondrial) slower ..... *Mulloidichthys pflugeri*
6.	a.	Phosphoglucomutase slower 7
	b.	Phosphoglucomutase same ..... *Upeneus taeniopterus*
7.	a.	Lactate dehydrogenase C_4 faster ..... *Mulloidichthys vanicolensis*
	b.	Lactate dehydrogenase C_4 slower ..... *Mulloidichthys flavolineatus*

NOTE: All electrophoretic mobilities (using TC pH 6.9 buffer) are relative to those of the homologous isozymes of *Parupeneus porphyreus*.

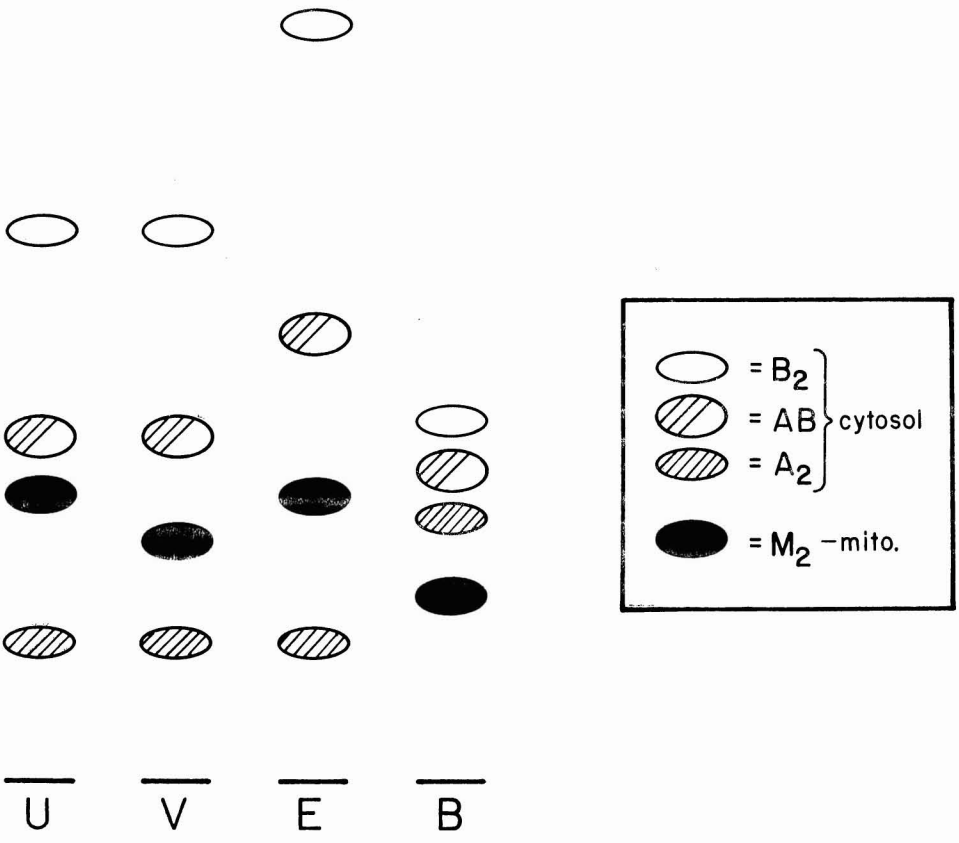

FIGURE 3. Schematic diagram of malate dehydrogenase (MDH) isozyme patterns for four species of *Synodus* based on mobilities observed in vertical starch gels using TC pH 6.9 buffer. U = *S. ulae*, V = *S. variegatus*, E = *S. englemani*, and B = *S. binotatus*. The anode is toward the top of the figure.

TABLE 4

ESTIMATES OF GENETIC SIMILARITY, I (Nei 1978), ABOVE THE DIAGONAL AND GENETIC DISTANCE, $D = -\ln I$, BELOW THE DIAGONAL FOR FIVE SPECIES OF LIZARDFISHES

	Synodus ulae	*Synodus variegatus*	*Synodus englemani*	*Synodus binotatus*	*Saurida gracilis*
Synodus ulae ($\bar{N} = 12$)	—	0.54	0.41	0.31	0.17
Synodus variegatus ($\bar{N} = 9$)	0.61	—	0.45	0.28	0.17
Synodus englemani ($\bar{N} = 4$)	0.89	0.80	—	0.24	0.17
Synodus binotatus ($\bar{N} = 3$)	1.17	1.28	1.42	—	0.17
Saurida gracilis ($\bar{N} = 9$)	1.78	1.75	1.76	1.76	—

NOTE: $\bar{N}$ = average number of individuals screened per locus.

specific isozyme phenotypes for the malate dehydrogenases (MDH) of the four species of *Synodus* studied. In this case, *Synodus binotatus* was different from the other species at all three MDH loci. The remaining three species (*S. ulae*, *S. variegatus*, and *S. englemani*) shared the same electromorph (mobility class) for MDH-A_2, while two of them (*S. ulae* and *S. variegatus*) exhibited the same electromorph of MDH-B_2 and two (*S. ulae* and *S. englemani*) possessed the same electromorph of the mitochondrial MDH isozyme.

Genetic variability in the lizardfishes appeared low ($\bar{P}_{0.95} < 0.05$; $\bar{H} < 0.02$), although fewer than ten specimens of each species were analyzed. Values of genetic similarity and distance derived from pairwise comparisons of the four *Synodus* and one *Saurida* species are presented in Table 4. Distance values ranged from a low of 0.61 between *Synodus ulae* and *S. variegatus*, the two species most similar in appearance, to values of at least 1.75 between *Saurida gracilis* and each of the *Synodus* species.

A dendrogram depicting species interrelationships based on the genetic distance values is presented in Figure 4. There was strong agreement between the estimate of relationships based on this genetic parameter and that based on morphological similarity. Also of interest was the fact that the genetic distance values at the nodes connecting taxa were spread rather evenly over the range 0–2.0. That is, the estimated genetic distance between genera (*Synodus* versus *Saurida*) was not substantially larger than that between the most divergent *Synodus* species.

A survey of the literature of electrophoretic estimates of intergroup genetic differentiation for freshwater and marine fishes (Avise and Ayala 1976, Avise and Smith 1977, Buth 1979, 1980, Buth and Burr 1978, Buth, Burr, and Schenk 1980, Johnson 1975, Kornfield et al. 1979, Ryman et al. 1979, Smith, Francis, and Paul 1978, Turner 1974, Utter, Allendorf, and Hodgins 1973, Vawter et al. 1980, Winans 1980) reveals the values given in Table 5. Although the ranges of values for each taxonomic level overlap, the mean values are reasonably distinct. Thus, assuming the taxonomy of the groups involved is not in error, these values serve as a useful reference point for interpreting other such data, especially as they pertain to taxonomically questionable groups. In this regard, the observed values for species and genera of Hawaiian goatfishes fall within the expected ranges. However, the value of interspecific and intergeneric comparisons of lizardfishes and the single interspecific comparison of bonefishes exceed the highest values observed in these other studies. These inconsistencies emphasize the fundamental independence of the rates of morphological and biochemical evolution and caution against rigid quantitative interpretation of genetic distance estimates in terms of the formal taxonomy of organisms.

CONCLUSIONS

Reproductive isolation can be considered the cornerstone upon which the concept of biological species rests. Given that reproduc-

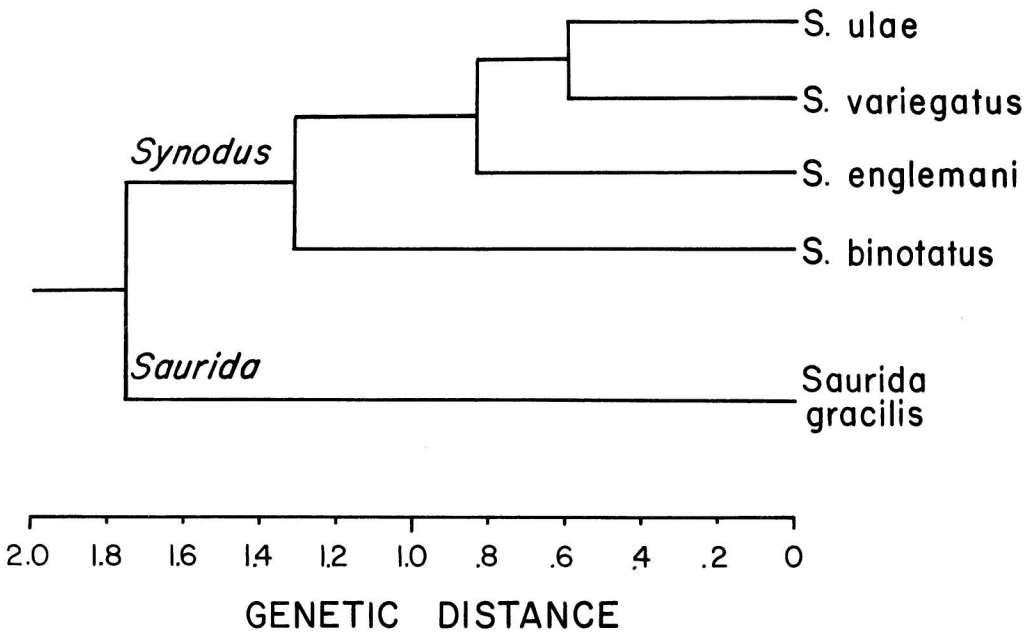

FIGURE 4. Dendrogram (UPGMA, Sokal and Sneath 1963) of genetic distance (Nei 1978) based on all possible pairwise comparisons of the five species of lizardfishes examined.

TABLE 5

AVERAGE GENETIC DIFFERENTIATION AMONG FISH GROUPS

	POPULATIONS	SPECIES	GENERA
Average genetic similarity, *I*	0.97	0.75	0.40
Average genetic distance, *D*	0.05	0.30	0.90
Range of *D* values	0.002–0.065	0.025–0.609	0.580–1.21

tive isolation through time is expected to result in the progressive genetic differentiation of separate species, the demonstration of qualitative genetic differences between sympatric populations is a sufficient (although not a necessary) criterion by which to recognize distinct species. Perhaps the simplest and one of the most direct approaches for determining genetic similarity or distance between groups is the technique of gel electrophoresis of proteins. Using this approach, the demonstration of fixed allelic differences between sympatric groups provides unambiguous genetic evidence of complete reproductive isolation.

Virtually simultaneously with the discovery that many enzymes exist in isozymic form, it was recognized that animals exhibit species-specific isozyme patterns (Markert and Moller 1959). The application of this observation to systematic studies, particularly involving the identification and description of fish species, was quickly recognized and exploited (Allendorf and Utter 1979, Avise and Smith 1977, Buth 1979, 1980, Daly and Richardson 1980, Ferguson and Mason 1981, Herzberg and Pasteur 1975, Johnson 1975, Lundstrom 1977, Manwell and Baker 1970, Miller and El-Tawil 1974, Page and Whitt 1973, Smith and

Robertson 1981, Smith, Wood, and Benson 1979, Tsuyuki and Roberts 1965, Turner and Liu 1976, Vawter et al. 1980).

The electrophoretic demonstration of fixed allelic differences between pairs of species immediately suggests another powerful use of the electrophoretic technique—the identification of F_1 interspecific hybrids. Indeed, the general morphological intermediacy of most interspecific hybrids makes identification based on anatomical characters difficult at best. The hybrid electrophoretic phenotype, on the other hand, is a distinctive characteristic that makes hybrid identification simple, fast, and reliable. Numerous studies of fish hybridization have utilized this approach with considerable success (Abramoff, Darnell, and Balsano 1968, Aspinwall and Tsuyuki 1968, Brassington and Ferguson 1976, Clayton, Harris, and Tretiak 1973, Hulata, Rothbard, and Avtalion 1981, Manwell, Baker, and Childers 1963, Metcalf et al. 1972, Nyman 1970, Reinitz 1977, Tsuyuki and Roberts 1965, Turner et al. 1980, Vrijenhoek 1972, Wheat, Whitt, and Childers 1973, Wheat et al. 1971).

Several investigations have been directed at describing and understanding the isozymic changes that occur during early development in fishes (e.g., Champion and Whitt 1976*a*, *b*, Shaklee et al. 1974, Whitt 1981, Wright, Heckman, and Atherton 1975). These studies have shown that, although the particular gene loci expressed at any given time change during development, allelic isozymes are expressed in embryos and larvae just as they are in adults. Therefore, it is possible to use unique, species-specific alleles as markers to identify (to species) juvenile, larval, and even embryonic stages of fishes (Gardiner 1974, Sidell, Otto, and Powers 1978, Smith and Crossland 1977, Smith, Benson, and Frentzos 1980).

Electrophoretic estimates of genetic differentiation can also be used to estimate divergence times for cladistic events. This application, which depends upon the existence of a molecular clock (Maxson and Wilson 1974, Nei 1971), seems to give reasonably accurate estimates of absolute divergence times for several pairs of fish species (Gorman and Kim 1977, Vawter et al. 1980) and would be expected to be even more accurate for estimating relative divergence times for closely similar taxa.

The following guidelines summarize our recommendations regarding the application of electrophoretic studies of proteins to questions of speciation and evolution.

1. Advantages of Electrophoretic Data

Qualitative electrophoretic characters (e.g., electromorph mobility classes) are generally the result of the codominant expression of alleles at a single genetic locus. This property allows the genotype of an individual to be directly inferred from its biochemical phenotype. Because environmental variables such as temperature, salinity, oxygen tension, etc., usually have no effect on the qualitative expression of electrophoretic characters in fishes (Shaklee et al. 1977, Somero 1975; but see also Baldwin 1971), specimens having different environmental histories can be compared. Furthermore, since it seems likely that at least some electrophoretic variation may be selectively neutral, such characters are unlikely to exhibit significant convergence—a well-known problem with many morphological characters, which confuses systematic interpretation.

2. Test in Sympatry

The demonstration of fixed allelic differences among samples from sympatric populations is evidence of complete reproductive isolation and thus establishes the existence of independent gene pools (= separate species). However, the test is not reciprocal. Failure to demonstrate fixed differences (especially when the number of loci examined is low) cannot be used to establish the conspecific status of groups.

3. Test in Allopatry

The demonstration of apparently fixed allelic differences among allopatric samples is not a particularly robust test of speciation, because geographic and/or temporal variation in allelic composition may generate significant differentiation among conspecific populations.

Closely spaced sampling strengthens this test. This lack of robustness is not a problem of the technique; rather, it reflects a fundamental inadequacy of the biological species concept in dealing with allopatric populations.

4. Distribution of Effort

Maximizing the number of gene loci screened (rather than using large numbers of individuals per group) provides the most efficient means for obtaining accurate estimates of genetic distance between groups and testing the conspecific status of two or more groups. This strategy is particularly appropriate for studying organisms such as fishes, which generally have relatively low levels of polymorphism and heterozygosity, because the interlocus component (fixed differences at some loci versus identity at others) makes a much larger contribution to the estimate of genetic distance than does the intralocus variability (due to the inaccurate estimation of allele frequencies at polymorphic loci using small numbers of individuals). This result is not unexpected given the U-shaped distributions seen (for most species of animals) when the frequency of loci is plotted against genetic similarity.

5. Semiquantitative Interpretation of Results

(*A*) Because estimates of genetic distance usually have relatively large standard errors associated with them (Nei 1978), one must be cautious not to overinterpret small differences, whether they occur in tables of genetic distance or in dendrograms derived from such values. The magnitude of the standard errors is usually great enough to accommodate several alternative dendrograms of group interrelationships (some of which may be substantially different from that generated as most parsimonious).

(*B*) There is no ultimate quantitative criterion against which to measure observed values of genetic distance in resolving taxonomic questions. However, comparison of values of genetic distance with those published for other closely related groups of organisms can serve as a useful frame of reference in interpreting results relative to questions of the taxonomic status of the groups under investigation.

6. Construction of Biochemical Keys

Electrophoretic data on fixed allelic differences among species can serve as a useful basis for constructing species identification keys, especially when the species under study are so morphologically similar as to preclude the use of anatomical characters in species identification. Such biochemical keys are particularly useful in the identification of isolated tissue samples (e.g., processed fish fillets or other animal products), which lack normal morphological characteristics, and early life history stages (e.g., juveniles, larvae, or embryos), which may be morphologically unidentifiable.

7. Identification of F_1 Interspecific Hybrids

When two species are shown to possess numerous fixed allelic differences, the electrophoretic demonstration of heterozygous phenotypes (for the two parental alleles) at all these loci in one or more individuals is a robust and unambiguous means of identifying these fish as F_1 interspecific hybrids.

8. Estimation of Divergence Times

Given that, on the average, proteins may evolve at roughly constant rates, electrophoretic estimates of genetic distance can be used to approximate the absolute time of evolutionary divergence of two lineages if calibration is possible. When such estimates are for similar groups of organisms (which have had similar evolutionary histories) and are based on data for a large number of gene loci, they should provide reasonably accurate estimates of the relative times of divergence of the different groups even without accurate calibration.

9. Resolution of Nomenclatural Problems

(*A*) The combined use of electrophoretic studies of fresh or frozen material with multivariate morphometric analyses of both fresh and preserved museum type specimens provides an unusually powerful and general

approach to resolving questions of the formal taxonomy of species. This multidisciplinary approach is particularly fruitful when the nomenclature of the species in question is based on historical type specimens that cannot be subjected to electrophoretic analysis.

(*B*) When the recognition of a new species is based in part or whole upon electrophoretic characters, a complete discussion of the diagnostic biochemical characters should become a part of the published species description (Miller and El-Tawil 1974, Shaklee and Tamaru 1981, Waples 1981). In such cases, we recommend that intact paratype specimens or isolated tissues from paratypes or the holotype be deposited as frozen reference material (at −80°C) with an appropriate museum.

10. Limitations on Applicability

For questions concerning the interrelationships of higher taxa (above the level of species), biochemical data sets may provide useful insights (Shaklee and Whitt 1981) but should not be used to the exclusion of other information (data on anatomical characters, behavior, developmental patterns, karyotypes, etc.). Electrophoretic data have the advantage of being almost entirely genetic but the disadvantage of representing a relatively small, and possibly nonrepresentative, set of gene loci.

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