A TAXONOMIC APPROACH TO EVALUATION OF THE CHARGE STATE MODEL USING TWELVE SPECIES OF SEA ANEMONE

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ABSTRACT

The charge-state model of electrophoretic variation was tested by comparing the distances between nearest electromorphs of five enzyme loci within polymorphic species and among pooled species of sea anemone. If the charge-state model is generally true, and in particular if it allows linear distance between electromorphs to be used as a measure of genetic distance, then electromorphs of different species should be on the same "mobility ladder". Therefore, distances between adjacent electromorphs should be approximately equal for the two sets of comparisons. It was found that distances between adjacent electromorphs for each locus were significantly smaller for the pooled comparisons than within polymorphic species. Thus, it was concluded that much of the variation detected among different species does not conform to the charge-state model, and therefore that distance between electromorphs *per se* would not be a good measure of genetic distance. However, the charge-state model does appear to adequately account for most of the variation existing as common polymorphisms within species, or between very closely related species. Possible reasons for this apparent difference in the nature of the variation seen within and among species are discussed.

THE charge-state, or ladder-rung model was proposed by OHTA and KIMURA (1973) to describe electrophoretic variation more realistically than did the infinite-alleles model of KIMURA and CROW (1964). The charge-state model assumes that the electrophoretic mobility of a protein is solely a function of its net electrostatic charge and that mobility is not affected by the protein's tertiary structure. Substitution by an amino acid with a different charge results in a stepwise shift of the protein to an adjacent electromorph class. Electromorphs, therefore, are separated by discrete jumps of unit value.

The charge-state model has been of fundamental importance in developing the theory that most observed electrophoretic variation is adaptively neutral (KIMURA 1968; KING and JUKES 1969; KIMURA and OHTA 1971). It has been used to explain the similarity of alleleic profiles in geographically isolated populations without invoking the action of natural selection and for comparisons of predicted and observed levels of heterozygosity and number of alleles. The charge-state model also has important implications for systematics, because this model predicts that there should be an increase in electrophoretic separation with an

increase in patristic distance (RICHARDSON, RICHARDSON and SMOUSE 1975; WEHRHAHN 1975; BROWN, MARSHALL and WEIR 1975; RICHARDSON and SMOUSE 1976). Thus, the average difference in mobility could be used as a measure of taxonomic distance (FLAKE and LENNINGTON 1977; BROWN, MARSHALL and WEIR 1981). Whereas other genetic distance measures (e.g., NEI 1972) rely simply on whether or not two alleles are different, the proposed relationship between taxonomic distance and electrophoretic distance would allow the investigator to quantify *how* different they are.

Considering the importance of the charge-state model, there have been relatively few studies testing its validity. JOHNSON (1974, 1977) criticized the model as being biochemically totally unrealistic. He suggested that conformation, not electrostatic charge, is the most important determinant of electrophoretic mobility. COBBS and PRAKASH (1977), using the same procedures as those of JOHNSON, came to opposite conclusions. RAMSHAW and EANES (1978) used isoelectric focusing, rather than gel-sieving, and also concluded that for the esterase-5 locus of *Drosophila pseudoobscura*, electromorphs were mainly the result of charge differences. BROWN, MARSHALL and WEIR (1981) concluded that although the charge-state model might not be totally accurate, it was robust and approximately correct. Using variants of human hemoglobin of known amino acid sequence, RAMSHAW, COYNE and LEWONTIN (1979) and FUERST and FERRELL (1980) examined the ability of electrophoresis to detect variants, and studied the underlying biochemical basis for the mobility classes detected. RAMSHAW, COYNE and LEWONTIN (1979) concluded that for human Hb A the charge-state model is wrong. FUERST and FERRELL (1980) found that 60–70% of the variants could have their mobilities predicted on the basis of net charge alone, ignoring tertiary structure. They also compared human Hb A with nonhuman hemoglobins, and found that deviation from the charge-state model of electromorph mobilities increased as the number of amino acid differences increased. This casts doubt on the expectation of a simple relationship between electrophoretic distance and taxonomic distance.

The studies on hemoglobin variants of known structure are excellent for furthering our understanding of the mechanisms that can lead to nonstepwise mobility differences between proteins, but they do not directly address the question of whether mobility *per se* is of taxonomic utility. This is because the use of mobility differences for systematics depends on *most* (not necessarily all) proteins behaving in a stepwise fashion. It may be that hemoglobins (or esterases or myoglobins) are peculiar, but that most other proteins routinely assayed electrophoretically fit the charge-state model quite well. A more appropriate test of the model, from the viewpoint of systematics, is to compare electrophoretic profiles from several taxa, using a number of proteins. RICHARDSON and SMOUSE (1976) compared seven loci for several species of the *Drosophila mulleri* complex, and concluded that perturbations from the charge-state model were minor, and electrophoretic distance in this group had great taxonomic utility. However, the group of species they examined is a very closely related complex of semispecies, subspecies, and sibling species. It might be that the fit of their data to the model reflects this close relationship. It would be worthwhile

to test the validity of the charge-state model over a broader taxonomic range, preferably comparing conspecific populations, congeneric species, and species from several different genera. The study reported here encompasses such a taxonomic range, using 12 species of sea anemone, representing 6 genera, of the family Actiniidae.

The charge-state model was tested by making both intra- and interspecific comparisons of the distances between electromorphs at several polymorphic loci for these 12 species of sea anemone. The basis of the test is the expectation that mobility differences caused solely by net charge changes should be stepwise, and these should accumulate. If the charge-state model is valid, electromorphs found within species and in different species are expected to be on the same "ladder" of mobility states. Thus, the average distance between adjacent electromorphs for all species pooled should be greater than or equal to the average distance within a polymorphic species. However, distances among species would also be greater if small, nonstepwise mobility differences tended to accumulate within species while large, stepwise differences caused by net charge changes characterized different species. Thus, finding that distances among species were greater than distances within species would not necessarily validate the charge-state model. On the other hand, finding that the average distance between adjacent electromorphs among species was smaller than that found within species would constitute convincing evidence that the charge-state model is incorrect. Therefore, the experiments were designed in such a way as to test whether or not distances among electromorphs from different species are smaller than such distances within species.

MATERIALS AND METHODS

The species used in this study are 12 species from 6 genera in the family Actiniidae: *Bunodosoma cavernata*, *Bunodosoma granulifera*, *Bunodosoma californica*, *Anthopleura stellula*, *Anthopleura pallida*, *Anthopleura carneola*, *Bunodactis texaensis*, *Bunodactis stelloides*, *Bunodactis* sp., *Epiactis prolifera*, *Phyllactis conquilega*, and *Actinia tenebrosa*. The relationships among these were previously studied (McCommas 1982a) using (in part) Nei's standard genetic distance (Nei 1972, 1975). *B. cavernata* is represented by four populations on the Gulf coast, three populations from the Atlantic coast, and one population from Puerto Rico. *B. granulifera* is represented by collections from populations at Puerto Rico, Curacao, Panama, and Grand Cayman. Details of collection sites for all species can be found in McCommas (1982a).

All anemones were frozen at −60° before processing. Each individual was then thawed, refrozen, thawed, and rinsed in deionized water to remove mucus, and blotted dry. After being minced with scissors, each animal was mixed with buffer (100 ml seawater filtered through a 0.2 μm Millipore filter, 1.0 ml 1% NAD solution, 1.0 ml 1% NADP solution, and 1.0 g polyvinylpyrrolidone) in the proportion of one part buffer to three parts tissue. The samples were centrifuged at 44,500 × g at 4° for 30 min, after which the supernatant was collected. Electrophoretic apparatus and procedures were similar to those described by Selander *et al.* (1971). The starch used was Electrostarch, lots 307 and 392. Although two starch lots were used during the course of the study, relative mobilities of electromorphs of any given enzyme were determined on only one lot. The following electrophoretic buffers were used in this study, following recipes and terminology given in Selander *et al.* (1971): lithium hydroxide, discontinuous Tris-citrate (Poulik), continuous Tris-citrate I, and Tris-versene-borate.

The loci chosen for this study had to meet the following criteria: 1) they had to be polymorphic in three or more species, and 2) they had to have very sharp bands. The loci meeting these

requirements fell into two groups: those whose average within-species distances between adjacent electromorphs were about 2 mm, and those having average within-species distances of 4 mm or greater. Since the purpose of the experiments was to test whether or not among-species distances are less than distances within species, only the latter group of loci was used. This does not bias the test in favor of rejecting the null hypothesis since, if the charge-state model is correct, the distances among species should be greater than or equal to the distances within species regardless of how large the distances within species are. Nor is the test biased by the greater number of electromorphs that will be found for several pooled species compared to a single species. It might be suspected that, because of the finite space of an electrophoretic gel, increasing the number of electromorphs would necessarily reduce the distance between them. However, if the charge-state model is strictly true, then "new" electromorphs that are found when further species are examined should be on the same mobility ladder.

The following five loci fulfilled all requirements: superoxide dismutase (*Sod*), glycerate-2-dehydrogenase (*G2dh*), octanol dehydrogenase (*Odh*), esterase-1 (*Est-1*), and esterase-2 (*Est-2*). Stain recipes, usually with slight modifications, were taken from several sources: SHAW and PRASAD (1970), SELANDER *et al.* (1971), and AYALA *et al.* (1972). The substrate for *Est-1* was naphthol AS-D acetate; that for *Est-2* was 4-methylumbelliferyl acetate. The use of esterase loci for a study like this might be questioned on the grounds that homology of the zones of esterase activity commonly observed using common staining procedures is uncertain. In common with most organisms, if a substrate such as alpha-naphthyl acetate is used, these species show many zones of activity. However, for each of the two substrates employed here, only a single zone of activity is observed. This substrate specificity and the taxonomic affinity of the species used makes it very likely that homologous loci are, in fact, being compared.

The procedure used to determine mobilities was a series of matched qualitative comparisons, allowing even very small electrophoretic differences to be reliably detected (RAMSHAW, COYNE and LEWONTIN 1979). Samples were run as replicates side-by-side with others of the same or similar mobility. The order of pairs was changed until each electromorph had been run directly next to the electromorph(s) closest to it in mobility. In this way, an unambiguous order for the mobilities was determined.

For purposes of statistical analysis, distances were measured from one electromorph to the electromorph that was anodally closest to it in mobility. Because of unequal variances, the significance of differences in distances was tested using the t' statistic (SOKAL and ROHLF 1969: 374–375).

RESULTS AND DISCUSSION

Table 1 lists all electromorphs found for each locus for each species. Data for populations of *B. cavernata* and populations of *B. granulifera* were pooled. Figures 1 to 5 illustrate the electrophoretic variation in the five loci studied. The left-hand portion of each figure presents all electromorphs found in the 12 species, in order of increasing mobility. On the right are the electromorphs found in each species that was polymorphic for that locus. Computation of t' values showed that distances between nearest electromorphs within species were significantly greater for every locus than were the distances between nearest electromorphs pooled over all species (for *Sod* and *G2dh*, $P < 0.01$; for *Odh*, *Est-1*, and *Est-2*, $P << 0.001$). Clearly, the "steps" between electromorphs from different species were not the same as the "steps" within species.

It is highly improbable that the increments in mobility of electromorphs from different species represent unit charge differences; if they did, it would mean that electromorphs within a species were several steps (mutations) apart, with no intervening mobilities. More likely, the large differences in mobility of electromorphs within species are primarily caused by unit charge changes and

TABLE 1

Electromorph mobilities for each locus of each species

Species	Locus				
	Sod	*G2dh*	*Odh*	*Est-1*	*Est-2*
B. cavernata	100,111	95,100	93,100,107,112	92,96,100,104,108	95,100
B. granulifera	100,111	99,107	106,111	97,101,105	93,100,105,110
B. californica	111	107	103,111	97,101,105	105
A. stellula	100	104	96	95	100
A. pallida	111	96	96,102	110	104
A. carneola	111	99	82,89,96	102	97
B. texaensis	111	96.5,105,111	96	98,101,105,108,115	95,103,108
Bunodactis sp.	90,101	107	88,95	92	95,101
B. stelloides	111	97	75,81	105	95,101
E. prolifera	111	98	106	87	97
P. conquilega	102,112	108	92	112	104
A. tenebrosa	110	104	68	113	91,101

the small differences in electromorphs from different species are caused by other factors. These factors could include conformational changes in the molecule, differences in the pK values of substituted amino acids, and partial net charges caused by shielding of internal amino acid substitutions and effects of neighboring residues (RAMSHAW, COYNE and LEWONTIN 1979; RETZIOS and THATCHER 1979).

The *Sod* locus (Fig. 1) is a good example of the types of mobility differences seen within and among species. The step distance within species is very large (about 11 mm). When different polymorphic species are compared, it is seen that some of their "ladders" are displaced from each other by a small amount, presumably caused by conformational and/or partial charge differences. Thus, when all species are pooled, the electromorphs fall into three widely separated mobility classes, with small variations from the median mobility being detected in two of the classes. At the other loci, where more variation is detected, there is a correspondingly greater amount of "interdigitation" of mobility ladders from different species. Thus, when all species are pooled for comparison, the average distance between neighboring electromorphs is very small, relative to the distances observed within polymorphic species.

There are several possible mechanisms that could give rise to this pattern of small nonstepwise mobility differences in interspecific comparisons. Since the charge of an amino acid is affected by neighboring residues, identical substitutions in different parts of the molecule could result in slightly different overall net charges. If the rate of gene flow is much greater than the mutation rate, then electromorphs of a given mobility in different populations of a species probably represent a single mutation. But, assuming substitutions are random, different species would probably acquire any given amino acid substitution in different parts of the molecule, and thus have slightly different net charges.

Alternatively, some of the nonstepwise mobility differences could be caused by conformational differences between two electromorphs. Conformational

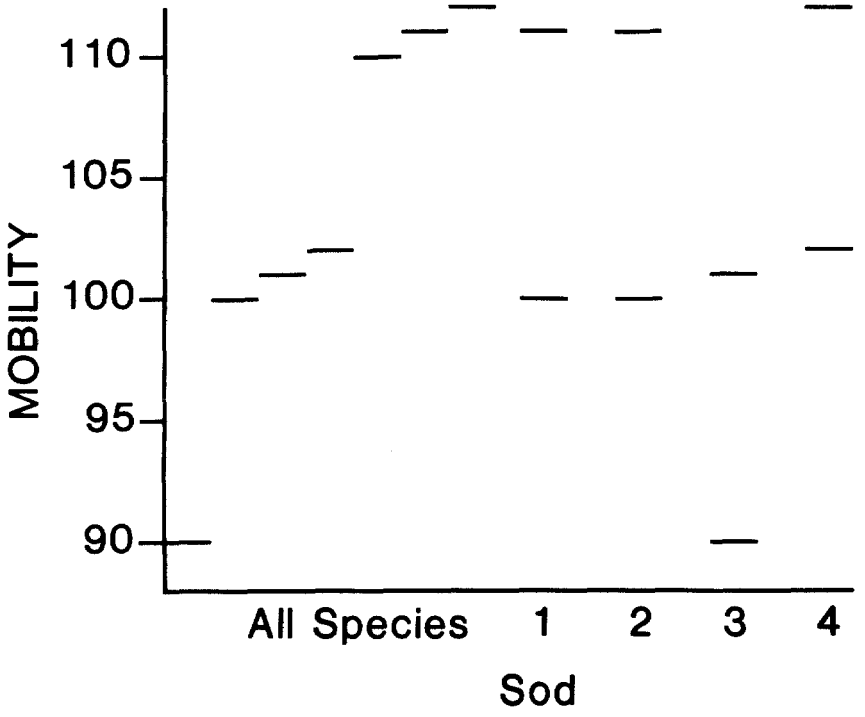

FIGURE 1.—Mobilities of *Sod* electromorphs for all species pooled and within each polymorphic species. Species codes: 1, *B. cavernata*; 2, *B. granulifera*; 3, *Bunodactis* sp.; 4, *P. conquilega*.

changes are more likely to have effects on the active site than are substitutions which only change the net electrostatic charge of the molecule. This is because the functioning of the active site depends to a great extent on its geometry. Since the active site is responsible for the catalytic properties of the enzyme molecule, conformational changes will probably be deleterious. In the view of RICHARDSON, RICHARDSON and SMOUSE (1975), such changes are expected to produce "genetic revolutions", and those that survived to fixation would be both adaptive and rare. An alternative explanation is that because conformational changes are more likely to be deleterious than surface charge changes, there is a smaller subset of mutations causing conformational changes that are adaptively neutral. Therefore fewer such mutations would be fixed in a given amount of time. The results of any of these explanations is that nonstepwise differences are more likely to be encountered at the interspecific level than they are as polymorphisms within species.

The results of this study agree with these predictions. Examination of Figures 1 to 5 shows that the variation within species generally appears to be in good agreement with the charge-state model. Comparison of populations of *B. cavernata*, *B. granulifera*, and *B. californica* further substantiates the view of divergence from the charge-state model with taxonomic divergence. Populations of *B. cavernata* in the Gulf of Mexico have diverged from populations on the

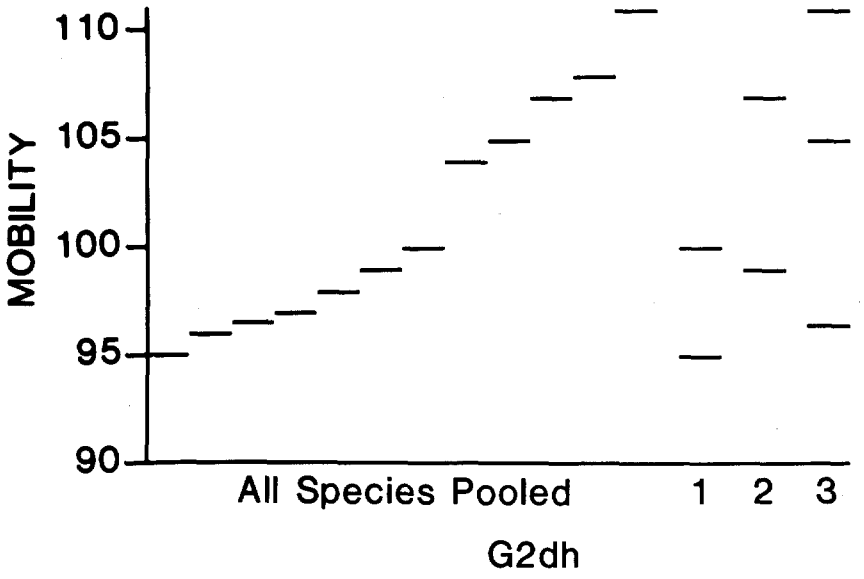

FIGURE 2.—Mobilities of *G2dh* electromorphs for all species pooled and within each polymorphic species. Species codes: 1, *B. cavernata*; 2, *B. granulifera*; 3, *B. texaensis*.

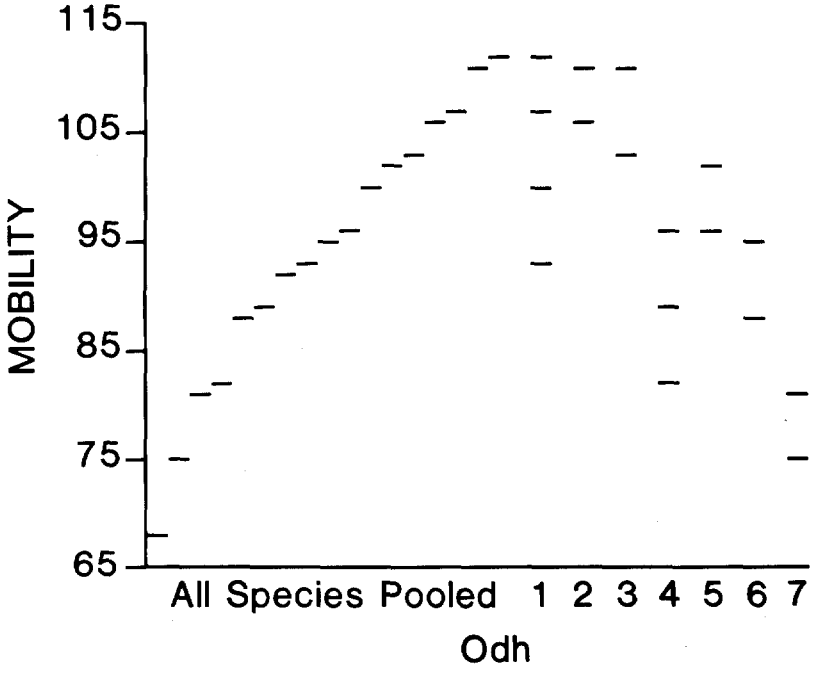

FIGURE 3.—Mobilites of *Odh* electromorphs for all species pooled and within each polymorphic species. Species codes: 1, *B. cavernata*; 2, *B. granulifera*; 3, *B. californica*; 4, *A. carneola*; 5, *A. pallida*; 6, *Bunodactis* sp.; 7, *B. stelloides*.

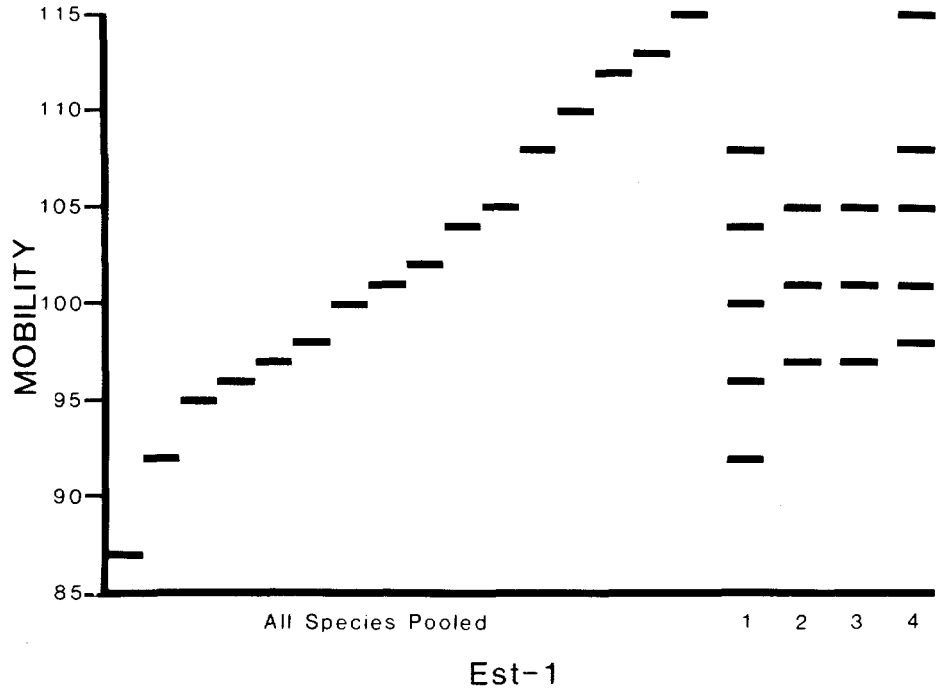

FIGURE 4.—Mobilities of *Est-1* electromorphs for all species pooled and within each polymorphic species. Species codes: 1, *B. cavernata*; 2, *B. granulifera*; 3, *B. californica*; 4, *B. texaensis*.

Atlantic coast substantially at several loci (NEI's D = 0.284; McCOMMAS 1982b). But, if the mobilities of electromorphs from the two areas are pooled and compared with those polymorphic within an area (analogously to the inter- and intraspecific comparisons), there are no significant differences in the distances between adjacent electromorphs. That is, the electromorphs in both areas are on the same mobility ladder. The same is true for the four populations of *B. granulifera* sampled in this study. When *B. granulifera* is compared with *B. californica*, its presumed geminate species (McCOMMAS 1982b), one apparent nonstepwise difference is found at the *Odh* locus. On the other hand, *B. granulifera* and *B. cavernata*, with a NEI'S genetic distance of 1.67 (McCOMMAS 1982a), show nonstepwise differences at every locus except *Sod* (which is obviously the most evolutionarily conservative locus studied here).

This pattern is consistent with the results of RICHARDSON and SMOUSE (1976). They found that electromorphs within a species were regularly-spaced and ladder-like, whereas some of the electrophoretic variation among species deviated from the charge-state model. The reason for the better overall fit of their data to the ladder model could be that they examined a closely related species complex within one genus, perhaps comparable to the *B. granulifera-B. californica* comparisons, or the comparisons of Atlantic and Gulf *B. cavernata* populations.

How do the results of this study, suggesting that variation within species basically fits the charge-state model, correspond to the results of RAMSHAW,

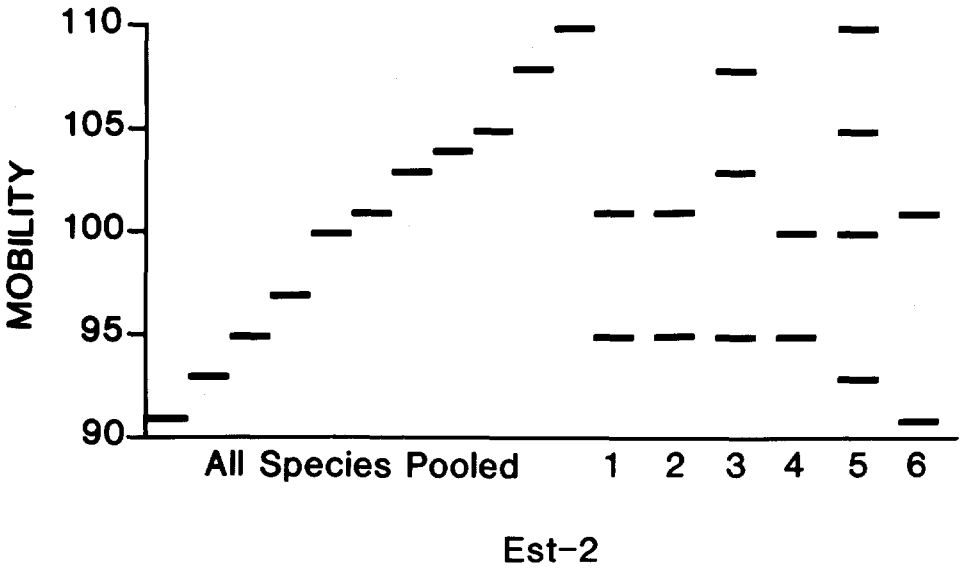

FIGURE 5.—Mobilities of *Est-2* electromorphs for all species pooled and within each polymorphic species. Species codes: 1, *B. stelloides*; 2, *Bunodactis* sp.; 3, *B. texaensis*; 4, *B. cavernata*; 5, *B. granulifera*; 6, *A. tenebrosa*.

COYNE and LEWONTIN (1979) and FUERST and FERRELL (1980)? In both investigations a small proportion of nonstepwise variants within humans for Hb A was found. However, the apparent discrepancies between the previous and present results are probably not serious. Many of the Hb A variants were discovered because they were pathological; alleles coding for such maladaptive proteins would not reach high frequencies in natural populations because of selection against them. Other Hb A variants, present in very low frequencies in the population, were found as the result of massive screenings designed to find electrophoretic variants. Equally rare variants would probably not have been encountered in the much smaller samples used in this study. Finally, some of the variants found within species in this study may have a nonstepwise component "superimposed" on a unit charge change. For example, three of the electromorphs of *Est-2* in *B. granulifera* (Figure 5) are separated by 5 mm, but the slowest electromorph (93) is 7 mm from its nearest electromorph (100). *B. cavernata* also has the 100-morph, but its slowest electromorph is 95, which is the usual 5 mm slower. Since both electromorphs 93 and 95 can't represent a single charge difference, one of them (probably the 93) has some additional confounding influence. Thus, even variation within species in this study is not always perfectly stepwise.

If the results of this study are generally true, it invalidates the idea that increased electrophoretic distance should correspond with increased taxonomic distance except perhaps in comparison of conspecific populations or very closely related species. This lack of correspondence between electrophoretic distance and taxonomic distance is well illustrated in Figures 1 to 5. It is obvious from these figures that the smallest increments between electromorphs are

found in different species. The mixture of stepwise and nonstepwise differences seen here also makes it difficult, if not impossible, to say how many mutational steps may have occurred between two species, or exactly what the pathway of molecular evolution was in a given instance. Although the results of this study rule out the use of electrophoretic separation *per se* as an indicator of taxonomic affinity, they increase confidence in the conclusions reached in systematics studies using electrophoretic data and the more widely-employed measures of genetic distance. Those approaches, both phenetic and cladistic, are based on the assumption that two electromorphs are identical if they share the same mobility. But the charge-state model predicts that many of these electromorphs are heterogeneous charge classes composed of nonidentical proteins. This study's demonstration of the sensitivity of electrophoresis to detecting differences in proteins other than those caused by unit charge changes means that the bands seen on gels need not be assumed to be heterogeneous charge classes. Thus, it is more likely than would be the case if the charge-state model were strictly true that two electromorphs that have identical mobilities are, in fact, the same. However, in order to take advantage of this sensitivity, the electromorphs must be compared in some way similar to that used here so that small differences in mobility can be reliably detected.

The results of this study suggest that the charge-state model is a good model to use for analyzing electrophoretic variation within species or among very closely related species. However, for studies of variation within a more taxonomically diverse assemblage of organisms, electrophoretic variation might best be modeled by an approach like that of LI (1976), who divided mutations affecting mobility of a protein into stepwise and nonstepwise mutations. He showed that even if only 5% of mutations are nonstepwise, the results are considerably different from those predicted by the charge-state model. In this regard, it is interesting to note that recent analysis of allele-frequency distributions from natural populations of many species consistently fit the infinite alleles model better than the charge-state model (CHAKRABORTY, FUERST and NEI 1980). It may be that nonstepwise electrophoretic variation is much more widespread than is commonly thought.

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