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Genetics

Genetic divergence between rodent species assessed by using two-dimensional electrophoresis

(protein differentiation/O'Farrell's technique/molecular evolution/systematics)

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ABSTRACT **O'Farrell's technique of two-dimensional gel electrophoresis (2-DGE) has previously been applied to the study of intrapopulation genetic variation. This approach assays a larger, and in part nonoverlapping, cohort of protein encoding loci compared to conventional one-dimensional electrophoretic procedures (SGE) and has revealed substantially lower levels of mean heterozygosity. Here we extend this approach to analyze levels and patterns of genetic differentiation between species. We have used 2-DGE to compare an average of 189 polypeptides between six species of wild mice representing levels of evolutionary divergence ranging from different subspecies to different families. The magnitude of protein divergence estimated by 2-DGE was on the average only about one-half that predicted by SGE. This discrepancy may result from differences in sensitivities between the techniques or differences in the mean level of variation and divergence between the sets of loci assayed by the two methods. Nonetheless, the ranking of genetic distances by 2-DGE was identical to that by SGE. Thus, these results support the use of the simpler SGE techniques to estimate relative levels of genetic divergence.**

Knowledge of the amount of genetic variation within and between natural populations is central to an understanding of evolutionary processes. Most early attempts to assess genetic variability were based on analyses of segregation patterns in progeny of particular crosses, and they suffered from the inherent bias that only genes that exhibited variation could be recognized (1). In the mid 1960s, one-dimensional electrophoretic techniques were applied to the problem of quantifying genetic variability and divergence in protein products of individual loci (2–7). Because these new techniques could sample an array of about 20–50 polymorphic and monomorphic loci, chosen without known bias with respect to level of variation, the temptation was great to extrapolate results to estimate total genomic variation. The assumption that the soluble proteins assayed by standard gel electrophoresis (SGE) provide a representative sample of the genome has recently come under increased empirical attack. The proteins assayed in conventional electrophoretic surveys may be biased toward a more variable class of gene products (8–16).

Two-dimensional gel electrophoresis (2-DGE), as introduced by O'Farrell in 1975 (17), has allowed examination of a substantially increased number of protein-encoding loci. In 2-DGE, a mixture of denatured proteins is separated first on the basis of charge by isoelectric focusing and then on the basis of molecular weight by $NaDodSO_4$ slab gel electrophoresis. The technique is capable of separating as many as 1100 individual peptides from crude cell extracts on a single gel (17). The ability of 2-DGE to resolve most protein variation resulting from charge-change is well documented (17–19), as is the typical Mendelian behavior of the variants examined to date (9, 13, 20, 21).

2-DGE techniques have been used to assay membrane and other structural proteins in addition to some water-soluble, largely enzymatic, proteins assayed by SGE (8, 9, 13, 20). In 2-DGE, proteins are detected by general protein stains, or by autoradiography, and represent the more abundant proteins of a cell or tissue. Hence, the proteins assayed by SGE compose a small and only partially overlapping set of the proteins assayed by 2-DGE. Recent applications of 2-DGE to the analysis of intrapopulation variation have revealed substantially less genic heterozygosity than had been estimated by SGE (8, 10–12). As noted by Leigh Brown and Langley (8), these results again "throw open the question of the level of genetic variability in nature."

In this study, we extended 2-DGE techniques to analyze levels of genetic differentiation between populations. We examined protein divergence between six species of wild mice representing levels of evolutionary divergence ranging from different subspecies to different families. The objectives of this study are: (*i*) to assess the potential of 2-DGE for estimating genetic divergence and evolutionary relatedness; and (*ii*) to compare estimates of genetic differentiation based on 2-DGE with those derived from SGE, and to examine possible reasons for any disparity.

MATERIALS AND METHODS

Mice were live-trapped from natural populations in the following localities: (*i*) family Cricetidae—*Peromyscus maniculatus*, Union County, GA ($n = 5$), Rutland County, VT ($n = 2$), Curry County, OR ($n = 3$); *Peromyscus leucopus*, Union County, GA ($n = 4$); *Peromyscus boylii*, Amador County, CA ($n = 1$); *Ochrotomys nuttalli*, Clarke County, GA ($n = 4$); *Sigmodon hispidus*, Clarke County, GA ($n = 4$); (*ii*) family Muridae—*Mus musculus*, Pickaway County, OH ($n = 4$). Only adult, apparently healthy animals were used.

Samples for 2-DGE were prepared by grinding 100 mg of fresh liver in a glass tissue grinder containing 300 μl of lysis buffer (17). After centrifugation at $1000 \times g$ for 10 min, the supernatant was drawn off and diluted to a final tissue-to-lysis buffer ratio of 1:8 (wt/vol). 2-DGE was performed essentially according to O'Farrell (17).

Chemicals were obtained from the sources listed by O'Farrell (17) with the following exceptions: ampholytes and acrylamide, from Bio-Rad; *N*, *N'*-methylenebisacrylamide and glycine, from Sigma; and Nonidet P-40, from Bethesda Research Laboratories (Rockville, MD). Total ampholyte concentration was 2% and consisted of one part pH 3–10 and four parts pH 5–7 ampholytes. Gels were prerun according to O'Farrell (17), after which

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Abbreviations: 2-DGE, two-dimensional gel electrophoresis (O'Farrell's technique); SGE, standard gel electrophoresis.

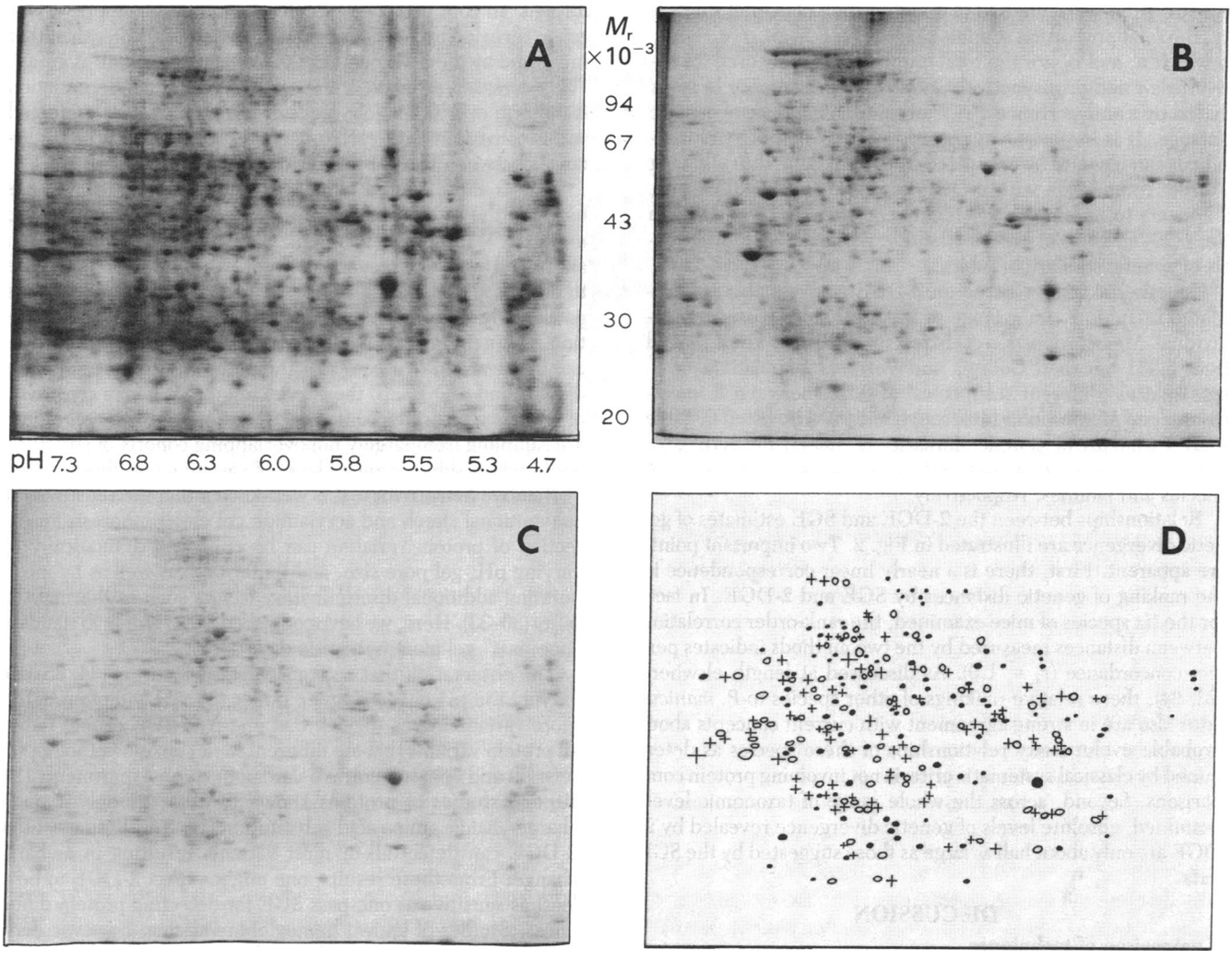

FIG. 1. Two-dimensional gels of liver samples from *P. maniculatus* (*A*) and *O. nuttalli* (*B*). A third gel (shown in *C*, diagrammed in *D*) contained a mixture of liver samples from the two species shown in *A* and *B*. Spots scored as unique to *P. maniculatus* or *O. nuttalli* are indicated by "+" or "O," respectively. Spots scored as common to both species are indicated by solid shapes. Molecular weight standards (from Pharmacia) were phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,100).

a total of 40 μl of sample was loaded onto each gel and overlayed with 20 μl of sample overlay buffer (17). Gels were run for 8200 V-hr (usually at 510 V) followed by 1 hr at 800 V. The isoelectric focusing gels were stained according to the method described by Jackle (22). This procedure allows visualization of the quality of the gel resolution and, by rapidly fixing the focused proteins, results in sharper spots in the second-dimension separation.

Discontinuous $NaDodSO_4$ gel electrophoresis was carried out in 10% acrylamide (17). Gels were run at 40 mA per gel for approximately 2.5 hr. Slab gels were fixed for 30 min in 50% methanol/10% trichloroacetic acid, stained for 40 min in the same solution containing 0.2% brilliant blue R, and destained in 25% methanol/10% acetic acid.

To facilitate comparisons among the protein "constellations," samples from each pair of organisms were run on 2-DGE gels both separately and in mixture. Gels were photographed with 35-mm Kodak high-contrast copy film, and prints were made on Kodak fine grained positive film. The transparent prints from samples run separately could be directly overlaid and scored for spot differences and similarities. Scoring was confirmed by examining gels run with equal mixtures of the two samples. *P. maniculatus* from Georgia was arbitrarily chosen as a standard against which all other samples were compared.

Starch gel electrophoretic data were drawn largely from published studies (23, 24) which used conventional SGE techniques (25–28). Additional comparisons were made among *P. maniculatus* populations and between *P. maniculatus* and *M. musculus* at 30 loci, by using similar methods.

RESULTS

Examples of protein constellations revealed by 2-DGE of individual liver samples of *P. maniculatus* and *O. nuttalli* are shown in Fig. 1. Also illustrated are a gel run with a mixture of liver samples from the two species and a diagram of the gels indicating spots scored same or different. Spots were observed over most of the molecular weight and pH ranges used, although the best resolution was usually obtained in central regions of the gel. In any given gel, 300–400 proteins could be detected by general protein staining, but only those spots showing clarity and good resolution were utilized. All comparisons were scored independently by both authors.

Except in conspecific comparisons, it is usually difficult to identify homologous allelic products with certainty on these two-dimensional gels. Direct estimates of per-locus genetic similarity therefore are not easily obtained. We chose a simple

and straightforward method of quantification of overall similarity, the total proportion of spots shared (F): $F = 2n_{xy}/(n_x + n_y)$, in which n_x and n_y are the total number of spots scored for individuals x and y, respectively, and n_{xy} is the number of spots shared by x and y. Hence $1 - F$ becomes a measure of genetic distance. It is important to note that F between pairs of individuals can likewise be calculated from SGE data, thus allowing a direct comparison with 2-DGE results. Also, for SGE gels, F appears to be empirically highly correlated ($r = 0.99$) with Nei's (29) measure of genetic identity, particularly at lower levels of genetic similarity (Table 1).

Genetic similarities based on 2-DGE analyses also are presented in Table 1. An average of 189 spots were scored per individual. Mean proportion of shared spots ($\bar{F}$) for 2-DGE ranged from 0.951 between Georgia and Vermont populations of *P. maniculatus* (different subspecies) to 0.504 between *P. maniculatus* and *M. musculus* (different families). Also listed in Table 1 are estimates of genetic similarity (F) based on SGE gels. These range from 0.806 to 0.061 in comparisons between subspecies and families, respectively.

Relationships between the 2-DGE and SGE estimates of genetic divergence are illustrated in Fig. 2. Two important points are apparent. First, there is a nearly linear correspondence in the ranking of genetic distances by SGE and 2-DGE. In fact, for the six species of mice examined, the rank-order correlation between distances measured by the two methods indicates perfect concordance ($r_s = 1.0$). As discussed at length elswhere (23, 24), these relative rankings of other species to *P. maniculatus* also are in strong agreement with current concepts about probable evolutionary relationships of these species as determined by classical systematic criteria not involving protein comparisons. Second, across the whole range of taxonomic levels examined, absolute levels of genetic divergence revealed by 2-DGE are only about half as large as those suggested by the SGE data.

DISCUSSION

Comparison of techniques

In our hands, 2-DGE techniques assay nearly 1 order of magnitude more proteins (and presumably loci) than do conventional techniques of SGE. Nonetheless, the relative ranking of genetic distances by 2-DGE and SGE proved to be highly concordant. In contrast, the absolute magnitude of genetic divergence revealed by 2-DGE appears to be much smaller than that suggested by the SGE data (on the average, about half as great). This decreased estimate of the extent of genetic differentiation is uniform over the whole range of taxonomic levels examined and is consistent with earlier 2-DGE estimates of protein distances between two subspecies of *Mus* (13).

Table 1. Genetic similarities (F) between rodents based on 2-DGE and SGE

Comparison (state)	2-DGE		SGE	
	F*	$\bar{F}$	F	I†
P. manic. (GA) vs. *P.m.* (VT)	0.952	0.951	0.806	0.951
	0.949			
P.m. (GA) vs. *P.m.* (OR)	0.927	0.916	0.788	0.950
	0.905			
P.m. (GA) vs. *P. leucopus*	0.856	0.853	0.698‡	0.802‡
	0.850		0.706§	0.738§
P.m. (GA) vs. *P. boylii*	0.740	0.812	0.571‡	0.637‡
	0.883			
P.m. (GA) vs. *O. nuttalli*	0.581	0.599	0.250§	0.245§
	0.617			
P.m. (GA) vs. *S. hispidus*	0.491	0.547	0.121§	0.107§
	0.602			
P.m. (GA) vs. *M. musculus*	0.504	0.504	0.061	0.067
	0.504			

* Proportion of spots shared, as scored by C.F.A. (upper value) and J.C.A. (lower value).
† Nei's (29) genetic identity.
‡ Calculated from data of Avise *et al.* (23).
§ Calculated from data of Patton *et al.* (24).

Relative Sensitivities. It is well-known that the sensitivity of conventional starch and acrylamide gel electrophoresis for detection of protein variation can be enhanced dramatically by varying pH, gel pore size, and buffer type as well as by incorporating additional discriminatory factors such as thermal stability (30–33). Here, we have compared 2-DGE only to standard "one-pass" gel electrophoretic data.

The observation that native isoelectric focusing (34, 35) and varying the pH of the running buffer (30, 31) will often detect more variation does suggest that one-pass SGE cannot detect all protein variants that are different in net charge (although see refs. 30 and 36). In contrast, carbamoylation experiments (18, 19) and studies of proteins known to differ by only a single charge-change amino acid substitution (17, 37, 38) suggest that 2-DGE can detect all or most variants resulting in a charge change. From these results, one might expect 2-DGE to be at least as sensitive as one-pass SGE for detecting protein differences. Studies of known hemoglobin variants, however, have shown some charge-equivalent substitutions to be distinguishable by SGE (31, 39). Whether this class of substitutions is detectable in the denaturing conditions of 2-DGE remains to be examined.

To our knowledge, only two direct empirical comparisons of the number of known variants detected by 2-DGE and by one-

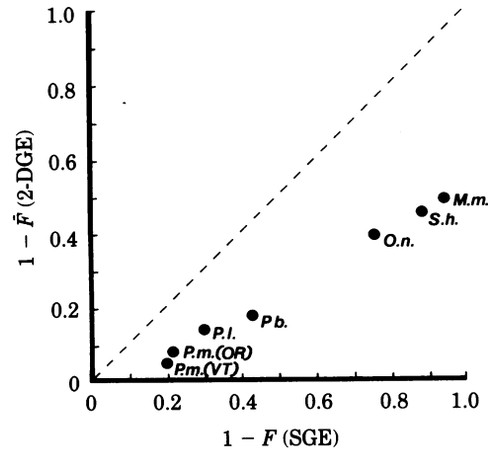

FIG. 2. Relationship between genetic distances ($1 - \bar{F}$) observed by 2-DGE and SGE techniques. Each point denotes the comparison between *P. maniculatus* from Georgia and the indicated species (see Table 1). The dashed line represents the expectation if both methods revealed identical patterns and levels of differentiation.

pass SGE have been published. Anderson and Anderson (40) found three human transferrin variants detectable by SGE to be distinct on 2-DGE. In contrast, Leigh Brown and Langley (8) reported that, for *Drosophila* α-glycerophosphate dehydrogenase, only the major charge differences representing the slow/fast polymorphism were detectable by 2-DGE; several variants detected by certain conditions of SGE could not be discriminated by 2-DGE. Recently, T. McLellan and colleagues (personal communication) have extended this latter comparison with similar results.

Recent studies (41) of 2-DGE variation in human α_2 HS glycoprotein also demonstrated the detection of allelic protein variants differing in molecular weight. Because these involved large molecular weight shifts (due to sequence differences in carbohydrate attachment sites), presumably these changes would be detectable by SGE as well.

Clearly, more data are needed. The magnitude of any sensitivity bias is still unclear. Nonetheless, results of the above studies, taken in sum, suggest that although 2-DGE might be less sensitive to individual protein variation and differences than SGE, this explanation alone may not be sufficient to account for the substantial differences in levels of protein differentiation revealed by the two methods.

Proteins Assayed. A second possibility is that the class of proteins assayed by 2-DGE is truly less variable, on the average, than that assayed by SGE. Several authors have suggested that ribosomal, regulatory, membrane-associated, and structural proteins may show lower levels of variability due to the greater steric constraints of their intermolecular interactions (e.g., refs. 11, 12, 14, 16, and 42). At least some of these proteins are probably well represented among the abundant proteins assayed by 2-DGE, but they are not typically included in SGE surveys which assay water-soluble, mainly enzymatic, proteins. In support of the hypothesis of steric constraints, significantly less polymorphism has been reported at loci coding for multimeric enzymes (which form intermolecular hybrid molecules) than at loci coding for monomeric enzymes (refs. 43–45; but see ref. 46). Also, ribosomal proteins show a striking lack of variation within and between species of animals, plants, and bacteria (16, 47, 48).

2-DGE is also capable of assaying some of the enzymes of intermediary metabolism assayed by SGE. An advantage of utilizing 2-DGE techniques to estimate protein variation and differentiation is that at least some representatives of each of a broader array of protein classes can be monitored. Thus, 2-DGE techniques may provide a more balanced view of variation at protein-encoding loci throughout the genome. This is not to imply that 2-DGE techniques themselves are without sampling bias. For example, protein abundance itself conceivably could be biased with respect to total structural gene variation (11, 14); and most 2-DGE techniques have so far examined primarily proteins with isoelectric points in the pH range 5–7.

Ironically, recent suggestions, from 2-DGE analyses, that SGE techniques may have overestimated structural genome variation and divergence come at precisely the time when other lines of evidence imply that SGE techniques have grossly underestimated genome variation. Detailed studies of several enzyme systems, involving a greater array of assay conditions, have uncovered considerably more allelic diversity than had been disclosed by SGE (e.g., refs. 30–33). In the future, it will be of interest to apply some of these refined techniques to some of the structural proteins now monitored by 2-DGE.

Applications to systematics

What is the potential of 2-DGE as a survey tool for estimating genetic differentiation and systematic relationships? Although 2-DGE is more difficult and time-consuming than SGE, the advantage of scoring between 100 and 600 proteins on a single gel is considerable. On the average, we have scored 189 protein spots per individual, reflecting 150 or more loci. Using a more sensitive double-label autoradiography technique, McConkey *et al.* (10) examined as many as 600 peptides on a single gel. The 2-DGE technique shares with SGE a general objectivity in scoring: the spots are scored either the same or different according to migration position. We have introduced a relatively simple, inexpensive, and reliable method of scoring 2-DGE gels which includes the use of transparent high-contrast prints in combination with double-sample gels to verify spot migration. This procedure contributes significantly to the identification of protein differences because the spots can often be seen to change position relative to background constellations.

Nonetheless, many problems remain in interpreting 2-DGE gels. Protein spots occupying similar or identical positions on gels from different individuals can vary greatly in intensity, and these differences could arise from any of several sources including regulatory differences in gene expression, differences in quality of sample preparation and protein solubilization, and "chance" poor resolution of proteins in a particular region of the 2-DGE gel. [One of us (J.C.A.) was consistently more conservative in counting only the heavier protein spots, and this presumably contributed to the different scores obtained by the two authors in some species comparisons (Table 1)]. Another disadvantage of the 2-DGE technique relative to SGE as a tool for systematic surveys is that the loci (and their homologies) controlling the spot constellations remain unknown. This effectively precludes the use of some powerful methods of phylogenetic reconstruction which rely upon the identification of homologous, qualitative character states such as allelic products of a known locus (24, 49).

Notwithstanding these difficulties, it is apparent from our results that the relative ordering of genetic distances among related species is highly correlated between the 2-DGE and SGE data sets. Inferences from 2-DGE and SGE about relative evolutionary relationships of these mice species are thus concordant.

Overall, the results of this study are important for several reasons. First, they suggest that the magnitude of structural gene differentiation between species may be less than previously estimated from the suite of mainly enzymatic proteins routinely assayed by SGE. However, the contributions of technical bias, relative to inherent differences in classes of proteins assayed, to this observation remain unresolved. Second, although the problems associated with running, scoring, and interpreting the gels may limit general application, 2-DGE can be a useful adjunctive tool in systematics. Third, the results of this study strongly support the use of the simpler and more straightforward SGE techniques to estimate relative genetic divergence; the 15–30 loci routinely assayed by SGE are apparently reliable indicators of relative divergence at a much larger set of protein-encoding loci.

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