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Is the polymorphism of protein amounts related to phenotypic variability? A comparison of two-dimensional electrophoresis data with morphological traits in maize

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Summary. The hypothesis that the quantitative variations in gene product levels could be a more important basis for morphological and adaptative change than the classical qualitative variability revealed by electrophoretic techniques was studied by comparing five maize lines from three sets of variables: (i) qualitative variations of proteins (presence/absence) revealed by two-dimensional polyacrylamide gel electrophoresis (2D PAGE), at a physiological seedling stage; (ii) quantitative variations in proteins (spots more or less intense) revealed by 2D PAGE, at the same physiological stage; (iii) general combining abilities of fourteen heritable, morphological or agronomical characters measured at various juvenile and adult stages. Distances between lines were defined, based on qualitative and quantitative variations of proteins. These distances do not appear to be correlated and do not give the same patterns of divergence between lines, as shown by principal coordinate analyses. Mahalanobis distances computed from the general combining abilities of the morphological characters are significantly correlated ($r=0.75$) to quantitative but not to qualitative distances. The comparison of the first planes of the principal coordinate analyses performed on the three kinds of distances clearly confirms this finding. Our results are discussed in connection with the possible genetic meaning of the two molecular distances and with the hypothesis that regulatory processes are primarily implicated in morphological variation.

Key words: Maize – Protein polymorphism – Morphological variation – Genetic distances – Two-dimensional electrophoresis

Introduction

During the past 20 years, various techniques have been developed to analyze genetic variability at the molecular level: allozyme electrophoresis, sequencing of proteins and nucleic acids, digestion by restriction enzymes, etc.

Numerous studies have tried to understand the phenotypic or adaptive significance of molecular polymorphism (Lewontin 1974; Nevo 1978; El-Kassaby 1982; Koehn et al. 1983; Nevo 1983; etc.). Surprisingly, in most cases this molecular variability appears not to be related to morphological variation either on the interspecific or the intraspecific level (Kimura 1968, 1983; King and Wilson 1975; Gottlieb 1977, 1981; Avise and Aquadro 1982; Wheeler and Guries 1982; Cabrera et al. 1983; Damerval and de Vienne 1985; etc.). In the case of populations under artificial selection this poor correlation has also been documented (Stuber et al. 1980; Stuber et al. 1982; Kahler 1983).

These studies are concerned with the discontinuous, qualitative variability of protein or DNA sequences. Yet it is reasonable to think that increasing attention might be paid to quantitative variability at the molecular level. Indeed, various observations suggest that in natural populations several polymorphic loci distributed throughout the genome may affect the amount of a given protein (McDonald and Ayala 1978; Barnes and Birley 1978; Paigen 1979; Laurie-Ahlberg et al. 1980, 1982; Scandalios and Baum 1982). Some authors have shown that the factors controlling the activity of particular enzymes do not respond to selection in the same way as the structural genes (Powell and Andjelkovic 1983; Matsuo and Yamazaki 1984; Powell and Amato 1984). For evolutionary change and for adaptation, the polymorphism of such modifiers could provide an important and, above all, a specific source of variation (Wilson 1976; Wilson et al. 1977; McDonald et al. 1977; Turner et al. 1979; McIntyre 1982; McDonald 1983; Dickinson et al. 1984). In this connection Powell (1979) and Powell et al. (1980) compared the diversity of midgut specific expression of *Drosophila* amylase with the diversity of amylase allozymes within and between some populations and species. They found no significant correlation between these two diversities.

Our general purpose is to examine the idea that the organization of genetic variability among individuals may be different for qualitative and quantitative molecular polymorphisms, and to study the possible relationships between the factors controlling protein amounts and phenotypic variation. The molecular markers used are obtained by two-dimensional electrophoresis of denatured proteins (O'Farrell 1975; Garrels 1979). This technique reveals several hundreds of gene products, whereby it is possible to investigate not only the "qualitative" variability (presence/absence of spots between genotypes) but also the "quantitative" variability, through the variations of spot intensities: by comparing genotypes genetic differences in factors controlling protein quantities can thus indirectly be scored (Klose and Feller 1981; Klose 1982; Zivy et al. 1983, 1984; Bahrman et al. 1985; Colas des Francs and Thiellement 1985; Anderson et al. 1985).

A study of genetic distances between five maize lines allowed the comparison of these two kinds of variability. We show here that when high resolution two-dimensional gels are used, both are large, and, above all, the variations in the amounts of proteins reveal a new, specific genetic variability which is different from that issued from the qualitative variation. Moreover, the Mahalanobis distances computed from morphological and agronomical characters appear clearly correlated to the "quantitative" but not the "qualitative" distances. This result is discussed with respect to the possible genetic meaning of these two molecular variabilities.

Material and methods

Plant material

Five maize lines involved in a diallel cross were studied: W 117, Co 125 (dent type), F 188 (flint-dent type), F 1254 and F 1772 (flint type). These lines were chosen for their different kernel types (dent, flint, or dent-flint) and because they belong to different groups of combining abilities. Kernels were harvested at the same place in the same year to avoid environmental effects. They were germinated in the dark. When the coronary roots appeared at the first node (at about ten days), the coleoptile and the first leaf were cut off. Each sample consisted of tissue from three different plantlets.

Protein extraction and electrophoresis

Proteins were extracted according to the method of Harrison and Black (1982) and Bahrman et al. (1985). This drastic method prevents protease action during extraction (Colas des Francs et al. 1985).

Rod gels for isoelectric focusing (IEF) were 19.5 cm long and 1 mm in diameter. The gel mixture was 9.2 M urea, 4% Triton X-100, 2.84% acrylamide, 0.16% N,N′ methylene bisacrylamide, 4% carrier ampholytes (0.8% LKB pH 3.5 to 10, 1.6% LKB pH 5 to 7, 1.6% LKB pH 5 to 8) 0.1% Temed, 0.015% ammonium persulfate. The anolyte was 10 mM orthophosporic acid, and the catholyte was a 20 mM degassed sodium hydroxide solution. Ten microliters of the sample were laid onto the top of the gels with 5 µl of overlay solution (twice diluted solubilization solution); the gels were run at 900 V for 24 h (21,600 Vh); they were then equilibrated 2 min in the O'Farrell's SDS sample buffer (O'Farrell 1975) without 2-mercaptoethanol and with sucrose instead of glycerol.

The SDS-electrophoresis was performed with the apparatus described by Garrels (1979) according the procedure used in Damerval et al. (1986). For each line, two separately extracted samples were run at least twice, in order to provide four good quality gels.

Staining and scoring procedure

The staining procedure was as described by Oakley et al. (1980), except for the revelator which was diluted twice. Four good quality 2D-gels per line were scored visually by two observers. Over 20% of spots per line were discarded as non-reproducible (mostly faint spots). For the qualitative comparison of the genotypes, we also eliminated two dense regions (in zones 21 and 33) and additional spots whose variation could not be precisely ascertained because of proximity effects. To estimate the quantitative variability, it was impossible to examine visually 20 gels at a time (5 lines, 4 gels per line) as would be needed to take into account the intra and interline variations of intensities, for each spot. As we were only interested in the relative *mean* values of variations between lines, we chose a different scoring method.

For each line, we chose a 2D gel so that the five 2D gels retained (one per line) had very similar staining levels, and compared *together* these 2D gels. We considered as quantitative differences only the cases in which the spots very clearly displayed several levels of intensity among the lines where they were present: a *slight* difference in spot intensity between two lines was not taken into account if the intensity of the spot in another line lay between the two intensities observed. In most cases we could define only two levels, rarely three, never more. This procedure minimizes the number of differences but makes them more reliable. Moreover, if there was any doubt, we concurrently examined the replicates of the gels compared, and if uncertainty remained, we always favoured the hypothesis of absence of variation. In spite of these precautions, some errors certainly remain. However, we cannot imagine any systematic bias giving rise to the important relative differences of quantitative variations between lines (see below). Moreover, statistical confirmations of the robustness of our scoring appear also in "Results" and "Discussion".

Parameters relative to molecular distances

When comparing two lines, 3 situations may occur for one spot: present/absent, present in the two lines but with different intensities according to the criterion previously defined, and present in both lines with the same intensity. Let the numbers of spots in each of these situations be respectively p, q, and c. Two types of distances between lines can thus be defined, the "qualitative" distances $d_P = p/(p+q+c)$ and the "quantitative" distances $d_Q = q/(q+c)$.

Biometrical and morphological characters

The 5 lines studied for protein variation belong to a diallel design implicating 12 lines. The lines and their single cross progeny were studied under field conditions in Lusignan in 1981. The design was a 8×9 lattice with 5 replicates; the plots were 4.5 m long single rows with 35 plants, 0.8 m apart (Hébert 1986). The diallel cross allowed the estimation of general combining abilities (GCA) according to Garretsen and Keuls (1978) for the 5 lines studied for protein variation.

Twenty-nine characters were measured, covering the whole plant cycle, in order to study the expression of heterosis during development (Hébert 1986). The fourteen more heritable ones were used in the present study, to ascertain that the differences between lines have a genetic significance:

– Mean rates of leaf appearance were calculated as the ratio of the number of leaves produced during a given period of time to the sum of degree-days received during the same period; this last parameter was estimated as the mean of minimum and maximum soil temperature at 10 cm, with a lower threshold of 6 °C (Cauderon 1958). Rate values were measured at the four visible leaves and six visible leaves stages for the so-called young-type leaves (ligule not visible), and at the 14 visible leaves stage for the adult-type (ligule visible).

– The global rate of leaf appearence was determined as the linear regression coefficient of the number of leaves on the sum of degree-days from the emergence to the 14 leaves stage: it was calculated for young-type and adult-type leaves.

– The origin of the previous regression line, determined only for adult-type leaves, is a measure of the number of leaves that can be seen just after coleoptile breakage. This value can be related to the number of leaf-primordia of the embryo.

– Vigor was visually estimated at the 6–8 leaves stage by a global indice.

These seven first characters constituted variables of early growth.

Dates of 50% shedding tassels and 50% silking plants, ear insertion height, total number of leaves, total height, surface of the flag leaf and dry matter content at the harvest constituted the seven adult characters.

Mahalanobis distances (D^2) (Mahalanobis 1936) were computed between lines, each line being represented by a vector whose elements are the principal components of the GCA of the variables considered (Camussi and Ottaviano 1981).

Correlations between distances

The values of a given distance matrix are not independent. Therefore, the classical test of significance of the correlation coefficient is not valid when the two variables considered are distances. So we used a permutation test (Brunel and Rodolphe 1985). We computed the correlation coefficients for the 120 (5!) possible permutations of the lines. The percentage of values which exceed the one observed allows to estimate the probability that the correlation is greater than this threshold value. This is an unilateral test of the hypothesis of no relation against the hypothesis of a positive relation.

Results

Relationships between qualitative and quantitative distances

A 2D gel and the global map of all the reproducible spots scored are shown in Fig. 1. One thousand and sixty-seven spots were numbered. Only 258 were common to the five lines with the same intensity. Seventy-six per cent were variable, either in intensity (18%), in presence/absence (48%), or in both of them (10%).

Table 1. Distances between lines. Above diagonal, qualitative distances; below diagonal, quantitative distances

	Co 125	W 117	F 188	F 1254	F 1772
Co 125		0.248	0.251	0.385	0.259
W 117	0.173		0.313	0.423	0.300
F 188	0.191	0.238		0.379	0.311
F 1254	0.203	0.226	0.241		0.409
F 1772	0.268	0.274	0.229	0.267	

The qualitative (d_P) and quantitative (d_Q) distances were computed for the 10 line pairs (Table 1) and principal coordinate analyses of the distance matrices (Gower 1966) were performed.

The comparison of the first planes for d_P and d_Q, which respectively represent 69.2% and 67.8% of the variance, displayed remarkable differences (Figs. 2a and b). The most important one concerned the relative positions of F 1254 and F 1772, which were reversed. This discrepancy persisted in the dendrograms obtained by the mean aggregation method (Fig. 2c, d). Also, the dent lines W 117 and Co 125 were closer according to d_Q than d_P. The low value of the correlation between the two transformed variables Arcsin $\sqrt{d_P}$ and Arcsin $\sqrt{d_Q}$ ($r=0.28$) confirmed these observations: the permutation test gives 26 values out of 120 greater than 0.28, providing an estimate of the probability $P=0.22$ that r be greater than 0.28 under the hypothesis of no link (Fig. 3).

The stability of the classifications was examined by additive three-way analysis of variance (ANOVA) on the two transformed distances, with a fixed effects model. The gels were divided into 16 zones (Fig. 1b) according to molecular mass (Mr) and isoelectric point (pI). This design was also chosen for estimating possible Mr or pI effects. The factors were the line pair (10 levels), the Mr (4 levels) and the pI (4 levels). The F values (Table 2) for the line pair effect were both highly significant and d_P better discriminated than d_Q (14.25 vs 2.72). Since the correlation between the two types of distances was not significant, the classifications of the lines were not statistically identical. The mean distances were ranked as follows (global type I risk: 10%) for d_P:

Co 125/ W 117 | Co 125/ F 188 | Co 125/ F 1772 | W 117/ F 1772 | F 188/ F 1772 | W 117/ F 188 | F 188/ F 1254 | Co 125/ F 1254 | W 117/ F 1254

and for d_Q

Co 125/ W 117 | Co 125/ F 188 | Co 125/ F 1254 | F 188/ F 1772 | F 188/ F 1254 | W 117/ F 1254 | W 117/ F 188 | Co 125/ F 1772 | W 117/ F 1772 | F 1772

As expected, the differences in the ranking order were mainly due to the relative values of the line pairs including F 1254 and F 1772.

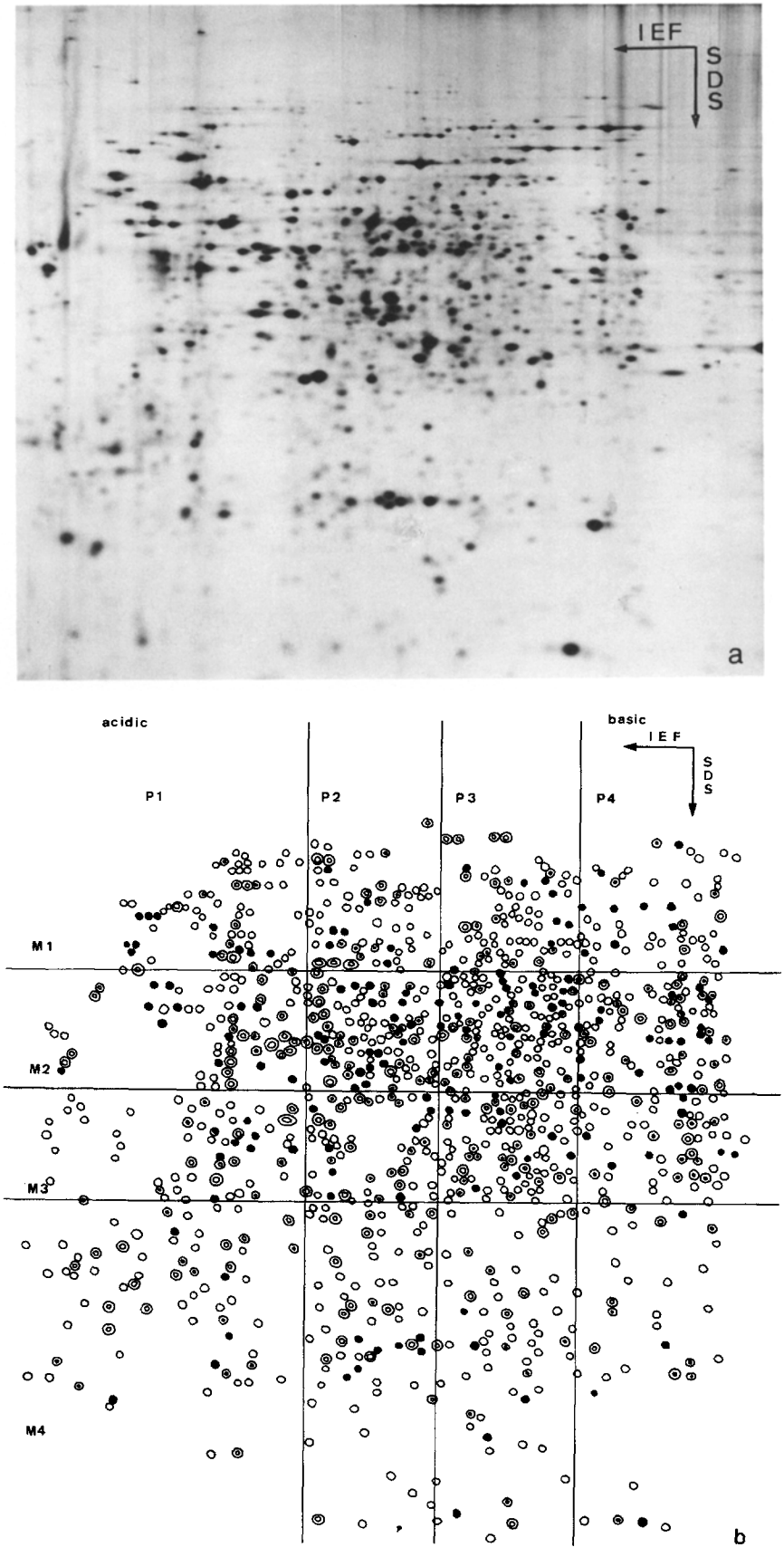

Fig. 1. **a** Two-dimensional gel of line Co 125. **b** Global map of the 1,067 reproducible spots in the 5 lines. The horizontal lines delimit 4 zones of molecular mass (M1 to M4) and the vertical lines 4 zones of isoelectric point (P1 to P4). Double circles: spots present in the 5 lines with the same intensity. Open circles: spots present in at most 4 lines with the same intensity. Black spots: spots present in the 5 lines with different intensities. Circled black spots: spots present in at most 4 lines with different intensities

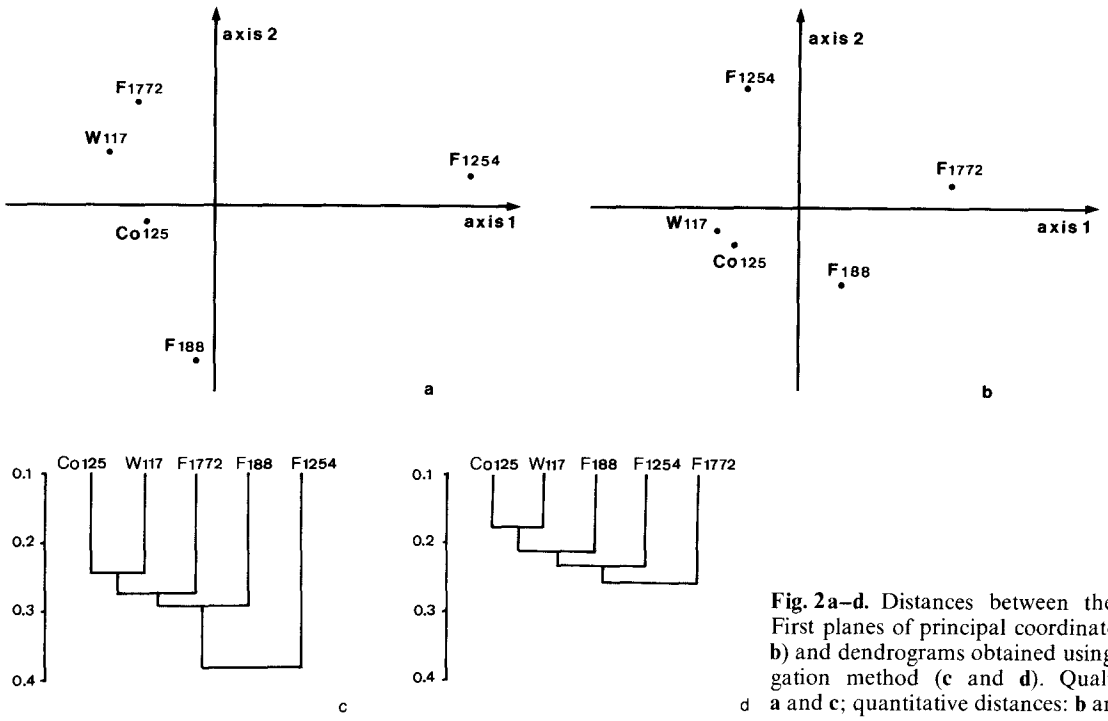

Fig. 2a–d. Distances between the 5 maize lines. First planes of principal coordinate analyses (**a** and **b**) and dendrograms obtained using the mean aggregation method (**c** and **d**). Qualitative distances: **a** and **c**; quantitative distances: **b** and **d**

Table 2. F values of 3-way analysis of variance performed on Arcsin $\sqrt{d_P}$ and Arcsin $\sqrt{d_Q}$

Factor	F	
	Arcsin $\sqrt{d_P}$	Arcsin $\sqrt{d_Q}$
Line pair	14.25**	2.72**
Molecular mass	15.53**	2.49**
Isoelectric point	4.79**	2.09^{NS}

** Significant at 1% level; NS: not significant

The effects of Mr and pI were also highly significant, except for pI on d_Q (Table 2), with the following ranking orders:

M2 M1 M4 M3 P2 P1 P4 P3

for d_P, and

M4 M1 M2 M3

for d_Q.

In view of these results, we first examined the possibility of a spot density effect (Rosenblum et al. 1983). In the dense regions, overlapping of spots could cause a decrease of total variability and/or an increase of quantitative variability relative to qualitative variability. From the 16 zones defined, we computed the correlation coefficient between spot density and percentage of variable spots (qualitative and/or quantitative): $r=-0.12$ ($P \leqq 0.65$). The correlation coefficient between spot density and the ratio of the number of quantitatively variable spots to the total number of variable spots is $r=0.34$ ($P \leqq 0.20$). Otherwise, we verified that gaps in the pH gradient (Tollaksen et al. 1981) cannot account for the pI effect on d_P (not shown). Thus the zone effect is not due to these two technical artefacts. Other untestable factors (quality of resolution) or genetic reasons could be involved. Nevertheless, it is worthwhile noting that the ranking orders of Mr according to d_P and d_Q were different, confirming the genetic specificity of these distances.

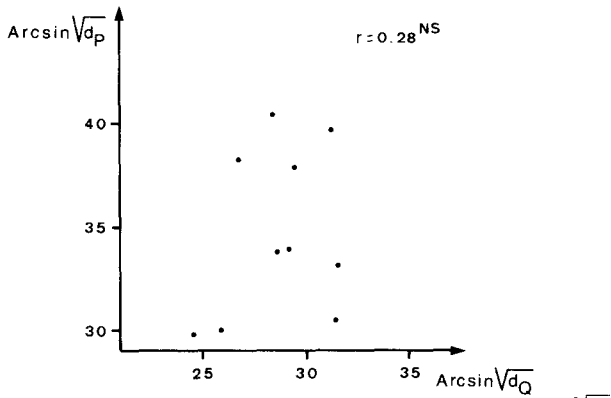

Fig. 3. Correlation between qualitative distance Arcsin $\sqrt{d_P}$ and quantitative distance Arcsin $\sqrt{d_Q}$

The main question about the Mr and pI effects is whether or not they significantly modified the classifications of the lines according to the zones: one-way ANOVAs were performed on each Mr and pI zone, with the line pair as factor. These are low power ANOVAs because in each Mr (resp. pI) zone the levels of the factor pI (resp. Mr) are taken as repetitions. For d_P as well as d_Q, the correlations between distances corresponding to zone couples were then computed.

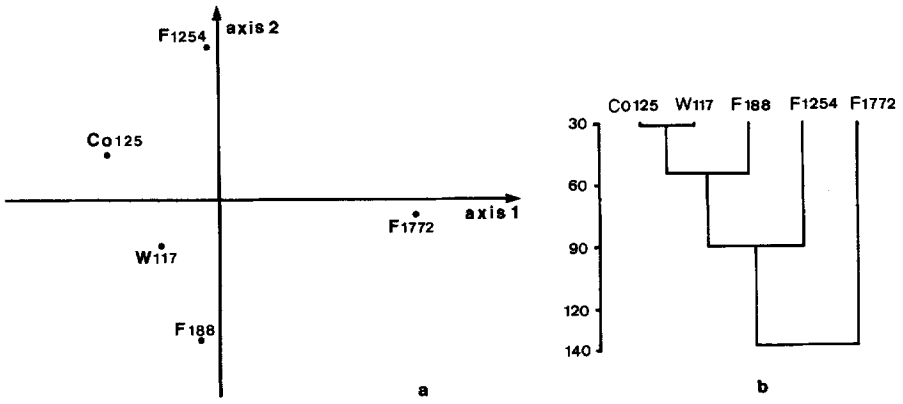

Fig. 4. First plane of the principal coordinate analysis performed from D^2 matrix (**a**), and dendrogram obtained using the mean aggregation method (**b**)

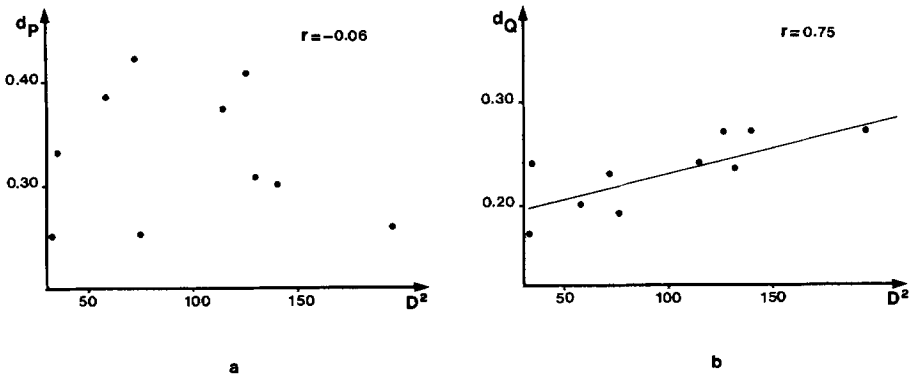

Fig. 5a. Correlation between d_P and D^2; $r = -0.06^{NS}$. **b** Correlation between d_Q and D^2; $r = +0.75$ ($P \leq 0.04$)

Table 3. Mahalanobis distances between the maize lines computed from the general combining abilities of 14 heritable biometrical variables

	Co 125	W 117	F 188	F 1 254
Co 125				
W 117	31.34			
F 188	76.02	33.6		
F 1 254	58.79	72.46	115.76	
F 1 772	196.43	140.68	130.84	127.35

The results indicated that for d_P the line pair effect was significant in three Mr zones and three pI zones, with significant correlations between their distances. Thus, there was no zone effect on classification. For d_Q the F values were significant in only one zone, M4, at the 5% level. Considering the low power of these ANOVAs, this was not inconsistent with the highly significant F value obtained in the threeway ANOVA.

Comparison with the morphological distances

The Mahalanobis distances (D^2) between the five Maize lines, computed from the general combining abilities (GCA) of 14 heritable biometrical variables, are given in Table 3. Figure 4a displays the first plane of the principal coordinate analysis performed on the D^2 matrix. This plane represents 88.1% of the variance.

The discrimination between the lines obtained for D^2 is very similar to that obtained for d_Q, but not to that obtained for d_P (Figs. 2 and 4). The correlation between D^2 and d_Q is $r = 0.75$ while the correlation between D^2 and d_P is $r = -0.06$ (Fig. 5). Using the permutation test for the correlation between D^2 and d_Q, we found only five values out of 120 greater than $+0.75$, and zero less than -0.75. Thus, the estimated probability, under the hypothesis of no link, that r be greater than $| 0.75 |$ is 0.04. With an α risk of 0.05, a link exists between D^2 and d_Q, but not between D^2 and d_P.

Juvenile and adult characters were considered together to compute D^2. If each subset of characters is considered separately, no significant correlation is obtained either with d_P or with d_Q (not shown). Thus, the high correlation between D^2 and d_Q is not due to a particular subset of variables, but concerns the juvenile as well as the adult characters.

Discussion

A design involving five maize lines was used to compare various distances computed from molecular and morphological characters.

Our molecular distances relies on a partitioning of the protein variability issued from two-dimensional electro-

phoresis into "qualitative" and "quantitative". We obtained highly significant F values for line pair effect on qualitative distance (d_P) and quantitative distance (d_Q), and these two distances appear not to be related. From a statistical point of view, this result confirms a posteriori the reliability of our scoring procedure: a large number of errors would have led to an increase of residual variance incompatible with the significant F values.

The large qualitative variability contrasts with the first results obtained by two-dimensional electrophoresis, even though d_P cannot directly be compared to the parameters of variability in other studies (Leigh Brown and Langley 1979; McConkey et al. 1979; Racine and Langley 1980; Aquadro and Avise 1981; Ohnishi et al. 1982; McLellan et al. 1983). If one considers that a "true" genetic qualitative distance (d_G) should be defined by the ratio of the number of loci with different alleles to the total number of loci and if one considers that our qualitative variability is solely due to allelism of protein-encoding loci (which is questionable), in these conditions it is easy to show that d_P overestimates the genetic distance d_G according to the relation $d_G = d_P/(2 - d_P)$. Instead of ranging from 0.25 to 0.41, the distances would go from 0.14 to 0.26. These values remain high, and of course this hyperbolic transformation has no influence on our results, since it does not modify the ranking order of the lines. Actually, some presence/absence variations may correspond to quantitative variations where the faint spot was below the level of detection. This could be another cause of overestimation of qualitative variation. However, this phenomenon is probably slight, otherwise we would not have observed differences between d_P and d_Q classifications. We think that our high resolution two-dimensional technique probably partly accounts for the high variability obtained: recent observations on wheat in our laboratory showed that technical improvements may increase the differences between two lines 2.5 fold relative to first studies (Damerval et al. 1986; see also Rosenblum et al. 1983).

A great variation in gene product levels was scored. Until now this phenomenon has been described in very few species from two-dimensional electrophoresis (Klose and Putz 1983; Zivy et al. 1983, 1984) but it probably has a general value (Paigen 1979; Scandalios and Baum 1982; Tepper et al. 1982). As we used large gels, the overlapping of spots was minimized, so gene product level variation was probably only scarcely contaminated by qualitative variation. On the contrary, it was certainly underestimated because of the misclassification of some variations as qualitative (see above) and above all because of the rough visual analysis of gels. According to N. L. Anderson (pers. commun.) visual comparison of spots allows detection of only 200% or 300% intensity differences, while automatic analysis would detect below 50% intensity differences (Anderson et al. 1985). Thus, a part of the quantitative variation is not detected in our analysis. Inversely we are sure to work on a reliable subset of quantitative variation.

The two kinds of molecular distances did not give the same information on the relationships between lines. This result was all the more robust as it was obtained from many gene products and there were ambiguities in the identification of the two types of variability. In any case their specificity clearly appears when they are compared to the Mahalanobis distances computed from 14 heritable morphological or agronomical variables. The quantitative distances are significantly correlated to morphological distances: as the juvenile and the adult variables were both needed to produce the high correlation, we assume that d_Q is a global parameter, including the variation of a large number of genes, although it was measured at only one early stage. On the contrary, the qualitative distances seem completely independant from morphological distances.

For reasoning purposes, the genome is classically dissected in two classes of genes, the structural and the regulatory genes (see discussion about this partitioning in McIntyre 1982). Any amino acid substitution modifying the pI or the apparent molecular mass (De Jong et al. 1978), as well as structural aberrations such as small deletions, insertions, etc., lead to the presence/absence variation of spots. If the regulatory genes are generally defined as any gene that affects the amount, the tissue distribution or the developmental profile of another gene product (McIntyre 1982; Hedrick and McDonald 1980), their genetic variation will appear as a quantitative variation of spots. However, there are evident ambiguities in this categorization: (i) regulatory effects producing repression or activation, as well as changes in post-translational modifications should appear as qualitative variations; (ii) according to Zuckerkandl (1978), Klose (1982) and Mukai et al. (1984), most regulatory elements are situated in noncoding regions, but McIntyre (1982) has suggested that the regulatory processes are mediated by proteins themselves (processing enzymes, kinases, proteases . . .). In the latter case, regulatory variation may appear as both quantitative and qualitative spot variation; (iii) mutations in a structural gene may result in modifications of its level of expression or may influence gene product stability, giving rise to quantitative variation, possibly along with qualitative variation.

Because of these ambiguities, we cannot state that our quantitative and qualitative variations respectively represent regulatory and structural gene variation. But this semantic question seems to us not to be pertinent. Our main result is that the genetic factors responsible for protein amount variations, whatever their molecular origin, do not discriminate the lines in a way similar to the factors causing presence/absence variation. This reinforces the idea that the evolutionary forces could act in a different way according to the types of genes (Powell 1979; Powell et al. 1980; Matsuo and Yamazaki 1984).

The hypothesis that the regulatory variation (in a very general sense) could play an important role in explaining variability at the organismal level has already been suggested (King and Wilson 1975). Until

now, however, very little work has been devoted to this question. Klose and Putz (1983) failed to find a relationship between 2D electrophoregram patterns of trisomic mouse embryos and morphological aberrations. Yamazaki and Matsuo (1984) and Matsuo and Yamazaki (1984) showed that inducibility of α-amylase of *Drosophila melanogaster* was correlated to fitness components using starchy food. Our result takes directly into account the variations of gene products; as we have worked with several hundreds of molecular variables, it strongly suggests the idea that the variability of factors controlling protein quantities may be an important basis for morphological variation. If it can be generally applied, it could represent a new aspect in population and evolutionary genetics.

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