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Isozyme parameters in genetic evaluation in Brassica – A tribute to Haldane's vision on the synergy between biochemistry and genetics*

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Isozymes as biochemical markers possess distinct advantages over molecular markers like RFLP in breeding for quantitative trait (QT) improvement. Five parameters exhaustive of isozyme variation, viz. number of bands, relative absorption, standard error (relative absorption), relative mobility, and standard error (relative mobility) (IST) were conceived. The two standard error parameters are new and not used so far. They are crucial in accounting for environmental contribution to isozyme variation. In this paper, varieties were arranged into a set of base groups on a genetic basis using six QTs. The grouping was done afresh on IST and a commonality index was used to measure the efficiency of IST in genetic differentiation. Using two sets of experiments in Brassica representing four species, and the enzyme esterase, it was shown that IST based on esterase alone was quite efficient in identifying the base genetic groups. The results confirm Prof. J. B. S. Haldane's vision that integration of biochemical and genetic concepts on properly designed experiments, logically conceived data and analysis will provide novel avenues for innovative methods of improvement.

FROM a biochemist to a maverick philosopher and celebrated population geneticist, Professor J. B. S. Haldane is a legend in Science. His keen perception and innovative approaches to experimental inferences vitalized the field of population genetics. For instance, Haldane noted as far back as 1932 that selection of extremes for traits governed by single diallelic gene will result in a progressive increase and hence a preferential selection of recessives over time¹. He could also visualize the synergistic strength of multi-disciplinary data in drawing comprehensive inferences. At that time he felt that 'it is not unreasonable to expect that enzymes will be found among the immediate products of gene action. A still further speculation is that the process by which genes produce their immediate products is the same as that by which they reproduce themselves.'²

No doubt his speculations have taken a strong shape in the recent past. Biochemistry and molecular biology have provided some positive leads to predictive plant breeding. However, projected progress using some of them like, for instance, quantitative trait (QT) improvement using RFLP markers, can admit of a critical review³.

QTs are known to be under polygenic control. They show continuous variation. The many genes controlling

QTs usually have small main but significant interaction effects. Genotype \times environment interaction is a rule in the expression of QTs. Yet the use of isozyme markers in genetic characterization has been well-experimented and documented^{4,5}. However, if a meaningful association is sought between markers and QTs, the markers must (i) have a phenotypic expression showing continuous or quantitative or quantifiable variation, and (ii) be responsive to environment. Isozymes answer such requirements in many respects compared to DNA markers (Table 1).

Criticisms on isozymes as markers centre generally around the following: (i) appearance of bands on gels can be influenced by several factors which prevent accurate assessment of band homology among genotypes; for instance, enzymes coded by different genes may have similar mobilities and may produce overlapping bands, while artifacts of the extraction procedure such as influence of oxidized phenols on proteins can also change band number; (ii) the non-genetic approach to characterization renders isozyme markers inefficient in breeding and genetic studies⁶.

Yet, for a relative evaluation of genetic potential, the above criticisms are less important and a genetic approach to characterization renders isozyme markers efficient in breeding and genetic studies. Enzyme bands specified by allelic genes (allozymes) can be distinguished from those specified by non-allelic genes, biochemical artifacts can be detected and the genetic basis

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Table 1. Some desired properties of isozyme markers contrasting DNA markers

Isozymes	DNA markers
Deal only with coding DNA	Deal with entire DNA (~90% junk DNA)
Relevant to gene expression (at polypeptide level)	Randomly cleaved/synthesized nucleotide chain not relevant to gene or its functional products
Environment sensitive	Environment irrelevant
More representative of genetic variation	Little relevant to genetic variation
Association with QTs conceivable	Association with QTs far fetched

of band patterns have also been described⁷. Further, the efficiency of genetic characterization of parents by isozyme markers can be checked by an analysis of F₁ progeny and F₂ segregation patterns. Isozyme data generation can be standardized to be fit for relative evaluation by choosing the most suitable plant tissue, the method and appropriate stage of sampling, the enzymes that have high differentiation potential and the associated protocols to obtain distinct band patterns. Once this standardization is completed and facilities for isozyme assay of large number of samples are set, isozyme data can be obtained with as much ease as QT data.

In a project on isozymes as biochemical markers for germplasm characterization and genetic enhancement in Brassicas, such isozyme data based on a few enzymes were generated. As a first step electrophoretic gel patterns were parametrized to give five basic QTs – number of bands, relative mobility, standard error (relative mobility), relative absorption, standard error (relative absorption)⁸, to be denoted by IST hereafter. The gel patterns were deciphered and the mobility and absorption were estimated accurately by a laser densitometer using a standard Gelscan XL software. This step is as important as setting five parameters, two of which (standard errors of relative mobility and relative absorption) were new and not used in such studies. The two parameters take into account intra-varietal (plant to plant) and environmental (among replications, seasons, locations) variation. The five isozyme parameters thus acquire a status equal to QTs. The two standard error parameters place the generally overlooked degrees of mobility and densities of seemingly similar bands of various genotypes in a plane amenable for genetic interpretation. On this plane, isozyme data collected on genotypes raised in a replicated field design on samples of plants from each plot qualify to be conceived as co-varying with a set of QTs. This conceivable correlation between isozyme data and QTs defining plant yield or performance is the base on which isozymes derive strength to predict plant performance and select segregants. This covariation of isozyme QTs with morphological traits is thus a logical derivation directly contrasting the articu-

lated association of static and invariant DNA marker data with extremely dynamic and environment-responsive QTs including yield or its components.

This paper is restricted to evaluating this proposition using data on esterase isozyme from some accessions of Brassica species. Without elaborating methods of analysis⁸ the relative performance of the accessions has been assessed and discussed.

Material and methods

Application of standard procedures for esterase analysis

Seed germination. 50–100 seeds of each accession were washed in distilled water thrice and allowed to imbibe water for 30 min. These seeds were then germinated on filter paper in sterile petri plates in a controlled environment chamber. Seedlings were grown in a seed germinator in dark in a temperature of 37°C maintaining relative humidity at 80%. There was complete germination in general; in some cases, it was approximately 80%. Seven-day-old seedlings were used for extraction.

Extraction procedure. Ten seedlings including roots were homogenized using cold pestle and mortar, kept in ice, in ice cold extraction buffer. The buffer was 50 mM Tris-HCl, pH 7.4 containing 0.05 M ascorbic acid. The crude homogenate was then centrifuged at 12000 rpm for 15 min at 4°C to clear the cellular debris. The supernatant was of pale yellow colour and clear. The supernatant was transferred to new eppendorf tubes, aliquoted and stored in a refrigerator for up to an hour at the most.

The proteins were separated by electrophoresis on polyacrylamide gel using standardized parameters of temperature and power supply on a series of vertical dual gel electrophoresis equipment connected to water circulated cooling system. The gels were exposed to esterase substrate (α naphthyl acetate) and standard protocol procedures were followed for staining and fixing gels. The gels were scanned on a laser densitometer (Pharmacia LKB, Ultrosan XL) for precise data of absorption at each band.

Data analysis

Data generated on the parameters, number of bands, relative mobility (RM) and relative absorption (RA) by Gelscan XL software were in 'DIF' format. They were retrieved and used in computing standard errors of RM and RA utilizing a software developed in the project (Arunachalam 1995, unpublished).

Before assessing the merit of the five isozyme parameters in assessing the performance of test entities, one must define unequivocally, the term

'performance'. Here, the performance of varieties has been defined by a set of QTs related to yield – namely – number of primary branches, number of secondary branches, yield per primary branch, yield per secondary branch, grain : shoot ratio and days to 50% flowering. These six QTs (CQT) were used jointly to index the base performance of varieties against which the potential of IST was evaluated. The six QTs were earlier identified on a stepwise multiple regression of plant yield on various QTs to have a coefficient of determination of about 90%.

Two methods were used to order the diverse performance of the accessions:

Multivariate mean differentiation (MMD). In this method, the performance of each variety was defined by 'k' traits. Based on (i) a performance score after lsd grouping, (ii) multi-trait differences tested by Hotelling's T^2 and (iii) a 'performance index' (PI) assigned on (i) and (ii)⁹, the varieties were ranked on their descending order of performance.

Multivariate divergence pattern (MDP). Multivariate distance statistic, D^2 has been widely used in assessing genetic divergence between varieties. The D^2 values between pairs of varieties were aligned in four divergence classes (DC1 to DC4) following the method developed by Arunachalam and Bandyopadhyay¹⁰ where, it was also suggested that the frequency of heterosis would be high when parents of crosses were preferentially selected from intermediate divergence classes (DC3 and DC2). Based on these concepts, a score of +1 for D^2 comparisons falling in DC3 and DC2, 0 for those in DC1 and -1 for those in DC4 were allotted. The total of the scores across characters provided 'alignment index' (AI) for each variety.

The PI based on multivariate mean test and AI based on genetic divergence comparison were set for each variety for CQT and IST separately. A commonality index was developed to assess the potential of IST in correctly identifying the performance given by CQT.

In general, it cannot be expected that an identical ranking of varieties will result (based on any of the two methods) on CQT and IST. However if an objective grouping can be made, it is possible that there would be an extent of commonality in the composition of groups obtained on CQT and IST. Therefore the varieties were set in three groups based on descending order of performance.

Group	MMD	MDP
	Genotypes occurring in	Genotypes with [®]
I	0–25%	$AI \geq (m + s)$
II	26–75%	$(m - s) \leq AI < (m + s)$
III	76–100%	$AI < (m - s)$

[®]: Order based on ranked performance indices; AI: Alignment index; m: mean AI; s: standard error AI.

The efficiency of the methods was checked in two sets of experimental material in Brassica.

Experiment 1. On 12 genotypes representing the species, *B. campestris* (AA), *B. juncea* (AABB), *B. napus* (AACC) and *B. carinata* (BBCC) and was repeated twice.

Experiment 2. On 41 genotypes representing the same four species with more number of entries per species.

Thus adequate variability between and within Brassica species was selected in the two experiments. This will enable one to examine whether isozyme markers could mark variation at inter- and intra-species levels, convincingly.

Results and discussion

Striking results were obtained from the two experiments. The most salient ones are summarized and discussed in the light of recent literature.

The variability in Experiment 2 was higher than in Experiment 1 though the two experiments consisted of the same four species. Such differences are common with most QTs of interest in breeding experiments and methods developed must be applicable across such experimental noise to provide comparable results.

The commonality index of IST on multivariate mean differentiation, was 2/12 in Experiment 1 and 16/36 in Experiment 2. But commonality index on multivariate divergence pattern, improved to 4/12 in Experiment 1 and more substantially to 23/36 in Experiment 2. The results are striking particularly in the context that this study is based on only one enzyme, esterase. The efficiency, on logic, is expected to be higher when data on more isozymes are included.

So far in the published literature gel patterns are analysed on simple differences in means over the presence or absence of bands and at times, on the thickness of bands generally interpreted as allelic dose effect. A scoring process on the presence or absence of bands (and such criteria in other cases too) is used in a parsimony or dendrogram analysis for interpretation. They do not take into account environmental interaction in the expression of markers like isozymes used in this study. On the other hand, molecular markers like DNA, RNA, etc. do not vary with environment, leading one to question the logic of assuming a possible association between molecular markers and expressed QTs. The premises on which isozyme variation is used in this study are strong to conceive such an association. The published reports on the use of isozyme markers in genetic differentiation^{11,12} converge to the same conclusion though the analysis was based on traditional dendrogram comparisons.

The results of this study provide a valid method of first stage selection, say, of F₂ segregants in the laboratory. A large population can be screened on isozyme data and only those suspected to differ in the desired direction from parents grown in field up to maturity. This would be time- and resource-efficient and cut breeding costs. Further data on a few isozymes selected to optimize efficient differentiation can be used for even isozyme fingerprinting of genetic variation, after establishing their stability across environments.

We believe to have thus provided an evidence for Prof. J. B. S. Haldane's vision that biochemical data used on judicious logic and appropriate methods of analysis can contribute to the quality of genetic inferences.

In conclusion, we recall Haldane's remarks¹³ which are relevant even today: 'We see that a geneticist cannot possibly neglect biochemistry. I hope that I have also shown that the study of genetics is not without value to the biochemist.' It is time that logical planes of interaction between biochemists, molecular biologists, geneticists and plant breeders are carved for new concepts to stay against the swift current of increasing sources of dynamic variation.

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