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Biochemical genetic markers in sugarcane

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Summary. Isozyme variation was used to identify biochemical markers of potential utility in sugarcane genetics and breeding. Electrophoretic polymorphism was surveyed for nine enzymes among 39 wild and noble sugarcane clones, belonging to the species most closely related to modern varieties. Up to 114 distinct bands showing presence versus absence type of variation were revealed and used for qualitative characterization of the materials. Multivariate analysis of the data isolated the *Erianthus* clone sampled and separated the *Saccharum spontaneum* clones from the *S. robustum* and *S. officinarum* clones; the latter two were not differentiated from one another. The analysis of self-progenies of a 2n=112 *S. spontaneum* and of a commercial variety showed examples of mono- and polyfactorial segregations. Within the progeny of the variety, co-segregation of two isozymes frequent in *S. spontaneum* led to them being assigned to a single chromosome initially contributed by a *S. spontaneum* donor. This illustrates how combined survey of ancestral species and segregation analysis in modern breeding materials should permit using the lack of interspecific cross-over to establish linkage groups in a sugarcane genome.

Key words: Sugarcane – *Saccharum* – Isozymes – Taxonomy – Linkage group

Introduction

Genetic markers provided by natural molecular variation are more and more used in plant genetics and breeding. In this field, sugarcane is lagging far behind the other crops of comparable economic importance. This is due to its genetic complexity. Modern sugarcane varieties result from interspecific hybridization and may contain more than 100 chromosomes contributed by up to five different species (Heinz 1987). The largest number of chromosomes (>80) was contributed by *Saccharum officinarum*, itself commonly thought to be an octoploid with 10 as a basic chromosome number (Roach and Daniels 1987). Most others were contributed by a few *S. spontaneum* parents (Walker 1987). Isozyme variation has been little used, although it was first discussed as early as 1969 (Heinz 1969). Taxonomic inferences were restricted to the confirmation of the genus *Saccharum*, as encompassing *S. officinarum*, *S. spontaneum*, *S. robustum*, *S. sinense*, and *S. edule* (Waldron and Glasziou 1971). Practical application has been basically limited to the test of the identity of varietal materials and the verification of the hybrid origin of offsprings after a cross. More recently, the use of Restriction Fragment Length Polymorphisms (RFLPs) has been initiated with a ribosomal DNA (rDNA) probe, to differentiate between *S. sinense* and *S. barberi* (Wood 1987). As part of an effort to identify molecular genetic markers usable in sugarcane breeding, we surveyed isozyme variation in a sample of wild and noble clones, representing the species thought to be the closest relatives of the modern commercial clones. Due to the high ploidy level of these materials, a definite genetic interpretation of the variation is not yet available. However, the scope for practical applications can already be illustrated.

Materials and methods

The materials used to survey diversity consisted of noble canes *S. officinarum* and wild canes *S. robustum*, *S. spontaneum* and *Erianthus* sect. *Ripidium*. The noble canes were from the IRAT working collection in Guadeloupe and the wild canes were pro-

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Table 1. Materials surveyed for all enzymes

Species and name	Origin	Chromosome no.
S. officinarum	Indonesia, New Guinea	80
Batavia		
Biscuit		
Congo		
Crystallina		
Formosa		
Gua 871		
Lousier		
S. robustum	Indonesia, New Guinea	
IJ 76-547		76 (1)
IM 76-234		
IS 76-138		60 (2)
MOL 4503		60 (3)
NG 57-238		
NG 77-21		60 (2)
NG 77-54		96 (2)
NG 77-94		100 (1)
NG 77-107		60 (2)
NG 77-108		
NG 77-122		98 (2)
NG 77-136		88–92 (2, 1)
NG 77-148		80 (1)
NG 77-230		80 (1)
US 57-159-20		
S. spontaneum		
Co local	India	
SES 14	India	64 (4)
SES 91	India	96 (4)
SES 178	India	64 (4)
SES 197A	India	60 (4)
SES 264	India	60 (4)
SES 278	India	64 (4)
SES 289A	India	54 (4)
SES 317	India	56 (4)
US 56-19-3	Thailand	
IK 76-67	Indonesia	
IK 76-86	Indonesia	
Pasoeroean	Indonesia	112 (5)
Formosa 4	Taiwan	
Tainan 96	Taiwan	96 (2)
Isiolo	Kenya	124 (6)
Erianthus		
IK 76-48	Indonesia	

(1) Sreenivasan and Sreenivasan (1984); (2) WICSCBS (1981); (3) Daniels and Roach (1987); (4) Panje and Babu (1960); (5) Daniels et al. (1980); (6) Sreenivasan and Jagathesan (1975)

vided by the West Indies Central Sugar Cane Breeding Station, Barbados. Isozyme variation was surveyed for nine enzymes on a variable number of clones, depending on the enzyme. A set of 39 clones, including representatives of all species (Table 1), was assayed for all enzymes. Three plants were analysed for each clone. A second batch of materials was used for preliminary studies of isozyme segregations. It consisted of self-progenies of the *S. spontaneum* clone, Pasoeroean, and of the commercial variety R570. The Pasoeroean materials (37 clones) were produced by IRAT in Guadeloupe. The R570 materials (21 clones) were provided by the Centre d'Essai, de Recherche et de Formation (CERF), La Réunion.

Isozyme analyses were conducted at the Laboratoire d'Analyse du Génome des Espèces Tropicales (AGETROP) of CIRAD. Enzyme extraction was practised on the blades of just-expanded leaves collected from one-month-old plants, for all enzyme systems except for one kind of esterase (EST A, see below), which was extracted from the non-chlorophylliferous part of the expanding leaf. The procedures for enzyme extraction were as described by Feldmann (1985). Four electrophoretic migration systems were used: Al, A2 (polyacrylamid gels) and S1 (starch gel) were those described by Feldmann; S2 was a TRIS-Histidine pH 8.0 starch gel as described by Second (1982). Enzyme staining was practised as advised by Feldmann (1984).

The Factor Analysis of Correspondences (FAC) method (Benzecri 1973) was used to obtain a synthetic picture of the organization of molecular variation. This method treats qualitative data. Each clone is taken as a taxonomic unit, and each isozyme band is considered as a character, with presence and absence as the two possible modalities. The FAC identifies several independent axes (eigenvectors) that account for the largest part of the variation. These axes are linear combinations of the characters, and each clone can be located along the axes.

Results

Molecular diversity

Most enzymes revealed much variation. Examples of banding patterns are given in Fig. 1. A diagrammatic representation of all the patterns observed and their occurrence in the various species samples are given in Fig. 2.

Aminopeptidase (AMP). The AMP were revealed with several substrates, namely, Leucyl-B Naphthylamide (L), Valyl-B Naphthylamide (V), Arginyl-B Naphthylamide (Ar), and alanyl-B Naphthylamide (Al) (Fig. 1 a). The complete set of bands obtained with L could be split into four types, according to their intensities with the various substrates. Only the faster bands displayed usable variation in our electrophoretic conditions. Two types could be distinguished: (1) those which displayed the same activity with both L and V, little activity with Al, and no activity with Ar, and (2) those which appeared most intense with L, faint with V and Al, and absent with Ar. Eleven and ten bands, respectively, were present for some clones and absent for others, and the relative intensities of the bands were repeatable. Only the presence versus absence type of variation is shown in Fig. 2 a1 and a2. For both types 1 and 2, variation was very restricted within *S. officinarum*, whereas it was very high in *S. robustum* and *S. spontaneum*.

Amylase (AMY). AMY zymograms (Fig. 1 b) were composed of major repeatable bands and of fainter "secondary bands", which appeared inconsistent upon the various repeats of the analyses. The latter bands are in

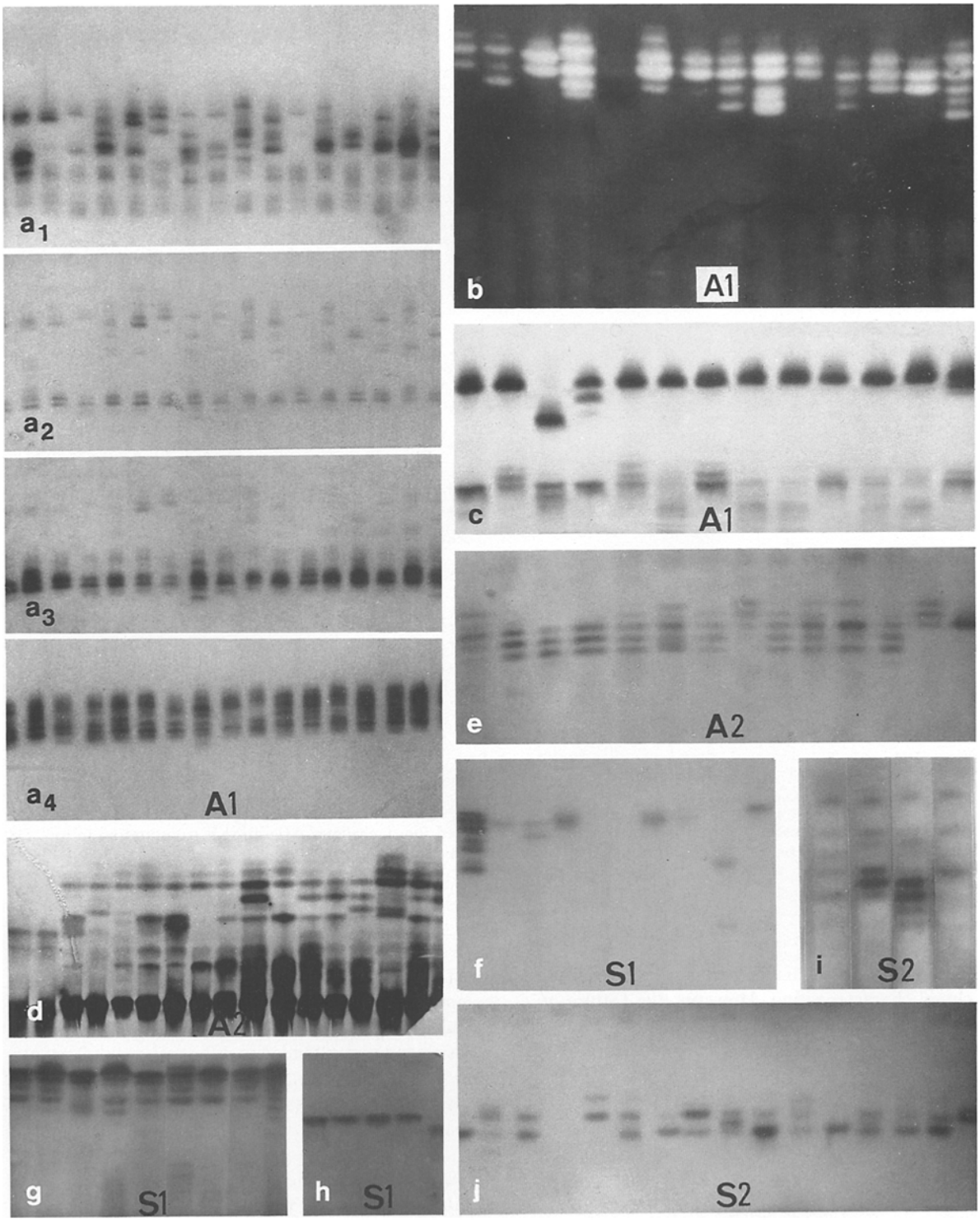

Fig. 1 a–j. Isozyme variation among sugarcane clones: **a1** L-AMP; **a2** V-AMP; **a3** Al-AMP; **a4** Ar-AMP; **b** AMY; **c** GOT; **d** POX; **e** EST A; **f** EST B; **g** MDH A; **h** MDH B; **i** ADH; **j** PGI. Four electrophoretic migration systems were used (A1, A2, S1, S2 – see text). Migration is from bottom to top

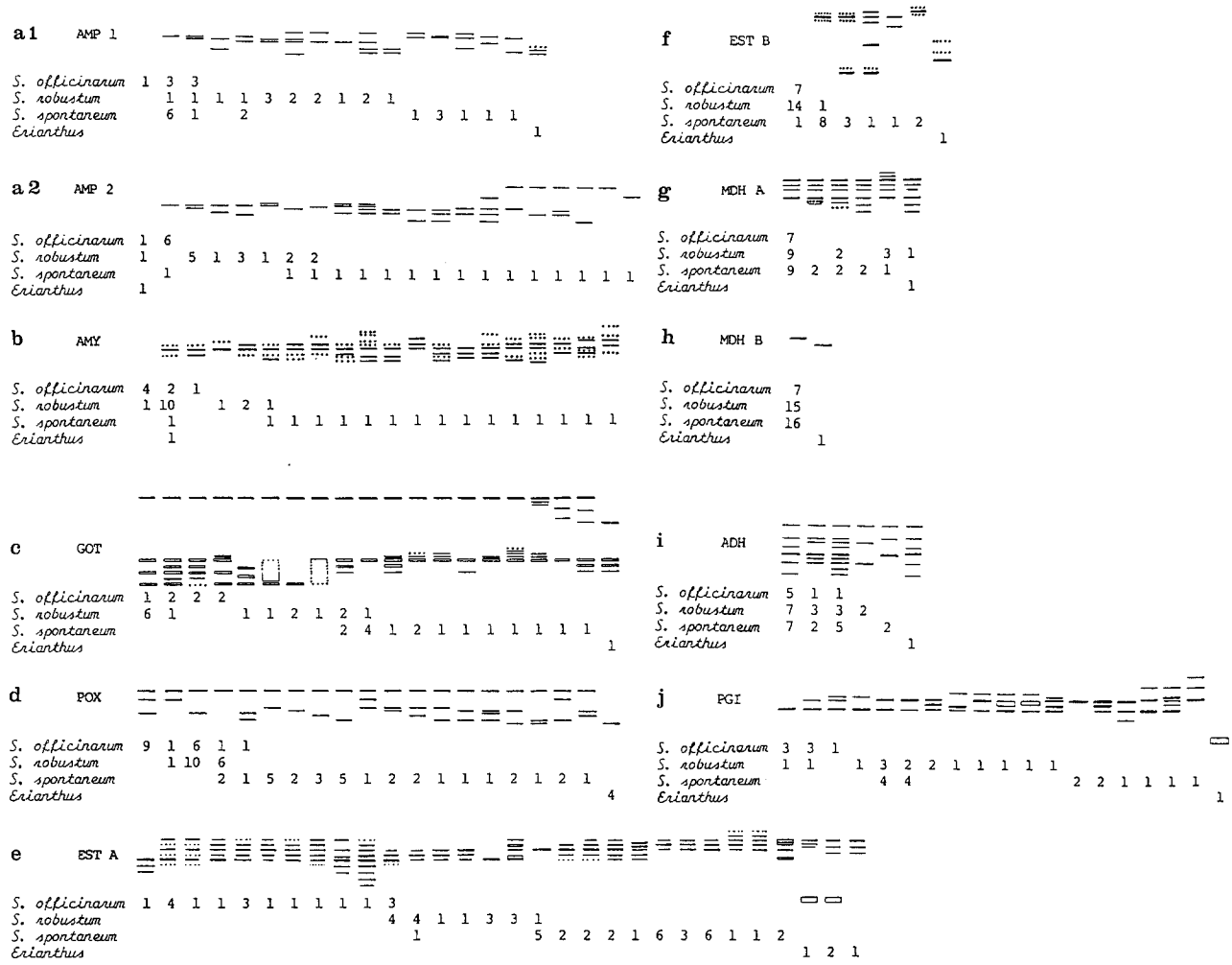

Fig. 2a–j. Diagrammatic representation of the various zymograms observed for all enzymes. Migration is from bottom to top. The *dotted lines* correspond to secondary bands. The numbers below the zymograms indicate their occurrence in the various species samples surveyed

dotted lines in Fig. 2b. Such inconsistency hampered accurate clonal characterization. However, the feature of the variation of the major bands made it useful for interspecific comparisons. Variation was low in *S. officinarum* and *S. robustum*, and high in *S. spontaneum*.

Aminotransferase (glutamate oxaloacetate transaminase: GOT). GOT zymograms were composed of two regions (Figs. 1c and 2c). The faster migratory region displayed simple variation, with a total of five types of zymogram. A single-banded type characterized all *S. officinarum* and *S. robustum* clones and most *S. spontaneum* clones. Three three-banded zymograms were also found in *S. spontaneum*. Another single-banded type characterized the four *Erianthus* clones tested. The second region, closer to the origin, was more complex. Most zymograms displayed discrete bands, and might correspond to patterns produced by dimeric enzymes coded by multiple alleles (commonly found in plants for GOT), sometimes with unequal dosages; two clones displayed smears. Variation in this second region tended to differentiate the *S. spontaneum* clones from the *S. officinarum* and *S. robustum* clones, the latter two often displaying the slower migrating bands.

Peroxidase (POX). The POX zymograms displayed repeatable variation in the region of faster migration (Fig. 1d). Eight variable bands could be identified (Fig. 2d). The presence versus the absence of the bands was repeatable, while the distribution of the intensity of the bands was not. *S. officinarum* and *S. robustum* clones mostly shared three bands, whereas *S. spontaneum* was more variable. The four *Erianthus* clones tested displayed a single characteristic pattern.

Esterase (EST). The EST isozymes from the non-chlorophylliferous part of expanding leaves (EST A) were resolved in polyacrylamid gels. Many thin bands were observed (Fig. 1e). Their relative intensity was repeatable, so that in many cases, several types of zymograms could be distinguished, although they consisted of the same migratory bands (Fig. 2e). Most zymograms appeared interpretable as resulting from the dimerisation of multiple protomers present in variable proportions. As a rule, *S. robustum* displayed slower bands than *S. spontaneum*. *S. officinarum* clones displayed bands with a wider range of migration distances. The four *Erianthus* clones displayed a pattern organization of the fast migratory region comparable to that of the *Saccharum* clones, although with specific levels, and three of them displayed an additional very slow thick band.

The EST isozymes from green leaf blades (EST B) were resolved in starch gels. They appeared as a set of red bands (Fig. 1f), which correspond to enzymes more active with the B-form of the naphthyl acetate substrate. Seven major bands could be identified (Fig. 2f), which were sometimes accompanied by slightly slower or faster and less intense secondary bands. All *S. officinarum* and most *S. robustum* clones displayed no band, whereas most *S. spontaneum* exhibited one or several bands. The *Erianthus* clone displayed a specific band.

Malate dehydrogenase, NAD dependent (MDH A). MDH A zymograms (Fig. 1g) were classified into six types (Fig. 2g). A single zymogram characterized most *Saccharum* clones. Among the other five, one displayed two additional fast bands and four displayed two additional slow bands. The banding patterns suggested that MDH A are dimeric enzymes.

Malate dehydrogenase, NADP dependent (MDH B). MDH B zymograms consisted of a single band (Figs. 1h and 2h). Two types were found, one characterizing all the *Saccharum* clones, the other, with the slower band, characterizing the *Erianthus* clone.

Alcohol dehydrogenase (ADH). ADH zymograms (Figs. 1i and 2i) in green leaves comprised two variable zones and one fast band common to all clones. The slow migratory zone displayed a type of variation characteristic of dimeric enzymes, with three possible homodimers and interaction heterodimers at intermediate migration distances. The second migratory zone could be deduced from the first zone, as corresponding to the heterodimers of interaction between the first zone and the faster band. The relative intensities of the bands were found to be slightly variable upon the repeats. When the non-chlorophylliferous part of leaf was used, only zone 1 appeared, with different band intensities.

Phosphoglucose isomerase (PGI). PGI patterns showed much interclonal variation (Fig. 1j). A total of 19 patterns could be tentatively distinguished (Fig. 2j). *S. officinarum* displayed only three types, whereas *S. robustum* and *S. spontaneum* were quite variable. The *Erianthus* clone had a specific pattern.

Global pattern of interclonal variation

Each enzyme taken individually brings about particular information as to the mutual interrelationships between the various species samples surveyed. A synthetic view of these relationships was obtained using multivariate analysis. A FAC was performed on the matrix (clones × isozyme bands). For the GOT of clones NG 57-238 and NG 77-230, which appeared as smears, the smear was considered as the sum of all the bands observed in the same region among the other clones.

A total of 114 isozyme bands were considered, which made 228 possible modalities. Axes 1, 2, and 3 of the FAC accounted for 13.3%, 12.0%, and 7.0% of the whole variation, respectively. The distributions of the clones along axis 1 and in plane (2, 3) are shown in Fig. 3. The distribution along axis 1 isolated the *Erianthus* clone from the *Saccharum* clones. The distribution in plane (2, 3) separated the *S. spontaneum* clones from the *S. robustum* and *S. officinarum* clones. The latter two species had very overlapping distributions, while that of *S. officinarum* was much more restricted.

A second FAC was performed after discarding the putative interaction heterodimeric bands for GOT, EST A, ADH, and MDH. It yielded interclonal variation patterns very similar to those of the first FAC.

Segregation studies

Segregations were studied among 37 plants of the selfed progeny of the *S. spontaneum* clone Pasoeroean for AMP type 1, AMY, GOT, EST B, PGI, ADH, and MDH. There was no variation for GOT, ADH, and MDH. There was variation for AMY, but no homogeneous classes of zymograms could be distinguished, which suggested complex inheritance. There was discrete variation for AMP, EST B, and PGI.

For AMP type 1, the parental clone displayed two bands, the slower one being more intense than the faster one. Both bands segregated (Fig. 4a). Out of the 37 plants tested, the slow band was absent in 3 cases, whereas the fast band was absent in 9 cases. The relative intensities of the two bands were variable among the plants which possessed both, this most likely reflecting different dosages of the genes.

For EST B, two bands could be observed (Fig. 4b): the slow band displayed an unambiguous absence (11 cases) versus presence (26 cases) variation, compatible

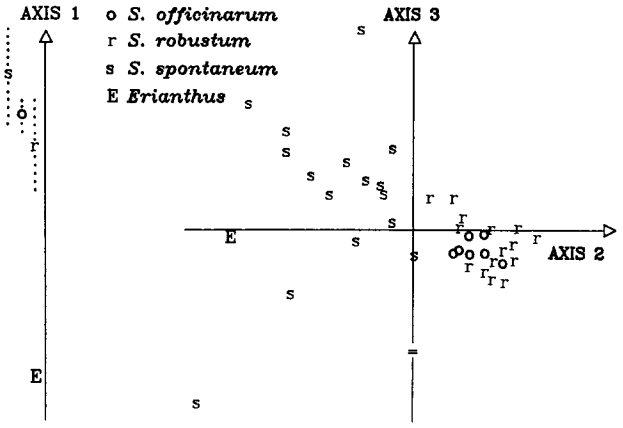

Fig. 3. Distribution of 39 sugarcane clones along axis 1 and in plane (2, 3) of a factor analysis of correspondences of isozyme variation

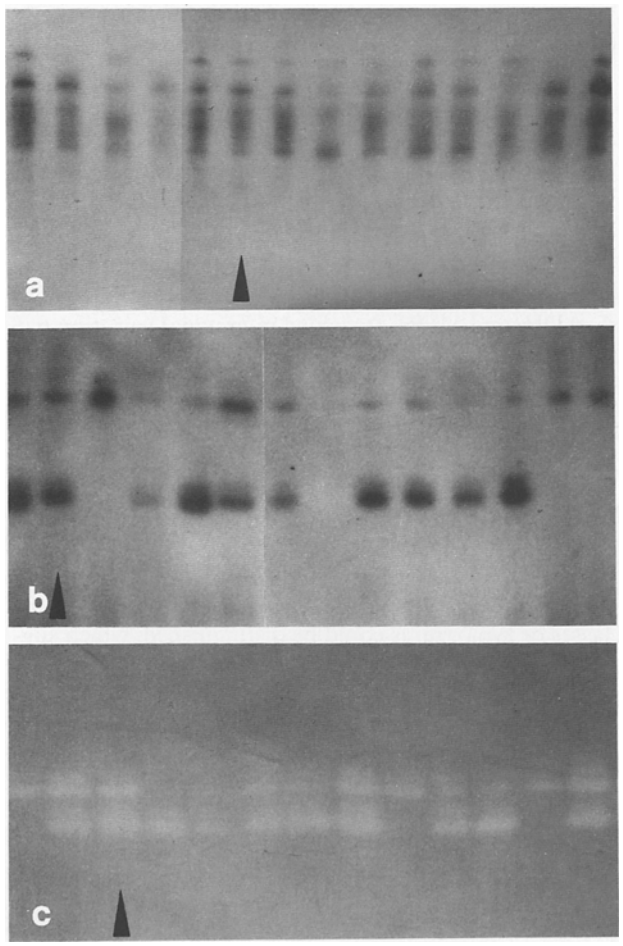

Fig. 4a–c. Isozyme segregations in self-progenies: **a** AMP 1 for *S. spontaneum* clone Pasoeroean; **b** EST B for *S. spontaneum* clone Pasoeroean; **c** AMY for variety R570. The marks show the parental zymograms

with a monogenic segregation; the fast band displayed a continuous intensity distribution, making it impossible to distinguish between absence and presence of the band.

PGI zymograms displayed absence versus presence type of variation only for the slowest band. Eight cases of absence were recorded among the 37 plants, which suggests it might be controlled by a single gene.

Segregations were also studied among 21 plants obtained by selfing variety R570. EST A and AMP showed continuous variation in the relative intensities of the bands, making it impossible to distinguish discrete types. There was clear-cut variation for AMY and POX.

For AMY (Fig. 4c), the slower major band was absent in 8 plants and present in 13 plants. The faster band showed continuous variation.

For POX, two bands segregated in a presence versus absence way. The faster one was absent in 6 plants and present in 15 plants. The slower one was absent in 8 plants and present in 13 plants; these two sets were the same as those for the slower band of AMY. There was, thus, complete cosegregation of the two isozymes.

Discussion

Molecular variation in the sample surveyed is very extensive. The electrophoretic banding patterns usually consist of multiple bands of unequal intensities. This is consistent with the high level of polyploïdy present in the various groups of sugarcanes; a given locus exists in many copies in the genome, many alleles can coexist in the same plant, and their dosages may be very different. This brings about practical difficulties in characterizing the clones, due to the high number of bands which may migrate at close distances, and to the occurrence of faint bands when the corresponding allele has few copies in the genome. Thus, there is a risk of oversimplification when drawing the diagrams and some information may be lost. Despite such restriction, the method proves valuable both for use in taxonomic studies and as a source of marker genes.

Molecular variation in our sample permitted good differentiation among the species surveyed. The *Erianthus* clone was strongly differentiated from all *Saccharum* clones; it displayed a unique pattern for most enzymes. The *S. spontaneum* clones were separated from the *S. robustum* and *S. officinarum* clones; many markers were found only in *S. spontaneum,* viz., some POX, AMY, or EST B bands, and there were strong frequency differences for others, viz., EST A and GOT bands. Only *S. officinarum* and *S. robustum* were not separated from one another by the multivariate analysis. The distribution of *S. officinarum* was included in that of *S. robustum*. This supports the current hypothesis that *S. robustum* is the closest relative of *S. officinarum* (Roach and Daniels

1987). The *S. robustum* clones displayed markers absent from *S. officinarum* mainly for AMP, PGI, and ADH. It is noteworthy, however, that some *S. officinarum* clones displayed GOT and EST A bands, absent from the *S. robustum* sample surveyed but present in the *S. spontaneum* sample. Going further into comparisons among the various species to draw taxonomic and evolutionary inferences would require survey of a much wider sample of clones. Despite the limitation of our sample, it can be anticipated that isozyme variation will be a very helpful criterion for broadening the genetic base used in breeding modern varieties.

The segregation studies illustrate the use of isozymes as genetic markers. The analysis of the self-progeny of Pasoeroean (2n = 112) showed examples of monogenic segregations. The system of AMP type 1 suggests the possibility of associating presence versus absence segregation records and gene dosage effects, in order to investigate the patterns of inheritance in sugarcane. Use of dimeric enzymes would facilitate the analysis because it amplifies the dosage effects. EST A isozymes may constitute an efficient system, since they are probably dimers and have multiple alleles. The analysis of the self-progeny of R570 was practised on a small number of plants. However, three probable monogenic segregations were uncovered; all three concerned markers specific to or most frequent in *S. spontaneum*. It is, therefore, likely that their segregation is related to the segregation of a chromosome initially contributed by a *S. spontaneum* parent. Two of the three bands co-segregate completely; they are, hence, most probably located on the same *S. spontaneum* chromosome. They need not be closely linked on the chromosome, since there is no recombination between the *S. officinarum* and the *S. spontaneum* chromosomes (Roach 1984).

This example highlights an efficient means for identifying linkage groups in the *S. spontaneum* genome, applicable to isozymes as well as to RFLPs. The segregation of *S. spontaneum* specific markers in progenies of commercial varieties will enable them to be assigned to various linkage groups. The optimal material would be a variety which has a single copy of each basic *S. spontaneum* chromosome; it is unlikely to be easily found. However, using a sufficient number of varieties should permit finding as many linkage groups as there are basic chromosomes. This knowledge would be a first step towards extensive use of genetic markers in surgarcane, which could open a wide range of new perspectives. A direct application derives from the possibility to monitor segregation of *S. spontaneum* chromosomes in breeding materials with a single marker on each chromosome (Roughan et al. 1971), and to try and relate it to agronomic characters.

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