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Genetic similarity among five species of *Saccharum* based on isozyme and RAPD markers

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Abstract

Genetic relationship among *Saccharum* species clones was assessed using six isozyme systems and thirteen RAPD primers. Multimeric isozyme profile and polymorphic RAPD markers reflected the genetic complexity of the genus. Clones could be distinguished from one another only based on the esterase profile though species affinities could not be detected using this system. Clonal distinction and intraspecies similarities could be understood using a combination of six isozyme systems studied. Low genetic dissimilarity index within *Saccharum* and overlapping of isozyme pattern of a few clones with other species were suggestive of continuous variation across *Saccharum* species. Species affinities as revealed through dendrogram constructed based on Phylogenetic Analysis Using Parsimony indicated closeness between *S. barberi* and *S. sinense* and between *S. officinarum* and *S. robustum* clones. Relationship between *S. spontaneum* with *S. barberi* and *S. sinense* observed through RAPD analysis supported *S. spontaneum* based origin of the species. The results of isozyme and RAPD analyses were comparable and agreed to the classically derived evolutionary affinities of the genus.

Key words: Isozyme, RAPD, *Saccharum* species, genetic similarity, dendrogram

Introduction

Saccharum L. is a genus characterized by long crop duration of an year, with high polyploidy, heterozygosity, chromosome mosaicism, continuous morphological variation among its members and quantitative characters highly influenced by the environment. Improvement in sugarcane is largely through interspecific hybridization involving three cultivated species (*S. officinarum*, *S. barberi* and *S. sinense*) and two wild species (*S. robustum* and *S. spontaneum*), the first four being the major contributors. Interrelationship among *Saccharum* species provides essential information to estimate potential hybridization range, fertility and characters of economic importance [1]. Such studies based on morphological, cytological and ethnological parameters have given rise to several hypotheses, the widely accepted being *S. officinarum* or noble canes to have been originated from *S. robustum*, *S. spontaneum* as the most primitive species and *S. barberi* and *S. sinense* as introgression products of *S. officinarum* with other members of *Saccharum* complex principally *S. spontaneum* in India and China respectively, but the finer details are still inconclusive [2]. The advantage of molecular markers like isozymes and Random Amplified Polymorphic DNA (RAPD) in screening a large number of genotypes or accessions in a short time independent of environment has been proved in several crops. The present study was conducted by employing six isozyme systems and 13 random DNA decamer primers through RAPD on a selected set of clones from different Saccharum species to understand their interrelationship and to explore the possibility of fingerprinting *Saccharum* species clones based on isozyme polymorphism.

Materials and methods

Fourteen clones belonging to five species of *Saccharum* were taken for the study, of which eleven clones viz. 57NG110, 57NG77, Baragua (*S. officinarum*), Pathri, Saretha (*S. barberi*), Lal Khadi, Kheli (*S. sinense*), NG 77-221, IJ76-414 (*S. robustum*) and SES 515/7 and SES 594 (*S. spontaneum*) were used commonly for all the isozyme systems. These clones were selected as representative clones of each group based on a metroglyph analysis on morphological and yield and quality characters carried out on 61 clones of *Saccharum* species and hybrid clones [3]. Clones selected for Random Amplified Polymorphic DNA (RAPD) included five typical clones viz. 57NG110, Khakai, Pathri, NG 77-221 and SES 515/7 representing *S. officinarum*, *S. sinense*, *S. barberi*, *S. robustum* and *S. spontaneum* respectively.

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The enzyme systems studied were peroxidase [4], esterase [4], polyphenol oxidase [5], acid phosphatase [6], α amylase [7] and phosphorylase [7]. One gram of fresh leaf sample from young fully opened (third) leaf was ground in liquid nitrogen and extracted with 1 ml of suitable extraction buffer (0.1 M Sodium phosphate buffer (pH 7.2) was used for peroxidase, polyphenol oxidase, α amylase and phosphorylase, sodium phosphate buffer (pH 9.5) for esterase and citrate buffer (pH 5.3) for acid phosphatase respectively). The extract was centrifuged at 10,000 rpm in a refrigerated centrifuge for fifteen minutes and the supernatant served as the enzyme source for electrophoresis. The experiment was repeated, wherever necessary, to get well stained gels with good resolution of bands. DNA for RAPD analysis was extracted following the method of Walbot [8]. Young immature leaf roles were taken from the shoot tips, ground in liquid nitrogen, homogenized in grinding buffer, pellet resuspended in suspension buffer and incubated at 65°C for 20 minutes. To this 7.5M ammonium acetate was added and incubated on ice, supernatant was collected by centrifuging and DNA precipitated in alcohol and pelleted. DNA thus collected was purified through RNAse treatment, phenol:chloroform extraction and ethanol precipitation and quantified through agarose gel electrophoresis. The reaction mixture for RAPD consisted of 75ng sample DNA, 10 × Taq buffer-2.5µl, 200mM dNTPS-1µl, primer-40ng, 2.5 µm $MgCl_2$-2 µl, Taq polymerase-1 unit and made upto 20µl with sterile distilled water. Amplification was carried out on a thermal cycler (PTC 100 TM Programmable Thermal Controller, M. J. Research, Inc., USA) with initial denaturation at 94°C for 5 minutes, followed by 45 cycles of denaturation at 92°C for 1 minute, annealing at 35°C for 1 minute, and primer extension at 72°C for 2 minutes. A final extension at 72°C for 7 minutes was given at the end of the cycles and the samples were held at 4°C till retrieval [9]. 10µl of amplified product was loaded on 1.5% agarose gel and electrophoresed at 50 mAmp.

Table 1. Operon primers used in RAPD analysis and their base sequences

No.	Primer No.	Base sequence
1.	OPA 18	5' AGGTGACCGT
2.	OPB 03	5' CATCCCUCTG
3.	OPC 10	5' TGTCTGGGTG
4.	OPC 15	5' GACGGATCAG
5.	OPE 09	5' CTTCACCGGG
6.	OPF 02	5' GAGGATCCCT
7.	OPF 07	5' CCGATATCCC
8.	OPF 09	5' CCAAGCTTCC
9.	OPF 16	5' GGAGTACTGG
10.	OPF 18	5' TTCCCGGGTT
11.	OPG17	5'ACGACCGAGA
12.	OPH 07	5' CTGCATCGTG
13.	OPI 15	5' TCATCCGAGG

The gel was stained in ethidium bromide, visualized and photographed. In all, thirteen decamer primers were taken for the study. Primer number and base sequences are given in Table 1.

Scoring

Each band corresponding to an isozyme was designated using the enzyme code (PRX for peroxidase, EST for esterase, PPO for polyphenol oxidase, AMY for α amylase, PRL for phosphorylase and ACP for acid phosphatase) followed by a number indicating the relative mobility of the band. Relative mobility (Rm) is calculated as the ratio of the distance of the band from the origin to the distance of the dye front expressed in percentage [IO]. RAPD bands were designated based on their molecular weight calculated using the kilobase (Kb) ladder used as marker.

Presence of each band on the gel was scored as 1 and its absence as 0. Genetic similarity between every pair of clones studied was represented as similarity index (SI) estimated as Nei's index [11]. Dissimilarity index (DS) indicating divergence between any two samples was estimated as DS = (1-SI). Dendrogram depicting the relationship of the clones were constructed using Phylogenetic Analysis Using Parsimony (PAUP) version 3.0 [12] for inferring the phylogenics of the genus in a McIntosh IIci computer. The length of the rays of the dendrogram indicated genetic diversity of the accessions taken for analysis.

Results and discussion

Peroxidase

Thirteen *Saccharum* species clones studied showed 19 isozymes of which six viz. PRX 5, PRX 50, PRX 53 and PRX 59 were present in all the clones (Fig 1a, Fig 2a). *S. officinarum* clone 57NG110 showed a simpler peroxidase profile. Baragua varied from it due to the presence of isozymes like PRX 12, PRX 21, PRX 39 and PRX 46. These isozymes were absent in other *S. officinarum* clones but present in one or more clones of other *Saccharum* species, indicating that the clone is an atypical *S. officinarum* with genome contribution from other species its genetic organization. Zymogram pattern of Saretha and Pathri of *S. barberi* were similar and so too that of *S. sinense* clones, Khakai and Kheli suggesting intraspecific similarity.

Esterases

Leaf esterases of twelve *Saccharum* species clones were visualized as dark brown bands on a colourless background (Fig. 1b, Fig. 2b). There were a total of seventeen isozymes and the clones showed similarity with regard to four isozymes viz. EST 30, EST 83, EST 85 and EST 89. The slowest migrating isozyme, EST 24 was present only in Khakai (*S. sinense*) and

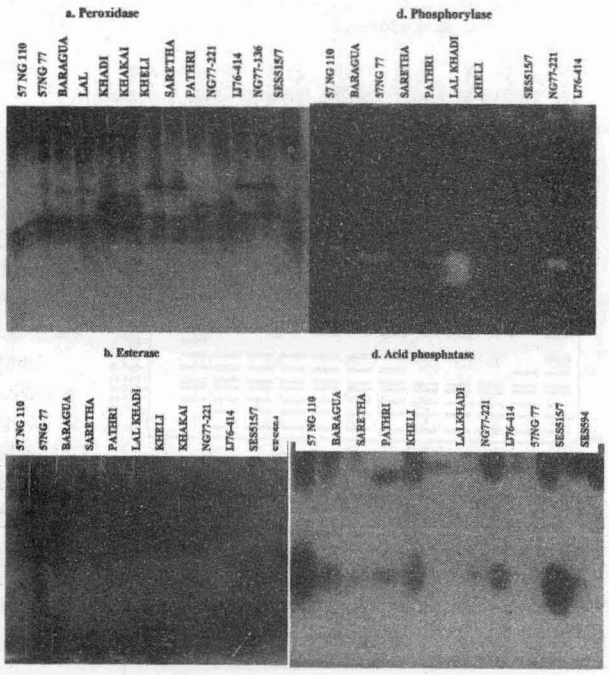

Fig. 1. Isozyme profile observed in a seven per cent polyacrylamide gel showing polymorphism among Saccharum species clones

in IJ76-414 (*S. robustum*). The typical *S. officinarum* clone 57NG110 showed a more complex esterase profile with fourteen isozymes and the other two *S. officinarum* clones studied were distinctly different due to the absence of specific isozyme markers like EST 65, EST 68 and EST 75. *S. spontaneum* and *S. robustum* clones exhibited a relatively simpler esterase profile. Baragua again varied from other *S. officinarum* clones.

Esterase pattern of all the clones studied were different from one another. Saretha and Pathri (*S. barberi*) and Khakai and Kheli (*S. sinense*) with similar peroxidase profile differed for esterases. However, esterase zymogram profile of clones did not indicate species affinities, leading to the inference that esterases may not be used in species identification in *Saccharum*. A similar conclusion was made earlier [2] where within species and between species variations were similar.

Polyphenol oxidase

Seventeen different isozymes were observed as light bands on a colourless background in thirteen *Saccharum* species clones (Fig. 2c). Isozymes PPO 1, PPO 5, PPO 9, PPO 12 and PPO 44 were present in all the clones. The absence of PPO 19 and presence of PPO 41, PPO 56, PPO 59 and PPO 63 made Baragua distinct with more similarity with *S. barberi*, *S. sinense* and *S. robustum*, in line with peroxidase profile of the clone showing its interspecific origin. Unlike esterase, the *S. officinarum* clones 57NG110 and 57NG77 had a more similar PPO profile with variation only in the PPO 73 and PPO 76 isozymes. Saretha and Pathri with similar peroxidase profile were also different with more isozymes in Saretha. The presence of PPO 73 and PPO 76 in *S. barberi* clones made them distinct from *S. sinense* clones. Similarly, *S. robustum* and *S. officinarum* were similar for most of the isozymes. In general, the clones exhibited more amount of uniformity in zymogram pattern for this isozyme system.

Acid phosphatase

Ten different isozymes were observed as reddish brown bands in eleven clones studied (Fig. 1c, Fig. 2d). Two isozymes viz. ACP 2 and ACP 70 were present in all the clones. Pathri (*S. barberi*) exhibited the simplest acid phosphatase profile. *S. spontaneum* clones were divergent from the rest of the species. Presence of an isozyme ACP 43 in Kheli distinguished it from the other *S. sinense* clone viz. Lal Khadi, while the *S. officinarum* clones 57NG110 and 57NG77 had similar acid phosphatase profile. Another clone of the same species viz. Baragua had a similar isozyme profile as that of Saretha (*S. barberi*). The presence of an isozyme ACP 53 in *S. officinarum*, *S. robustum* and *S. spontaneum* was significant considering the existing knowledge on phylogenetic interrelationship among these species [Heinz, 1987]. This enzyme system thus gave informative markers for phylogenetical studies, but clonal identification based on this system was not possible due to less diversity among the clones studied.

Phosphorylase

Phosphorylase system was relatively simple in *Saccharum* with just four different isozymes visualized as colourless bands in a dark blue background (Fig. 1d, Fig. 2e). *S. sinense* clones Lal Khadi and Kheli with four different isozymes were the most complex and with 100 per cent similarity. Baragua was simple with a single isozyme like the typical *S. officinarum* clone 57NG110 and the two *S. spontaneum* clones. *S. barberi* and *S. robustum* clones studied could not be differentiated using this system. Due to the presence of fewer isozymes and less variability among clones for phosphorylase, this enzyme might not be useful in screening species clones. However, *S. sinense* clones could well be distinguished from the other members of the genus, especially from its closest relative viz. *S. barberi*.

α amylase

Eleven *Saccharum* species clones were studied for α amylase system and the isozymes were observed as colourless bands on a blue background (Fig. 2f). Out of ten different isozymes, only one viz. AMY 6 was present in all the clones. Presence of two isozymes viz. AMY 6 and AMY 8 in *S. spontaneum* and *S. sinense* clones indicated their genetic similarity. Similar isozyme profile of *S. robustum* clones NG77-221 and

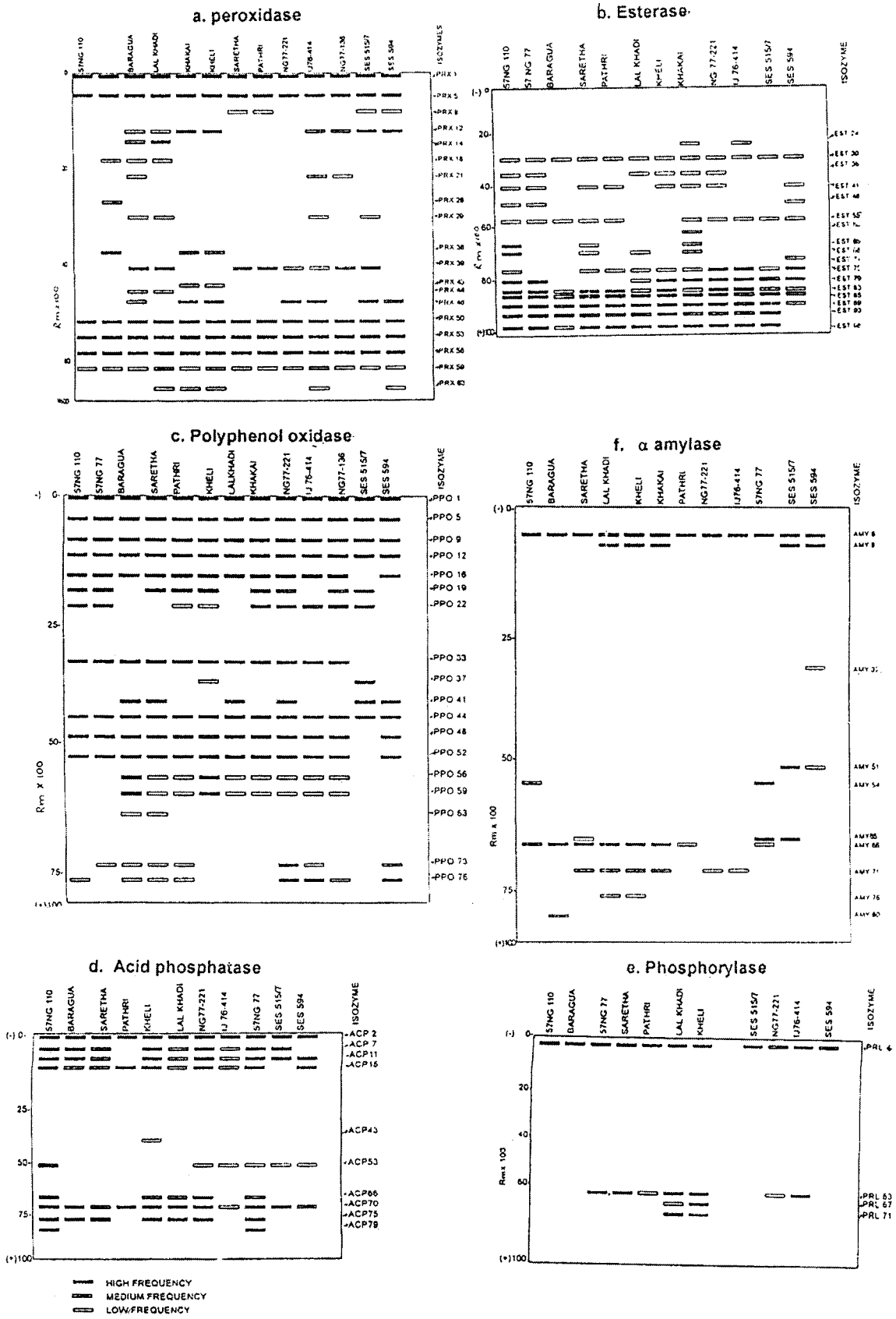

Fig. 2. Zymograms indicating isozyme polymorphism among Saccharum species clones

Table 2. Number of isozymes observed in eleven *Saccharum* species clones for six isozyme systems

Clone	Perox-idase	Ester-ase	Poly-phenol oxi-dase	Acid phos-pha-tase	α amy-lase	Phos-phoryla se	Total
57NG77	9	11	12	9	4	2	47
57NG110	6	14	12	9	3	1	45
Baragua	14	7	15	6	3	1	46
Saretha	8	11	16	6	4	2	47
Pathri	8	9	15	3	2	2	39
Lal Khadi	13	10	12	7	5	4	51
Kheli	11	10	14	8	5	4	52
IJ76-414	12	10	14	6	2	2	46
NG77-221	8	11	16	8	2	2	47
SES515/7	11	9	9	5	4	1	39
SES 594	10	10	10	5	4	1	40
Total	110	112	145	72	38	22	

Table 3. Similarity index among sugarcane species accessions based on six isozyme systems

	Similarity index										
Clone	57NG110	Baragua	Kheli	Lal Khadi	Saretha	Pathri	NG77-221	IJ76-414	SES515/7	SES 594	57NG77
57NG110	1.00										
Baragua	0.79	1.00									
Kheli	0.76	0.76	1.00								
Lal Khadi	0.61	0.78	0.78	1.00							
Saretha	0.77	0.69	0.71	0.82	1.00						
Pathri	0.75	0.73	0.71	0.77	0.84	1.00					
NG77-221	0.76	0.65	0.61	0.73	0.75	0.79	1.00				
IJ76-414	0.72	0.67	0.65	0.65	0.74	0.81	0.84	1.00			
SES 515/7	0.75	0.69	0.65	0.68	0.74	0.68	0.69	0.67	1.00		
SES 594	0.79	0.72	0.72	0.69	0.78	0.65	0.65	0.68	0.67	1.00	
57NG77	0.71	0.72	0.68	0.71	0.78	0.69	0.69	0.71	0.75	0.79	1.00

IJ76-416 and that of *S. sinense* clones Lal Khadi and Kheli were important, reflecting intraspecific relationship. However, *S. spontaneum* clones were distinct due to the presence of AMY 51 and absence of AMY 66, AMY 71 and AMY 80 and remained the most diverse species for this isozyme system.

Combined analysis of Saccharum species for six isozyme systems

Eleven clones from five species of *Saccharum* tested commonly for six isozyme systems were considered for combined analysis of genetic diversity. Number of isozymes observed in these clones is given in Table 2. Altogether 78 isozymes were observed in six isozyme system with a mean of thirteen isozymes. Multimeric peroxidase profile in *Saccharum* was a reflection of the complex genetic organization of the genus. Among *Saccharum* species clones, *S. sinense* clones viz. Lal Khadi and Kheli recorded more number of isozymes (51 and 52 respectively), while lowest number was in the *S. spontaneum* clones. More number of isozymes in these species reflected their complex genetic constitution. This was in agreement to the earlier studies that suggested a complicated genetic origin of *S. sinense* with introgression of *S. spontaneum*, *Miscanthus* and *Erianthus* in their origin [2]. Similarity index between all possible pairs of eleven clones for six isozyme systems (Table 3) showed a range of 0.61 between Kheli and NG77-221 and between 57NG110 and Lal Khadi to 0.84 between Saretha and Pathri and between NG77-221 and IJ76-414. It was also clear from the dendrogram that *S. barberi* clones and the *S. robustum* clones were the most similar. Lal Khadi showed similarity with *S. barberi*, so also 57NG110 with Baragua and SES594 with the *S. officinarum* clone 57NG77. SES515/7 was the most diverse clone. Dendrogram depicting genetic dissimilarity was shown in Fig. 3. Genetic dissimilarity between *S. officinarum* and *S. robustum* was low supporting the earlier views of origin of *S. officinarum* from *S. robustum*. Two species, *S. barberi* and *S. sinense* were close together with the lowest level of dissimilarity. However, in spite of close genetic similarity, these two species could be distinguished on the basis of isozyme polymorphism. *S. sinense* had a relatively complex genetic constitution than *S. barberi*. These two species showed relationship with *S. spontaneum* indicating its role in their evolution.

Isozyme approach is thus identified as a valuable means for distinguishing species accessions of sugarcane through determination of isozyme polymorphism. It was also observed that a single isozyme by itself would not be useful to detect the full range of variability within or among species. This study on six isozymes was probably optimum to bring out species differences as revealed through dendrograms, which also reflected the phylogenetic classification and overall similarity among the species. The study on isozymes has got a wide applicability in crops like

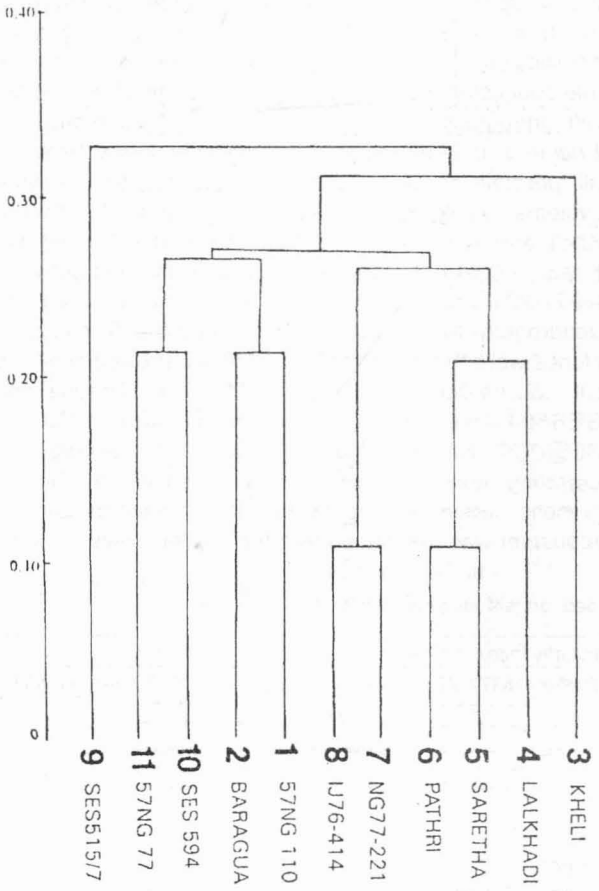

Fig. 3. Dendrogram based on dissimilarity indices for six isozyme systems

sugarcane in the effective analysis of tracing the phylogeny and evolution [13].

RAPD analysis of Saccharum species

RAPD technique revealed differences among *Saccharum* species (Fig. 4). Number of scorable markers was more in 57NG110 (*S. officinarum*) with 82 bands followed by 81 in Khakai (*S. sinense*, 79 in SES515/7 (*S. spontaneum*), 65 in NG77-221 (*S. robustum*) and 52 in Pathri (*S. barberi* with an average of 10.3 bands per primer. Out of these, 15 bands were common to all the species clones and more than 50 per cent of the bands in each clone were dissimilar between any two species, while a few bands are unique to each species clone (Table 4). Similarity index between each pair of clones for RAPD profile is presented in Table 5. Pathri (*S. barberi*) and Khakai (*S. sinense*) showed a higher similarity index (0.70) and *S. robustum* was more similar to *S. officinarum*, in line with evolutionary affinities. The most dissimilar pair was 57NG110 and

Table 4. Number of common, dissimilar and unique RAPD markers in *Saccharum* species clones

Sl. No.	Clone	Number of RAPD Markers			
		Common	Dissimilar	Unique	Total
1	57NG110	15 (18.3)	49 (59.7)	18 (22.0)	82
2	NG77-221	15 (23.0)	44(67.8)	6(9.2)	65
3	SES515/7	15 (19.0)	58 (73.4)	6(7.6)	79
4	Pathri	15 (28.8)	35 (67.3)	2(3.9)	52
5	Khakai	15 (18.5)	58 (71.6)	8 (9.9)	81

Figures in parentheses indicate percentage

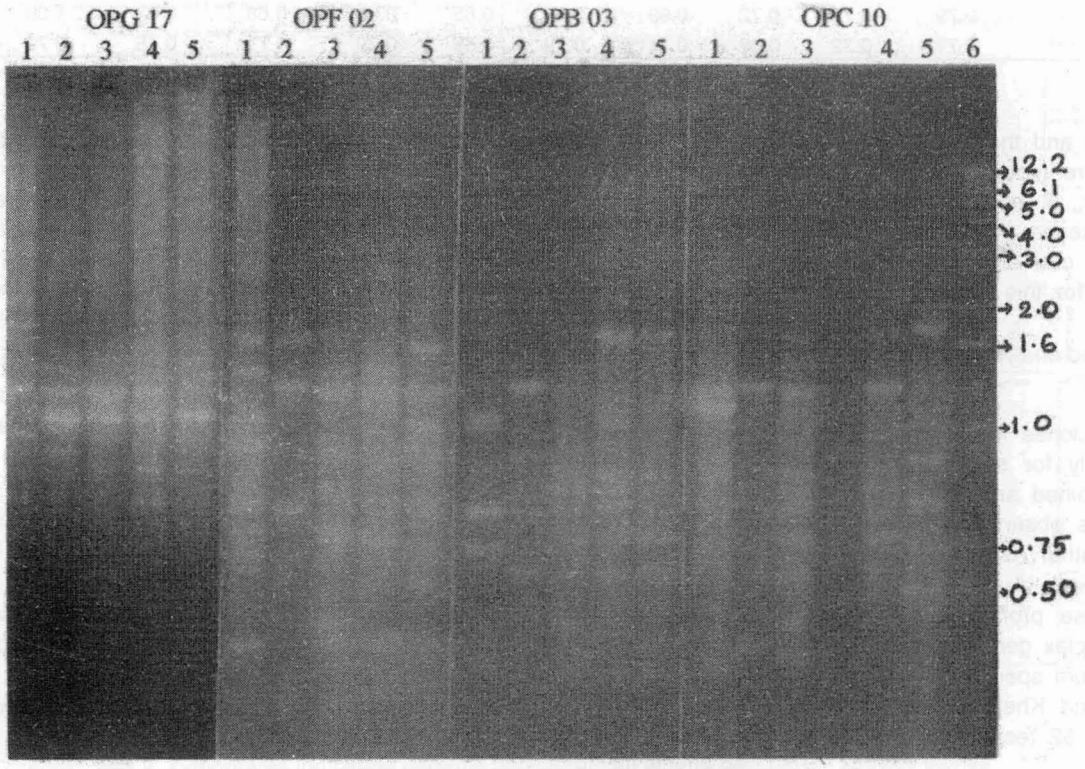

Fig. 4. Ethidium bromide gel stained 1.5 per cent agarose gel showing RAPD profile of *Saccharum* species clones based on random decamer primers Lane 1-57NG110, 2-NG77-221, 3-SES515/7, 4-Pathri, 5-Khakai, 6-Kb ladder

Table 5. Similarity indices of *Saccharum* species clones based on RAPD

	Similarity index				
Clone	57NG110	NG77-221	SES515/7	Pathri	Khakai
57NG110	1.00	-	-	-	-
NG77-221	0.66	1.00	-	-	-
SES 515/7	0.65	0.65	1.00	-	-
Pathri	0.34	0.61	0.65	1.00	-
Khakai	0.59	0.62	0.65	0.70	1.00

Pathri (0.34). Dendrogram showing the interrelationship among the clones (Fig. 5) indicated that *S. spontaneum* was the most diverse clone. This clone showed more resemblance with *S. sinense* and *S. barberi* than with *S. officinarum* and *S. robustum* clones indicating the similarity between the genomes of these species and thereby supporting the concept of *S. spontaneum* based origin of *S. barberi* and *S. sinense*. The value of RAPD approach was supported by the close similarity between the dendrograms based on RAPD results and taxonomic relationship.

Comparison of isozyme and RAPD results

The results of isozyme and RAPD studies were comparable with each other and with taxonomic and phylogenetic classification in *Saccharum* and also agreed to the previous concepts of evolution of the genus. These techniques were also found useful in quantifying the genetic diversity in the genus *Saccharum* which finds applications in applied sugarcane breeding aimed at genetic base broadening of modern sugarcane cultivars and in basic studies of elucidating the genetic architecture of *Saccharum* species.

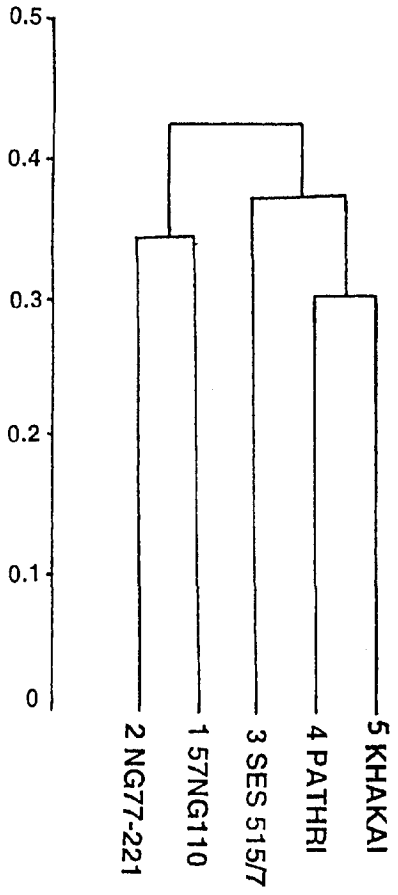

Fig. 5. Dendrogram based on dissimilarity indices among Saccharum species accessions for RAPD

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