

Assessment of Genetic Diversity within Wild Sugarcane Germplasm using Randomly Amplified Polymorphic DNA Techniques (RAPD)

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ABSTRACT. This investigation was carried out with the objective of assessment of genetic diversity within locally collected wild relatives of genus *Saccharum* using Randomly Amplified Polymorphic DNA (RAPD) techniques. Methods for speedy extraction of representative DNA, its amplification by Polymerase Chain Reaction (PCR) to reveal reproducible bands have been investigated and established. The pre-screening of sixty primers using two bulk DNA samples representing *S. spontaneum* and *Erianthus* accessions constituting forty four wild accessions used in this investigation, allowed the selection of nine primers which revealed polymorphism and reproducible banding profiles for the characterisation of all forty four accessions. The analysis of these banding profiles revealed that the genetic diversity of the investigated accessions ranged from 0% to 69.23%. It was possible to identify the genetically diverse accessions as well as duplication of similar accessions from the collection. This investigation showed that the RAPD technique could be used effectively in germplasm identification in sugarcane in order to assist plant breeders to utilise the available germplasm effectively.

INTRODUCTION

Sugarcane belongs to the genus *Saccharum*. It is an economically important polysomatic, highly heterozygous, clonally propagated plant. The genus *Saccharum* is a genetically complex one, characterised by high chromosome numbers and suspected to be of autopolyploid in origin (Angeliq *et al.*, 1993) causing difficulties in breeding.

Since the nobilization strategy of modern sugarcane varieties is almost exclusively 'backcross derivatives' of only a few *Saccharum* species

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(Price, 1965; Berding and Roach, 1987), limited degree of genetic diversity exists between these varieties (Berding and Roach, 1987). This would have serious implications for sugarcane breeding programmes, where varietal improvement, through incorporation of new genetic material has become essential.

Genetic resources are at the center of an effective programme in plant breeding and in developing a fundamental understanding of adaptation. Therefore, phenomena, conservation, characterisation and documentation of wild relatives of sugarcane are inevitable to allow exploitation of available potential and to prevent further reduction in DNA diversity of sugarcane.

Among the DNA fingerprinting techniques, Randomly Amplified Polymorphic DNAs (RAPDs) (Welsh and McClelland, 1990; Williams *et al.*, 1990) have proven to be powerful tools over the conventional approach of morphological concept in the assessment of genetic variation of plant populations, and in the elucidation of genetic relationships among accessions within a species. One of the major advantages of RAPD is that it can be utilised without prior knowledge of the genome. RAPDs have been used to determine DNA diversity in a range of crops including rice (Ko *et al.*, 1994), cassava (Marmey *et al.*, 1994), sugarcane (Harvey *et al.*, 1994) and even in polyploid species such as *Saccharum spontaneum* (Al-Janabi *et al.*, 1993).

In this study, RAPD technology was used in the analysis of genetic diversity within and between locally collected wild sugarcane accessions and the imported wild accessions (*Saccharum spontaneum* and *Erianthus*) available in the germplasm of the Sugarcane Research Institute, Sri Lanka.

MATERIALS AND METHODS

Plant material

Forty-four sugarcane wild accessions were analysed in this study. Of the twenty six accessions of *S. spontaneum*, thirteen were collected from Horton plains, seven from the Knuckles range, one from Peradeniya and five were imported. Of eighteen *Erianthus* spp. fourteen were imported while the other four were collected from the Horton plains and Knuckles range (Table 1).

Table 1. Wild sugarcane accessions.

	Place of collection	Name of the accession	
<i>Saccharum spontaneum</i>	Peradeniya	Spontaneum ¹	
	Imported	SES 182 ²	FIJI 52 ⁵
		IND 81-64 ³	IK 76-88 ⁶
		SES 304 ⁴	
	Horton plains	SLC 92-77 ⁷	SLC 92-95 ¹⁴
		SLC 92-85 ⁸	SLC 92-86 ¹⁵
		SLC 92-87 ⁹	SLC 92-76 ¹⁶
		SLC 92-82 ¹⁰	SLC 92-96 ¹⁷
		SLC 92-98 ¹¹	SLC 92-94 ¹⁸
		SLC 92-78 ¹²	SLC 92-90 ⁴¹
		SLC 92-92 ¹³	
	Knuckles range	SLC 92-61 ¹⁹	SLC 92-51 ²³
		SLC 92-62 ²⁰	SLC 92-60 ²⁴
		SLC 92-64 ²¹	SLC 92-33 ²⁵
		SLC 92-66 ²²	
<i>Erianthus</i>	Imported	IS 76-73 ²⁷	IS 77-222 ³³
		IK 76-92 ²⁸	IS 76-215 ³⁵
		IK 76-78 ²⁹	IS 76-216 ³⁶
	Horton plains and Knuckles range	MOL 50-99 ³⁰	IND 81-80 ³⁷
		MOL 49-43 ³¹	SES 7 ³⁸
		FIJI 51 ³²	MINDANAO-1 ³⁹
		MINDANAO-2 ³⁴	
	Horton plains and Knuckles range	SLC 92-81 ⁴⁰	SLC 92-79 ⁴³
		SLC 92-65 ⁴²	SLC 92-80 ⁴⁴

Number appearing with the accession number represents the respective lane number in Plate 1.

All the accessions were planted in polyethylene pots under greenhouse conditions at the Sugarcane Research Institute's research fields at Uda Walawa.

DNA extraction and assessment

DNA was extracted from seven-month old plants using the protocol described by Tai and Tanksley (1991). Plants of *S. spontaneum* spp. and *Erianthus* spp were cut at the base and the leaf blades were trimmed to obtain immature leaf roll tissues for DNA isolation (Honeycutt *et al.*, 1992).

Immature leaf roll tissues from three individual plants (stools) were pooled and ground in a cold mortar in liquid nitrogen to form a fine powder. The leaf powder was collected into a 15 ml centrifuge tube and incubated at 65°C for 15 min after adding 0.7 ml of pre-heated (65°C) extraction buffer (10 ml of 1 M Tris-HCL pH 8.0, 10 ml of 0.5 M EDTA, 2.922 g of sodium chloride, 12.5 ml of 10% SDS, and 0.38 g of sodium bisulfite for 100 ml of buffer). Into these incubated samples 250 µl of 5 M potassium acetate was added and left for 20-40 min on ice. The extracts were collected into 2 ml centrifuge tubes after centrifuging at 13,000 rpm for 10 min. The DNA was precipitated with isopropanol and centrifuged at 13,000 rpm for 5 min. The supernatant was discarded and the pellet was washed with 70% ethanol. The pellet was resuspended in 300 µl of TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 7.4), and 150 µl of 7.4 M ammonium acetate was added to precipitate RNA and centrifuged at 13,000 rpm to precipitate RNA. Then the supernatant was removed and DNA was precipitated with isopropanol. DNA was pelleted by centrifuging at 13,000 rpm for 5 min and the pellet was washed in 70% and 100% ethanol, respectively after discarding the supernatant. The DNA pellet was dissolved in 100 µl of TE buffer. The extracted DNA was purified by phenol chloroform extraction followed by ethanol precipitation and the quality and the quantity of DNA was determined by agarose gel electrophoresis.

DNA amplification

Amplification parameters including template DNA, primer, Taq DNA polymerase, Mg₂SO₄ and number of thermal cycles and temperature were varied in several experiments to determine the optimal conditions.

Standard 20 μ l reaction mixtures contained 15 ng of template DNA, 1 \times thermal buffer, 3 μ M of ten-mer primer, 0.5 units of Vent (exo-) DNA polymerase, 0.2 mM Mg_2SO_4 and 2 mM of each dNTP. PCR reactions were carried out in Techne Progene thermal cycler using the following temperature profile:

Initial Denaturation	at 92°C	1 min
Followed by 30 cycles of Denaturation	at 92°C	40 sec
Annealing	at 48°C	1 min
Extension	at 72°C	1.5 min
Final extension	at 72°C	10 min

The amplification products were electrophoresed through 1.5% (w/v) agarose gels at 5.6 V/cm in the presence of ethidium bromide (0.5 μ g/ml) and the banding patterns were visualised under UV light and photo documented.

Primer screening for germplasm characterisation

Sixty different UBC (Primers obtain from University of British Columbia, Canada) and Operon (Primers obtain from Operon Technologies) primers were screened using two bulked DNA samples, representing the DNA of *S. spontaneum* accessions and the DNA of *Erianthus* accessions respectively with the concentration of 5 ng/ μ l as template DNA. The reactions were carried out three times to confirm the reproducibility of the amplified DNA fragments and nine primers were selected for the assessment of genetic diversity of wild sugarcane germplasm.

RAPD analysis of germplasm accessions

Nine ten-base-pair oligonucleotide primers were selected based on the primer screening reactions. PCR reactions with the optimum conditions found in this study were carried out using the DNA of all accessions under investigation with different selected primers.

Data analysis

Polymorphic fragments were scored for their presence (1) and absence (0) in the respective individuals. Based on the data generated from polymorphic fragments, an index of genetic similarity (F) or genetic distance was calculated (1-F) (Nei and Li, 1979) using RAPD-distance computer package (Armstrong *et al.*, 1995), as follows,

$$F = 2 \times N_{NB1} / (N_A + N_B)$$

where, N_A = the number of bands in accession A, N_B = the number of bands in accession B, N_{NB1} = the number of bands present (scored 1) in both accessions A and B. The distance matrix was then used by the same package to construct phenogram (Unweighted Pair Group Method with Arithmetic Mean Method of clustering (UPGMA)).

RESULTS AND DISCUSSION

Preparation of template DNA

For a large-scale RAPD analysis, it is desirable to use a method for DNA isolation that allows fast extraction of DNA from a large number of samples while ensuring uniformity of yield and purity of samples and minimising the risk of cross contamination. The main advantage of using RAPD technology is that it requires minute amounts of template DNA which need not be ultra-pure in terms of protein contamination (Caetano-Anolles *et al.*, 1991a, b; Hardrys *et al.*, 1992). The method modified by Tai and Tanksley (1991) was used in this study. This produces DNA samples with the concentration between 29.50 and 596.10 ng/ μ l. The advantage of this DNA extraction protocol was that multiple extractions could be carried out in a relatively shorter period of time, without sacrificing DNA yields and purity.

Optimisation of RAPD protocol

The sensitivity of RAPD-PCR technology to changes in experimental parameters is well known (Munthali *et al.*, 1992). The effect of several parameters were examined to optimise RAPD assay for sugarcane wild accessions, and it was found that the conditions explained in the section of materials and methods of this study produced the best amplification of sugarcane wild accessions.

Genetic diversity between wild sugarcane accessions determined by the RAPD methodology

A set of sixty, ten-mer primers were tested using two bulked DNA samples of *S. spontaneum* and *Erianthus* and nine primers were then selected as they produced the most clearly resolvable, consistent banding profiles over repeated runs with at least a few polymorphisms detectable. Sequences and the source of the selected primers for DNA diversity analysis in this study is given in Table 2.

Table 2. Sequences and the source of the selected primers for DNA diversity analysis.

Primer designation	Sequence (5'-3')
UBC 03*	CCT GGG CTT A
UBC 86	GGG GGG AAG G
UBC 97	ATC TGC GAG C
UBC 614	GTA GTC TCG C
UBC 623	TGC GGG ACT G
UBC 643	ATA AGC GGT G
UBC 690	TAA TCC GGT C
OC 13*	AAG CCT CGT C
OC 17	TTC CCC CCA G

* Primers were named as follows;
 UBC - obtained from the University of British Columbia
 OC - obtained from the Operon Technologies

The RAPD analysis of 44 wild sugarcane accessions employing nine selected ten-mer primers yielded 113 reproducible bands of which 109 were polymorphic. The average number of polymorphic bands per primer was 12.56. Plate 1 shows the amplification profiles generated by primer UBC 643.

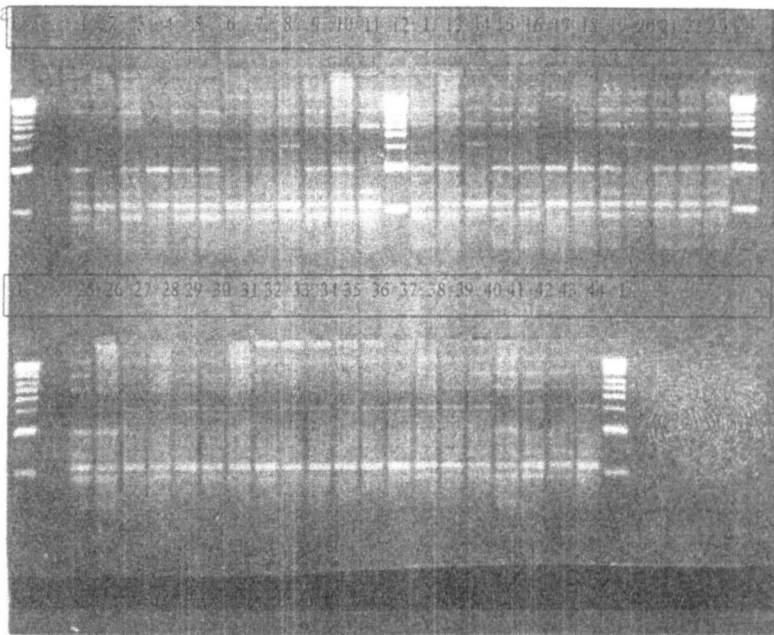


Plate 1. RAPD profile generated using UBC643.

[Note: L = 1 kb ladder; 1-44 = tested accessions as listed in Table 1]

The DNA diversity measures between the 44 accessions expressed as percent of genetic distance ranged from 0% (between IK 77-222 and Mindanao-2, IK 77-222 and IS 76-215, Mindanao-2 and IS 76-215) to 69.23% (SLC 92-78 and SLC 92-87) (Figure 1).

The 44 accessions analysed formed one major cluster and elucidates SLC 92-78 at the average genetic distance of 1.46 level. The major group is sub clustered into two groups, A and B at the average genetic distance of 1.32. As distinguished by morphological features, group A was mainly composed of *S. spontaneum* accessions while one imported *Erianthus* accession namely NG 77-95 was also included in the same cluster. Group B was mainly composed of *Erianthus* spp. including six *S. spontaneum* accessions, namely SES 182, SLC 92-77, SLC 92-85, SLC 92-87, SLC 92-86 and SLC 92-64 (Figure 2).

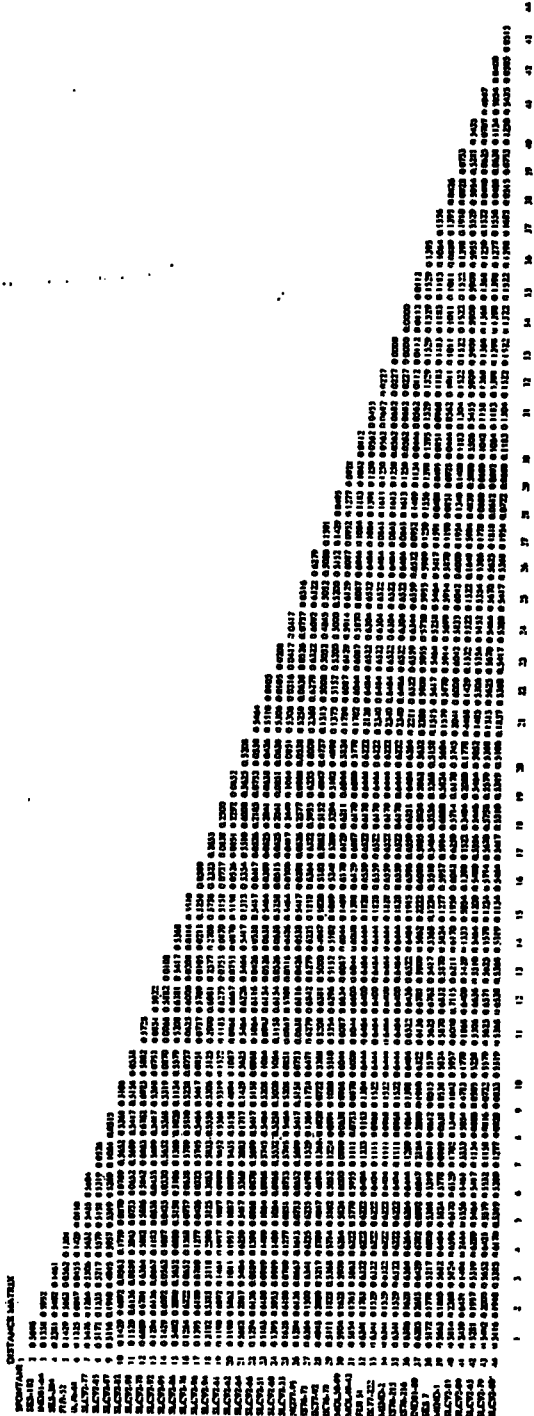


Figure 1. Distance matrix generated using RAPD profiles.

Figure 2 clearly shows that the DNA diversity among the accessions in group A is somewhat homogeneous whereas the accessions in group B show more heterogeneity in DNA diversity.

Among the *S. spontaneum* accessions clustered into group A, SES 304, SLC 92-94, Spontaneum, SLC 92-90 are clearly distinguished from the other accessions even at the average genetic distance between clusters of 0.3. SES 304 was a *S. spontaneum* accession introduced from Fiji islands while SLC 92-94 and SLC 92-90 were collected from Horton plains and *Spontaneum* was collected from Peradeniya.

The remaining sub cluster of group A can be further divided into four sub groups while elucidating another two accessions collected from Knuckles range namely SLC 92-61 and SLC 92-62 at the average genetic distance between the clusters of 0.1. These four sub clusters clearly show the geographical distribution of the collected accessions. SLC 92-66, SLC 92-51 and SLC 92-60 are *S. spontaneum* accessions collected from Knuckles range of Sri Lanka and among those accessions the genetic distances ranged from 0.0105 to 0.0316 (Figure 1). Therefore these three accessions could be identified as duplications. It also indicated that the *S. spontaneum* accessions namely SLC 92-82 and SLC 92-98 which were collected from Horton plains are again clustered into one and the genetic distance between those is 0.0538. IND 81-64, FIJI 52 and IK 76-88 are *S. spontaneum* accessions, which were imported to Sri Lanka from Fiji islands. They formed one sub cluster and again IND 81-64 shows diversity from the other two accessions.

Figure 1 also revealed that the accessions SES 182 and SLC 92-64 which were *S. spontaneum* imported and collected from Knuckles range respectively, are genetically diverse at the average genetic distance between clusters at 0.4 level. At the average genetic distance between clusters of 0.2, the imported *Erianthus* accession of IS 76-73 and *S. spontaneum* accessions collected from Horton plains, SLC 92-77 and SLC 92-86 were identified as genetically distinct accessions. The remaining accessions were clustered into four sub clusters, all of which consists of *Erianthus* spp. These results also revealed that at a very low level of average distance between clusters, these accessions could be separated further. In group B there are five *S. spontaneum* accessions, which were genetically closely related to *Erianthus* spp. This would enable the RAPD technique to identify the closely related accessions of related genera even though they exhibit different morphological features.

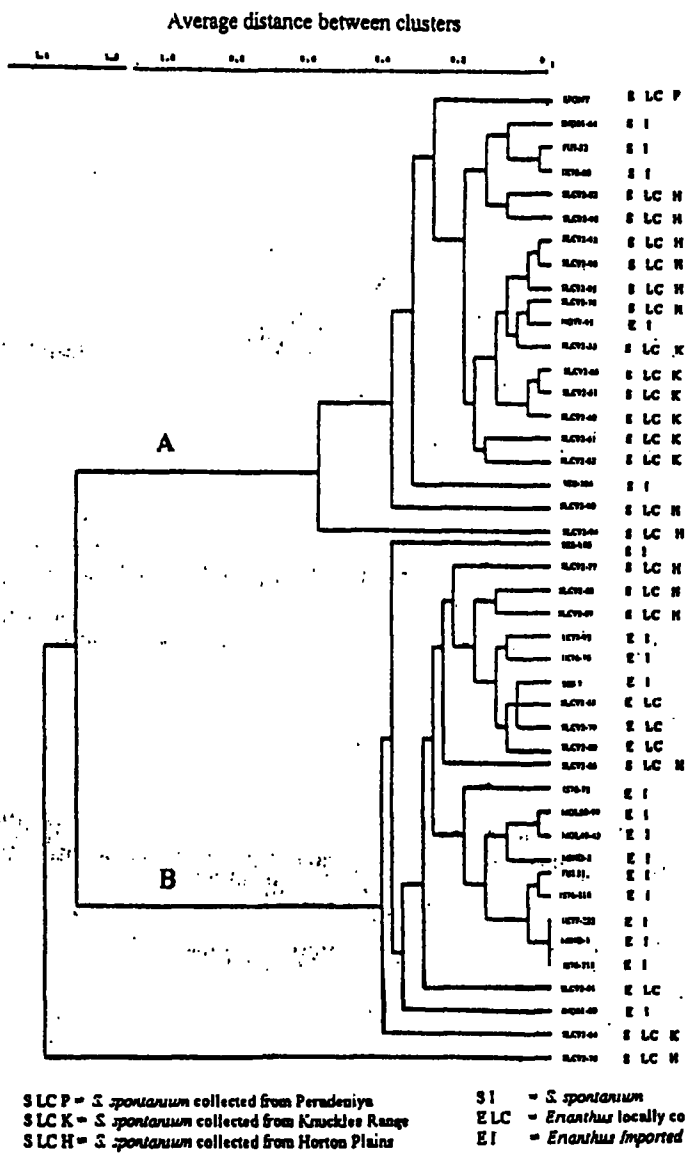


Figure 2. Phenogram showing DNA diversity between germplasm accessions. Average distance between clusters.

CONCLUSIONS

The forty four wild accessions studies showed significant DNA diversity. SLC 92-78 collected from Horton plains of Sri Lanka was found to be a distinct *S. spontaneum* accession and this could be used as one of the parents in interspecific crosses to create highly variable offspring.

Among the morphologically similar *S. spontaneum* accessions, namely Spontaneum (collected from Peradeniya), SES-304 (imported), SLC 92-90, SLC 92-94, SLC 92-77, SLC 92-86 (collected from Horton plains) and SLC 92-61, SLC 92-61, SLC 92-64 (collected from Knuckles range) were found to be genetically distinct at low genetic distances.

Morphologically indistinguishable *Erianthus* accessions such as SES 182, IND 81-80, IS 76-73 (imported) and SLC 92-81 (collected from Horton plains) were discovered as distinct accessions at low genetic distances.

The accessions remaining in the sub clusters at very low genetic distances could be identified as duplicates.

These findings have an immediate practical application for sugarcane breeders as the RAPD technology provides a fast, efficient and reliable approach for characterisation of sugarcane germplasm for effective management of collections in terms of identification of duplicates, the proper utilisation of genetically diverse accessions in crossing programme, and to formulate a meaningful accession exchange programme between other countries to enhance the germplasm.

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