

PROGRESS TOWARDS A FINGERTYPING DATABASE FOR SUGARCANE VARIETIES

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Abstract

There is a growing need for crop breeders to register novel germplasm with the International Union for Protection of new Varieties of Plants (UPOV) and to obtain Intellectual Property Rights. The UPOV is currently compiling a series of guidelines for identifying sugarcane varieties based on morphological features. However, such phenotypic characters may be unreliable due to environmental influences. Effective DNA profiling would enhance the repertoire of characters for varietal discrimination and provide increased creditability. The feasibility of using the relatively new Amplified Fragment Length Polymorphism (AFLP) technique for characterising sugarcane germplasm was investigated. Of seven primer combinations tested, one was found capable of distinguishing 20 varieties in a reproducible manner. This augurs well for the development of an AFLP-based fingertyping database for sugarcane.

Introduction

One of the challenges facing breeders of sugarcane and other crops is that of identification of new varieties. Before they can be marketed, newly bred varieties of crop plants are required to undergo statutory testing, part of which requires that varieties are shown to be distinct from others (D), that they demonstrate uniformity (U) and stability (S) in the characteristics used to distinguish them (Law *et al.*, 1998). This DUS-testing forms the basis on which Intellectual Property Protection known as Plant Breeders Rights (PBR) are granted. UPOV is currently putting together a series of morphological features to be used as guidelines for identification of sugarcane varieties. However, phenotypic characters may be questionable as they are influenced by factors such as age of the plant material and the environment. The ability to distinguish and identify sugarcane varieties at a molecular level would not only serve as a tool for DUS-testing, but would also eliminate uncertainties that are often associated with phenotypic descriptions. Moreover, DNA fingerprinting could be used for validation of varietal occurrence in the field, or in breeding programmes where it is desirable to check parental identity.

A novel Polymerase Chain Reaction (PCR)-based fingerprinting technique, AFLP, has recently been developed (Vos *et al.*, 1995). This technique is based on selective amplification of genomic restriction fragments and combines high res-

olution, reproducibility and sensitivity. The AFLP technique can be used to generate fingerprints from DNA of any origin or complexity without prior sequence knowledge. These characteristics make AFLP a powerful fingerprinting tool.

The objective of this study was to evaluate use of the AFLP technique to discriminate sugarcane varieties as a preliminary step towards developing a genetic fingerprinting database for the industry.

Material and Methods

DNA was extracted from a set of 20 sugarcane varieties according to the method described by Harvey and Hockett (1998). DNA was then quantified spectrophotometrically at 260 nm and the integrity and purity of the extract determined by agarose gel electrophoresis. Samples were stored at -20°C .

The AFLP assay was performed using a kit according to the manufacturer's instructions (Gibco Life Technologies Inc.). Sugarcane genomic DNA (250 ng) was digested using EcoRI and MseI restriction endonucleases at 37°C for 2 h then samples were incubated at 70°C for 15 minutes to inactivate the enzymes. DNA fragments were then ligated to EcoRI and MseI adaptors for 2 h at 20°C . After terminating the reaction, the ligation mixture was diluted 10-fold with sterile TE buffer (10 mM TrisCl (pH 8,0), 0,1 mM EDTA), and the fragments were pre-amplified (GeneAmp 9700, Perkin-Elmer) using a temperature profile of 94°C for 30 s, 56°C for 60 s and 70°C for 60 s (20 PCR cycles). An aliquot (5 μl) of the pre-amplified product was analysed on a 1% (w/v) agarose gel to test success of the reaction. Pre-amplified fragments were then selectively amplified with MseI and ^{32}P -labeled EcoRI primers each with three selective nucleotides (Table 1). The PCR amplification profile included one cycle at 94°C for 30 s, 65°C for 30 s and 72°C for 60 s, after which the annealing temperature was lowered by $0,7^{\circ}\text{C}$ for 12 successive cycles. This was followed by 23 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 60 s. Sub-samples (5 μl) of the PCR-product were then separated by electrophoresis through a denaturing 5% (w/v) polyacrylamide DNA sequencing gel containing 7,5M urea. Autoradiographs were obtained by exposing Kodak BioMax MR-2 film to the dried gel for three to six days at -85°C . After development of X-rays, varieties were visually scored on a light box for presence or absence of bands. Each experiment was repeated at least once to establish reproducibility.

Results and Discussion

DNA was extracted from a sample of 5-6g leaf roll tissue. Using the described extraction protocol (Harvey and Hockett, 1998), high quality DNA with total concentrations varying from 0,05-2,60 μ g/ μ l was obtained. These variations did not influence fingerprinting profiles, which is in accordance with the findings of Vos *et al.*, (1995).

To produce AFLP fingerprints, restriction fragments were generated using a rare cutting enzyme, EcoRI, and a frequent cutter, MseI, followed by ligation of adapters. For amplification of a reduced range of restriction fragments, a two-step amplification strategy was carried out. During the first step, a set of EcoRI and MseI primers with one selective nucleotide each was used to generate large quantities of secondary template. Products of the pre-amplification step, viewed on an agarose gel, appeared as a smear of fragments ranging from approximately 50-1900 bp (results not shown). Once pre-amplification was confirmed, a more selective amplification was carried out using sets of EcoRI and MseI primers with three selective nucleotides each, as shown in Table 1. Different primer combinations were assessed in order to obtain a single combination that could be used to identify all 20 varieties.

Table 1. Primer combinations used to determine % polymorphic bands (A), number of unique polymorphisms (B) and the number of sugarcane varieties distinguished (C). E represents EcoRI and M represents MseI endonuclease.

Primer combination (PC)		A	B	C
E-AAG + M-CAA	I	62	4	14
E-AAG + M-CAG	II	51	4	14
E-AAC + M-CAA	III	72	8	20
E-AAC + M-CAC	IV	55	6	-
E-ACA + M-CAA	V	41	2	10
E-ACC + M-CAT	VI	70	6	18
E-ACC + M-CTT	VII	55	2	12

Of the seven primer combinations analysed, all but one yielded more than 50% polymorphic bands (Table 1). Depending on the primer combination, 75-100 bands were resolved per single lane (Figure 1). Under the reaction conditions described, the size of amplified DNA fragments ranged from 50 to 500 bp relative to a size ladder (not shown). Replication using the same set of primers yielded similar polymorphisms and DNA fingerprint profiles. This reproducibility is possible because of the nature of AFLP technique, which requires stringent reaction conditions for primer annealing (Vos *et al.*, 1995).

After screening only seven primer combinations as shown in Table 1, it was found that a single primer combination (PC III) could be used to distinguish all 20 varieties. Discrimination was based on presence of unique polymorphisms (Table 1; Figure 1) as well as band variation amongst varieties (Figure 1). With these primers, AFLPs were used

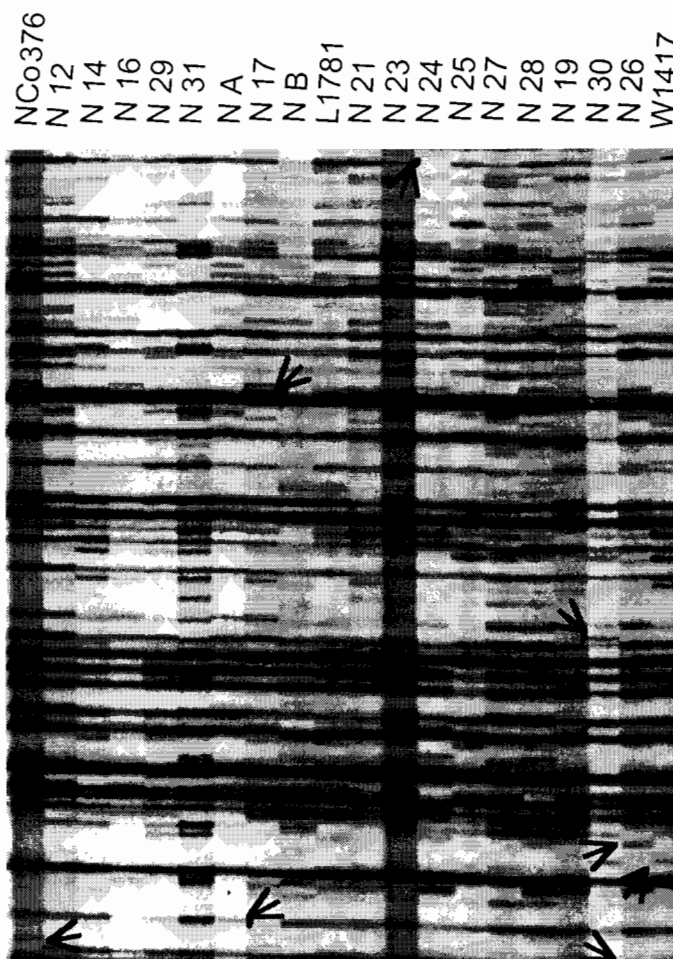


Figure 1. AFLP fingerprints of 20 sugarcane varieties generated with primer combination III (Table 1). Arrows have been used to indicate polymorphisms that are unique to individual varieties.

successfully to establish an identity for each sugarcane variety. The ability to use one PC to distinguish between different sugarcane varieties shown in this study has proven that AFLP is a highly powerful fingerprinting technique, as demonstrated elsewhere (Vos *et al.*, 1995; Breyne *et al.*, 1997).

The use of Restriction Fragment Length Polymorphisms (RFLPs) (D'Hont *et al.*, 1995; Harvey *et al.*, 1998) and Random Amplified Polymorphic DNA (RAPDs) (Harvey and Botha, 1996) for identification of sugarcane varieties and/or hybrids has been reported previously. However, such techniques have numerous limitations. Although reliable, the need for relatively large amounts of good quality DNA and the low potential for automation with regard to processing large numbers of samples is a major problem with RFLPs. The reproducibility of RAPDs, especially in relation to inter-laboratory comparisons, has been questioned (Breyne *et al.*, 1997). Establishment of a reliable, high resolution fingerprinting technique such as AFLP was therefore necessary to complement existing methods of identification.

The use of DNA fingerprints has not yet been accepted as a standard DUS-testing technique. However, the fact that pro-

tein fractionation by electrophoresis has been incorporated into tests for barley, wheat and maize varieties (Law *et al.*, 1998) bears much promise for the future use of DNA profiles for such tests.

The establishment of a sensitive, reproducible fingerprinting technique, as shown in this study, has potential application in the development of a system for varietal identification in sugarcane.

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