E

BMT/7/6
ORIGINAL: English
DATE: October 22, 2001

UPOV

INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS

GENEVA

WORKING GROUP ON BIOCHEMICAL AND MOLECULAR TECHNIQUES AND DNA-PROFILING IN PARTICULAR

Seventh Session
Hanover, Germany, November 21 to 23, 2001

DNA PROFILING IN SUGARCANE: IMPLICATIONS FOR VARIETAL PROTECTION

prepared by experts from Australia

DNA PROFILING IN SUGARCANE: IMPLICATIONS FOR VARIETAL PROTECTION

G. Piperidis[1], M. C. Cox[2], D. Appo[3], A. Rattey[1] and C. Kimbeng[3]

Bureau of Sugar Experiment Stations:
[1] P.O. BOX 86, INDOOROOPILLY, QLD, 4068
[2] PRIVATE BAG 4, BUNDABERG DC, QLD, 4670
[3] PMB 57, MACKAY MAIL CENTRE, QLD, 4741

Abstract

Since 1995, the Bureau of Sugar Experiment Stations (BSES) in Australia has protected all new sugarcane varieties released under the Australian Plant Breeder's Rights (PBR) Act (1994). The process of obtaining protection requires comparison of new varieties with the most similar varieties of common knowledge. Over 50 plant, stem, and leaf morphological measurements are taken for the comparison to demonstrate distinctness, uniformity, and stability. In recent years, information on the DNA profile of the new variety has been included in sugarcane PBR applications as additional information. Here, we show that the morphological and DNA data of a number of sugarcane varieties of disputed identity are in complete agreement. We describe the DNA profiling method used for sugarcane variety identification and discuss the potential for international collaboration to establish a standardised protocol for sugarcane variety identification using the database developed at BSES to store and retrieve the DNA profile data. DNA profiles could be used in place of the morphological data currently used to demonstrate distinctness, uniformity, and stability of new sugarcane varieties.

Introduction

Sugarcane is a complex, highly polyploid and aneuploid interspecific hybrid. Most modern sugarcane hybrids are derived from *Saccharum officinarum* (noble canes) and *S. spontaneum*. Chromosome numbers vary from 100 to 120, with approximately 70-80% derived from *S. officinarum* and the rest from *S. spontaneum*, either as complete or recombined chromosomes. Sugarcane is a tropical grass (Family *Poaceae*) with a C4 photosynthetic pathway.

The Bureau of Sugar Experiment Stations (BSES) has breeding and selection programs in five regions of Queensland and northern New South Wales in Australia. Since 1995, all new sugarcane varieties released by BSES have been protected under the Australian Plant Breeders Rights (PBR) Act (1994). The Australian PBR Act conforms to the 1991 revision of the UPOV convention. Currently we have 31 'Q' varieties protected, 28 with 'grant' status (full protection) and three with 'accepted' status (provisional protection).

The process of applying for PBR in sugarcane has been described previously (Cox *et al.,* 2001). In summary, a Part 1 application is submitted to establish a *prima facie* case that the variety exists and is distinct from all other varieties of common knowledge. To obtain a grant of PBR, the applicant must verify the claim, normally by conducting a comparative trial that

includes the new variety and the most similar varieties of common knowledge and, if possible, the parents. The comparative trial is conducted to demonstrate distinctness, uniformity and stability (DUS). Morphological measurements (both quantitative and qualitative) are taken, using over 50 plant, stem and leaf characteristics. The description and a photograph comparing the new variety with similar varieties is published in the Plant Varieties Journal, followed by a six-month period for objection or comment. Propagating material of the new variety is deposited in the Australian Sugar Cane Genetic Resource Centre at Meringa (17°04'south, 145º45'east), Australia. Upon successful completion of all the requirements, resolution of objections (if any) and payment of the certificate fee, a Certificate of Plant Breeder's Rights is issued by the PBR Office.

Until recently, the morphological descriptors used had been developed within BSES, based on the original work by Artschwager (1939) and, more recently, Gallagher (1997). However, in 2000, test guidelines for sugarcane, drafted by Brazil, were discussed at a UPOV meeting of 'Technical Working Party for Agricultural Crops' in Sweden. Following significant input by BSES at that meeting and subsequently, the test guidelines were finalised at a similar meeting in Mexico in 2001. The new test guidelines will be used in future to determine DUS.

While PBR is granted on the basis of morphological description (DUS), additional characters, such as DNA markers, may also be included in the application. In recent years, microsatellite markers have become increasingly popular as the marker system used in many crops for variety identification (Bligh *et al*., 1999), pedigree verification (Pestsova *et al*., 2000), and genetic mapping studies (Kong *et al*., 2000). BSES has identified a set of five microsatellite primers (Simple Sequence Repeats or SSRs) that currently can uniquely identify all 50 sugarcane varieties screened. This is noted for each new PBR application. In this paper, we describe our current methods of DNA profiling and the development of a database to store and retrieve marker data. The potential for international collaboration to establish a standardised protocol using a common set of microsatellite markers for sugarcane identification is also discussed. In addition, we compare morphological and DNA evidence to substantiate that:

1. The variety Q96 propagated at Meringa is not the true Q96 grown commercially in the Burdekin (mislabelling has occurred).
2. The variety Q96 and the seedling 89A3112 are actually the same clone.
3. The variety Q117 and the seedling 92A9006 are actually the same clone.

MATERIALS AND METHODS

Comparison of morphological data and DNA profiling

The opportunity was taken to include in the comparative trial a number of varieties that we suspected had been mislabelled. Q96 growing on the station at Meringa (Q96_Mer), was found to have a different DNA profile to the Q96 grown commercially (Q96_Farm), even though they were quite similar in stalk height, thickness, and colour. Q96 was reintroduced to Meringa from another station in Queensland around 1993, as the variety had been discarded from Meringa.

Two seedlings selected in the Burdekin (19°34'south, 147°19'east) program appeared to be very similar to two commercial varieties used as standards in selection trials. In the Burdekin, breeders are usually faced with large, heavily lodged plots of seedlings from which they select

individual clones. It is possible under these difficult conditions for errors to be made and selections could sometimes inadvertently be taken from standard variety plots instead of seedling plots. The clone 89A3112 was very similar in appearance to Q96 (Q96_Farm) while the clone 92A9006 appeared to be very similar to Q117. DNA was extracted from these varieties to enable a comparison of their DNA profiles.

All of these varieties were included in the comparative trial planted at Meringa in August 2000. A total of 35 clones, including varieties for PBR application, their comparators and some parents, were planted in a randomised complete block design with three replications. Plots were single row, 10 m in length with 1.5 m between rows. Data for 50 morphological traits (28 quantitative and 22 qualitative) were measured or recorded in May 2001. Ten quantitative traits were based on measurement of 12 stalks per replicate and were subjected to analysis of variance using SAS (SAS Institute, 1997), while other quantitative traits were visually rated. Descriptive traits were assessed on a total of six stalks, two from each replicate. Measurement data was converted to a 1 to 9 scale (eg very short, very short to short, short, short to medium, medium, medium to long, long, long to very long and very long). For some traits, especially those visually assessed, the 1-9 scale was reduced to three (3, 5, 7) or five (1, 3, 5, 7, 9) categories (Table 1). The plant part, trait, method and scale of assessment are shown in Table 1. The collective data from the 28 quantitative traits were subjected to clustering analysis using a 32-bit PC version of Watson *et al*.'s (1995) GEBEI package.

Table 1. Quantitative traits assessed and used in the clustering analysis

Plant part	*Trait*	*Method*	*Scale*
Plant	growth habit	Visual	1, 3, 5, 7, 9
	tillering	Visual	1, 3, 5, 7, 9
	leaf canopy	Visual	1-9
	suckering	Visual	1-9
Stalk	height	Measurement	1-9
	adherence of sheath	Visual	1-9
Internode	alignment	Visual	1-9
	length bud side	Measurement	1-9
	length opposite bud	Measurement	1-9
	width perpendicular	Measurement	1-9
	width dissecting	Measurement	1-9
	wax covering	Visual	1-9
	wax band width	Visual	3, 5, 7
	bud groove length	Visual	1-9
	bud groove depth	Visual	1-9
	root band width	Visual	3, 5, 7
	bud prominence	Visual	1-9
	bud width	Visual	1-9
Leaf	lamina length	Measurement	1-9
	lamina width (lw)	Measurement	1-9
	midrib width (mw)	Measurement	1-9
	lw:mw ratio	Measurement	1-9
	leaf sheath length	Measurement	1-9
	hair group 57 density	Visual	1-9
	hair group 57 length	Visual	1-9
	ligule height	Visual	3, 5, 7
	hair group 61 density	Visual	1-9
	hair group 61 length	Visual	1-9

DNA profiling

The International Sugarcane Microsatellite Consortium was instigated by the Centre for Plant Conservation Genetics (CPCG), Lismore, in 1998 with the aim of isolating and characterising microsatellite markers for use in sugarcane (Cordeiro *et al*., 2000). More than 250 microsatellites have been identified, and primers designed to amplify sugarcane microsatellite markers have been distributed to all participating organisations in Australia, Brazil, Colombia, France, Mauritius, Philippines, South Africa, and USA. As part of this Consortium, we have used the markers to develop DNA profiles of Australian sugarcane varieties and basic germplasm to assist in more efficient utilisation and conservation of the Australian Sugar Industry's genetic resources.

The initial phase of developing a DNA profiling system is to validate the utility of the markers to be used. For this purpose DNA was extracted from 20 sugarcane varieties sampled from two different locations, and duplicate Polymerase Chain Reactions (PCRs) were performed to confirm the robustness and repeatability of the SSR markers. The markers were separated on large polyacrylamide sequencing gels and detected using ^{33}P. The amplification patterns proved to be reliable and repeatable under these conditions. The microsatellite primers were then tested to determine which would be most useful for variety identification. Initially, 100 primers were tested against four sugarcane varieties: Q96, Q110, Q117 and Q124. For routine DNA profiling, only polymorphic primers that generated relatively simple banding patterns that were easy to interpret were selected. Due to the complex nature of the sugarcane genome, many of the primers resulted in complex overlapping profile patterns that were difficult to interpret and these were not considered for DNA profiling.

Five primer pairs were selected for routine DNA profiling. These primers generated a total of 25 polymorphic markers suitable for DNA profiling and variety identification. With these primers we have been able to distinguish among all 50 Q varieties tested, some of which are full siblings (progeny from the same parental cross). Further, as few as two primer pairs were needed to discriminate between any two of the Q canes tested.

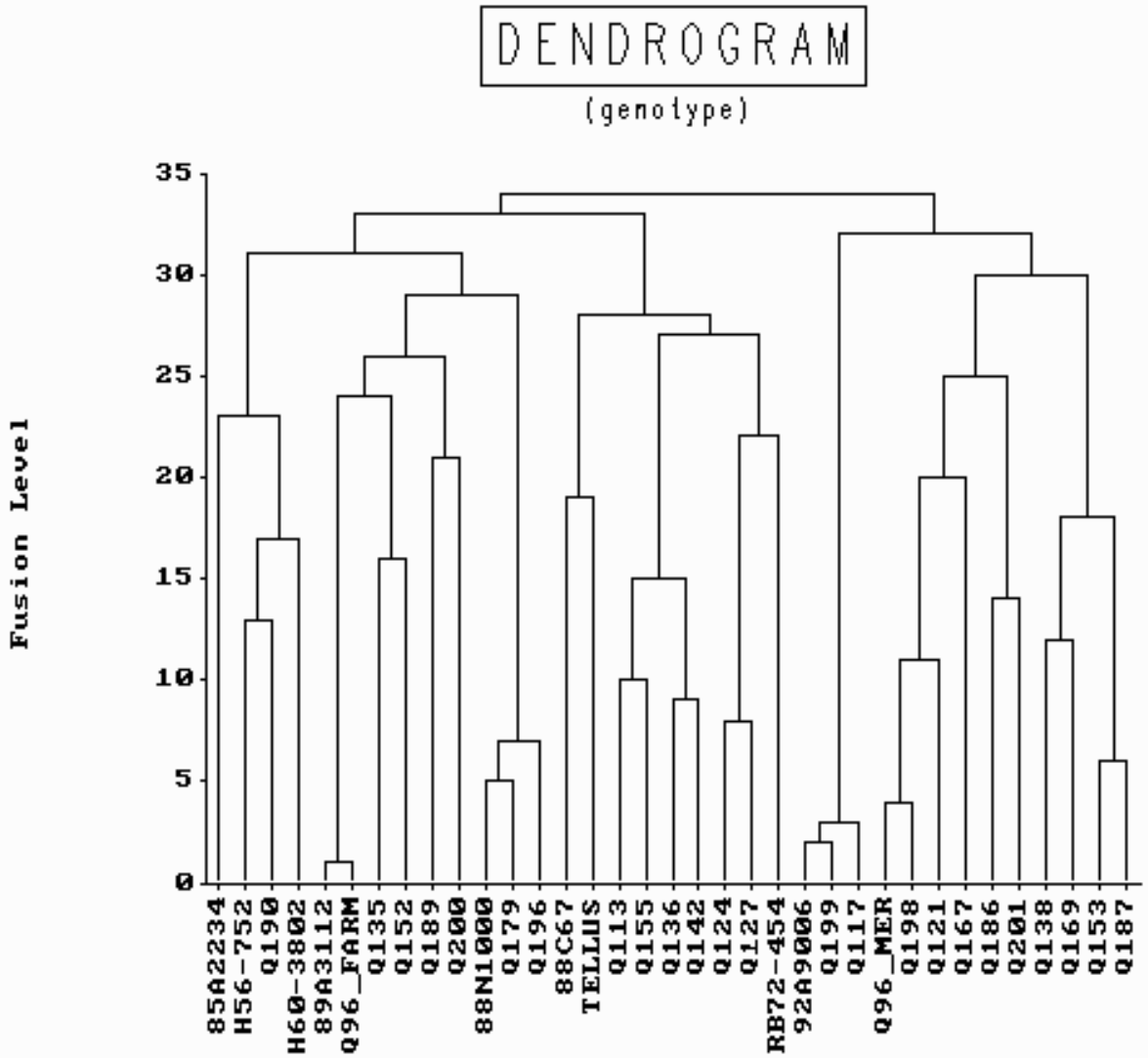
During investigations into the different DNA profiles of Q96_Mer and Q96_Farm, leaf samples of Q96 were sent from ten different locations for DNA profiling. This allowed an assessment of the uniformity of sugarcane DNA profiles across different locations.

The SSR markers were scored as present/absent and the information generated for each variety was manually entered into a database developed using Microsoft® ACCESS 97.

RESULTS AND DISCUSSION

Clustering analysis was applied to compare the similarity of varieties based on the quantitative morphological data gathered from the comparative trial. The resulting dendrogram is shown in Figure 1.

Figure 1. Dendrogram showing similarity of clones based on clustering analysis of 28 quantitative traits



The first two clones to fuse in the dendrogram (ie the most similar clones) were Q96_Farm and 89A3112 (fusion level 1). Q96_Mer was very different from both of these clones, having separated at the first major split into two clonal groups. A group of three clones, 92A9006, Q199 and Q117, were the next to fuse (fusion groups 2 and 3).

Qualitative data, not included in the clustering analysis, were then compared between Q96_Farm, 89A3112 and Q96_Mer, and between Q117, 92A9006 and Q199 (Tables 2 and 3).

Table 2. Comparison of non-quantitative traits for Q96_Farm, 89A3112 and Q96_Mer

Trait	Q96_Farm	89A3112	Q96_Mer
Alignment of internodes	Medium to strongly zigzagged	Strongly zigzagged	Medium to strongly zigzagged
Internode shape	Cylindrical	Cylindrical to slightly bobbin-shaped	Bobbin-shaped
Internode cross-section	Oval	Oval	Oval
Dewaxed colour (exposed)	Greyed-orange (177A) and greyed-purple (187A)	Yellow-green (147A) and greyed-purple (187A)	Greyed-orange (166A) and greyed-purple (187A)
Dewaxed colour (unexposed)	Yellow-green (146C, 152D and 153B)	Yellow-green (153D)	Greyed-yellow (160B)
Bud shape	Oval to triangular pointed	Triangular pointed	Pentagonal
Bud – position of base	Near	Near	Near
Bud – position of tip	Above	Level to above	Level
Leaf scar prominence	Prominent	Medium to prominent	Medium to prominent
Growth ring	Depressed	Depressed	Flush
Auricle prominence	Medium	Medium	Inconspicuous
Auricle shape – ULP	Lanceolate	Deltoid to lanceolate	Deltoid
Auricle size – ULP	Small	Small to medium	Small to medium
Auricle shape – OLP	Lanceolate	Deltoid to lanceolate	Transitional
Auricle size – OLP	Small	Small to medium	-

Table 3. Comparison of non-quantitative traits for Q117, 92A9006 and Q199

Trait	Q117	92A9006	Q199
Alignment of internodes	Medium zigzagged	Medium zigzagged	Weakly zigzagged
Internode shape	Concave-convex	Cylindrical to concave-convex	Bobbin-shaped to concave-convex
Dewaxed colour (exposed)	Yellow-green (144A to 146A)	Yellow-green (146A)	Yellow-green (148A) & greyed-purple (187A)
Dewaxed colour (unexposed)	Yellow-green (145A and 151D)	Yellow-green (146D)	Yellow-green (151D to 152B)
Bud shape	Oval to triangular pointed	Triangular pointed	Ovate to triangular pointed
Bud – position of base	Near	Near	Medium
Bud – position of tip	Level	Level	Level
Leaf scar prominence	Medium to prominent	Medium to prominent	Medium
Growth ring	Flush	Flush	Flush to swollen
Auricle prominence	Medium	Prominent	Absent
Auricle shape – ULP	Lanceolate	Lanceolate	Transitional
Auricle size – ULP	Medium	Medium to large	-
Auricle shape – OLP	Deltoid	Deltoid	Transitional
Auricle size – OLP	Medium	Medium to large	-

The quantitative (Figure 1) and qualitative (Table 2) morphological data indicate there are no major differences between Q96_Farm and 89A3112. Q96_Mer was shown to be quite distinct from both Q96_Farm and 89A3112 for quantitative traits (Figure 1) and also for a number of qualitative traits – dewaxed colour unexposed, bud shape, growth ring, auricle prominence and shape of OLP auricle (Table 2).

DNA profiles also were generated for Q96_Farm, Q96_Mer, and 89A3112 using four microsatellite primer pairs. The DNA profiles of Q96_Farm and 89A3112 were identical for all four primer pairs. The DNA profile of Q96_Mer, however, was different at almost 60% (11/19) of the markers selected for DNA profiling. Thus, the morphological and DNA profile data are in complete agreement for these three varieties.

Q117 and 92A9006 also are very similar for both quantitative and qualitative traits (Figure 1 and Table 3). Q199 is similar to Q117 and 92A9006 in many traits, but is clearly differentiated by dewaxed colour (exposed), bud position of base, and all auricle characteristics (Table 3). In addition, there were minor differences in alignment of internodes, leaf scar prominence and growth ring. Again, this matches precisely the DNA profile data using four microsatellite primers, which indicated Q117 and 92A9006 were identical. Further, Q199 was different from Q117 at over 50% (9/17) of the markers selected for DNA profiling.

DNA testing of Q96 sampled from ten different locations resulted in separation of the samples into two distinct groups. The first group consisted of three Q96 samples with identical DNA profiles; Q96_Mer and the samples from Bundaberg and Samford. This was verified by the fact that the Q96 from Bundaberg and Samford were sourced from Meringa after it was reintroduced there in 1993. The second group of seven samples, which included Q96_Farm, also had identical DNA profiles, thus confirming the uniformity of sugarcane DNA profiles across different locations.

The amount of data generated from DNA profiling sugarcane varieties requires a data management system to enable storage, access, and linkage of the microsatellite data in a safe and secure manner. A database was developed using Microsoft® ACCESS 97 specifically for this purpose. Currently, there are 50 unique sugarcane DNA profiles stored in this database, which has a facility for matching DNA profiles from clones of unknown or disputed origin. Primer sequence information and PCR conditions for the SSR markers used for DNA profiling are stored within the database with hyperlinks to gel images to enable comparison between gel images. This database has the potential to ensure uniformity between sugarcane DNA profiling laboratories world-wide. Indeed, at the International Society of Sugar Cane Technologists meeting held in Brisbane, Australia (17-21 September, 2001), ten organisations from eight countries have agreed to cooperate and use this database as the basis for generating a world-wide standardised protocol for DNA profiling in sugarcane identification.

Conclusions

Microsatellite markers can be used to unambiguously identify sugarcane varieties. At BSES, microsatellite DNA profiles of all new varieties are currently submitted as additional information in PBR applications. The DNA profile data are consistent with the morphological data collected in comparative trials to show distinctness, and could therefore

be used in place of the morphological data used for PBR applications of new sugarcane varieties.

References

Artschwager, E. 1939. Illustrated outline for use in taxonomic description of sugarcane varieties. Proc. Int. Soc. Sugar Cane Technol. 6:116-128.

Bligh, H.F.J., Blackhall, N.W., Edwards, K.J., and McClung, A.M. 1999. Using Amplified Fragment Length Polymorphisms and Simple Sequence Length Polymorphisms to Identify Cultivars of Brown and White Milled Rice. Crop Sci. 39:1715-1721.

Cordeiro, G.M., Taylor, G.O., and Henry, R.J. 2000. Characterisation of microsatellite markers from sugarcane (*Saccharum* sp.), a highly polyploid species. Plant Science 155: 161-168.

Cox, M.C., Piperidis, G., Kimbeng, C.A. and Hansen, P.B. 2001. Plant Breeder's Rights – protecting the sugar industry's investment in varietal development. Proc. Aust. Soc. Sugar Cane Technol. 23:142-147.

Gallagher, D.J. 1997. Optimised descriptors recommended for Australian sugarcane germplasm (*Saccharum* spp. hybrid). Aust. J. Agric. Res. 48:775-779.

Kong, L., Dong, J., and Hart, G.E. 2000. Characteristics, linkage-map positions, and allelic differentiation of *Sorghum bicolor* (L.) Moench DNA simple-sequence repeats (SSRs). Theor. Appl. Genet. 101:438-448.

Pestsova, E., Salina, E., Börner, A., Korzun, V., Maystrenko, O.I., and Röder, M.S. 2000. Microsatellites confirm the authenticity of inter-varietal chromosome substitution lines of wheat (*Triticum aestivum* L.). Theor. Appl. Genet. 101:95-99.

SAS Institute Inc. 1997. SAS/STAT Release 6.12. SAS Inst Inc., Cary NC, USA.

Watson, S.L., DeLacy, I.H., Podlich, D.W., and Basford, K. E. 1995. GEBEI An analysis package using agglomerative hierarchical classificatory and SVD ordination procedure for genotype x environment data. Centre for Statistical Research Report, Department of Agriculture, The University of Queensland, Australia.

[End of document]