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New *Saccharum* hybrids in *S. spontaneum* cytoplasm developed through a combination of conventional and molecular breeding approaches

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Abstract

Identification of sugarcane F_1 hybrids is difficult when selections are based solely on morphological traits. Our objective was to combine morphological traits and molecular marker analysis to select F_1 hybrids from two separate crosses between Djatiroto, a clone of *Saccharum spontaneum*, and elite sugarcane clones, LCP 85-384 (Cross 97-3144) and CP 62-258 (Cross 97-3146). The maternal inflorescences of Djatiroto were emasculated by submersion in a circulating 45°C hot-water tank for 10 min to minimize self-fertilization. Cross 97-3144 produced 4.7 g of seeds with 338 viable seeds per gram and Cross 97-3146 produced 2.4 g of seeds with 166 viable seeds per gram. After greenhouse germination, 96 progeny from each cross were evaluated in a field plot. Evaluations were conducted on the ratoon crops for stalk diameter (mm), juice Brix (percentage soluble solids), and a randomly amplified polymorphic DNA (RAPD) marker OPA-11-366 that was reproducibly amplified through PCR from the elite clones, but not the maternal *S. spontaneum* clone. Fifty progeny (52.1%) from Cross 97-3144 and 36 progeny (37.5%) from Cross 97-3146 inherited the RAPD marker. Five putative F_1 progeny were selected from each cross, namely US 99-43, US 99-44, US 99-45, US 99-46 and US 99-47 from Cross 97-3144, and US 99-48, US 99-49, US 99-50, US 99-51 and US 99-52 from Cross 97-3146, based on their relatively larger stalk diameter, higher Brix and inheritance of the RAPD marker. The hybrid nature of these selected progeny was verified with sugarcane microsatellite markers. This is the first report of the development of *Saccharum* hybrids with the cytoplasm of *S. spontaneum* for breeding purpose through a combination of conventional and molecular breeding approaches. Availability of these F_1 hybrids could enhance the genetic diversity of *Saccharum* germplasm and has enabled sugarcane geneticists and breeders to explore the possible contribution of *S. spontaneum* cytoplasm in the development of new sugarcane cultivars.

Keywords: microsatellite; RAPD; *Saccharum spontaneum* cytoplasm; sugarcane

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Introduction

Elite sugarcane clones (*Saccharum* L. spp. hybrids) are believed to be interspecific hybrids derived principally from *S. officinarum* L. (noble cane) (Linnaeus, 1753; Grassl, 1969), *S. robustum* Brandes and Jesw. ex Grassl (Grassl, 1946; Price, 1960; Arceneaux, 1967) and *S. spontaneum* L. (Linnaeus, 1771). These are complex aneu-polyploids with chromosome numbers up to $2n = 120$ (Burner and Legendre, 1993a; D'Hont *et al.*, 1994; Burner, 1997). All sugarcane clones cultivated around the world to date have their cytoplasm derived from the noble cane. Based on pedigree analysis of 114 cultivars, Arceneaux (1967) reported that the genetic base of sugarcane cultivars was severely limited. While as many as 19 clones of *S. officinarum* were found in the genealogies of these sugarcane cultivars, only four *S. spontaneum* clones were represented in the nuclear genome. Recent molecular data further confirmed that elite sugarcane clones had little genetic diversity in their nuclear genome (Harvey *et al.*, 1994; Huckett and Botha, 1995) and no detectable chloroplast diversity due to their cytoplasmic monoculture from *S. officinarum* (D'Hont *et al.*, 1993; Al-Janabi *et al.*, 1994; Melloto-Passarin *et al.*, 2004).

Wild clones of *S. sponotaneum* are genetically diverse, extremely vigorous, and hybridize readily with most other elite (commercial or near-commercial) and exotic sugarcane relatives (Price, 1957; Artschwager and Brandes, 1958; Chu *et al.*, 1962; Rao and Vijayalakshmi, 1963; Dunckelman and Breaux, 1969; Kandasami *et al.*, 1983; Nagatomi and Ohshiro, 1983; Tai, 1989; Burner and Legendre, 1993a; Tai *et al.*, 1995; Pan *et al.*, 2004). These clones possess excellent stubble vigour and longevity, morphological and cytological variability, and disease and insect resistance (Dunckelman and Legendre, 1982; Sreenivasan *et al.*, 1987). Since Arceneaux's report (1967), sugarcane breeders have made intensive use of the nuclear genome of *S. spontaneum* germplasm (Dunckelman and Legendre, 1982; Miller and Tai, 1992; Burner and Legendre, 1993a, b). To our knowledge, there has been no report on the use of the cytoplasmic genome of *S. spontaneum* clones in sugarcane breeding. Neither has any genetic stock with *S. spontaneum* cytoplasm ever been released. In conventional sugarcane breeding, clones of either elite cultivars or *S. officinarum* are typically used as the maternal parents because they tend to be less male-fertile than the clones of *S. spontaneum*. More importantly, *S. officinarum* transfers $2n + n$ gametes to progeny of these types of crosses (Dunckelman and Legendre, 1982; Burner and Legendre, 1993b). *Saccharum spontaneum* clones are also considered noxious weeds with substantial self-fertilization and vigorous rhizomes (Tai *et al.*, 1994). Sugarcane breeders are advisedly hesitant to introduce to the field a population that may contain selfed progeny of *S. spontaneum*. The lack of nuclear or cytoplasmic sterility and impracticality of hand emasculation due to tiny floret size and the presence of callus hairs in all *Saccharum* species are probably the third reason. Lastly, there have been no reliable species-specific molecular markers available for sugarcane breeders until recently (D'Hont *et al.*, 1995; Piperidis *et al.*, 2000; Pan *et al.*, 2001, 2003a, b).

Prior to the discovery of molecular markers, F_1 progeny were selected based solely on phenotypic evaluations in Louisiana (Dunckelman and Legendre, 1982; Burner and Legendre, 1993b). This type of conventional selection is not efficient as many, if not all, economic traits are quantitative in nature, particularly in the case of F_1 hybrids where selection decisions are often subjective and based on morphology of genotypes evaluated for a year or less in non-replicated plots in the early stage of selection (Tai, 1989; Burner and Legendre, 1993b; Burner, 1997). Hybrid selection is also compromised as F_1 and selfed progeny share similar morphology (Gill and Grassl, 1986). Clearly, objectivity is required to verify hybrids for proper assignment of parentage, especially those derived through *S. spontaneum* cytoplasm due to regulatory concerns. Since the mid-1990s, several molecular marker types, both species- and trait-specific, have been developed for use in sugarcane breeding. These include PCR markers from the 5S-rDNA spacer region (D'Hont *et al.*, 1995; Besse *et al.*, 1996; Piperidis *et al.*, 2000; Pan *et al.*, 2001), RFLP markers (Ming *et al.*, 2002), RAPD markers (Sobral and Honeycutt, 1993; Harvey *et al.*, 1994; Msomi and Botha, 1994; Huckett and Botha, 1995; McIntyre and Jackson, 1995; Sills *et al.*, 1995; Harvey and Botha, 1996; Mudge *et al.*, 1996; Burner *et al.*, 1997; Pan *et al.*, 1997, 2003b), AFLP markers (Besse *et al.*, 1998; Hoarau *et al.*, 2001) and microsatellite markers (Cordeiro *et al.*, 2000, 2001, 2003; da Silva, 2001; Pan *et al.*, 2003a, b).

While PCR markers from the 5S-rDNA spacer region were suited to identify intergeneric sugarcane hybrids derived from *Erianthus* and *S. giganteum*, these markers were unable to differentiate among elite sugarcane clones and closely related wild species, such as *S. officinarum* and *S. spontaneum* (D'Hont *et al.*, 1995; Besse *et al.*, 1996; Pan *et al.*, 2000, 2001; Piperidis *et al.*, 2000). Other marker types such as RAPDs or microsatellites, in contrast, show a great deal of genetic variability among these taxa and are therefore more suited to discriminate between inter-specific or inter-clonal hybrids. The objective of this study was to produce and select F_1 hybrids from *S. spontaneum* × elite sugarcane clones using a combination of conventional and molecular approaches.

Materials and methods

Crossing procedure

Crossing, seed processing, germination and seedling transplanting were conducted according to Dunckelman and Legendre (1982). Two inter-specific crosses were made at USDA-ARS, Sugarcane Research Unit, Houma, LA in the autumn of 1997 between a *S. spontaneum* clone, Djatiroto, and elite clones, LCP 85-384 (Milligan *et al.*, 1994) (Cross 97-3144) and CP 62-258 (JD Miller, personal communication) (Cross 97-3146). In Cross 97-3144, two tassels of Djatiroto were pollinated by four tassels of LCP 85-384. In Cross 97-3146, one tassel of Djatiroto was pollinated by two tassels of CP 62-258. Prior to crossing, dehiscing flowers in the distal region and immature flowers in the proximal region of the three maternal tassels were removed, keeping only the florets in the middle region of the tassels. These tassels were emasculated by submersion into a 45°C circulating water bath for 10 min, following a protocol modified from Machado *et al.* (1995) and Nagai (1984). Crosses were set up in isolation cubicles immediately after hot-water treatment. After 4 weeks, seeds were harvested, dehumidified at 37°C for 3 days and stored at −20°C. Viable seeds per gram were obtained by germinating 0.5 g of seeds and counting the number of seedlings 2 weeks after germination (Dunckelman and Legendre, 1982).

Nursery, morphological observation and extraction of total nucleic acids

In April 1998, about 130 seedlings from each cross were transplanted at 0.46 m intra-row and 1.8 m inter-row spacing in a non-replicated nursery at USDA-ARS, Sugarcane Research Unit, Houma, LA. The nursery had been fallowed for several years to minimize volunteer sugarcane plants. In the first-ratoon crop in 1999, 96 progeny from each cross were measured for stalk diameter and juice Brix (percentage soluble solids). Leaf whorls were collected from the plants of Djatiroto, CP 62-258, LCP 85-384 and numbered progeny for total nucleic acid extraction according to Pan *et al.* (2000).

RAPD-PCR

Primer OPA-11 (5′-CAATCGCCGT, Operon Technologies Inc., Alameda, CA) was chosen based on a previous study (Pan *et al.*, 2004). Sources of the primer, PCR reaction mix, thermal cycling programme and agarose gel electrophoresis were according to Burner *et al.* (1997). Gel images were taken on a NucleoVision Gel Documentation Station using GelExpert™ software (Nucleotech Corp., Hayward, CA). The gel images were manually scored for the presence and absence of the OPA-11-366 RAPD marker.

Selection of putative F_1 hybrids

Putative F_1 hybrids were selected based on phenotypic traits and the RAPD marker. Phenotypic traits included stalk diameter and juice Brix (percentage soluble solids). Stalk diameter (mm) of the fifth internode from the ground was measured from three stalks per progeny both in June and November 1999. Stalk weight (g) was measured from 10 stalks per progeny in December 2000. Juice Brix values were determined by a hand refractometer on juice extracted from the fifth internode from the ground in September and November 1999 and by an RFM-190 refractometer (Bellingham and Stanley, Lawrenceville, GA) on press mill juice in December 2000. Fibre content (%) was obtained in December 2000 using the method described in Legendre (1992). The frequency distribution of progeny with or without the OPA-11-366 marker was tested using χ^2 tests (Steel and Torrie, 1980).

Microsatellite amplification and analysis

LCP 85-384, CP 65-258 and the 10 putative F_1 hybrids were fingerprinted with the three polymorphic sugarcane microsatellites, SMC334BS, SMC336BS and MCSA068G08 (Cordeiro *et al.*, 2003; Pan *et al.*, 2003b) (Table 1). PCR reactions, thermal cycling programmes and fragment analysis were conducted according to Pan *et al.* (2003a) using either the ABI PRISM 310 genetic analyser system (Applied Biosystems, Foster City, CA) or the CEQ8000 genetic analyser system (Beckman-Coulter, Fullerton, CA).

Results and discussion

Cross 97-3144 produced 4.7 g of seeds with 338 viable seeds per gram. The total number of seeds from Cross 3146 weighed 2.4 g with 166 viable seeds per gram. These were considered to be within the range of typical seed yields from regular sugarcane crosses. Pollen viability of Djatiroto was not tested after hot-water treatment, although pollen stainability in I_2-KI was 91–100%. Under normal conditions, sugarcane clones with high pollen stainability tend to have a high seed set and are rated by breeders as 'males' whereas those with low pollen stainability are rated as 'females' (Burner and Legendre, 1993b). However, this assumption may not be true when tassels are subject to a hot-water treatment. As this

Table 1. A description of the sugarcane microsatellite markers used to confirm hybrids[a]

Microsatellite	Repeat motif		Primer sequence (5′ → 3′)	Optimum T_m/T_a (°C)	Dye
SMC334BS	$(TG)_{36}$		Under confidentiality agreement[b]	53.4/58	6-FAM™
SMC336BS	$(TG)_{23}(AG)_{19}$		Under confidentiality agreement[b]	52.7/58	HEX™
MCSA068G08	$(CAG)_6$	Forward	CTA ATG CCA TGC CCC AGA GG	57.2/62	NED™
		Reverse	GCT GGT GAT GTC GCC CAT CT		

[a]For each microsatellite, the repeat motif and length are shown. Where unencumbered by confidentiality agreements, the primer sequence is given. The melting (T_m) and annealing temperatures (T_a) were calculated using the software program MacVectorTM 6.0.
[b]Contact R. J. Henry (rhenry@scu.edu.au) regarding agreement.

was an initial trial using a *S. spontaneum* clone as the maternal parent, the effect of this emasculation method was unknown. Alternative treatments of 50°C for 4.5–10 min have been reported (Nagai, 1984; Machado *et al.*, 1995), however, in our hands, seed yield following hot-water treatment varied widely regardless of parental clone, water temperature and length of treatment. It is also known that the ambient temperature during tassel elongation and anthesis has a significant effect on male fertility (Dunckelman and Legendre, 1982). Sugarcane breeders often depend on cool ambient air temperatures to minimize the pollen fertility (Pan *et al.*, 2003b). A systematic study on the effect of hot-water treatment may be required to better control the issue of male fertility of sugarcane tassels used as females.

Of the 96 progeny from Cross 97-3144 (Djatiroto × LCP 85-384), 50 (52.1%) inherited the paternal OPA-11-366 marker (data not shown). An example is shown in Fig. 1, where 11 out of the 22 progeny have inherited the OPA-11-366 RAPD marker (indicated with an arrowhead) (lanes 1, 2, 4, 7, 8, 10,12, 16, 17, 20 and 21). From Cross 97-3146 (Djatiroto × CP 62-258), only 36 of the 96 progeny (37.5%) inherited the OPA-11-366 marker. χ^2 tests indicated a 1:1 segregation (with: without the RAPD marker) among the progeny from Cross 97-3144 but a 1:2 segregation (with: without the RAPD marker) for Cross 97-3146. It is unclear why more progeny from Cross 97-3144 inherited the RAPD marker than those from Cross 97-3146, despite random seed sampling, germination and seedling transplanting. Seeds from several other crosses often contained selfs and contaminated progeny (Pan *et al.*, 2003b). However, this was not an issue with this study as the main focus was to produce and select F_1 hybrids with their cytoplasm derived from *S. spontaneum*. The presence of the paternal RAPD marker in a progeny was sufficient to consider it an F_1 hybrid, but those without the RAPD marker may also be hybrids.

The ratio, however, indicates that both elite parents, LCP 85-384 and CP 62-258, are heterozygous for the OPA-11-336 marker. This finding is consistent with a number of reports that RAPD bands are sexually transmissible in crop species (Welsh *et al.*, 1991; Lanham *et al.*, 1992; Marshall *et al.*, 1994; Huckett and Botha, 1995). Huckett and Botha (1995) have demonstrated that RAPD bands are transmitted in a Mendelian fashion among elite sugarcane crosses and are useful for validating and tracing parentage. A limitation of RAPD, however,

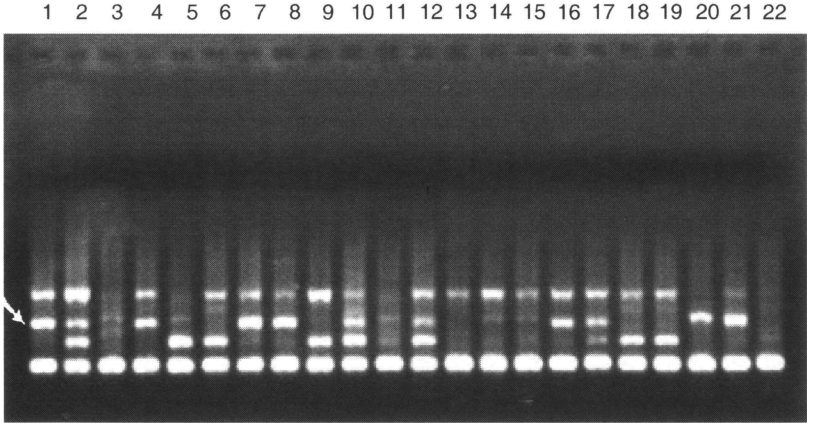

Fig. 1. Fractionation of amplified RAPD-PCR products from 22 progeny (nursery stake numbers 23–44, from left to right) of Cross 97-3144 (Djatiroto × LCP 85-384) in a 1.5% Synergel/agarose binary gel containing 0.5 μg/ml ethidium bromide. The arrow marks the RAPD marker OPA-11-336. The typical banding pattern of OPA-11-336 for LCP 85-384 is in lane 2 and for Djatiroto in lane 22.

is that they are usually dominant in other crops and, as such, homozygotes cannot be distinguished from heterozygotes without progeny tests (Sobral and Honeycutt, 1994).

Substantial variation was observed for stalk diameter and Brix within each progeny population (data not shown). Readings for the two elite parental clones and the *S. spontaneum* clone were obtained from greenhouse-grown stalks due to their non-availability in the nursery. Mean stalk diameters of 11.3, 17.8 and 22 mm and mean Brix values of 15.0%, 17.2% and 18.5% were observed for Djatiroto, LCP 85-384 and CP 62-258, respectively (Table 2). In November 1999, five F_1 hybrids that carried the RAPD marker were selected from each cross, based on their relatively larger stalk diameters and Brix values (Table 2). These were US 99-43, US 99-44, US 99-45, US 99-46 and US 99-47 from Cross 97-3144, and US 99-48, US 99-49, US 99-50, US 99-51 and US 99-52 from Cross 97-3146. Vegetative cuttings of these selections were made and the resulting plantlets were either included in the backcrossing programme or transplanted into the field for evaluation. Mean stalk weight (g), juice Brix (%) and fibre (%) values of the 10 selected progeny are presented in Table 2.

Overall, the presence of the RAPD marker was not correlated with either higher or lower values of stalk diameter or Brix (data not shown). The main use of the marker was to aid in the selection of the best F_1 individual for further genetic improvement. Stalk diameter is a moderately heritable trait (Milligan *et al.*, 1990) that is also positively correlated with kg-Brix (Miller, 1977). Increasing sucrose content is still a top objective for sugarcane breeders. During 2000–2002, several BC_1 and BC_2 populations of these *Saccharum* F_1 hybrids were produced with a *S. spontaneum* cytoplasm. These populations have been established in the breeding nursery for agronomic evaluation and selection. Efforts are also being made for further backcrossing of selected BC_2 clones with elite sugarcane clones for continued genetic improvement.

The F_1 hybrid nature of all 10 progeny were strongly supported by their microsatellite fingerprints (Tables 3 and 4). One example is shown in Fig. 2. For Cross 97-3144, the *S. spontaneum* clone Djatiroto produced six unique microsatellite alleles (4-159, 6-159, 6-173, 6-175, 8-186 and 8-191) that were not found in LCP 85-384; LCP 85-384 produced four unique microsatellite alleles (4-161, 6-171, 8-180 and 8-194) that were not found in *S. spontaneum* clone Djatiroto. All five F_1 hybrids inherited at least one allele that was unique to its maternal parent. In addition, two of the paternal alleles, 6-171 and 8-180, were also present in these F_1 hybrids. Allele 4-161 was inherited by US 99-43, US 99-44 and US 99-45 and allele 8-194 was inherited by US 99-43 and US 99-46. None of the progeny produced an allele of non-parental origin (Table 3). Similarly, for Cross 97-3146, Djatiroto had seven unique alleles (4-162, 6-161, 6-169, 6-175, 8-177, 8-186 and 8-188) whereas CP 62-258 had five unique microsatellite alleles (4-154, 4-166, 6-155, 8-180 and 8-199). Each F_1 hybrid inherited at least three of the Djatiroto-specific alleles. Two of the male-specific alleles, 4-166 and 8-180, were present in

Table 2. Description of 10 *Saccharum* F_1 hybrids in the cytoplasm of a *Saccharum spontaneum* clone Djatiroto

US assignment	Cross No.[a]	Field tag No.	Stalk diameter (mm)[b]	Stalk weight (g)[c]	Juice Brix (%)[d]	Fibre (%)[e]	OPA-11-366[f]
LCP 85-384			17.8	1193.0	17.2/21.4	11.3	+
CP 62-258			22	N/A	18.5	N/A	+
Djatiroto			11.3	N/A	15[g]	N/A	−
US 99-43	97-3144	3	13.7/20.5	816.5	17.4/18.0/13.9	14.9	+
US 99-44	97-3144	29	17.7/13.2	544.3	18.4/16.4/15.7	20.7	+
US 99-45	97-3144	43	12.7/13.9	272.2	17.6/17.8/15.9	12.1	+
US 99-46	97-3144	91	15.0/12.7	499.0	20.0/19.0/15.1	15.9	+
US 99-47	97-3144	95	16.3/13.6	317.5	18.2/15.1/14.1	15.4	+
US 99-48	97-3146	146	16.3/15.2	680.4	17.0/18.3/17.3	22.1	+
US 99-49	97-3146	154	16.0/17.5	861.8	17.6/19.0/14.7	15.4	+
US 99-50	97-3146	167	16.7/17.7	544.3	14.0/17.8/14.2	20.9	+
US 99-51	97-3146	182	17.0/16.1	771.1	16.0/17.0/14.7	15.9	+
US 99-52	97-3146	205	17.7/18.9	635.0	16.8/17.5/15.6	16.5	+

[a]Cross 97-3144 (Djatiroto × LCP 85-384); Cross 97-3146 (Djatiroto × CP 62-258).
[b]Mean of two measurements in: June 1999/November 1999.
[c]Mean of 10 stalks in December 2000.
[d]Three readings in: September 1999/November 1999/December 2000.
[e]Mean of measurements in December 2000.
[f]+, presence; −, absence.
[g]Measurements were made on stalks grown in cans.

Table 3. Confirmation of sugarcane inter-specific hybrids from Cross 97-3144 using microsatellite markers

	SSR allele[a]															
Clone	4-145	4-159	4-161	6-159	6-166	6-169	6-171	6-173	6-175	8-177	8-180	8-183	8-186	8-188	8-191	8-194
Djatiroto[b]	1	1	0	1	1	1	0	1	1	1	0	1	1	1	1	0
LCP 85-384[c]	1	0	1	0	1	1	1	0	0	1	1	1	0	1	0	1
US 99-43	1	0	1	1	0	1	1	0	0	1	1	0	0	1	1	1
US 99-44	1	0	1	1	1	1	1	0	0	1	1	1	1	1	0	0
US 99-45	1	1	1	1	0	0	1	1	1	1	1	1	0	1	1	0
US 99-46	1	1	0	0	1	1	1	1	1	1	1	1	1	1	0	1
US 99-47	1	0	0	0	1	1	1	0	1	1	1	1	0	1	0	0

Fragment analysis was conducted with the ABI PRISM 310 genetic analyser system.
[a]An allele is represented as #-### where the first # represents a particular microsatellite (4 = SMC334BS; 6 = SMC336BS; 8 = MCSA068G08) and the last three ### represent allele size in base pairs.
[b]Dajiroto: a *Saccharum spontaneum* clone as the maternal parent.
[c]LCP 85-384: an elite sugarcane cultivar as the paternal parent.

Table 4. Confirmation of sugarcane inter-specific hybrids from Cross 97-3146 using microsatellite markers

	SSR allele[a]															
Clone	4-150	4-154	4-162	4-166	6-155	6-161	6-169	6-171	6-175	6-177	8-177	8-180	8-183	8-186	8-188	8-191	8-199
Djatiroto[b]	1	0	1	0	0	1	1	1	1	1	1	0	1	1	1	1	0
CP 62-258[c]	1	1	0	1	1	0	0	1	0	1	0	1	1	0	0	1	1
US 99-48	1	1	1	1	1	1	1	1	0	0	0	1	1	1	0	1	1
US 99-49	1	1	1	1	1	0	1	0	1	1	0	1	0	1	1	1	0
US 99-50	1	0	1	1	0	1	1	1	0	1	1	1	1	0	0	1	1
US 99-51	1	1	0	1	1	0	1	1	0	1	1	1	1	0	1	1	1
US 99-52	1	1	1	1	0	0	1	1	0	1	0	1	1	1	1	1	1

Fragment analysis was conducted with the CEQ8000 genetic analyser system.
[a]An allele is represented as #-### where the first # represents a particular microsatellite (4 = SMC334BS; 6 = SMC336BS; 8 = MCSA068G08) and the last three ### represent allele size in base pairs.
[b]Dajiroto: a *Saccharum spontaneum* clone as the maternal parent.
[c]CP 62-258: an elite sugarcane cultivar as the paternal parent.

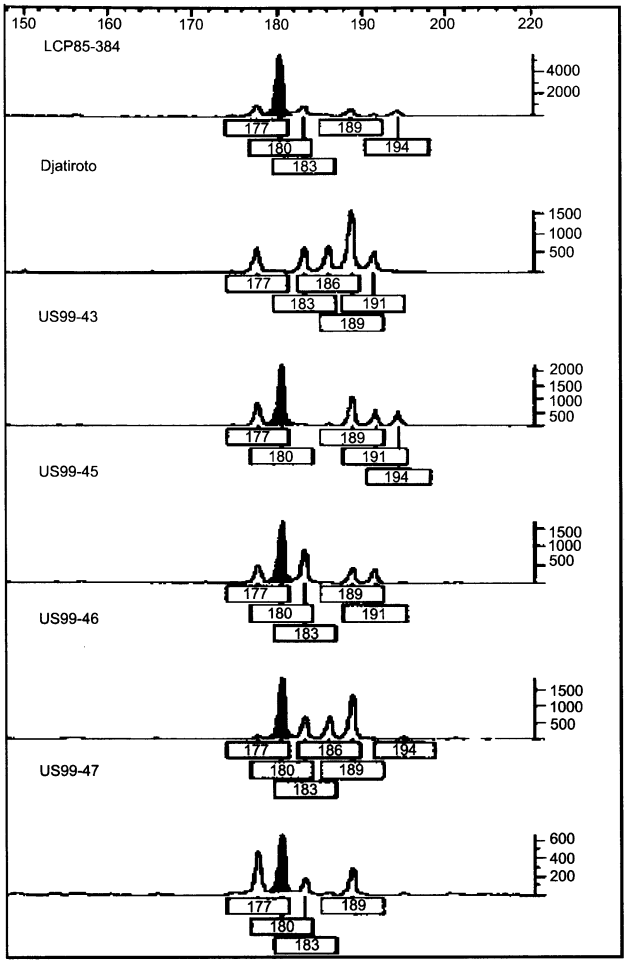

Fig. 2. Sugarcane microsatellite MCSA068G08 fingerprints for clone LCP 85-384, *Saccharum spontaneum* clone Djatiroto and four F_1 progeny US 99-43, US 99-45, US 99-46 and US 99-47. The microsatellite products were labelled during PCR amplification with the fluorescent dye NED™, separated by capillary electrophoresis on the ABI PRISM 310 genetic analyser. The sizes of the microsatellites were determined by the GeneScan and Genotyper software (Applied Biosystems) according to size standard GeneScan®-500 size standards (TAMRA). The top scale shows fragment sizes in base pairs; the *y*-axis, shown on the right, depicts the fluorescence intensity (or relative yield for each amplified microsatellite). The male-specific allele 8-180 is shown in solid peaks.

all five F_1 hybrids. Allele 4-154 was inherited by all selected hybrids except US 99-50; allele 6-155 was inherited by US 99-48, US 99-49 and US 99-51, and allele 8-199 was inherited by all selected hybrids except US 99-49. Again, none of the progeny produced an allele that was of non-parental origin (Table 4). Although there were size shifts for the apparent same allele between the two genetic analyser systems, the sizing was consistent between different runs when using the same system.

In summary, interspecific *Saccharum* hybrids in the cytoplasm of *S. spontaneum* were developed and confirmed with the aid of both conventional and molecular breeding approaches. The presence of both RAPD and microsatellite alleles from the paternal parents in these clones confirmed their hybrid nature. These F_1 hybrids were backcrossed as maternal parents with elite sugarcane clones to produce BC_1 and BC_2 lines for evaluation and selection. In 2003, more than 40 BC_1 lines were selected and advanced to first line trials; 12 BC_2 lines will be evaluated for selection in 2004. The addition of this new cyto-type of *Saccharum* germplasm to sugarcane breeding has allowed our sugarcane geneticists and breeders to explore the possible contribution of *S. spontaneum* cytoplasm in the development of new sugarcane cultivars.

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