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MOLECULAR GENOTYPING OF SUGARCANE CLONES WITH MICROSATELLITE DNA MARKERS

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ABSTRACT - Molecular genotypes of 27 sugarcane clones (*Saccharum* hybrids) were produced with nine sugarcane microsatellites. A total of 52 alleles were identified using a capillary electrophoresis system with 41 alleles displaying varying degrees of polymorphism and the remaining 11 being monomorphic. There were eight alleles for sugarcane microsatellite SMC286CS, five for SMC334BS, eight for SMC336BS, four for SMC713BS, five for mSSCIR5, five for mSSCIR33, five for MCSA042E08, four for MCSA053C10, and eight for MCSA068G08. Presence or absence of these 52 alleles from a clone allowed the assignment of its arbitrary microsatellite genotype. The genetic relatedness among these clones was assessed using the CLUSTAL W algorithm with DNAMAN® software based on their arbitrary genotypes. With the exception of four clones, CP 70-321, HoCP 91-555, L 97-137 and Q124, six groups of clones were identified that shared at least 76% homology between their microsatellite genotypes. The software program also produced a bootstrapped phylogenetic tree with branch patterns that in general coincided with the putative pedigrees of these clones. The derivation of molecular genotypes such as these has enabled sugarcane geneticists and breeders to verify the genetic pedigrees and purity of their sugarcane populations. These microsatellite genotypes can also aid in progeny selection and facilitate studies on allele transmission in this aneu-polyploidy crop.

KEY WORDS: Genetic diversity; Marker assisted selection; Phylogenetic trees.

INTRODUCTION

The cultivated forms of sugarcane (*Saccharum* hybrids) are believed to be aneu-polyploid or polyploid derivatives from *Saccharum officinarum* L. (noble cane) (Linnaeus, 1753; Grassl, 1969), *S. barberi* Jeswiet (Brandes, 1958), *S. sinense* Roxb. (Brandes, 1958; Roxburgh, 1819), *S. robustum* Brandes and Jeswiet ex Grassl (Grassl, 1946), and *S. spontaneum* L. (Linnaeus, 1771). Nonetheless, almost all sugarcane cultivars grown in the world today can be traced back to a few common progenitor clones (D'Hont *et al.*, 1995; Tew, 2000). Sugarcane breeders attempt to expand the genetic base of sugarcane cultivars by introducing agriculturally desirable traits from related wild species through introgression or basic breeding (Burner and Legendre, 1993; Legendre and Breaux, 1983; Tai, 1989). Such traits include increased sugar content, enhanced vigor and ratooning ability, disease and insect resistance, and cold tolerance. However, the efficiency of introgression in sugarcane has been low due to the technical difficulties in crossing and selection. Sugarcane flowers are miniscule, fragile, and perfect (Moore, 1987). Hand emasculation is impractical, and treating maternal inflorescence by immersion in either hot water (Divinagracia, 1980; Heinz and Tew, 1987; Krishnamurthi, 1977) or alcohol (Soeprijanto, 1989), or by exposure to cool atmosphere temperatures (Jimmy Miller, personal communication) does not guarantee complete pollen sterility. This often results in progeny population being mixtures of selfs and hybrids. Since visual selection for promising hybrids among these progeny populations is unreliable (Divinagracia, 1980; Heinz and Tew, 1987; Tai, 1989), there has been an increasing demand for both species- and trait-specific DNA markers in sugarcane breeding (Pan, 2001; Pan *et al.*, 2001).

A number of reports on various sugarcane molecular markers are available and include restriction fragment length polymorphism (RFLP) (Besse and McIntyre, 1996; Besse *et al.*, 1996; Burnquist, 1991;

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D'HONT *et al.*, 1993; GLASZMANN *et al.*, 1989, 1990; GRIVET *et al.*, 1996; MING *et al.*, 1998), random amplified polymorphic DNA (RAPD) (BURNER *et al.*, 1997; HARVEY and BOTHA, 1996; PAN *et al.*, 1997; PAN *et al.*, 2004b), amplified fragment length polymorphism (AFLP) (BESSE *et al.*, 1998), and genus-specific polymerase chain reaction (PCR) markers from the 5S rRNA locus (D'HONT *et al.*, 1995; PAN *et al.*, 2000; PAN *et al.*, 2001; PIPERIDIS *et al.*, 2000). A few RAPD fingerprints also were reported on sugarcane clones (HARVEY and BOTHA, 1996; PAN *et al.*, 1997). Microsatellite markers, also known as variable number tandem repeats (VNTRs) (JEFFREYS *et al.*, 1985), or simple sequence repeats (SSRs), or short tandem repeats (STRs) (WEBER and MAY, 1989; EDWARDS *et al.*, 1991) are short DNA fragments that contain various numbers of tandem repeat units of di-, tri-, or tetra-nucleotide motifs (EDWARDS *et al.*, 1991; POLYMEROPOULOS *et al.*, 1991). Although numerous reports on microsatellites in other crop species (for review, see CORDEIRO *et al.*, 2000, 2001) are available, only a few have been reported in sugarcane (CORDEIRO and HENRY, 2001; CORDEIRO *et al.*, 2000, 2001, 2003; DA SILVA, 2001; JANNOO *et al.*, 2001; PAN *et al.*, 2003; PIPERIDIS *et al.*, 2001). Recently, about 260 primer pairs were designed from microsatellite-harboring genomic sequences (CORDEIRO *et al.*, 2000), and an additional 35 primer pairs were developed based upon a private sugarcane EST database (CORDEIRO *et al.*, 2001). Two parallel studies were just reported, one on SSR genotypes of twenty-five Florida sugarcane clones (PAN *et al.*, 2003) and the other on the assessment of genetic diversity among 66 accessions of the genera *Saccharum*, Old World *Erianthus* Michx. Sect. *Ripidium*, North American *E. giganteus* (*S. giganteum*), *Sorghum* and *Miscanthus* in sugarcane germplasm using six SSR markers (CORDEIRO *et al.*, 2003).

The objectives of this study were to: 1) produce molecular genotypes for 27 sugarcane clones; 2) assess the extent of genetic variability among these clones based on these genotypes, and 3) validate the applicability of these microsatellite genotypes in sugarcane breeding, in particular verification of the pedigrees of sugarcane clones and genetic purity of their progeny populations.

MATERIAL AND METHODS

Plant Material and Nucleic Acid Extraction

Twenty-seven sugarcane clones were genotyped with microsatellite markers using a capillary electrophoresis system. Each clone is a unique line of sugarcane plants that are vegetatively propagated from a single seed-derived plant. A sugarcane clone can either be a variety or a breeding line. These included nine elite varieties grown in Louisiana: CP 65-357, CP 70-321, CP 72-370, HoCP 85-845, HoCP 91-555, LCP 82-89, LCP 85-384, LCP 86-454, and LHo 83-153, an Australian variety Q124, a French variety R570, and 16 non-varietal clones HoCP 96-509, HoCP 96-540, HoCP 97-606, HoCP 97-609, HoCP 98-718, HoCP 98-734, HoCP 98-741, HoCP 98-771, HoCP 98-776, HoCP 98-778, HoCP 98-781, L 95-462, L 97-128, L 97-137, L 98-207, L 98-209. LCP 85-384 (MILLIGAN *et al.*, 1995) is the current leading variety in Louisiana accounting for 81% of the total sugarcane crop acreage. HoCP 96-540 (TEW *et al.*, in press) and L 97-128 (Ken Gravois, Louisiana State University, personal communication) are two new clones with potential for commercial release in Louisiana. Eight of these clones that are claimed to be the progeny of other clones included in this study are listed in Table 1 for pedigree verification based on microsatellite genotypes.

TABLE 1 - *Putative pedigrees of eight sugarcane clones*[‡].

Sugarcane clone	Female parent	Male parent
HoCP 96-540	LCP 86-454	LCP 85-384
HoCP 97-609	LCP 85-384	CP 70-321
HoCP 98-734	LCP 85-384	LCP 86-454
HoCP 98-771	CP 89-831†	LCP 85-384
HoCP 98-776	LCP 85-384	CP 70-1133†
HoCP 98-781	CP 89-831†	LCP 82-89
L 98-207	LCP 86-454	LCP 85-384
L 98-209	LCP 86-454	LCP 85-384

‡ Microsatellite genotypes of SMC334BS, SMC336BS and MCSA068G08 are available from other related studies for CP 89-831 (unpublished) and CP 70-1133 (PAN *et al.*, 2003).

Total nucleic acids were extracted from the leaf tissue according to PAN *et al.* (2000). The nucleic acid pellet was dried in a DNA120 SpeedVac System (Savant Instruments, Inc., Holbrook, NY) prior to re-suspension in 250 µl sterile water with RNase A (40 µg per ml). The solution was re-extracted twice with chloroform/isoamyl alcohol (24/1), and the genomic DNA re-suspended in 250 µl sterile water. DNA concentration was determined on a Perkin Elmer UV/VIS Spectrophotometer Lambda BIO10 (Foster City, CA). Genomic DNA of Q124 and R570 were gifts from J. Waldron (University of Queensland, Australia) and C. Kaye (CIRAD, France), respectively.

Primers, Reaction Mixture, and PCR Program

Nine microsatellites were selected for this study based on data of CORDEIRO *et al.* (2001) and preliminary testing (PAN, unpublished). The basic repeat unit, primer sequence, and annealing temperature are described in Table 2. Where unrestricted by confidentiality agreements, the primer sequences are also listed. The 5' end of the forward primers was labeled with one of three fluorescent phosphoramidite dyes, FAM, HEX, or TET (Applied Biosystems, Foster City, CA). PCR reactions were conducted on a GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA). The reaction volume was 25 µl containing 25 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 80 µM each of dATP, dTTP, dGTP, and dCTP, 0.2 µM each of respective forward and reverse primers, and 1 unit of *Taq* DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN).

TABLE 2 - *A description of the sugarcane microsatellite markers*†

Primer	Repeat motif		Primer Sequence (5′ → 3′)	Opt T_m/T_a (°C)
SMC286CS	$(TG)_{48}$		Under confidentiality agreement‡‡	51.7/55
SMC334BS	$(TG)_{36}$		Under confidentiality agreement‡‡	53.4/58
SMC336BS	$(TG)_{24}(AG)_{19}$		Under confidentiality agreement‡‡	52.7/58
SMC713BS	$(CAA)_6$		Under confidentiality agreement‡‡	53.9/55
MCSA042E08	$(GAT)_{13}$	Forwar Reverse	GTT GAG GGT GAA GCG GAT GG AGC CTC TGC CAC CAC TCC TC	55.1/62
MCSA053C10	$(CAG)_5$	Forward Reverse	CGA GCA TGG CGA GGA GTC CG GCA GGG CGA GGC GAG ATC AG	59.6/68
MCSA068G08	$(CAG)_6$	Forward Reverse	CTA ATG CCA TGC CCC AGA GG GCT GGT GAT GTC GCC CAT CT	57.2/62
mSSCIR5	$(GGC)_9$	Forward Reverse	GCA GCC TTG GTT CGG TCT ATG GCA TCC CTC GCC CTT CCT C	59.6/57
mSSCIR33	$(GT)_{14}(GA)_9N_6$ $(GA)_6N_8(GA)_9$	Reverse Forward	GCT CAT ATA TCT TCC TGG TC AGT GGT CTG GTG CTT TGG	48.7/57

‡ For each microsatellite, the repeat motif and length are shown. Where unencumbered by confidentiality agreements, the primer sequence is given. The melting (T_m°C) and annealing temperatures (T_a°C) were calculated using the software program MacVector™ 6.0.
‡‡ Contact GM Cordeiro or RJ Henry regarding agreement.

The PCR program was set for 95°C for 5 min; 30 cycles of 30 s at 94°C, 30 s at the annealing temperature (Table 2), 30 s at 72°C; and final extension at 72°C for 2 min. Ten µl of the PCR mixtures were tentatively examined by agarose gel (3%) electrophoresis, and if PCR reactions were proved successful, a poolplex mixture was assembled for sizing of PCR products on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) with the following run analysis conditions: Module GS STR POP4 C, 25 min run time in a 30 cm capillary-length-to-detector with a size standard GENESCAN 500-TAMRA, Local Southern Sizing Method. Each poolplex sample contained one µl each of three amplified microsatellite products with different fluorescence labels (FAM, HEX, and TET), 0.4 µl of GeneScan-500 TAMRA size standard (Applied Biosystems, Foster City, CA), and 20 µl deionized formamide (Applied Biosystems, Foster City, CA). Capillary electrophoresis data were captured by GeneScan software as GeneScan files (Applied Biosystems, Foster City, CA).

Construction of Microsatellite Genotypes and Homology Trees

The GeneScan files from the capillary electrophoresis system were analyzed with the GenoTyper® software (v3.7) (Applied Biosystems, Foster City, CA). An allele represents a unique DNA fragment that produces a measurable fluorescence peak during capillary electrophoresis. Allele sizes were assigned to peaks sizable by GenoTyper® except those considered either as "stutters", which generally differed structurally from the associated allele by a single repeat unit (Levinson and Gutman, 1987; Schlotterer and Tautz, 1992; Weber and May, 1989), or "pull-ups", which were false, irregularly shaped peaks due to poolplexing (E.A. Casanova, Applied Biosystems Inc., personal communication). The sizes reported here were, in general, one nucleotide larger than those of the genomic alleles due to an extra adenine nucleotide added to the 3′ ends of the PCR products by the polymerases (Clark, 1988; Holton and Graham, 1991; Hu, 1993; Marchuk *et al.*, 1991; Mead *et al.*, 1991).

Genotypes were constructed with the GenoTyper® software by exporting data into a tabular format or by an electropherogram. Presence of a microsatellite allele was arbitrarily given a score of "A" while a "C" indicated its absence. The distribution of alleles from all the nine microsatellites in a clone was then combined into an arbitrary sequence of As or Cs to give rise to its microsatellite genotype. The resulting 27 arbitrary sequences were treated as DNA sequences which were aligned with the CLUSTAL W algorithm (Feng and Doolittle, 1987; Thompson *et al.*, 1994) and a multiple sequence editor (MASED) of the DNAMAN® software package (Lynnon Biosoft, Vaudreuil, Quebec, Canada) (Pan *et al.*, 2000, 2003, 2004) to generate a pairwise homology/distance matrix and homology/phylogenetic trees with bootstrapping (confidence) values. The algorithm produces initially a homology matrix based on the observed divergence method, and then applies a correction method of Jukes and Cantor (1969) to align progressively all sequences according to the branching order in the phylogenetic tree using dynamic alignment method. The parameters set for the dynamic multiple sequence alignment were "10" for gap open penalty, "5" for gap extension penalty, and "40%" for delay divergent sequences. Bootstrapping values were obtained upon 1,000,000 trials.

RESULTS

Description of SSR Alleles and Genotypes

In general, the nine microsatellites were all polymorphic although MCSA053C10, MCSA042E08, and mSSCIR5 showed a lesser degree of polymorphism

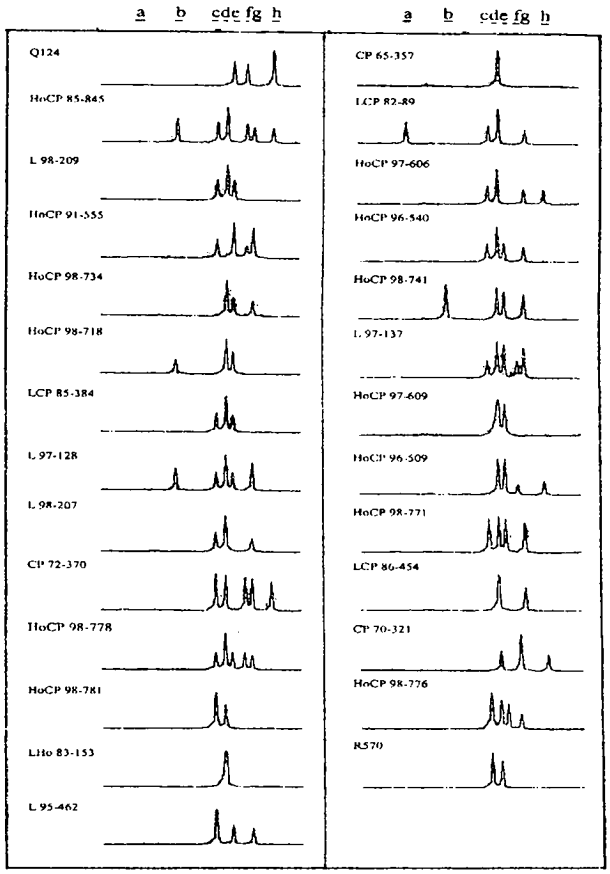

FIGURE 1 - Allele distribution of microsatellite SMC336BS in 27 sugarcane clones. Relative positions of the eight alleles (Table 3) are indicated on the top: a (allele 36-141), b (allele 36-153), c (allele 36-167), d (allele 36-169), e (allele 36-171), f (allele 36-175), g (allele 36-177), and h (allele 36 183). An allele is represented by the prefix number 36 representing the microsatellite SMC336BS followed by the allele size in base pairs.

by producing 9 of the 11 monomorphic alleles (see below). There were a total of 52 alleles and the number of alleles amplified from each microsatellite varied. There were eight alleles (86-129, 86-132, 86-135, 86-139, 86-142, 86-144, 86-146, and 86-149) for SMC286CS, five (34-145, 34-149, 34-160, 34-162, and 34-164) for SMC334BS, eight (36-141, 36-153, 36-167, 36-169, 36-171, 36-175, 36-177, and 36-183) for SMC336BS, four (13-119, 13-357, 13-360, and 13-369) for SMC713BS, five (42-123, 42-135, 42-151, 42-155, and 42-197) for MCSA042E08, four (53-143, 53-147, 53-150, and 53-153) for MCSA053C10, eight (68-177, 68-180, 68-183, 68-186, 68-189, 68-191, 68-194, and 68-200) for MCSA068G08, five (R5-145, R5-168, R5-365, R5-373, and R5-378) for mSSCIR5, and five (33-297, 33-320, 33-326, 33-330, and 33-335) for mSSCIR33. Eleven alleles, namely, 68-180, 13-119, 53-143, 53-147, 53-150, 42-123, 42-151, 42-155, 42-197, R5-373, and R5-378, were found in every clone. The remaining 41 alleles were polymorphic. Capillary electrophoregrams of the microsatellite SMC336BS for the 27 sugarcane clones are shown in Fig. 1.

The microsatellite genotypes are displayed in Table 3. Each sequence may be regarded as the genotype for the corresponding clone, be it a combination of either the 52 alleles from all nine microsatellites, or alleles from a particular microsatellite. As an example of the latter case, the genotype of the variety CP 65-357 derived from the microsatellite SMC334BS is "AACCC" where the two As represent the alleles 34-145 and 34-149 (see the row "CP 65-357", columns "34-145" through "34-164" in Table 3). Similarly, the genotype of the variety LCP 85-384 derived from the microsatellite SMC336BS is "CCAAACCC" where the three As represent the alleles 36-166, 36-169, and 36-171 (see the row "LCP 85-384", columns "36-141" through "36-183" in Table 3). Allele 36-141 was only found in clones HoCP 98-718 and LCP 82-89, while allele 36-169 was present in 25 clones except L 95-462 and Q124.

Description of the Homology/Phylogenetic Trees

Pairwise homology values ranged from 51.9% (between L 97-137 and the Australian variety Q124) to 88.5% (between L 98-209 and LCP 85-384) (homology matrix not shown). A narrower range of values was found between Louisiana clones and ranged from 59.6% (between CP 72-370 and HoCP 98-718, HoCP 91-555 and L 98-207, HoCP 97-609 and L 97-137) to 88.5% (between L 98-209 and LCP 85-384). Six groups of clones shared at least 76% homology (Fig. 2A). Group I included R570 and HoCP 98-781. Group II included HoCP 98-718 and HoCP 96-509. Group III included L 98-207, LCP 86-454, HoCP 97-606, HoCP 98-741, L 98-209, LCP 85-384, HoCP 97-609, HoCP 98-776, and HoCP 98-734. Group IV included CP 72-370, HoCP 96-540, HoCP 98-771, HoCP 98-778, HoCP 85-845, and L 97-128. Group V included L 95-462 and LCP 82-89. Group VI included LHo 83-153 and CP 65-357. Groups I and II joined at a homology level of 73%, so did Groups III, IV and V. At the same level, clone CP 70-321 joined the Group VI. Clones HoCP 91-555 and L 97-137 shared a 71% homology; while Q124 did not group with any clone at a homology level greater than 62%.

A bootstrapped phylogenetic tree is shown in Fig. 2B to depict the genetic relatedness among

TABLE 3 - *Arbitrary sequence derivation (genotype) of 27 sugarcane clones.*

† Allele code / Variety	34-145	34-149	34-160	34-162	34-164	36-141	36-153	36 167	36-169	36-171	36-175	36-177	36-183	68-177	68-180	68-183	68-186	68-189	68-191	68-194	68-200	86-129	86-132	86-135	86-139	86-142	86-144	86-146	86-149	13-119	13-357	13-360	13-369	53-143	53-147	53-150	53-153	42-123	42-135	42-151	42-155	42-197	R5-145	R5-168	R5-365	R5-373	R5-378	33-297	33-320	33-326	33-330	33-335
CP 65-357	A	A	C	C	C	C	C	C	A	C	C	C	C	A	A	A	A	A	C	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	A	A	A	A	A	A	A	A	A	A	C	C	C	C
CP 70-321	A	A	A	A	C	C	C	C	A	C	A	C	A	A	A	A	C	C	A	A	A	A	C	C	C	C	A	A	A	C	A	A	A	A	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A
CP 72-370	A	C	C	A	C	C	C	A	A	C	A	A	A	A	A	A	C	A	C	C	A	A	A	C	C	C	A	A	A	A	C	C	A	A	A	C	A	A	A	A	A	A	A	C	A	A	C	A	A	A
HoCP 85-845	A	C	C	A	C	C	A	A	A	C	A	A	A	A	A	A	A	C	A	C	A	C	A	C	C	A	A	C	A	A	A	C	A	A	A	C	A	A	A	A	A	A	A	C	A	A	C	A	C	A
HoCP 91-555	A	C	C	A	A	C	C	A	A	C	A	A	C	A	A	A	C	C	C	C	C	A	A	A	C	C	C	C	C	A	C	C	C	C	A	A	A	C	A	C	A	A	A	A	A	A	A	A	C	C	A
HoCP 96-509	A	C	C	A	C	C	C	C	A	A	A	C	A	A	A	A	A	C	A	C	C	A	A	C	A	C	C	A	C	C	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	A	A	A	A	C	A	C
HoCP 96-540	A	C	C	A	C	C	C	A	A	A	A	C	A	C	A	A	A	A	A	C	A	C	A	A	A	C	C	A	C	C	C	A	A	A	A	A	A	C	A	A	A	A	A	A	C	A	A	C	A	A	C	A
HoCP 97-606	A	C	C	C	C	C	C	A	A	C	C	A	A	A	A	A	A	A	A	A	C	A	C	A	C	A	A	A	C	A	A	C	A	A	A	A	A	C	A	A	A	A	A	A	A	C	A	A	C	A	C	A
HoCP 97-609	A	C	C	C	C	C	C	C	C	A	A	C	C	C	A	A	C	A	A	A	A	A	A	C	C	A	C	C	C	A	C	C	C	A	A	C	C	A	A	A	A	C	A	A	A	A	C	A	A	C	C	A	A
HoCP 98-718	A	C	C	A	A	A	C	C	A	A	C	C	C	A	A	A	A	C	A	C	A	C	A	C	C	A	C	C	C	A	A	A	A	A	A	C	A	A	A	A	A	A	A	A	A	A	C	C	C	A
HoCP 98-734	A	C	A	C	C	C	C	C	A	A	C	A	C	A	A	C	A	A	C	A	C	A	C	C	C	C	A	C	C	A	C	C	C	A	A	A	C	A	A	A	A	A	A	A	A	A	C	A	A	C	A
HoCP 98-741	A	C	C	A	C	C	A	C	A	A	C	A	C	A	A	A	A	A	A	A	A	C	A	A	C	C	A	C	C	A	C	A	C	A	A	A	C	A	A	A	A	A	A	A	A	A	C	C	A	A	A
HoCP 98-771	A	C	C	A	C	C	C	A	A	A	C	A	C	A	A	A	C	C	C	C	C	A	A	A	C	C	A	A	C	A	A	A	A	A	A	A	C	A	A	A	A	A	A	C	A	A	C	C	A	A	A
HoCP 98-776	A	A	C	A	C	C	C	A	A	A	A	C	C	A	A	A	A	A	A	A	A	A	C	A	C	C	C	A	A	A	A	C	C	C	A	A	A	C	A	A	A	A	A	A	C	A	A	C	C	A	C	A
HoCP 98-778	A	C	C	C	C	C	C	C	A	A	A	A	A	C	A	A	A	A	A	C	C	A	A	A	A	C	C	A	A	C	A	A	C	A	C	A	C	A	A	A	C	A	A	A	A	A	A	A	C	A	C	A
HoCP 98-781	A	C	C	A	A	A	C	C	A	A	C	C	C	C	A	A	A	C	A	C	A	C	A	C	C	C	A	A	A	C	A	A	A	C	A	A	A	C	A	A	A	A	A	A	A	A	A	C	A	C	A	A
L 95-462	A	C	C	C	C	C	C	C	A	C	A	C	A	C	A	A	C	A	A	A	A	C	A	A	A	C	A	A	C	A	A	C	A	A	A	C	C	C	A	A	A	C	A	A	A	A	A	A	A	C	C	A
L 97-128	A	A	C	C	C	C	C	A	A	A	A	C	A	C	A	A	A	A	A	C	C	C	A	C	A	C	C	A	A	C	A	A	A	C	A	A	A	C	A	C	A	A	A	A	C	A	A	A	C	C	C	A
L 97-137	A	A	A	A	C	C	C	A	A	A	A	A	C	C	A	A	C	A	C	A	C	A	A	A	A	A	C	A	A	A	C	A	C	A	C	A	C	A	A	A	C	C	C	A	A	A	A	C	C	A
L 98-207	A	C	A	C	C	C	C	A	A	A	C	C	A	C	A	A	A	A	A	A	A	A	A	C	C	C	A	A	A	C	A	A	A	A	A	A	A	C	A	A	A	A	A	A	A	A	A	C	C	A	C	A
L 98-209	A	C	A	C	C	C	C	A	A	A	C	C	C	A	A	A	A	A	C	C	A	A	C	A	C	C	A	C	C	A	C	A	C	A	A	A	C	A	A	A	A	A	A	A	A	A	A	C	C	C	A	A
LCP 82-89	A	C	C	C	C	A	C	A	A	C	C	A	C	A	A	A	C	A	A	A	C	A	C	A	C	A	C	C	A	A	C	A	C	A	A	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
LCP 85-384	A	C	A	C	C	C	C	A	A	A	C	C	C	A	A	A	A	A	A	A	A	A	C	A	C	C	A	A	C	A	A	A	A	A	A	A	C	A	A	A	A	A	A	C	A	A	C	C	C	A	A
LCP 86-454	A	C	A	A	C	C	C	C	A	C	C	A	C	A	A	A	A	A	A	A	A	A	A	C	A	A	A	C	A	A	A	C	A	A	A	C	A	A	A	A	A	A	A	A	A	C	A	A	A	A	A
LHo 83-153	A	A	C	C	C	C	C	C	C	A	C	C	C	C	A	A	A	C	C	C	A	A	A	A	A	A	C	C	A	A	A	A	A	C	C	A	A	A	C	A	A	A	A	A	A	A	A	C	A	A	A
R 570	C	C	C	A	C	C	C	A	A	C	C	C	C	A	A	A	C	C	A	C	C	C	C	C	C	C	A	A	C	C	A	A	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A
Q 124	A	A	C	A	C	C	C	C	C	A	A	C	A	A	A	A	C	A	A	C	C	C	C	C	C	C	A	A	C	A	A	A	A	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	C	A	A	A

† An allele is represented by a prefix number representing a particular microsatellite (34 = SMC334BS; 36 = SMC336BS; 68 = MCSA068G08; 86 = SMC286CS; 13 = SMC713BS; 53 = MCSA053C10; 42 = MCSA042E08; R5 = mSSCIR5; and 33 = mSSCIR33) followed by the allele size in base pairs. The letter "A" represents the presence of the allele and the letter "C" its absence.

these 27 clones. Again, the Australian sugarcane variety Q124 stood alone, followed by CP 65-357 and HoCP 96-509. The remaining 25 clones were divided into five clusters: Cluster I included CP 70-321 and LHo 83-153; Cluster II included CP 72-370 and HoCP 98-771; Cluster III included the French variety R570, HoCP 98-781 and HoCP 98-718; Cluster IV included HoCP 85-845, HoCP 97-606, HoCP 91-555, L 97-137, HoCP 98-778, L 97-128, and HoCP 96-540; Cluster V included L 98-207, LCP 86-454, L 95-462, LCP 82-89, and HoCP 98-734; and Cluster VI included HoCP 98-741, HoCP97-609, L 98-209, LCP 85-384, and HoCP 98-776. However, the bootstrapping values were all below 30.

Application of Microsatellite Genotyping in Sugarcane Breeding

Because of the reproducibility and genetic stability, the validity of these microsatellite genotypes to apply in sugarcane breeding has been demonstrated in the following two cases. The first case dealt with sugarcane variety registration and pedigree verification. For example, HoCP 96-540 is the latest variety released to the Louisiana sugarcane farmers. It was selected from progeny of LCP 86-454 and LCP 85-384 (Table 1). Of the 34 defined alleles from HoCP 96-540, 26 were found in both parents, 5 were found in its maternal parent LCP 86-454, and 3 were found in its paternal parent LCP 85-384 (Table 3). No non-parental alleles were found in HoCP 96-540 indicating that it probably was a progeny of its two parents. The same was true for clones HoCP 97-609, HoCP 98-734, HoCP 98-771, L98-207, and L 98-209. For HoCP 98-776 and HoCP 98-781, however, this was not the case. HoCP 98-776 produced two alleles, 36-175 and 68-200, which were not found in either of its parents. Another allele 34-164 was produced by HoCP 98-781 but not by either parent, LCP 85-384 or LCP 82-89 (Tables 1, 3). These non-parental alleles must come through contaminated pollen source.

The second case dealt with the genetic identity of sugarcane vegetative cuttings to ensure sugarcane population quality. Availability of microsatellite genotypes allowed the sugarcane breeders to screen their populations for genetic identity. In 2002, samples of vegetative cuttings of LCP 85-384 from different field plots in Louisiana were assessed with three of the nine SSR markers used in this study, namely, SMC334BS, SMC336BS, and MCSA068G08. Identical microsatellite genotypes were produced by the three vegetative cutting samples of LCP 85-384. In addition, the three SSR markers were also used to check for the genetic purity of two sugarcane populations at the USDA-ARS, SRU at Houma, LA. One population, which was developed for a borer resistance inheritance study, had about 16% of the individuals that produced non-parental microsatellite alleles and therefore were contaminants. About 8% of the individuals from another population derived from self-pollination of LCP 85-384 were contaminants. These contaminants were discarded from the two populations.

DISCUSSION

The efficiency of conventional sugarcane breeding in Louisiana, whether commercial (Breaux and Legendre, 1983) or basic/introgressive (Burner and Legendre, 1993; Legendre and Breaux, 1983; Tai, 1989) is low due to the technical difficulties in crossing sugarcane and progeny selection. Elite sugarcane clones do not generally flower naturally in Louisiana but must be induced to flower by artificial photoperiod treatment. Emasculation has been another technical challenge. Although it has become a routine practice at the USDA, Sugarcane Research Unit to treat the maternal flowers with hot water at 50°C for 5 min (Divinagracia, 1980; Heinz and Tew, 1987; Krishnamurthi, 1977), complete pollen sterility is not assured. Hence, progeny populations often consist of a mixture of hybrids and selfs. Field selection by cane breeders is a costly process that spans, on average, 14 years on a large area of land as selections are based largely on morphological characteristics or juice quality that require multiple year and location tests (Breaux and Legendre, 1983; Legendre and Breaux, 1983). A system that allows for early detection of hybrids or selfs will therefore improve the efficiency of sugarcane breeding.

As with other crops, there has been an increasing adoption of molecular markers in sugarcane breeding programs to improve efficiency worldwide. There have been reports on sugarcane marker projects, mainly for the construction of linkage maps (Burnquist, 1991; Grivet *et al.*, 1996; Ming *et al.*, 1998) or genetic variability assessment (Besse and McIntyre, 1996; Besse *et al.*, 1996; Besse *et al.*, 1998; Burner *et al.*, 1997; Da Silva, 2001; D'Hont *et al.*, 1993; Glaszmann *et al.*, 1990; Harvey and Botha, 1996; Pan *et al.*, 2003, 2004; Paterson *et al.*, 1995). The first report on marker-assisted selection (D'Hont *et al.*, 1995) described the presence of PCR products from both par-

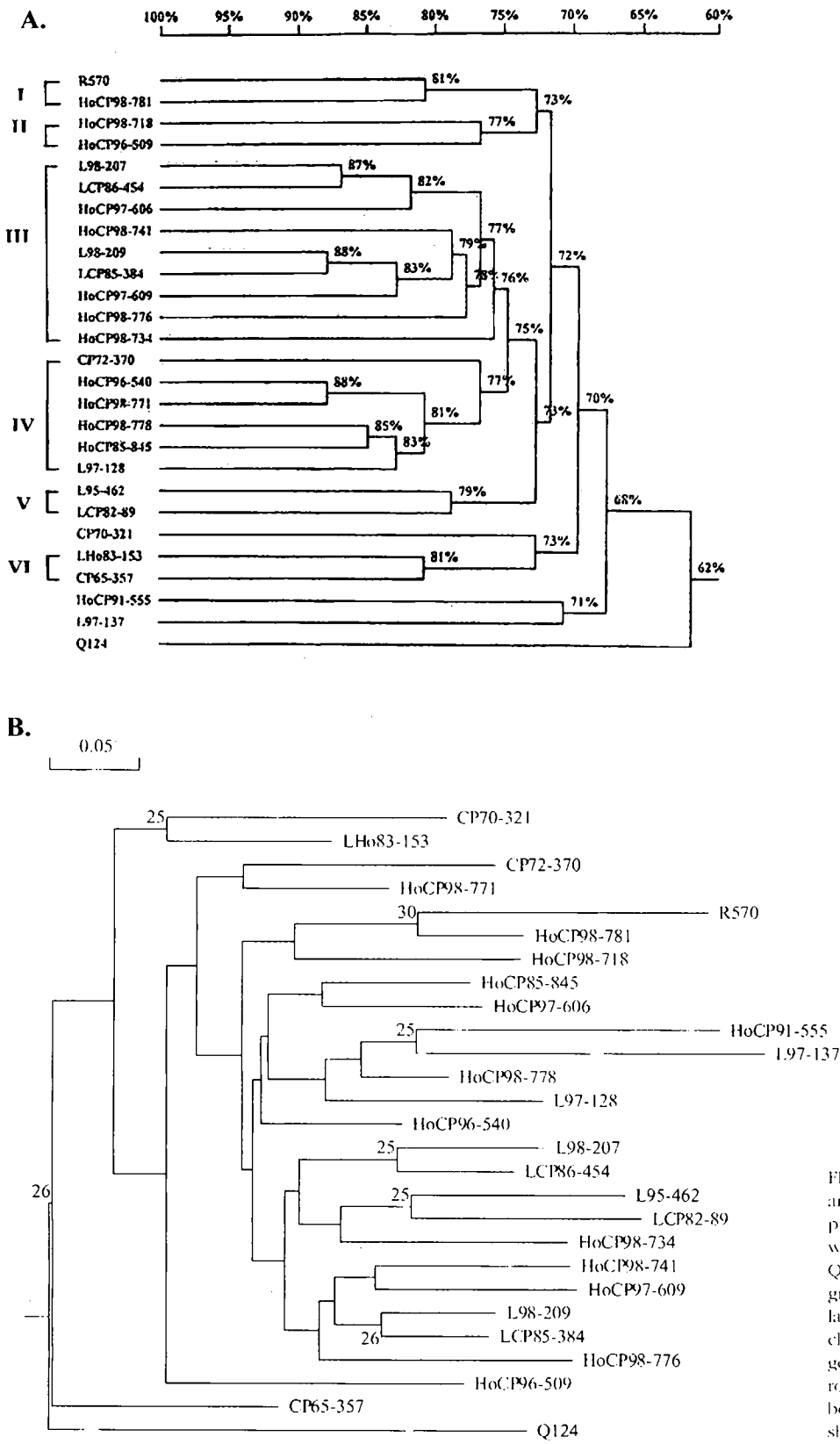

FIGURE 2 - Homology (**Panel A**) and phylogenetic (**Panel B**) trees produced by the DNAMAN® software (Lynnon Biosoft, Vaudreuil, Quebec, Canada) showing the degree of similarity (%) and genetic relatedness between the 27 sugarcane clones based on their microsatellite genotypes. The phylogenetic tree is rooted with a length of 60 with bootstrapping values greater than 20 shown on the branches.

ents (*S. officinarum* X *Erianthus arundinaceous*) in hybrid progeny using the generic primers PI and PII from the 5S rRNA locus (Cox *et al.*, 1992). Research on the PI/PII primers has been extended by the development of *Erianthus* spp.- and *S. giganteum*-specific PCR primers (Pan *et al.*, 2000; Pan *et al.*, 2001). A cultivar-specific RAPD marker, OPA11-366, has also been identified for use in identifying F_1 hybrids derived from the crosses between a *S. spontaneum* clone Djatiroto and the sugarcane varieties LCP 85-384 and CP 62-258 (Pan *et al.*, 2004). The recent development of sugarcane microsatellite primers (Cordeiro *et al.*, 2000; Cordeiro *et al.*, 2001) has led to at least three reports on sugarcane variety genotyping with these markers (Hack *et al.*, 2002; Jannoo *et al.*, 2001; Piperidis *et al.*, 2001). These studies were, however, based on a polyacrylamide gel electrophoresis system that used either autoradiography or silver- or SYBR® Gold-staining in the detection of microsatellite products. Although these methods are less expensive and perhaps more accessible, issues related to resolution, background, accuracy, and to some extent, tediousness, do occur.

The data presented in this report and another parallel study (Pan *et al.*, 2003) was based on a capillary electrophoresis system; the use of three fluorescence dyes allowed post-PCR poolplexing of products. In addition, size standards labeled with a red dye were incorporated to each poolplexed sample to allow accurate size determination. The only drawback of poolplexing was the infrequent appearance of false peaks due to "pull-up" signals, particularly when the samples were overloaded. However, these are easily distinguished through their irregular shapes or when viewing poolplexed samples simultaneously. In general, data produced from a capillary electrophoresis system has higher resolution, less background, and greater accuracy in sizing.

Each of the nine microsatellites used in this study presumably targets a specific locus and is more or less polymorphic. Although co-dominant for many diploid crops in nature, the transmission mechanism of these microsatellite markers in sugarcane still remains uncertain. Due to its high aneuploidy and the difficulty in distinguishing alleles from homoeologous chromosomes, it is quite difficult to determine heterozygosity from homozygosity at any particular locus (Cordeiro *et al.*, 2003). Nonetheless, this study has demonstrated the potential use of microsatellite markers in sugarcane breeding. Only a tiny amount of DNA template (usually at nanogram scale) was needed. DNA shearing, which often occurs as a result of quick preparation methods, does not affect the PCR amplification of the relatively small size products (140 to 350 bp). This is a significant and practical factor when screening a large number of progeny. The high ploidy level of sugarcane does provide high allele numbers with a small number of markers, thereby increasing the likelihood of useful polymorphisms. Indeed, none of the 27 clones shared identical genotypes in this study while 21 of the 25 Florida clones had different genotypes even with only three SSR markers (Pan *et al.*, 2003).

In almost all genotyping papers, the number "1" is used to denote the presence of a marker and the number "0" for its absence. The software package, NTSYSpc (Exeter Software, Setauket, NY) may be used to generate a similarity/distance matrix along with a dendrogram. Bootstrapping or confidence values are, however, not produced with NTSYSpc. The substitution of the letters "A" or "C" in place of the numbers "1"or "0" respectively allows arbitrary sequences to be created for analysis with the software package DNAMAN® (Lynnon Biosoft, Vaudreuil, Quebec, Canada) that has the capability of generating pairwise homology/distance matrice and dendrograms with bootstrapping values. The homology tree (Fig. 2A) showed six groups of clones that shared at least 76% of homology within each group; while in the phylogenetic tree (Fig. 2B) the clones were clustered in a pattern that in general coincided with the putative pedigrees of these clones. For example, homology group III contained the leading Louisiana variety, LCP 85-384, and six of the other eight clones that had LCP 85-384 as its putative parent. However, this represented our preliminary attempt to use microsatellite genotyping data to assess the genetic relatedness of sugarcane clones. The fact that all bootstrapping values were under 30 indicated that our inter-clonal relatedness assessment is of limited usage, due primarily to the small number of microsatellite markers used in this study.

Nine of the 25 clones as well as two *S. spontaneum* clones (Djatiroto and SES84/58) from another study (Cordeiro *et al.*, 2003) were also genotyped with the genetic analyzers ABI PRIZM 3100, ABI PRIZM 3700 (Applied Biosystems, Foster City, CA), or CEQ8000 (Beckman-Coulter, Fullerton, CA). The same number of alleles was observed on these samples although a size shift by 1 to 3 bp was observed when using different instruments (unpublished). However, this would not be a problem in genotyping experiments as long as the same instrument is used.

Sugarcane plants are indeed difficult to manhandle during crossing and there is always opportunity for pollen contamination. There also is the risk of mix-ups in handling of stem sections during propagation. As sugarcane is vegetatively propagated, an error could have large consequences. We have shown that the microsatellite genotyping technology can be a good tool to ensure the genetic identity of a particular sugarcane clone. This was documented in two similar studies. In one study, a Florida sugarcane clone, CP 84-1198, produced three microsatellite alleles (34-158, 34-160, and 36-166) that were not found in either of its parents, CP 70-1133 and CP 72-2086. In addition, one vegetative cutting sample of CP 96-1602 produced a different SMC336B8 genotype that also showed severe smut symptoms than other two cutting samples of CP 96-1602 with no smut. It also produced different RAPD fingerprints, indicating a planting error during field trials (Pan *et al.*, 2003). In another study, Hack *et al.* (2002) found the recorded pedigree of Cross AA40 was invalid upon genotyping with both RFLP and microsatellite markers.

The microsatellite genotypes of 27 sugarcane clones reported here should provide Louisiana sugarcane breeders with additional molecular tools to identify and select their F_1 hybrids. The grouping pattern derived from this study will also give sugarcane breeders an idea how these clones relate to one another. It also becomes possible for sugarcane geneticists to conduct allelic inheritance studies in such a complex aneu-polyploidy crop as sugarcane.

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