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Sugarcane Variety Identification through DNA Fingerprinting With Microsatellites Markers

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ABSTRACT

Accurate identification of sugarcane (*Saccharum* spp.) varieties and clones is primordial for efficient genetic breeding programs. Because sugarcane is normally vegetatively propagated, the unit of cultivation and breeding is a clone, resulting in each cultivar being represented by a single genotype. Various molecular procedures can be employed to determine identity and relationships in plants, based on DNA differences. We have used microsatellite markers in sugarcane (Saccharum spp.) for variety identification in order to determine if the variety planted in commercial fields at several counties in South Texas was in fact the one expected. This procedure was conducted for two cultivars (TCP87-3388 and TCP89-3505) and one elite clone (TCP98-4454). We found identification problems in all three cases, based on genetic differences between the variety planted and the authentic one expected. We have also reported a new microsatellite marker, derived from a signal transduction gene, that is hypervariable and highly informative.

Additional Index Words: *Simple sequence repeats*

Sugarcane (*Saccharum spp*) variety improvement is based on development and crossing of elite clones. In the USA, the World Collection of Sugarcane and related grasses is maintained at the USDA-ARS Clonal Germplasm Repository, Miami, FL. Modern sugarcane cultivars have originated from complex hybridization events (the so called 'Nobilization Process') between *Saccharum officinarum*, *S. barberi, S. sinense* and the wild related species *S. spontaneum* (Sreenivasan et al., 1987). Even tough different species were involved in this process, most, if not all modern sugarcane varieties grown in the world, are derived from a few genotypes (d'Hont et al, 1996), resulting in a very narrow genetic base. Efforts to broadening the genetic base require the characterization of sugarcane germplasm using morphological traits such as leaf, stalk and inflorescence characteristics (Daniels and Roach, 1987), as well as sugar composition (Tai and Miller, 2002).

A better understanding of the genetic diversity and inter-relationships among modern cultivars will facilitate the exploitation of these genotypes for the genetic improvement of sugarcane. Traditional methods, which combined agronomic and morphological characteristics, have been useful in identifying and describing differences between members of the *Saccharum* complex (Skinner et al. 1987). Molecular markers have been used to characterize basic germplasm (Alwala et al., 2006), but not elite clones or cultivars.

Polymerase chain reaction (PCR) markers are useful to survey genetic diversity in populations for which little data is available. Among the different types of PCR markers, microsatellites are advantageous, given their ability to detect multiple alleles, combined with single locus inheritance (Cordeiro et al., 2001).

One problem that many plant breeders find is that varieties found in several parts of the same country or region can have the same name but may not be the same genotype. Fingerprinting can determine if varieties with the same name are genetically identical. This information helps sugarcane breeders and growers to decide which plants to use as seed source.

The monitoring of sugarcane fields in the Rio Grande Valley (RGV) for the presence of ineligible varieties that may be visually indistinguishable from designated varieties, is important to protect the integrity of such fields. Presently, in this region, different sugarcane fields of the commercial varieties TCP87-3388 (Irvine et al., 1997), TCP89-3505 (Scott

et al., 2005) and the elite clone TCP98-4454 have been suspected to carry a different variety, based upon observations of inconsistent agronomic performance, in the case of the TCP87-3388 (Steve Bearden, personal communication), and presence of smut (*Ustilago scitaminea*) in the case of TCP89-3505 and TCP98-4454, both considered resistant to this disease.

The comparison of the variety present on each location, based on the phenotype (agronomic performance and morphology) is complicated by environmental effects, known to affect quantitative traits such as yield production. DNA fingerprinting in sugarcane is based on the fact that, being vegetatively propagated, each individual variety has a unique genetic profile, revealed through its DNA. The fingerprinting bands from one variety can be compared to the bands from other varieties to detect similarities and differences.

The objective of this study was to characterize different varieties at the molecular level (DNA Fingerprint) by applying microsatellite markers, in order to confirm three sugarcane genotypes (TCP87-3388, TCP89-3505 and TCP98-4454) grown on different commercial fields. This is the first report of DNA markers used for variety identification on commercial sugarcane fields in Texas.

MATERIALS AND METHODS

Electronic Search of Microsatellites. The presence of microsatellites was investigated within ESTs identified in the SUCEST database (Rossi et al., 2003) using the software SSRIT - Simple Sequence Repeat Identification Tool- which finds all perfect simple sequence repeats (SSRs) in a given sequence and is available at http://www.gramene.org/gramene/searches/ssrtool (Temnykh et al., 2001). PCR primer pairs were designed using the Primer3 program (Rozen and Skaletzky, 1996) and were synthesized with an infrared modification (either Irdye700 or Irdye800) for visualization of their PCR products in a NEN DNA analyzer 4300S (Li-Cor, Inc. Lincoln, NE).

DNA Extraction and Microsatellite Analysis. Genomic DNA was extracted from leaf tissue using the Qiagen DNEasy kit which required the following items: 1) Centrifuge Qiagen model 4-15C; 2) Plate Rotor 2 x 96 for 2 Qiagen 96-well plates, 56/60 Hz and 3) tungsten carbide beads 3-mm (200).

Fingerprinting analyses involving microsatellite markers were performed at the Texas Agricultural Experiment Station, Weslaco Center. We utilized microsatellite markers obtained both from expressed sequence tag – EST (da Silva, 2001) and genomic libraries (Cordeiro et al., 2001) of sugarcane. PCR reactions were performed in a MJ PTC-100 (MJ Research, Inc. Waltham, PA) thermocycler under the same conditions as described in the literature (Cordeiro et al., 2001; da Silva, 2001).

RESULTS AND DISCUSSION

Due to the fact that all plants of each sugarcane variety have the same genotype, any differences in DNA detected among plants considered to be identical, means that the plants are not identical. We obtained samples of DNA from leaf tissue and used microsatellite markers to produce a "fingerprint" that is depicted as a series of bands of varying size, similar to a bar code. A total of 10 microsatellite markers were used (four genomic and six EST- derived), producing a total of 134 DNA fragments or bands (Table 1).

Table 1 – Genomic and EST- derived microsatellite markers used for fingerprinting of TCP87-3388, TCP89-3505 and TCP98-4454.

Primer	# of scored bands on TCP87-3388	Total # of bands observed
mSSCIR12	8	27
mSSCIR17	11	22
EST-SSR15	3	9
EST-SSR81	25	28
EST-SSR80*	6	7
SMC869*	20	20
EST-SSR5*	9	9
EST-SSR29a*	3	3
EST-SSR7*	5	5
mSSCIR23*	3	4
Total number of bands	93	134

*Primers not used on all the samples. Number of samples used: EST-SSR80 = 71; SMC869 = 40; CIR23 = 11EST-SSR5, EST-SSR29a, EST-SSR7 = 4.

DNA-based fingerprinting analysis was performed on leaf samples collected from each field, to determine the identity of the variety present in that field. PCR amplification of all loci analyzed for this study was robust and reliable, as long as primer-

specific annealing temperatures were utilized. We used polyacrylamide gel electrophoresis and microsatellite-based systems for sugarcane fingerprinting using the Li-Cor 4300 DNA Analysis System (Li-Cor, Lincoln, NE), which uses two-color infrared fluorescence detection (IR 700 and IR 800) to generate two images, allowing high throughput. To successfully monitor the sugarcane fields in the Rio Grande Valley, a method for high throughput DNA extraction was necessary, given the high number of samples analyzed.

Fingerprinting of TCP87-3388. Leaf samples of sugarcane plants, growing in four different fields identified as containing the variety TCP87-3388, were collected on October 2005. Microsatellite markers found in the original TCP87-3388 variety were applied to those samples (Figure 1). This analysis showed that the variety planted on three of those fields had the same DNA fingerprint as the known TCP87-3388 standard, but one field had a different fingerprint, indicating a different variety. These preliminary results suggested the need for a variety identification survey of all fields containing TCP87-3388, in order to support the integrity of the variety quality assurance system.

Given the high number of samples to be analyzed (241) there was a need for a high throughput DNA extraction method. We opted to use the Qiagen method, which yielded high quality DNA.

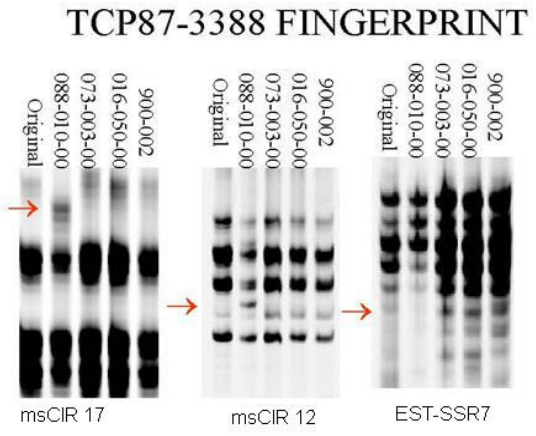

Fig. 1. Fingerprint of TCP87-3388 from 4 different fields (088-010-00; 073-003-00; 016-050-00 and 900-002), in comparison with DNA from the original TCP87-3388. Panels were generated by 3 Microsatellite markers (EST-SSR29a; msCIR12 and EST-SSR7).

Results from the microsatellite analysis indicated 20 fields, out of 241 sampled, contained a variety different from the authentic TCP87-3388 (Table 2).

Fingerprinting of TCP89-3505 and TCP98-4454. The occurrence of smut on one field (RGVSG) of the newly released cultivar TCP89-3505 and two fields (RGVSG and Madero) of the elite clone TCP98-4454, raised the question as to whether those fields contained the authentic variety, since occurrence of smut had not

Table 2. Fields containing a variety other than TCP87-3388, based on the results obtained with the Microsatellite markers mSSCIR12 and mSSCIR17.

Field	Sampling Date	Location
1. 016-060-00	03/16/06	CAMERON CO.
2. 024-023-00	3/27/06	CAMERON CO
3. 049-014-00	03/16/06	HIDALGO CO
4. 049-019-00	03/16/06	HIDALGO CO
5. 088-010-00	03/16/06	CAMERON CO
6. 092-005-00	03/16/06	HIDALGO CO
7. 096-002-00	02/16/06	HIDALGO CO
8. 099-004-00	03/16/06	WILLACY CO
9. 123-001-00	02/10/06	PAD 162
10. 107-011-00	03/20/06	WILLACY CO
11. 221-009-00	02/10/06	MERCEDES
12. 232-127-00	02/16/06	HIDALGO CO
13. 261-034-00	02/10/06	CAMION RD
14. 280-026-00	03/28/06	CAMERON CO
15. 316-018-00	02/16/06	HIDALGO CO
16. 323-017-00	03/22/06	HIDALGO CO
17. 327-006-00	02/10/06	CAMION RD
18. 328-008-00	03/16/06	HIDALGO CO
19. 521-038-00	02/10/06	WILLACY CO
20. 521-063-00	03/22/06	WILACY CO

been previously reported on TCP89-3505, and had only been previously detected on a very low intensity on TCP98-4454. To address these questions, we conducted a DNA Fingerprinting analysis with microsatellites.

The results obtained with the five microsatellite markers used (data not shown) indicated that the DNA fingerprint of the plants showing smut whips was different from that of authentic TCP98-4454. Based on this result, we decided to include in the analysis the DNA of the same variety grown on other fields, as well as the DNA of the variety TCP98-4445. Figure 2 shows the fingerprinting obtained with these two genotypes. The results indicated that the fingerprint of: 1) TCP89-3505 grown at the RGVSG is different from the "original" TCP89-3505; 2) TCP98-4454 grown at the RGVSG with smut symptoms is different from the authentic TCP98-4454 and the same as TCP98-4454 grown in the following places: Madero (also with smut symptoms), Rio Farms 232-095; East end Valley Acres; East field; "Plant Cane" and Rio Farms 142.

We chose Microsatellites for our fingerprinting analysis because, as PCR markers, they are easy to use ; being the product of specific primers, they are stable and being locus-specific, they are transferable across genotypes within the species (da Silva 2001). Microsatellites have also been the preferred method for fingerprinting, differentiation, and genetic analysis in other perennial species such as *Populus* (Rahman and Rajora, 2002). In this work, Microsatellite DNA markers were used for genetic fingerprinting and proposed for identification, classification, certification, and registration of clones, cultivars, and varieties as well as genetic resource management and protection of *Populus* breeders' rights.

Microsatellites are hypervariable single-locus markers that have previously been shown to be highly informative in the identification of multiple varieties, for crops such as wheat (*Triticum aestivum*; Plaschke et al. 1995; Bryan et al. 1997), and tomato (*Lycopersicon esculentum*; Bredemeijer et al. 1998). The potential of genetic markers for cultivar identification in plants has prompted several studies, even for fraud detection in ornamentals (Becher et al., 2000).

A recent survey of the TIGR- SOGI (*Saccharum officinarum* Gene Index) database revealed 254,635 ESTs for *S. officinarum*, (da Silva, Unpublished Data) representing a source of microsatellites. These EST-derived markers represent a potentially powerful tool for fingerprinting analysis (da Silva and Bressiani, 2005). Because our group is interested in developing EST-SSRs for stress resistance studies, we performed a search on the SOGI database for ESTs involved on signal transduction, a phenomenon that is important

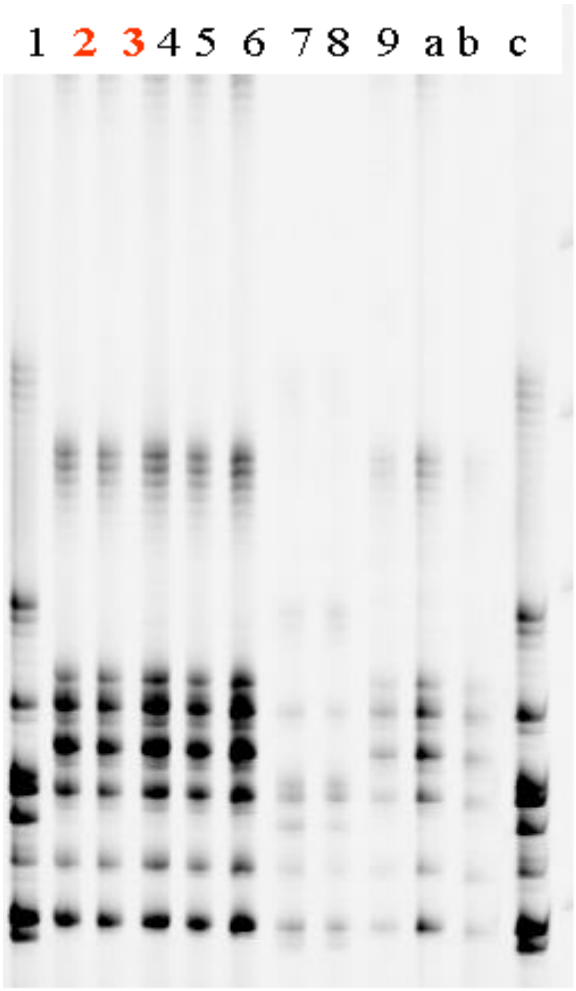

Fig. 2. TCP98-4454 Fingerprinting with marker msCIR12.;1- TCP98-4454 Original; 2- TCP98-4454 Mill (smut); 3 TCP98-- 4454 Madero (smut); 4- TCP98- 4454 Rio Farms 232-095; 5- TCP98-4454 East end Valley Acres; 6- TCP98- 4454 East Field; 7- TCP98- 4445 East Field; 8- TCP98-4445 Valley Acres; 9- TCP98-4454 Plant Cane; a- TCP98- 4454 Rio Farms 142; b- TCP98-4454 Byron; c- TCP98-4445 Rio Farms 142.

for stress response. This search revealed a total of 115 ESTs. One of these, the EST SCJFLR1035E04.g, obtained from a leaf roll cDNA library (Vettore et al., 2001) and annotated as phytochrome A signal transduction 1 protein [Arabidopsis thaliana] gi|8777405|dbj|, presents 2 repeat units (Table 3). One of these units, the di-nucleotide AT repeated 23 times, is a hyper-variable repeat (Temnykh et al., 2001), that was informative enough for fingerprinting analysis.

Signal transduction is a phenomenon that occurs in plants when they are exposed to stresses such as

Table 3 – Sugarcane EST-SSR markers developed from a gene coding for the `signal transduction 1 protein`.

MARKER	SSR	PRIMERS*	EST ANNOTATION
EST-SSR81	$(AT)_{23}$	F: TTCTGCGTGGCACTGACTAC R: ACAAAGGGCATCCTTTCTGA	phytochrome A signal transduction 1 protein

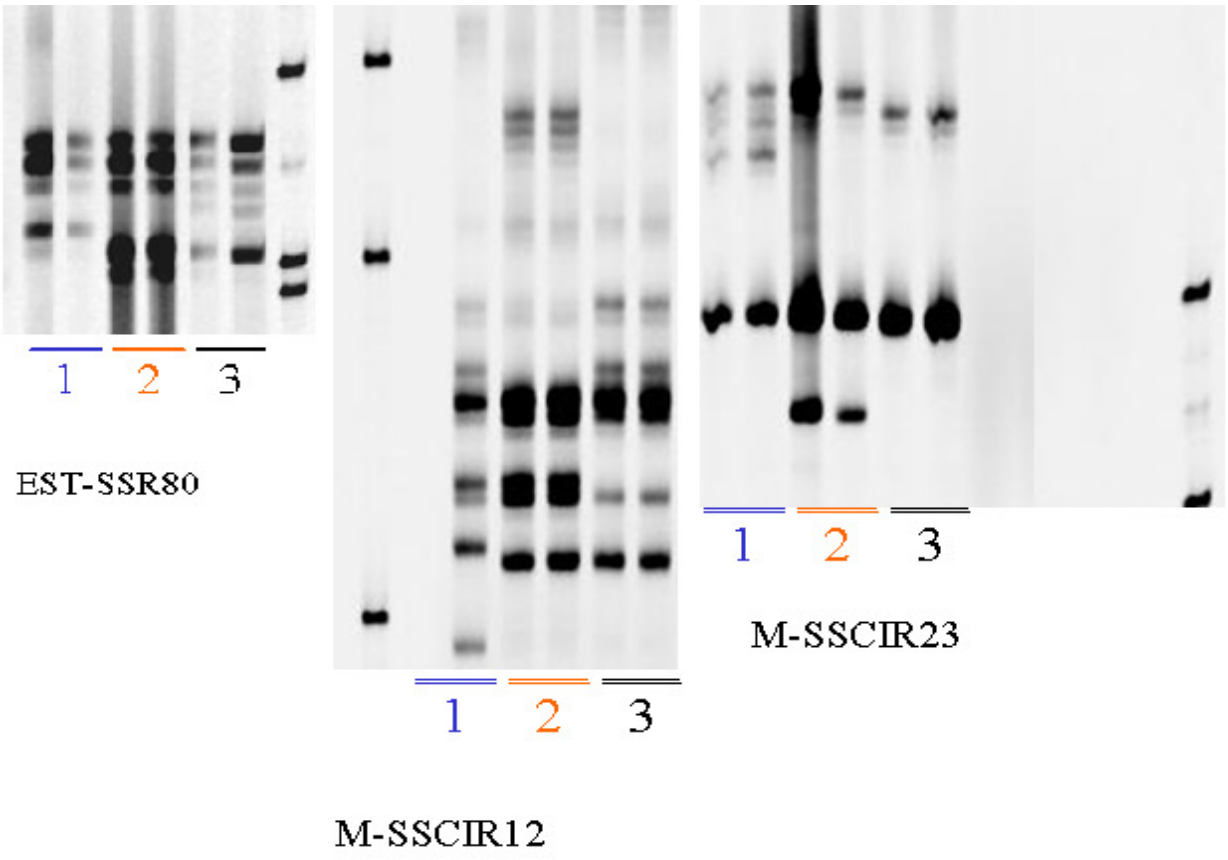

Fig. 3. TCP89-3505 Fingerprinting with genomic- and EST- derived microsatellite markers. Sample 1: Original; Sample 2 grown at the RGVSG and Sample 3 CP72-1210: Markers used were EST-SSR80; msCIR12 and msCIR23. Each sample is shown in two lanes, side by side.

temperature, light, water, nutrient and microbe-plant interactions which induce cellular responses locally and/or throughout the plant (Souza et al., 2001). Because plants are constantly attacked by a wide variety of malignant microorganisms, they have developed an array of responses to survive these pathogenic attacks. Sensing and transducing the presence of a particular microorganism is necessary for an effective response, leading to a specialized gene expression to confer disease resistance on the plant. A number of resistance genes are induced by hormones when plants are exposed to pathogens (Reymond and Farmer, 1998). The fact that a hypervariable repeat is present within a signal transduction protein leads to the speculation that this EST-SSR may produce markers associated to a stress response.

In this paper, we compared the microsatellite bands obtained from the sugarcane plants present in numerous fields to microsatellite bands obtained from the authentic sugarcane variety believed to be present in each field. A single difference in the resulting DNA fingerprintings indicated that the variety present on a

particular field was not the one expected.

The sugarcane variety DNA Fingerprinting profile generated was highly replicable, allowing identification of fields truly containing the authentic variety, supporting the integrity of RGV's variety quality assurance system.

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LITERATURE CITED

Alwala S, Suman A, Arro JA, Veremis JC, and Kimbeng CA (2006) Target Region Amplification Polymorphism (TRAP) for Assessing Genetic Diversity in Sugarcane Germplasm Collections Crop Sci 46: 448-455.

Becher SA, Steinmetz K, Weising K, Boury S, Peltier D, Renou J-P, Kahl G and Wolff K (2000) Microsatellites for cultivar identification in Pelargonium Theor Appl Genet 101:643–651.

Bredemeijer GMM, Arens P, Wouters D, Visser D and Vosman B (1998) The use of semi-automated fluorescent microsatellite analysis for tomato cultivar identification. Theor Appl Genet 97:584–590

Bryan GJ, Collins AJ, Stephenson P, Orry A, Smith JB and Gale MD (1997) Isolation and characterisation of microsatellites from hexaploid bread wheat. Theor Appl Genet 94:557–563

Cordeiro, GM, Taylor GO and Henry RJ (2001) Characterization of SSR Markers from Sugarcane (*Saccharum spp.*), a highly polyploid species. Plant Science 155: 161-168.

D'Hont A, Grivet L, Feldmann, P Rao PS, and Berding N (1996) Characterization of the Double Genome Structure of Modern Sugarcane Cultivars (*Saccharum spp.*) by molecular cytogenetics. Molecular General Genetics: 405-413.

Da Silva JA and Solís-Gracia N (2006) Development of Simple Sequence Repeat Markers from Genes Related to Stress Resistance in Sugarcane. Subtropical Plant Science. Subtropical Plant Science 58:5-11.

Da Silva, JA and Bressiani JA (2005) Sucrose Synthase EST-derived RFLP marker associated to sugar content in elite sugarcane progeny. Genetics and Molecular Biology 28, 2: 294-298.

Da Silva, J A (2001) Preliminary Analysis of Microsatellite Markers derived from Sugarcane ESTs. Genetics and Molecular Biology 24 (1-4): 155-15.

Daniels J and Roach BT (1987) Taxonomy and Evolution. In: Sugarcane Improvement Through Breeding. Don Heinz (ed). Elsevier, Amsterdan 603 pp.

Irvine, JE, Miller JD and Legendre BL (1997) Registration of 'TCP87-3388' sugarcane. Crop Sci. 37: 1019.

Plaschke J, Ganal MW, and Röder MS (1995) Detection of genetic diversity in closely related bread wheat using microsatellite markers. Theor Appl Genet 91:1001–1007.

Rahman MH and Rajora OP (2002) Microsatellite DNA fingerprinting, differentiation, and genetic relationships of clones, cultivars, and varieties of six poplar species from three sections of the genus Populus. Genome 45(6): 1083–1094.

Reymond, P and Farmer, EE (1998) Jasmonate and salicylate as global signals for defense gene expression. *Curr. Opin. Plant Biol. 1*: 404-411.

Rossi M, Araujo PG, Paulet F, Garsmeur O, Dias VM, Chen H, Van Sluys M-A and d'Hont A (2003) Genomic distribution and characterization of EST-derived resistance gene analogs (RGAs) in sugar cane. Mol Gen Genomics 269: 406-419.

Rozen S and Skaletzky HJ (1996) PRIMER3. http:// wwwgenome. wi.mit.edu/genome_software/other/ primer3.html.

Scott, A, da Silva JA, Rozeff N, Hernandez E, Legendre BL, and Miller JD (2005) Registration of 'TCP89-3505' Sugarcane. Crop Science 45:2120.

Skinner JC, Hogarth DM and Wu KK (1987) Selection Methods, Criteria and Indices In: Sugarcane Improvement Through Breeding. Don Heinz (ed). Elsevier, Amsterdan 603 pp.

Souza GM, Simoes ACQ, Oliveira KC, Garay HM, Fiorin LC, Gomes FS, Nishiyama-Jr MY and da Silva A (2001) The sugarcane signal transduction (SUCAST) catalogue: prospecting signal transduction in sugarcane. Genetics and Molecular Biology, 24 (1-4), 25-34.

Sreenivasan TV, Ahloowalia BS and Heinz DJ (1987) Cytogenetics In: Sugarcane Improvement Through Breeding. Don Heinz (ed). Elsevier, Amsterdan 603 pp.

Tai PYP and Miler JD (2002) Germplasm diversity among four sugarcane species for sugar composition. Crop Sci. 42: 958-964.

Temnykh S, DeClerck G., Lukashova A., Lipovich L, Cartinhour S and McCouch S (2001) Computational and experimental analysis of SSRs in rice (*Oryza sativa* L.): Frequency, length variation, transposon association, and genetic marker potential. Genome Research 11: 1441-1452.

Vettore, AL, da Silva F, Kemper E L and Arruda, P (2001) The libraries that made SUCEST. Genet. Mol. Biol., vol.24, 1-4, p.1-7.