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Biparental crosses confirmed by SSR with Mendelian inheritance in sugarcane breeding

María F. Perera*, María B. García**, Carolina Díaz Romero**, María I. Cuenya**, María P. Filippone* and Atilio P. Castagnaro*

ABSTRACT

In sugarcane (*Saccharum* spp.) breeding programs, parents used in crosses are classified as male or female based on the relative amounts of viable pollen produced. High pollen production favored by environmental conditions reduces "female inflorescence" availability and restricts the possibility of cross combinations. However, male parents could be employed as female parents when an efficient emasculation treatment is used. An ideal approach for hybridity testing is using molecular markers, especially microsatellites (SSR). To determine the effectiveness of an emasculation treatment (immersion of the panicle in water at 50ºC for five minutes) employed in the Sugarcane Breeding Program of Estación Experimental Agroindustrial Obispo Colombres (EEAOC), Tucumán, Argentina, six cross combinations (selfings and reciprocals) between two varieties commonly used as males, LCP 85-384 and RA 87-3, were evaluated by using SSRs. Samples were amplified with one primer pair that produced seven polymorphic and three monomorphic bands between the two progenitors. While Mendelian segregation may be difficult to observe in the progeny of a complex polyploid like sugarcane, the analysis showed that each marker segregated in a Mendelian fashion (as evaluated by χ^2 tests, $P \leq 0.05$) for each cross combination. Results indicated that the emasculation treatment was successful and that SSRs made it possible to identify true hybrid progeny routinely in sugarcane breeding.

Key words: hybridity testing, hot-water emasculation treatment, segregation analysis, SSR, sugarcane breeding program.

RESUMEN

Confirmación de cruzamientos biparentales mediante marcadores microsatélites con herencia mendeliana en el mejoramiento de caña de azúcar

En los programas de mejoramiento de caña de azúcar (*Saccharum* spp.), los progenitores empleados en los cruzamientos se clasifican como masculinos o femeninos según las cantidades relativas de polen viable producido. La alta producción de polen, favorecida por las condiciones ambientales, reduce la disponibilidad de inflorescencias femeninas y restringe la posibilidad de combinación en los cruzamientos. Sin embargo, los progenitores masculinos pueden ser empleados como progenitores femeninos cuando se aplica un tratamiento de emasculación efectivo. Una aproximación ideal para determinar la hibridez consiste en la utilización de marcadores moleculares, especialmente los microsatélites (SSR). Para determinar la efectividad de un tratamiento de emasculación (inmersión de la panoja en agua a 50ºC durante cinco minutos), utilizado en el Programa de Mejoramiento Génetico de Caña de Azúcar (PMGCA) de la Estación Experimental Agroindustrial Obispo Colombres (EEAOC), en Tucumán, R. Argentina, seis combinaciones de cruzamientos (autofecundaciones y recíprocos) entre dos variedades comúnmente usadas como progenitores masculinos, LCP 85-384 and RA 87-3, fueron evaluadas mediante SSRs. Las muestras fueron amplificadas con un par de cebadores que produjeron siete bandas polimórficas y tres monomórficas entre los dos progenitores. Mientras que la segregación mendeliana puede ser difícil de observar en la progenie de un poliploide complejo como la caña de azúcar, el análisis mostró que cada marcador presentó herencia mendeliana (tal como se evaluó por pruebas χ^2, $P \leq 0{,}05$) para cada combinación de cruzamiento. Los resultados indicaron que el tratamiento de emasculación fue exitoso y que los SSRs hicieron posible la identificación rutinaria de los verdaderos híbridos en la progenie obtenida por mejoramiento en caña de azúcar.

Palabras clave: determinación de hibridez, tratamiento de emasculación con agua caliente, análisis de segregación, SSR, programa de mejoramiento de caña de azúcar.

*Sección Biotecnología, EEAOC. franciscaperera@eeaoc.org.ar
**Sección Caña de Azúcar, EEAOC.

INTRODUCTION

Commercial sugarcane varieties derive from an artificial species obtained by crossing different species of *Saccharum* genus, characterized by a high degree of polyploidy and frequent aneuploidy (Cordeiro *et al*., 2000). These cultivars are developed following three procedures: i) assembling a described parental clone population; ii) generating variable progenies by cross-pollination; and iii) selecting useful clones (Hogarth and Berding, 2005). The most vigorous parents for producing varieties to further increase sugar and fiber production are evaluated and selected (McIntyre and Jackson, 2001). In Tucumán, Argentina, the Estación Experimental Agroindustrial Obispo Colombres (EEAOC) has conducted a sugarcane breeding program since 1968. It takes at least eleven years to complete a sugarcane breeding cycle, starting with the crossing between elite clones (bi-parental), continuing with selection, advancement, testing, and ending with the release of a new variety. Photoperiod houses need to be used to induce flowering in this sub-tropical area, where natural flowering is sub-optimum because low temperatures affect flower initiation, as well as pollen fertility (Hogarth and Berding, 2005).

Inflorescences are hermaphrodite panicles, in which both pollen quantity and fertility depend on genotype and environmental conditions. Hence, parents are classified as male or female based on the relative amounts of viable pollen produced (McIntyre and Jackson, 2001). The high pollen production of some genotypes, favored by high temperatures, reduces "female inflorescence" availability and restricts the number of cross combinations possible (Berding, 1981). However, emasculation allows employing male parents as female parents and extending the range and direction of clones that can be used in breeding programs. This technique implies pollen sterilization by immersion of the panicle in hot water, cold water, chemical products or steam (Machado *et al*., 1995). Also, the emasculation treatment is an alternative to overcome self-pollination (Pan *et al*., 2003).

Progeny hybridity can be detected in different ways, such as seed characterization, but the characteristics are unreliable as they are largely controlled by the maternal parent. Also, isozymes are sometimes employed (Shoda *et al*., 1999); however, these biochemical markers are of limited use, as they can be affected by developmental stage and environment, and can only be assayed with considerable tissue material. Genetic fingerprinting is therefore an ideal approach to hybridity testing (Romero *et al*., 2009a). Several markers have been used to characterize genuine hybrids from *Saccharum* and *Erianthus* (Zhang *et al*., 2004) and to correctly identify true hybrids derived from sugarcane crosses (Edmé *et al*., 2006; Manigbas and Villegas, 2004; McIntyre and Jackson, 2001; Pan *et al*., 2003). Markers should segregate in a Mendelian fashion to correctly assess the hybrids. A segregation analysis is also required to construct a map, and markers showing segregation distortion are considered skewed or eliminated (Grivet *et al*., 1996).

This paper aims to determine the effectiveness of an emasculation treatment by immersion of the panicle in hot water, in crosses between two commercial varieties commonly used as males, by using SSR markers with Mendelian segregation. Specific markers were identified for each genotype and used to screen the hybrid progeny in each cross combination.

MATERIALS AND METHODS

Emasculation treatment

Sugarcane commercial varieties LCP 85-384 and RA 87-3, commonly used as males, were grown and maintained at the EEAOC. The main characteristics of both varieties are presented in Table 1.

Flowering was induced in photoperiod houses in November, 2009. The photoperiod treatment, previously

Table 1. Main characteristics of LCP 85-384 and RA 87-3.

Characteristics*		LCP 85-384	RA 87-3
Origin		Louisiana (E.E.U.U.)	Argentina
Total area planted (ha) in Tucumán		65.18	7.95
Average tons of sugarcane/ha		64.9	75.55
Average tons of sugar/ha (July)		9.8	9.7
Behaviour against diseases	**Rust**	Susceptible	Resistant
	Leaf scald	Resistant	Moderately resistant
	Sugarcane mosaic virus	Resistant	Moderately susceptible
	Ratoon stunting disease	Susceptible	Susceptible
	Pokkah boeng	Moderately resistant	Susceptible
	Red streak	Moderately resistant	Susceptible
	Smut	Resistant	Moderately resistant

Source: Romero *et al.* (2009b).

optimized, had a commencing day length of 12 h 30 min during 60 days. A decrement of 1 min/day day length was adopted for the following 40 days. After flowering in March 2010, the emasculation treatment was applied. It implied pollen sterilization by immersion of the panicle of the two varieties in a hot water tank, at 50ºC for five minutes. Then, plants were maintained in a greenhouse until flowers opened (three to seven days) and pollen absence was evaluated with a magnifying glass. Controlled crosses were set up using standard procedures in the local sugarcane breeding program. In brief, stalks with female (emasculated males) and male inflorescences were cut at the base and moved to cubicles with male inflorescences placed above the female inflorescences during 15 days. The stalks were gently shaken daily at around 10:00 am to favour pollen transfer. Each cross was made in duplicate. The female tassels were bagged and stored for a month to complete seed formation. Seeds were stored in paper bags at -18ºC. One gram of seeds per cross combination was sown in trays with a mixture of substrate (three parts of soil, two of mulch and one of perlite), until seedlings emerged. After 21 days, seedlings were transferred to trays with individual cells. Seedlings were held under natural light conditions in a greenhouse and under temperatures ranging from 25ºC to 28ºC. Table 2 presents the cross combinations studied, the average number of seedlings obtained/g of seed, and the number of evaluated seedlings.

DNA isolation

Fresh leaves of two-month old seedlings from 120 samples belonging to different cross combinations and leaves of the two commercial varieties were collected and frozen at -70ºC. Then, 200 mg of leaves of each sample were placed in liquid nitrogen and ground in a mortar to a fine powder. DNA was extracted by using a modification of Aljanabi *et al.* (1999) method. RNA was removed with Rnase. DNA quality was estimated by agarose gel electrophoresis and its concentration was determined spectrophotometrically.

SSR

Fifteen SSR primer pairs (Cordeiro *et al*., 2000; D´Hont, 2005; unpublished sequence) (Table 3) that had been previously checked were evaluated so as to choose the one that produced an appropriate molecular profile.

Table 3. Repeat motifs of the primers used to evaluate the sugarcane genotypes.

Primer	Repeat motifs
SMC222CG$_a$	$(CA)_{24}$
SMC226CG	$(CA)_{10}$
SMC248CG	$(TTA)_6$
SMC319CG	$(CA)_{17}$
SMC477CG	$(CA)_{31}$
SMC863CG	$(TC)_9$
MSCIIR1$_b$	$(GT)_{18}(GA)_{31}$
MSCIIR12	$(GA)_{13}$
MSCIIR14	$(GA)_{22}$
MSCIIR15	$(GA)_{22}$
MSCIIR16	$(GA)_{18}$
MSCIIR19	$(GA)_{23}$
MSCIIR27	$(GA)_{18}$
MSCIIR34	$(GA)_{20}$
MSCIIR68	$(GT)_{26}GC(GT)_{12}$

The MSCIIR19 primer pair (D´Hont, 2005; unpublished sequence) was chosen. Reaction mix (20 µl) contained: 10 ng DNA, 1X buffer (Promega Corp.), 100 µM dNTPs (Amersham Biosciences), 1.2 µM primers (Sigma-Aldrich Co.), 2.5 mM MgCl2 (Promega Corp.), and 0.5 units Taq DNA polymerase (Promega Corp.). Cycling parameters were: one cycle at 94°C (5 min); 30 cycles at 94ºC (45 s), 52ºC (30 s) and 72ºC (1 min); and 1 cycle at 72ºC (3 min). SSR products were separated by electrophoresis on 6% polyacrylamide denaturing gel and visualized through the silver staining system (Caetano-Anollés and Gresshoff, 1994). Although SSRs are classified as co-dominant type markers, they were treated as dominant markers in this study to analyze the highly complex *Saccharum* genome. Segregation analysis was performed with χ^2 test ($P \leq 0.05$).

RESULTS AND DISCUSSION

After the emasculation treatment, the absence of pollen in the two varieties, RA 87-3 and LCP 85-384, was

Table 2. Sugarcane cross combinations studied in the emasculation experiment.

	Combinations	Average number of seedlings obtained/g of seed	Number of evaluated seedlings
Crosses	LCP 85-384 emasculated x RA 87-3	129	43
	RA 87-3 emasculated x LCP 85-384	139	44
Selfings	LCP 85-384	23	22
	RA 87-3	14	11
	LCP 85-384 emasculated	0	0
	RA 87-3 emasculated	0	0

confirmed with a magnifying glass, whereas the presence of pollen was detected in the same varieties without treatment. The crosses were performed successfully and the number of seedlings obtained/g of seed is shown in Table 2. No viable seeds were obtained when LCP 85-384 and RA 87-3 were emasculated and selfed. DNA of the progeny of each cross combination was screened with the primer that best differentiated the parents, and the presence or absence of the specific markers was scored. The SSR primer (MSCIIR19), chosen out of the 15 previously screened (Perera *et al*., 2012), amplified seven polymorphic and three monomorphic fragments between the two varieties (Figure 1), whereas the remaining 14 primer pairs produced fewer polymorphic bands.

Molecular markers, especially SSRs, make it possible to determine self-pollination easily and accurately and to identify true hybrid progeny routinely, even in breeding program early stages (Zhang *et al*., 2004). Also, Manigbas and Villegas (2004) correctly identified the true hybrids derived from sugarcane crosses with one SSR. With this molecular hybridity test, the conventional sugarcane breeding cycle would be optimized (Manigbas and Villegas, 2004).

A segregation analysis was conducted to detect hybrids and possible distortions, especially taking into account sugarcane polyploid structure. It showed that each marker segregated in a Mendelian fashion (as evaluated by $\chi 2$ tests, $P \leq 0.05$) for each cross (Table 4) and selfing (Table 5). As SSR bands were considered dominant (and sugarcane is highly heterozygous) only half of the hybrid progeny (1:1) on average inherited each male or female-specific SSR band in the crossings, and a 3:1 ratio did so in the selfings (McIntyre and Jackson, 2001). For monomorphic bands in the crossings, segregation is expected to be 3:1, considering that sugarcane is highly heterozygous. Thus, it was important to use a sufficient number of male-specific bands to secure a reasonable probability in detecting hybrids. McIntyre and Jackson (2001) and Manigbas and Villegas (2004) have proved that RAPD and SSR markers can be a powerful tool to efficiently detect true hybrids. However, only the presence of female and male specific markers was scored to confirm the hybrid nature of the progeny without the employment of a statistical test.

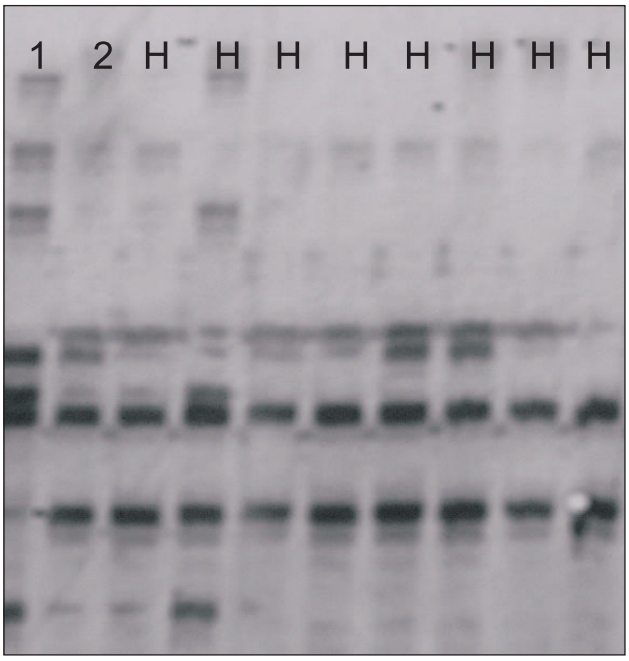

Figure 1. SSR molecular profiles of LCP 85-384 (1), RA 87-3 (2) and eight hybrids (H) in polyacrilamide gel.

Taking into consideration how markers segregated in the hybrids, out of the six markers presented in LCP 85-384, four were heterozygous (markers 5, 6, 8 and 10) and two were homozygous (markers 2 and 3), because the latter two did not segregate in the hybrids when LCP 85-384 was selfed (Table 5). Out of the seven markers present in RA 87-3, five were heterozygous (markers 1, 4, 7, 8 and 9) and two were homozygous (markers 3 and 5) (Table 5). Out of the three monomorphic bands between the two varieties, two did not segregate (markers 3 and 5) because, as it was aforementioned, they were both homozigous in RA 87-3, the same as marker 3 in LCP 85-384. The monomorphic marker 8 segregated in a 3:1 ratio as expected. Although only a limited number of SSR markers have been studied, this survey suggests that these markers with Mendelian inheritance were enough to allow detecting hybrids, even in spite of the polyploid nature of sugarcane.

Self-pollination can occur even after hot-water treatment to emasculate sugarcane (Pan *et al*., 2003). However, the implementation of an effective emasculation method allows the expansion of bi-parental crosses, so cultivars commonly used as males can be employed as females. This method could also help determine the influence of cytoplasmic inheritance, although some studies using molecular markers in sugarcane showed lack of cytoplasmic diversity among commercial varieties (D´Hont *et al*., 1993).

Results showed that the treatment was successful in the complete emasculation of RA 87-3 and LCP 85-384 varieties, without causing a serious reduction in both stigma and ovary viability, as reported by the Hawaiian Sugar Planters' Association (HSPA, 1984). Moreover, this method is simpler, faster and cheaper than other emasculation techniques (Machado *et al*., 1995), with the additional asset that the SSR technique allows assessing the fidelity of sugarcane crosses (Tew and Pan, 2005).

Table 4. Segregation analysis (χ2 test, *P*≤0.05) for each cross combination between two sugarcane varieties.

Genotyping			LCP 85-384 emasculated x RA 87-3				RA 87-3 emasculated x LCP 85-384			
Marker	LCP 85-384	RA 87-3	Expected segregation	Observed segregation	χ2	Probability (%)	Expected segregation	Observed segregation	χ2	Probability (%)
1		__	1:1	19:24	0.581	44.5766ns [a]	1:1	20:24	0.364	54.6494ns
2	__		1:1	Without segregation			1:1	Without segregation		
3	__	__	3:1	Without segregation			3:1	Without segregation		
4		__	1:1	20:23	0.209	64.7315ns	1:1	22:22	0.00	100.0ns
5	__	__	3:1	Without segregation			3:1	Without segregation		
6	__		1:1	20:23	0.209	64.7315ns	1:1	20:24	0.364	54.6494ns
7		__	1:1	23:20	0.209	64.7315ns	1:1	21:23	0.091	76.3025ns
8	__	__	3:1	32:11:00	0.209	64.7315ns	3:1	32:12:00	0.091	76.3025ns
9		__	1:1	23:20	0.209	64.7315ns	1:1	23:21	0.091	76.3025ns
10	__		1:1	27:16:00	2.814	9.3448ns	1:1	25:19:00	0.818	36.5712ns

[a]ns: not significant.

Table 5. Segregation analysis (χ2 test, *P*≤0.05) in the self progeny of two sugarcane varieties.

	LCP 85-384 selfed					RA 87-3 selfed				
Marker	**LCP 85-384**	**Expected segregation**	**Observed segregation**	**χ2**	**Probability (%)**	**RA 87-3**	**Expected segregation**	**Observed segregation**	**χ2**	**Probability (%)**
1						__	3:1	6:5	2.455	11.7185ns
2	__	3:1	Without segregation							
3	__	3:1	Without segregation			__	3:1	Without segregation		
4						__	3:1	7:4	0.758	38.4088ns
5	__	3:1	15:7	0.545	46.0181ns[a]	__	3:1	Without segregation		
6	__	3:1	15:7	0.545	46.0181ns					
7						__	3:1	7:4	0.758	38.4088ns
8	__	3:1	15:7	0.545	46.0181ns	__	3:1	7:4	0.758	38.4088ns
9						__	3:1	7:4	0.758	38.4088ns
10	__	3:1	15:7	0.545	46.0181ns					

[a]ns: not significant.

CONCLUSIONS

The immersion of RA 87-3 and LCP 85-384 panicles in water at 50ºC for five minutes was successful as an emasculation treatment. The implementation of this treatment in the local breeding program allowed widening the range of possibilities for genotype crosses.

The SSR markers with Mendelian inheritance made it possible to identify true hybrid progeny routinely in sugarcane breeding.

Therefore, both tools implemented jointly will improve the efficiency of sugarcane breeding programs.

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CITED REFERENCE

Aljanabi, S.; L. Forget and A. Dookun. 1999. An improved and rapid protocol for the isolation of polysaccharide and polyphenol-free sugarcane DNA. Plant Mol. Biol. Rep. 17 (3): 1-8.

Berding, N. 1981. Improved flowering and pollen fertility in sugarcane under increased night temperatures. Crop Sci. 21 (6): 863-867.

Caetano-Anollés, G. and P. M. Gresshoff. 1994. Staining nucleic acids with silver: an alternative to radioisotopic and fluorescent labeling. Promega Notes Magazine 45: 13.

Cordeiro, G. M.; G. O. Taylor and R. J. Henry. 2000. Characterisation of microsatellite markers from sugarcane (*Saccharum* sp.), a highly polyploid species. Plant Sci. 155 (2): 161-168.

D´Hont, A.; Y. H. Lu; P. Feldmann and J. C. Glazmann. 1993. Cytoplasmic diversity in sugarcane revealed by heterologous probes. Sugar Cane 1: 12-15.

Edmé, S. J.; N. G. Glynn and J. C. Comstock. 2006. Genetic segregation of microsatellite markers in *Saccharum officinarum* and *S. Spontaneum*. Heredity 97 (5): 366–375.

Grivet, L.; A. D'Hont; D. Roques; P. Feldmann; C. Lanaud and C. Glaszmann. 1996. RFLP mapping in cultivated sugarcane (*Saccharum* spp.): genome organization in a highly polyploid and aneuploid interspecific hybrid. Genetics 142 (3): 987-1000.

Hawaiian Sugar Planters' Association (HSPA). 1984. Breeding and selection. Experiment Station Annual Report: 1-11.

Hogarth, D. M. and N. Berding. 2005. Breeding for a better industry: conventional breeding. In: Proc. ISSCT Congress, 25, Guatemala, Guatemala, 2005, pp. 472-481.

Machado, J. R.; D. I. Walker; J. A. Bressiani; A. Goncalves and da J. Silva. 1995. Emasculation of sugarcane tassels using hot water. In: Proc. ISSCT Congress, 22, Cartagena, Colombia, 1995. pp. 346–348.

Manigbas, N. L. and L. C. Villegas. 2004. Microsatellite markers in hybridity tests to identify true hybrids of sugarcane. Phil. Crop Sci. 29 (2): 23-32.

McIntyre, C. L. and P. A. Jackson. 2001. Low level of selfing found in a sample of crosses in Australian sugarcane breeding programs. Euphytica 117 (3): 245–249.

Pan, Y.; T. L. Tew; M. P. Grisham; E. P. Richard Jr.; W. H. White and J. Veremis. 2003. Selection of interspecific sugarcane hybrids using microsatellite DNA markers. In: Proc. ISSCT Congress, 23, New Delhi, India, 2003, pp. 103.

Perera, M. F.; M. E. Arias; D. Costilla; A. C. Luque; M. B. García; M. I. Cuenya; J. Racedo; S. Ostengo; M. P. Filippone and A. P. Castagnaro. 2012. Evaluation of genetic diversity in sugarcane cultivars based on DNA markers and morphological traits. Euphytica 185 (3): 491–510.

Romero, G.; C. Adeva and Z. Battad. 2009a. Genetic fingerprinting: advancing the frontiers of crop biology research. Philippine Science Letters. [On line]. 2 (1): 8-13. Available from http://philsciletters.org/May%202,%202009/Genetic%20fingerprinting%20Advancing%20the%20frontiers%20of%20crop%20biology%20research%20-%20Romero%20et%20al..pdf (accessed 5 October 2012).

Romero, E. R.; P. A. Digonzelli y J. Scandaliaris. 2009b. Manual del cañero. 1. ed. EEAOC, Las Talitas, R. Argentina.

Shoda, M.; T. Nagamine; T. Terauchi; F. Akamine and A. Sugimoto. 1999. Isozyme application for variety identification and progeny hybridity in Japanese sugarcane. Breed Sci. 49 (2): 89-95.

Tew, T. L. and Y. Pan. 2005. Molecular assessment of the fidelity of sugarcane crosses with high-throughput microsatellite genotyping. J. Amer. Soc. Sugar Cane Technol. 25: 119.

Zhang, M. Q.; X. F. Zheng; A. L. Yu; J. S. Xu and H. Zheng. 2004. Molecular marker application in sugarcane. Sugar Tech. 6 (4): 251–259.