Optimal use of SSRs for Establishing Genetic Relationships and Variety Identification in a Collection of Sugarcane Hybrids

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Simple sequence repeat (SSR) analysis was carried out to assess genetic diversity and to establish genetic relatedness among sugarcane hybrids in a collection of 62 genotypes. Molecular data were also used to determine the discriminating power and utility of different SSR primers for sugarcane genotype identification and to find the optimal primer combination to ensure unambiguous identification. In 62 sugarcane hybrids, a total of 107 loci were detected with 13 SSR primers. The percentage of polymorphic loci was quite high (94%), with the NKS23, mSSCIR19 and NKS34 primers revealing the highest levels of polymorphism. The dissimilarity coefficients ranged from 0.064 to 0.68 with a mean of 0.46. The majority-rule consensus tree showed eight main clusters supported by more than 50% occurrences with several subgroups with the maximum bootstrap score (100%). A total of 315 banding patterns were detected. Discrimination power analysis revealed that the efficiency of a given primer does not depend only on the number of bands or the banding pattern but also on the frequency of differences between patterns generated by the primers. The primers NKS23 and mSSCIR19 generated patterns at the same frequency (isofrequencies) and have a maximal discriminating power. PIC values close to 1 (0.719 to 0.985) demonstrated that, in spite of the low number of primers, these SSR were sufficiently polymorphic, discriminating, and informative and will be useful in sugarcane variety registration and in genetic identity tests.

Key Words: Discrimination power, Genetic Diversity, SSR, Sugarcane

Germplasm collections are important in genetic conservation and as a component of plant improvement programs by providing plant breeders with sources of useful traits. Both cultivated and wild species are, commonly maintained in germplasm banks. The screening and the evaluation of the genetic diversity in these collections is essential to sustain scientific-based actions, not only to optimize and facilitate the breeding process but also to provide management strategies to maintain high degree of variability of the breeding materials. Given the number of genotypes present in collections, it is important to unambiguously distinguish between accessions to clarify synonyms and for properly attending rights protection.

The effectiveness in exploring diversity varies with the nature of traits (morphological, genealogical records, biochemical, chromosomal or molecular characters). However, it has been suggested that the underlying genetic diversity in cultivated sugarcane is quite narrow. Most of the sugarcane varieties share common ancestors derived from early few crosses involving *Saccharum officinarum* and *S. spontaneum* in the early nobilization process. Many Indian cultivars have been introduced and used in crosses in most sugarcane breeding programs in USA. For instance many Co cultivars were ancestral parent of LCP 85-384, several generations back (Liu *et al*., 2011). Compared to other crops, sugarcane breeding programs have historically been slow to progress because of the low efficiency and technical difficulties in crossing and selection processes (Chen *et al.*, 2009) as well as the narrow genetic base from which the original material was used in founding crosses. A common aim for many sugarcane breeding programs in the world is to increase the genetic base by introducing variability from related wild species, thus, it is necessary to accurately assess the genetic variability currently available in germplasm banks. Many methods have been used to evaluate genetic variability and genetic relationships among breeding materials in this crop. Although recent studies have been based on pedigrees, this approach is often constrained by the availability of accurate and complete information. The traditional methods that combine agronomical and morphological traits are not effective for this purpose because many of these characters are influenced by

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environmental factors and, thus, do not reflect underlying diversity. Morphological markers do not allow for reliable discrimination of sugarcane accessions when they are closely related. Molecular markers, however, do permit discrimination among commercial cultivars and provide accurate estimations of their genetic relationships. In the complex polyploid aneuploid sugarcane (*Saccharum* spp.), which harbors in excess of 100 chromosomes, interpretation and analysis of data generated by amplification of simple sequence repeats (SSRs) is more difficult than it is in diploid species (Glynn *et al.,* 2009). The multiple copies of homologous chromosomes present in the sugarcane genome make SSR manual band scoring difficult. However, the hypervariability of SSRs among related organisms make them an informative and excellent choice of marker for a wide range of applications in this crop, including genotype identification and analysis of genetic diversity (Pan *et al.,* 2007; Cordeiro *et al.,* 2003). Thus, to differentiate large numbers of sugarcane materials and establish genetic relationships among them, it is important to detect those SSRs that are easily scored and have a sufficient level of polymorphism. These parameters will not only ensure a low cost of analysis but also guarantee genotype discrimination and reduce the risk of confusion of any of these genotypes with a randomly chosen accession from a larger sample of the germplasm bank (Belaj *et al.*, 2004).

Tessier *et al.,* (1999) developed the parameter *Discriminating Power* by means of which the efficiency of a given primer used alone or in combination with others can be evaluated for the identification of varieties.

The objectives of this study were as follows: (1) to assess genetic diversity among sugarcane hybrids in the sugarcane breeding collection at INTA (Argentina), (2) to establish genetic relatedness among these materials, (3) to determine the discriminating power and effectiveness of different SSR primers for sugarcane genotype identification and (4) to determine the optimal SSR primer combination to ensure unambiguous identification of a set of sugarcane genotypes.

Materials and Methods

Plant Material

Sixty two sugarcane hybrids from a breeding collection at INTA (Tucumán, Argentina) mostly derived from *S officinarum* and *S. spontaneaum* were included in this study (Table 1). These genotypes are of interest for breeding purposes in Argentina due to their adaptability to subtropical areas (short cycle and early maturity). Some of these materials are or were used as commercial varieties in Argentina. Materials investigated are listed in Table 1. From these, F97-786, F97-395 and F98-70, are suspected to be duplicates of some other varieties studied based on field morphological resemblance. TUC77-42b is a suspected duplicate of TUC77-42 collected in a different site.

DNA Extraction

Total genomic DNA was extracted from young leaves according to Doyle and Doyle, (1987).

SSR Amplification

Based on the consistency of band patterns obtained in a previous study, 13 SSR primers were chosen (Table 2). Polymerase chain reactions (PCRs) were performed with 20 µl reaction mixes consisting of 6 ng of template DNA,

Table1. Sugarcane varieties included in the genetic variability analysis and country of origin

Varieties	Country of Origin	Varieties	Country of Origin
LCP85-384	Louisiana, USA	US74-1011	Louisiana, USA
LCP86-454	Louisiana, USA	US74--1015	Louisiana, USA
LCP85-376	Louisiana, USA	US72-1289	Louisiana, USA
NCO310	Natal, South Africa	L75-33	Louisiana, USA
HoCP85-845	Louisiana, USA	TCP81-3067	Tucumán, Argentina
HoCP92-648	Louisiana, USA	TCP87-388	Tucumán, Argentina
HoCP92-645	Louisiana, USA	NA78-724	Salta, Argentina
HoCP92-624	Louisiana, USA	NA84-3471	Salta, Argentina
HoCP89-888	Louisiana, USA	NA63-90	Salta, Argentina
HoCP91-552	Louisiana, USA	NA76-128	Salta, Argentina
HoCP92-631	Louisiana, USA	NA73-2596	Salta, Argentina
HoCP91-555	Louisiana, USA	NA88-948	Salta, Argentina
HoCP88-739	Louisiana, USA	NA73-1454	Salta, Argentina
HoCP90-941	Louisiana, USA	CP48-103	Louisiana, USA
CP70-1133	Louisiana, USA	TUC67-24	Tucumán, Argentina
CP79-1380	Louisiana, USA	TUC71-7	Tucumán, Argentina
CP79-318	Louisiana, USA	TUC68-18	Tucumán, Argentina
CP65-350	Louisiana, USA	TUC67-24	Tucumán, Argentina
CP57-603	Louisiana, USA	TUC79-9	Tucumán, Argentina
CP57-614	Louisiana, USA	TUC78-39	Tucumán, Argentina
CP72-2086	Louisiana, USA	TUC72-4	Tucumán, Argentina
CP66-346	Louisiana, USA	TUC69-2	Tucumán, Argentina
CP62-258	Louisiana, USA	L91-281	Louisiana, USA
FAM81-820	Tucumán, Argentina	RA89-686	Tucumán, Argentina
FAM83-11	Tucumán, Argentina	RA87-2	Tucumán, Argentina
TUC80-7	Tucumán, Argentina	RA91-209	Tucumán, Argentina
TUC72-16	Tucumán, Argentina	RA93-154	Tucumán, Argentina
TUC74-6	Tucumán, Argentina	CP88-1834	Louisiana, USA
CP87-357	Louisiana, USA	F98-70	Tucumán, Argentina
TUC71-7	Tucumán, Argentina	F97-395	Tucumán, Argentina
TUC68-18	Tucumán, Argentina	F97-786	Tucumán, Argentina
TUCCP77-42	Tucumán, Argentina	CP65-357	Louisiana, USA
TUCCP77-42b	Tucumán, Argentina		

Table 2. SSR primers used for genotyping 62 sugarcane accessions from the INTA Sugarcane Breeding Collection (Tucumán, Argentina)

SSR	Repit Motif	Size Range (bp)	Annealing Tm (ºC)	Forward Primer sequence (5` to 3`) Reverse Primer sequence (5` to 3`)
NKS26	$(TG)_{18}$	194-164	54	GTT CTC GAC ATG GGC CTA CT CTG CAC TTT CGG TCC TTT TT
mSSCIR19	$(GA)_{23}$	130-160	48	GGT TCC AAA ATA CAC AAA CAA TCT TAT CTA CGC ACT T
NKS38	$(AG)_{15}$	92-292	55	TGA ACT CGG CAA CAG TTT TT CCC ACC AAG TCG TTC TGA AT
NKS 23	$(GA)_{18}$	113-498	54	TAA ACC CCC GAA AAA GAA CC TCC GGA GGT AGA TCC ATT TG
NKS34	$(GT)_{18}$ $(A)_{31}$	131-214	58	CGT CTT GTG GAT TGG ATT GG TGG ATT GCT CAG GTG TTT CA
mSSCIR16	$(GA)_{18}$	130-300	54	TGG GGA GGG CTG ACT AGA GGC GGT ATA TAT GCT GTG
SMC703BS	$(CA)_{12}$	186-229	62	GCC TTT CTC CAA ACC AAT TAG T GTT GTT TAT GGA ATG GTG AGG A
mSSCIR3	$(GT)_{28}$	171-187	60	AAT GCT CCC ACA CCA AAT GC GGA CTA CTC CAC AAT GAT GC
mSSCIR18	$(GA)_{23}$	170-200	52	GGG TGT TCT GTT GAG CA GAG GTA GGA GGG AGT GTT
SMC766BS	$(CA)_{20}$ $(GA)_{16}$	170-270	60	TTA CTC GGC TGG GTT TTG TTC TAA GAA TCG TTC GCT CCA GC
SMC7CUQ	$(CA)_{10}$ $(C)_4$	160-170	60	GCC AAA GCA AGG GTC ACT AGA AGC TCT ATC AGT TGA AAC CGA
mSSCIR78	$(GTT)_6$	150-310	48	TGCCTTAAC CGT GAC ATC GAGGACGAGGAGCAGAA
mSSCIR34	(GA)	130-300	56	ATCGCCTCCACTAAATAAT TTGTCTTTGCTTCCTCCTC

0.1 mM dNTPs, 0.25 mM SSR primers (forward and reverse), 1 U of GoTaq DNA Polymerase (Promega) and 1X reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$). PCRs were carried out in a thermal cycler (Techne TC-412) using a program of 94ºC for 4 min and 35 cycles of 45 sec at 94ºC, 45 sec at 56ºC and 1 min at 72ºC. Each of the amplifications was repeated at least twice by independent PCR to examine the reproducibility and confirm band patterns. Amplification products were separated on 6% denaturing polyacrylamide gels using a GibcoBRL Model S2 Sequencing Gel Electrophoresis Apparatus (Life Technologies, Paisley, Scotland). Electrophoresis was carried out for 1.5 h at 60 W. At the end of electrophoresis, the gels were stained with silver nitrate according to Creste *et al.,* (2001). The resulting banding pattern was scored manually. Only consistent bands with strong intensity were considered for the analysis.

Data Analysis

Each band was treated as a unit locus, and the genotype was scored for the presence (1) or absence (0) of a band.

To measure the amount of genetic diversity, the polymorphism or rate of polymorphism was estimated as the proportion of polymorphic bands over the total number of markers per SSR primer.

Genetic dissimilarities among all possible pairs of sugarcane accessions were calculated as $d=1-s_{ij}$, where s_{ij} corresponds to the Jaccard similarity coefficient. Based on dissimilarity matrices, a hierarchical cluster analysis was performed using the UPGMA (Unweighted Pair Group Method using Arithmetic Average), and the relationships between all pairs of genotypes were visualized as a majority rule consensus tree (consensus threshold 50%). The cluster analysis was validated by bootstrap analysis with 1000 replications using FAMD software (Schluter and Harris, 2006). According to Highton, (1993) the limit of 50% was considered to indicate statistical support for the topology at a node in the bootstrap consensus tree.

To select the subset of SSR primers that produce informative profiles and to determine the discriminating power of each marker, the efficiency of a SSR primer

was evaluated using the following parameters for each assay unit (*U*) (the product of PCR amplification obtained with one set of primers) (Belaj *et al.*, 2004):

1. Number of polymorphic bands (n_p)
2. Number of non-polymorphic bands (n_{np})
3. Average number of polymorphic bands per assay unit (n_p/U)
4. Number of loci (*L*): Although SSRs are classified as co-dominant markers, given the highly complex genome of *Saccharum*, they have been treated as dominant markers, and each SSR marker represents a single locus (Cordeiro *et al.*, 2003).
5. Number of banding pattern for each SSR primer (T_p)
6. Confusion probability (C_j) on the jth assay unit: $c_j = \sum_{i=1}^{j} P_i \frac{(Npi-1)}{N-1}$, where p_i is the frequency of the ith pattern; *N*, sample size; *I*, total number of patterns generated by the primer
7. Discriminating power (D_j) of the jth assay unit: $D_j = 1 - C_j = 1 - \sum_{i=1}^{j} P_i \frac{(Npi-1)}{N-1}$
8. Limit of D_j as *N* tends toward infinity: $D_L = lim\ (D_j) = 1 - \sum_{i=1}^{j} P_i^2$
9. Effective number of patterns per assay unit: $P = \frac{1}{1-Dj}$

The optimal set of primer combinations for identification purposes was evaluated as described by Belaj *et al.*, (2004). In the set of 62 accessions, it is possible to find N(N-1)/2 different pairs; thus, the theoretical number of non-distinguishable pairs of genotypes is given by x_k= [N(N-1)/2]C_k, where C_k is the Joint confusion probability and is a product of the C_j of each primer under the independence hypothesis.

Results and Discussion

Even though SSRs are classified as co-dominant markers, their treatment as dominant markers was necessary to analyze the highly complex genome of *Saccharum*. All of the tested primers successfully amplified DNA from the Sugarcane Breeding Collection at INTA. With 13 SSR primers, 107 loci were detected in the set of 62 sugarcane varieties. The number of loci per marker ranged from 4 to 13 (Table 3). Marker size ranged between 110 to 310 bp. Primers that generated substantial polymorphism among the sugarcane accessions were detected. Of 107 fragments scored, 100 were polymorphic (94%), and the seven remaining showed monomorphic patterns (4.9%). The primers NKS23, mSSCIR19 and NKS34 generated the highest levels of polymorphism with 13, 12 and 12 polymorphic fragments, respectively. Earlier studies reported similar levels of polymorphism (Aitken *et al.*, 2006; Creste *et al.*, 2010; Singh *et al.*, 2010; Choperena *et al.*, 2009). These authors have estimated the polymorphism by AFLP or SSR markers. Although these markers have different distributions along nuclear DNA, the *P* values do not significantly differ. The high percentage of polymorphism can be explained because of the complex polyploid aneuploid in sugarcane. Working with EST-SSR, Ukoskit *et al.*, (2012) described high values of percentage of polymorphism (93.7%). They suggested that different chromosome numbers could

Table 3. Comparison of informativeness obtained from a set of 12 SSR primers in 62 sugarcane genotypes. Number of polymorphic bands (n_p), Number of non-polymorphic bands (n_{np}), Number of loci (*L*), Number of banding pattern for each SSR primer (T_p), Confusion probability (C_j), Effective number of patterns per assay unit (*P*). Primer discriminating power calculated from the sample of 62 sugarcane accessions (D_j) and estimated as *N* tends toward infinity (D_L). Number of varieties unique identified (*VI*)

SSR	n_p	n_{np}	*L*	T_p	C_j	D_j	D_L	*P*	Order of primers based on *Dj* and D_L	VI
NKS26	6	1	7	13	0.264	0.736	0.719	3.566	10	6
mSSCIR19	12	0	12	54	0.005	0.995	0.976	42.195	2	48
NKS38	8	0	8	25	0.025	0.975	0.963	26.676	4	12
NKS 23	13	0	13	59	0.000	1.000	0.985	68.907	1	56
NKS34	12	0	12	41	0.052	0.948	0.931	14.479	5	32
mSSCIR16	10	0	10	34	0.094	0.906	0.874	7.937	7	21
SMC703BS	3	2	5	6	0.268	0.732	0.720	3.571	12	0
mSSCIR3	5	0	5	12	0.245	0.755	0.740	3.850	11	6
mSSCIR18	6	0	6	15	0.080	0.920	0.904	10.441	6	0
SMC766BS	4	0	4	9	0.227	0.773	0.760	4.169	9	3
SMC7CUQ	6	3	9	13	0.182	0.818	0.805	5.141	8	5
mSSCIR78	10	0	10	34	0.017	0.983	0.968	31.480	3	22

explain their results. Chromosome numbers were not investigated in our materials. According to Liu *et al.*, (2011) SSR markers developed for one country might not work in another country due to differences in the genetic background of the genotypes. The set of SSR marker used in this research showed a high degree of transferability to the hybrid materials included (100% successfully amplified).

Fig. 1. shows the fingerprint of the 107 SSR loci. The illustration offers a graphic visualization of the variability estimated among the 62 sugarcane genotypes included in the study.

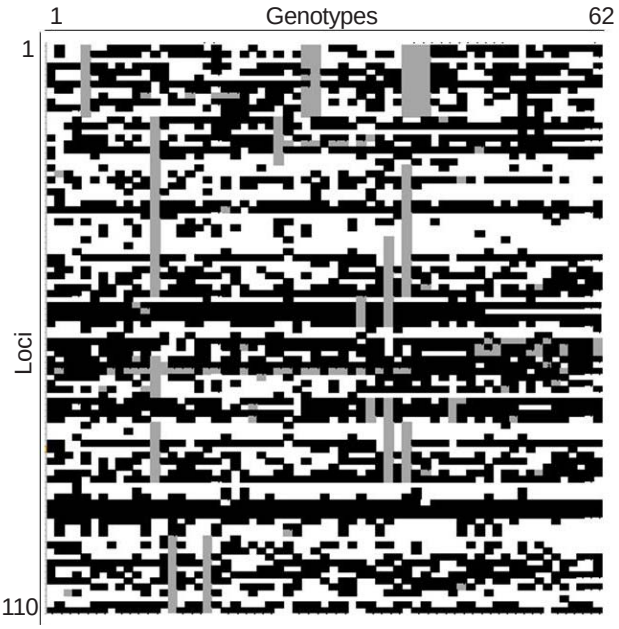

Fig. 1. SSR fingerprints of sugarcane hybrids from INTA Breeding Collection (Argentina). Shared absent bands (0) are shown in black; the white color indicates shared bands, (1) and the grey squares indicate missing data

The analysis of genetic relationships was carried out considering 58 sugarcane genotypes because individuals with more than 10% data missing were discarded. Loci with more than 10% missing data were also removed from the data matrix for the estimation of genetic dissimilarities. A histogram of pairwise dissimilarity for the remaining 58 sugarcane hybrids from the SSR data is presented in Figure 2 and indicates a normal distribution with a mean of 0.46. The dissimilarity coefficients ranged from 0.064 (CP65-357; F97-786) to 0.68 (CP57-614; NA73-1454). The majority of the dissimilarity coefficients were observed between 0.5 and 0.6, and most of the SSR-based pairwise comparisons exhibited genetic dissimilarities higher than 0.53. Table 4 shows the dissimilarity values between pairs of sugarcane hybrids.

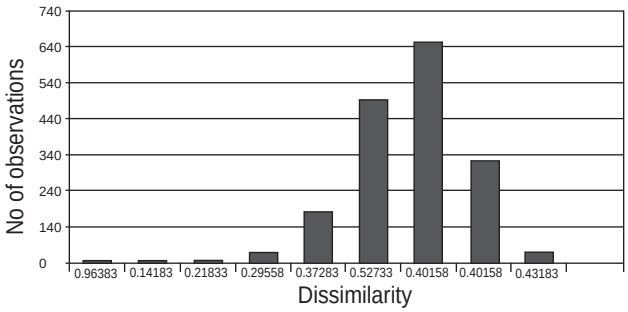

Fig. 2. Frequency of genetic dissimilarity among pairwise combinations of 56 sugarcane accessions based on SSR data.

The bootstrap analysis was summarized with a majority-rule consensus tree, considering the limit of 50% to indicate statistical support for the topology at a particular node (Fig. 3). Eight clusters were supported by more than 50% occurrences. Cluster VII and VIII and several subgroups within clusters I, II and III were supported by the maximum bootstrap score (100%), indicating strong support for these nodes. Cluster I contained the majority of sugarcane hybrids (19 genotypes), with 71% of bootstrap value. Ten of the twelve "TUC" varieties from EEAOC-Tucumán (Argentina) were grouped together, coinciding with their place of origin. The pairs F97-786/CP65-357 and TUC77-42b/F98-70, which were suspected to be duplicate genotypes, showed near expected low dissimilarity (0.064 and 0.13, respectively); and were grouped together, as supported by 100% occurrences. The PIC values were so high that even duplicate genotypes were differentiated at molecular level. In respect to clones TUC77-42 and TUC77-42b the dissimilarity value obtained (0.32) indicate that they are not the same genotype.

The remaining four branches were found to be unresolved (<50%) (LCP85-384, NA78-724, HoCP90-941 and NA63-90).

The number of banding patterns per marker (T_p) was highly variable, from 6 up to 59 (Table 3). For the 12 SSR tested, the total number of banding patterns was 315. Coinciding with the highest number of banding patterns, the microsatellite NKS23 allowed identification of the most unique accessions (56). However, SMC776BS, with only 4 different banding patterns, was able to discriminate 3 genotypes, which was better than mSSCIR18, with 6 banding patterns, which failed to identify any of the 62 sugarcane varieties studied. This information and the discrimination power analysis revealed that the efficiency of a given primer does not depend only on the number of bands or the banding pattern. These

Table 4. Genetic dissimilarities estimated for 58 sugarcane varieties based on SSR markers

	LCP65-384	LCP86-454	LCP85-376	NCo310	NoCP85-845	HoCP92-643	HoCP92-645	HoCP92-624	HoCP89-888	HoCP91-552	HoCP92-631	HoCP91-555	HoCP90-941	US74-1011	US74-1015	US72-1289	L75-33	TCP81-3067	TCP87-368	NA84-3013
LCP65-384	0																			
LCP56-454	0.45	0																		
LCP85-375	0.53	0.35	0																	
NCo310	0.54	0.54	0.33	0																
HoCP85-845	0.52	0.37	0.38	0.51	0															
HoCP92-648	0.38	0.58	0.54	0.5	0.53	0														
HoCP92-645	0.49	0.49	0.54	0.57	0.39	0.45	0													
HoCP92-624	0.4	0.45	0.51	0.52	0.49	0.4	0.44	0												
HoCP89-888	0.5	0.57	0.53	0.54	0.53	0.46	0.45	0.45	0											
HoCP91-552	0.43	0.46	0.46	0.54	0.39	0.35	0.38	0.4	0.38	0										
HoCP92-631	0.51	0.47	0.52	0.52	0.46	0.49	0.37	0.46	0.44	0.53	0									
HoCP91-555	0.53	0.42	0.43	0.51	0.43	0.46	0.45	0.45	0.52	0.47	0.42	0								
HoCP90-941	0.41	0.52	0.6	0.59	0.57	0.49	0.54	0.46	0.58	0.48	0.52	0.55	0							
US74-1011	0.56	0.43	0.5	0.54	0.38	0.52	0.41	0.46	0.44	0.49	0.36	0.41	0.52	0						
US74-1015	0.51	0.47	0.58	0.55	0.49	0.52	0.46	0.46	0.46	0.48	0.42	0.49	0.56	0.37	0					
US72-1282	0.59	0.48	0.51	0.58	0.45	0.53	0.49	0.48	0.5	0.45	0.38	0.54	0.58	0.37	0.44	0				
L75-33	0.41	0.47	0.49	0.55	0.4	0.47	0.4	0.38	0.48	0.48	0.32	0.47	0.49	0.42	0.47	0.44	0			
TCP81-3067	0.43	0.38	0.42	0.49	0.47	0.46	0.48	0.46	0.46	0.38	0.52	0.56	0.43	0.52	0.48	0.54	0.45	0		
TFP87-388	0.48	0.53	0.68	0.63	0.54	0.48	0.45	0.47	0.57	0.52	0.41	0.56	0.43	0.47	0.43	0.52	0.46	0.51	0	
NA84-3013	0.52	0.48	0.43	0.33	0.51	0.48	0.55	0.48	0.57	0.54	0.43	0.47	0.62	0.47	0.51	0.52	0.53	0.51	0.59	0
NA78-724	0.5	0.57	0.53	0.56	0.47	0.53	0.51	0.52	0.43	0.48	0.48	0.54	0.53	0.53	0.55	0.5	0.38	0.53	0.59	0.52
NA84-3471	0.56	0.57	0.55	0.46	0.55	0.43	0.48	0.46	0.54	0.54	0.43	0.55	0.63	0.47	0.55	0.56	0.52	0.49	0.61	0.38
NA63-90	0.43	0.60	0.49	0.41	0.55	0.35	0.55	0.42	0.45	0.47	0.47	0.42	0.47	0.54	0.52	0.59	0.42	0.45	0.54	0.49
NA76-128	0.4	0.55	0.51	0.49	0.52	0.23	0.49	0.4	0.47	0.45	0.47	0.47	0.56	0.46	0.56	0.5	0.47	0.47	0.51	0.4
NA73-2596	0.42	0.52	0.52	0.48	0.47	0.34	0.37	0.39	0.42	0.42	0.39	0.43	0.43	0.49+	0.52	0.51	0.48	0.44	0.49	0.46
NA88-948	0.5	0.46	0.56	0.57	0.55	0.43	0.36	0.37	0.5	0.48	0.38	0.54	0.49	0.51	0.48	0.47	0.47	0.39	0.42	0.5
NA73-1454	0.64	0.57	0.45	0.33	0.54	0.51	0.59	0.55	0.57	0.54	0.6	0.52	0.6	0.56	0.51	0.57	0.58	0.48	0.63	0.44
CP48-103	0.48	0.54	0.61	0.6	0.47	0.37	0.49	0.51	0.56	0.51	0.52	0.48	0.59	0.59	0.49	0.6	0.52	0.54	0.42	0.55
CP68-350	0.37	0.52	0.6	0.59	0.57	0.4	0.35	0.4	0.45	0.43	0.35	0.51	0.49	0.51	0.45	0.52	0.44	0.43	0.33	0.55
CP70-1133	0.44	0.39	0.49	0.48	0.53	0.44	0.47	0.31	0.52	0.44	0.44	0.47	0.47	0.49	0.5	0.53	0.44	0.34	0.53	0.46
CP79-1380	0.4	0.51	0.48	0.44	0.45	0.43	0.47	0.39	0.57	0.48	0.48	0.49	0.46	0.46	0.46	0.51	0.51	0.42	0.55	0.42
CP79-318	0.43	0.54	0.49	0.52	0.47	0.43	0.41	0.34	0.46	0.42	0.39	0.44	0.51	0.5	0.47	0.46	0.3	0.49	0.51	0.51
CP65-350	0.4	0.38	0.46	0.48	0.36	0.44	0.37	0.37	0.45	0.42	0.36	0.34	0.42	0.36	0.46	0.45	0.36	0.4	0.45	0.45
CP57-603	0.49	0.5	0.58	0.53	0.54	0.52	0.46	0.44	0.58	0.44	0.48	0.51	0.5	0.53	0.45	0.54	0.52	0.44	0.49	0.49
CP57-614	0.41	0.51	0.63	0.65	0.59	0.48	0.49	0.4	0.53	0.53	0.48	0.58	0.44	0.5	0.56	0.58	0.45	0.42	0.46	0.61
CP72-2086	0.34	0.41	0.51	0.54	0.48	0.5	0.48	0.38	0.47	0.45	0.47	0.53	0.48	0.52	0.58	0.52	0.39	0.37	0.56	0.52
CP66-346	0.46	0.41	0.37	047	0.34	0.48	0.42	0.5	0.48	0.41	0.44	0.45	0.59	0.44	0.44	0.42	0.44	0.44	0.51	0.49
FAM81-820	0.49	0.58	0.52	0.53	0.49	0.45	0.48	0.42	0.54	0.49	0.44	0.43	0.52	0.49	0.5	0.47	0.38	0.56	0.52	0.54
TUC80-7	0.44	0.4	0.28	0.35	0.33	0.45	0.39	0.44	0.48	0.39	0.41	0.35	0.52	0.35	0.44	0.5	0.35	0.38	0.56	0.38
TUC72-16	0.35	0.52	0.48	0.47	0.42	0.32	0.37	0.33	0.37	0.29	0.42	0.46	0.45	0.47	0.4	0.43	0.29	0.4	0.44	0.5
TUC74-6	0.44	0.5	0.47	0.6	0.49	0.45	0.54	0.41	0.52	0.47	0.43	0.45	0.5	0.55	0.48	0.52	0.42	0.5	0.47	0.44
TUC71-7	0.41	0.45	0.44	0.52	0.32	0.41	0.42	0.45	0.5	0.41	0.44	0.49	0.51	0.45	0.45	0.48	0.33	0.39	0.48	0.5
TUC68-18	0.49	0.5	0.58	0.53	0.57	0.45	0.58	0.52	0.51	0.47	0.5	0.57	0.48	0.57	0.48	0.53	0.45	0.41	0.46	0.55
TUC67-24	0.37	0.42	0.51	0.49	0.33	0.33	0.39	0.39	0.4	0.37	0.35	0.38	0.48	0.38	0.38	0.42	0.41	0.45	0.33	0.45
TUC77-42	0.42	0.45	0.42	0.43	0.39	0.36	0.41	0.33	0.44	0.34	0.43	0.42	0.48	0.44	0.45	0.52	0.32	0.37	0.49	0.52
TUC77-42	0.47	0.43	0.53	0.58	0.25	0.46	0.43	0.46	0.56	0.49	0.43	0.5	0.5	0.43	0.46	0.46	0.39	0.5	0.4	0.52
TUC78-39	0.51	0.59	0.52	0.47	0.55	0.52	0.47	0.38	0.52	0.48	0.52	0.48	0.56	0.52	0.52	0.54	0.51	0.55	0.53	0.46
TUC72-4	0.35	0.49	0.57	0.57	0.4	0.43	0.44	0.4	0.46	0.46	0.46	0.44	0.48	0.51	0.5	0.56	0.51	0.55	0.41	0.58
TUC69-2	0.44	0.45	0.5	0.52	0.3	0.43	0.45	0.39	0.5	0.43	0.37	0.47	0.48	0.44	0.45	0.43	0.32	0.43	0.43	0.52
L91-ZB1	0.35	0.42	0.52	0.53	0.48	0.44	0.43	0.36	0.47	0.44	0.4	0.5	0.45	0.49	0.52	0.52	0.25	0.4	0.45	0.55
RA89-686	0.49	0.57	0.55	0.41	0.55	0.42	0.53	0.52	0.54	0.49	0.5	0.51	0.57	0.55	0.5	0.6	0.55	0.46	0.53	0.38
RA87-2	0.39	0.54	0.56	0.55	0.51	0.31	0.49	0.38	0.55	0.48	0.45	0.5	0.48	0.53	0.48	0.54	0.39	0.39	0.47	0.52
RA91-209	0.51	0.56	0.54	0.5	0.57	0.38	0.55	0.48	0.55	0.5	0.56	0.55	0.5	0.57	0.55	0.55	0.61	0.47	0.46	0.41
RA93-154	0.5	0.58	0.51	0.52	0.59	0.43	0.49	0.47	0.5	0.48	0.53	0.5	0.47	0.58	0.56	0.56	0.51	0.5	0.6	0.52
CP88-1834	0.52	0.37	0.47	0.55	0.36	0.54	0.45	0.46	0.46	0.44	0.37	0.5	0.52	0.43	0.44	0.38	0.38	0.43	0.42	0.51
F98-70	0.5	0.5	0.55	0.6	0.42	0.44	0.48	0.46	0.47	0.47	0.48	0.46	0.57	0.47	0.46	0.48	0.39	0.51	0.53	0.55
F97-395	0.4	0.51	0.58	0.56	0.5	0.48	0.48	0.42	0.45	0.43	0.41	0.52	0.46	0.5	0.46	0.48	0.29	0.42	0.5	0.59
F97-786	0.44	0.52	0.55	0.56	0.44	0.46	0.48	0.44	0.47	0.47	0.34	0.49	0.52	0.43	0.45	0.49	0.25	0.45	0.47	0.56
CF85-357	0.44	0.54	0.57	0.58	0.46	0.47	0.44	0.44	0.44	0.44	0.38	0.52	0.52	0.46	0.45	0.47	0.25	0.48	0.49	0.58
TUC77-426	0.45	0.58	0.63	0.63	0.44	0.47	0.46	0.44	0.5	0.47	0.52	0.54	0.53	0.54	0.47	0.52	0.43	0.51	0.52	0.6

Contd .....

	NA78-724	NA84-3471	NA63-90	NA76-128	NA73-2596	NA88-948	NA73-164	CP48-103	CP68-360	CP70-1133	CP79-1380	CP79-318	CP65-360	CPS7-603	CPS7-614	CP72-2086	CP66-346	FAAM1-820	TUCRO-7	TUC72-16	TUC74-6	TUC71-7	TUC68-18	TUC67-24
NA78-724	0																							
NA84-3471	053	0																						
NA63-90	0.46	0.37	0																					
NA76-128	0.48	0.33	0.35	0																				
NA73-2596	0.41	0.38	0.34	0.34	0																			
NA33-948	0.54	0.46	0.56	0.47	0.55	0																		
NA93-1454	0.59	0.52	0.45	0.55	0.56	0.55	0																	
CP48-103	0.62	0.55	0.47	0.46	0.45	0.54	0.6	0																
CP68-350	0.51	0.51	0.47	0.43	0.34	0.25	0.65	0.47	0															
CP70-1133	0.53	0.46	0.45	0.44	0.44	0.43	0.54	0.48	0.48	0														
CP79-1380	0.59	0.44	0.36	0.46	0.39	0.49	0.55	0.51	0.46	0.35	0													
CP79-318	0.42	0.46	0.3	0.48	0.44	0.53	0.49	0.49	0.48	0.43	0.39	0												
CP65-350	0.53	0.52	0.41	0.42	0.41	0.48	0.54	0.48	0.44	0.31	0.33	0.43	0											
CP57-603	0.64	0.53	0.49	0.55	0.51	0.42	0.52	0.57	0.43	0.44	0.44	0.5	0.35	0										
CP57-614	0.55	0.56	0.55	0.55	0.36	0.53	0.68	0.56	0.33	0.47	0.45	0.53	0.48	0.56	0									
CP72-2056	0.47	0.52	0.5	0.47	0.4	0.48	0.61	0.52	0.49	0.25	0.51	0.44	0.57	0.55	0.5	0								
CP66-346	0.47	0.52	0.49	0.5	0.47	0.46	0.45	0.46	0.52	0.5	0.5	0.43	0.4	0.51	0.53	0.52	0							
FAM81-820	0.49	0.51	0.45	0.47	0.44	0.52	0.5	0.53	0.51	0.47	0.47	0.37	0.4	0.57	0.51	0.48	0.41	0						
TUC80-7	0.48	0.4	0.37	0.44	0.42	0.49	0.48	0.53	0.52	0.48	0.57	0.39	0.51	0.44	0.54	0.51	0.29	0.42	0					
TUC72-16	0.4	0.45	0.33	0.41	0.37	0.48	0.46	0.42	0.41	0.47	0.4	0.21	0.36	0.51	0.43	0.44	0.32	0.37	0.33	0				
TUC74-6	0.58	0.58	0.44	0.42	0.45	0.48	0.59	0.44	0.49	0.5	0.47	0.42	0.41	0.51	0.54	0.52	0.47	0.45	0.44	0.44	0			
TUC71-7	0.55	0.49	0.41	0.36	0.44	0.52	0.57	0.44	0.48	0.51	0.42	0.42	0.34	0.46	0.56	0.49	0.34	0.45	0.23	0.28	0.33	0		
TUC68-18	0.58	0.53	0.35	0.48	0.44	0.54	0.52	0.44	0.47	0.57	0.52	0.51	0.46	0.53	0.54	0.54	0.48	0.48	0.45	0.39	0.37	0.52	0	
TUC67-24	0.46	0.48	0.44	0.38	0.32	0.46	0.54	0.24	0.39	0.42	0.45	0.43	0.36	0.53	0.46	0.4	0.38	0.48	0.4	0.22	0.41	0.36	0.41	0
TUC79-9	0.47	0.51	0.4	0.47	0.38	0.45	0.49	0.53	0.35	0.41	0.41	0.32	0.35	0.46	0.44	0.43	0.42	0.44	0.3	0.23	0.51	0.37	0.46	0.38
TUC77-42	0.54	0.59	0.54	0.46	0.51	0.5	0.63	0.45	0.49	0.5	0.43	0.5	0.34	0.56	0.58	0.53	0.42	0.53	0.39	0.41	0.46	0.35	0.53	0.34
TUC78-39	0.5	0.5	0.44	0.52	0.44	0.5	0.49	0.61	0.45	0.45	0.43	0.35	0.49	0.45	0.51	0.57	0.5	0.47	0.45	0.44	0.57	0.56	0.59	0.56
TUC72-4	0.53	0.6	0.44	0.51	0.45	0.51	0.63	0.58	0.44	0.53	0.49	0.44	0.57	0.56	0.48	0.52	0.44	0.57	0.43	0.35	0.45	0.42	0.48	0.28
TUo69-2	0.52	0.54	0.48	0.45	0.42	0.45	0.57	0.45	0.42	0.43	0.43	0.44	0.52	0.54	0.41	0.5	0.35	0.42	0.34	0.27	0.41	0.25	0.44	0.33
L91-281	0.52	0.53	0.48	0.48	0.43	0.41	0.61	0.54	0.38	0.36	0.46	0.39	0.35	0.46	0.28	0.37	0.45	0.43	0.46	0.55	0.47	0.39	0.52	0.4
RA59-686	0.51	0.43	0.43	0.44	0.33	0.44	0.47	0.53	0.43	0.47	0.49	0.52	0.52	0.51	0.45	0.56	0.49	0.55	0.69	0.42	0.53	0.52	0.49	0.45
RA87-2	0.57	0.46	0.4	0.59	0.4	0.46	0.54	0.47	0.48	0.39	0.39	0.39	0.4	0.48	0.41	0.42	0.46	0.35	0.46	0.36	0.35	0.34	0.41	0.43
RA91-209	0.57	0.47	0.44	0.4	0.32	0.42	0.54	0.52	0.47	0.54	0.4	0.52	0.54	0.56	0.5	0.58	0.51	0.52	0.51	0.49	0.44	0.44	0.48	0.45
RA93-154	0.55	0.54	0.46	0.44	0.47	0.43	0.5	0.59	0.53	0.51	0.53	0.38	0.51	0.56	0.54	0.57	0.48	0.47	0.48	0.44	0.49	0.46	0.58	0.57
CP85-1534	0.5	0.55	0.52	0.48	0.45	0.42	0.56	0.52	0.43	0.48	0.48	0.45	0.41	0.52	0.46	0.49	0.36	0.4	0.42	0.36	0.4	0.35	0.44	0.39
P98-70	0.54	0.61	0.54	0.45	0.53	0.52	0.58	0.54	0.5	0.51	0.53	0.45	0.41	0.54	0.55	0.55	0.54	0.43	0.39	0.34	0.46	0.35	0.55	0.44
P97-395	0.4	0.54	0.45	0.56	0.47	0.45	0.58	0.58	0.44	0.36	0.45	0.25	0.4	0.49	0.38	0.38	0.43	0.42	0.47	0.3	0.52	0.48	0.5	0.46
P97-786	0.59	0.51	0.39	0.43	0.41	0.5	0.51	0.5	0.46	0.44	0.49	0.35	0.59	0.50	0.49	0.43	0.45	0.38	0.41	0.35	0.46	0.35	0.43	0.41
CP65-357	0.36	0.55	0.45	0.47	0.44	0.53	0.59	0.59	0.48	0.46	0.52	0.5	0.39	0.55	0.52	0.45	0.42	0.33	0.42	0.31	0.46	0.55	0.43	0.44
TUC77-42b	0.6	0.62	0.55	0.53	0.52	0.53	0.61	0.49	0.5	0.51	0.51	0.46	0.44	0.55	0.54	0.51	0.5	0.44	0.47	0.37	0.47	0.38	0.5	0.44

	TUC79-9	TUC77-42	TUC78-39	TUC72-4	TUC69-2	L91-281	RA89-686	RA87-2	RA91-209	RA93-154	CP88-1834	F98-70	F97-395	F97-786	CP85-357	TUC77-42b
TUC79-9	0															
TUC77-42	0.4	0														
TUC78-39	0.41	0.58	0													
TUC72-4	042	0.33	0.55	0												
TUC69-2	0.36	0.35	0.52	0.33	0											
L91-281	0.32	0.47	0.46	0.41	0.31	0										
RA89-686	0.43	0.6	0.43	0.53	0.46	0.49	0									
RA87-2	0.37	0.47	0.48	0.46	0.32	0.27	0.45	0								
RA91-209	0.53	0.56	0.5	0.54	0.46	0.49	0.38	0.37	0							
RA93-154	0.44	0.58	0.41	0.59	0.47	0.42	0.43	0.38	0.42	0						
CP55-1834	0.42	0.39	0.45	0.47	0.26	0.32	0.52	0.35	0.41	0.47	0					
F96-70	0.37	0.28	0.51	0.4	0.35	0.39	0.53	0.41	0.55	0.42	0.33	0				
F97-395	0.39	0.52	0.39	0.45	0.37	0.23	0.49	0.35	0.56	0.42	0.37	0.44	0			
F97-786	0.38	0.45	0.49	0.47	0.3	0.35	0.48	0.37	0.53	0.46	0.36	0.43	0.22	0		
CP85-357	0.41	0.48	0.47	0.47	0.31	0.38	0.51	0.37	0.55	0.44	0.33	0.4	0.2	0.06	0	
TUC77-42b	0.41	0.32	0.51	0.37	0.38	0.42	0.56	0.39	0.57	0.49	0.4	0.13	0.44	0.45	0.41	0

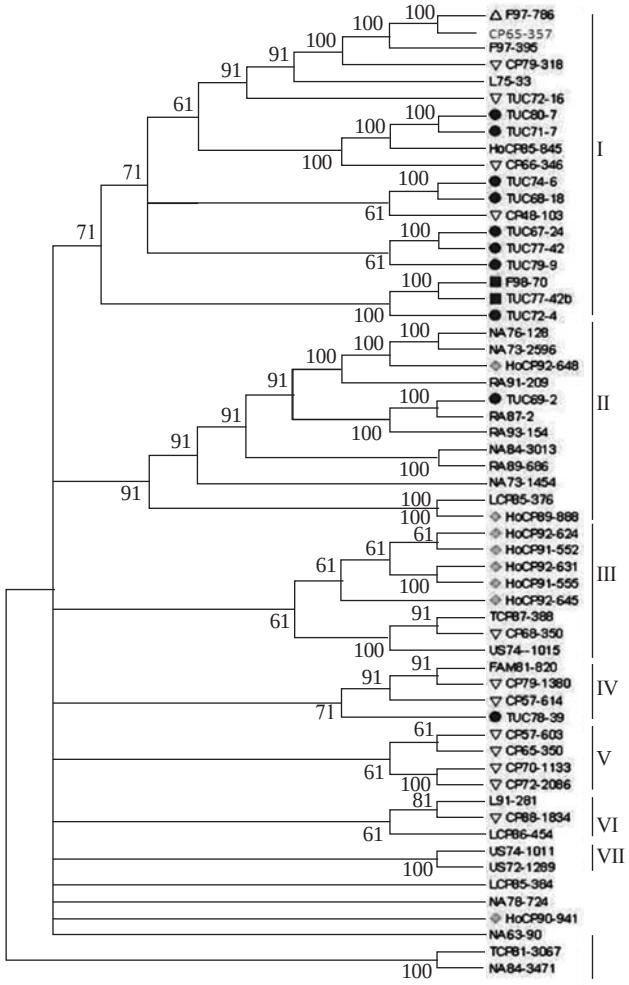

Fig. 3. Unrooted phylogenetic tree depicting the genetic relationships among the *Saccharum* based on genetic dissimilarity coefficients. The numbers indicate the percentages with which a given branch is supported in 1000 bootstrap replications. The consensus tree only shows a topology but does not display branch lengths. ▲ Indicate two duplicate accessions in the collection. ● ▽ ◊ Indicates accessions from EEAOC-Tucumán (Argentina), Canal Point (Florida-Houma)-USDA and Houma (Louisiana) Canal Point (Fla)-USDA, respectively.

Table 5. Primers optimal combination for the identification of a set sugarcane accessions from INTA Breeding Collection. Joint confusion probability (C_k) for the five most discriminating primers. Theoretical number of non-differentiated pairs of genotypes (X_k) for a given combination of k primers on a set of 62 accessions (1891 pairs) estimated under the hypothesis of independence of the considered primers patterns. Because cero values for Cj were found, a correction was applied (C_{Jc}=C_jx0.001)

SSR	C_j	C_{jc}	C_k	X_k
NKS 23	0.000	0.001	1.00×10^{-3}	4,3
mSSCIR19	0.005	0.006	6.00×10^{-6}	2.5×10^{-2}
mSSCIR78	0.017	0.018	1.08×10^{-7}	4.6×10^{-4}
NKS38	0.025	0.026	2.00×10^{-9}	8.5×10^{-6}
NKS34	0.052	0.053	1.48×10^{-10}	6.3×10^{-7}

findings are consistent with those of Tessier *et al.*, (1999). Using random amplified polymorphic DNA, they found that discriminating power depends on the frequency differences between patterns generated by the primers. They found that a primer has maximal discriminating power when it generates patterns at the same frequency (the isofrequency situation). In our case, the primers with the maximal discriminating power were NKS23 and mSSCIR19, which generated different numbers of banding patterns but were nearly isofrequent (0.0003 to 0.01). In contrast, primers NKS16 and SMC703BS showed very different frequencies (0.06 to 0.44) in their banding patterns, which account for their lower discriminating powers, confirming that the efficiency of SSR loci is also subject to this rule.

Low values for confusion probability (Cj) were obtained. They ranged from 0 (NKS23) to 0.268 (SMC703S). As it is negatively correlated, the microsatellite NKS23 showed the highest value of discriminating capacity (Dj) supporting the significant utility of this SSR primer in sugarcane variety identification. However, there are other primers with high Dj values (>0.9) that are also effective (Table 3).

The DL value ranked from 0.719 to 0.985, with a mean of 0.865. DL value is an extension of the Polymorphism Information Content (PIC) (Anderson *et al.*, 1993), which is determined by the frequencies of different banding patterns generated by a primer. Our results are in the range of those values reported by Pan (2006) and Creste *et al.*, (2010), 0.75 and 0.82 respectively. Moreover, the PIC values for SSRs used in this study were higher than those mean PIC values reported by Marconi *et al.*, (2011) and Banumathi *et al.*, (2010), 0.69 and 0.66 respectively. Liu *et al.*, (2011) recognized that the PIC value of any SSR marker is not constant and change with the number of samples, the more diverse the panel of genotypes, the higher the PIC values. Banumathi *et al.*, (2010) and Marconi *et al.*, (2011) worked with a panel of 48 and 18 clones respectively. The ability of a few markers to generate unique genetic profiles in sugarcane accessions is due to the complex polyploid nature of the genome, which allowed the detection of several allelic types in a single accession. DL values close to 1 demonstrated that, in spite of the low number of primers (12) used, these SSRs were sufficiently polymorphic and informative and should be useful in registration of sugarcane varieties and in genetic identity tests.

High values for effective number of patterns (*P*) per unit assay were detected. The highest value (68.90) corresponds to NKS23. Belaj *et al.,* (2003) showed that this parameter indicates the size of an ideal population in which all of the individuals can be distinguished. In our case, with NKS23, almost 69 parameters can be obtained when the population size tends to infinity (up to 69 varieties can be identified with the same primer). Relatively high *P* values illustrate SSR primer discrimination capacity when handling a large number of samples in sugarcane, which is very important for germplasm collections management where numerous genotypes need to be accurately characterized and identified.

The primers NKS 23, mSSCIR19, mSSCIR78, NKS38 and NKS34 were selected based on their high discriminating powers. The joint confusion probabilities (C_x) and theoretical numbers of indistinguishable pairs (X_k) estimated with these primers showed that the combination of the first two primers (NKS23 and mSSCIR19) is effective for discriminating the 62 sugarcane genotypes with a cumulative confusion probability value of 6.00 x 10^{-6}, with just 0.025 pairs of accessions from the theoretical 1891 pairs indistinguishable (Table 5). Increasing the number of combinations of primers decreases the theoretical number of indistinguishable pairs, but the cost of the analysis increases. For this reason, the most informative primers should be chosen to reduce the cost and the time of analysis and ensure reliable varietal identification.

Conclusions

SSRs were useful for assessing genetic variation and genetic relationships of materials in a sugarcane breeding collection at INTA. The polyploid nature of the sugarcane genome allows the detection of numerous SSR banding patterns, and thus a small number of microsatellite markers generate unique genetic profiles in sugarcane genotypes. The high PIC values and the high discriminating capacity of SSR primer combinations tested in this study suggest that SSRs would be suitable for use in genotype identification.

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