GMR

Changes on microsatellites of expressed sequence tag of sugarcane (*Saccharum* spp) during vegetative propagation

R. Augusto[1], R.C. Maranho[2], C.A. Mangolin[3], J.C. Bespalhok Filho[4] and M.F.P.S. Machado[3]

[1]Programa de Pós-Graduação em Genética e Melhoramento,
Universidade Estadual de Maringá, Maringá, PR, Brasil
[2]Programa de Pós-Graduação em Agronomia,
Universidade Estadual de Maringá, Maringá, PR, Brasil
[3]Departamento de Biotecnologia, Genética e Biologia Celular,
Universidade Estadual de Maringá, Maringá, PR, Brasil
[4]Departamento de Fitotecnia e Fitossanidade,
Universidade Federal do Paraná, Curitiba, PR, Brasil

Corresponding author: M.F.P.S. Machado
E-mail: mfpsmachado@uem.br

ABSTRACT. The reduction in sugarcane productivity in subsequent cutting stages may be related to a gradual decrease of the allele number and mean observed heterozygosity (H_O) in the sugarcane ratoon. This hypothesis was tested assessing the number of alleles and H_O values in 10 expressed sequence tag microsatellites (*Est-SSR* loci) of the sugarcane varieties RB72454 and RB867515 in different cutting stages. Changes of allele numbers in samples of different cutting stages were observed in seven and six *EstSSR* loci of the RB72454 and RB867515 varieties, respectively. Reduction of allele numbers was observed in the samples collected in the fourth and sixth cutting stages of the RB72454

variety. In contrast, an increase of the allele numbers was detected in the samples collected on fourth, sixth, and seventh cutting stages of the RB867515 variety. Unchanged allele numbers were observed only in *EstB41*, *EstC84*, and *EstB130* loci of the RB72454 variety, and *EstB41*, *EstC67*, *EstA68*, and *EstB130* loci of the RB867515 variety. The variety RB867515 has lower polymorphism and values of H_O than the RB72454 variety in different stages of cutting. At molecular level, in *Est-SSR* loci, the RB72454 variety showed higher changes in subsequent stages of cutting than RB867515. The similarities and divergences at molecular level between varieties RB72454 and RB867515 observed in the 10 *Est-SSR* loci during subsequent cutting stages can not explain the reduced productivity frequently observed after subsequent cutting stages but showed that phenotypic and physiological changes after each cutting stage are also accompanied by changes at genomic level.

Key words: *Est-SSR* polymorphism; Sugarcane varieties; Vegetative propagation; Reduced productivity; Genetic instability

INTRODUCTION

Microsatellite markers also known as DNA-simple sequence repeats (SSR loci) have been extensively used as molecular markers in sugarcane (*Saccharum* spp). Although microsatellites have co-dominant inheritance (Tautz, 1989; Weber and May, 1989), the analysis of SSR loci in sugarcane has been especially useful to evaluate genetic distances among sugarcane varieties (Singh et al., 2008, 2011; Oliveira et al., 2009; Duarte-Filho et al., 2010; Liu et al., 2011; Silva et al., 2012). The estimation of genetic diversity within each variety from the mean expected heterozygosity (H_E) is inaccurate since cultivated sugarcane varieties have one of the most complex plant genomes, carrying variable chromosome numbers (Grivet and Arruda, 2002).

The estimation of genetic diversity within each variety evaluated from the mean observed heterozygosity (H_O) is plausible but has been little investigated (Maranho et al., 2014). The genetic structure of nine sugarcane varieties using 15 primers for SSR loci identified RB72454 and RB867515 varieties as presenting the highest levels of H_O (Maranho et al., 2014). The RB72454 variety was widely cultivated in Brazil until 2000 and the RB867515 was considered the most widely grown and most cultivated until 2015 for its remarkable features: high productivity and high sucrose content.

Sugarcane cultures are maintained by vegetative propagation via stem cuttings or sections of the stalk. After planting the sugarcane seedlings, the first cut is accomplished within 12-18 months. The annual regrowth of the ratoons ensures the harvest in the cutting stages for about 4 years, or until reduced productivity when becomes necessary the replanting of the sugarcane field (Matos, 2011). The genetic stability in expressed loci for desirable agronomic characteristics is expected during the different cutting stages in order to maintain productivity in the sugarcane varieties. Our objective in the current study was to assess the H_O in expressed sequence tag microsatellites (*Est-SSR* loci) of the RB72454 and RB867515 varieties to verify if the number of alleles and the heterozygosity levels in each *Est-SSR* locus are kept during subsequent cutting stages. Changes in allele numbers may suggest the loss or the emergence

of new alleles while changes in H_O values may suggest variation in allele frequencies in SSR loci associated with agronomic characteristics of interest during the subsequent cutting stages of sugarcane cultures maintained by vegetative propagation.

MATERIAL AND METHODS

Sugarcane samples

The sugarcane varieties RB72454 and RB867515 analyzed in the present study were generated from a breeding program at Rede Interuniversitária para o Desenvolvimento do Setor Sucroenergético (RIDESA). The crossing between CP53-76 with an unknown genotype produced the RB72454 while RB867515 was obtained from a crossing between RB72454 with an unknown genotype. Both varieties have been cultivated in Northern Paraná State and supply the ethanol and sugar factories (Fazenda Junqueira, Alto Alegre District in Colorado, PR, Brazil). The selections of samples included 24 plants of the RB72454 variety (eight plants on each cutting stage - second, fourth, and sixth) and 31 plants of the RB867515 variety (seven plants on the second cutting stage and eight plants on fourth, sixth, and seventh cutting stages).

The RB867515 is the most cultivated variety in Brazil occupying 25% of the total area cultivated with sugarcane in the country while the RB72454 variety was widely cultivated in Brazil until 2000 (Oliveira et al., 2015). The main characteristics of these two cultivars that are especially important for ethanol and sugar production are high productivity and wide adaptability (RB72454) and high yield and sucrose content (RB867515).

Microsatellite selection

DNA was isolated from the shoots of young leaves of 24 plants of the RB72454 variety (eight plants on each cutting stage - second, fourth, and sixth) and 31 plants of the RB867515 variety (seven plants on the second cutting stage and eight plants on each cutting stage - fourth, sixth, and seventh) according to the procedure used by Hoisington et al. (1994). The five EST-SSR primers (Table 1) previously mapped for sugarcane by the International Sugarcane Microsatellite Consortium (ISMC) (Cordeiro et al., 2000; Singh et al., 2008; Oliveira et al., 2009) were synthesized by Invitrogen Technologies Corporation (USA) and used to analyze the DNA samples from each sugarcane variety. The homologies identified in the database (SUCEST) for the primers ESTB41, ESTC66, ESTC67, ESTC68, ESTC69, ESTC84, ESTC91, ESTB92, ESTB130, and ESTB145 are as follows: a hypothetical protein, a probable xyloglucan endotransglucosylase, β-amylase, fructose-1,6-bisphosphate aldolase, cellulose synthase 6, a putative acetyl-CoA C, a putative fructose-1,6-bisphosphatase, a putative sugar transporter, a putative auxin response factor 7a, and α-galactosidase-like protein, respectively. Polymerase chain reaction (PCR) was performed using the Techne TC-512 thermal cycler. The amplifications were performed by the Touchdown PCR (Td-PCR) program (Don et al., 1991) using primer-specific annealing temperatures (T°A).

For Td-PCR, 20 µL volumes containing 25 ng genomic DNA, 10 mM Tris-HCl at pH 8.8, 2.0 µL 10X reaction buffer (20 mM Tris-HCl at pH 8.0, 0.1 mM EDTA, 1.0 mM DTT, and 50% (v/v) glycerol), 2.5 mM $MgCl_2$, 0.1 mM of each dATP, dGTP, dCTP, and dTTP, 0.3 µL of each primer (F and R primers), and 1 U Taq polymerase (Invitrogen) were used.

Table 1. Sequences of the primers and temperatures of annealing used to amplified the DNA samples from RB72454 and RB867515 varieties; sequence simples repeated (SSR), base pairs, and homologies identified by database SUCEST for each *Est-SSR* locus.

Primer	Sequences	T °C	Locus	SSR	bp	Data base SUCEST
ESTB41	F: CATGGAGAGCTGGGCGACCTG R: GGCGGCGGCGAGGATGA	68	*EstB41*	$(CGA)_8$	163	Hypothetical protein
ESTC66	F: AGTACAGGCTGCTCTCAATCAA R: TCTGTCATCTGTGTTCGTTCTG	61	*EstC66*	$(CCGC)_3$	152	Probable xyloglucan endotransglucosylase
ESTC67	F: GAGCGAGCCAGTGAGGTCT R: CCCTCGTCTCGTCCTCCTT	59	*EstC67*	$(AGCC)_4$	157	β-amylase
ESTA68	F: ACAGTGTTGACCA GTAGGAAGAAT R: CAGGTACTTGGCGGTCTTG	TD	*EstA68*	$(CG)_7$	234	Fructose 1,6 bisphosphate aldolase
ESTC69	F:GAACGACGAGCAAGGGAAGGAATG R: CAATAAGGCAGGACGGAACAGATG	TD	*EstC69*	$(AAAC)_4$	159	Cellulose synthase 6
ESTC84	F: AAGCCGGGTTCCAGTCCAG R: GCAACCAAAAGGCTCAGAACAG	TD	*EstC84*	$(GCCT)_4$	204	Putative acetyl-CoA C
ESTC91	F: CGAGGACGATGTGGGAGAGG R: CTCACCTCCCCCAACACAGTC	54	*EstC91*	$(GCCG)_3$	259	Putative fructose 1,6 bisphosphatase
ESTB92	F: TCTGAATGGATGTCGCCCTGTG R: TTTGCGGGCTTCTCTGCTTTCT	TD	*EstB92*	$(TTC)_5$	241	Putative sugar transporter
ESTB130	F: GCCCAGGTAATTATCCAGACTC R: GCTGTTGCTCACTGGTTCC	54	*EstB130*	$(CAA)_{13}$	124	Putative auxin response factor 7a
ESTB145	F: GGGAAGCAAGCGAGAGCAGCAGAG R: GAGCGCGAGGCCGTTGTTGAG	68	*EstB145*	$(CCT)_5$	234	α-galactosidase-like protein

TD: touchdown; Don et al. (1991).

The initial denaturation at 94°C for 1 min was followed by 10 cycles of 1 min at 94°C, 1 min with initial temperature of 65°C and a reduction of 1°C per cycle, and 2 min at 72°C; and 20 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. The final extension was 5 min at 72°C. The PCR using specific temperatures for the primer annealing was 94°C for 5 min followed by 30 cycles of 94°C for 1 min and the T°A of each primer for 1 min, and the later cycles with 72°C for 1 min. The final extension was 15 min at 72°C.

After amplification, a total of 20 μL of each sample was separated by electrophoresis on a 4% agarose gel (50% agarose and 50% agarose Metaphor) (CAMBREX) containing 0.5X TBE buffer (44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA). All 72 samples amplified by a single SSR primer were run on the same gel at 60 V for 4 h. A 1-kb ladder (Invitrogen) was used as a molecular marker weight. The gels were stained using 0.5 μg/mL ethidium bromide, and the image was captured using Molecular Image LOCCUS L-PIX-HE using the Picasa 3 program.

Microsatellite analysis

Homozygous and heterozygous phenotypes for the different alleles were scored within each *Est-SSR* locus (*EstB41*, *EstC66*, *EstC67*, *EstA68*, *EstC69*, *EstC84*, *EstC91*, *EstB92*, *EstB130*, and *EstB145*) in each RB72454 and RB867515 sugarcane variety. The genetic variability within each sugarcane variety was represented by the number of alleles per locus and by the values of mean H_O. Polyploidy in sugarcane hinders the estimates of allele frequencies found in each locus as well as the values for average H_E for these loci. The polyploidy plants may show heterozygous phenotypes formed by more than two bands, which are products of three or more alleles in loci located on different chromosomes in the polyploidy genome. However, it is possible to detect the proportion of observed homozygous plants as well as the proportion of heterozygous plants containing two or more alleles, so it is possible to estimate the mean H_O for each locus.

RESULTS

Polymorphism in the 10 *Est-SSR* loci of both RB72454 (100%) and RB867515 (90%) sugarcane varieties were high. The absence of heterozygous plants and occurrence of one allele was observed only in *EstC67* (β-amylase; EC 3.2.1.2) locus of the RB867515 variety. The number of alleles and the values of mean H_O for each locus in the samples of RB72454 and RB867515 varieties in each cutting stage (second, fourth, and sixth for RB72454 and second, fourth, sixth, and seventh for RB867515) are shown in Table 2. The H_O values in 10 *Est-SSR* loci ranged from 0.50 to 0.575 for RB72454 and from 0.50 to 0.5571 for RB867515 varieties. The highest H_O value (100%) in samples of all cutting stages was observed in *EstC66* (a probable xyloglucan endotransglucosylase; EC 2.4.1.207 and/or EC 3.2.1.151) and *EstC69* (cellulose synthase 6; EC 2.4.1.29) loci of the RB72454 variety and in the *EstC66*, *EstC69*, and *EstC91* (a putative fructose-1,6-bisphosphatase; EC 3.1.3.11) loci of the RB867515 variety. The absence of heterozygous in samples of all cutting stages was observed in *EstB92* (a putative sugar transporter) and *EstB145* (α-galactosidase-like protein) loci of the RB72454 variety and in *EstB92*, *EstB145*, and *EstC67* loci of the RB867515 variety.

Table 2. Number of alleles (N_A) and mean observed heterozygosity (H_O) for each *Est-SSR* locus of the RB72454 and RB867515 varieties of sugarcane at different cutting stages.

Locus	RB72454						RB867515							
	N_A			H_O			N_A				H_O			
	2°.	4°.	6°.	2°.	4°.	6°.	2°.	4°.	6°.	7°.	2°.	4°.	6°.	7°.
EstB41	2.0	2.0	2.0	1.000	0.625	0.750	2	2	2	2	1.000	0.8750	0.6250	1.000
EstC66	4.0	2.0	4.0	1.000	1.000	1.000	2	4	2	4	1.000	1.000	1.000	1.000
EstC67	2.0	2.0	1.0	1.000	0.625	0.000	1	1	1	1	0.000	0.000	0.000	0.000
EstA68	2.0	2.0	1.0	0.125	0.250	0.250	2	2	2	2	0.5714	0.000	0.000	0.000
EstC69	4.0	4.0	2.0	1.000	1.000	1.000	2	2	4	4	1.000	1.000	1.000	1.000
EstC84	2.0	2.0	2.0	0.000	0.000	0.250	1	2	3	3	0.000	0.2500	0.500	0.2500
EstC91	4.0	4.0	3.0	0.875	1.000	1.000	3	3	4	4	1.000	1.000	1.000	1.000
EstB92	2.0	2.0	1.0	0.000	0.000	0.000	1	2	1	1	0.000	0.000	0.000	0.000
EstB130	2.0	2.0	2.0	0.750	1.000	1.000	2	2	2	2	1.000	1.000	0.8750	0.8750
EstB145	3.0	2.0	2.0	0.000	0.000	0.000	2	1	2	2	0.000	0.000	0.000	0.000
Mean	2.7	2.4	2.0	0.575	0.550	0.500	1.8	2.1	2.3	2.5	0.5571	0.5125	0.5000	0.5125

Changes of H_O values in samples from different cutting stages (second, fourth, and sixth in RB72454 variety, and second, fourth, sixth, and seventh in RB867515 variety) were observed in 6 of the 10 *Est-SSR* loci (*EstB41*, *EstC67*, *EstA68*, *EstC84*, *EstC91*, and *EstB130*) of the RB72454 variety (Table 2), and only in 4 of the 10 *Est-SSR* loci (*EstB41*, *EstA68*, *EstC84*, and *EstB130*) of the RB867515 variety. Thereby, the levels of H_O estimated for sugarcane, as well as the numbers of alleles detected, are dependent on the *Est-SSR* locus, cutting stage, and variety. While the *EstC67* locus is monomorphic in samples from second, fourth, sixth, and seventh cutting stages of the RB867515 variety, it is polymorphic suggesting different allele frequencies in samples from second, fourth, and sixth cutting stages of the RB72454 variety.

Changes of allele numbers in samples of different cutting stages were observed in 7 (*EstC66*, *EstC67*, *EstA68*, *EstC69*, *EstC91*, *EstB92*, and *EstB145*) and 6 (*EstC66*, *EstC69*, *EstC84*, *EstC91*, *EstB92*, and *EstB145*) *Est-SSR* loci of the RB72454 and RB867515 varieties, respectively. Reduction of allele numbers was observed in the *Est-SSR* loci of the samples collected in the fourth and sixth cutting stages of the RB72454 variety (Table 2). In contrast, an increase of the allele numbers was detected in the *Est-SSR* loci of the samples collected on

fourth, sixth, and seventh cutting stages of the RB867515 variety. Unchanged allele numbers in samples of different cutting stages were observed only in *EstB41*, *EstC84*, and *EstB130* loci of the RB72454 variety, and *EstB41*, *EstC67*, *EstA68*, and *EstB130* loci of the RB867515 variety.

Accordingly, the variety RB867515 has lower polymorphism and values of H_O than the RB72454 variety in different stages of cutting. So, the variety RB72454 can be characterized as having higher H_O than the RB867515 variety in the SSR contained within expressed sequences (loci *EstB41*, *EstC66*, *EstC67*, *EstA68*, *EstC69*, *EstC84*, *EstC91*, *EstB92*, *EstB130*, and *EstB145*). The RB72454 variety has also the highest number of *Est-SSR* loci with changes of allele number and values of H_O in different cutting stages. At molecular level, in SSR of expressed sequences, the RB72454 variety showed higher changes in subsequent stages of cutting than RB867515.

DISCUSSION

Despite high polymorphism, lower H_O values were detected in *Est-SSR* loci of both varieties RB72454 (P = 100%; H_O = 0.50-0.575) and RB867515 (P = 90%; H_O = 0.50-0.5571) than in SSR of noncoding loci as previously reported by Maranho et al. (2014) for RB72454 (P = 100%; H_O = 0.833) and RB867515 varieties (P = 93.75%; H_O = 0.7920). In contrast to the lowest values of H_O detected in the 10 *Est-SSR* loci, the highest H_O values were detected in SSR from the locus probably coding xyloglucan endotransglucosylase (EC 2.4.1.207 and/or EC 3.2.1.151) (locus *EstC66*) and in SSR from the locus coding a cellulose synthase (EC 2.4.1.29) (locus *EstC69*). The xyloglucan endotransglucosylase as well as the cellulose synthase are enzymes directly involved in the construction, growth, and cell wall extensibility (Takeda et al., 2002; Doblin et al., 2002; Festucci-Buselli et al., 2007; Miedes et al., 2011; Hara et al., 2014) since the long polysaccharide xyloglucan can form hydrogen bonds with cellulose microfibrils. The xyloglucan endotransglucosylase/ hydrolase genes are also involved with defense and pathogen-resistance mechanisms (Sharmin et al., 2012; Olsen et al., 2016). So that the *EstC66* and *EstC69* loci in the RB72454 and RB867515 varieties are examples of important coding sequences associated with agronomic characteristics of interest, which contain SSR presenting 100% H_O. High polymorphism in SSR of the cellulose synthase locus observed also in other sugarcane varieties was used to explain the high variability of substrates used in pretreatment and in enzymatic hydrolysis processes of their respective bagasse fibers in order to obtain the fermentable sugars to produce ethanol (Augusto et al., 2015). The SSR in the *EstC91* locus for a putative fructose-1,6-bisphosphatase also showed 100% H_O in the RB867515 variety. The fructose 1,6-bisphosphatase (EC 3.1.3.11) is an important enzyme that had a positive effect on the pathway of carbon fixation and photosynthesis (Chueca et al., 2002; Tamoi et al., 2006).

Despite the high degree of relatedness between the varieties RB72454 and RB867515 (RB72454 variety is the female parent of the RB867515 variety), differences in the H_O values are observed at the *EstC67*, *EstC68*, *EstC84*, *EstC91*, and *EstB130* loci in samples collected in different cutting stages. Pronounced differences at molecular level between the varieties RB72454 and RB867515 are observed in the subsequent cutting stages. While the mean number of alleles decreased from the second to the sixth cutting stage in the RB72454 variety, an increase of the mean number of alleles has occurred from the second to the seventh

cutting stage in the 10 *Est-SSR* loci of the RB867515 variety. Changes in the allele frequencies (indicated by different values of H_O) in six *Est-SSR* loci (*EstB41*, *EstC67*, *EstA68*, *EstC84*, *EstC91*, and *EstB130*) of the RB72454 variety and four *Est-SSR* loci (*EstB41*, *EstA68*, *EstC84*, and *EstB130*) of the RB867515 variety also point out the molecular divergence between both varieties. The different number of chromosomes of both varieties should be one of the factors that may be used to justify a divergence at molecular level. The chromosome number of the RB72454 variety (2n = 112; amplitude of 108 -114) is higher than the number reported for the RB867515 variety (2n = 110; amplitude 105-114).

Within each variety RB72454 and RB867515, somatic mutations may lead to the appearance of new alleles while somatic recombination may lead to loss of alleles and/or reduction of heterozygous phenotypes in the vegetative propagation after each cutting stage. Somatic mutations in vegetative-propagated plants are relatively frequent natural events reported since the 90s of the last century (Franks et al., 2002; Vouillamoz et al., 2003), which have been recently evident at DNA level (Vezzulli et al., 2012). The occurrence of somatic recombination in addition to somatic mutations in grapes that were vegetatively propagated was reported as an event that may promote gene homozygosis in the heterozygous condition (Oliveira-Collet et al., 2005). It is possible that transposable genetic elements described in the genomes of *S. officinarum* and *S. spontaneum* (de Souza et al., 2013), can mediate the chromosomal rearrangements in the varieties of *Saccharum* spp. Epigenetic variability due to differential methylation levels in a complex genome as the sugarcane may also lead to genotypic and phenotypic instability (Marfil et al., 2009). Also due to differential methylation events, mechanisms of successive silencing of genes, such as the ones observed in *in vitro* vegetative propagation (Nocarova et al., 2010), may lead to a reduction of heterozygous phenotypes. Such alterations (one or more than one) imply in genetic instability and deserve further investigation.

The similarities and divergences at molecular level between varieties RB72454 and RB867515 observed in the 10 *Est-SSR* loci during subsequent cutting stages can not explain the reduced productivity frequently observed after subsequent cutting stages but showed that phenotypic and physiological changes after each cutting stage are also accompanied by changes at genomic level.

There are few features of interest to the producer (harvest time, closing between lines, growth rate, exigency of environments, until industrialization period) that differentiate RB72454 and RB867515 varieties. Both varieties have high sucrose content and medium content of fibers (Daros et al., 2015). The similar pattern for fiber content in RB72454 and RB867515 varieties is consistent with the same level of heterozygosity detected in the *EstC66* (probable xyloglucan endotransglucosylase) and *EstC69* (cellulose synthase 6) loci and with the genetic stability observed at these loci during the cutting stages. The samples of RB72454 variety showed higher polymorphism, higher values of H_O, and higher number of *Est-SRR* loci with changes in the subsequent cutting stages than the RB867515 variety.

The high heterozygosity of the RB72454 variety is consistent with its utility as parental in breeding programs. Santos et al. (2012) listed the RB72454 variety as parental (female or male) in more of 30% of crosses for generation of new varieties of the germplasm bank of Serra do Ouro, maintained by the Genetic Improvement Program for Sugarcane at the Federal University of Alagoas, Brazil. According to Daros et al. (2015) RB72454 gave rise to 25 new varieties RB and it was one of the most cultivated varieties in Brazil until 2010, when the area was reduced due to 'orange rust' infestation.

Although the heterozygosity of the RB867515 in 10 loci *Est-SSR* is somewhat lower than the observed in the RB72454 variety, the H_O of the RB867515 seems to be sufficient to confer a high adaptive potential to the RB867515 variety. The RB867515 is the most cultivated variety in Brazil occupying 25% of the total area cultivated with sugarcane in the country. The 2015 census showed the RB867515 variety in: i) first place in 46.6% of states (regions South, Southeast, and Midwest) where sugarcane is cultivated; ii) second place in 33.33% of the states (Northeast region); and iii) third place in 20% of the states (also in Northeast region) (Oliveira et al., 2015).

Although the real factors related with the changes of number of alleles and values of H_O in the different cutting stages of each RB72545 and RB867515 varieties are unknown, the evidence of such changes have important practical implications. It is needed prudence in studies to evaluate the genetic similarity between pairs of sugarcane genotypes destined for breeding programs. Different values of genetic identity may be expected, e.g., in comparison between samples of RB72454 variety in the second cutting stage (Na = 2.7; H_O = 0.5750) with the RB867515 variety from the same cutting stage (Na = 1.8; H_O = 0.5571) or from the sixth cutting stage (Na = 2.3; H_O = 0.5000).

The estimated values for the 10 *Est-SSR* loci of the RB72454 and RB867515 varieties confirmed our expectations to find lower values than those described in SSR of non-coding DNA regions. On the other hand, it was possible to identify SSR with 100% of heterozygosity at loci which encode important enzymes associated with agronomic traits of interest. The high heterozygosity in these loci seems to be maintained in samples of subsequent cutting stages (second, fourth and sixth cutting stages in RB72454 variety and second, fourth, sixth, and seventh cutting stages in RB867515 variety), while in others *Est-SSR* loci occurred absence of heterozygous or variations in the number of alleles and of the H_O values during the vegetative propagation of sugarcane. The values of H_O at the 10 *Est-SSR* loci of both varieties RB72454 and RB867515 has decreased from the second to the sixth cutting stage suggesting that a decrease on the mean H_O may be related to reduction in sugarcane productivity in subsequent cutting stages during its vegetative propagation. According to this, there is now the suspicion that the reduction in sugarcane productivity in subsequent cutting stages may be due to a gradual decrease of the mean H_O in the sugarcane ratoon.

The conclusion and recommendation from our study is that the comparison among different sugarcane varieties in order to find the contrasting genotypes should be made using varieties in the same cutting stage since that changes in the number of alleles and in the allele frequencies (indicated by different values of H_O) has been observed in expressed sequence tag microsatellites (*Est-SSR* loci) of sugarcane in different cutting stages during the vegetative propagation. Therefore, it is needed prudence in studies to evaluate the genetic similarity between pairs of sugarcane genotypes destined for breeding programs. Moreover, polymorphisms in *Est-SSR* loci associated with sugar and ethanol production during the sugarcane vegetative propagation may mark different sugar and ethanol production in different cutting stages.

ACKNOWLEDGMENTS

REFERENCES

Augusto R, Maranho RC, Mangolin CA and Pires da Silva Machado MdeF (2015). High polymorphism in *Est-SSR* loci for cellulose synthase and b-amylase of sugarcane varieties (*Saccharum* spp.) used by the industrial sector for ethanol production. *Appl. Biochem. Biotechnol.* 175: 965-973. - http://dx.doi.org/10.1007/s12010-014-1340-1

Chueca A, Sahrawy M, Pagano EA and López Gorgé J (2002). Chloroplast fructose-1,6-bisphosphatase: structure and function. *Photosynth. Res.* 74: 235-249. - http://dx.doi.org/10.1023/A:1021243110495

Cordeiro GM, Taylor GO and Henry RJ (2000). Characterisation of microsatellite markers from sugarcane (*Saccharum* sp.), a highly polyploid species. *Plant Sci.* 155: 161-168. - http://dx.doi.org/10.1016/S0168-9452(00)00208-9

Daros E, Oliveira RA and Barbosa GVS (2015). *45 anos de variedades RB de cana-de-açúcar. 25 anos de RIDESA.* Editora Graciosa, Curitiba.

de Souza MC, Silva JN and Almeida C (2013). Differential detection of transposable elements between *Saccharum* species. *Genet. Mol. Biol.* 36: 408-412. - http://dx.doi.org/10.1590/S1415-47572013005000030

Doblin MS, Kurek I, Jacob-Wilk D and Delmer DP (2002). Cellulose biosynthesis in plants: from genes to rosettes. *Plant Cell Physiol.* 43: 1407-1420. - http://dx.doi.org/10.1093/pcp/pcf164

Don RH, Cox PT, Wainwright BJ, Baker K, et al. (1991). 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res.* 19: 4008. - http://dx.doi.org/10.1093/nar/19.14.4008

Duarte-Filho LSC, Silva PP, Santos JM, Barbosa GVS, et al. (2010). Genetic similarity among genotypes of sugarcane estimated by SSR and coefficient of parentage. *Sugar Tech* 12: 145-149. http://dx.doi.org/10.1007/s12355-010-0028-2

Festucci-Buselli RA, Otoni WC and Joshi CP (2007). Structure, organization, and functions of cellulose synthase complexes in higher plants. *Braz. J. Plant Physiol.* 19: 1-13. http://dx.doi.org/10.1590/S1677-04202007000100001

Franks T, Botta R, Thomas MR and Franks J (2002). Chimerism in grapevines: implications for cultivar identity, ancestry and genetic improvement. *Theor. Appl. Genet.* 104: 192-199. - http://dx.doi.org/10.1007/s001220100683

Grivet L and Arruda P (2002). Sugarcane genomics: depicting the complex genome of an important tropical crop. *Curr. Opin. Plant Biol.* 5: 122-127. - http://dx.doi.org/10.1016/S1369-5266(02)00234-0

Hara Y, Yokoyama R, Osakabe K, Toki S, et al. (2014). Function of xyloglucan endotransglucosylase/hydrolases in rice. *Ann. Bot. (Lond.)* 114: 1309-1318. - http://dx.doi.org/10.1093/aob/mct292

Hoisington D, Khairallah M and González-Léon D (1994). Laboratory Protocols: CIMMYT Applied Molecular Genetics Laboratory. 2nd edn. CIMMYT, Mexico, D.F.

Liu P, Que Y and Pan Y-B (2011). Highly polymorphic microsatellite DNA markers for sugarcane germplasm evaluation and variety identity testing. *Sugar Tech* 13: 129-136. http://dx.doi.org/10.1007/s12355-011-0077-1

Maranho RC, Augusto R, Mangolin CA and Machado MFPS (2014). Use of differential levels of mean observed heterozygosity in microsatellite loci of commercial varieties of sugarcane (*Saccharum* spp). *Genet. Mol. Res.* 13: 10130-10141. - http://dx.doi.org/10.4238/2014.December.4.7

Marfil CF, Camadro EL and Masuelli RW (2009). Phenotypic instability and epigenetic variability in a diploid potato of hybrid origin, *Solanum ruiz-lealii. BMC Plant Biol.* 9: 21. - http://dx.doi.org/10.1186/1471-2229-9-21

Matos CRA (2011). Secretaria do Meio Ambiente/Coordenadoria de Biodiversidade e Recursos Naturais. Etanol e Biodiesel. SMA, São Paulo.

Miedes E, Zarra I, Hoson T, Herbers K, et al. (2011). Xyloglucan endotransglucosylase and cell wall extensibility. *J. Plant Physiol.* 168: 196-203. - http://dx.doi.org/10.1016/j.jplph.2010.06.029

Nocarova E, Opatrny Z and Fischer L (2010). Successive silencing of tandem reporter genes in potato (*Solanum tuberosum*) over 5 years of vegetative propagation. *Ann. Bot. (Lond.)* 106: 565-572. - http://dx.doi.org/10.1093/aob/mcq153

Oliveira KM, Pinto LR, Marconi TG, Mollinari M, et al. (2009). Characterization of new polymorphic functional markers for sugarcane. *Genome* 52: 191-209. - http://dx.doi.org/10.1139/G08-105

Oliveira RA, Daros E and Hoffmann HP (2015). Liberação Nacional de Variedades RB de Cana-de-açúcar. Editora Graciosa, Curitiba.

Oliveira-Collet SA, Collet MA and Machado MFPS (2005). Differential gene expression for isozymes in somatic mutants of *Vitis vinifera* L. (Vitaceae). *Biochem. Syst. Ecol.* 33: 691-703. http://dx.doi.org/10.1016/j.bse.2004.12.016

Olsen S, Striberny B, Hollmann J, Schwacke R, et al. (2016). Getting ready for host invasion: elevated expression and action of xyloglucan endotransglucosylases/hydrolases in developing haustoria of the holoparasitic angiosperm *Cuscuta. J. Exp. Bot.* 67: 695-708. - http://dx.doi.org/10.1093/jxb/erv482

Santos JM, Duarte Filho LSC, Soriano ML, Silva PP, et al. (2012). Genetic diversity of the main progenitors of sugarcane from the RIDESA germplasm bank using SSR markers. *Ind. Crops Prod.* 40: 145-150. http://dx.doi.org/10.1016/j.indcrop.2012.03.005

Sharmin S, Azam MS, Islam MS, Sajib AA, et al. (2012). Xyloglucan endotransglycosylase/hydrolase genes from a

susceptible and resistant jute species show opposite expression pattern following *Macrophomina phaseolina* infection. *Commun. Integr. Biol.* 5: 598-606. - http://dx.doi.org/10.4161/cib.21422

Silva DC, Duarte Filho LSC, Santos JM, Barbosa GV, et al. (2012). DNA fingerprinting based on simple sequence repeat (SSR) markers in sugarcane clones from the breeding program RIDESA. *Afr. J. Biotechnol.* 11: 4722-4728.

Singh RK, Srivastava S, Singh SP, Sharma ML, et al. (2008). Indentification of new microsatellites DNA markers for sugar and related traits in sugarcane. *Sugar Tech* 10: 327-333. http://dx.doi.org/10.1007/s12355-008-0058-1

Singh RK, Singh RB, Singh SP and Sharma ML (2011). Identification of sugarcane microsatellites associated to sugar content in sugarcane and transferability to other cereal genomes. *Euphytica* 182: 335-354. http://dx.doi.org/10.1007/s10681-011-0484-0

Takeda T, Furuta Y, Awano T, Mizuno K, et al. (2002). Suppression and acceleration of cell elongation by integration of xyloglucans in pea stem segments. *Proc. Natl. Acad. Sci. USA* 99: 9055-9060. - http://dx.doi.org/10.1073/pnas.132080299

Tamoi M, Nagaoka M, Miyagawa Y and Shigeoka S (2006). Contribution of fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase to the photosynthetic rate and carbon flow in the Calvin cycle in transgenic plants. *Plant Cell Physiol.* 47: 380-390. - http://dx.doi.org/10.1093/pcp/pcj004

Tautz D (1989). Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Res.* 17: 6463-6471. - http://dx.doi.org/10.1093/nar/17.16.6463

Vezzulli S, Leonardelli L, Malossini U, Stefanini M, et al. (2012). Pinot blanc and Pinot gris arose as independent somatic mutations of Pinot noir. *J. Exp. Bot.* 63: 6359-6369. - http://dx.doi.org/10.1093/jxb/ers290

Vouillamoz J, Maigre D and Meredith CP (2003). Microsatellite analysis of ancient alpine grape cultivars: pedigree reconstruction of *Vitis vinifera* L. 'Cornalin du Valais'. *Theor. Appl. Genet.* 107: 448-454. - http://dx.doi.org/10.1007/s00122-003-1265-5

Weber JL and May PE (1989). Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *Am. J. Hum. Genet.* 44: 388-396. -