Genetic divergence and admixture of ancestral genome groups in the sugarcane variety 'RB867515' (*Saccharum* spp)

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ABSTRACT. We analyzed 80 plants of the sugarcane (*Saccharum* spp) variety 'RB867515' in order to investigate its diversity and genetic structure at the molecular level. Four simple sequence repeat (SSR) loci (UGSM51, SMC1237, SEGMS1069, and UGSM38) and five expressed sequence tag (EST)-SSR loci (ESTA68, ESTB92, ESTB145, ESTC66, and ESTC84) were used as molecular markers. The polymorphic loci rate was 66.6%. A total of 17 alleles and an average of 1.88 alleles/locus were detected. The number of alleles in the EST-SSR loci was lower

than the number of alleles in the SSRs of non-expressed loci. The mean observed heterozygosity among the nine SSR loci was 0.3291. Genetic structure analysis showed that 'RB867515' contains alleles from three ancestral groups (K = 3), but there is little admixing of alleles in the same plant (from 0.8 to 17.3%); only 1.88% of the plants shared alleles from two or three groups. ESTB92, ESTC84, and UGSM38 were monomorphic, but there was evidence of polymorphism in ESTA68, ESTB145, ESTC66, UGSM51, SMC1237, and SEGMS1069, indicating that 'RB867515' has variability at the molecular level and the potential to be used as a parent in breeding programs. The molecular variability observed in 'RB867515' indicates that the clone terminology that is used to identify this cultivar is inconsistent with the original meaning of "clone", which is defined as a sample of genetically identical plants.

Key words: Genetic variability; Molecular polymorphism; 'RB867515' sugarcane variety; EST-SSR loci

INTRODUCTION

The use of sugarcane (*Saccharum* spp) as a source of sugar and ethanol has been very important to the Brazilian economy. In 2010/2011, 624 million tons of sugarcane were produced and used for the production of 33 million tons of sugar and 27.6 billion liters of ethanol (http://jornalcana.com.br/). In 2013/14, sugarcane cultivation continued to expand to about 314,000 ha, equivalent to an increase of 3.7% compared to the 2012/13 crop (CONAB, 2015; http://www.conab.gov.br). In 2014/15, 152.80 million tons of sugar and 231.57 million liters of ethanol were produced up to the first quarter of 2015 (http://unica.com.br/). From April to July 2016, the amount of sugarcane crushed was 16.07% higher than during the same period in 2015 because of the increased cultivation area. The sugar and ethanol production sector in Brazil consists of 53 units, with a prediction of 170 units in 2015 in the Brazilian Central-South region (http://unica.com.br/).

Sugarcane bagasse is used as a source of electricity and to produce second-generation ethanol (2G). Bagasse burned in boilers generates electricity for mills, and surplus energy. The enzymatic hydrolysis of bagasse to obtain 2G ethanol is being practiced in Brazil by two companies in the states of Alagoas and São Paulo. Mills will expand their production of sugarcane-related products to sugarcane diesel, bio-kerosene for aviation, biodegradable plastics, pharmaceuticals, and polymers for the manufacture of cosmetics and fragrances, in addition to 2G ethanol (Daros et al., 2015). Bagasse from sugarcane has also been used to produce cellulose film (Ruzene et al., 2009), and as an additive material in cement production (Sousa, 2009). Sugarcane molasses are used for fermentation, and the vinasse is used as a fertilizer. Therefore, sugarcane is a renewable energy source for the country (Lee and Bressan, 2006).

The wide use of sugarcane has stimulated interest in increasing its production, and in the development of new varieties that are adapted to heterogeneous regions of Brazil. For breeding programs, it is important to know the genetic diversity and how the commercial varieties of sugarcane are genetically structured. High genetic diversity is important for breeding programs, while genetic uniformity is desirable for the industrial sector. Breeding programs can conduct molecular diagnoses to investigate the genetic diversity of sugarcane

by using markers that are revealed by polymerase chain reactions (PCRs). Simple sequence repeats (SSRs), also known as microsatellite markers, have been used to evaluate the genetic diversity of many plant species. Microsatellites are effective due to their polymorphisms, co-dominant inheritance, ease of detection by PCR, relative abundance, extensive coverage of the genome, and the requirement for only small amounts of DNA (Powell et al., 1996; Faleiro, 2007; Lopes et al., 2014). Microsatellite markers have been used to study the genetic diversity of sugarcane since the beginning of the 21st century (Cordeiro et al., 2000, 2001; Cordeiro and Henry, 2001; Pan et al., 2003; Pinto et al., 2004; Maranho et al., 2014; Augusto et al., 2015). These markers have been associated with sugarcane diseases (Wei et al., 2006) and sugar content (Singh et al., 2008). SSRs that are located in sequences that are transcribed in the genome and have been developed from expressed sequence tags (ESTs) are important, because they indicate direct associations between genes and traits of agronomic interest. EST-SSR analysis is a simple method of studying the expressed part of the genome, even in organisms with large, complex, and highly redundant genomes, such as sugarcane (Sterky and Lundeberg, 2000; Augusto et al., 2015).

SSRs and EST-SSRs have often been used to assess genetic diversity among different varieties of sugarcane, in order to find contrasting genotypes for breeding programs (Santos et al., 2012); however, the genetic diversity within each variety has been little studied (Maranho et al., 2014). There are indications that certain varieties have a high level of heterozygosity, and there is high genetic divergence between plants that are grown in different soil and climatic regions (Augusto et al., 2015). In studies using molecular markers in sugarcane, the main concern is to compare different varieties. A mixture of genomic DNA from various plants of each variety, or a small number of samples representing each variety (5-10), is used, and a large number of alleles and high level of heterozygosity have been reported based on the analysis of a small number of samples (Maranho et al., 2014). Therefore, the present study investigated polymorphisms in SSR and EST-SSR loci in the sugarcane variety ‘RB867515’ using 80 plants as samples. ‘RB867515’ was the most cultivated sugarcane variety in 2015 because of its remarkable characteristics, such as high productivity and high sucrose content. Evaluating the degree of SSR polymorphisms in ‘RB867515’ may indicate its potential in breeding programs, or in the industrial sector.

MATERIAL AND METHODS

‘RB867515’ sugarcane variety

The origin of the ‘RB867515’ variety, which was developed by RIDESA (Rede Inter Universitária para o Desenvolvimento do Setor Sucroalcooleiro), is shown in Figure 1. Eighty samples were collected from plants during the fourth cutting stage that were grown in a cultivated area in Mandaguaçu (23°20’53’’S, 52°5’42’’W, state of Paraná, southern Brazil). Samples were randomly collected from different plants according to the Fukuda and Otsubo (2003) model by performing a zigzag route (Figure 2), in order to cover a homogeneous area of the field. The youngest leaves of each clump (with a small amount of fibers) were selected to facilitate DNA extraction. The samples were packed in aluminum foil, stored in ice, and transferred to the laboratory, where they were kept at -80°C until DNA extraction.

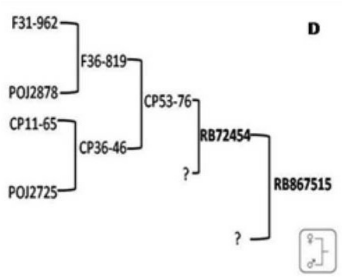

Figure 1. Genealogy of the 'RB867515' variety of sugarcane. Source: RIDESA (2010).

Figure 2. Sampling area in Mandaguaçu, Paraná State, southern Brazil. The samples from the 'RB867515' variety of sugarcane were randomly collected by the zigzag method in order to cover a homogeneous area of the field.

DNA isolation and amplification

DNA was isolated according to the protocol described by Aljanabi et al. (1999), and modified by increasing the NaCl concentration to 5 M. The youngest leaves (100 mg) were frozen with liquid nitrogen and transferred to a 2-mL microtube and homogenized in 300 µL extraction buffer [200 mM Tris-HCl and 50 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0], 100 µL NaCl (5 M), 2% cetyltrimethylammonium bromide, 0.06% sodium sulfite, 5% *N*-lauryl sarcosine, and 10% polyvinylpyrrolidone-40. The mixture was incubated for 60 min at 65°C, and subsequently we followed the protocol described by Aljanabi et al. (1999).

Four SSR primers (UGSM51, SMC1237, SEGMS1069, and UGSM38) and five EST-SSR primers (ESTA68, ESTB92, ESTB145, ESTC66, and ESTC84) were used for DNA amplification. Nine SSR sugarcane primers that had been mapped by the International Sugarcane Microsatellite Consortium (Cordeiro et al., 2000; Singh et al., 2008; Oliveira et al., 2009) were synthesized by Invitrogen Technologies Corporation (USA) and used for the amplification of the DNA samples. The EST-SSR sequences were obtained from the libraries of expressed sequences (EST-SSR) developed by Oliveira et al. (2009) and Pinto et al. (2004). PCR was performed using a Techne TC-512 thermal cycler. The amplifications were performed using the touchdown (TD) PCR program (Don et al., 1991) using a primer-specific annealing temperature (TA) (Table 1).

For TD-PCR, 20-µL volumes containing 13.1 µL Milli-Q water (Millipore Corporation), 1.5 µL genomic DNA (10 ng/µL), 0.4 µL of each primer (forward and reverse; 10 µM) (Invitrogen), 0.8 µL of each dNTP (dATP, dGTP, dCTP, and dTTP; 0.1 mM), 1.6 µL $MgCl_2$ (2 mM), 0.2 µL (1 U) Platinum® *Taq* DNA polymerase (Invitrogen), and 2.0 µL 1X reaction buffer (Invitrogen) were used.

Table 1. Primers used in the amplification of sugarcane genomic DNA.

Primer	T (°C)	Sequence	Number of alleles
SMC1237FL	57	5'-TTACGAACACCCACCTA-3' (F) 5'-GCGCGAGGTAACCTACTGAA-3' (R)	3
SEGMS1069	50	5'-CGGGAATTCGATTTCATGGGTTC-3' (F) 5'-GACTACCTAAGCATCGTCCTC 3' (R)	2
UGSM38	55	5'-CCGAGTGATGATGTGATGT 3' (F) 5'-GGGACAACTAATGTAAGTGATT 3' (R)	1
UGSM59	55	5'-GTGAACGACTCCATCGCC 3' (F) 5'-TTGAAACGAGCGTAAATAAGA 3' (R)	2
ESTC66	61	5'-AGTACAGGCTGCTCTCAATCAA 3' (F) 5'-TCTGTCATCTGTGTTCGTTCTG 3' (R)	3
ESTA68	TD	5'-ACAGTGTTGACCAGTAGGAAGAAT 3' (F) 5'-CAGGTACTACTTGGCGGTCTTG 3' (R)	2
ESTC84	TD	5'-AAGCCGGGTTCCAGTCCAG 3' (F) 5'-GCAACCAAAAGGCTCAGACAG 3' (R)	1
ESTB92	TD	5'-TCTGAATGGATGTCGCCCGTG 3' (F) 5'-TTTGCGGGCTTCTCTGCTTTCT 3' (R)	1
ESTB145	68	5'-GGGAAGCAAGGGAGAGCAGCAGAG 3' (F) 5'-GAGCCGCGAGGCCTTGTTGAG 3' (R)	2

TD, touchdown (Don et al., 1991); T, temperature.

The PCR conditions were as follows: initial denaturation at 94°C for 1 min; 10 cycles of 1 min at 94°C, 1 min with an 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 each at 94°C, 1 min at 55°C, and 2 min at 72°C. The final extension was 5 min at 72°C. The specific temperature for primer annealing was 94°C for 5 min followed by 30 cycles at 94°C for 1 min and TA of each primer for 1 min, and later cycles at 72°C for 1 min. The final extension was 15 min at 72°C.

After amplification, 20 μL of each sample was separated by electrophoresis on a 4% agarose gel (50% agarose and 50% MetaPhor™ agarose, Cambrex) containing 0.5X TBE buffer (89 M Tris, 89 M boric acid, and 2 M EDTA). All 80 samples were amplified by a single SSR primer and run on the same gel at 60 V for 4 h. A 1-kb ladder (Invitrogen) was used as a weight molecular marker. Gels were stained using 0.5 μg/L ethidium bromide, and images were captured using a Molecular Image Locus L-PIX-HE with the Picasa 3 program.

Microsatellite analysis

Homozygous and heterozygous phenotypes for the different alleles were scored within each SSR (UGSM51, SMC1237, SEGMS1069, and UGSM38) and EST-SSR (ESTA68, ESTB92, ESTB145, ESTC66, and ESTC84) loci. Polyploid plants may exhibit 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. Therefore, it is possible to detect the proportion of observed homozygous plants as well as the proportion of heterozygous plants that contain two or more alleles, and estimate the mean observed heterozygosity for each locus.

For the genetic structure analysis, the plants were scored for the presence or absence of SSR and EST-SSR markers (a score of 1 was assigned for presence and 0 for absence of a homologous band), and the data were inserted into a binary data matrix as discrete variables. Polymorphisms among the SSR and EST-SSR markers were analyzed using STRUCTURE software 2.0 (Pritchard and Wen, 2003), which evaluated the level of genetic admixture between the samples (Pritchard et al., 2000). The genotypes were clustered, and the number of clusters (K) ranged from 2 to 5. The genotypes were tested using the admixture model

with a burn-in period of 10,000 iterations followed by 100,000 Markov chain Monte Carlo iterations, in order to verify the presence or absence of SSR and EST-SSR markers (bands) across the samples. The true number of populations (K) is often identified using the maximal value of Δ(K) returned by the software, and the most probable number (K) of subpopulations was identified as described by Evanno et al. (2005). The graphical output of STRUCTURE was taken as input data for STRUCTURE HARVESTER, which is a web-based program for visualizing STRUCTURE output and implementing the Evanno et al. (2005) method to obtain a graphical representation of the results (Earl and von Holdt, 2012).

RESULTS AND DISCUSSION

The analysis of nine SSR loci in 80 samples of 'RB867515' at the fourth cutting stage revealed three alleles in ESTC66 and SMC1237 and two alleles in ESTA68, ESTB145, UGSM59, and SEGMS1069, while ESTB92, ESTC84, and UGSM38 were monomorphic. The polymorphism rate was 66.6%, with a total of 17 alleles and an average of 1.88 alleles/locus. A lower number of alleles (1.8 alleles/locus) was detected in the EST-SSRs than in the SSRs, which were not expressed (2.0 alleles/locus).

The number of alleles, as well as the mean observed heterozygosity (H_O = 0.3291), in the nine SSR loci was lower than the value previously reported in the same variety at the same cutting stage, when 13 SSR loci were analyzed (Augusto, 2013). The number of alleles per locus (N_A = 2.1) and the mean observed heterozygosity (H_O = 0.5225) reported by Augusto (2013) were higher in 'RB867515' in Alto Alegre Sugar and Alcohol Plant (Usina de Açúcar e Alcool Alto Alegre) in Colorado City, State of Paraná, Brazil. The number of alleles in ESTA92 (2), ESTB145 (4), ESTC66 (4), and ESTC84 (3) was higher in 15 samples of 'RB867515' at the fourth cutting stage in Colorado City (Augusto, 2013) than in 80 plants of the same variety and cutting stage [ESTA92 (1), ESTB145 (2), ESTC66 (3), and ESTC84 (1)] in Mandaguaçu City. The different number of alleles per locus and different values of H_O for the same variety at the same cutting stage is an indication that the genetic variability of 'RB867515' differs in different areas of cultivation.

High genetic divergence was detected in 'RB82579' samples at the second cutting stage in the states of Paraná and Pernambuco due to different allele frequencies in ESTA68, ESTC66, ESTC67, ESTC69, and ESTC91 (Augusto et al., 2015). An analysis of the same loci in 'SP81-3250' at the second cutting stage in both Paraná and Pernambuco revealed moderate genetic divergence, suggesting that the genetic variability of sugarcane cultivars depends upon the growing region and the variety (Augusto et al., 2015).

'SP81-3250' has been characterized by sugarcane producers as a stable clone, with wide adaptability and high productivity; these characteristics are consistent with the genetic stability of the variety grown in different regions. In the present study, the genetic stability of 'RB867515' seemed to be low, and its polymorphism rate was variable within the area of cultivation in the same state (Mandaguaçu and Colorado are only 58 km apart). The environmental conditions and soil types in the two regions (Mandaguaçu and Colorado) are similar, and cannot explain the different genetic variability of 'RB867515' at the same cutting stage when grown in both regions. Future studies should investigate why the same variety grown in similar geographical areas may have different genetic diversity. A different number of alleles or different H_O values for the same variety in the two areas may be due to the different 'RB867515' genotypes used in the establishment of the cultivated areas. Variable

frequencies of aneuploidy may also generate different numbers of alleles or different H_O values in the same variety. Polyploidy has been described as the main driving force in the divergence and biodiversity of angiosperms (Leitch and Leitch, 2008). Aneuploidy in somatic cells of polyploid organisms, such as sugarcane, can be stimulated by various stressors. Different stress conditions may produce different genotypes by different selection processes. Previous studies have shown that stress in *in vitro* cultures of 'RB867515' meristems increases genetic diversity. The polymorphism rate in random amplified segments of DNA was three times higher in *in vitro* cultured meristems than in plants propagated in the field by rhizomes (Silva et al., 2008). Among sugarcane plants propagated as crops, it is possible that manpower, equipment, and the different tools used for cutting may represent mechanical stressors, which are different in each growing area.

The differential allele frequencies between ESTA68, ESTB92, ESTB145, ESTC66, ESTC84, UGSM51, SMC1237, SEGMS1069, and UGSM38 in the 80 'RB867515' samples categorized the plants into three ancestral groups, according to model-based Bayesian statistics (Figure 3). The optimal K value determined by the Bayesian analysis indicated that the plants were grouped into three clusters (ΔK2 = 0.00, ΔK3 = 4.4901, ΔK4 = 1.9905, ΔK5 = 0.0574, and ΔK6 = 0.000). The bar plot obtained for the K value (K = 3; ΔK3 = 4.4901) was consistent with very little mixing of alleles between groups 2 and 3 (Figure 3).

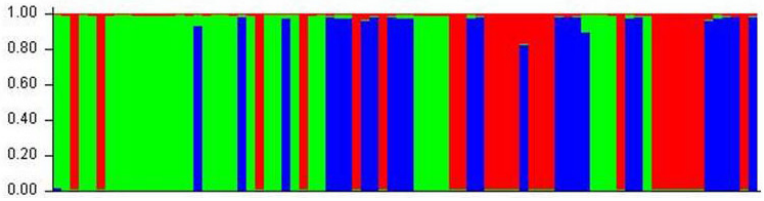

Figure 3. Bar plot based on simple sequence repeat and simple sequence repeat-expressed tag sequence markers for 80 samples of the 'RB867515' variety of sugarcane within the K clusters. Each plant is represented by a single vertical bar that is broken into three colored segments (red, green, and blue). Each color represents the proportion of ancestral alleles in the genome for each individual, which is represented by a vertical bar.

Figure 3 shows that 29.2% of the plants were in the red group, 40.2% were in the green group, and 30.6% were in the blue group. Figure 3 also shows that few plants shared alleles from groups 2 and 3 (1.88%). Each plant had one group of three predominant alleles. 'RB867515' seems to have been formed by a set of plants containing alleles of three ancestral groups at ESTA68, ESTB92, ESTB145, ESTC66, ESTC84, UGSM51, SMC1237, SEGMS1069, and UGSM38, but there was little mixing of alleles in the same plant (0.8 to 17.3%). In the genealogy described for 'RB867515', at least eight different genotypes exist (Figure 1), but the selection proceeded with alleles from only three ancestral groups for the nine SSR loci. The interbreeding of certain "noble" hybrids derived from a very small number of *S. spontaneum* plants as parents is common in sugarcane breeding programs (Bremer, 1961; Walker, 1987) and could explain the reduced number of ancestral genomes in improved varieties such as 'RB867515'. A genetic basis consisting of only four genotypes (Figure 1) in addition of: i) non-random mating; ii) the selection of plants with characteristics of agronomic interest, regardless of the genotypes in the microsatellite loci; iii) vegetative propagation; and iv) polyploidy with a variable number of chromosomes (range 105-114) are factors that may have contributed to the small amount of mixing between the ancestral groups of 'RB867515'.

Lower H_O values were obtained in the SSRs in the expressed sequences (ESTC66, ESTA68, ESTC84, ESTB92, and ESTB145; H_O = 0.2025) than in the SSRs in non-coding regions (UGMS51, UGMS38, SEGMS1069, and SEGMS1237; H_O = 0.4873). A high H_O level has also been detected in SSR loci in non-coding regions of other sugarcane varieties (Maranho et al., 2014). As EST-SSRs are in expressed sequences of the genome, a greater genetic stability may be expected in the EST-SSRs of cultivated sugarcane varieties. ESTA68, ESTB92, ESTB145, ESTC66, and ESTC84 correspond to DNA sequences that are expressed in the sugarcane genome. According to homologies in the SUCEST database (http://sucest-fun.org/index.php/projects/sucest), ESTA68, ESTB92, and ESTB145 encode fructose 1,6-diphosphate aldose (EC 4.1.2.13), a protein related to sugar transport, and an enzyme with a similar activity to α-galactosidase (EC 3.2.1.22), respectively, while ESTC66 and ESTC84 encode the enzymes xyloglucan endoglycosidase (EC 3.2.1.4) and acetyl Co-A carboxylase (EC 6.4.1.2), respectively.

As these enzymes are related to metabolism, it is possible that the SSRs contained in these loci may have been selected in association with quantitative features of interest in 'RB867515'. This is a hypothesis that requires further investigation. The analysis of nine SSR loci (ESTA68, ESTB92, ESTB145, ESTC66, ESTC84, UGSM51, SMC1237, SEGMS1069, and UGSM38) suggests that 'RB867515' is variable at the molecular level, and the terminology "clone", which is used to identify this cultivar, is not consistent with the original meaning of the word, which is defined as a sample of genetically identical copies. The 'RB867515' sugarcane clone is not formed by a group of genetically identical plants, so it has genetic variability and the potential for use in breeding programs. Genetic divergence in this sugarcane variety is caused by the presence of three groups of ancestral alleles, which are distributed in a relatively homogeneous manner in each plant.

Conflicts of interest

The authors declare no conflict of interest.

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