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Genetic diversity of *Saccharum* complex using ISSR markers

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ABSTRACT. Sugarcane (*Saccharum* sp, Poaceae) is native to Southeast Asia, and due to growing demand as raw material, its cultivation recently expanded to new frontiers. The genetic diversity analysis is essential for targeting strategies in the formation and maintenance of a germplasm. This study aimed to assess the genetic diversity of 26 accessions of sugarcane from the Active Germplasm Bank of Embrapa Coastal Tablelands, using inter-simple sequence repeat (ISSR) molecular markers. Sixteen primers were used, resulting in 87 fragments with 91.13% of polymorphism. The similarity of the individuals ranged between 0.22 and 0.87. Individuals RB867515 and RB92579 were closer genetically, and the most distant ones were PI240785 and NSL 291970. Four distinct clusters were formed, using UPGMA. This information can be used to prioritize the selection of accessions for the conduction of hybridization in breeding and germplasm exchange actions.

Key words: *S. officinarum*; *S. robustum*; *S. sinensis*; *S. spontaneum*; ISSR molecular markers

INTRODUCTION

Sugarcane (*Saccharum* sp) is a plant of the Poaceae family, native to Southeast Asia, and cultivated in tropical and subtropical regions (Casu et al., 2004). Its cultivation expanded to new frontiers recently due to growing demand as raw material (biomass) for ethanol production and power generation, as a source of several new added-value products along with sugar production (Santos et al., 2012).

The genus *Saccharum* includes six species: noble cane, *S. officinarum* (2n = 80), two wild species, *S. robustum* (2n = 60-80) and *S. spontaneum* (2n = 40-128), and three secondary species, *S. barberi* (2n = 81-124), *S. sinense* (2n = 111-120) and *S. edule* (2n = 60, 70, 80). *S. officinarum*, *S. spontaneum* and *S. robustum* represent the basic species, and *S. barberi* and *S. sinense* are secondary species probably derived from the hybridization of *S. officinarum* and *S. spontaneum*. All species cited along with the genera *Erianthus*, *Miscanthus*, *Narenga*, and *Sclerostachya* constitute the "*Saccharum* complex" (Daniels et al., 1975).

As there is the need to diversify the genetic basis of modern cultivars to meet an increasingly competitive market, mainly because they derive from exclusive backcrosses involving a few clones of *S. officinarum* and *S. spontaneum* (Shrivastava and Srivastava, 2016), it is extremely important to characterize cultivars worldwide (Almeida et al., 2009).

As a result, breeding programs of sugarcane have emerged worldwide. In Brazil, the Brazilian Agricultural Research Corporation (EMBRAPA) started to study energy derived from sugarcane to provide genetic material with high levels of biomass adapted to different regions of cultivation. Embrapa Coastal Tablelands located in Sergipe, Northeast region, has kept 128 accessions in an Active Germplasm Bank, including the genera *Saccharum*, *Erianthus*, and *Miscanthus*, derived from collections in Brazil and from imports and exchanges with international institutions, where the molecular characterization for the identification of genetic diversity has been held.

The possibility of accessing the genetic variability directly at DNA level enables precise techniques that assist the process of intellectual protection of genetic materials. The molecular markers are important tools for the breeding of plants and aim at the mapping of genes, genetic diversity analysis, disease diagnostics, and taxonomic and evolutionary studies (Wünsch and Hormaza, 2007). In sugarcane, some studies have been conducted to identify the genetic diversity among genotypes using AFLP (Selvi et al., 2006), RAPD (Khan et al., 2009; Ullah et al., 2013), chloroplast microsatellite markers (coSSR) (Raj et al., 2016), SSR (Pandey et al., 2011) and inter-simple sequence repeat (ISSR) (Srisvastava and Gupta, 2008; Almeida et al., 2009; Devarumath et al., 2012; Rao et al., 2016). RAPD and ISSR have been amplified for water stress tolerance in sugarcane varieties (Fahmy et al., 2008).

The aim of this study was to assess the genetic diversity of sugarcane accessions from the Active Germplasm Bank of Embrapa Coastal Tablelands using ISSR molecular markers, for selection of accessions aiming the conduction of hybridization in breeding programs and germplasm exchange.

MATERIAL AND METHODS

Twenty-four sugarcane accessions established *in vitro* were used, from the second subculture, provided by the National Center for Genetic Resources Preservation/ARS/ USDA, Fort Collins, CO, USA, and two varieties "RB" from Inter-University Network for

the Development of Sugarcane Industry, characterized as varieties cultivated in Brazil (Table 1). All accessions are from the Active Germplasm Bank of the *Saccharum* Complex from Embrapa Coastal Tablelands, located in the Experimental Field Jorge Prado Sobral, located in Nossa Senhora das Dores, Sergipe, Brazil, (10°29'27''S; 37°11'34''W).

Young leaves from 26 accessions were collected, identified, packed in Styrofoam plastic bags with ice, and stored at -80°C at the Molecular Biology Laboratory of Embrapa Coastal Tablelands.

Table 1. Characteristics of 26 accessions of "*Saccharum* Complex" from the Active Germplasm Bank of Embrapa Coastal Tablelands, Brazil.

Number	Identification of accession	Species
1	Q 45866	*Saccharum officinarum*
2	Q 46199	*Saccharum robustum*
3	Q 45251	*Saccharum robustum*
4	Q 42509	*Saccharum* spp
5	Q 45911	*Saccharum officinarum*
6	Q 45864	*Saccharum officinarum*
7	Q 44830	*Saccharum officinarum*
8	Q 45337	*Saccharum* hybrid
9	Q 45416	*Saccharum* spp
10	Q 44890	*Saccharum officinarum*
11	Q 44833	*Saccharum officinarum*
12	Q 45869	*Saccharum officinarum*
13	Q 42433	*Saccharum* hybrid
14	PI 240785 Q 45923 NG 57-208	*Saccharum robustum*
15	PI 197800 Sumatra #2	*Saccharum spontaneum*
16	Q 36830 MOL 6091	*Saccharum* sp
17	GH-49 UNKR65P35	*Saccharum robustum*
18	MIA 35303 MOL 6091	*Saccharum robustum*
19	NSL 291970 Glagh 1286	*Saccharum spontaneum*
20	PI 88652 NG 28213	*Saccharum officinarum*
21	MIA 35301 MOL 6089	*Saccharum robustum*
22	RB 867515	*Saccharum* sp
23	RB 92579	*Saccharum* sp
24	PI 495109 Q46207 IN 84-045	*Saccharum robustum*
25	PI 286657 SES-006	*Saccharum spontaneum*
26	PI 29109	*Saccharum sinensis*

DNA extraction followed the 2% cetyltrimethyl ammonium bromide (CTAB) protocol (Romano and Brasileiro, 1999). Seventeen ISSR primers were used (University of British Columbia, Vancouver, Canada) to detect polymorphism at a concentration of 15 ng/mL. Primers were randomly selected. Polymerase chain reaction (PCR) amplifications were carried out in a thermocycler (Veriti, Applied Biosystems). Samples were initially subjected to denaturation at 94°C for 4 min, followed by 37 amplification cycles. During each cycle, samples were subjected to denaturation at 94°C for 1 min, annealing at different temperatures for 2 min, and extension at 72°C for 2 min.

Each ISSR reaction was carried out in a final volume of 20 mL containing 1.5 mL 15 ng/mL DNA; 0.2 mL Taq DNA polymerase (Invitrogen); 0.6 mL $MgCl_2$; 0.4 mL dNTP; 1 mL primer (oligonucleotides); 2 mL buffer; and 14.3 mL ultrapure water.

Fragments were visualized on 2% agarose gel (1X TBE; 89 mM Tris; 89 mM boric acid; 2.5 mM EDTA, pH 8.3) in a horizontal electrophoresis system run at a constant voltage of 182V, 91 mA, and 17 W for 115 min. For size standardization of bands, 5 µL 100-bp molecular weight marker was used. Gels were stained with ethidium bromide solution (0.02 mL/mL water), for approximately 30 min, and then visualized under UV light, using the Loccus L-pix HE photodocumentation device (Loccus Biotecnologia, Brazil).

Gel analysis resulted in a binary matrix according to the presence (1) or absence (0) of fluorescent bands. The percentage of polymorphic loci was calculated from the number of amplified bands. Similarity coefficients were calculated using the Jaccard index (Jaccard, 1908).

To determine the minimum number of amplified fragments needed for studies on genetic diversity, estimates were obtained from correlation (r) of similarity matrix values and the stress value (E), which indicates the adjustment between the original matrix and the simulated matrix. The optimal number of fragments was calculated by means of the GENES software (Cruz, 2007) and considered satisfactory for the analysis when the stress value was less than 0.05 (Kruskal, 1964) and the correlation closer to 1.

From the similarity matrix, a dendrogram was generated by the unweighted pair-group method with arithmetic mean (UPGMA). Bootstrapping was carried out with 10,000 replicates using the FreeTree software (Pavlícek et al., 1999). The TreeView software was also used to generate the dendrogram (Pavlícek et al., 1999). Samples were clustered considering the principal coordinates analysis (PCoA), with the aid of the Genalex v.p software (Peakall and Smouse, 2006). The Shannon index (I) (Brown and Weir, 1983) and the expected heterozygosity (H_E) (Lynch and Milligan, 1994) were also estimated using the Genalex 6.3 software (Peakall and Smouse, 2006).

RESULTS AND DISCUSSION

The individuals were analyzed based on 87 loci obtained from 16 ISSR primers. The optimal number to obtain the desired precision in the analysis of genetic diversity from *Saccharum* sp was after 83 fragments, when the stress value was 0.055 and the correlation (r) 0.991 (Figure 1).
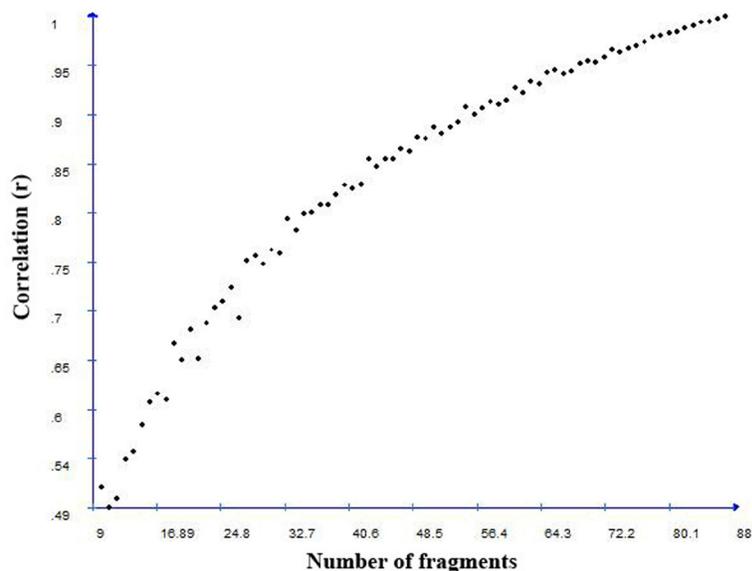

Figure 1. Correlation coefficient and number of fragments using ISSR markers between 26 individuals of sugarcane belonging to Embrapa Coastal Tablelands based on genetic similarities obtained by the bootstrap method.

In this study, there was a directly proportional relationship between the number of amplified fragments and the correlation magnitude of the values of the original similarity matrix obtained from resampling with different numbers of amplified fragments. Thus, from the optimal number of fragments obtained (83), the correlation coefficient value approached the maximum value, which proves the consistency of the data with the number of primers used and the number of fragments obtained, being sufficient for the analyses of genetic diversity.

Among the 16 primers tested in the amplification of samples, 87 loci were generated, of which 80 primers were polymorphic (91.13%). In each ISSR reaction, the total number of amplified fragments ranged from three (UBC-828, UBC-845, UBC-855) to twelve (UBC-815) (Table 2 and Figure 2).

Table 2. Inter-simple sequence repeat (ISSR) primers used in 26 sugarcane accessions from the Active Germplasm Bank of Embrapa Coastal Tablelands, Brazil, with their respective sequences, annealing temperature (AT), number of fragmented bands (NFB), polymorphism percentage (%P), Shannon Index (I), and expected heterozygosity (H_E).

Primers	Sequence (5'-3')	AT (°C)	NFB	%P	I	H_E
807	AGA GAG AGA GAG AGA GT	47	4	100	0.53	0.36
812	GAG AGA GAG AGA GAG AA	54.8	7	100	0.48	0.31
815	CTC TTC TCT CTC TCT CTG	47.6	12	100	0.49	0.32
816	CAC ACA CAC ACA CAC AT	54.8	5	100	0.37	0.22
818	CAC ACA CAC ACA CAC AG	57.2	4	100	0.44	0.28
825	ACA CAC ACA CAC ACA CT	54.8	7	85.7	0.43	0.29
826	ACA CAC ACA CAC ACA CC	57.2	5	100	0.48	0.31
827	ACA CAC ACA CAC ACA CG	57.2	5	100	0.60	0.41
828	TGT GTG TGT GTG TGT GA	54.8	3	100	0.24	0.14
835	AGA GAG AGA GAG AGA GYC	58.8	7	100	0.56	0.38
841	GAG AGA GAG AGA GAG AYC	48.5	5	20	0.12	0.08
845	CTC TCT CTC TCT CTC TRG	48.1	3	66.7	0.38	0.26
851	GTG TGT GTG TGT GTG TYG	49.2	4	100	0.67	0.48
855	ACA CAC ACA CAC ACY T	53.1	3	100	0.60	0.41
856	ACA CAC ACA CAC ACA CYA	56.4	7	85.7	0.43	0.28
887	DVD TCT CTC TCT CTC CT	55.6	6	100	0.42	0.27
Total average			5.44	91.13	0.45	0.30
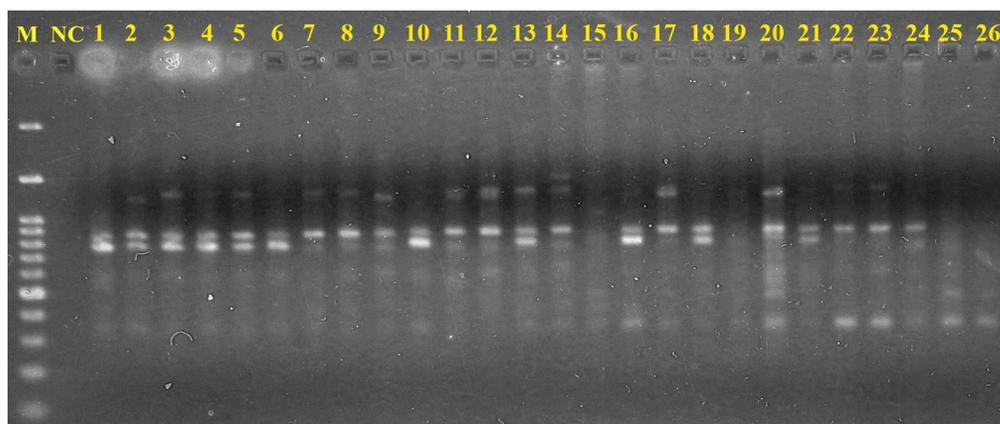
Figure 2. Electrophoretic profiles of inter simple sequence repeats of 26 sugarcane accessions amplified in agarose gel using the primer UBC 887. Embrapa Coastal Tablelands, Brazil. *Lane M* = 100-bp marker; *lane NC* = negative control; *lanes 1* to *26* = DNA samples.

Several markers have been used for studies on sugarcane diversity. AFLP, which presents advantages such as the detection of the highest number of locus, was used by Selvi et al. (2006) in species of the genus *Saccharum* and obtained 1323 bands with 12 pairs of primers, from which 1122 (84.8%) were polymorphic. Ullah et al. (2013) used eight RAPD markers and observed 73.5% polymorphism between the five varieties studied. In a study conducted with 20 accessions of *S. officinarum* in Pakistan, Khan et al. (2009) showed 86.8% polymorphism for 188 of the 210 polymorphic fragments obtained by the use of 21 RAPD primers. Raj et al. (2016), analyzing the genetic diversity of the *Saccharum* complex, using coSSR, found polymorphic amplifications (100%) in nine primers by amplifying 54 fragments. Pandey et al. (2011), however, found 148 polymorphic bands in five SSR primers.

Almeida et al. (2009) found high polymorphism (95%) using seven ISSR primers; of the 56 fragments obtained, 53 were polymorphic. Srivastava and Gupta (2008), when analyzing the diversity of 40 genotypes of sugarcane, found 79 bands amplified by 10 ISSR primers, from which 62 (78.48%) were polymorphic and 17 were monomorphic. Devarumath et al. (2012) found the production of 65 amplified fragments, with 96.5% polymorphism, in 13 ISSR primers used for PCR amplification. Rao et al. (2016), for ISSR analysis, used 19 primers in 14 genotypes of sugarcane for amplification of the PCR, a total of 164 bands were marked with 109 polymorphic bands and 55 monomorphic bands. These results, as well as in this study, prove the efficiency of ISSR when compared with other markers, revealing a high degree of polymorphism.

The I ranged from 0.12 to 0.67, with an average of 0.45. The H_E ranged from 0.08 to 0.48, with an average of 0.30 (Table 3). Those indexes showed intermediate levels of genetic diversity. However, for some initiators, such as 827, 851 and 855, the values of I and H_E can be considered high, showing high variability. According to Giustina et al. (2014), the value of I can vary between 0 and 1, with a value of 1 indicating the highest diversity of a population. The values found in this study are close to those verified by Nayak et al. (2014) in *S. spontaneum*, *S. officinarum*, *S. hybrid*, *S. barberi*, *S. robustum*, and *S. sinense*, in which the values of I found were between 0.38 and 0.49 and of H_E were between 0.23 and 0.30.

The polymorphic information content (PIC) indicates how much the marker used shows of polymorphic information in studies on genetic diversity. Values above 0.5 are highly informative; those between 0.25 and 0.50 are moderately informative; and values below 0.25 are considered slightly informative (Botstein et al. 1980). In this study, the PIC value was 0.28, considered moderately informative.

The Jaccard similarity coefficient ranged between 0.22 and 0.87, with an average of 0.49, which indicates a relatively high genetic diversity. The accessions RB867515 and RB92579, both *Saccharum* sp, were the most similar genetically, with 0.87 index. On the other hand, the pair of accessions PI240785 and NSL 291970, *S. robustum* and *S. spontaneum*, respectively, showed less genetic similarity (0.22).

The polymorphism identified by the markers was used to create a matrix of genetic distance. Four main clusters were identified in UPGMA analysis based on the Jaccard coefficient (Figure 3). The further individual, PI240785, is in the cluster C1. Most of the accessions (all from Queensland, Australia) was allocated in a main cluster C2, with several subclusters, Q45866 and Q46199 being the closest ones (0.77) of that group.

The second main cluster, C3, has eight separate individuals in subclusters, with individuals RB867515 and RB92579 possessing greater similarity (0.87). The cluster C4, however, was isolated from others and had all accessions of *S. spontaneum* and *S. sinensis* separated into two subclusters with similarity of 0.73 between PI286657 and PI29109.

Table 3. Matrix generated based on the Jaccard similarity coefficient of 26 sugarcane accessions from the Active Germplasm Bank of Embrapa Coastal Tablelands, Brazil.

|	Q45866	Q46199	Q45251	Q42509	Q45911	Q45864	Q44830	Q45337	Q45416	Q44890	Q44833	Q45869	Q42433	PI240785	PI197800	Q36830	GH-49	MIA35303	NSL29197970	PI88652	MIA35301	RB867515	RB92579	PI495109	PI286657	PI29109
Q45866	-																									
Q46199	0.77	-																								
Q45251	0.57	0.58	-																							
Q42509	0.64	0.70	0.52	-																						
Q45911	0.46	0.52	0.47	0.60	-																					
Q45864	0.74	0.71	0.52	0.58	0.54	-																				
Q44830	0.60	0.65	0.55	0.61	0.54	0.66	-																			
Q45337	0.60	0.61	0.48	0.46	0.40	0.58	0.58	-																		
Q45416	0.59	0.52	0.56	0.51	0.46	0.56	0.56	0.63	-																	
Q44890	0.61	0.61	0.58	0.54	0.54	0.64	0.58	0.53	0.54	-																
Q44833	0.56	0.57	0.63	0.49	0.49	0.57	0.64	0.65	0.55	0.67	-															
Q45869	0.57	0.60	0.54	0.48	0.48	0.60	0.57	0.52	0.52	0.50	0.54	-														
Q42433	0.45	0.51	0.52	0.53	0.48	0.48	0.60	0.63	0.60	0.60	0.54	0.53	-													
PI240785	0.41	0.44	0.34	0.43	0.40	0.47	0.45	0.54	0.46	0.48	0.43	0.41	0.42	-												
PI197800	0.45	0.48	0.39	0.45	0.35	0.44	0.40	0.46	0.39	0.31	0.33	0.43	0.34	0.33	-											
Q36830	0.44	0.54	0.47	0.53	0.44	0.45	0.46	0.52	0.38	0.46	0.39	0.42	0.43	0.41	0.53	-										
GH-49	0.46	0.57	0.42	0.45	0.43	0.54	0.54	0.57	0.39	0.51	0.46	0.48	0.42	0.38	0.40	0.47	-									
MIA35303	0.49	0.45	0.45	0.49	0.47	0.45	0.44	0.53	0.44	0.52	0.41	0.41	0.43	0.24	0.41	0.50	0.57	-								
NSL29197970	0.34	0.38	0.34	0.38	0.35	0.33	0.31	0.31	0.32	0.32	0.33	0.36	0.31	0.22	0.62	0.42	0.41	0.48	-							
PI88652	0.41	0.50	0.48	0.46	0.33	0.40	0.44	0.43	0.42	0.46	0.47	0.39	0.40	0.35	0.38	0.42	0.47	0.55	0.48	-						
MIA35301	0.50	0.56	0.49	0.53	0.45	0.48	0.47	0.48	0.40	0.60	0.48	0.37	0.40	0.42	0.36	0.51	0.54	0.68	0.49	0.56	-					
RB867515	0.52	0.59	0.48	0.57	0.53	0.55	0.52	0.55	0.55	0.57	0.53	0.56	0.49	0.45	0.47	0.52	0.53	0.57	0.45	0.60	0.52	-				
RB92579	0.52	0.59	0.51	0.57	0.50	0.54	0.52	0.57	0.55	0.52	0.48	0.56	0.47	0.45	0.49	0.55	0.50	0.54	0.46	0.54	0.49	0.87	-			
PI495109	0.48	0.53	0.44	0.50	0.45	0.53	0.53	0.58	0.39	0.53	0.51	0.52	0.50	0.45	0.45	0.53	0.54	0.52	0.41	0.46	0.50	0.62	0.62	-		
PI286657	0.27	0.35	0.27	0.34	0.31	0.29	0.31	0.37	0.27	0.27	0.28	0.27	0.23	0.25	0.39	0.41	0.40	0.43	0.49	0.46	0.43	0.39	0.42	0.37	-	
PI29109	0.34	0.46	0.32	0.40	0.35	0.36	0.36	0.40	0.32	0.35	0.33	0.32	0.29	0.26	0.45	0.51	0.45	0.49	0.46	0.52	0.49	0.52	0.49	0.47	0.73	-

Despite the large distribution of accessions in most groups, indicating great genetic diversity, these were not separated by a group of the same species in each subcluster, which can be explained by the existence of common alleles among different species, since they are of the same genus. Similar results were found by Raj et al. (2016), in which there was a close relationship among the different species that constitute the *Saccharum* complex; with *S. spontaneum*, however, contributing to greater diversity. According to the authors, *S. spontaneum* is known to be a highly variable species regarding morphology, geographical distribution, and chromosome number (2n = 48-128). High divergence of *S. spontaneum* from the rest of the species *Saccharum* was also reported by Alwala et al. (2006).

Selvi et al. (2006), however, found divergent results, in which the genus *Saccharum* was organized in a large cluster, but with each group of species in separate subclusters.
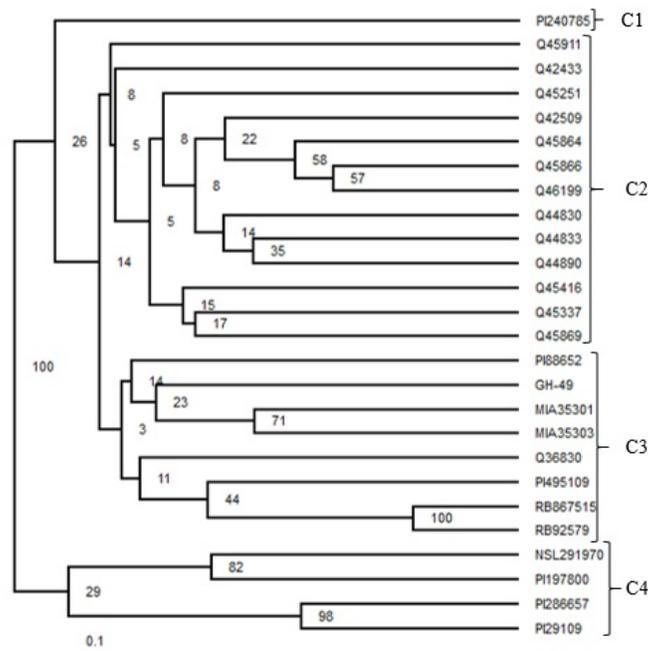
Figure 3. Phylogenetic representation of unweighted pair group method using arithmetic averages (UPGMA) clustering estimated by the genetic similarity of the Jaccard coefficient and by the bootstrap analysis (10,000X) for 26 sugarcane accessions from the Active Germplasm Bank of Embrapa Coastal Tablelands, Brazil.

The genetic distances were also assessed by the PCoA (Figure 4). Four clusters were identified among the different accessions studied, and the amount of the first two main components was 49.30%. These results stress the efficiency of ISSR in genetic diversity detection among the accessions of sugarcane. According to the results of UPGMA, some pairs of individuals (RB867515/RB92579, PI286657/PI29109, Q45866/Q46199, Q44833/Q44890, Q45337/Q45869) were genetically very close in PCoA, showing that the use of more than one clustering method prevents interference to be adopted in the allocation of materials within a specific genotypic subcluster (Silva et al., 2011).
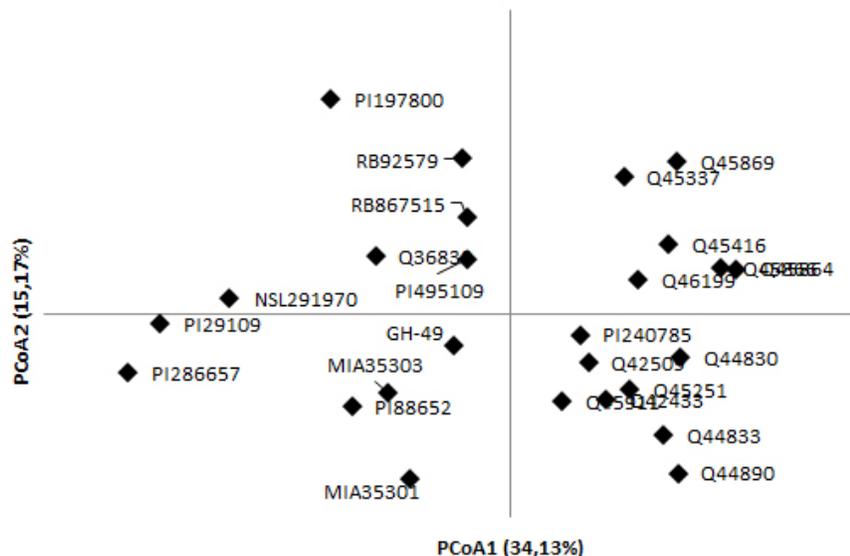
Figure 4. Principal coordinates analysis (PCoA) of 26 sugarcane accessions from the Active Germplasm Bank of Embrapa Coastal Tablelands, Brazil.

CONCLUSIONS

The genetic diversity of sugarcane genotypes from the Active Germplasm Bank of Embrapa Coastal Tablelands is considered high. The pair of individuals with less genetic similarity was PI240785 and NSL 291970, *S. robustum* and *S. spontaneum* respectively.

The results found in this study showed the level of genetic resolution and reliability obtained by analysis with ISSR molecular markers allowed the discrimination of different genetically accessions, which could be used for the management of genetic resources of the species and, once selected, will be important for breeding programs aimed at selection of superior genotypes.

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