Assessment of Genetic Diversity in Contrasting Sugarcane Varieties Using Inter-Simple Sequence Repeat (ISSR) Markers

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ABSTRACT

Sugarcane is an important tropical crop, responsible for two thirds of the world sugar production, gaining actually importance as a source of biofuel. Drought tolerance is a very important feature considering the actual climate change scenario throughout the world. This study aimed to determine the genetic diversity between sugarcane varieties with contrasting features under drought. For this purpose, twelve ISSR primers were used to characterize nine sugarcane varieties under cultivation in different countries including selected drought resistant material from Northeast Brazil and two varieties from India as contrasting genotypes. 317 scorable bands were generated, among which 301 comprised polymorphic markers, with an average of 25 polymorphic bands per primer. In the generated dendrogram the accessions were placed in clusters, where cluster A included two varieties from India (Co331 and Co419), and B comprised plants eight Brazilian accessions and a “Barbado” variety. Within this clade, drought tolerant and susceptible varieties were clearly separated. The present evaluation revealed important contrasting parental candidates regarding their drought response, very promising for future mapping approaches aiming the identification of quantitative trait loci (QTLs) associated to drought in sugarcane. The selected primers were used for the first time in sugarcane, representing valuable tools for future evaluations, with emphasis to diversity characterization and genetic mapping.

Keywords: Saccharum, Genetic Variability, ISSR Marker, Drought Tolerance

1. Introduction

Sugarcane is among the most important industrial crops of tropical and subtropical regions and is cultivated in more than 90 countries around the globe primarily for its ability to store high concentrations of sugar and more recently for the production of ethanol, very demanded as biofuel. Modern sugarcane are complex hybrids derived largely from the interspecific crosses involving Saccharum officinarum L. (2n = 80) and the wild species S. spontaneum L. (2n = 40 - 128) [1]

The choice of the variety is one of the most important factors in sugarcane breeding and production. Different varieties have different yield potentials, pest and disease resistance and are bred for different ecological and econo-
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Rental lines in sugarcane breeding programs have been defined on the basis of agronomic characters and pedigree records, using bi-parental crosses or polycrosses between elite genotypes. However, the lack of genealogy data and the inadequate identification of some genotypes have impaired an accurate estimation of the genetic diversity (GD) among sugarcane accessions based on pedigree data. In addition, the continuous selection for the same traits such as sucrose content in breeding programs has caused a reduction in GD, limiting further advances in sugarcane breeding [11].

Several different molecular markers have been used in previous studies that have examined diversity among sugarcane cultivars from different regions worldwide. These include RFLP [12], RAPD [13,14], AFLP [15,16], SSR [17] and TRAP [16]. As shown in the different approaches carried out, the employment of molecular markers bring significant improvements to assist in the association of traits with genetic markers and genetic maps, helping in the achievement of significant yield increases in breeding programs [8].

The objective of this study was to establish genetic diversity within a collection of selected sugarcane varieties with important contrasting features under drought conditions, identified in breeding programs in northeastern Brazil, a region that includes extensive semi-arid areas with few agronomic activities. This approach is important to identify contrasting parental candidates for future mapping approaches aiming to identity QTLs associated to drought tolerance/susceptibility and also for the development of comparative expression essays regarding the same traits.

2. Material and Methods

2.1. Plant Material

This study evaluated nine sugarcane varieties already cultivated in different countries and widely used in breeding stations located in northeast Brazil. These comprise eight Brazilian sugarcane varieties (SP79-1011, SP70-1143, SP78-4764, RB98710, RB943365, RB763710, RB75126 and RB863129) from two breeding programs (COPERSUCAR: Sugar and Alcohol Production Syndicate of the São Paulo State, and RIDESA: Inter University Network for Development of Sugar and Alcohol Sector) and one Barbado variety. Plants of SP (COPERSUCAR) and RB (RIDESA) varieties have been maintained by conventional bud propagation in the experimental area of the Carpina Sugarcane Experimental Station (EECAC). All eight SP and RB varieties have important agronomic traits, representing promising material for the development of new cultivars. Additionally, two Indian sugarcane varieties (Co331 and Co419), often cultivated under a wide range of agroclimatic conditions, were employed, representing external contrasting material still not used in Brazilian breeding programs (Table 1).

2.2. DNA Isolation

DNA was isolated from young leaf tissues using a CTAB (cetyl-trimethyl-ammoniumbromide) protocol [18], with minor modifications [19]. Contaminating polysaccharides were selectively precipitated [20] and DNA concentrations were determined comparatively by electrophoresis.
in agarose gel 1.2% using known amounts of phage λ-DNA as a reference.

2.3. ISSR Analysis

The ISSR amplification reactions contained 15 ng of genomic DNA, 2.0 μL 10 × buffer, 2.5 mM MgCl₂, 200 μM of each dNTP (Fermentas), 50 μM primers and 0.5 U Taq DNA polymerase (Invitrogen), with the final volume adjusted to 20 μL with H₂O bidest. The amplification reaction was carried out in an Eppendorf Mastercycler Gradient or Techn TC-412 thermal cycler. The reaction included an initial denaturation step of 4 min at 94°C, followed by 30 cycles, each consisting of a denaturation step of 30 s at 94°C, annealing of 1 min at 50.4°C to 60.5°C (depending on the primer) and an extension of 2 min at 72°C. PCR was terminated with a final extension of 7 min at 72°C. ISSR reaction products were separated on 1.8% horizontal agarose gels, in TBE buffer with a voltage of 5 V/cm and visualized under ultraviolet light after staining in 0.5 μg/mL ethidium bromide. Digital photo documentation was taken for each gel. The 100 bp DNA ladder plus molecular weight marker was used to compare the molecular weight of amplified products. Twelve ISSR primers previously selected for Poaceae species, common cordgrass [21], barley [22] and rice [23] were chosen and ordered from MWG-Biotech for application on sugarcane varieties (Table 2).

2.4. Data Analysis

Polymorphic ISSR markers were scored as binary data: presence (1) or absence (0). Only clearly resolved bands were used in the genetic analysis. The genetic similarity among the varieties was calculated by Jaccard Similarity Coefficient using NTSYS-pc 2.1 software [24]. A dendrogram was constructed based on genetic distance using neighbor-joining method and bootstrap analysis (1000 replicates) with MEGA program (Molecular Evolutionary Genetic Analysis), Version 4 for Windows [25].

3. Results

3.1. PCR Analysis

The ISSR analysis, carried out in 11 varieties, produced 317 bands, from which 301 (94.9%) were polymorphic among the sugarcane varieties, with an average of 25 ISSR polymorphic bands per primer. All primers amplified fragments, with a number of amplicons varying from 39 (primer 857) to 14 (primers 807 and 815) fragments per reaction, with sizes varying from ~200 bp to ~2.0 kb (Table 2). No single band was specific to any individual variety, nor to a given feature. Some primers produced polymorphic bands specific to a set of genotypes (Figure 1).

Table 2. List of ISSR primers used, including their nucleotide sequence, annealing temperature, number of total and polymorphic bands, as well as percentage of polymorphic bands.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Temp.</th>
<th>5'-3’ Sequence</th>
<th>Total # bands</th>
<th># Polym. bands</th>
<th>% Polym.</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBC 857</td>
<td>54.0</td>
<td>5’ACACACACACACACACACACAG3’</td>
<td>41</td>
<td>39</td>
<td>90.2</td>
</tr>
<tr>
<td>UBC 834</td>
<td>52.8</td>
<td>5’AGAGAGAGAGAGAGAGAGAGYT3’</td>
<td>37</td>
<td>35</td>
<td>94.6</td>
</tr>
<tr>
<td>UBC 848</td>
<td>54.0</td>
<td>5’ACACACACACACACACACACARG3’</td>
<td>37</td>
<td>35</td>
<td>94.6</td>
</tr>
<tr>
<td>UBC 810</td>
<td>50.4</td>
<td>5’GAGAGAGAGAGAGAGAT3’</td>
<td>32</td>
<td>31</td>
<td>96.9</td>
</tr>
<tr>
<td>UBC855</td>
<td>52.0</td>
<td>5’ACACACACACACACACACACYT3’</td>
<td>32</td>
<td>30</td>
<td>93.7</td>
</tr>
<tr>
<td>UBC 811</td>
<td>52.8</td>
<td>5’GAGAGAGAGAGAGAGAC3’</td>
<td>27</td>
<td>26</td>
<td>96.3</td>
</tr>
<tr>
<td>UBC 812</td>
<td>52.0</td>
<td>5’GAGAGAGAGAGAGAGAGAGAA3’</td>
<td>22</td>
<td>21</td>
<td>95.4</td>
</tr>
<tr>
<td>K18</td>
<td>54.0</td>
<td>5’DVHCACACACACACACACAC3’</td>
<td>22</td>
<td>21</td>
<td>95.4</td>
</tr>
<tr>
<td>UBC 828</td>
<td>52.8</td>
<td>5’TGTGTGTGTGTGTGTGA3’</td>
<td>18</td>
<td>18</td>
<td>100</td>
</tr>
<tr>
<td>UBC823</td>
<td>52.0</td>
<td>5’CTCTCTCTCTCTCTCTCT3’</td>
<td>18</td>
<td>17</td>
<td>94.4</td>
</tr>
<tr>
<td>UBC 807</td>
<td>52.0</td>
<td>5’AGAGAGAGAGAGAGAGAGAGT3’</td>
<td>16</td>
<td>14</td>
<td>87.6</td>
</tr>
<tr>
<td>UBC 815</td>
<td>54.0</td>
<td>5’CTCTCTCTCTCTCTCTCT3’</td>
<td>15</td>
<td>14</td>
<td>93.3</td>
</tr>
</tbody>
</table>

ISSR primers were obtained from the University of British Colombia (Ayres and Strong 2001, Fernández et al. 2002, Jeung et al. 2005) R = (A,G); Y = (C,T); D = (A,G,T) (i.e. not C); H = (A,C,T) (i.e. not G), V = (A,C,G) (i.e. not T). Legend for abbreviations: Temp. = Annealing temperature; # = number; Polym. = Polymorphic/Polymorphism.

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3.2. Clustering Analysis

The neighbor-joining method generated a dendrogram with two main clusters, where A included two varieties from India (Co331 and Co419) and B comprised one Barbado and eight Brazilian varieties. The drought susceptible accession RB94-3365 occupied a basal position considering the upper branch. The majority of sugarcane varieties clearly grouped into two major clusters in the dendrogram (Figure 2). The first branch included all tolerant varieties divided into two groups. The first group comprised two subgroups comprising the varieties SP79-1011; SP-70-1143; RB98710 and the second one with RB763710; RB863129 varieties. Within this group the accessions RB763710 and RB863129 grouped with high bootstrap value (100). The second branch included all drought susceptible genotypes plus the variety B8008 in a basal position.

Genetic similarity estimates varied from 0.14 (between RB763710 and Co419) to 0.54 (between RB863129 and RB763710) (Table 3). Low coefficients of similarity were detected among sugarcane varieties, with an average similarity value of 0.25. The lowest similarity coefficient among Brazilian varieties was detected between RB763710 and SP78-4764 (15.8%), also reflecting their contrasting performance under drought (tolerance and sensitivity to water stress, respectively) (Table 1).

4. Discussion

Similar results were reported for other accessions during the evaluation of the genetic diversity of sugarcane varieties with ISSR markers [1] with 78.48% of the bands produced being polymorphic. However, the result obtained in the present study bear higher polymorphism levels than those previously reported by other authors using RAPD [13,26-28].

The high polymorphism level detected by ISSR markers was expected due to the use of the external Indian varieties and also considering the segmental allopolyploid nature of sugarcane, generally attributed to the interspecific hybridization crosses used during breeding programs that generated the actual breeding accessions. The nature of ISSR, targeting regions especially rich in microsatellites may also justify the higher level of polymorphism, since those regions are known to accumulate a larger number of mutations due to DNA polymerase slippage during replication and unequal crossing-over [29]. Despite of that, researchers who have compared RAPD and ISSR methods have found that ISSR markers exhibit higher level of reproducibility, when compared to RAPD [30-32].

Reduced coefficient of similarity between RB763710 and SP78-4764 varieties of sugarcane has been observed by other authors. Specific regions of the sugarcane genome related to drought tolerance, rather than the entire genome, were sampled to evaluate the genetic variability of candidate parents for breeding purposes [11]. In their study, the lowest similarity value for drought was obtained among the genotypes IACSP95-2078 and SP86-42 (0.44), illustrating that this cross would probably result in the highest variability for drought among the genotypes sampled. The SP86-42 variety presents excellent performance in the Brazilian areas of “Cerrado” vegetation.

Figure 1. Examples of ISSR amplification results in sugarcane. (a) Amplicons using primer ISSR 815; (b) amplicons using the primers ISSR 810 and 812. M: 100 bp ladder (molecular weight marker). Arrows indicate major polymorphic sites. Order of the genotypes: RB763710, RB863129, SP79-1011, SP70-1143, RB98710, B8008, RB75126, SP78-4764, RB943365, Co331, Co419.

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Figure 2. Consensus tree of the analyzed sugarcane accessions based on ISSR data after the Neighbor Joining analysis (complete deletion and p-distance) using MEGA program. Bar indicates genetic distance. Bootstrap values > 80 [1000 replicates] are depicted on the branches. Black circles represent drought tolerant varieties and triangles represent susceptible varieties.

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Table 3. Similarity matrix among the sugarcane varieties obtained by Jaccard Similarity Coefficient using NTSYS-pc 2.1 software.

<table>
<thead>
<tr>
<th></th>
<th>RB793710</th>
<th>RB863129</th>
<th>SP79-1011</th>
<th>SP70-1143</th>
<th>RB98780</th>
<th>B8008</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB793710</td>
<td>0.5346535</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RB863129</td>
<td></td>
<td>0.2586207</td>
<td></td>
<td>0.5638298</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP79-1011</td>
<td>0.2338710</td>
<td>0.2786885</td>
<td>0.3738318</td>
<td>0.4528302</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP70-1143</td>
<td>0.2540984</td>
<td>0.2868852</td>
<td>0.2649573</td>
<td>0.3025210</td>
<td>0.4090909</td>
<td></td>
</tr>
<tr>
<td>RB98780</td>
<td>0.2320000</td>
<td>0.266412</td>
<td>0.2338710</td>
<td>0.2307692</td>
<td>0.2800000</td>
<td>0.4907407</td>
</tr>
<tr>
<td>B8008</td>
<td></td>
<td>0.1587302</td>
<td>0.2173913</td>
<td>0.193077</td>
<td>0.2204724</td>
<td></td>
</tr>
<tr>
<td>RB75126</td>
<td>0.1666667</td>
<td></td>
<td>0.1984733</td>
<td>0.2032520</td>
<td>0.1869919</td>
<td></td>
</tr>
<tr>
<td>SP78-4764</td>
<td>0.1451613</td>
<td></td>
<td>0.1600000</td>
<td>0.1826087</td>
<td>0.1532258</td>
<td></td>
</tr>
<tr>
<td>RB943365</td>
<td>0.1600000</td>
<td>0.1472868</td>
<td>0.1680672</td>
<td>0.1680000</td>
<td>0.1854839</td>
<td></td>
</tr>
<tr>
<td>Co331</td>
<td>0.1451613</td>
<td></td>
<td>0.1600000</td>
<td>0.1826087</td>
<td>0.1532258</td>
<td></td>
</tr>
<tr>
<td>Co419</td>
<td>0.1692308</td>
<td></td>
<td>0.1880342</td>
<td>0.4000000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(fire adapted savannas characterized by poor and mineralized soils), being also grown in drought-prone environments.

Data analysis using the neighbor-joining method generated a dendrogram with two main clusters that grouped individuals that share the same gene pool of origin. Cluster “A” included two varieties whose genomic background is mainly from plants used in breeding programs from India (Co331 and Co419), and cluster “B” included eight Brazilian varieties and one Barbado variety. All accessions included in this last cluster have been used as parental in crosses carried out in Brazilian breeding programs aiming to generate drought tolerant material.

The accession RB94-3365 is the genetic basis of all analyzed Brazilian varieties, due to its high productivity, despite of drought susceptibility, justifying its basal position considering the upper branch. The majority of sugarcane varieties clearly grouped into two major clusters in the dendrogram with a bootstrap of 99. Within this group, the first branch emerged with bootstrap 84, including all tolerant varieties divided into two groups.

The first group within the upper cluster included two subgroups, one comprising three (SP79-1011; SP-70-1143; RB98710) and the second with two (RB763710; RB863129) accessions. Within this branch, RB763710 and RB863129 grouped with a bootstrap of 100. Besides being tolerant to drought, both varieties (RB763710 and SP79-1011) are sources of resistance against rust and present contrasting maturation time, growth habit and sugar yield potentials, being very useful candidates in future breeding programs.

The second branch within the upper cluster presented bootstrap 80 and included all drought susceptible genotypes, with the Barbado variety (B8008) in a basal position, a material identified as source of resistance against rust and bearing high sugar production.

Differential responses to abiotic stress were observed among clusters. The association of drought-tolerance present in RB varieties (RB863129, RB763710 and RB98710) and SP varieties (SP70-1143 and SP79-1011) in the same branch, was supported by high bootstrap scores (84), indicating that these accessions are also in-
intersting for breeding of this feature [33,34]. Considering salinity sensitive varieties, two groups had been formed: the first containing one variety (RB943365) and the second with three varieties (SP78-4764, RB75126 and B8008). This is promising, especially considering that other authors have shown that the genetic variations identified with aid of molecular markers are useful in evaluating upland accessions for drought-tolerance with related morphology [35].

The same was observed regarding sugarcane genotypes with contrasting response to red rot disease [36]. The authors reported the usefulness of ISSR markers to separate red-rot disease resistant, moderately resistant and susceptible among sugarcane genotypes. The sugarcane accessions Co 8011, Co 86010, Co 85061, Co 62198, Co 86032, GSBT 9 and Co 8014 that are resistant to moderately resistant to red rot disease were grouped into one cluster, while remaining genotypes Co 7804, Co 62175, Co 8371 and Co 671, susceptible to red rot disease, were grouped into another cluster.

Several authors have reported high genetic similarity among sugarcane varieties using RAPD [13,14,27]. This genetic homogeneity, in spite of regional adaptation and selection history, is probable due to the repeated use of few sets of nearly related varieties. In addition, considering the supposed narrow genetic basis of sugarcane varieties based on RAPD markers, one may suppose that other techniques have to be tested in the search for more efficient polymorphism identification. For example, using AFLP to analyze commercial sugarcane varieties grown under tropical and subtropical regions of India the level of genetic diversity among the tropical and subtropical cultivars was much higher than most of the pairwise diversity measures within each of these two adaptive groups. The AFLP-based clustering of the cultivars also corresponded well with their known pedigree [37]. This method is really more effective than RAPD, but presents the disadvantage of being more expensive and time consuming than ISSR.

The results obtained here using ISSR were comparable to other studies with Poaceae. In a study using AFLP and ISSR molecular markers to evaluate the genetic diversity and relationships of 56 waxy rice accessions, a single ISSR primer BDB(AC)7 produced a total of 88 bands ranging from 200 to 600 bp, including 77 polymorphic bands [38]. The average polymorphism of the total ISSR markers was 92.2%, much higher than that observed for AFLP.

Similarly, authors reported the usefulness of ISSR in surveying genetic variation of 46 barley accessions [32]. For this purpose, 18 primers were used, generating a total of 107 bands with 105 (98.13%) polymorphic bands, with two to 10 polymorphic bands per primer.

The present results revealed considerable levels of genetic diversity to design crosses for mapping purposes employing linkage analysis aiming to identify QTLs with aid of DNA markers considering the analyzed varieties. Moreover, unambiguous discrimination and identification of cultivars using ISSR markers are of significance in the context of establishing clone fidelity, cultivar certification and germplasm management.

The here tested ISSR primers were tested for the first time in sugarcane and presented better performance regarding the detection of polymorphisms in this crop, than previously used ISSR primers. Especially considering the narrow genetic basis of many existing genetic banks in sugarcane, they represent additional possibilities for diversity screening. In the case of the present evaluation they helped in the planning of futures crosses using drought tolerant varieties with the most productive drought sensible cultivars, aiming the direct breeding of this important feature, as well as for the identification of most contrasting drought tolerant and sensible materials for a cross aiming the construction of a genetic map for QTL identification.

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