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Genotype-Specific Microsatellite (SSR) Markers for the Sugarcane Germplasm from the Karst Region of Guizhou, China

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Abstract

Genetic variability among sugarcane genotypes from the Karst region of China was evaluated using genotype-specific microsatellite (SSR) markers. Eighteen sugarcane genotypes including 13 active cultivars and five elite QT-series clones bred locally were screened for genetic variability with 21 SSR primer pairs. All the primer pairs were highly polymorphic and amplified a total of 167 alleles with an average of eight alleles per primer pair. The average polymorphism information content (PIC) value was 0.86 with a range of 0.68 and 0.92. A UPGMA dendrogram categorized the 18 sugarcane genotypes into three major groups containing three, ten and five genotypes, respectively. No geographical affinity was observed among genotypes within the same group. Eight SSR primer pairs produced cultivar-specific alleles, of which five alleles were unique to the QT-series clones, namely, SMC334BS-165 and SMC851MS-145 in QT 08-558, mSSCIR43-229 in QT 4, SM597CS-182 in QT 08-536 and SMC7CUQ-168 in QT 06-212. The clone-specific SSR alleles will be useful in identifying elite QT-series clones for use in the sugarcane crossing programs in China.

Keywords

Sugarcane, Clone-Specific SSR Markers, Genetic Diversity, Karst Region

1. Introduction

Modern sugarcane cultivars (*Saccharum* hybrids spp., $2n = 110 - 130$) are inter-specific, highly polyploid and aneuploid hybrid derivatives of a few progenitor *Saccharum*

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clones with a genome size of around 7.5 - 10 Gb. Due to high level of heterozygosity and complexity of its genome, sugarcane molecular research has been confronted with unique challenges not encountered in other major crops, resulting in a slow progress in sugarcane molecular breeding. Nonetheless, many researchers have made an effort to evaluate different sources of sugarcane germplasm with various DNA markers, including 5S rRNA intergenic transcribed spacer (ITS) [1], restriction fragment length polymorphism (RFLP) [2] [3], random amplified polymorphic DNA (RAPD) [4] [5] [6] [7], amplified fragment length polymorphism (AFLP) [8] [9] [10], simple sequence repeats (SSR) [8] [11] [12] [13] [14], target region amplification polymorphism (TRAP) [15] [16], conserved-intron scanning marker (CISP) [17] [18] and single nucleotide polymorphism (SNP) [19] [20]. Sugarcane breeders have successfully found some cultivar-specific, species-specific and trait-specific DNA makers to accelerate the breeding process [13] [21] [22] [23] [24] [25].

Among PCR-based marker systems, SSR is a powerful marker system once developed for a particular organism due to its ubiquitous distribution across the entire genome, high polymorphism, and reliability. Molecular breeding scientists have successfully developed sugarcane SSRs including 221 genomic-SSRs developed by the International Sugarcane Microsatellite Consortium (ISMC) [11], 402 expressed sequence tags (EST)-derived SSRs developed by da Silva [12], 837 EST-SSRs from SUCEST by Souza's group [25] [26] [27] [28] [29], and 702 SSRs mined from both genomic and expressed sequences by the Indian Agricultural Research Institute (IARI) [30]. Some of the sugarcane SSR markers were applied to paternity analysis [31], genetic diversity assessment [32] [33] [34], genetic linkage map construction [28] [35], germplasm evaluation and variety identity testing [13] [36]. In addition, SSR technology combined with capillary electrophoresis and fluorescence detection system shows better performance in genotyping analysis due to higher accuracy and detection power [36]. Pan *et al.* employed this detection system to successfully identify sugarcane clones that had been mis-labeled [37]. Recently, this detection system also enabled the segregation analysis of microsatellite (SSR) markers in sugarcane polyploids [38] [39].

Karst represents special landscape geographic regions formed from the dissolution of soluble rocks. China is one of the countries in the world with a large distribution of Karst landscape that can be further classified as tropical and sub-tropical Karst region, plateau Karst region, or mountain Karst region. Guizhou represents the typical plateau Karst landform in China with an average altitude of 1100 meters above the sea level. Sugarcane has been grown in Guizhou for more than 60 years, normally in the valley or on hilly slopes 375 - 900 meters above the sea level. In the local sugarcane growing area, the annual mean temperature is 17.0˚C - 20.2˚C, the average annual precipitation is 1200 mm, and 345 - 365 days are frost-free. In 2013, sugarcane growing area was around 30,000 hectares in Guizhou alone, which was reduced gradually in the past two years due to lower sugar price in the market. Since the 19th century, elite sugarcane germplasm has been introduced into Guizhou from other sugarcane research institutions in China as well as other countries for evaluation and selection. Approximately 20

sugarcane cultivars are currently being used in Guizhou sugarcane production, of which, six cultivars account for more than 80% of the planted area in Guizhou; each with unique traits. For example, ROC21, ROC22 and ROC25 from Taiwan have overall good traits; YT 94-128 is early maturing and drought resistant; Xuan 50 has multi-tillers and thin stalks; and QT4 is a locally bred variety specifically for black sugar production.

The goal of this study was to evaluate the extent of genetic variability among the sugarcane germplasm involved in sugarcane breeding and production in Guizhou with highly polymorphic SSR primer pairs. The expected results will provide not only a better understanding of the genetic base of sugarcanes germplasm, but also useful information to assign cross parents for the breeding program in Guizhou.

2. Materials and Methods

2.1. Plant Materials

Eighteen sugarcane genotypes were involved in this study, including 13 cultivars that were widely grown for sugar production in Guizhou, China and five elite clones that were selected through conventional breeding by the Guizhou Institute of Subtropical Crops (GISC) (**Table 1**). Healthy apical leaves were collected from the plants of the 18 genotypes from the germplasm nursery at GISC, which were stored at −20˚C prior to DNA extraction. DNA isolation was conducted according to Pan *et al.* [1]. DNA concentrations were measured on a Shimadzu Model UV-1601 spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) and DNA quality was assessed by 0.8% agarose gel electrophoresis using diluted uncut λ DNA as the standard. Final DNA concentration was adjusted to five ng/μL and stored at −20˚C for SSR analysis.

2.2. SSR Primer, PCR, and Fragment Analysis

SSR analysis was conducted with 21 highly polymorphic ISMC SSR primer pairs following the protocol of Pan [13]. PCR amplification reactions were conducted in a total volume of 10 μL, containing 10 ng of DNA, 0.2 mM each of FAM-labeled forward primer and unlabeled reverse primer, 80 mM each of dNTP, 10 mM Tris-HCl, 2.5 mM $MgCl_2$, 50 mM KCl, and 1 U Taq DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN, USA). The thermo-cycle program was subsequently conducted as follows: 95˚C for 5 mins, followed by 30 cycles of 94˚C for 30 s, annealing temperature for each pair of primers for 30 s, 72˚C for 30 s, followed by 72˚C for 2 min. FAM-labeled PCR fragments along with the GS500 size standards were separated through capillary electrophoresis (CE) on the DNA Analyzer ABI 3730XL (Applied Biosystems, Foster City, CA, USA) to generate <.fsa> GeneScan files.

2.3. Data Analysis

The GeneMapper software v3.0 (Applied Biosystems, Foster City, CA, USA) was used to analyze the GeneScan files through visualization of capillary electrophoregrams of

Table 1. A list of sugarcane genotypes involved in the study.

Name*	Origin	Pedigree
FN38	Fujian, China	Yuetang 83-257 × Yuetang 83-271
GT11	Guangxi, China	CP49-50 × Co419
GT27	Guangxi, China	Yuetang 85-1622 × Yacheng 73-512
GT29	Guangxi, China	Yacheng 94-46 × ROC22
GT31	Guangxi, China	Yuetang 91-976 × ROC1
QT4	Guizhou, China	Nco310 × CP36-105
ROC21	Taiwan, China	70-3792 × ROC163
ROC22	Taiwan, China	ROC5 × 69-463
ROC25	Taiwan, China	79-6048 × 69-463
YT00-236	Guangdong, China	Yuenong 73-204 × CP 72-1210
YT60	Guangdong, China	Yuetang 92-1287 × Yuetang 93-159
YT94-128	Guangdong, China	Zhanzhe 80-101 × ROC1
QT06-156	Guizhou, China	ROC10 × CP 57-614
QT06-212	Guizhou, China	ROC10 × CP 57-614
QT08-526	Guizhou, China	ROC10 × Yacheng 84-125
QT08-536	Guizhou, China	ROC10 × Yacheng 84-125
QT08-558	Guizhou, China	Neijiang 00-118 × Guitang 94-119
Xuan 50	Brazil	(Unknown)

*FN = Funong; GT = Guitang; QT = Qiantang; ROC = Taiwan, China; YT = Yuetang; Xuan 50 = RB72-454.

SSR alleles with sizes automatically calibrated using the GS500 size standards. Irregular Peaks such as "stutters", "pull-ups", "dinosaur tails", and "minus-A peaks" were not scored according to Pan [13]. The presence of a regular peak was scored as 1 and its absence was scored as 0. The polymorphism information content (PIC) of each SSR primer pair was calculated according to the formula of Milbourne *et al.* [40]: PIC = 1 - ΣPg^2, where Pg is the frequency of an individual genotype. Genetic distance between each genotype was calculated using the Jaccard similarity coefficient and then a dendrogram was constructed using NTSYSpc-2.2 [41] software on the basis of unweighted pair group method with arithmetic means (UPGMA). A homology tree was built using DNAMAN® (Lynnon Co., Quebec, Canada) to analyze the genetic homology relationship among the 18 sugarcane genotypes where the presence of a regular peak was recorded as A and its absence was recorded as C [42].

3. Results and Discussion

3.1. Characteristics of SSR Polymorphism

All sugarcane SSRs used in this study were polymorphic yielding a total of 167 alleles, of which 148 alleles (88.6%) were polymorphic. Figure 1 is an example of SSR profiles

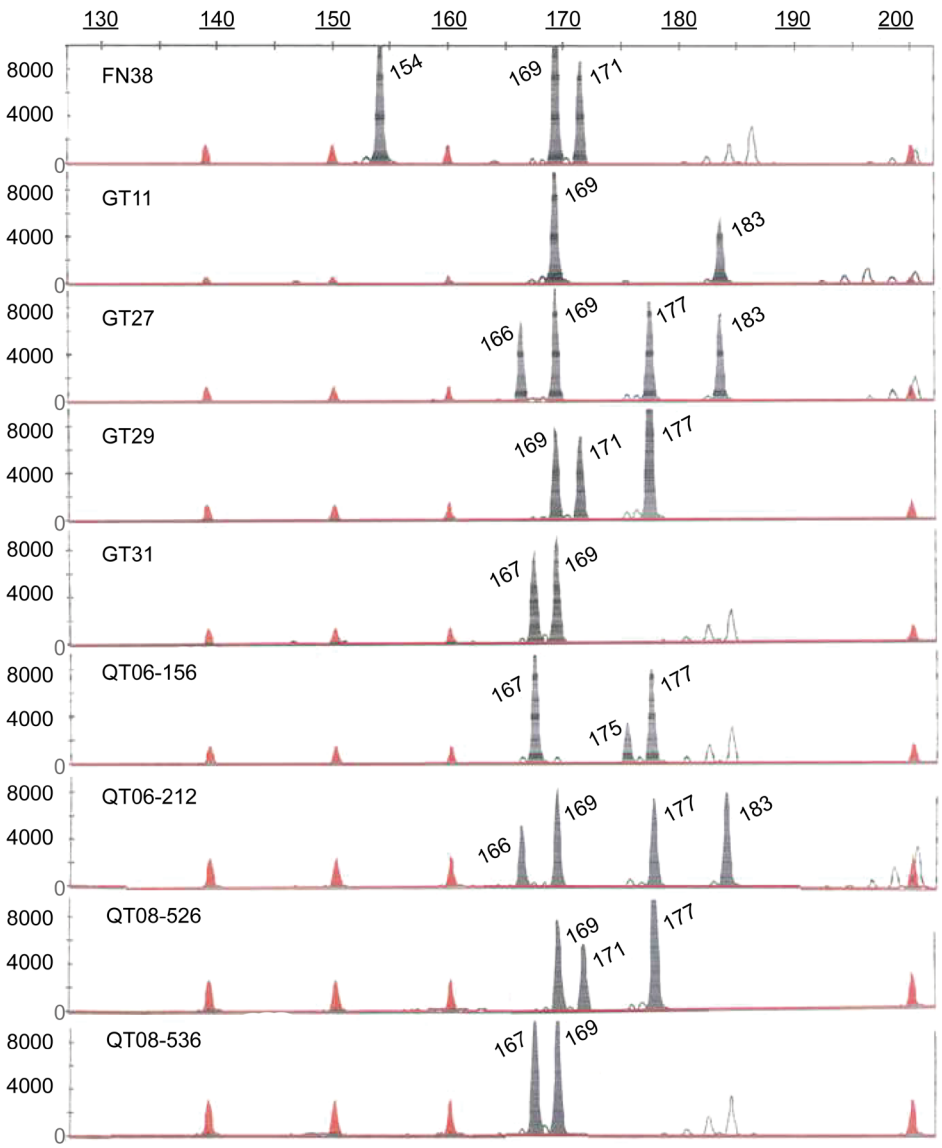

Figure 1. Discriminative SSR profiles of nine sugarcane genotypes produced by SSR primer pair SMC336BS. The Y-axis value represents the fluorescence strength or the relative yield of amplified DNA fragment. The X-axis value represents DNA fragment sizes in base pairs.

of nine sugarcane genotypes based on marker SMC336BS. The number of alleles produced by each SSR primer pair ranged from four to twelve with an average of eight alleles per primer pair (**Table 2**), slightly higher in comparison with Hameed's study (6.85) [43] but lower than the average number of alleles per primer pair reported by Chandra (9.2) [21] and Liang (15.6) [44] using the same set of SSR makers. SMC24DUQ and SMC597CS both produced maximum number of alleles, of which 10 were polymorphic. Although SMC486CG and SMC569CS produced the minimum number of alleles, all alleles were polymorphic. The percentage of polymorphic bands for each SSR primer pair was in the range of 66.7% and 100%. Eight SSR primer pairs (38.1%), namely, SMC278CS, SMC31CUQ, SMC334BS, SMC336BS, SMC486CG,

Table 2. Genetic diversity among 18 sugarcane genotypes as revealed by SSR primer pairs.

Primer Pair	Total # Bands	# Polymorphic Bands (%)	SSR Size Range (bp)	PIC
SMC119CG	10	9 (90.0)	106-228	0.91
SMC1604SA	8	7 (87.5)	106-127	0.91
SMC18SA	6	4 (66.7)	137-165	0.82
SMC24DUQ	12	10 (83.3)	126-151	0.91
SMC278CS	9	9 (100)	140-182	0.91
SMC31CUQ	9	9 (100)	138-179	0.92
SMC334BS	10	10 (100)	147-165	0.92
SMC336BS	10	10 (100)	141-187	0.91
SMC36BUQ	6	5 (83.3)	104-255	0.86
SMC486CG	4	4 (100)	225-242	0.81
SMC569CS	4	4 (100)	167-222	0.78
SMC7CUQ	7	5 71.4)	158-172	0.83
SMC597CS	12	10 (83.3)	144-182	0.91
SMC703BS	7	5 (71.4)	202-220	0.83
SMC851MS	9	8 (88.9)	127-145	0.9
mSSCIR66	7	7 (100)	127-156	0.9
mSSCIR3	9	8 (88.9)	169-198	0.88
SMC1751CL	6	4 (66.7)	137-154	0.68
SMC22DUQ	7	6 (85.7)	147-163	0.88
mSSCIR43	10	10 (100)	169-252	0.91
mSSCIR74	5	4 (80)	217-229	0.78
Total	167	148		
Mean	8	7		0.86

SMC569CS, mSSCIR66, produced 100% polymorphic alleles. The PIC values of the 21 SSR primer pairs ranged from 0.68 to 0.92 with an average of 0.86. SMC31CUQ and SMC334BS were the most informative primer pairs producing the highest PIC value of 0.92.

Eight SSRs were found to be cultivar-specific for nine sugarcane genotypes, amplifying a total of 11 unique SSR alleles. Of which, five were specific to QT-series clones, namely, SMC851MS-145, mSSCIR43-229, SM597CS-182, SMC7CUQ-168, and SMC334BS-165. Primer pair SMC851MS produced three cultivar-specific alleles, namely, SMC851MS-127 in Xuan 50, SMC851MS-143 in FN 38, and SMC851MS-145 in QT 08-558. Primer pair mSSCIR43 produced two cultivar-specific alleles, namely, mSSCIR43-229 in QT 4 and mSSCIR43-240 in GT 31. Each of the remaining six SSR primer pairs amplified only one cultivar-specific allele, namely, SM597CS-182 in QT

08-536, SMC7CUQ-168 in QT 06-212, SMC334BS-165 in QT 08-558, SMC24DUQ-151 in FN 38, SMC1604SA-106 in GT 11, and SMC119CG-223 in YT 60. These results implies that a fewer number of primer pair combinations among these eight SSR primer pairs will be able to identify sugarcane genotypes within a large scale of sugarcane germplasm evaluation. Moreover, these primer pairs are also primer pairs of choice for pedigree verification in sugarcane polycross or hybrid identification in bi-parental crosses when the nine sugarcane genotypes are used as the parents.

The performances of each SSR primer pair may vary on different sugarcane cultivars because these cultivars may have different chromosome makeups. The 21 SSR primer pairs used in the present study were chosen from 67 highly polymorphic SSR primer pairs based on the evaluation of five elite U.S. germplasm [13]. These primer pairs were able to distinguish among cultivars and breeding lines in the U.S. [42]. These SSR primer pairs also can detect genetic variations among sugarcane clones originated from China, Bangladesh, South Africa, Mexico, India, and related wild species [21] [44] [45]. Therefore, these 21 SSR primer pairs can be considered as core SSR primer pairs for sugarcane molecular characterization worldwide. However, Pan suggested that these SSR primer pairs be tested first on a local elite sugarcane germplasm collection developed under certain geographical environment prior to a full-scale genotyping exercise [13].

It is the first report on evaluating genomic diversity among sugarcane cultivars growing in Karst region in China, the 21 SSR primer pairs showed highly polymorphic among both material sugarcane and elite clones, which indicates these 21 SSR primer pairs are suitable for genotyping sugarcane clones bred in Karst climate in China.

3.2. Genetic Diversity

Homology analysis showed there were 64-98% homologies among the 18 sugarcane genotypes (**Figure 2**). Two pairs of genotypes, namely, ROC21 and YT 00-236 and YT 94-128 and ROC25, shared the highest homology value of 98%, followed by ROC22 and YT-60 (97%). The homology values shared between the QT-series sugarcane genotypes were between 67% and 76%. A UPGMA dendrogram was constructed to reveal the extent of genetic diversity among the 18 sugarcane genotypes. Genetic similarity coefficients for all the genotypes ranged from 0.64 to 0.98. The 18 sugarcane genotypes were clustered into three major groups, namely, A, B and C (**Figure 3**). Group B was the largest group with 10 genotypes (55.6%), followed by Group C with five genotypes (27.8%) and Group A with three genotypes (16.7%). Group B was further divided into two sub-groups, BI and BII. The Sub-group BI consisted of two genotypes, GT11 and QT08-558; while the Sub-group BII included eight genotypes. Genotype ROC21 and YT00-236 in Group C were almost genetically identical. In Group B, both ROC25 and YT94-128 and ROC22 and YT60 were genetically close to each other. This was probably due to the fact that ROC-series cultivars of Taiwan were first introduced into Guangdong province, where these cultivars were used either as cross parents to breed the YT-series clones or directly as cultivars if they well adapted to local climates.

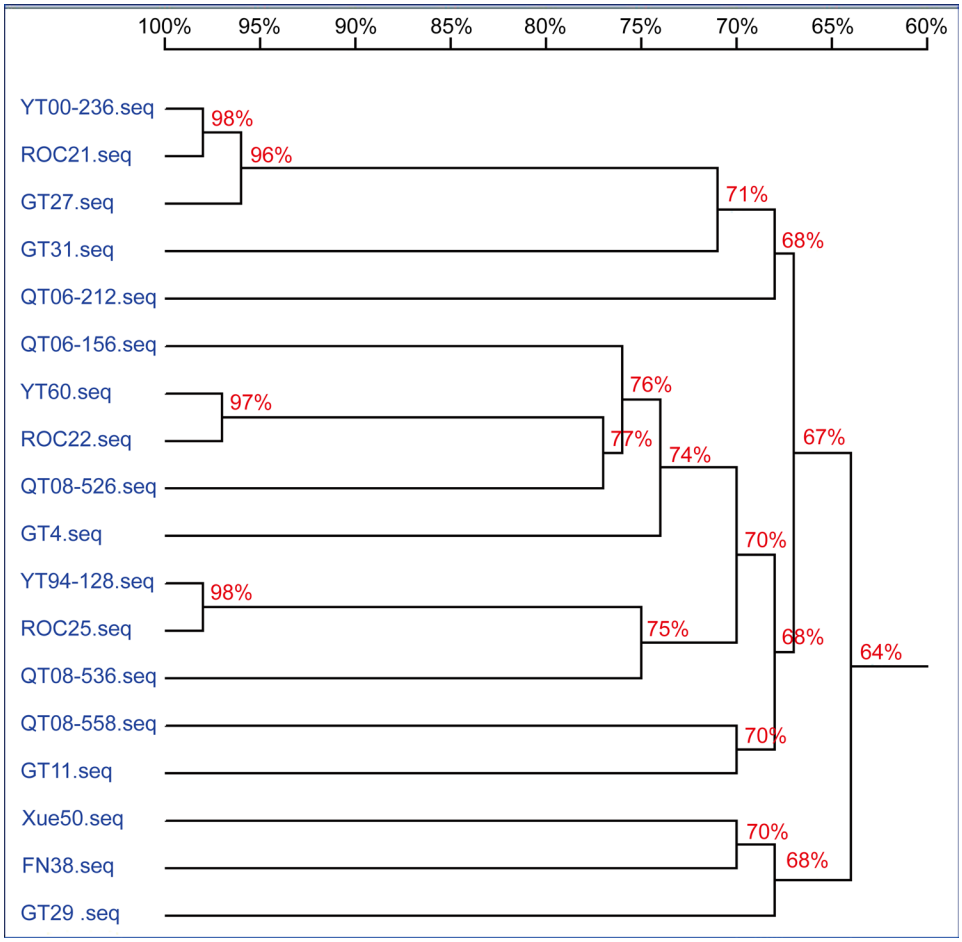

Figure 2. A homology tree of 18 sugarcane genotypes based on SSR marker data.

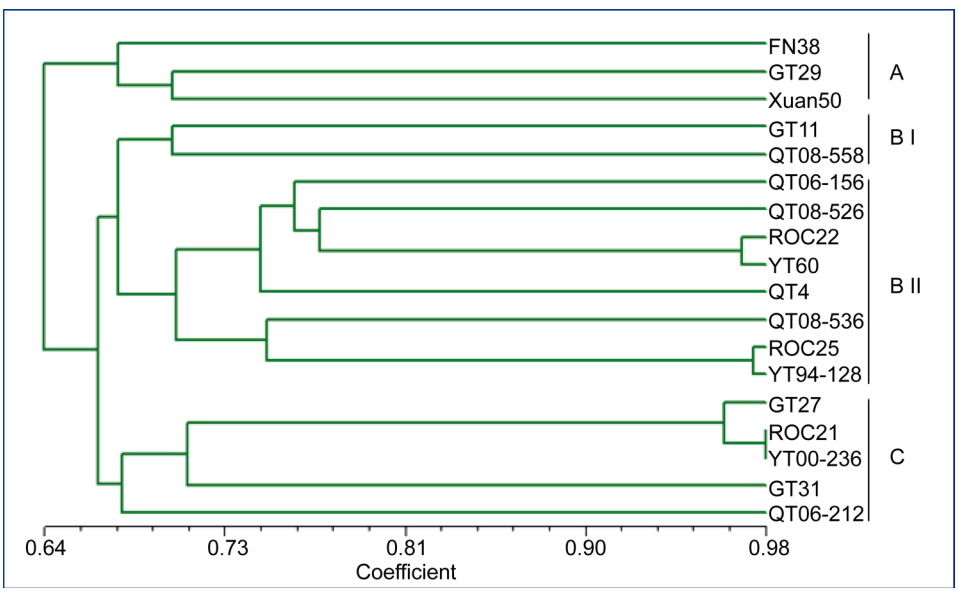

Figure 3. A UPGMA dendrogram showing genetic relationships among 18 sugarcane genotypes based on Jaccard similarity coefficients.

The 18 sugarcane genotypes did not group in accordance with their geographical origins. One reason might be that these sugarcane genotypes were descendants of the early sugarcane Noblization breeding program, thus, sharing a genetic base of a few common progenitor clones. Our result was in agreement with those from Nair's and Liang's studies [4] [42].

4. Conclusion

It is the first report on SSR-based molecular evaluation of genetic variability among sugarcane genotypes from the Karst region of China. All 21 SSR primer pairs were highly polymorphic. Eight SSR primer pairs produced 11 genotype-specific SSR alleles from nine sugarcane genotypes and are capable of distinguishing the nine sugarcane genotypes from one another. Five out of eight SSR primer pairs produced alleles that were unique to the QT-series clones. These eight SSR primer pairs can be very useful in sugarcane variety identity tests. Based on the genetic similarity values, the 18 sugarcane genotypes were clustered into three major groups, but the grouping occurred irrespective of geographical origins. The results from this study help understand the genetic base of sugarcanes germplasm adapted to the Karst region and future assignment of cross parents for the breeding program in Guizhou.

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