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SSR Marker-Assisted Management of Parental Germplasm in Sugarcane (*Saccharum* spp. hybrids) Breeding Programs

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Abstract: Sugarcane (*Saccharum* spp. hybrids) is an important sugar and bioenergy crop with a high aneuploidy, complex genomes and extreme heterozygosity. A good understanding of genetic diversity and population structure among sugarcane parental lines is a prerequisite for sugarcane improvement through breeding. In order to understand genetic characteristics of parental lines used in sugarcane breeding programs in China, 150 of the most popular accessions were analyzed with 21 fluorescence-labeled simple sequence repeats (SSR) markers and high-performance capillary electrophoresis (HPCE). A total of 226 SSR alleles of high-resolution capacity were identified. Among the series obtained from different origins, the YC-series, which contained eight unique alleles, had the highest genetic diversity. Based on the population structure analysis, the principal coordinate analysis (PCoA) and phylogenetic analysis, the 150 accessions were clustered into two distinct sub-populations (Pop1 and Pop2). Pop1 contained the majority of clones introduced to China (including 28/29 CP-series accessions) while accessions native to China clustered in Pop2. The analysis of molecular variance (AMOVA), fixation index (*Fst*) value and gene flow (*Nm*) value all indicated the very low genetic differentiation between the two groups. This study illustrated that fluorescence-labeled SSR markers combined with high-performance capillary electrophoresis (HPCE) could be a very useful tool for genotyping of the polyploidy sugarcane. The results provided valuable information for sugarcane breeders to better manage the parental germplasm, choose the best parents to cross, and produce the best progeny to evaluate and select for new cultivar(s).

Keywords: sugarcane; parental line; population structure; plant breeding; genetic diversity; simple sequence repeats (SSR)

1. Introduction

Sugarcane cultivars are allopolyploids with highly heterozygous and complex genomes, which render a slow progress in breeding. To date, most commercial sugarcane varieties can be traced back to a limited number of popular cultivars belonging to either the POJ- or Co-series, which represent a very narrow genetic base [1]. Therefore, it is important for sugarcane breeders to fully understand the genetic relationship among parental lines and to choose elite parents of different genetic background for crossing in order to broaden the genetic diversity of sugarcane population [2].

Hainan sugarcane breeding station (HSBS) is the primary sugarcane crossing facility in Mainland China. It produces nearly all the seeds for sugarcane breeders in China every year [3]. HSBS has

more than 2000 germplasm materials. Currently, thousands of new elite sugarcane genotypes are created by breeders each year. The utilization of these ever-increasing germplasm materials is a daunting challenge. Parental selection is a crucial step for good quality cross-breeding. Therefore, breeding materials should be adequately evaluated by different analytical methods to ensure their genetic suitability.

In the past, sugarcane breeders studied the genetic differences of parents mainly from the aspects of the genetic relationship, geographical origin and morphology. The genetic differences of sugarcane parents cannot really be reflected by pedigree because of mixed pollen, selfing and seed admixture [4]. Although morphological traits can be evaluated, these traits are easily influenced by the environment and may not reflect the real genetic diversity of sugarcane germplasm resources [5]. DNA molecular markers with high stability, multiple quantity and high polymorphism are more suitable for evaluating sugarcane germplasm collection [1]. With the rapid development of biotechnology, sugarcane researchers have utilized different types of DNA molecular markers, including amplified fragment length polymorphisms (AFLP) [1,5], restriction fragment length polymorphisms (RFLP) [6,7], random amplification of polymorphic DNAs (RAPD) [8,9], single nucleotide polymorphism (SNP) [10], simple sequence repeats (SSRs) [11], inter simple sequence repeat (ISSRs) [12,13], expressed sequence tag-simple sequence repeat (EST-SSRs) [14–16], 5S rRNA intergenic spacers [17], start codon targeted (SCoT) [18], target region amplification polymorphism (TRAP) [5,19,20], and cleaved amplified polymorphism sequences (CAPS) [21] for evaluating sugarcane germplasm.

Among PCR-based markers, SSR (microsatellite) markers are considered one of the most efficient markers for plant breeding due to large quantity, low dosage, co-dominant, reliability and multi-allelic detecting [22]. SSR markers have been used widely to study sugarcane genetic diversity and population structure [22–24], variety identity [25], genetic map [26,27], and genetic association [28–30]. Furthermore, fluorescence-labeled SSR markers combined with high-performance capillary electrophoresis (HPCE) have manifested better performance in genotyping of polyploid sugarcane, due to higher accuracy and better detection power [22–24,31–37].

Now, this paper reports a study that was designed to manage the parental germplasm of the sugarcane breeding programs in China through the microsatellite (SSR) DNA fingerprinting using fluorescence-labeled SSR primers and the high-performance capillary electrophoresis (HPCE) system. The results will help sugarcane breeders better manage the parental germplam, choose cross parents, design cross combinations, and produce high quality seedlings for the selection and development of elite varieties.

2. Materials and Methods

2.1. Plant Materials

One hundred and fifty parental clones were chosen for this study, based on the number of lines used most often in crossing from 2014 to 2018 in all Chinese sugarcane breeding programs (Table 1 and S1). These included 32 of clones from foreign origin, 109 clones from the China Mainland, and nine ROC-series clones from China Taiwan. Among the 32 foreign clones, one was from India (Co-series), 29 were from the U.S. (CP-series) and two were from Thailand (K-series). Among the 109 clones from China Mainland, four were from the Dehong Sugarcane Research Institute, Yunnan Province (DZ-series); 11 were from the Fujian Agriculture and Forestry University, Fujian Province (FN-series); two were from the Jiangxi Sugarcane Research Institute, Jiangxi Province (GN-series); 21 were from the Guangxi Academy of Agricultural Sciences, Guangxi Province (GT-series); six were from the Liucheng Academy of Agricultural Sciences, Guangxi Province (LC-series); six were from the Neijiang Academy of Agricultural Sciences, Sichuan Province (NJ-series); 18 were from the Hainan Sugarcane Breeding Station of Guangzhou Sugarcane Industry Research Institute, Hainan Province (YC-series); 29 were from the Guangzhou Sugarcane Industry Research Institute, Guangdong Province (YT-series); 10 were from the Yunnan Academy of Agricultural Sciences, Yunnan Province (YZ-series) and two were from

other breeding units in China Mainland (one from Sichuan Research Institute of Sugar Crops, Sichuan Province and one from the Guangdong Academy of Agricultural Sciences, Guangdong Province).

Table 1. The 150 sugarcane accessions used in the experiment.

No.	Accession	Series	No.	Accession	Series	No.	Accession	Series
1	Co1001	Co	51	GZ75-65	GN	101	YC06-92	YC
2	CP57-614	CP	52	HoCP00-1142	CP	102	YC07-65	YC
3	CP67-412	CP	53	HoCP00-2218	CP	103	YC07-71	YC
4	CP72-1210	CP	54	HoCP01-517	CP	104	YC09-13	YC
5	CP72-2086	CP	55	HoCP01-564	CP	105	YC71-374	YC
6	CP80-1827	CP	56	HoCP02-610	CP	106	YC94-46	YC
7	CP81-1254	CP	57	HoCP02-623	CP	107	YC97-24	YC
8	CP84-1198	CP	58	HoCP03-704	CP	108	YC97-40	YC
9	CP89-2143	CP	59	HoCP03-708	CP	109	YC98-2	YC
10	CP93-1382	CP	60	HoCP03-716	CP	110	YC98-27	YC
11	CP93-1634	CP	61	HoCP05-902	CP	111	YN73-204	YN
12	CP94-1100	CP	62	HoCP07-612	CP	112	YT00-236	YT
13	CT89-103	CT	63	HoCP07-613	CP	113	YT00-318	YT
14	DZ03-83	DZ	64	HoCP07-617	CP	114	YT00-319	YT
15	DZ05-61	DZ	65	HoCP91-555	CP	115	YT01-120	YT
16	DZ06-51	DZ	66	HoCP92-648	CP	116	YT01-125	YT
17	DZ93-88	DZ	67	HoCP93-746	CP	117	YT01-71	YT
18	FN02-6404	FN	68	HoCP95-988	CP	118	YT03-373	YT
19	FN02-6427	FN	69	K5	K	119	YT03-393	YT
20	FN05-2848	FN	70	K86-110	K	120	YT85-177	YT
21	FN0711	FN	71	LC03-1137	LC	121	YT86-368	YT
22	FN0712	FN	72	LC03-182	LC	122	YT89-240	YT
23	FN0713	FN	73	LC04-256	LC	123	YT91-976	YT
24	FN0717	FN	74	LC05-128	LC	124	YT92-1287	YT
25	FN91-23	FN	75	LC05-136	LC	125	YT93-124	YT
26	FN92-4621	FN	76	LC05-291	LC	126	YT93-159	YT
27	FN95-1702	FN	77	LCP85-384	CP	127	YT94-128	YT
28	FN99-20169	FN	78	NJ00-118	NJ	128	YT96-86	YT
29	GN95-108	GN	79	NJ00-15	NJ	129	YT97-20	YT
30	GT00-122	GT	80	NJ03-218	NJ	130	YT97-76	YT
31	GT02-1156	GT	81	NJ07-13	NJ	131	YT99-66	YT
32	GT02-208	GT	82	NJ86-117	NJ	132	YZ02-2540	YZ
33	GT02-281	GT	83	NJ92-244	NJ	133	YZ02-588	YZ
34	GT02-467	GT	84	ROC1	ROC	134	YZ03-194	YZ
35	GT02-761	GT	85	ROC10	ROC	135	YZ07-100	YZ
36	GT02-901	GT	86	ROC16	ROC	136	YZ07-49	YZ
37	GT03-11	GT	87	ROC20	ROC	137	YZ89-7	YZ
38	GT03-1403	GT	88	ROC22	ROC	138	YZ94-343	YZ
39	GT03-2112	GT	89	ROC23	ROC	139	YZ94-375	YZ
40	GT03-3005	GT	90	ROC25	ROC	140	YZ99-601	YZ
41	GT03-3089	GT	91	ROC26	ROC	141	YZ99-91	YZ
42	GT03-8	GT	92	ROC28	ROC	142	ZZ33	YT
43	GT03-91	GT	93	YC04-55	YC	143	ZZ41	YT
44	GT05-3084	GT	94	YC05-64	YC	144	ZZ43	YT
45	GT05-3595	GT	95	YC06-111	YC	145	ZZ45	YT
46	GT73-167	GT	96	YC06-140	YC	146	ZZ49	YT
47	GT89-5	GT	97	YC06-166	YC	147	ZZ50	YT
48	GT92-66	GT	98	YC06-61	YC	148	ZZ80-101	YT
49	GT94-119	GT	99	YC06-63	YC	149	ZZ90-76	YT
50	GT96-154	GT	100	YC06-91	YC	150	ZZ92-126	YT

2.2. SSR Genotyping

Young leaf tissues were collected from three individual clones, rinsed with 75% ethanol, and kept at −80 °C prior to DNA extraction. The genomic DNA was extracted from leaf tissues using the cetyl trimethyl ammonium bromide (CTAB) method [38] with minor modifications. The quality and concentration of DNA were measured using the UV-Vis Spectrophotometer Q5000 of Quawell (Quawell Technology, Inc. San Jose, CA, USA) and diluted to 20 ng/μL. A set of 21 SSR primer pairs (Table 1) with stable and clear amplification was selected from previous reports [3,11,33,39–42]. All forward primers were labeled with a fluorescence dye, 6-carboxy-fluorescein (FAM) or Hexachlorofluorescein (HEX). PCR reactions were performed with the following cycling condition: 95 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, then primer-specific annealing temperature (Tm) for 90 s, 65 °C for 30 s, followed by one cycle at 65 °C for 10 min. The annealing temperatures for the 21 primer pairs were optimized separately, ranging from 49 °C to 62 °C (Table 2). The amplified PCR products were checked by a 3% agarose gel electrophoresis. High-performance capillary electrophoreses (HPCE) was conducted on the ABI 3730XL DNA analyzer (Applied Biosystems, Inc. Foster City, CA, USA) to generate GeneScan files. The GeneScan files were analyzed using the GeneMarker V2.2 software (SoftGenetics, LLC. State College, PA, USA) to show SSR DNA fragments (alleles) and the sizes of these fragments were calibrated automatically against the GeneScan500 size standards. Due to the polyploidy nature of sugarcane, the SSR alleles had to be manually called first and the score sheet was manually rechecked according to Pan [43]. The presence of an allele was scored as "1" and its absence scored as "0". SSR alleles were named using a combination of primer name and allele size.

Table 2. The 21 simple sequence repeat (SSR) markers used in this study.

Primer Name	Type [a]	Repeat Motif	Primer Sequence (5′-3′)	Annealing Temperatures (°C)
mSSCIR36	G-SSR	$(GA)_{18}GT$ $(GA)_4$	CAACAATAACTTAACTGGTA CTGTCCTTTTTATTCTCTTT	52
mSSCIR46	G-SSR	$(GT)_{10}$	ATGCTCCGCTTCTCACTC AAGGGGAAAATGAAAACC	52
mSSCIR74	G-SSR	$(CGC)_9$	GCGCAAGCCACACTGAGA ACGCAACGCAAAACAACG	56
SCM4	E-SSR	$(CGGAT)_4$	CATTGTTCTGTGCCTGCT CCGTTTCCCTTCCTTCCC	52
SCM7	E-SSR	(GCAC)4	ACGGTGCTCTTCACTGCT GGGCATACTTCCTCCTCTAC	60
SCM18	E-SSR	$(ATAC)_3$	CATCAGTATCATTTCATCTTGG CAGTCACAGTCGGGTAGA	60
SMC1825LA	G-SSR	$(TG)_{11}$	CACGTCCTTCCGCCTTGA TCATCGTTCGTCGCACTG	56
SMC286CS	G-SSR	$(TG)_{43}$	TCAAATGGGACCTTATTGGAG TCCCTCGATCTCCGTTGTT	52
SMC477CG	G-SSR	$(CA)_{31}$	CCAACAACGAATTGTGCATGT CCTGGTTGGCTACCTGTCTTCA	60
SMC486CG	G-SSR	$(CA)_{14}$	GAAATTGCCTCCCAGGATTA CCAACTTGAGAATTGAGATTCG	60
SMC569CS	G-SSR	$(TG)_{37}$	GCGATGGTTCCTATGCAACTT TTCGTGGCTGAGATTCACACTA	60
SMC597CS	G-SSR	$(AG)_{31}$	GCACACCACTCGAATAACGGAT AGTATATCGTCCCTGGCATTCA	52
SMC334BS	G-SSR	$(TG)_{36}$	CAATTCTGACCGTGCAAAGAT CGATGAGCTTGATTGCGAATG	60
SMC36BUQ	G-SSR	$(TTG)_7$	GGGTTTCATCTCTAGCCTACC TCAGTAGCAGAGTCAGACGCTT	56

Table 2. *Cont.*

Primer Name	Type [a]	Repeat Motif	Primer Sequence (5′-3′)	Annealing Temperatures (°C)
SMC7CUQ	G-SSR	$(CA)_{10}(C)_4$	GCCAAAGCAAGGGTCACTAGA AGCTCTATCAGTTGAAACCGA	60
SEGM285	G-SSR	$(GCAC)_4$	AAGAAGAAGACTGAGAAGAACACT TAGCAACAACTTAATTTAGCAATC	56
UGSM345	E-SSR	$(TG)_6$	CTGTACTGGTATTACATGTGACCT TCTACTAATCACAAGAGAAGATGC	60
UGSM10	E-SSR	$(GGC)_{11}$	GCTACTATGGACAACAGGG ATGAAGAGACGAGACGAAGA	56
UGSuM50	E-SSR	$(TC)_{14}$	CTACTGCCGAGGAAAGATCG GGAAAAGTTTGTGGCAAGGA	56
MCSA068G08	E-SSR	$(CAG)_6$	CTAATGCCATGCCCCAGAGG GCTGGTGATGTCGCCCATCT	56
MCSA176C01	E-SSR	$(GGT)_5$	GAGTCAGTTGGTGCCGAGATTG GAACAGGTTAAAGCCCATGTC	56

[a] G-SSR: SSR primer pair designed from genomic sequence; E-SSR: SSR primer pair designed from UniGene or cDNA sequences.

2.3. Genetic Diversity Analysis

Qualitative allelic data matrix was constructed and formatted using the DataFormatter software [44]. The PowerMarker v3.25 software [45] was used to calculate allele frequency, number of alleles per locus, polymorphism information content (PIC), the gene diversity index (*h*), Shannon's information index (*I*), and percentage of polymorphic loci (*PPL*) of each marker. The resolving power of the primer (*Rp*) [46] was calculated using allele frequencies. The probability of identity (*PI*) [23] was computed using the CERVUS v3.0 software [47]. Unique (Series-specific) alleles were estimated using GeneALEx v6.502 [48,49].

2.4. Population Structure Analysis

The model-based program Structure v2.3.4 [50] was used to analyze the population structure involving the 226 alleles amplified by the 21 SSR primer pairs. The number of populations (*K*) was set from one to 10, and at each *K* value, ten runs were conducted separately with 50,000 iterations of burn-in length and 50,000 Markov Chain Monte Carlo (MCMC). Then, the best K value was estimated using Evanno's Δ*K* method [51] with an online tool, Structure Harvester [52]. An individual Q matrix was generated by CLUMPP v1.1.2 [53]. Parental clones with membership probabilities greater than 0.5 were identified as the same group [54]. A Principal Coordinate Analysis (PCoA) map was generated based on the genetic distances between pairs of clones by GeneALEx v6.502 [48,49]. An unrooted phylogenetic tree was constructed based on the neighbor-joining (NJ) method and the genetic distance matrix using PowerMarker v3.25 [45] and adjusted with MEGA v6.06 [55].

2.5. Differentiation Analysis and Genetic Diversity Indices

Analysis of Molecular Variance (AMOVA) was conducted to find the genetic differentiation within and among subpopulations using GeneALEx v6.502 [48,49]. From AMOVA, the fixation index (*Fst*) and gene flow (*Nm*) within the population was also acquired. In addition, genetic diversity indices, including number of different alleles (*Na*), number of effective alleles (*Ne*), Shannon's information index (*I*), observed heterozygosity (*Ho*), expected heterozygosity (*He*), unbiased expected heterozygosity (*uHe*), and percentage of polymorphic loci (*PPL*) of different sub-groups were also calculated using GeneALEx v6.502 [48,49].

3. Results

3.1. Polymorphism Revealed by SSR Genotyping

The 21 SSR primer pairs amplified a total of 226 alleles with an average of 10.8 alleles per primer pair (Table 2). Of the 226 alleles, 220 alleles were polymorphic and the other six alleles could be amplified in each clone. The number of alleles amplified by one primer pair ranged from five by MCSA176C01 to 25 by SCM4. The mean PIC value of each SSR primer pair ranged from 0.15 to 0.29 with an average of 0.23. The probability of identity (*PI*) of the 21 markers was all very low, which ranged from 0.000001 (mSSCIR36) to 0.071332 (SMC569CS) with an average of 0.015532. For the 21 primers pairs, the resolving power of the primer (*Rp*) was relatively high, ranging from 3.68 (SMC569CS) to 21.01 (mSSCIR36) with an average of 9.14. The mean number of alleles and the mean PIC value of genomic SSRs were 10.6 and 0.23, and were 9.8 and 0.23 for EST SSRs, respectively (Table 3).

Table 3. Genetic diversity parameters of 150 of the most popular parental clones from sugarcane hybrid breeding programs.

Primer Name	Allele (No.)	Product Size (bp)	Range of PIC [a] Values	Mean of PIC Values	*PI* [b]	*RP* [c]
mSSCIR36	21	127–168	0.01–0.38	0.15	0.000001	7.09
mSSCIR46	12	146–177	0.01–0.37	0.15	0.002858	13.04
mSSCIR74	6	215–228	0.00–0.37	0.17	0.042135	4.69
SCM4	25	92–209	0.01–0.37	0.17	0.000087	4.16
SCM7	7	155–188	0.03–0.37	0.18	0.048672	3.68
SCM18	9	226–251	0.00–0.38	0.19	0.010157	8.67
SMC1825LA	10	91–119	0.01–0.37	0.20	0.001240	6.53
SMC286CS	13	128–152	0.01–0.37	0.21	0.000411	7.31
SMC477CG	15	115–134	0.00–0.36	0.21	0.000125	4.11
SMC486CG	7	222–243	0.06–0.36	0.22	0.051066	4.88
SMC569CS	6	166–220	0.04–0.38	0.24	0.071332	14.05
SMC597CS	14	143–166	0.03–0.37	0.24	0.000034	10.99
SMC334BS	12	145–163	0.01–0.38	0.24	0.000140	6.27
SMC36BUQ	12	103–251	0.00–0.37	0.25	0.010448	7.49
SMC7CUQ	7	156–170	0.00–0.37	0.26	0.024118	9.76
SEGM285	13	306–389	0.03–0.38	0.26	0.000143	21.01
UGSM345	8	320–334	0.01–0.38	0.27	0.005772	13.68
UGSM10	10	97–125	0.00–0.38	0.28	0.005289	9.31
UGSuM50	6	123–139	0.05–0.38	0.28	0.023095	6.24
MCSA068G08	8	179–202	0.06–0.38	0.29	0.003035	15.57
MCSA176C01	5	427–440	0.11–0.38	0.29	0.026013	13.31

[a] PIC: Polymorphism information content; [b] *PI*: Probability of identity; [c] *RP*: Resolving power.

3.2. Genetic Diversity

The gene diversity (*h*) of the polymorphic allele ranged from 0.013 to 0.500 with an average of 0.282. The Shannon's information index (*I*) of the polymorphic allele ranged from 0.010 to 0.534 with an average of 0.261. Among the different series of sugarcane parental lines, the highest values of both gene diversity (*h*) and Shannon's information index (*I*) were found in the YC-series (0.261, 0.397), followed by the YT-series (0.254, 0.386,) and the GT-series (0.251, 0.376) (Table 3), indicating that the YC-series is genetically more diverse than the other series. The average percentages of polymorphic allele for the YT-, YC-, and CP-series were 0.814, 0.805 and 0.743, respectively. Alleles were identified that were unique to the 12 distinct germplasm groups (Table 4).

Table 4. Gene diversity, Shannon's information index, percentage of polymorphic loci and series-specific alleles of different series.

Series	Sample Size	*h* [a]	*I* [b]	*PPL* [c]	Series-Specific Alleles
CP	29	0.239	0.361	0.743	SCM7-188, SCM18-238, SMC486CG-225, SMC486CG-233
DZ	4	0.235	0.341	0.562	
FN	11	0.245	0.365	0.677	mSSCIR46-153
GN	2	0.148	0.205	0.296	
GT	21	0.251	0.376	0.721	
LC	6	0.197	0.293	0.522	
NJ	6	0.205	0.302	0.527	SMC36BUQ-125
ROC	9	0.201	0.301	0.558	SMC36BUQ-184, SEGM285-359
K	2	0.164	0.227	0.327	
YC	18	0.261	0.397	0.805	mSSCIR46-146, mSSCIR46-149, SCM7-175, SMC569CS-174, SMC569CS-202, SMC36BUQ-106, SMC36BUQ-132, UGSM10-113
YT	29	0.254	0.386	0.814	SMC36BUQ-105, SMC36BUQ-139
YZ	10	0.241	0.358	0.650	
Mean		0.176	0.261	0.480	

[a] *h*, Gene diversity; [b] *I*, Shannon's information index; [c] *PPL*, percentage of polymorphic loci.

3.3. Population Structure and Phylogeny

The *K*-value was used to estimate the number of clusters of the clones based on the genotypic data. A continuous gradual increase was observed in the log-likelihood of *K*-value (LnP(*K*)) with the increase of *K*-value (Figure 1B and Table S2). The number of clusters (*K*) was plotted against Delta *K* (Δ*K*), which revealed a sharp peak at *K* = 2 (Figure 1A and Table S2). The optimal K-value was *K* = 2, which revealed that the highest probability for the presence of two sub-populations (Pop1 and Pop2) among the 150 sugarcane clones (Figure 1C); Pop1 consisted of 50 clones and Pop2 contained 100 clones (Table S3). Pop1 clones were mainly introduction accessions and most of the Pop2 clones were from Mainland China.

In accordance with the population structure results, PCoA also showed two clusters with the first three axes together explained 20.04% of cumulative variation. In the PCoA plot, the first and second principal coordinates accounted for 8.41% and 6.71% of the total variations, respectively (Figure 2). Furthermore, the unrooted neighbor-joining phylogenetic tree (Figure 3) also showed two clusters. One cluster contained most of the clones of Pop1; the other cluster contained most of the clones of Pop2. However, the admixture of clones between the two sub-populations does exist. Few accessions (YC98-27, GT03-2112 and FN0717) native to China were clustered into Pop1 while several others (HoCP01-517, ROC10, ROC16, K5, ROC25, ROC22, ROC1) introduced to China Mainland were grouped into Pop2.

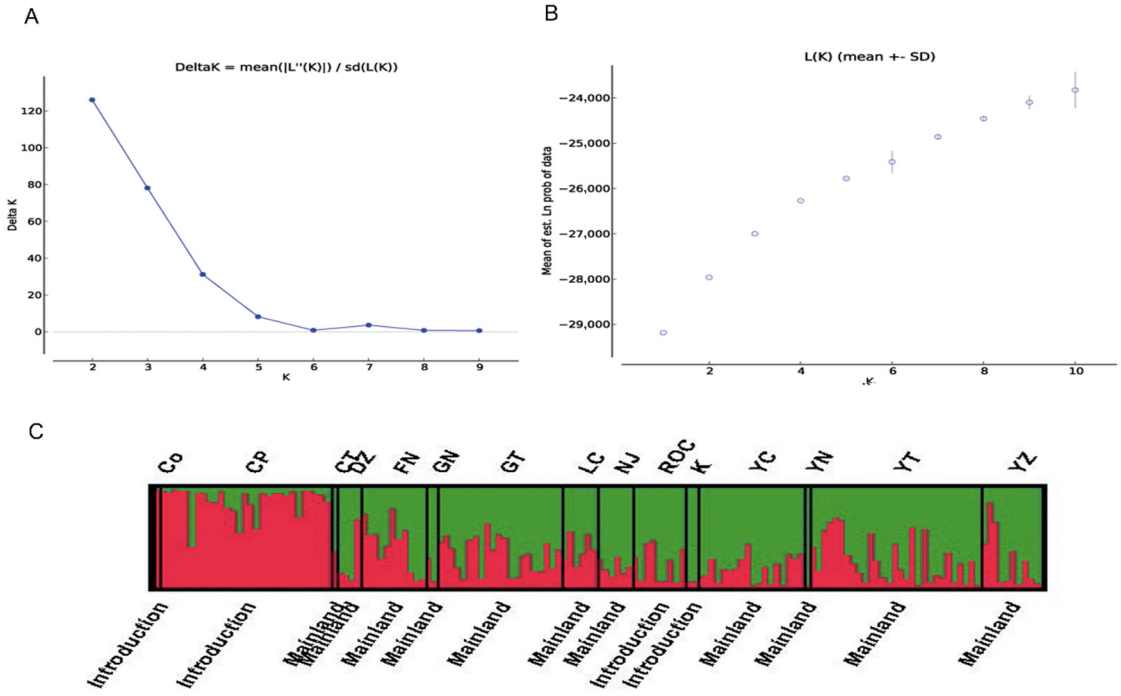

Figure 1. (**A**) Delta *K* (Δ*K*) for different numbers of subpopulations (*K*); (**B**) average log-likelihood *K*-value (LnP(*K*)) against the number of *K*; (**C**) the population structure of 150 most popular parental clones in the hybrid breeding programs in China based on the distribution of 226 SSR alleles among these clones. Pop1 clones are coded in red and Pop2 clones in green.

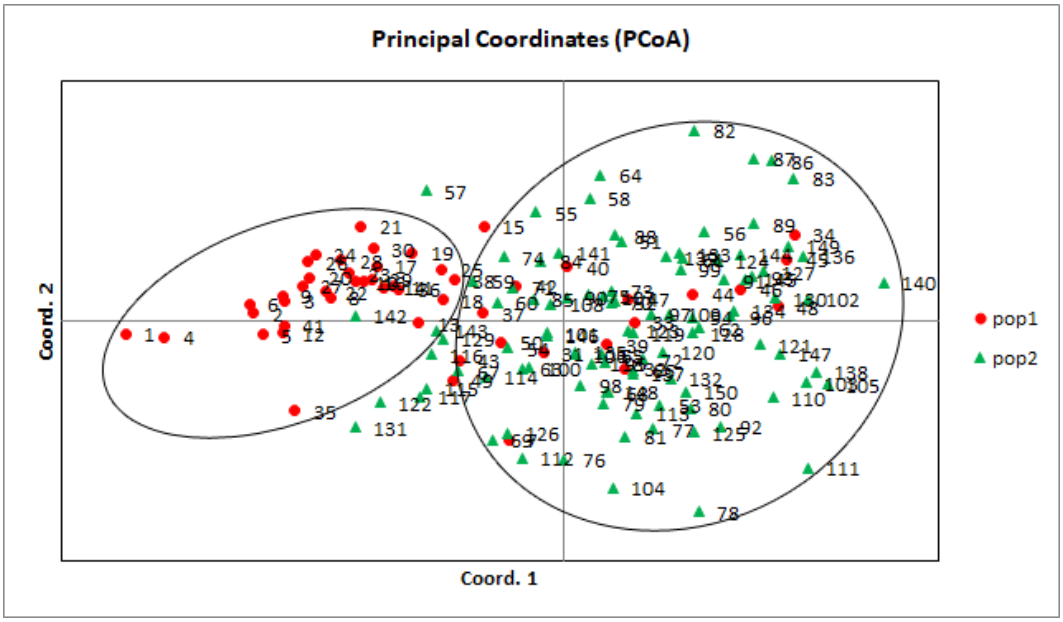

Figure 2. Principal coordinates analysis (PCoA) scatter plots. Red circles represent the Pop1 clones and green triangles the Pop2 clones.

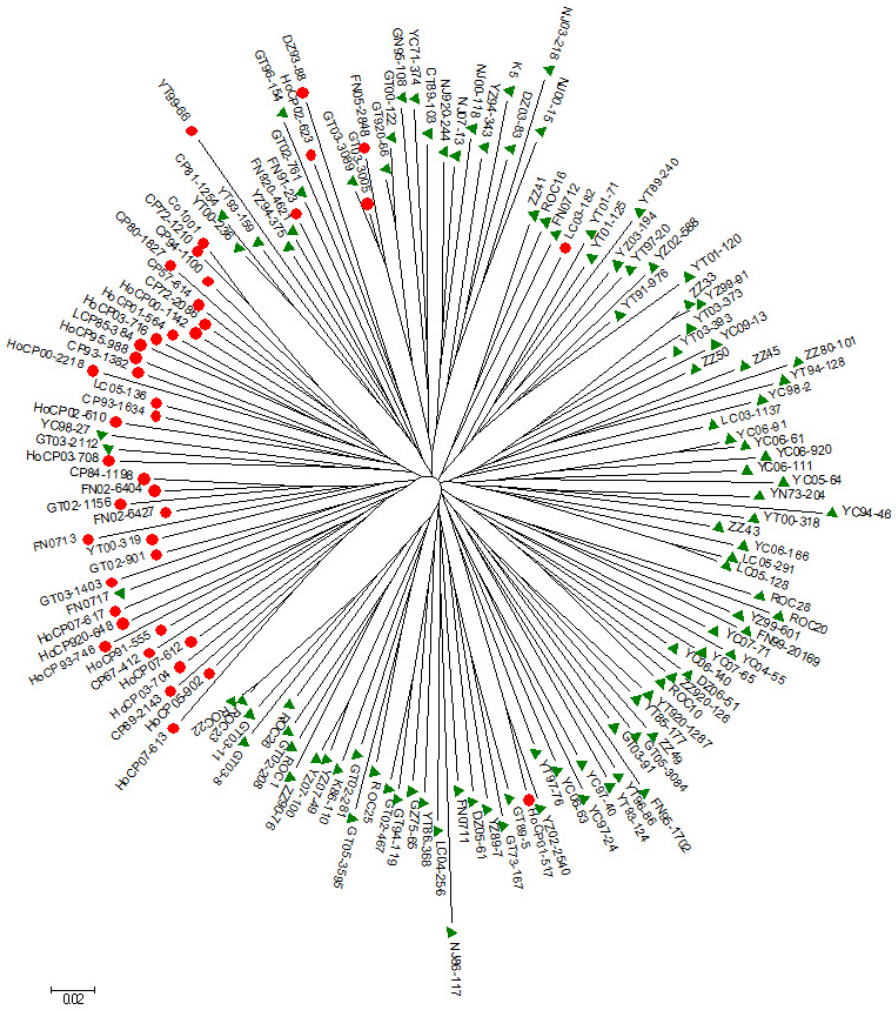

Figure 3. A neighbor-joining phylogenetic tree based on the pair-wise genetic distance between 150 most popular parental clones from hybrid breeding programs in China. Red circles represent the Pop1 clones and green triangles the Pop2 clones.

3.4. Genetic Differentiation and Allelic Pattern Across Populations

The two sub-populations Pop1 and Pop2 identified by the Structure analysis were subjected to the GeneALEx analysis to calculate the values of Analysis of Molecular Variance (AMOVA), *Nei*'s genetic distance and genetic diversity indices (Table 5). The variation value within the sub-populations (95% of total variation) was significantly higher than that between the sub-populations (5% of total variation). In addition, a high gene flow (*Nm* = 4.981) and a low fixation index value (*Fst* = 0.048) were obtained on the basis of *Nei*'s genetic distance analysis.

Table 5. Analysis of molecular variance (AMOVA) of SSR-based genetic variation between and within two sub-populations of Pop1 and Pop2.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares	Estimated Variance	Percentage of Variation
Between sub-Pops	1	546.240	546.240	6.308	5%
Within sub-Pop	148	18,601.600	125.686	125.686	95%
Total	149	19,147.840		131.995	100%
Fixation Index	*Fst* = 0.048				
Gene Flow	*Nm* = 4.981				

The mean value of the number of different alleles (*Na*) and effective alleles (*Ne*) of the two sub-populations were 1.885 ± 0.015 and 1.462 ± 0.017, respectively. The mean values for *I*, *He* and *uHe* among the 150 parental clones were 0.413 ± 0.011, 0.272 ± 0.008 and 0.274 ± 0.009, respectively. Pop2 (*I* = 0.423 ± 0.016, *He* = 0.278 ± 0.012, and *uHe* = 0.278 ± 0.012) showed higher levels of genetic diversity than Pop1 (*I* = 0.403 ± 0.017, *He* = 0.267 ± 0.012, and *uHe* = 0.269 ± 0.012). The percentage of polymorphic loci per population (*PPL*) ranged from 83.63% (Pop1) to 93.36% (Pop2) with an average of 88.50% (Figure 4).

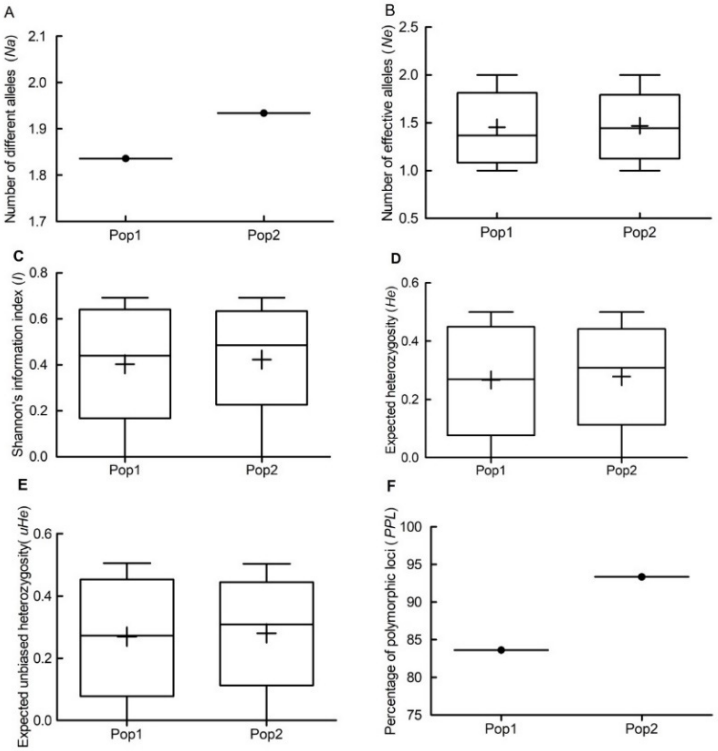

Figure 4. Allelic pattern of SSR across the two sub-populations Pop1 and Pop2. (**A**) Number of SSR alleles (*Na*); (**B**) number of effective SSR alleles (*Ne*); (**C**) Shannon's information index (*I*); (**D**) expected heterozygosity (*He*); (**E**) expected unbiased heterozygosity (*uHe*); and (**F**) percentage of polymorphic loci (*PPL*).

4. Discussion

Cross hybridization has become the main breeding method for the sugarcane variety improvement. In the traditional sugarcane cross-breeding process, selecting parental clones for crossing is the most important step. Only parental clones sharing a highly level of genetic diversity and complementarity can generate high quality seedling populations [56,57]. Since the 1950s, some sugarcane cultivars from America and China Taiwan have played a very important role in China's sugarcane cross-breeding programs [3]. Meanwhile, some new elite sugarcane parents are being created and utilized by the breeders every year. To make informed crossing choices, the genetic relationship among the parental clones involved in the latest sugarcane cross-breeding programs should be clarified.

In this study, we used 21 pairs of SSR primers to investigate the genetic diversity and population structure of 150 of the most commonly used parental clones. These primer pairs amplified 226 alleles, of which 97.3% were polymorphic. The mean PIC and the gene diversity (*h*) of the polymorphic alleles were 0.23 and 0.28, respectively, which were lower than the values reported on the "World Collections of Sugarcane and Related Grasses" (WGSRG) (PIC = 0.2568, *h* = 0.310) [23]. This may be largely due to the number of accessions involved in the world collection study. The WCSRG study involved 1002 highly diverse accessions, belonging to nine species, whereas only 150 clones were used in this study.

Since 2000, a large number of genomic SSR and EST-SSR markers has been developed and applied effectively in estimating genetic diversity in the sugarcane [16,35,39,41,58]. After a lot of screening and identification (unpublished), we selected the best 21 primer pairs from these reports, including eight EST-SSR and 13 genomic SSR. We found that the number and mean PIC value of the EST-SSR alleles were lower than those of the genomic SSR alleles (Table 2). This can be due to the fact that the EST-SSR alleles are located in more conserved regions of the genome [16].

The probability of identity (*PI*) is an individual identification estimator that shows the probability of two different accessions sharing the same genotypes at one specific locus in a population [23]. In this study, the *PI* values of all SSR primer pairs were very low, ranging from 0.000001 (mSSCIR36) to 0.071332 (SMC569CS) (Table 2). The combined *PI* value for all markers was 9.04×10^{-57}, indicating that these 21 SSR primer pairs are able to distinguish the 150 parental clones. The resolving power of the primer pair (*Rp*) is an index, which explains the primer pair's ability to identify different genotypes. *Rp* is related to the distribution of alleles within the sampled genotypes [46] and has been found to correlate strongly with the genotype in evaluating 34 potato cultivars using four primers [46]. The mean *Rp* value (9.135) of the 21 SSR primer pairs is much higher than other studies, such as 2.37 by [59] and 2.2 by [12], indicating these primer pairs are more informative and could identify more cultivars.

Based on geographic origin, the 150 clones were sorted into 15 series. Among these series, the genetic diversity (*h*) indices ranged from 0 to 0.261 and the Shannon's information index (*I*) ranged from 0 to 0.397. At the series level, the YC-series had the highest genetic diversity ($h = 0.261$, $I = 0.397$), which was similar to the previous results reported by You et al. [35,60]. The YC-series clones are from the Hainan Sugarcane Breeding Station of Guangzhou Sugarcane Industry Research Institute in Sanya city, Hainan province, where the primary sugarcane crossing facility of China is located. The YC-series clones were selected from crosses involving indigenous clones, foreign clones, and clones of closely related *Saccharum* species and genera [35]. Furthermore, the YC-series also had the greatest number of eight series-specific alleles. Only four, two, one, and one unique alleles were found in the CP-series, YT-series, ROC-series, FN-series and NJ-series clones, respectively. Series-specific alleles are the alleles found only in a single population among a broader collection of populations [61,62]. These alleles have been proven to be informative for population genetic studies [63,64] and we may use these alleles for variety identification and marker assisted selection.

The 150 parental clones were classified into two groups (Pop1 and Pop2) based on the PCoA, phylogenetic analysis and population structure analysis. Pop1 contained the majority of foreign accessions with the membership probabilities of >0.5, while most accessions from Mainland China were assigned to Pop2. Certain specific target traits intentionally selected by different germplasm collectors or breeders might also contribute to the population structure [54]. However, admixture of clones between the two sub-populations do exist (Figures 1–3). For example, one out of the 29 CP-series clones, nine ROC-series clones and two K-series clones clustered into Pop2, but the majority of introduction clones clustered into Pop1. Likewise, one out of four DZ-series, five out of 11 FN-series, four out of 21 GT-series, two out of six LC-series, seven out of 29 YT-series, and two out 10 YZ-series clones clustered into Pop1, while the majority of the clones from Mainland China clustered into Pop2. This might be due to genetic exchange among different series, or the similar threshold (Pop1: 0.5098, Pop2: 0.4902) (Table S3) resulting in several clones to be clustered completely into a certain group (Pop1 or Pop1), while others being clustered into both groups.

The utilization data was based the most widely used 150 parental clones of sugarcane breeding programs in China during the recent five years. These included 32 of clones from foreign origin, 109 clones from the China Mainland, and nine ROC-series clones from China Taiwan. Among the 32 foreign clones, only one was from India (Co1001), two were from Thailand (K5 and K86-110) while the majority of them (29/32) were from the US (CP-series). Co1001 has been used as parental line extensively in the sugarcane breeding programs in the world. Some sugarcane cultivars, including the CP-series and China Mainland clones, were the progenies of Co-series varieties. Compared to clones from China Mainland, the CP-series clones may have closer genetic distance with the Co-series. So CP-series clones

and Co-series clone can be clustered into Pop1. K5 and K86-110, which were from Thailand, were two of the most widely used parental clones in China. Some clones from China Mainland were the progenies of K5 and K86-110. Clones from China mainland may have the closer genetic distance with the two clones to be clustered into Pop2. The ROC-series varieties have been used as major cultivars in China Mainland accounting for greater than 80% of sugarcane planting areas [24]. In addition, the ROC-series accessions were also the most widely used parents in China Mainland during the recent five years (Table S1). In our study, the ROC-series accessions were clustered into Pop2 because of their closer genetic distance with China Mainland's clones. It is suggested that less attention be continually paid on the utilization of ROC-series accessions in China Mainland's sugarcane breeding programs.

Fixation index (*Fst*) measures the genetic distance between populations. An *Fst* value of zero indicates no differentiation between the sub-populations, while one indicates complete differentiation [65]. An *Fst* value less than 0.05 is considered no differentiation, while an *Fst* value greater than 0.15 is considered significant in differentiating populations [66]. In this study, the *Fst* value between the two sub-populations was 0.048 (Table 5), which was low and would indicate a very low genetic differentiation. This is consistent with the results obtained from the AMOVA, where the genetic variation within sub-populations (95%) was significantly higher than between sub-populations (5%). Gene flow (*Nm*) is the transfer of genetic variation from one population to another. If the value is less than one, then the gene exchange would be limited between sub-populations [67].In this study, the *Nm* value was high, 4.981 suggesting that a high level of genetic exchange may have occurred and this can result in a low genetic differentiation between the two sub-populations. Since the genetic diversity indices of Pop2, such as the number of different alleles (*Na*), effective alleles (*Ne*), *I*, *He* and *uHe*, were all higher than those of Pop1, Pop2 is more diverse than Pop1.

Selecting genetically distant accessions from Pop1 and Pop2 for crossing parents in sugarcane breeding programs will potentially lead to elite varieties with broadened genetic bases. Almost all the CP-series clones from the US were clustered into Pop1. These clones have been used extensively as parental lines in the sugarcane breeding programs in China; some have become or are elite progenitors of Chinese cultivars [67]. In addition, this study shows that several YC-series clones are also good crossing parents with a high level of genetic diversity.

5. Conclusions

Using a high-performance capillary electrophoresis (HPCE) detection system, the most widely used 150 sugarcane parental clones from 15 different series were fingerprinted with 21 SSR primer pairs. A total of 226 SSR alleles were identified and the distribution of these SSR alleles were subjected to genetic variation, phylogeny, population structure, and principal coordinate analyses. The results showed that the parental lines were clustered into two distinct groups, Pop1 and Pop2. Pop1 contained the majority of foreign clones, while Pop2 consisted of the majority of accessions from Mainland China. Genetic differentiation between the two groups was low. The YC-series clones of Pop2 displayed a high level of genetic diversity and the CP-series clones were elite parents of several Chinese cultivars. The introduction and utilization of more clones of the YC- and CP-series into China's sugarcane breeding programs will broaden the genetic base of breeding germplasm and produce high quality seedlings for selection and development of elite varieties.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/9/8/449/s1, Table S1: Utilization data of the most widely used 150 parental clones from sugarcane hybrid breeding programs in China during the recent five years. Table S2: Tabulated *K* values of 150 most popular parental clones from sugarcane hybrid breeding programs in China at $K = 1$ to 10. Table S3: Sub-population assignment of the 150 most popular parental clones from the sugarcane breeding programs in China based on the *Q* values.

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