SRAP analysis of genetic diversity of nine native populations of wild sugarcane, *Saccharum spontaneum*, from Sichuan, China

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ABSTRACT. *Saccharum spontaneum* is a wild sugarcane species that is native to and widely distributed in China. It has been extensively used in sugarcane breeding programs, and is being tested for the development of bioenergy cultivars. In order to provide basic information for the exploitation of this species, we analyzed genetic variation among and within native *S. spontaneum* populations collected from Sichuan, China. Eighty plants from nine native populations were sampled. Twenty-one sequence-related amplified polymorphism primer pairs generated 235 clearly scorable bands, of which 185 were polymorphic (78.7%). Nei's genetic diversity was 0.2801 and Shannon's information index was 0.4155 across the populations. Genetic diversity parameters, G_{ST} value (0.2088) and N_m value (1.8944), showed that the genetic variation within populations was greater than that among populations. In the

cluster analysis, one major grouping was formed by populations from Ya'an and another one by populations from Sichuan basin; a population from Baoxing formed a single cluster. In order to fully comprehend the genetic diversity of cold-tolerant local germplasm in this species, germplasm should be collected from the heterogeneous environments along the northern regions of this species' distribution. The germplasm that we collected should be a valuable resource for *Saccharum* breeding.

Key words: *Saccharum spontaneum*; SRAP; Genetic diversity; Diversity index; Genetic structure

INTRODUCTION

Saccharum spontaneum is the major wild species extensively used in sugarcane genetic improvement and cultivar development worldwide for its resistance to abiotic and biotic stresses (Ming et al., 2006). More recently, *S. spontaneum* crossed with selected sugarcane clones (*S. officinarum* or *Saccharum* hybrids) has been deployed to produce early generation hybrids selected for sugar-fiber energy cane or fiber-only energy cane as a bioenergy crop (Tew and Cobill, 2010). Hybrids are selected for adaptation to more temperate environments and production of higher biomass yields compared to traditional sugarcane cultivars. New germplasm in *S. spontaneum* will provide genetic materials with considerable potential, needed for new bioenergy cultivar development. More specifically those collected from more temperate and humid regions can contribute to desirable genes for cold tolerance and disease resistance.

S. spontaneum is widespread in China within a vast geographic range of northern latitudes 18°~33° and east longitudes 97°~122°, covering the provinces of Anhui, Fujian, Guangdong, Guangxi, Guizhou, Hainan, Henan, Hubei, Hunan, Jiangsu, Jiangxi, Shaanxi, Sichuan, Taiwan, Xinjiang, Xizang, Yunnan, and Zhejiang (Chen and Phillips, 2006). An early report indicated that most clones of Chinese *S. spontaneum* from southwest provinces were 2n = 80, and some 2n = 64, in addition to rare ones being 2n = 68 or 116 (Chen et al., 1981). Chinese native *S. spontaneum* possesses a large variation in tillering ability, photosynthetic efficiency and dry matter accumulative ability (Xie et al., 2007). Using random amplified polymorphic DNA (RAPD) markers, Zhang et al. (2010) studied the genetic diversity of local *S. spontaneum* accessions collected from the Guangxi Zhuangzu Autonomous Region, a southern province in China. Zhang et al. (2006) also reported large phenotypic variation for seven selected quantitative traits of the same germplasm collections from Guangxi. We collected native *S. spontaneum* germplasm in 2008 from the Sichuan Province representing more northern regions of the species' adaptation. As the regions are humid and more temperate than Guangxi, our collections may contain desirable traits encompassing cold tolerance and disease resistance. However, the genetic diversity of our germplasm collections was unknown.

Sequence-related amplified polymorphism (SRAP) was developed as a reliable molecular marker system based on simple PCRs of genomic DNA (Li and Quiros, 2001). SRAP analyzes DNA polymorphisms with amplifying open reading frames using specifically designed primers. The objective of the current investigation was to analyze genetic variation among natural *S. spontaneum* populations collected from Sichuan based on SRAP.

MATERIAL AND METHODS

Plant materials

Experimental plant materials of native *S. spontaneum* were collected from the main valleys of the Min River, Qingyi River and Tuo River in Sichuan Province, China, in September 2008 (Figure 1). According to the sampling method of collecting different populations by Jing and Lu (2003), each local population was sampled by collecting leaf tissues from 5 to 15 randomly selected individual plants, *in situ*, on the basis of the population size at a selected location. Ramets of each sampled plant were dug up and transplanted to an observation nursery for morphological characterization experiments (data not shown). Totally, we collected samples and germplasm of nine populations consisting of 80 individuals. Location and habitat conditions of the nine populations are given in Table 1.
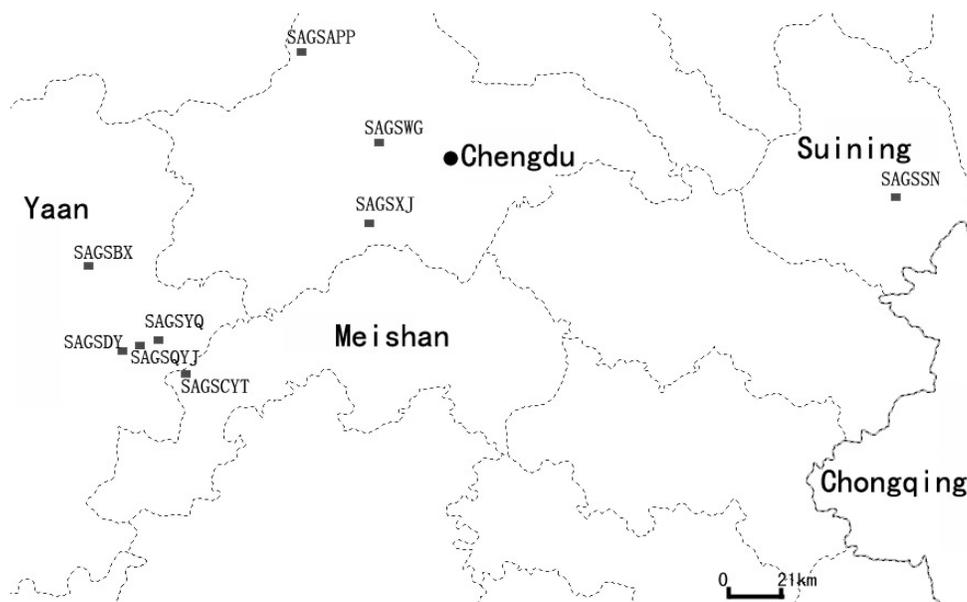
Figure 1. Local map of Sichuan, China, showing sampling locations.

Table 1. Locations and habitat characters of 9 *Saccharum spontaneum* populations collected from Sichuan Province in China.

Identity	Location	Altitude (m)	Latitude	Longitude	Habitat	Individuals per population
SAGSQYJ	Qingyi River, Yaan	562	29°59′	103°00′	Riverside	15
SAGSDY	Duoyun, Yaan	593	29°59′	102°57′	Roadside	7
SAGSBX	Baoxing, Yaan	795	30°15′	102°49′	Riverside	9
SAGSYQ	Yaoqiao, Yaan	543	30°01′	103°35′	Riverside	5
SAGSCYT	Caoyutan, Yaan	486	29°51′	103°14′	Riverside	10
SAGSZPP	Zipingpu, Dujiangyan	737	31°01′	103°35′	Riverside	8
SAGSXJ	Xinjin, Chengdu	532	30°23′	103°48′	Slope shrub	6
SAGSWJ	Wenjiang, Chengdu	525	30°40′	103°46′	Riverside	10
SAGSSN	Dongchan, Suining	310	30°32′	105°32′	Slope shrub	10

DNA isolation

Fresh and young leaves of *S. spontaneum* were hand collected from individual selected plants as individual samples, and each sample was dried separately using self-indicating silica gel. These leaf tissues were taken back to the laboratory and stored in a freezer at -80°C for use. Genomic DNA was isolated from the collected leaf tissues using the modified CTAB method of Doyle (1991). Integrity and quality of DNA samples were evaluated by electrophoresis on 0.8% agarose gels containing 0.5 μg/mL ethidium bromide and quantified in a UV spectrophotometer. Qualified DNA samples were stored in a refrigerator at 4°C for PCR.

SRAP-PCR procedure

The volume per PCR was 20 μL. The reaction chemical components included 4 μL 10 ng/μL DNA, 2 μL 10X PCR buffer, 2 μL 25 mM Mg^{2+}, 2.4 μL 2.5 mM dNTPs, 0.2 μL 5 U/μL Taq DNA polymerase, 1 μL 10 mM upstream primer, 1 μL 10 mM downstream primer, and 7.6 μL of sterile water. One hundred and twenty primer pairs of 10 upstream primers and 12 downstream primers published by Li and Quiros (2001) were screened for effective amplification, clear bands, high polymorphisms and reliability. The primers were synthesized by Shanghai Biochemical Engineering Technology (Shanghai, China). The SRAP markers were amplified using the following thermal cycle parameters: 5 min at 94°C; 5 cycles of 94°C for 1 min, 35°C for 1 min, and 72°C for 2 min; 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min; extension of 5 min at 72°C; and a final storage at 4°C. The PCR products were separated and visualized using 2% agarose gel electrophoresis.

Data analysis

Clearly amplified PCR bands were visually scored as "1" for presence of one band or "0" for absence of any band in a specific sample. The percentage of polymorphic bands (PPB), Shannon's information index (I), Nei's gene diversity (h), genetic diversity within populations (H_S), total genetic diversity (H_T), genetic differentiation coefficient (G_{ST}), gene flow estimates (N_m), and the Nei's genetic distance were calculated using POPGENE (Yeh and Boyle, 1999). Cluster analysis (UPGMA dendrogram) of the different populations was performed with the SHAN module in the NTSYS software using Nei's genetic distances (Rohlf, 2000).

RESULTS

SRAP primer selection and analysis

Twenty-one SRAP primer combinations (PCs) were selected from 120 PCs in a screening test (data not shown). The selected SRAP PCs were used in the PCR amplifications of 80 *S. spontaneum* DNA samples of 9 populations. PCR bands were scored if they were clearly readable. The PCs yielded a total of 235 scored bands, of which 185 were polymorphic. The number of amplified and scored bands for each primer pair varied from 4 to 13, with an average of 8.81 (Table 2).

Table 2. Primer sequences used in SRAP analyses of *Saccharum spontaneum* and their amplification parameters.

Primer combinations*	Total bands	Polymorphic bands	Percent polymorphic bands
em1+me8	10	8	80.00
em1+me9	9	8	88.89
em2+me9	8	4	50.00
em3+me2	11	9	81.82
em3+me3	11	8	72.73
em3+me4	12	12	100.00
em5+me3	9	7	77.78
em3+me6	11	10	90.91
em3+me7	12	11	91.67
em5+me4	13	12	92.31
em5+me9	15	13	86.67
em7+me5	11	9	81.82
em8+me5	11	6	54.55
em8+me6	9	6	66.67
em8+me8	9	4	44.44
em9+me2	11	7	63.64
em9+me3	11	9	81.82
em9+me5	12	10	83.33
em9+me7	14	11	78.57
em9+me9	13	11	84.62
em10+me3	13	10	76.92
Mean	11.19	8.81	77.45
Total	235	185	78.72

*Primer sequence information published by Li and Quiros (2001).

Genetic diversity and population relationship

The PPB among individuals across all populations was 78.72%. PPB within populations showed a large range. For example, population SAGSQYJ had the highest value of PPB at 70.21%, followed by 62.13% for SAGSBX and SAGSCYT. The lowest PPB value was 52.77% for population SAGSYQ. The resultant mean PPB within populations was 59.43% (Table 3).

Table 3. Genetic diversity of 9 populations of *Saccharum spontaneum*.

Population	No. of polymorphic bands	Percent polymorphic bands	Observed number of alleles	Effective number of alleles	Nei's gene diversity (h)	Shannon's information index (I)
SAGSQYJ	165	70.21	1.6936	1.3980	0.2347	0.3539
SAGSDY	134	57.02	1.5660	1.3827	0.2206	0.3249
SAGSBX	146	62.13	1.6170	1.4102	0.2347	0.3466
SAGSYQ	124	52.77	1.5277	1.3812	0.2158	0.3147
SAGSCYT	146	62.13	1.6170	1.3631	0.2150	0.3236
SAGSZPP	130	55.32	1.5532	1.3532	0.2056	0.3056
SAGSXJ	126	53.62	1.5277	1.3523	0.2035	0.3007
SAGSWJ	145	61.70	1.6170	1.3977	0.2309	0.3425
SAGSSN	141	60.00	1.6000	1.4102	0.2334	0.3430
Mean	139	59.43	1.5910	1.3832	0.2216	0.3284
Species	235	78.72	1.7872	1.4854	0.2801	0.4155
SD	13	5.44	0.0533	0.0228	0.0124	0.0191

The H_T of the *S. spontaneum* populations was 0.2801 by the Nei's gene diversity (h). The Nei's gene diversity values of the 9 populations ranged from 0.2035 to 0.2347, with an average of 0.2216. The H_T by Shannon's index (I) was estimated to be 0.4155 higher than Nei's

index, and Shannon's index of the 9 populations varied between 0.3007 and 0.3539, with an average of 0.3284. However, Shannon's index and Nei's index were parallel if compared. Of the nine populations, SAGSQYJ had the highest gene diversity ($h = 0.2347$, $I = 0.3539$) and SAGSXJ had the lowest ($h = 0.2035$, $I = 0.3007$) (Table 3).

Given the assumption of genetic equilibrium, H_S within the nine *S. spontaneum* populations was 0.2216, while the H_T among the populations was 0.2801. The G_{ST} and N_m were 0.2088 and 1.8944, respectively. Genetic similarity coefficients among the 9 *S. spontaneum* populations are presented in Table 4. Pairwise genetic similarity coefficients between populations varied from 0.91 (SAGSZPP and SAGSBX, SAGSCYT and SAGSBX) to 0.96 (SAGSWJ and SAGSSN, SAGSQYJ and SAGSYQ, SAGSXJ and SAGSWJ).

Table 4. Nei's genetic similarity coefficients between 9 populations of *Saccharum spontaneum* collected in Sichuan, China.

Population (No.)	2	3	4	5	6	7	8	9
SAGSQYJ (1)	0.94	0.93	0.96	0.95	0.94	0.94	0.94	0.94
SAGSDY (2)		0.92	0.95	0.94	0.93	0.93	0.93	0.92
SAGSBX (3)			0.92	0.91	0.91	0.92	0.94	0.92
SAGSYQ (4)				0.94	0.92	0.93	0.92	0.92
SAGSCYT (5)					0.93	0.92	0.93	0.92
SAGSZPP (6)						0.95	0.94	0.95
SAGSXJ (7)							0.96	0.94
SAGSWJ (8)								0.96
SAGSSN (9)								

Cluster analysis generated a UPGMA dendrogram given in Figure 2. Three major clusters were identified at the similarity level of 0.93. The first cluster was composed of SAGSQYJ, SAGSYQ, SAGSDY, and SAGSCYT. The second cluster contained SAGSZPP, SAGSXJ, SAGSWJ, and SAGSSN. Population SAGSBX formed one distinct cluster.
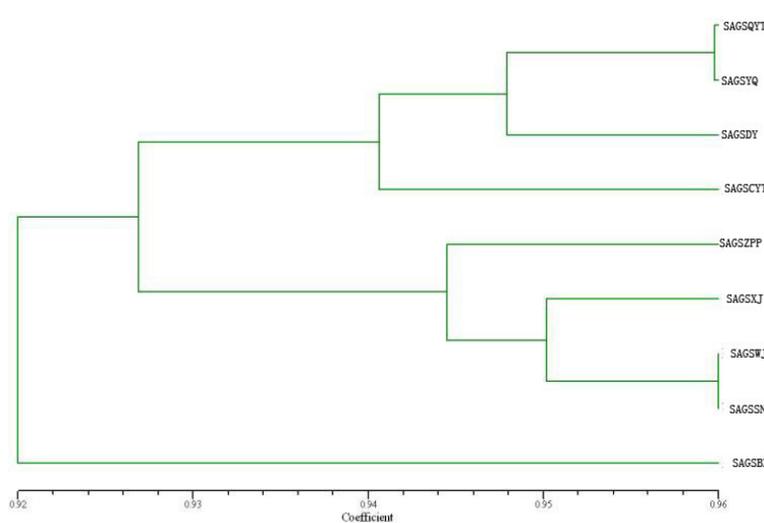
Figure 2. UPGMA dendrogram for 9 populations of *Saccharum spontaneum* based on Nei's genetic distance.

DISCUSSION

Of all *Saccharum* species, *S. spontaneum* has very wide geographic distribution from tropics to subtropics, resulting in the most diverse germplasm, which has been substantially used in sugarcane breeding programs (Ming et al., 2006), and are being deployed in the development of bioenergy cultivars (Tew and Cobill, 2010). Genetically diverse germplasm of *S. spontaneum* can effectively contribute to disease resistance, cold tolerance, insect resistance, and tillering potential to new breeding products. Pan et al. (2004) used 157 polymorphic RAPD markers in an assessment of the genetic diversity of an *S. spontaneum* collection of 33 accessions from India, Indonesia, Australia, Taiwan, Philippines, and the USA, maintained at the USDA-ARS Southern Regional Research Center, Sugarcane Research Unit, in Houma, LA, USA. They reported pairwise genetic homology coefficients in the accessions ranging from 0.65 to 0.89. Mary et al. (2006) used 410 polymorphic DNA markers in an analysis of the genetic diversity of 40 *S. spontaneum* accessions collected from four different geographic regions in India. Their results indicated that pairwise genetic similarity coefficients between the native accessions varied from 0.40 to 0.70, and that genetic diversity was associated with geographic origin. Using 84 polymorphic RAPD markers, Zhang et al. (2010) reported the genetic diversity of 89 *S. spontaneum* accessions collected from five different ecological regions of Guangxi, China. They found that the local germplasm was genetically diverse with pairwise similarity coefficients being from 0.26 to 0.91. In our current study, SRAP PCR amplification of nine *S. spontaneum* wild populations generated 185 polymorphic markers. The results indicated that the PPB of the nine populations was 78.82%. The SRAP results in this study were comparable to those of the RAPD study by Yang et al. (1998) and Zhang et al. (2010), which were 72.5 and 74.84%, respectively.

However, we assessed the genetic diversity of native *S. spontaneum* germplasm using populations instead of individual clones used by the above cited investigations. Therefore, we found that the Nei's genetic diversity parameter at the species level was 0.2801 higher than that (0.2216) at the population levels. According to the findings of Nybom and Bartish (2000), Nei's genetic diversity of monocotyledons is in an average of 0.190. Total Nei's genetic diversity and genetic diversity within populations in our study were both higher than the average value of monocotyledons, and therefore, we can conclude that the native *S. spontaneum* collected in Sichuan, China, was genetically diverse.

Hamrick and Godt (1989) pointed out that the majority (ca. 90%) of genetic variation of outcrossing wind-pollinated species involving 165 genera resided within populations, while the remaining small portion of genetic diversity resided between populations. We found that the genetic variation within populations (0.7912) is much greater than that between populations (0.2088), indicating that the local *S. spontaneum* germplasm was largely outcrossing in nature. Wright (1951) pointed out that gene flow would be able to resist the effect of genetic drift within populations and prevent the differentiation of populations as the value of $N_m > 1$. The genetic drift can lead to genetic differentiation among populations with $N_m < 1$. The N_m value in this study was 1.8944, indicating that there was no significant genetic differentiation between the 9 populations, substantiating the notion that *S. spontaneum* plants had cross-pollinating reproduction.

It is not difficult to find in the dendrogram (Figure 2) that the clusters were closely related to their geographic origins and surrounding geographic environments. The populations

collected along the Qingyi River in Ya'an formed one major cluster except one population from Baoxing. Although the location in Baoxing is close to the others in Ya'an, the population SAGSBX was not clustered with the remaining populations from Ya'an. Baoxing is located in the upper stream of the Qingyi River and its relatively colder climate due to its high altitude may have an impact on genetic differentiation. The second major cluster consisted of populations collected from Dujiangyan, Xinjin, Wenjiang, and Suining. The four locations belong to the west-central part of Sichuan basin, i.e., similar climates. Therefore, it appears that geographic distance and environmental factors including local climates interplay in the formation of the genetic structure and geographic patterns of the native *S. spontaneum* populations. The results clearly indicated that fuller sampling of the genetic diversity of cold-tolerant local germplasm in the species requires comprehensive germplasm collection in heterogeneous environments along the northern regions of the species distribution. The germplasm should be a valuable source in *Saccharum* breeding.

ACKNOWLEDGMENTS

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