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Analysis of genetic diversity among wild bermudagrass germplasm from southwest China using SSR markers

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ABSTRACT. Fifty-five wild accessions of bermudagrass (*Cynodon dactylon*) were collected from southwest China (Sichuan, Chongqing, Yunnan, Guizhou, and Tibet), and their genetic diversity was analyzed using simple sequence repeat markers. A total of 267 polymorphic bands were detected with 18 primer combinations. The genetic similarity among the accessions ranged from 0.688 to 0.894 with an average of 0.797. All 55 wild accessions were clustered into 7 eco-geographic groups. Our data showed that the dendrogram was almost in accordance with geographic distribution, and accessions from the same collection sites tended to be clustered into the same group. A genetic differentiation analysis revealed that the percentage of genetic variance was 70.07 and 29.93% within and among groups, respectively. Finally, we discuss the implications of these results for *C. dactylon* in southwest China.

Key words: *Cynodon dactylon*; SSR; Genetic diversity; Cluster analysis

INTRODUCTION

Cynodon dactylon, a perennial herbaceous plant of the grass subfamily Eragrostoideae, tribe Chlorideae, is a well-known turfgrass that grows in tropic, subtropic, and temperate zones (Harlan and De Wet, 1968; Taliaferro, 1995). It originates from Africa and is wildly distributed in South America, Africa, Europe, and South Asia (Dong and Shen, 2003). In China, *C. dactylon* is mostly distributed along and to the south of the Yellow River Valley (Tan, 1993).

Under natural growth conditions, *C. dactylon* has a seed-setting rate of 2.8-43.2, whereas the rate for self-pollination is only 0.01-8.09 (Richardson et al., 1978), suggesting higher genetic variation in natural populations. Over the past decades, several studies have examined morphology, physiology, and biochemistry in order to assess the diversity of *C. dactylon* (Liu et al., 2007). Modern techniques using DNA makers have been extensively used in studies of the genetic diversity of *C. dactylon*. Roodt et al. (2002) used the random amplified polymorphic DNA (RAPD) technique to determine the genetic relationship among *C. dactylon* cultivars in the South Africa. Caetano-Anollés et al. (1997) and Caetano-Anollés (1998) used DNA fingerprinting techniques to investigate the genetic relationships among *C. dactylon* and the DAF method to analyze the genomes of 2 varieties of Tifgreen and Tifdrawf. Mehmet et al. (2002) also used RAPD and DAF to study the hybrid intraspecific and interspecific genetic variation of *C. dactylon*. Using AFLP, Wu et al. (2004) reported the Nei's genetic similarity coefficients of the accessions to be 0.53-0.98 among *C. dactylon* in 11 different countries in Asia, Africa, Australia, and Oceania. They suggested that more representative samples from the areas of origin are needed to improve the accuracy of this estimate. Liu (2006) and Liu et al. (2007, 2008) analyzed the genetic diversity of the bermudagrass accessions in southwest China by RAPD, ISSRs, and RAMP. However, a large number of wild *C. dactylon* germplasms grow in southwest China; therefore, their genetic diversity deserves additional study at the DNA level.

Microsatellites or simple sequence repeats (SSRs) are repetitive sequences dispersed throughout the eukaryotic genome that have high mutation rates (Oliveira et al., 2006). This can produce high variability in the length of amplified DNA and high levels of heterozygosity (Ellegreen, 2004). These techniques have been successfully applied to the analysis of genetic diversity, relationships, and construction of genetic linkage maps in a wide range of species (e.g., *Oysia japonica*, Sorghum hybrid, Sudangrass, and Sorghum) (Guo et al., 2007; Zhan et al., 2008; Lu et al., 2009). However, few studies have used SSR to investigate the genetic diversity of wild *C. dactylon*.

To analyze and evaluate the genetic diversity of *C. dactylon* in southwest China, we collected 55 accessions of wild *C. dactylon* and studied their SSR polymorphisms. Our data may provide information for gathering, conserving, identifying, analyzing relationships, and breeding of *C. dactylon*.

MATERIAL AND METHODS

Plant materials

Fifty-five wild accessions of *C. dactylon* were sampled from southwestern China. A complete list of accession descriptions and putative geographical origins are provided in Table 1 and Figure 1. All genotypes were clone lines, which possess a unique genetic background.

Table 1. Wild *Cynodon dactylon* L. Pers. accessions tested in this study.

Order	Accession No.	Origin	Habit	Altitude (m)
1	Sau9933	Wenchuan, Sichuan	Roadside	1210
2	Sau9935	Maoxian, Sichuan	Wasteland	1480
3	Sau9936	Maoxian, Sichuan	Wasteland	1460
4	Sau02011	Wenchuan, Sichuan	Roadside	1310
5	Sau02012	Jinchuan, Sichuan	Roadside	2150
6	Sau02015	Jinchuan, Sichuan	Roadside	1310
7	Sau02004	Leibo, Sichuan	Flood land	1200
8	Sau02005	Huidong, Sichuan	Roadside	-
9	Sau02006	Ningnan, Sichuan	Roadside	-
10	Sau0085	Xichang, Sichuan	Flood land	1380
11	Sau02053	Xichang, Sichuan	Flood land	1380
12	Sau0088	Mianning, Sichuan	Field ridge	1774
13	Sau02033	Yuexi, Sichuan	Roadside	-
14	Sau0099	Panzhihua, Sichuan	Roadside	1100
15	Sau0098	Panzhihua, Sichuan	Woodland	1120
16	Ly97017	Panzhihua, Sichuan	Grassland	1200
17	Sau02028	Miyi, Sichuan	Hillside	1620
18	Sau0095	Yanbian, Sichuan	Field ridge	1150
19	Sau9918	Ya'an, Sichuan	Grassland	600
20	Sau02055	baoxing, Sichuan	Hillside	1010
21	Sau02060	Yingjing, Sichuan	Roadside	720
22	Sau02061	Tianquan, Sichuan	Riverside	740
23	Sau02064	Lushan, Sichuan	Hillside	630
24	Sau02065	Lushan, Sichuan	Riverside	685
25	Sau9927	Yibin, Sichuan	Flood land	240
26	Sau9922	Yibin, Sichuan	Riverside	255
27	Sau9931	Yibin, Sichuan	Flood land	245
28	Sau9924	Yibin, Sichuan	Roadside	340
29	Sau9926	Yibin, Sichuan	Wasteland	260
30	Sau02041	Yibin, Sichuan	Riverside	250
31	Sau02042	Yibin, Sichuan	Riverside	250
32	Sau02045	Liangping, Chongqing	Riverside	400
33	Sau02046	Liangping, Chongqing	Roadside	380
34	Sau9942	Changshou, Chongqing	Woodland	305
35	Sau9945	Changshou, Chongqing	Flood land	140
36	Sau9953	Hechuan, Sichuan	Flood land	130
37	Sau9947	Jialing, Chongqing	Woodland	230
38	Sau02050	Wanzhou, Chongqing	Shipside	150
39	Sau02048	Wanzhou, Chongqing	Roadside	490
40	Sau06001	Yunyang, Chongqing	Flood land	160
41	Sau02019	Libo, Guizhou	Flood land	370
42	Sau02020	Libo, Guizhou	Flood land	360
43	Sau02022	Dushan, Guizhou	Roadside	950
44	Sau02023	Dushan, Guizhou	Field ridge	970
45	Sau02024	Dushan, Guizhou	Roadside	810
46	Sau02025	Dushan, Guizhou	Roadside	890
47	Sau03001	Bayi, Tibet	Garden	3080
48	Sau03002	Chayu, Tibet	Roadside	2550
49	Sau03003	Chayu, Tibet	Roadside	2460
50	Sau03004	Chayu, Tibet	Roadside	2030
51	Sau03005	Chayu, Tibet	Roadside	1750
52	Sau02026	Xiaoshao, Yunnan	Groove	1900
53	Sau02027	Xiaoshao, Yunnan	Roadside	1910
54	Ly98010	Kunming, Yunnan	Roadside	1720
55	Sau02054	Qiaojia, Yunnan	Roadside	841

DNA extraction

DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Reichardt and Rogers, 1997). DNA concentration was estimated by the fluorescence intensities of ethidium bromide-stained samples on 0.8% agarose gels.

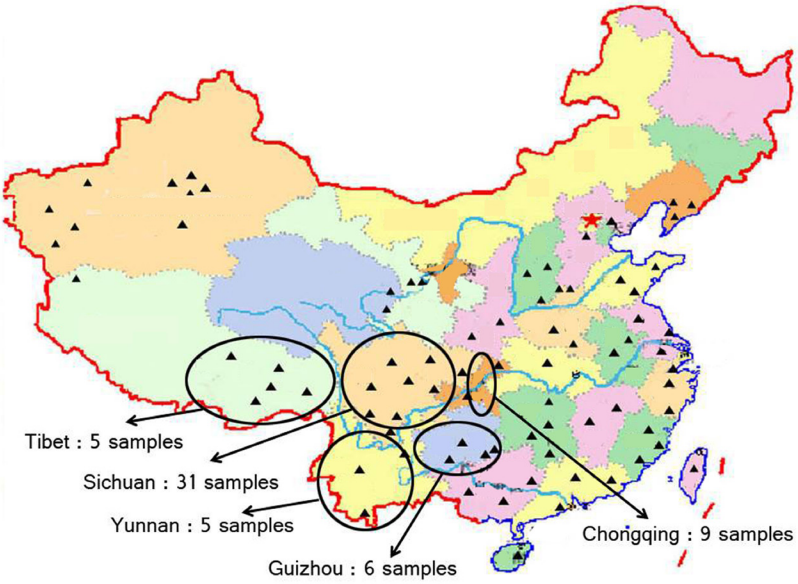

Figure 1. Chinese map showing the collection sites of the 55 samples of *Cynodon dactylon* L. Pers.

Polymerase chain reaction (PCR) amplification

A total of 18 primer pairs were used to assay the 55 bermudagrass accessions; these were EST-SSR primers from main cereal crops (Wang et al., 2005). Primer banding patterns that were difficult to score and those that failed to amplify consistently in all genotypes were excluded. Consequently, only 18 combinations were selected (Table 2). For SSR amplification, the total volume of the PCR was 15 µL and contained 40 ng template DNA, 1.5 mM Mg^{2+}, 1 U Taq DNA polymerase (Takara Biotechnology Co. Ltd., China), 240 nM dNTP, and 0.4 µM primer. PCR was performed using the following conditions: 94°C for 4 min; 10 cycles at 94°C for 1 min, 53°C for 30 s, and 72°C for 40 s with a 0.5°C decrease in annealing temperature per cycle; 35 cycles at 94°C for 1 min, 45°C for 30 s, and 72°C for 40 s; an extension at 72°C for 10 min, and storage at 4°C. The amplified fragments were separated on 6% denatured polyacrylamide gels (acrylamide:bisacrylamide, 19:1) in 1X TBE. After electrophoresis, the gel was stained with $AgNO_3$ solution using a previously reported method (Xu et al., 2002) and photographed using the Gel Doc XR system (Bio-Rad Laboratories).

Data analysis

Unequivocally and consistently reproducible amplified SSR bands were scored as present (1) and absent (0). Smeared and weak bands were excluded. Fragments of the same molecular weight were considered to represent the same locus. To analyze the suitability of the SSR markers for evaluating genetic profiles of *C. dactylon*, the performance of the markers was measured using polymorphic information content (PIC). The PIC value for each locus was calculated as proposed by Roldán-Ruiz et al. (2000):

$$PIC_i = 2f_i (1 - f_i)$$

where PIC_i is the PIC of the locus I; f_i is the frequency of the amplified fragments (band present), and $1 - f_i$ is the frequency of non-amplified fragments (band absent). The frequency was calculated as the ratio between the number of amplified bands at each locus and the total number of accessions. The PIC of each primer was calculated using the average PIC value from all bands of each primer pair.

The POPGENE software was also used to calculate the Shannon diversity index for SSR phenotypic data using the formula:

$$H_O = -\sum \pi i \ln \pi i$$

where πi is the frequency of a given SSR fragment. H_O was calculated at 2 levels: the average diversity within geographic groups (H_{group}) and the total diversity (H_{sp}) (Wachira et al., 1995). The proportion of diversity among populations was estimated as $(H_{sp} - H_{group}) / H_{sp}$ (Persson et al., 2001).

An unweighted pair-group method using arithmetic average (UPGMA) dendrogram was constructed based on the matrix of Nei's unbiased genetic distance (Nei and Li, 1979). Principal coordinates analyses were conducted to ordinate relationships among populations with Nei's distance matrix (NTSYS-pc version 2.11x; Rohlf, 2000). In order to test whether there was a correlation between genetic distances and geographical distances (km) among populations, a Mantel test was performed using the NTSYS-pc program 2.11x with 3000 permutations.

RESULTS AND DISCUSSION

SSR polymorphism

Amplification from 55 *C. dactylon* DNA samples using the 18 primer pairs (Table 2) produced a total of 353 DNA bands, of which 267 were polymorphic (average of 19.61 bands per primer pair, Table 3). Polymorphic bands corresponding to 75.10% ranged from 50 to 1700 bp in size. To determine the PIC values of each primer pair, we analyzed the mean PIC values for all loci. As a result, we obtained high PIC values for the primers SG26 (0.4565) and SG22 (0.4339) and a low PIC value for the primer W17 (0.1668) (Table 3). The average PIC value for a primer pair was 0.3307. A comparison of the frequency of polymorphic bands at each locus with the average PIC value of each locus showed that a greater number of polymorphic bands were associated with lower PIC values. While the results were similar to those obtained using RAPD markers (Liu, 2006), they identified a higher degree of genetic polymorphism than ISSRs and RAMP markers (Liu et al., 2007, 2008). This suggests that utilizing SSR marker is more feasible and effective. These results suggested that there is a rich genetic diversity among the wild *C. dactylon* based on SSR markers. SSR fingerprint patterns in *C. dactylon* L. Pers. amplified by primer SG26 numbers 1-55 are shown in Figure 2.

Genetic similarity analysis

Nei-Li genetic similarity coefficients were calculated using the NTSYS-pc2.10t soft-

ware, and a dendrogram was constructed by UPGMA. Our data showed that there were significant differences in genetic diversity between the samples tested. Nei's genetic similarity coefficient of the accessions tested ranged from 0.688 to 0.894. The average Nei's coefficient was 0.797, and the transformer was 0.206. The similarity coefficient matrix showed that *C. dactylon* collected from Leibo, Sichuan (Sau02004), and Huidong, Sichuan (Sau02005), had the lowest genetic similarity coefficient and the largest genetic distance, while *C. dactylon* collected from Chayu, Tibet (Sau03005), and Xiaoshao, Yunnan (Sau02027), had the highest degree of genetic similarity and the smallest genetic distance.

Table 2. Primer sequences used in SSR analyses of *Cynodon dactylon* L. Pers.

Primers	Sequences (5'-3')	Sequences (5'-3')
SG26	TGGCGGACATCCTATT	GGAGAGCCCGTCACTT
W6	GTCCAGCTCTCGGATCTTGG	TGCATCCAAACAAGCCATGC
M31	GAAGTCGCTGATGAGAACGTAACC	GCTAGCTAGTGTGAGTTCTTCCGC
S1	TCACCAGACCACCAGCTTC	GAGAACGGGCCAAGGTACT
M26	CTGTCGTAAGAGCGCCAACAG	GTCTGAACGATGAACAGTACACGC
SG3	CCAACCGAGTCGCTGATG	GTGGACTCTGTCGGGGCACTG
SG22	TGGGCAGGGTATCTAACTGA	GCC TTTTTCTGAGCCTTGA
M22	GAGAGGTCGTCGTCGCTACTG	GAGACCAGATTCTTGGAACGGTAA
SG20	TGTATGGCCTAGCTTATCT	CAACAAGCCAACCTAAA
SG25	CCTCCTTTTCCTCCTCCTCCC	TCAGAATCCTAGCCACCGTTG
R45	ATCGTTGCCTCGTAACAACC	ACCTGCAACTGCCAGAAGAG
R38	GTCATCTACCACACCCAGCC	CTTGGTCCAACCCGAACTTA
S11	GAGGGCGTACAGGAAGAACA	CCGAGAAGGACTTGGTGAAG
SG29	AATGAGGAAAATATGAAACAAGTACCAA	AATAACAAGCGCAACTATATGAACAATAAA
W17	GTGGCAGGCAGGCAAGCAAG	TGACGAGCTCATCGTCGTAG
S5	GTGGACGATGGATGGATCA	ATCACCACTGCCTCTCACAA
R2	AAGTCCGTCGACAGGATGAG	GCT GCTCTTCCTTGTGGCTA
M48	TGGACGATCTGCTTCTTCAGG	GAAGGCTTCTTCCTCGAGTAGGTC

Table 3. Amplification results from 18 primer combinations.

Primer pairs	Total No. of polymorphic bands	No. of polymorphic bands	Polymorphic bands (%)	PIC
SG26	18.00	16.00	88.89	0.4565
W6	21.00	14.00	66.67	0.3512
M31	25.00	17.00	68.00	0.3464
S1	20.00	12.00	60.00	0.3284
M26	25.00	19.00	76.00	0.3832
SG3	17.00	14.00	82.35	0.3749
SG22	26.00	25.00	96.15	0.4339
M22	16.00	12.00	75.00	0.3797
SG20	16.00	13.00	81.25	0.2585
SG25	19.00	18.00	94.74	0.4120
R45	14.00	11.00	78.57	0.3016
R38	15.00	12.00	80.00	0.3012
S11	22.00	15.00	68.18	0.2347
SG29	22.00	21.00	95.45	0.3560
W17	14.00	7.00	50.00	0.1668
S5	19.00	8.00	42.11	0.3026
R2	19.00	10.00	52.63	0.2783
M48	24.00	23.00	95.83	0.2859
Total	353.00	267.00		
Average	19.61	14.83	75.10	0.3307

PIC = polymorphic information content.

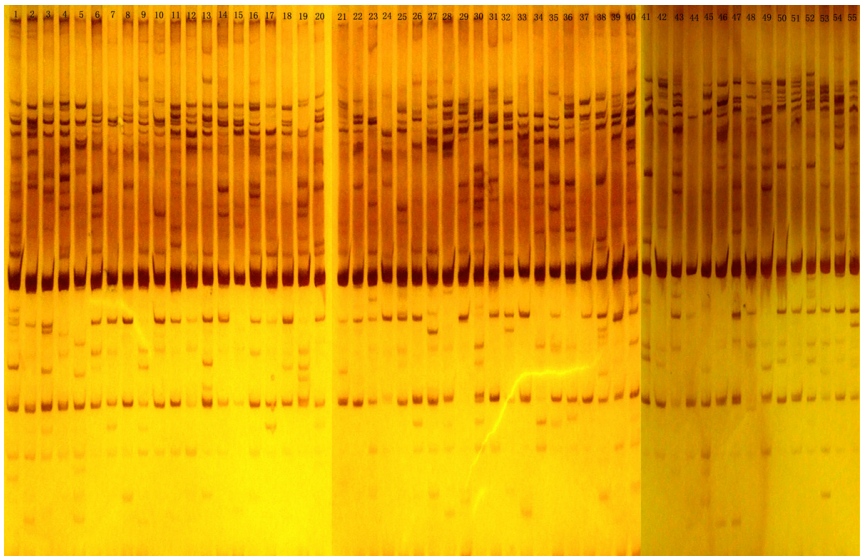

Figure 2. SSR fingerprint patterns in *Cynodon dactylon* L. Pers. amplified by primer SG26. *Lanes 1* to *55* are the same as described in Table 1.

According to Zhao Ruzhi's division of the physico-geographical regionalization in southwest region (Zhao, 1997) and the different ecological and geographical environments of the sampling places, 55 *C. dactylon* materials were divided into 7 eco-geographical groups: Aba Sichuan, Yaan Sichuan, Panzhihua-Xichang Sichuan, Yibin Sichuan, Chongqing, Yunnan, and Guizhou, Tibet (Table 4). The genetic similarity (GS) and distance (GD = 1 - GS) of the 7 eco-geographical groups represent the level of genetic diversity within the group. We found an increase in GD between the samples within a group, which was correlated with increased genetic diversity. As shown in Table 4, the Yunnan and Guizhou groups had the highest average GD, Shannon index, and Simpson index (Nei's gene diversity).

Table 4. Genetic polymorphism indexes of 6 eco-geographical groups of *Cynodon dactylon* L. Pers.

Groups	GD	D	H_O	$H_{between}$	H_{group}	H_{sp}	H_{within}
A'ba, Sichuan	0.1830	0.1872	0.2783	0.3805	0.2666	0.7007	0.2993
Ya'an, Sichua	0.1944	0.1935	0.2910				
Panxi region	0.1862	0.1615	0.2423				
Yibin, Sichua	0.1835	0.1836	0.2736				
Chongqing	0.1855	0.1678	0.2522				
Yunnan and Guizhou	0.2199	0.2228	0.3286				
Tibet	0.2125	0.1330	0.2002				

GD = genetic distance; D = Simpson index; H = Shannon index.

For the various eco-geographical groups, the species level of phenotypic diversity (H_{sp}) was 0.2666, while the population level of phenotypic diversity was 0.3805 (H_{group}). Phenotypic diversity within and among groups was 0.7007 (H_{within}) and 0.2993 ($H_{between}$), respectively. These results are consistent with those obtained by SRAP analysis (Yi et al., 2008).

Cluster analysis

Based on the genetic similarity coefficient, 55 samples were tested by UPGMA cluster analysis (Figure 3). UPGMA cluster analysis indicated that when GS = 0.81, 55 wild accessions were clustered into 6 groups. This suggested that there was a strong correlation between the genetic relationships and growing locations. In general, samples from the same eco-geographical environment were clustered into a single group. Cluster I contained samples from Yunnan, which had similar climates. The samples from the Tibet region formed one group, cluster III, which was in the alpine cold meadow of the edge of the Tibetan plateau at a height of 1700-3000 m above sea level. Thus, this environment differed from other groups and was classified into its own branch. The sample from Yibin Sichuan (Sau992629) differed from the others. Sample 29 (Sau9926) also differed from the others. The leaf texture was smooth; the height of plant was slightly below average, and the branch density was high. This sample was clustered in a group by itself, forming Cluster II. These results are consistent with those of Yi et al. (2008). Samples from Panzhihua-Xichang Sichuan and some samples from Aba Sichuan formed cluster IV. Cluster V contained samples from Guizhou. However, this correlation was not applicable to other groups. Cluster VI contained various sample types and had a total of 32 samples, including those from Chongqing, Sichuan, Aba Yaan, Yibin, and others. Similar results were reported by Liu et al. (2007), who analyzed 42 accessions by ISSR. This might have resulted from the number of collected samples.

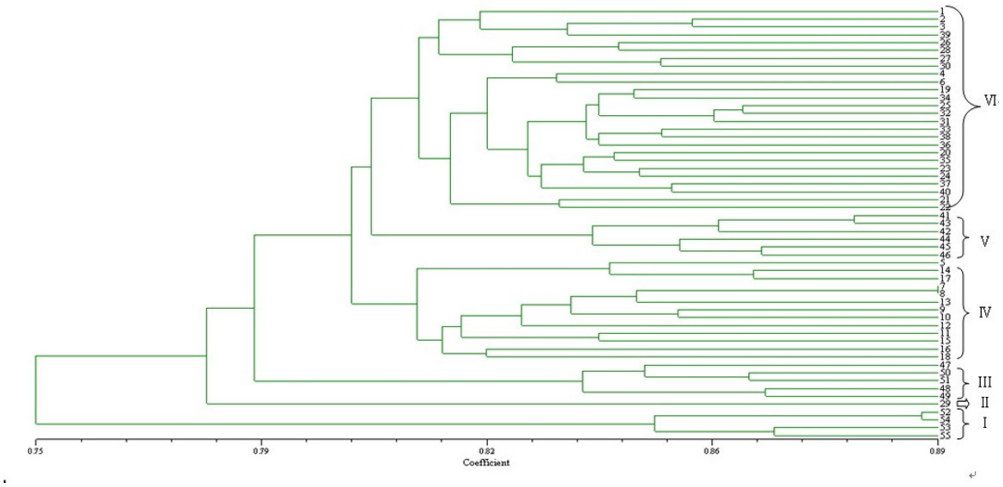

Figure 3. UPGMA dendrogram for *Cynodon dactylon* L. Pers. based on Nei-Li's genetic similarity coefficients.

Cluster analysis of eco-geographical group

To examine the relationships among all eco-geographical groups, NTSYS-PC was used for cluster analysis of UPGMA. The 8 tested eco-geographical groups were re-clustered into 7 main groups (Figure 4).

Groups from ABa, YaAn, and YiBin, belonging to Sichuan Province or Tibet Province, were still individually clustered. In contrast, the groups from Yunnan and Guizhou, which were

both from the Yungui Plateau, were clustered into a single group. Further, samples from the same eco-geographical environment were generally clustered into a single group, which was consistent with the clustering results. However, this correlation was not applicable to other groups. The Aba and Yaan regions were grouped together. The Panzhihua-Xichang and Chongqing regions were grouped together. Panzhihua and Xichang regions are located in the central subtropic geographical and climate zone; they have a unique climate and environment, called the dry-and-hot valley. Due to a vertical zonal change, its high terrain and deep valley represents a special terrain in South China. This region has a south subtropical climate in the subtropical climate zone, while Chongqing belongs to the central subtropical humid climate. However, Panzhihua, Xichang region, and Chongqing region were grouped together. Similar results in *C. dactylon* were reported by Yi et al. (2008). A possible explanation for this result could be that the NTSYS-pc cluster analysis artificially divided the groups. The samples from Aba, Panzhihua, and Xichang belonging to Sichuan were clustered together, which similarly did not follow this rule. This was probably due to the mixed-mating breeding system and high levels of asexual reproduction in bermudagrass. Additionally, human activities and flood scouring may have increased reproduction in different places.

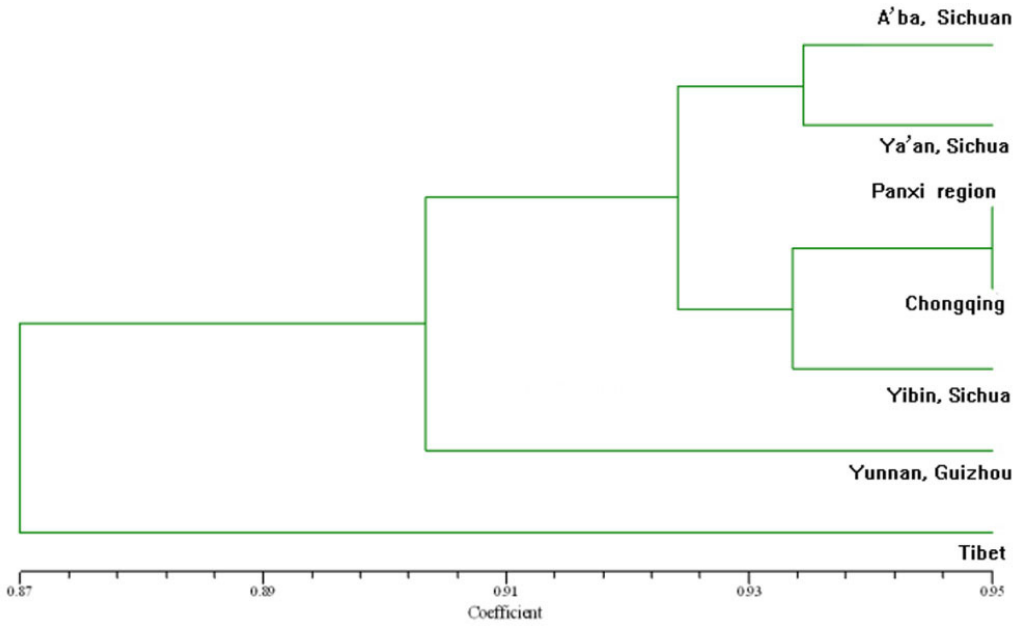

Figure 4. Dendrogram of 7 eco-geographical groups based on Nei's unbiased measures of genetic identity.

Our analysis of the genetic diversity using SSRs may contribute to protection plans and breeding of new varieties of *C. dactylon*. As described above, there are abundant wild *C. dactylon* in southwest China. However, because it has a high capability of agamogenesis and a rapid growth rate, the genetic diversity of *C. dactylon* has been reduced in recent years by human activity and flooding. We have shown that SSRs provide a rapid and effective tool for resolving the genetic polymorphisms in *C. dactylon*. Our study should help identify different lines and enable breeding of new cultivars of *C. dactylon* in the future.

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