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Assessment of genetic diversity of bermudagrass germplasm from southwest China and Africa by using AFLP markers

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ABSTRACT. *Cynodon dactylon* (L.) Pers. var. *dactylon* (common bermudagrass) is widely distributed geographically between approximately 45°N and 45°S latitude, penetrating to approximately 53°N latitude in Europe. The extensive variation of morphological and adaptive characteristics of the taxon has been substantially documented, but information is lacking on DNA molecular variation in geographically disparate forms. The genetic diversity of 51 wild accessions of bermudagrass from southwest China (Sichuan, Chongqing, Yunnan, Guizhou, and Tibet) and 8 African bermudagrass was analyzed using amplified fragment length polymorphism molecular markers. A total of 670 polymorphic bands were detected with 11 primer combinations, of which 663 (98.74%) bands were found to be polymorphic. The genetic similarity among the accessions ranged from 0.64-0.96 with an average of 0.78. All 59 wild accessions were clustered into 5 eco-geographic groups, and nearly all accessions from the same area were classified into the same group and were found to be associated with their geographical distributions. Therefore, complex geographical and

ecological environments are important factors for the genetic structure and geographical distribution of *C. dactylon*.

Key words: AFLP marker; Bermudagrass; Genetic structure

INTRODUCTION

Cynodon dactylon (L.) Pers. var. *dactylon* (common bermudagrass), which shows widespread distribution in warmer parts of the world, is used as livestock herbage and turf and is the most important member of the genus *Cynodon*. It originates from Africa and is widely distributed in South America, Africa, Europe, and South Asia (Langdon, 1954). In China, bermudagrass is mainly distributed along the southern region of the Yellow River Valley (Zhang et al., 2003).

In the natural growth condition, *C. dactylon* var. *dactylon* has a seed-setting rate ranging 2.8-43.2, whereas the rate of self-pollination is only 0.01-8.09 (Burton, 1965), suggesting it has a higher genetic variation in its natural population. Therefore, the natural genetic variation of bermudagrass is very significant (Richardson et al., 1978). Recent years, with the deterioration of ecological environment, plant populations have rapidly decreased, which could result in lost for several excellent genes.

Polymerase chain reaction (PCR)-based DNA fingerprinting techniques based on the analysis of information-rich nucleic acid molecules have been used to study genetic diversity, relatedness, phylogeny, and identifying off-types of cultivars in turfgrass (Caetano-Anollés, 1998). A number of researchers have employed randomly amplified polymorphic DNA (Iqbal et al., 2008; Zhang et al., 2011; Huo et al., 2013) and DNA amplified fingerprinting procedures (Caetano-Anollés et al., 1995, 1997; Karaca et al., 2002; Zhang et al., 2013). Recently, the amplified fragment length polymorphism (AFLP) DNA method (Vos et al., 1995) has been used to detect genetic diversity among forage bermudagrass cultivars (Karaca et al., 2002), to quantify the genetic variation of *Cynodon transvaalensis* and its relatedness to hexaploid *C. dactylon* var. *dactylon* (Wu et al., 2005), and to detect genetic analyses of Chinese *C. dactylon* var. *dactylon* accessions (Wu et al., 2006). A large number of wild *C. dactylon* var. *dactylon* germplasm grow in southwest China; however, there have been few studies examining the genetic diversity of wild *C. dactylon* var. *dactylon* in this region. In this study, AFLP molecular markers were used to analyze and evaluate the genetic diversity and relationship of *C. dactylon* var. *dactylon* in southwest of China and Africa. Our data provide methods and a theoretical basis for further gathering, collection, identification, relationship analysis, and breeding of wild *C. dactylon* var. *dactylon*.

MATERIAL AND METHODS

Plant material

Fifty-nine wild accessions of *C. dactylon* var. *dactylon* were collected from 5 provinces in southwest China (Sichuan, Chongqing, Yunnan, Guizhou, and Tibet) and Africa (Table 1). A healthy growing branch was collected from each sample, and a wet towel wrapped was wrapped around the branch for transport to the test base before preparing cuttings for the reproduction of clones.

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

Order	Accession number	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	Sau9947	Jialing, Chongqing	Woodland	230
37	Sau02050	Wanzhou, Chongqing	Shipside	150
38	Sau02048	Wanzhou, Chongqing	Roadside	490
39	Sau02019	Libo, Guizhou	Flood land	370
40	Sau02020	Libo, Guizhou	Flood land	360
41	Sau02022	Dushan, Guizhou	Roadside	950
42	Sau02023	Dushan, Guizhou	Field ridge	970
43	Sau02024	Dushan, Guizhou	Roadside	810
44	Sau02025	Dushan, Guizhou	Roadside	890
45	Sau03001	Bayi, Tibet	Garden	3080
46	Sau03002	Chayu, Tibet	Roadside	2550
47	Sau03003	Chayu, Tibet	Roadside	2460
48	Sau02026	Xiaoshao, Yunnan	Groove	1900
49	Sau02027	Xiaoshao, Yunnan	Roadside	1910
50	Ly98010	Kunming, Yunnan	Roadside	1720
51	Sau02054	Qiaojia, Yunnan	Roadside	841
52	19712D	Africa	Roadside	-
53	13318D	Africa	Roadside	-
54	15014D	Africa	Roadside	-
55	16717D	East Africa	Roadside	-
56	16741D	Africa	Roadside	-
57	19710D	Africa	Roadside	-
58	15725D	Africa	Roadside	-
59	16708D	East Africa	Roadside	-

DNA extraction and PCR amplification

DNA samples were isolated from fresh leaf tissues of the *C. dactylon* var. *dactylon*

plants with DNeasy plant mini kit from QIAGEN, Inc. (Hilden, Germany). The AFLP analysis was performed as described by Vos et al. (1995) with minor modifications (Bai et al., 1999). The AFLP procedure followed Wu et al. (2005). Primer sequences are shown in Table 2.

Table 2. Primer sequences used in AFLP analyses of *Cynodon dactylon* var. *dactylon*.

*Eco*RI	Sequences (5'-3')	*Mse*I	Sequences (5'-3')
E-AAC	GACTGCGTACCAATTCAAC	M-CTC	GATGAGTCCTGAGTAACTC
E-ACA	GACTGCGTACCAATTCACA	M-CTG	GATGAGTCCTGAGTAACTG
E-ACT	GACTGCGTACCAATTCACT	M-CAC	GATGAGTCCTGAGTAACAC
E-ACT	GACTGCGTACCAATTCACT	M-CTA	GATGAGTCCTGAGTAACTA
E-ACC	GACTGCGTACCAATTCACC	M-CAT	GATGAGTCCTGAGTAACAT
E-ACG	GACTGCGTACCAATTCACG	M-CTA	GATGAGTCCTGAGTAACTA
E-ACG	GACTGCGTACCAATTCACG	M-CTG	GATGAGTCCTGAGTAACTG
E-ACG	GACTGCGTACCAATTCACG	M-CTT	GATGAGTCCTGAGTAACTT
E-AGC	GACTGCGTACCAATTCAGC	M-CTG	GATGAGTCCTGAGTAACTG
E-ACG	GACTGCGTACCAATTCACG	M-CAC	GATGAGTCCTGAGTAACAC
E-ACA	GACTGCGTACCAATTCACA	M-CTT	GATGAGTCCTGAGTAACTT

Data analysis

Unequivocally and consistently reproducible amplified AFLP bands were scored as present (1) or absent (0). Smeared and weak bands were excluded. Fragments of the same molecular weight were considered to be the same locus.

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) and bootstrapping was conducted with 1000 replicates using the program TFPGA (Miller, 1999). The genetic similarity index was calculated as $GS = 2Nij/(Ni + Nj)$, where *Ni* was the bands appearing in the material (i). *Nj* indicated the bands appearing in the material (j). *Nij* were bands common in material (i) and material (j).

Principal components analysis on the genetic similarity index was conducted according to the first principal component and the second principal component, resulting in variation, and a 3-dimensional scatter plot was constructed.

RESULTS

AFLP polymorphism

Eleven AFLP selective amplification primer combinations produced a total of 670 bands among the 59 *C. dactylon* var. *dactylon* genotypes, with an average of 60.91 bands per primer combination (Table 3). Of the 670 bands scored, 663 (98.74%) were polymorphic, with an average of 60.27 polymorphic bands per primer combination. The primer combinations E-AGC-M-CTG and E-ACT-M-CAC amplified the largest (76) and smallest (39) numbers of total bands and polymorphic bands per gel, respectively (Table 3). Non-repeatable bands mainly included faint bands that showed up in some PCRs, but not in others. These results are consistent with previous reports regarding the reproducibility of AFLP markers (Zhang et al., 1999; Mian et al., 2002) and further confirm that the AFLP technique generates highly reproducible DNA profiles for *C. dactylon* var. *dactylon*. The size of polymorphic bands ranged from 100-500 base pairs. An AFLP gel with PCR products using primer combination E-AGC-M-CTG is shown in Figure 1.

Table 3. Amplification results from 10 primer combinations.

Primer pairs	Total No. of polymorphic bands	No. of polymorphic bands	Percentage of polymorphic bands (%)
E-AAC-M-CTC	42.00	40.00	95.24
E-ACA-M-CTG	64.00	63.00	98.44
E-ACT-M-CAC	39.00	38.00	97.44
E-ACT-M-CTA	53.00	52.00	98.11
E-ACC-M-CAT	67.00	66.00	98.51
E-ACG-M-CTA	67.00	67.00	100.00
E-ACG-M-CTG	62.00	61.00	98.39
E-ACG-M-CTT	62.00	62.00	100.00
E-AGC-M-CTG	76.00	76.00	100.00
E-ACG-M-CAC	76.00	76.00	100.00
E-ACA-M-CTT	62.00	62.00	100.00
Total	670.00	663.00	
Average	60.91	60.27	98.74

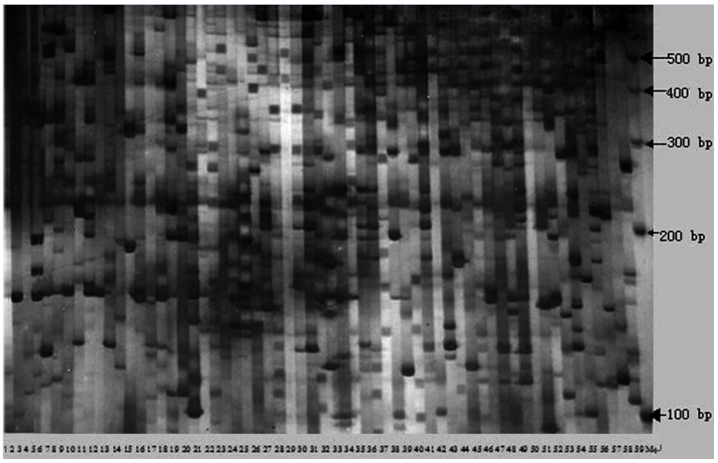

Figure 1. Fingerprinting patterns amplified using the primer combination E-AGC~M-CTG. *Lanes 1-59* = same as the order shown in Table 1.

Genetic similarity analysis

Nei-Li genetic similarity coefficients were calculated using the NTSYS-pc2.10t software and a dendrogram was constructed using UPGMA. We observed significant differences in genetic diversity between the tested materials. The Nei's genetic similarity coefficient of the tested accessions ranged from 0.64-0.96. The average Nei's coefficient was 0.78, and the transformer was 0.32. The similarity coefficient matrix revealed that *C. dactylon* var. *dactylon* collected from Xiaosao, Yunnan (Sau02026), and East Africa (16717D) had both the lowest genetic similarity coefficient and the largest genetic distance, while the 2 *C. dactylon* var. *dactylon* (Sau02023 and Sau02024) samples collected from Dushan and Guizhou showed the opposite results.

Cluster analysis

Based on the genetic similarity coefficient, 59 materials were tested using UPGMA cluster analysis (Figure 2). UPGMA cluster analysis indicated that when GS = 0.81, 59 wild

accessions were clustered into 5 groups. Cluster I contained the 31 materials from Sichuan and 2 materials (Sau9945 and Sau02050) from Chongqing. In cluster I, the 2 materials (Sau9945 and Sau02050) from Chongqing were clustered together first and the 31 materials from Sichuan were clustered into 1 subgroup. Cluster II contained the 2 materials (Sau02019 and Sau02020) from Guizhou and 5 materials from Chongqing. In cluster II, the 5 materials from Guizhou were clustered first, as they were the most closely related. Cluster III contained 4 materials from Yunnan and 4 materials from Guizhou. Three materials from Tibet composed cluster IV. The 8 materials from Africa composed cluster V.

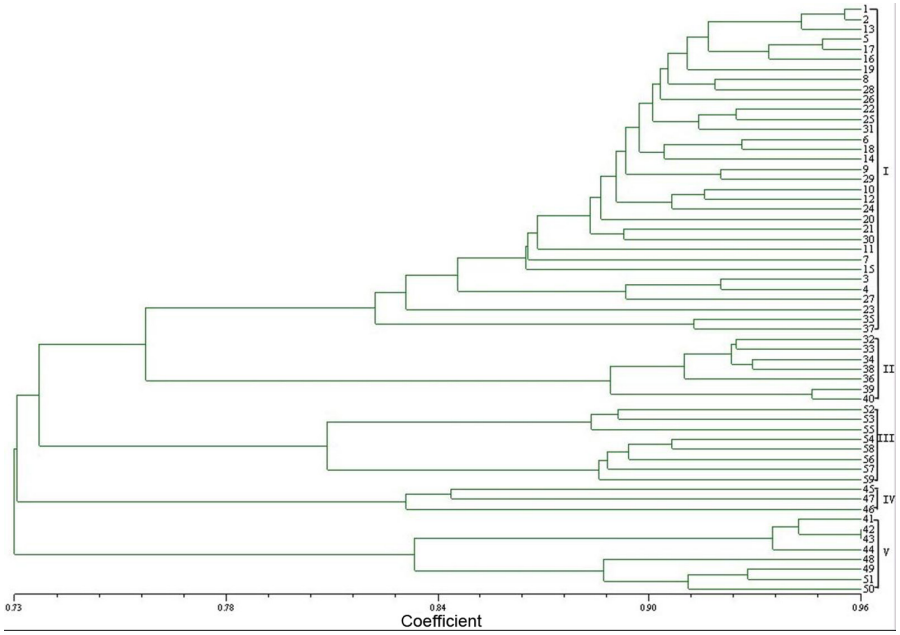

Figure 2. UPGMA dendrogram for *Cynodon dactylon* var. *dactylon* based on Nei-Li's genetic similarity coefficients.

Principal components analysis of *C. dactylon* var. *dactylon*

The genetic similarity index was evaluated using principal components analysis. Each point on the scatter diagram represents a material. The location of the material more directly reflects the genetic structure and genetic relationship of provenances. The proximity of the locations indicates a closer genetic distance; otherwise, a larger genetic distance was assumed. The 3 principal components showed that the genetic distances were 17.02, 16.68, and 16.21%, respectively.

Fifty-nine tested materials were used to construct the 3-dimensional scatter plot (Figure 3). These materials were considered to be a class, for which each location was close to the other. As a result, the 59 materials tested were roughly divided into 5 eco-geographical taxa. Figure 3 shows that the materials from China and the materials from Africa had a large distance, indicating that the genetic distance was also large. The materials from China showed significant genetic distance, while most materials from the same area in China showed lower differences. These results were consistent with those determined based on clustering.

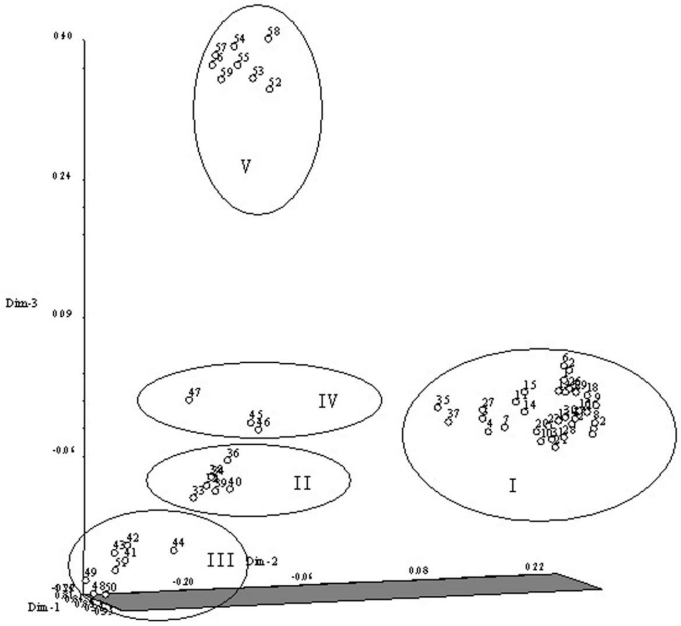

Figure 3. Principal component analysis based on AFLP patterns in *Cynodon dactylon* var. *dactylon*.

DISCUSSION

In this study, the extent of genetic relatedness among 59 tested materials was determined by the estimation of genetic distance measures, with clustering determined by UPGMA and principal component analysis. The results showed that the percentage of polymorphic bands was an average of 98.74%. Therefore, AFLP is a high-resolution method that can be used for detecting variation and polymorphisms among and within accessions of *C. dactylon* var. *dactylon*.

The relationship between genetic distance and geographical environment of species is widely examined based on the genetics of a plant population. Most studies have suggested that there is some correlation between the genes in species and the geographic distribution of the germplasm (Wilson et al., 2001; Liu, 2007, 2008; Yi et al., 2008). In this study, we observed a strong correlation between materials' genetic relationships and growing locations, which is consistent with the results of Wilson et al. (2001). In general, materials from the same eco-geographical environment can be clustered into 1 group. For example, the materials from Africa could be clearly separated from the materials from China because of the unique tropical desert climate in Africa. In addition, the materials from China were also generally separated into different eco-geographical environments. However, materials from different eco-geographical environments were clustered together. Two materials (Sau02019 and Sau02020) from Guizhou and 5 materials from Chongqing clustered together because all of these samples were collected from mountains at similar altitudes and eco-geographical environments. The 4 materials from Yunnan and 4 materials from Guizhou clustered together because they are located in the Yunnan-Guizhou Plateau, which has the same eco-geographical environment.

However, this correlation is not applicable to all groups. The 2 materials from Chongq-

ing clustered with the materials from Sichuan. The 6 materials from Guizhou did not cluster together. Two materials clustered with those from Chongqing, while the remaining 4 materials showed a closer genetic relationship with those from Yunnan. There are 3 explanations for this phenomenon. First, the materials were collected from different regions of same province at different altitudes and from different eco-geographical environments. This may account for the variations in the genotypes of materials from the same province. Second, bermudagrass uses a mixed-mating breeding system, which is dominated by asexual reproduction. Human activities and scouring of flood may have led to expanded reproduction in different regions. Third, genetic mutation has occurred throughout evolution of the species, and the mutants may be well-adapted to local natural environment. Therefore, appropriate measures must be used when isolating *C. dactylon* materials from different regions to avoid causing the variation in species because of hybrid populations.

Conservation implications

It is critical to recognize the genetic diversity and variation among and within accessions to choose relevant strategies for conservation and sampling management. Our analysis of the genetic diversity using the AFLP method may play an important role in protection plans and breeding of new *C. dactylon* var. *dactylon* varieties. Wild *C. dactylon* var. *dactylon* is abundant in China. However, because of its strong agamogenesis ability and rapid growth, *C. dactylon* var. *dactylon* genetic diversity has been reduced by human activity and flooding in recent years. Our results demonstrated that the AFLP method is a rapid and effective tool for resolving genetic polymorphisms in *C. dactylon* var. *dactylon*. Our results will be useful for establishing a germplasm resources center, identifying different lines, and breeding new cultivars of *C. dactylon* var. *dactylon*.

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