Genetic analysis for 57 accessions of *Cynodon dactylon* from 17 countries in 5 continents by SRAP markers

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Abstract

Sequence-related amplified polymorphism (SRAP) was used to distinguish between and explore the genetic relationships among 57 *Cynodon dactylon* accessions collected from 17 countries in 5 continents. Cluster analysis by the unweighted pair-group method of arithmetic averages showed 7 groups. The Chinese accessions, that were genetically very different from the others, formed 5 groups and displayed greater genetic variation than accessions from other geographic regions. The genetic similarity coefficients among the 57 accessions ranged from 0.53 to 0.97. Results obtained clearly indicated significant variation existed in wild *C. dactylon* genotypes, and the SRAP technique was a reliable tool for differentiating between *C. dactylon* genotypes and determining genetic relationships among them. To fully assess the available variation in *C. dactylon*, larger numbers of accessions should be collected from all major geographic regions, where the species occurs, especially from China and Australia.

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Introduction

Cynodon dactylon is a perennial grass species, widely distributed throughout the world between latitudes 45°N and 45°S (Harlan and de Wet 1969; Harlan 1970; Taliaferro 1995), and is used extensively as a pasture grass and turfgrass throughout this region.

In recent years, morphological markers have been used extensively in the estimation of the genetic variation within a range of grasses, namely *Cenchrus ciliaris* and *Cenchrus setigerus* (Pengelly *et al*. 1992), *Pennisetum purpureum* (van de Wouw *et al*. 1999), *Lablab purpureus* (Ewansiha *et al*. 2007), *Desmanthus virgatus* (Zabala *et al*. 2008), *Panicum* spp. (van de Wouw *et al*. 2008), *Cenchrus ciliaris* (Jorge *et al*. 2008) and *Cynodon* spp. (van de Wouw *et al*. 2009). Unlike morphological markers, molecular markers are not susceptible to environmental influences and thus provide a stable mechanism for determining relationships between plant groups. DNA molecular markers based on assessing nucleotide sequence variations and revealing polymorphism can be used to evaluate genetic variation and relatedness. Different DNA marker techniques, such as DNA fingerprinting (DAF) (Caetano-Anolles *et al*. 1995; Assefa *et al*. 1999; Yerramsetty *et al*. 2008), randomly amplified polymorphic DNA (RAPD) (Roodt *et al*. 2002; Etemadi *et al*. 2006), and amplified fragment length polymorphism (AFLP) (Zhang *et al.*1999; Wu *et al*. 2004), have been used in assessing the relatedness of *C. dactylon* accessions. These studies have demonstrated the utility of DNA profiling in assessing the degree of relatedness of *Cynodon* members, but none has focused on assessing variations within cosmopolitan *C. dactylon* accessions except Wu *et al*. (2004).

Sequence-related amplified polymorphism (SRAP) (Li and Quiros 2001) is aimed at preferentially amplifying open reading frames (ORF), which are coding sequences in the genome. It can disclose numerous co-dominant markers with large numbers of polymorphic loci and allow

easy isolation of bands for sequencing. This technique can generate more polymorphic fragments for revealing genetic diversity than the SSR, ISSR and RAPD markers (Budak *et al*. 2004b). Ferriol *et al*. (2003) showed that the information provided by SRAP markers was more consistent with the morphological variability and evolutionary history of the morphotypes than that from AFLP markers. As such, SRAP markers have been widely used in genotype identification, map construction, gene tagging and genomic and cDNA fingerprinting, *e.g*. in diversity analyses on buffalograss (Budak *et al*. 2004a; 2004b; 2004c).

Although previous research has provided preliminary data regarding genetic diversity at the species level, studies that discuss the systematic analysis of the genetic diversity and population structure of *C. dactylon* accessions throughout the world are limited. The aim of this study was to evaluate the genetic relationships between and genetic diversity among *C. dactylon* accessions, collected from 17 countries in 5 continents, based on SRAP markers.

Materials and methods

Plant materials

A total of 57 genotypes including 55 *C. dactylon* (Common bermudagrass) accessions and 2 cultivars (Tifway and Tifgreen) collected from 17 countries in 5 continents (Africa, Asia, South America, North America and Oceania) were used in this study (Table 1). Accessions were originally collected from grasslands, roadsides, seasides and open fields, and each was grown in three 20-cm diameter pots in the greenhouse under uniform conditions at the Tropical Crops Genetic Resources Institute, Chinese Academy of Tropical Agricultural Sciences (Hainan Island).

DNA extraction

Young and healthy leaf tissues (100 mg) of each genotype were collected from at least 2 pots (vegetative clones) of greenhouse grown plants.

Table 1. Geographic origins of 55 *C. dactylon* accessions and 2 cultivars in the present study.

No.	Accession or cultivar	Origin	Year of collection	No.	Accession or cultivar	Origin	Year of collection
1	Aus-01	Australia (Oceania)	2005	30	Vie-30	Vietnam (Asia)	2006
2	Bra-02	Brazil (South America)	2007	31	Vie-31	Vietnam (Asia)	2006
3	Bra-03	Brazil (South America)	2007	32	Vie-32	Vietnam (Asia)	2006
4	Con-04	Congo (Africa)	2008	33	Vie-33	Vietnam (Asia)	2006
5	Con-05	Congo (Africa)	2008	34	Vie-34	Vietnam (Asia)	2006
6	Con-06	Congo (Africa)	2008	35	Vie-35	Vietnam (Asia)	2006
7	Con-07	Congo (Africa)	2008	36	Vie-36	Vietnam (Asia)	2006
8	Con-08	Congo (Africa)	2008	37	Vie-37	Vietnam (Asia)	2008
9	Con-09	Congo (Africa)	2008	38	Zam-38	Zambia (Africa)	2008
10	Con-10	Congo (Africa)	2008	39	Pap-39	Papua New Guinea (Oceania)	2009
11	Col-11	Colombia (South America)	2007	40	Pap-40	Papua New Guinea (Oceania)	2009
12	Cos-12	Costa Rica (North America)	2006	41	Pap-41	Papua New Guinea (Oceania)	2009
13	Cos-13	Costa Rica (North America)	2006	42	Sin-42	Singapore (Asia)	2008
14	Cos-14	Costa Rica (North America)	2007	43	Sin-43	Singapore (Asia)	2008
15	Cam-15	Cambodia (Asia)	2007	44	Chi-44	China (Asia)	2006
16	Cam-16	Cambodia (Asia)	2007	45	Chi-45	China (Asia)	2006
17	Sou-17	South Africa (Africa)	2007	46	Chi-46	China (Asia)	2006
18	Mal-18	Malaysia (Asia)	2006	47	Chi-47	China (Asia)	2006
19	Sri-19	Sri Lanka (Asia)	2007	48	Chi-48	China (Asia)	2007
20	Sri-20	Sri Lanka (Asia)	2007	49	Chi-49	China (Asia)	2007
21	Sri-21	Sri Lanka (Asia)	2007	50	Chi-50	China (Asia)	2008
22	Sri-22	Sri Lanka (Asia)	2007	51	Chi-51	China (Asia)	2008
23	Sri-23	Sri Lanka (Asia)	2007	52	Chi-52	China (Asia)	2008
24	Sri-24	Sri Lanka (Asia)	2007	53	Chi-53	China (Asia)	2008
25	Tha-25	Thailand (Asia)	2006	54	Chi-54	China (Asia)	2006
26	Tha-26	Thailand (Asia)	2008	55	Chi-55	China (Asia)	2006
27	Tha-27	Thailand (Asia)	2008	56	Tifway	(Burton 1966)	2008
28	Indo-28	Indonesia (Asia)	2006	57	Tifgreen	(Hein 1961)	2008
29	Ind-29	India (Asia)	2008	–	–	–	–

Total genomic DNA was isolated following the modified hexadecyltrimethylammonium bromide (CTAB) DNA extraction procedure (Doyle and Doyle 1990). The quality and quantity of genomic DNA were determined visually from the band intensities following standard horizontal electrophoresis on 1.0% (w/v) agarose gels. The concentration was adjusted to 20 ng/μL for PCR amplification.

SRAP reactions

Forty-nine different primer combinations from Shenggong Inc. (Shanghai, China) were tested for PCR for the present study, including 7 forward and 7 reverse primers (Table 2) (Li and Quiros 2001). A total of 49 SRAP primer combinations were screened on 3 randomly selected accessions. Primers were excluded if their banding patterns were difficult to score or failed to amplify in the 3 randomly selected accessions. Of these 49 primer combinations, 15 combinations were chosen for further analysis on the basis of their good amplification capability (Table 3).

Each 25 μL PCR reaction mixture consisted of 80 ng genomic DNA, 0.4 μM primer, 250 μM dNTPs, 1.5 mM $MgCl_2$, 1.5 units of Taqpolymerase (TaKaRa Biotechnology, Dalian, China), and 2.5 μL 1×PCR buffer. The mixture was finally overlaid with 20–30 μL mineral oil. DNA amplification was carried out on a TaKaRa PCR Termal Cycler Dice TM (TaKaRa Biotechnology, Dalian, China) with the following conditions: initial denaturation at 94°C for 4 min, followed by 5 cycles of 1 min denaturation at 94°C, 1 min annealing at 35°C and 30 sec of elongation at 72°C. In the following 30 cycles, 1 min denaturation at 94°C, 1 min annealing at 50°C and 30 sec elongation at 72°C were performed, ending with an elongation step of 10 min at 72°C. The amplified products were kept at 4°C until they were loaded onto the gel. The amplification products were fractionated via 6% (w/v) polyacrylamide gel electrophoresis (PAGE) in 1×TBE (pH 8.0) at a constant 1800 V and room temperature for 3 h and then visualised using the simplified silver staining method (Xu *et al*. 2002).

Data analysis

SRAP bands throughout the gel profiles were scored visually as present (1) or absent (0) at least twice for each accession. Only reproducible and unambiguous SRAP fragments were used for scoring. Data were compiled in a binary data matrix using MS Excel and analysed using the Numerical Taxonomy and Multivariate Analysis System (NTSYS) program, version 2.1 (Exeter Software, Setauket, NY). Simple matching coefficients were computed using the SIM-

Table 2. The forward and reverse SRAP primer information for this study.

Name	Forward primer (3'–5')	Name	Reverse primer (3'–5')
Me1	TGAGTCCAAACCGGATA	Em1	GACTGCGTACGAATTAAT
Me5	TGAGTCCAAACCGGAAG	Em3	GACTGCGTACGAATTGAC
Me7	TGAGTCCAAACCGGTAG	Em4	GACTGCGTACGAATTTGA
Me8	TGAGTCCAAACCGGTAA	Em5	GACTGCGTACGAATTAAC
Me9	TGAGTCCAAACCGGTCC	Em6	GACTGCGTACGAATTGCA
Me10	TGAGTCCAAACCGGTGC	Em7	GACTGCGTACGAATTCGA
Me11	TGAGTCCAAACCGGT	Em8	GACTGCGTACGAATTCAA

Table 3. The 15 SRAP primer combinations used in this study.

No.	Primer combinations	No.	Primer combinations	No.	Primer combinations
1	Me1-Em1	6	Me5-Em5	11	Me10-Em1
2	Me1-Em3	7	Me7-Em7	12	Me10-Em4
3	Me1-Em6	8	Me8-Em8	13	Me10-Em6
4	Me1-Em8	9	Me9-Em1	14	Me11-Em5
5	Me5-Em1	10	Me9-Em5	15	Me11-Em8

QUAL module. Cluster analysis based on GSC using the Nei and Li distance (1979) was performed according to the unweighted pair-group method with arithmetic averaging (UPGMA) in the SAHN module. Principal coordinate analysis (PCoA) was performed to estimate the genetic distances among the major groups using the DCENTER and EIGEN modules of the NTSYS program.

Results

SRAP polymorphism

The 15 primer combinations amplified 439 reproducible fragments ranging in size from 200 to 1800 bp, of which 431 (98.3%) were polymorphic (Table 4). The highest number of amplification products was obtained with the primer combinations Me10-Em1 and Me11-Em8, and the lowest with Me7-Em7, while the average number of bands among the 15 primer combinations was 29.3. The number of polymorphic fragments for each primer combination varied from 24 to 36, with an average of 28.7.

Genetic diversity analysis

The genetic similarity coefficients (GSC) varied between 0.53 and 0.97 with an average of 0.72 among the 57 accessions. The lowest GSC (0.53) was between Chi-47 from China and Con-06 from Congo, which suggests that these were the least related accessions, while the highest GSC was 0.97, detected between accessions Sri-23 and Sri-24 from Sri Lanka, indicating a very close relationship.

The dendrogram grouped the 57 accessions into 7 main clusters (Figure 1) at the 0.73 similarity level, with most accessions from the same geographic locations or nearby regions tending to have high genetic similarity and clustering into the same subgroups or neighbouring subgroups.

Group I included only 1 accession collected from the roadside on Hainan Island, the most southern region of China, while Group II included 3 accessions collected from high elevations of Yunnan Province in south-west China. The GSC values among the accessions within these clusters were very similar, ranging from 0.82 to 0.86. Group III was comprised of 3 Chinese accessions from central China. The GSC in the group ranged from 0.79 to 0.84. Group IV consisted of 2 accessions from Jiangsu Province of east China with GSC of 0.82. Group V consisted of 3 accessions from Hainan Island, the tropical region of south China with GSC of 0.78 – 0.82, indicating close relationships within the group. Group VI included 2 accessions (one accession from South Africa, the other from Zambia), while Group VII contained the largest number of accessions (43), including the 2 cultivars (Tifway and Tifgreen).

Figure 2 presents the distribution of the different accessions according to the 2 principal axes of variation using Principal coordinate analysis. The percentages of variance revealed by

Table 4. Polymorphism detected by 15 SRAP primer combinations among 57 accessions of *C. dactylon.*

Primer combinations	Number of amplified bands	Number of polymorphic bands	Percentage of polymorphic bands (%)
Me1-Em1	26	26	100
Me1-Em3	32	32	100
Me1-Em6	29	27	93.1
Me1-Em8	25	25	100
Me5-Em1	32	28	87.5
Me5-Em5	27	27	100
Me7-Em7	24	24	100
Me8-Em8	26	25	96.2
Me9-Em1	34	34	100
Me9-Em5	31	31	100
Me10-Em1	36	35	97.2
Me10-Em4	31	31	100
Me10-Em6	24	24	100
Me11-Em5	26	26	100
Me11-Em8	36	36	100
Total	439	431	98.2

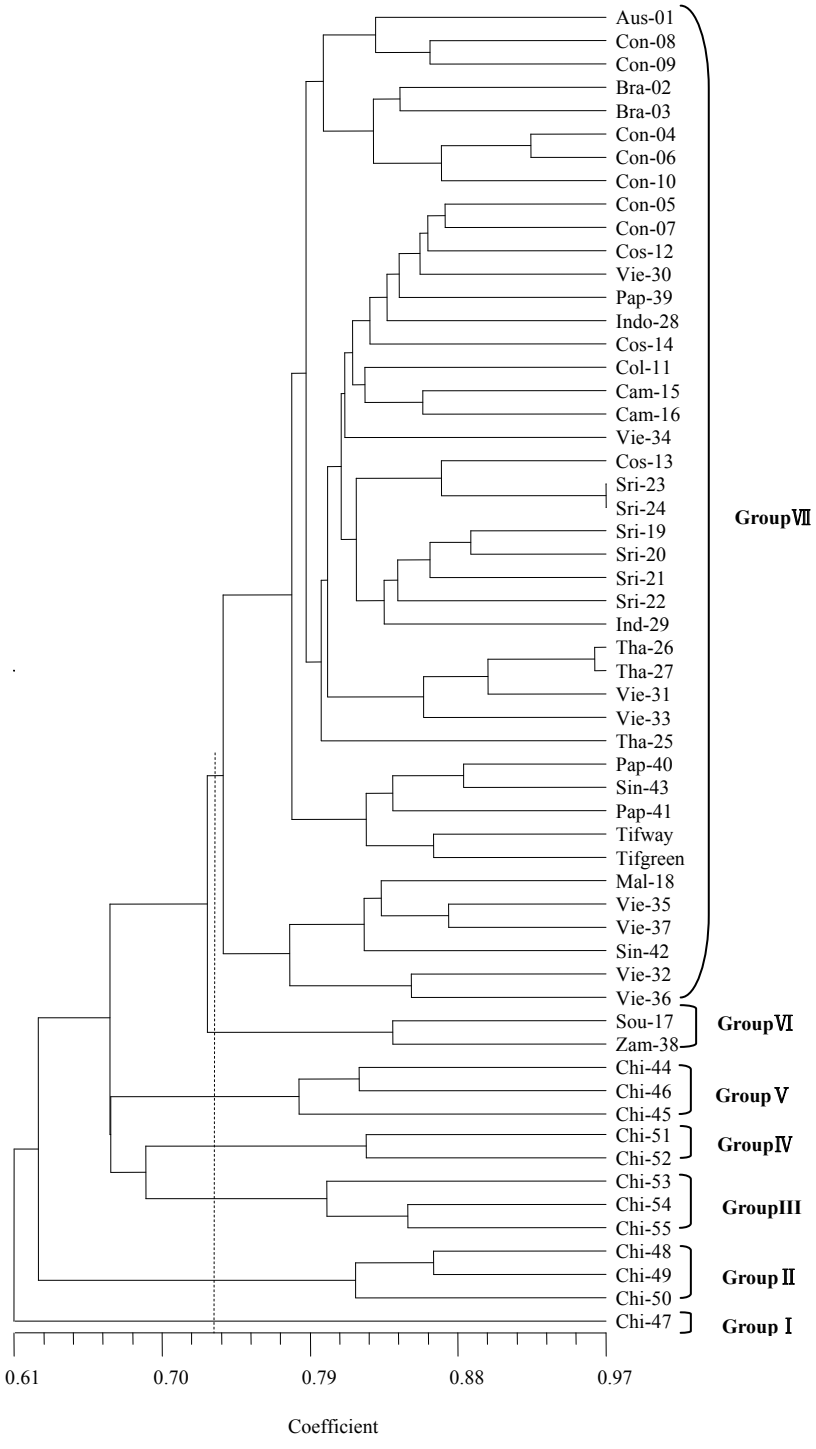

Figure 1. Dendrogram of 57 *C. dactylon* accessions resulting from cluster analysis (UPGMA) based on genetic similarity estimates from the SRAP marker analysis.

PCoA1 and PCoA2 were 73.7 and 3.1%, respectively. The PCoA separated the Chinese accessions into 3 distinct groups, while all remaining 45 accessions were grouped together. The groupings with PCoA were reasonably consistent with those from the cluster analysis. The main difference in groupings between the 2 methods was that cluster analysis grouped 8 accessions from east China, central China and south China into 3 groups, but PCoA clustered these 8 accessions into a single group.

Discussion

To the best of our knowledge, this is the first report where *C. dactylon* accessions collected from a very wide range of its natural distribution were evaluated using the SRAP marker technique. Previous studies examined only 28 accessions collected from 11 countries in 4 continents (Wu *et al*. 2004), while our study included 57 accessions collected from 17 countries in 5 continents. However, our study suffered the limitation that 36 accessions were collected in Asia, only 9 from Africa (7 from the Congo), and 1 from Australia. Wu *et al*. (2004) suggested that germplasm from all major geographic regions where the species occurs should be collected, to fully cover the available variation. Van de Wouw *et al*. (2009) supported this view and suggested that accessions from Australia and south-east Asia would be useful additions to the current germplasm. While our study examined a range of accessions from south-east Asia, it did little to address the shortage of material from Australia. We support the recommendations of Wu *et al*. (2004) and van de Wouw *et al*. (2009) and will continue to collect accessions from Africa, Australia and other major geographic regions where *C. dactylon* occurs for further evaluation.

Our finding, that the Chinese accessions were very distinct from the other accessions tested, forming 5 separate groups, supported the finding of Wu *et al*. (2004) that Chinese accessions were genetically different from accessions of *C. dactylon* from Africa, Europe and Australia. Yerramsetty *et al*. (2008) found wide genetic variation in the cultivars examined by using DAF and found that Chinese accessions grouped separately as well. The consistent findings from these studies indicate that Chinese accessions are unique and represent a distinct and valuable source of germplasm for developing/breeding new varieties of *C. dactylon* for commercial exploitation in China and other areas of the world.

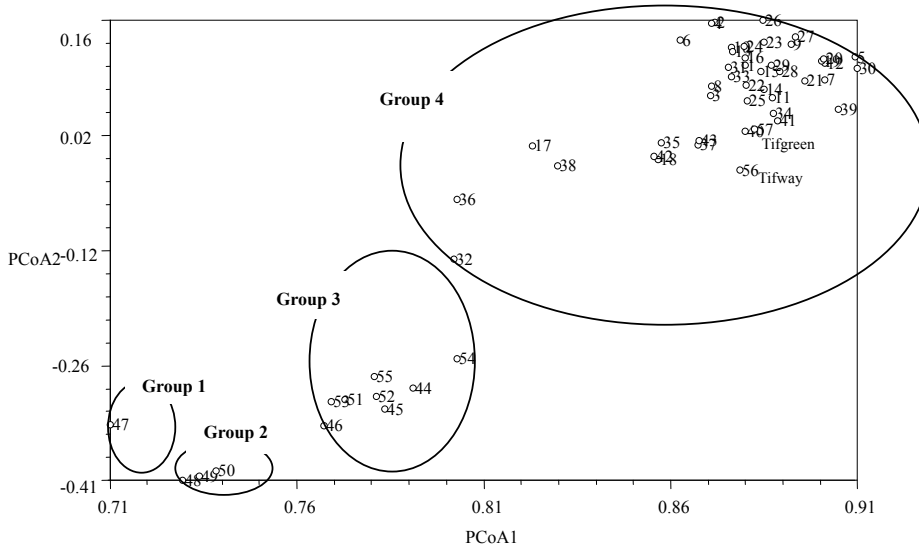

Figure 2. Principal coordinate analysis (PCoA) of 57 *C. dactylon* accessions based on the genetic similarity matrix derived from 439 SRAP markers. Notations: 1–57 were shown in Table 1.

The wide genetic variation in Chinese accessions relative to that displayed by accessions from other regions contrasts with the conclusion of Wu *et al*. (2004) that the greatest genetic variation existed in Africa. The wide genetic variation in Chinese accessions could be a reflection of the distinctly different environments from which they were collected. Chi-47 (Group I), collected from a very dry area of Hainan island, had low growth habit and short, broad leaves, with light leaf and stolon colour, and fine stolon internode diameter; Chi-44, Chi-45 and Chi-46 (Group II) from the seaside of Hainan Island, showed tall growth and long leaves; Chi-48, Chi-49 and Chi-50 (Group III) from very high elevations，showed tall growth, coarse texture and dark leaf colour and leaves were hairless; Chi-51 and Chi-52 (Group IV) from wetlands, had low growth habit, excellent texture and light leaf colour; Chi-53, Chi-54 and Chi-55 (Group V) from a hilly area, had the same stolon stem and spikelet colour, were tall growing, and had coarse texture and light leaf colour.

It was surprising that Chinese accessions were distinctly different from those collected in Vietnam, Thailand, Cambodia, Malaysia and other Asian countries. The main morphological differences were that the accessions collected in Vietnam, Thailand, Cambodia and other Asian countries had taller turf height, longer and wider leaves and longer internode and inflorescence lengths than Chinese accessions, but the Chinese accessions had darker stem and leaf colour. It was even more surprising that the non-Chinese accessions were not clearly separated genetically, which would suggest a high gene flow between continents. As a group, these accessions showed bigger leaves, greater turf height, more spikelets and longer internode length than the Chinese accessions. Chinese accessions had darker stem and leaf colour than the other accessions and were glabrous while other accessions displayed leaf hairs. Possible reasons for the limited variation between accessions from different continents included: firstly, cross-pollination might prohibit the formation of distinctly different genetic populations (Wang *et al*. 2009); secondly, the frequent exchanges of germplasm between different countries or regions could eliminate any differences between continents resulting from natural selection.

Like other molecular marker systems, the SRAP markers seemed to be useful for studying genetic relationships and the reproducibility of the polymorphic bands (Li and Ge 2001). This technique demonstrated high polymorphism (98.3%), which is consistent with previous reports regarding the polymorphism of SRAP markers (Wang *et al*. 2009). Our results add further evidence that the SRAP technique generates highly reproducible DNA profiles for *C. dactylon* accessions. Budak *et al*. (2004b) demonstrated that the polymorphism produced by the SRAP marker technique (95%) was higher than those by ISSR (81%), RAPD (79%) and SSR (87%). The polymorphism demonstrated in this study was similar to that by DAF (97%) (Yerramsetty *et al*. 2008) and higher than that by AFLP (75%) (Wu *et al*. 2004). The high levels of polymorphism may partially be attributed to the following: (a) the *C. dactylon* accessions have their own unique natural habitats since they were collected from a very wide range of its natural distribution; and (b) the SRAP marker technique is designed to amplify ORFs.

Genetic diversity among the 57 *C. dactylon* accessions in the present study, estimated using similarity coefficients, appeared to be relatively high, which is consistent with that observed by Caetano-Anolles *et al*. (1995), Assefa *et al*. (1999), Roodt *et al*. (2002) and Wu *et al*. (2004; 2006). This was supported by the wide separation of clusters obtained from PCoA, reinforcing the conclusions of other workers, *e.g*. Heering *et al*. (1996) and Chandra *et al.* (2004), that multivariate analysis, like cluster analysis and principal coordinate analysis, can be a useful tool in studying genetic diversity. Our study supports the conclusion of Wu *et al*. (2006) that substantial genetic variation existed in Chinese *C. dactylon* germplasm, which might be exploited to produce genetic improvement of the species.

In conclusion, accessions examined in this study were drawn from a wider geographic distribution than earlier studies, but there were limited numbers of samples from some key regions, *e.g*. Africa and Australia. Therefore, any further research on genetic variation in *C. dactylon* should involve extensive germplasm collections, especially in the main geographic regions of its distributional range, to correct these deficiencies. Since accessions from China were genetically different from those collected throughout the rest of this species' natural habitat, a wider sampling of material in this country seems warranted. The SRAP marker technique has advantages in terms

of convenience, good reproducibility and high polymorphism and could be used more extensively in genetics research.

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