Genetic relationships of bermudagrass (*Cynodon dactylon* var. *dactylon*) from different countries revealed by sequence-related amplified polymorphism (SRAP) analysis

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Bermudagrass [*Cynodon dactylon* (L.) Pers.] germplasm is genetically diverse and widely distributed between 45° N and 45° S latitudes in the world. This study was conducted to assess genetic variation and relatedness of 33 Chinese accessions of common bermudagrass (*C. dactylon* var. *dactylon*) and 22 cultivars developed in China, Australia and the USA by sequence-related amplified polymorphism (SRAP) markers. 30 primer combinations generated a total of 274 clearly scorable bands encompassing 268 that were polymorphic. Percentage of polymorphic loci (PPL) for the domestic and introduced accessions was 93 and 83%, respectively. Cluster analysis by unweighted pair-group method with arithmetic averages (UPGMA) based on the polymorphic markers indicated three distinct clusters. Genetic similarity coefficient (GSC) among the genotypes ranged from 0.57 to 0.97. Genetic diversity estimate, He, for the domestic and introduced accessions were 0.26 and 0.24, respectively. The results of this molecular characterization will be valuable for breeding new bermudagrass cultivars in the future.

Key words: *Cynodon dactylon* (L.) Pers., germplasm, sequence-related amplified polymorphism (SRAP), genetic relationship.

INTRODUCTION

Bermudagrass (*Cynodon* sp.) is the most important member of the Cynodonteae tribe and the Chloridoideae sub-family, within the grass (Poaceae) family (Clayton and Renvoize, 1986). Common bermudagrass is widely distributed between 45° N and 45° S latitudes, penetrating to approximately 53° N latitude in Europe (Harlan and de Wet 1969). Evidence from biosystematic studies of *Cynodon dactylon* var. *dactylon* suggested that it was a Eurasian grass until recent times (Harlan and de Wet 1969, Harlan, 1970a, b), and that a geographic area extending from West Pakistan to Turkey was a center of evolutionary activity for the taxon. Harlan (1970a) stated that the aggressive weedy races now widely distributed likely emerged from that center. Although *C. dactylon* (L.) Pers. is widely distributed in China, little information is available regarding the magnitude of genetic variation within the Chinese indigenous *Cynodon* (Wu et al., 2006), especially the genetic variation in Chinese bermudagrass accessions and introduced cultivars of different countries.

Several efforts have been made to examine genetic relationships between bermudagrass accessions and cultivars by using molecular markers to detect polymorphism in DNA extracted from bulked plant samples.

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Zhang et al. (1999) used amplified fragment length polymorphisms (AFLPs) to differentiate bermudagrass genotypes and determine genetic relationships among them, and Karaca et al. (2002) utilized the same marker to detect the genetic diversity among forage Bermudagrass cultivars. Wu et al. (2005, 2006) quantified the genetic variation of Cynodon transvaalenis and its relatedness to hexaploid C. dactylon, and analyzed the genetic relatedness of Chinese Cynodon accessions within and among different ploidy levels by AFLPs. DNA amplification fingerprinting (DAF) has been used to examine the relatedness of Cynodon cultivars from Australia (Ho et al., 1997), to assess the phylogenetic relationships among Cynodon species (Assefa et al., 1999) and hybrid offspring of different species (Caetano-Anollés et al., 1995, 1997), and to analyze the genetic relationships among off-types associated with vegetative propagated cultivars ‘Tifgreen’ and ‘Tifdwarf’ (Caetano-Anollés et al., 1998). Etemadi et al. (2006) demonstrated that randomly amplified polymorphic DNA (RAPD) could detect genetic diversity and certain ploidy levels of bermudagrass accessions, and little correlation was found between morphological characteristics and molecular analysis. Gulsen et al. (2009) demonstrated that different polyploidy Cynodon accessions could have higher diversity by different molecular markers. In China, Liu et al. (2007) and Yi et al. (2008) quantified the genetic diversity of bermudagrass accessions in southwestern provinces by inter simple sequence repeat (ISSR) and sequence-related amplified polymorphism (SRAP) molecular markers, respectively.

SRAP targets open reading frames (ORFs), which is the constitutive part of the functional genes, so SRAP may have more direct relationship with functional genes than other marker systems. As SRAP targets ORFs, and simple sequence repeat (SSR) targets SSRs, a genetic map with high-intensity that reflected the distribution of both ORFs and SSRs could be constructed when the two marker techniques were used together. In addition, when compared with other commonly used molecular markers, SRAP is more stable, reproducible and informative than RAPD and less complicated than AFLP.

When compared with SSR, SRAP primers can be used in almost all plants, which avoid the tedious work for developing the primers. Therefore, SRAP markers system has been used to investigate genetic diversity in plant species, including Brassica (Li and Quiros, 2001), cotton (Lin et al., 2004), turfgrass (Budak et al., 2004a, b), and other plant species. The results show that this marker is homogenously distributed in the genome and could produce higher polymorphism and more abundant information than those from ISSR, RAPD and SSR (Ferriol et al., 2003; Budak et al., 2004a). As for the application of SRAP markers in turfgrass, previous reports mainly focused on the investigations on the relationships among bermudagrass, zoysagrass (Zoysia sp.), centipedegrass [Eremochloa ophiuroides (Munro.) Hack], buffalograss [Buchloe dactyloides (Nutt.) Englem] and other grasses (Budak et al., 2004c; Yi et al., 2008). However, the degree to which bermudagrass accessions or cultivars from different countries or regions are genetically interrelated is unknown. An estimation of genetic diversity in those accessions or cultivars will provide important information for genetic diversification in breeding programs.

The objectives of this research were: (1) to assess the value of SRAP marker system for its ability to distinguish bermudagrass accessions; and (2) to describe the genetic variation of bermudagrass accessions from different countries or regions to study their genetic relationships.

MATERIALS AND METHODS

Plant materials included 55 bermudagrass accessions, including 33 wild C. dactylon var. dactylon accessions and 22 introduced cultivars originating from 4 countries (China, Australia, USA and India). The indigenous Chinese bermudagrass accessions were collected from 19 provinces ranging from tropical Hainan Island to the temperate climatic region around Xinjiang region (Table 1). All indigenous Chinese accessions were determined to be C. dactylon based on morphological characteristics, as described by Harlan et al. (1970a). Distinguishing characteristics of C. dactylon include racemes arranged in one whorl on inflorescences and subequal glumes with at least 3/4 of the length of spikelets.

The bermudagrass accessions were grown in the nursery at Institute of Botany, Jiangsu Province and Chinese Academy of Sciences, Nanjing, China. The seeded or vegetatively propagated bermudagrass cultivars were obtained from the suppliers listed in Table 1. All the wild accessions were collected by gathering healthy stolons from their origins (Table 1). Approximately 8 g healthy, mature and original seeds of seed-propagated cultivars were planted in plots of 1 x 1 m²; and approximately 140 healthy stem segments of vegetatively-propagated cultivars with two internodes were regularly planted in plots of 1 x 1 m². The distances between the plots of different cultivars were 50 cm. The plots of these cultivars were trimmed weekly or biweekly to prevent contamination of different cultivars. The plots were frequently mowed at a height of 50 mm to ensure the turf health and supply newly expanded leaf blades. Necessary irrigation, fertilization and fungicide application were made to ensure healthy turf.

Primer selection, amplification and detection

Genomic DNA of each bermudagrass accession was extracted from fresh and healthy leaves with sodium dodecyl sulfate (SDS) method (Wang et al., 2009). DNA concentrations of the samples were determined by ADNA with a known concentration as a reference, and were finally adjusted to 50 ng µL⁻¹ for PCR amplification (Wang et al., 2009). 90 SRAP primer combinations were applied for PCR amplification and analysis of 55 accessions (Table 2). Primers were excluded from the study if their banding patterns were difficult to score or failed to amplify in all accessions. Of these, 90 primer pairs and 30 primer combinations (Table 3) were selected for their consistent amplifications and clear banding patterns.

The reaction system for amplification was the same as the previously established protocols (Wang et al., 2009). Each 20 µl PCR reaction mixture consisted of 2 µl of 1× buffer (10 mM Tris-HCl pH 8.3, 5 mM KCl), 50 ng genomic DNA, 1.25 mM of MgCl₂, 250 µM of each dNTP (Invitrogen), 0.2 µM primer (Invitrogen) and 1.0 units of Taq DNA polymerase (Promega). The amplifications...
were performed with TC-412 (TECHNE Company, UK). The protocol of PCR amplification was as follows: 4 min of denaturing at 94°C, 1 min of degenerated at 94°C, 1 min annealed at 37°C and 10 s of elongated at 72°C. In the following 35 cycles, the annealing temperature was increased to 50°C, with a final elongation step of 7 min at 72°C. 10 µl of amplified products were fractionated on 8.0% non-denatured polyacrylamide gels using a Hoefer vertical-gel apparatus (JY-SCZ6). Gels consisted of acrylamide (19 acrylamide: 1 bisacrylamide) in 1×TBE buffer (90 mM Tris-boractic acid, 2 mM EDTA; pH 8.0). Gels were 0.75 mm in thickness and 16 × 18 cm in dimension. Electrophoresis conditions were held at 200 V for 2.5 h at room temperature. The gel was then subjected to rapid silver staining for detection (Wang et al., 2009).

Data analysis

Each SRAP band was visually coded as present (1) or absent (0). The distance matrix and dendrogram were constructed using the numerical taxonomy multivariate analysis system (NTSYS-pc) version 2.1 (Exeter Software, Setauket, N. Y.) software package. Genetic polymorphism (P-5%), Nei’s gene diversity (He) and Shannon’s information index were used to compute Nei’s standard genetic distance coefficients (Nei and Li, 1979) and to construct a UPGMA dendrogram within the SAHN module of the NTSYS program (Sneath and Sokal, 1973).

RESULTS

SRAP polymorphisms

The 30 pairs screened from 90 combinations were selected to amplify the genomic DNA of 55 accessions. The PCR products of all primer pairs showed polymorphism, with 6 to 12 polymorphic bands for each primer pair. The size of amplified fragments varied from 100 to 500 bp. Figure 1 shows an example of the results of amplification using primer combination Me4-Em7 with 55 accessions. A total of 274 bands were produced by 30 primer combinations, among which 98% were polymorphic, with 8.9 polymorphic bands per primer combination on average.

Based on the origins of the bermudagrass accessions, they were divided into domestic and introduced
acrossions. The results show that Percentage of polymorphic loci (PPLs) for domestic and introduced accessions were 83 and 93%, respectively, and $H_e$ were 0.24 and 0.26, respectively (Table 4).

Genetic diversity and relatedness

About 268 polymorphic bands were analyzed for genetic similarity coefficient (GSC) among the 55 bermudagrass accessions. The genetic diversity was relatively high among the accessions in this study. GSCs based on the SRAP data ranged from 0.57 to 0.97 from the 55 accessions. The lowest similarity coefficient (0.57) was between C158 and 'Xinnong No.1'. Accession C158 was collected in Shenzhen, Guangdong province of China. 'Xinnong No.1' was introduced from Xinjiang, China. The highest similarity coefficient was 0.97, detected between 'Cheyenne' and 'Pyramid', and 'Common' and C173. The three cultivars ('Cheyenne', 'Pyramid' and 'Common') were introduced from USA. Accession C173 was from Taiwan province of China.

Cluster analysis based on the GSCs separated the 55 bermudagrass accessions into three major groups: A, B and C (Figure 2). The variation patterns of the bermudagrass accessions appear to be associated with geographic origin. 'Wintergreen' and 'Kashi' were found to span the extremes of the dendrogram while the other accessions were distributed in between (Figure 2). Cluster A contained 'Wintergreen' and 'Windsorgreen' (Figure 2).

The GSC between 'Wintergreen' and 'Windsorgreen' is 0.80. 'Wintergreen' and 'Windsorgreen' are from Nyagan, NSW, Australian and 'Windsorgreen' were the offspring of 'Wintergreen' by radiation mutation (McMaugh, 1993).

Cluster B contained 48 accessions, making it the largest group. This cluster consisted of the majority of the wild accessions, which are two Chinese cultivars ('Yangjiang' and 'Nanjing') and 13 USA cultivars (Figure 2). The GSCs in the cluster ranged from 0.57 to 0.97. The 48 accessions were separated into four subgroups: I, II, III, and IV.

Subgroup I contained C134, C134M, C135, C610, C174, C672 and C788. Accessions C134, C135, C672, C174, C788 and C610 were collected from Shanxi, Jiangsu, Gansu, India, Tianjing and Sichuang, respectively. Accession C173 was from Taiwan province of China.

Subgroup II included 15 cultivars and 21 wild accessions. These were scattered into various sub-subgroups.
Figure 1. PCR amplification of bermudagrass genomic DNA from 55 genotypes. Lanes: 1, ‘Wintergreen’; 2, ‘Windsorgreen’; 3, C134; 4, C135; 5, C113; 6, C788; 7, ‘Common’; 8, C173; 9, C174; 10, ‘Cheyenne’; 11, ‘Pyramid’; 12, ‘Sahara’; 13, C177; 14, ‘Riviera’; 15, ‘Guymon’; 16, ‘Panama’; 17, ‘Sydeny’; 18, ‘Yuma’; 19, ‘Mohawk’; 20, ‘Sundevi Il’; 21, ‘Jackpot’; 22, C189-1; 23, ‘Yukon’; 24, ‘Yangjiang’; 25, ‘Tifton 10’; 26, C088; 27, C089; 28, C069; 29, C262; 30, C461; 31, C158; 32, C672; 33, C737; 34, C810; 35, C291; 36, ‘Primo’; 37, ‘Numex Sahara’; 38, C431; 39, C610; 40, C615; 41, C716; 42, C180; 43, C638; 44, C704; 45, C574; 46, C432; 47, C386; 48, C394; 49, C359; 50, ‘Nanjing’; 51, ‘Xinnong No.1’; 52, ‘Kashi’; 53, C224; 54, C224M; 55, C134M; M, 50 bp marker (Promega). One sequence-related amplified polymorphism (SRAP) primer combination, Me4-Em7 was assayed. The DNA samples were fractionated in 8% non-denaturing acrylamide gels stained with silver.

(Figure 2), and the same zone or nearly regions tended to have higher GSCs and cluster into the same sub-subgroups or neighbor sub-subgroups. As shown in Figure 1, most accessions were collected from western, eastern and southern provinces of China, including Jiangsu, Anhui, Shanghai, Guangxi and other neighboring provinces. However, six accessions (C177, C180, C574, C638, C704 and C716), which are from Sichuan, Henan and Hebei, formed sub-subgroups with accessions of eastern or southern regions. The GSC of ‘Cheyenne’ and ‘Pyramid’ cultivars was 0.97. Moreover, the amplified bands from the two cultivars were almost identical as shown in Figure 1. In this subgroup, ‘Yangjiang’ was the mutant C291, which is from Yangjian, Guangdong.

Subgroup III contained C189 (1), C158, C224 and C224M, which were all from southern provinces of China. Accessions C224 were collected from Hainan province, C224M was the offspring of C224 radiation mutation and C158 was collected from Guangdong province.

Subgroup IV only contained C615, which was collected from a football field in Chongqing, China (Figure 2).

Cluster C contained five cultivars, which were
from Oklahoma State University, Stillwater, OK, USA ('Riviera', ‘Yukon’ and ‘Guymon’) and Xinjiang, China (‘Xinnong No.1’ and ‘Kashi’), and the GSCs among the five cultivars ranged from 0.60 to 0.77 and averaged as 0.68.

DISCUSSION

SRAP marked advantages, polymorphism and stability

Currently, several molecular marker systems have been applied to the analysis of the genetic relationships of plants (Botstenin et al., 1980; Tautz, 1989; Williams et al., 1990; Vos et al., 1995). Each PCR-based marker technique has its own advantages and disadvantages. For instance, RAPD provides a simple PCR-based molecular tool for the evaluation of genetic variation, but its poor consistency and low reproducibility limit its use (Roodt et al., 2002). AFLP technology is now widely used for genomic fingerprinting due to its high multiplexing ratio (Vos et al., 1995; Zhang et al., 1999; Karaca et al., 2002). However, AFLP is complex, requires multiple steps, and has pseudo-polymorphism when methylation-sensitive restriction enzymes are used (Li and Quiros,
Genetic diversity of domestic and introduced bermudagrass accessions

Cynodon sp. originated in the old world where centers of diversity are distributed in parts of Africa and Eurasia (Harlan, 1970a), during the course of long-term evolution. C. dactylon (L.) Pers. is widely distributed in the world through natural and man-made-introduction and its aggressive growth characters, such as very strong vegetative propagation and anti-interference capability. A number of researchers have employed RAPD (Roodt et al., 2002), ISSR (Liu et al., 2006), DAF (Anderson et al., 2001), AFLP (Wu et al., 2004; 2006) and SRAP (Wang et al., 2009) to quantify genetic diversity of bermudagrass morphotypes than that found with AFLP markers. Budak et al. (2004c) comparative analysis of buffalograsses based on phylogenetic relationship using ISSRs, SSRs, RAPDs and SRAPs, showed that SRAP marker was homogenously distributed in the genome and could produce high polymorphism and more abundant information than ISSR, RAPD and SSR, and PPLs for SRAP, ISSR, RAPD and SSR was 95, 81, 79 and 87%, respectively. In this study, the PCR reaction mixture suitable for bermudagrass was set up according to the SRAP mixture of Brassica (Li and Quiros, 2001) with a few modifications (Wang et al., 2009). 30 selected from 90 SRAP primer combinations generated a total of 274 clear bands encompassing 268 (PPL = 97%) polymorphic.

Relationship of domestic and introduced accessions

Thirty SRAP primer pairs generated 274 bands, the average number of bands of per primer pair was 9.1 (Table 2). Cluster analysis by UPGMA separated the 55 accessions into distinct major groups: A, B and C. The results show that Group A includes Australian cultivars, that is ‘Wintergreen’ and ‘Windsorgreen’, ‘Windsorgreen’ is the offspring of ‘Wintergreen’ ^60^Co irradiation derivative (McMaugh, 1993). Australia has a unique climate conditions and the effect of the island, so that there are high genetic distance between bermudagrass accessions of Australia and those of China or USA, which could lead them to cluster different groups. Similarly, Wu et al. (2004) observed that accessions originating from Africa, Australia, Asia and Europe were separately clustered based on AFLPs. In this study, five cultivars, ‘Yangjing’, ‘Nanjing’, ‘Kashi’, ‘Tifton 10’ and ‘Xinnong No.1’, were collected from China. However, these cultivars did not belong to a group. ‘Tifton 10’, ‘Nanjing’ and ‘Yangjiang’ were collected from Shanghai, Jiangsu and Guangdong, respectively, which all belonged to the Yangtze River Delta region. Therefore, they had similar genetic background and were clustered together (Group B).
In this cluster, the accession C173 and ‘Common’ have the highest GSC (0.97), the morphological traits of accession C173 and ‘Common’ in the field plots were very similar (data not shown). Accession C173 was collected from Taiwan, with probably mutation of ‘Common’. Caetano-Anollés (1998b) analogously reported that triploid bermudagrass had genetic instability based on DAF and arbitrary signatures from amplification profiles (ASAP). Therefore, common bermudagrass may also easily produce similar phenomenon. Accession C615 in subgroup IV of Group B, may have unique climate in Chongqing, China or escaped out of an unknown cultivar (Figure 2) ‘Cheyenne’ and ‘Pyramid’ in the field were similar in this subgroup, and previous data revealed that they were both seed propagated cultivars (Samudio and Brede, 1998). The field on-spot record indicated that ‘Pyramid’ had excellent texture, delicate leaves and dark green color, but the ‘Pyramid’ used in this investigation had coarse texture and light green color, indicating that this cultivar might be contaminated. ‘Cheyenne’ and ‘Pyramid’ were adjacent cultivated in the nursery, which might give rise to cross-contamination. The GSC of ‘Cheyenne’ and ‘Pyramid’ was also 0.97, which further confirmed that Pyramid had been contaminated. The amplified bands from the two cultivars were almost identical as shown in Figure 1, indicating that this conjecture was reasonable.

In Group C, ‘Rivera’, ‘Guymon’ and ‘Yukon’ are from the same region and showed similar ecotype adaptability and high cold resistant, which led to clustering of different group with other USA cultivars. Yerramsetty et al. (2005) reported that many bermudagrass cultivars (‘Jackpot’, ‘Mohawk’, ‘Pyramid’, ‘Numex Sahara’ and other cultivars) were closely related to ‘Common’ by DAF and MHP-DAF (Minihairpin-DAF) markers. However, ‘Yukon’ was least genetically related to ‘Common’ (Tallaferro et al., 1983, 2003). The five cultivars in Group C all exhibited relatively strong cold resistance (data not reported), and were bred in cold regions. In fact, there is a wide zone existing between Xinjiang and other southern provinces, there is no distribution of bermudagrass, which perhaps resulted in distinct differences between accessions of Xinjiang and the other region on the morphological and molecular of characteristics (data not reported). Therefore, Xinjiang cultivars could solely cluster a group. In this context, Australia’s cultivars (‘Wintergreen’ and ‘Windsorgreen’) also have similar characteristics and led to cluster obviously different groups. Therefore, this showed that there is a wide genetic diversity among genotypes within C. dactylon. The results could be useful for ecotype selection in a breeding program.

The dendrogram resolved by SRAP markers in this study shows that most accessions from the same areas could form a subgroup, but a few accessions were not accorded. For example, there were a few accessions of southeast of China which have not clustered together. This showed that there was incomplete direct relationship between the origin of the accessions and molecular clusters, although the accessions were collected from different regions or discrete genetic differentiation within a region (Table 1). The reasons might be as follows: firstly, this might be genetic overlap occurring in the bermudagrass accessions from two different regions; secondly, this might be as a result of the open pollination behavior of Cynodon plants. Cross-pollination results in gene flow between natural populations, which probably prevents formation of distinctly differentiated genetic groups; thirdly, this might be as a result of the different countries or regions to exchange germplasm resources; lastly, the ploidy might lead to this result. Wu et al. (2006) reported that southeast provinces might be a unique climate type, which are relative to the frequency of occurrence of hexaploid and pentaploid of C. dactylon cytotypes. Gulsen et al. (2009) and Kang et al. (2007) had similar result by analyzing Turkey and Korean Cynodon accessions. On the basis of this study, we will further analyze Cynodon accessions of ploidy, in order to study the depth relationship among them, because ploidy distribution might be due to environmental effects, or the evolutionary and historical development of genotypes (Johnson and Riordan, 1998).

The results from this study show that the SRAP technique measure sufficient polymorphism for DNA typing, and may be a powerful tool for the genetic dissection of the bermudagrass genome. Chinese wild accessions and introduced cultivars all have maintained a broad degree of genetic diversity. Substantial genetic variation still exists within the Chinese bermudagrass gene pool, and selection within the gene pool is still feasible. The genetic relationships and distinctiveness obtained for the bermudagrass accessions in this study based on SRAPs should facilitate the selection of less related germplasm for intercrossing. At the same time, we would integrate molecular marker with many agronomical traits such as turf quality, cold resistance, drought resistance, acid resistance and other resistance, which could be useful to determine optimal breeding strategies, to ensure sustainable breeding programs in the future for various uses such as turf, forage, soil stabilization, and remediation, as well as to understand evolution of this warm season grass.

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Abbreviations

SRAP, Sequence-related amplified polymorphism; PPL, percentage of polymorphic loci; UPGMA, unweighted pair-group method with arithmetic average; ISSR, inter simple sequence repeat; SSR, Simple sequence repeats; RAPD, random amplified polymorphic DNA; GSC, genetic similarity coefficient; ORF, open reading frames; AFLP, amplified fragment length polymorphisms; He, Nei's gene diversity.

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