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Genetic variations in clonally propagated bermudagrass cultivars Identified by DNA fingerprinting

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Abstract

Clonally propagated bermudagrass constitute a major source of turf in the Southern United States. Most of parents for the clonally propagated bermudagrasses were initially introduced from other countries at some time in the past. Few of the cultivars were introduced from China by researchers in the Oklahoma State Agricultural Experimental Station. The objective of this DAF project was to determine the degree of genetic relatedness of these new introduced Chinese cultivars with the existing vegetative cultivars commonly grown in the United States by using DNA fingerprinting technique (DAF). A total of 89 bands were scored using four DAF primers. The cluster analysis was able to distinguish all the varieties studied into 6 distinct groups indicating the existence of wide genetic variations in the cultivars examined. Tifton 10 showed near similarity coefficient to the Chinese cultivars, the cultivar Tifton 10 was directly increased by the plant collection in Shanghai China by Glenn W. Burton. The closer grouping of Tifton 10 with the recently introduced Chinese accessions indicates the existence of similarity of germplasm within China. The cultivar Tifsport a radiation induced mutant from 'Midiron' bermudagrass and Tifway, were the most closely related varieties with an SC of 0.94. The two cultivars Tifsport and Tifway which are morphologically undistinguishable showed a very similar banding pattern indicating these cultivars to be nearly identical. The most distinct cultivars in this study were Midlawn and Quickstand which showed a SC of 0.72.

***Key Words*:** Genetic variation, polymerase, PCR, bermudagrass, primers, fingerprinting

Introduction

Bermudagrasses (*Cynodon* sp.) are long-lived perennials that are widely used for turf, livestock herbage, and soil stabilization in warm temperate tropical and subtropical regions of the world. Most turf bermudagrasses today are vegetatively propagated as sprigs or sod (Beard et al, 1973). There are nine species of bermudagrasses of which only two, *C. dactylon* and *C. transvalensis*, substantially contributes to the gene pools of today's modern-day turf grasses. Many of the of the most widely used bermudagrasses in the southern United States were developed and released in the mid-1950 and 60's originating from interspecific crosses of the diploid *C. transvalensis* Burtt-Davy (2n=2x=18 chromosomes) with the tetraploid *C. dactylon* (L.) Pers. var *dactylon* (2n=4x=36 chromosomes) forming a predominately sterile triploid hybrid (2n=3x=27) (Burton-GW, 1991) (Hanna WW, 1986) (Taliaferro, 1995). The fact that the parentage for these popular early varieties originated from

Table 1. Vegetative cultivars of bermudagrass studied in this project

No	Cultivar or accessions	Species	(2n)	Source and Reference
1	A 12205	Chinese Accession	N/A	Collected in Guangzhou province of China.
2	A 12250	Chinese Accession	N/A	Collected in Nanjing province of China
3	A 12198	Chinese Accession	N/A	Collected in Beijing province of China
4	Baby	Accession	N/A	Bladerunner Farms -NTEP
5	Midlawn	C.dactylon X C.transvaalensis	27	Kansas and OAES
6	OKC 18-4 (Patriot)	C.dactylon X C.transvaalensis	36	OAES, released as Patriot with C. dactylon parent Tifton 10
7	OKC 41-8	C.dactylon X C.transvaalensis	36	OAES , F1 hybrid with C. dactylon parent Tifton 10
8	OKC 70-18	C.dactylon X C.transvaalensis	27	OAES, F1 hybrid with C. dactylon accession from Austrailia
9	Quickstand	C.dactylon	36	Breeder class stock from Dr. A.J Powell, University of Kentucky. A single plant selection
10	Tifgreen	C.dactylon X C.transvaalensis	36	Breeder stock from Dr. Wayne Hanna, Tifton, GA
11	Tifsport	C.dactylon X C.transvaalensis	N/A	Breeder class stock from Dr. Wayne Hanna, Tifton, GA, Radiation induced mutation from Midiron
12	Tifton 10	C.dactylon	54	Breeder class stock from Dr. Wayne Hanna, Tifton, GA From Chinese accessions
13	Tifway	C.dactylon X C.transvaalensis	27	Breeder class stock from Dr. Wayne Hanna, Tifton, GA

germplasm from Southern Africa (Taliaferro, 1995), (Juska ,Hansen 1964) suggests that many of the most widely used vegetatively propagated bermudagrasses grown today may rely predominantly on a narrow geographically restricted genetic base.

Bermudagrasses comprise nine species in the *Cynodon* genus that are widely adapted and distributed across every continents from latitudes 45 North to 45 South (Harlan JR, 1970. Taliaferro, 1995). The diversity within this enormous gene pool is just beginning to be exploited. Exploitation and evaluation of germplasm for the continuous improvement of turf bermudagrasses is an ongoing pursuit by several breeding programs throughout the world. Recently, collections from China have been obtained and evaluated with respect to genetic relatedness with other accessions from around the world (Wu-YQ, et al, 2004) Results indicted that the Chinese accessions constitute a genetically distinct germplasm source when compared to European, African and Austrailian accessions, indicating a Hitherto unexploited source of genetic diversity for bermudagrass improvement. Research needs to be performed to evaluate the genetic relatedness of representative Chinese germplasm with currently grown US varieties.

In recent years molecular techniques have been developed to complement traditional morphological methods (Karp et al, 1997) in evaluating genetic diversity, including amplified fragment length polymorphisms (AFLP) (Vos et al, 1995) DNA amplification fingerprinting (DAF) (Caetano-Anolles et al, 1991) and random amplification of polymorphic DNA (RAPD) (Williams et al, 1990). All of these techniques have their strengths and weaknesses. AFLP is a powerful technique that is able to

distinguish between closely related genotypes, but usually requires expensive reagents, equipment and is fairly labor intensive. RAPD is more a simplified and easily perform PCR based technique, but lacks the discriminating power of DAF or AFLP. DAF is similar to RAPD in its simplicity, but possesses much higher discriminating power, does not require expensive reagents and can be performed with some commonly available lab equipment.

DAF is based on the PCR amplification of DNA fragments from genomic DNA using short 5-8 base oligonucleotide base pair primers (Caetano Anolles et al, 1992). The DAF procedure produces a wide range of amplification products of differing sizes. These products are subsequently separated from each other using polyacrylamide gel electrophoresis and visualized by DNA specific fluorescent dyes to reveal the bar code-like fragmentation pattern. The fragmentation pattern is highly characteristic of the genomic DNA sequence of each individual tested. To increase the resolving power of the DAF a technique that utilizes primers that are designed with a small minihairpin loop structure can be used to produce additional amplification products using previously amplified DAF amplicons as templates (Caetano Anolles et al, 1996). The MHP-DAF procedure dramatically increases the resolving power of the DAF technique in order to separate closely related species. Using both DAF and MHP-DAF in tandem allows for the effective resolution of closely and distantly related genotypes. Comparison of the fragmentation patterns among different genotypes using cluster analysis or bootstrapping methods allows for a clear and reliable determination of the genetic relationships. A better understanding of the genetic relationships among varieties is invaluable in helping turf breeder to develop new and improved cultivars (Caetano Anolles et al, 1997) and to broaden the genetic base of existing cultivars.

DAF has been widely used to study the genetic variations and relatedness of number of crop plants. In bermudagrass it has been used to examine the relatedness of the 18 *Cynodon* cultivars from Australia (Ho et al, 1997), to assess the diversity among *Cynodon* sp. and accessions (Assefa et al, 1999, Caetano Anolles et al, 1995), and hybrid derivatives of two species *C. transvaalensis, C. dactylon* (Caetano Anolles, et al 1995, 1997) and the analysis of genetic relatedness among off types associated with vegetative propagated cultivar Tifway (Caetano Anolles et al, 1997).

In this project we utilized DAF and MHP-DAF to determine the genetic relatedness among representatives Chinese accessions, US cultivars, and promising breeding lines with some Chinese parentage. Results confirm the suggestion that the Chinese varieties are very distinct from the currently grown US varieties.

Material and methods

Plant Materials

Ten bermudagrass cultivars (*Cynodon.* sp.) and three clonal accessions (*C. dactylon* var. *dactylon*) from China were used in the research (Table1). The bermudagrasses were grown in 15 cm diameter pot containing Metro mix 250 (Scotts-Sierra, Marysville, OH), fertilized with Peters Professional Peat-Lite (Scotts- Sierra, Marysville, OH) and Iron Chelate (Miller Chemical and Fertilizer Corp., Hanover, PA) to produce a robust and healthy vegetative growth. The plants were treated with the fungicide Chlorothalonil: [2,4,5,6-tetrachloroisophthalonitrile] (Tradename:Daconil)(Ortho group, Columbus, OH) at a rate of 4.2 ml/L and with [2-Methyl-2-(methylthio)propionaldehydeO-(methylcarbamoyl oxime] (trade name Temik, Rhone-Poulenc Ag Company, Research Triangle Park, NC). to reduce insect and fungal infestations.

DNA Isolation

Two grams of plant tissue was harvested for DNA isolation from leaf tissues. The leaf tissue was frozen in liquid nitrogen and ground in a mortar and pestle to a fine powder. The tissue was powdered and mixed to ensure a 100 mg sample would be representative of the two grams of leaf tissue. Genomic DNA was extracted using the DNeasy plant mini-extraction kit (Qiagen Inc, Valencia CA) as per directions provided by the supplier. The DNA concentration was assessed spectrophotometrically at 260 nm (Beckman Inc, Fullerton, CA) and quality was assessed by the ratio of 260 to 280 nm absorbance readings (Sambrook, 1989). If any of the 13 cultivars had a 260/280 ratio of less than 1.8 the entire batch was repeated for DNA extraction. The DNA was suspended to a final concentration of 5 ng/L in 0.5X TE. DNA quality was further assessed by TBE agarose gel electrophoresis to ensure that extracts showed no signs of DNA degradation.

Table 2. Sequence of the DAF and MHP-DAF and primers used in this study. MHP-DAF primers amplified templates previously amplified using DAF primer 9110 and 9111.

Primer Label	Primer Sequence
DAF 9110	CAGAAACGCC
DAF 9111	GAAACGCC
DAF 9112	GTAACGCC
DAF 9113	GTAACCCC
MHP-DAF 1	GCGAAGCGGA
MHP-DAF 2	GCGAAGCTACG
MHP-DAF 3	GCGAAGCCTA
MHP-DAF 4	GCGACAGCAGA

PCR amplification

Four DAF primers and four MHP-DAF primers (Table 2) were used in PCR amplification reaction mixtures in order to fingerprint the 13 bermudagrass cultivars used in this study. The PCR amplification mixture consisted of a final concentration of 2.5 U of Qiagen *Taq* polymerase (Qiagen Inc., Valencia, CA) 10X PCR buffer which included 1.5 mM MgCl2, 250 µM dNTP, 9 µM DAF primers (Integrated DNA Technologies Inc, AI), and 0.5 ng of template DNA, with the final volume made to 20 µl with sterile Milli Q water. The PCR mixtures were initially denatured at 94^{o} C for 60 seconds, denatured for 94^{o} C for 30 seconds, annealed at 30^{o} C and extended at 72^{o} C for 60 seconds. The program recycled for 39 times with final extension at 72^{o}C for 5 minutes. The PCR products were visualized on a 1% TBE agarose gel impregnated with ethidium bromide. The gel was examined to assure that the overall fingerprint intensity was nearly equal among all lanes. If PCR failed to amplify a fingerprint in any one of the 13 reactions then the entire set was re-run until the fingerprints were near equally amplified. Conditions for MHP-DAF were the same as for DAF except that 1 µL of DAF PCR product was diluted 1: 25 times with sterile distilled water and used instead of the genomic DNA template. Four MHP DAF primers are listed in Table 2 were used to amplify additional PCR fragments from a previously run DAF amplification mix that used either the 9110 or 9111 primers. The MHP-DAF PCR mixture consisted of the same ingredients as the DAF mixture above except that the primers were at 9 µM concentration. The amplification products were processed and analyzed the same way as the DAF amplification products.

Denaturing Polyacrylamide Electrophoresis:

PCR products were separated on a 20 cm long 6% acrylamide denaturing PAGE gel using a Bio Rad Protean II apparatus (Bio Rad, Richmond CA). The gel was made with Long Ranger Acrylamide to increase the resolving power of the separation (Cambrex Bio Science Rockland, Inc, ME), TBE and 7.1M urea. The PCR products was mixed with loading buffer containing bromo phenol blue, and loaded into the gel. Molecular markers were loaded in adjacent lanes. The gel was run 80 volts until the blue strain reached three-quarters of the length of the gel. The gel was removed and stained with SYBR gold staining solution (FMC Bioproducts, Rockland, ME) according to manufacturer directions, and photographed with a Bio Rad Gel Doc System.

Data Profiling and Analysis:

Electrophorectic bands of less than 1.5 kD were scored for their presence (1) or absence (0). The data was compiled in a Excel spreadsheet and imported into the NTSYS software version 2.0 (Exeter Software New York, NY) for cluster analysis. Similarity Coefficeients (Table 3) were computed by the SIMQUAL module. Cluster analysis was performed according to the unweighted pair group mean algorithm (UPGMA) within the SAHN module of the NTSYS program. The PCR reaction, electrophoresis separation, staining of gels, data profiling and analysis was replicated two to three times. Comparisons showed that there were either no differences, or only very minor differences, between replicated experiments.

Table 3. Similarity Coefficients for combinded DAF and MHP-DAF analysis

	A 12205	A 12250	A 19198	Baby	Midlawn	OKC 18-4	OKC 41-8	OKC 70-18	Quickstand	Tifgreen	Tifsport	Tifton 10	Tifway
A 12205	1.000												
A 12250	0.796	1.000											
A 19198	0.722	0.800	1.000										
Baby	0.522	0.578	0.607	1.000									
Midlawn	0.526	0.544	0.596	0.700	1.000								
OKC 18-4	0.674	0.693	0.744	0.596	0.607	1.000							
OKC 41-8	0.670	0.711	0.719	0.607	0.611	0.841	1.000						
OKC 70-18	0.652	0.693	0.767	0.648	0.630	0.793	0.811	1.000					
Quickstand	0.574	0.667	0.630	0.630	0.663	0.611	0.615	0.648	1.000				
Tifgreen	0.563	0.611	0.604	0.870	0.704	0.615	0.641	0.659	0.611	1.000			
Tifsport	0.578	0.544	0.596	0.559	0.644	0.622	0.604	0.615	0.544	0.607	1.000		
Tifton 10	0.685	0.689	0.719	0.585	0.589	0.781	0.748	0.678	0.667	0.589	0.641	1.000	
Tifway	0.570	0.552	0.589	0.574	0.644	0.630	0.619	0.615	0.574	0.622	0.933	0.656	1.000

Results and Discussion

DAF and MHP-DAF were used to determine the genetic relationships among 10 turf bermudagrass cultivars and three clonal accessions from China. A total of 89 and 181 bands for a total of 270 bands were scored for DAF and MHP-DAF, respectively. Of the 270 total bands 97% were polymorphic meaning that the band was present in at least one cultivar but lacking in others. DAF and MHP-DAF were able to differentiate all the cultivars based on their DNA fingerprint patterns with an overall average similarity index of (0.64). Because both DAF and DAF-MHP procedures showed very similar and consistent results we elected to combine both analyses. Genetic diversity among the selected varieties in this study was greater than that shown in a recent study of seeded varieties grown in the United States (Yerramsetty et al, 2005).

The combined results separated the cultivars studied into 4 distinct groups (Figure 2) consisting of: Group 1 A12205, A12250, A19198; Group 2 OKC 18-4, OKC 41-8, OKC 18-4; group 3 Baby and Tifgreen, Group 4 Tifsport and Tifway. In addition, three cultivars t were quite distinct from either of the four groupings, namely: Tifton 10, Midlawn, and Quickstand. Simialarity coefficients for each of these distinct cultivars averaged 0.67, 0.62, 0.62, respectively. Quickstand and Midlawn were the most distinct cultivars in this study.

Group one consisted of three accessions from China. These accessions were collected from the northern province of Beijing (A12198), the mid latitude coastal province of Nanjing (A12250) and the southern coastal province of Guangzhou (A12205). The overall average similarity coefficient was 0.77 for group one cultivars. There were 4 bands present in all three group 1 cultivars, but not detected in the rest. Group two consisted of three *C dactylon* x *C. transvalensis* cultivar hybrids, OKC 41-8, Patriot, and OKC 70-18 with an average group similarity coefficient of 0.81, all products of the Oklahoma State breeding program. Typically, hybrid bermudagrasses consist of multiple genomes with two genomes coming from the *C. dactylon* and one from *C. transvalensis* parent. The exception to this was Patriot which has three genomes from *C. dactylon* and one from *C. transvalensis*. Two of the three cultivars (Patriot and OKC 41-8) have Tifton 10 as a common *C. dactylon* parent. Tifton 10 originated as a selection by Dr. Burton from an earlier Chinese collection (Burton GW, 1991). The other cultivar in the group was 70-18 which is a F1 hybrid with the *C. dactylon* parent coming from Australia. The origin of the Australian parentage is unknown, but (Wu et al 2004) in a large AFLP fingerprinting study of Chinese accessions, found that Chinese accessions grouped most closely with accessions from Australia

than any others tested. This may explain why 70-18 with Australian parentage showed such close association to Patriot and OKC 41-8 which have substantial Chinese parentage. Group two had two polymorphic bands not detected in any other cultivar tested.

Group three consisted of the two closely related cultivars: TDS –BM1 known as 'Baby' and Tifgreen, with a similarity coefficient of 0.87. According to the patent application, the original selection for Baby was found in a home lawn in Las Cruces, N. Mexico. The patent originator listed Baby as a *C. dactylon* (Plant patents 9,976). Tifgreen is a natural triploid hybrid between *C. dactylon* and *C. transvalensis* which was expanded from an original selection from a golf course in North Carolina by Dr. Burton and released in 1956, becoming one of the premier industry standards for many years (Burton GW, 1991). Our DNA fingerprinting analysis indicated a close relationship between the triploid hybrid Tifgreen and Baby. The close genetic relationship along with morphological observations suggests that Baby may not be a *C. dactylon* afterall, but a variety more closely related to vegetative varieties such as Tifgreen. Three polymorphic bands were found that distinguishes Baby from Tifgreen and any other cultivar tested. The orginal patent application quotes a RAPD fingerprinting study where one marker distinguished the two cultivars (patent application).

Group four cultivars consisting of Tifsport and Tifway were the most closely related cultivars tested with a similarity index of 0.93. The triploid.hybrid Tifway is a natural chance hybrid developed by Dr. Burton (Burton, 1966), while Tifsport is reported to be a gamma radiation induced mutant from Midiron. Midiron was originally developed by Dr. Ray Keen (KSU) for high levels of cold tolerance. In the present DNA fingerprinting study, Tifsport was very closely related to Tifway. In a previous study looking at genetic background and spring dead spot resistance we found very similar results in that Tifsport was closely related to Tifway and quite distant from Midiron (Data not shown). Furthermore, an AFLP fingerprinting study of many triploid hybrid bermudagrasses, (Zhang et al, 1999) found that Tifway and Tifsport were also very closely related. The predominant evidence clearly indicates that Tifsport is much more closely related to Tifway than to Midiron. Producing new varieties by irradiation is an effective method for improving turfgrass when parent stock cannot be hybridized due to triploid

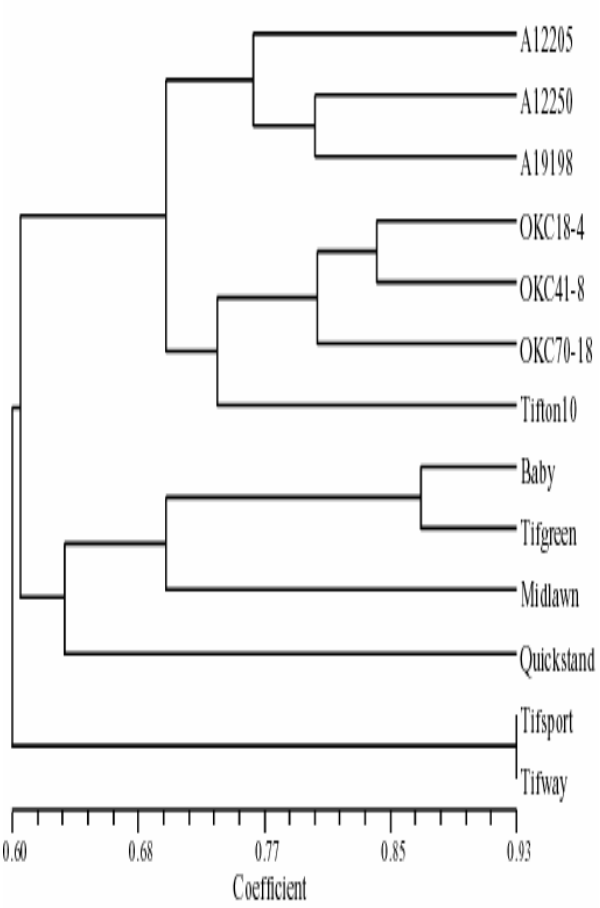
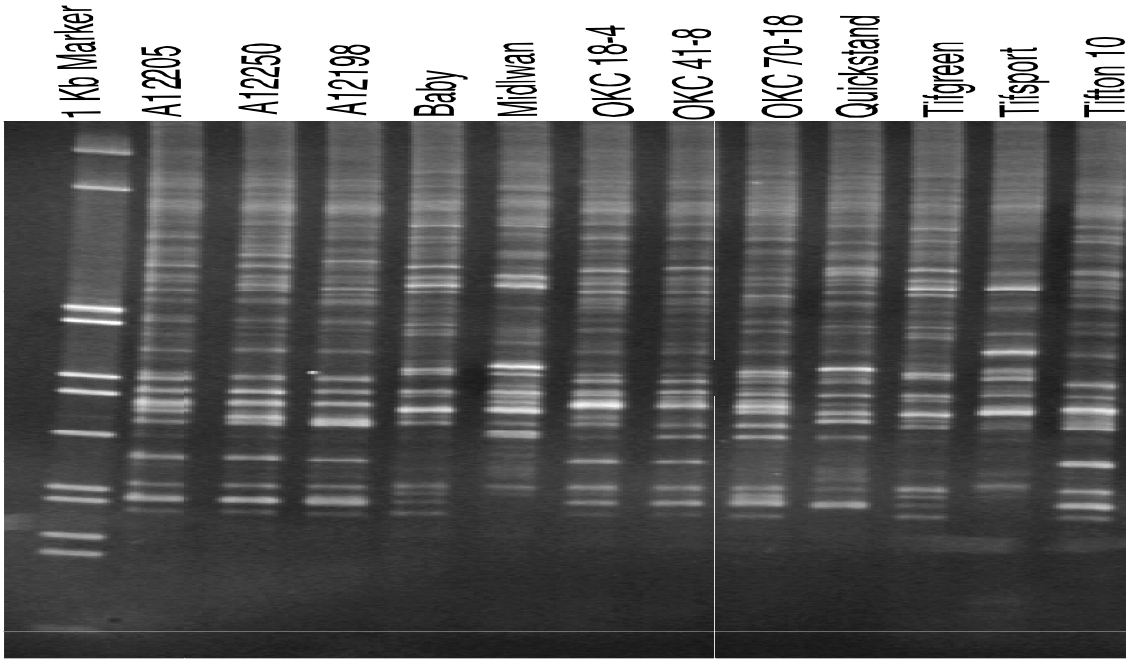


Fig 1. Dendrogram from DAF and MHP-DAF analysis of 13 vegetative cultivars of bermudagrass.

induced near sterility or poor seed set (Powell et al, 1974). How this differentiation between Tifsport and Midiron arose is not known, but can be due to genetic instabilities inherent in certain lines of vegetative propagated bermudagrasses (Caetano-Anolles, 1999), contamination of early genetic stocks or mistaken identity of original parental stock.

Midlawn and Quickstand and Tifton 10 were not closely related to any of the four groupings discussed above nor to each other. Midlawn developed by Kansas Experiment Station was originated as a natural interspecific sterile hybrid between tetraploid *C.dactylon* and diploid *C.transvaalensis*. Limited pedigree information for Quickstand indicates that it was found as a single plant selection growing in a field of 'Common' bermudagrass (Dennis Martin



personal communication). Both cultivars show high levels of cold tolerance. Tifton 10 as mentioned above came from a collection of Chinese varieties by Dr. Burton. Average similarity index for each were 0.62, 0.62, and 0.67, for Midlawn, Quickstand and Tifton 10, respectively. All three had 8, 9, and 5 bands present in each cultivar but absent in others.

The fact that the Chinese accessions grouped separately is indicative of their distinctness from the US cultivars tested in this study. In this study we find that the Chinese accessions were very distinct from the other cultivars grown in the USA. (Wu et al, 2004) examining the genetic relationship with 121 Chinese accessions found that they grouped very distinctly from collections from African, European and Australian bermudagrasses as well. These studies support the notion that the Chinese accessions represent a distinct and valuable source of germplasm for variety development which should be used to increase the genetic diversity of bermudagrasses used in the USA.

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