DNA FINGERPRINTING OF SELECTED CULTIVARS OF BERMUDAGRASS

By

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Master of Science

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1999

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December 2004

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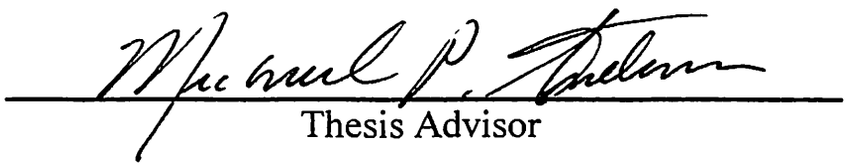
CULTIVARS OF BERMUDAGRASS

Thesis Approved:



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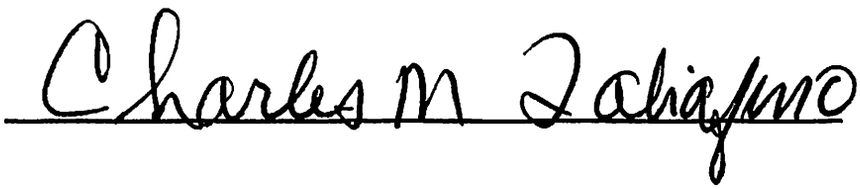

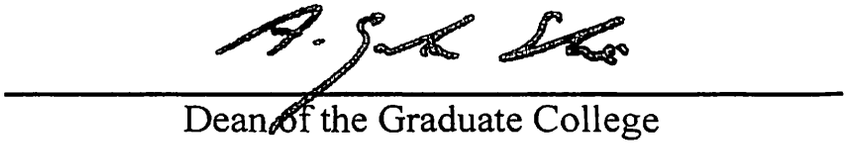

Dean of the Graduate College

PREFACE

This research was conducted to study the genetic variations among selected turf bermudagrass cultivars using the DNA Fingerprinting (DAF) technique. Two groups of cultivars were studied based on their reproductive mode i.e. whether propagated sexually or vegetatively. The research provides needed information on the genetic relatedness of current commercial cultivars and can be used to guide future programs in germplasm acquisition and breeding improvement.

Research was also undertaken to assess DNA profiles of turf bermudagrass cultivars differing in susceptibility to spring dead spot disease caused by *Ophiosphaerella herpotricha* using the DAF procedure. Results are anticipated to be useful in developing SDS disease resistant bermudagrass cultivars.

ACKNOWLEDGEMENTS

I am honored to have Dr. Michael Anderson as my major advisor. I wish to extend my sincere thanks and gratitude for his excellent supervision and innovative research ideas. His efforts and patience in imparting the research techniques and improving my scientific writing skills are very much appreciated.

I owe the credit of completing my research successfully to Dr. Charles Taliaferro. His guidance was an asset for me. Special thanks for the generous financial support he has been extending through out the completion of my graduate study. My special thanks to Dr. Dennis Martin for his valuable suggestions in this research work.

I sincerely thank Carole Anderson, for technical assistance. Thanks to colleagues Janice Hironaka and James Enis for their tremendous help in troubleshooting the experiments. Special thanks to Madhavi Dhulipala for sparing her valuable time in assisting me to score the gels. I also wish to acknowledge Gary Williams, Sharon Williams and Rose Edwards for maintenance of plants at the green house.

My efforts have been successful due to the constant encouragement of my mother Mrs. Sujatha and continuous support of my father Mr. Anju Babu. Thanks to my brother Vamsee Krishna for his moral support. Special thanks to my family members Mr and Mrs. Kamalavathi Prakasa Rao, Mr. and Mrs. Sakuntala Hanumantha Rao. Mr & Mrs. Manjula Durga Prasad, Mr. & Mrs. Lakshmi Mohan Rao

Thanks to my former principal investigator Dr.J.S Bentur, Dept of Entomology and Dr. N.P Sarma, Head Dept of Biotechnology from Directorate of Rice Research India, for encouraging me in every step in coming to United States for higher education.

Dedicated to my mother Mrs. Sujatha

FORMAT OF THESIS

This thesis is presented in the style of publication in the Crop Science journal and the format is divided into three independent chapters (Chapter 1, 2 and 3). Each chapter is complete in itself having abstract, introduction, materials and methods, results and discussion and literature cited sections in addition to tables and figures.

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Chapter 1

Introduction and Literature Review

History of bermudagrass:

Grasses currently used for turf began to evolve during the Cretaceous and early Tertiary times which date back 70 million years (Harlan, 1956). From historical times humans have been using grasses to beautify royal residences and gardens. One of the earliest uses of turfgrasses was in the garden carpets of King Chosroes of Persia in the fifth century AD. During the rule of the Moguls in the mid 1500's in India turf was used in landscaping the Taj Mahal (Goethe, 1955). In China, turf was used by the emperors in their pleasure gardens and parks (Malone, 1934). The first golf courses were established in Scotland in the 12th century utilizing a grassy playing surface. In more modern times the worlds first turf research was initiated at the Olcott turf gardens in Connecticut in 1885 (Olcott 1890). The United States Golf Association (USGA), a major promoter of 'the game', established its Green Section in 1920 to develop and promote advisory and

research programs on turfgrass varieties. Today the turfgrass industry is a $ 40 billion annual business utilizing a wide range of grass species for human enjoyment (NTEP,2004).

The exact date when bermudagrass was first introduced into the US is not clear but one of the earliest records is from the diary of Thomas Spalding, owner of Sapeloe Island, Georgia. The entry found in his dairy states that "Bermudagrass was brought to Savannah in 1751 by the then Governor Henry Ellis". (Burton and Hanna, 1995) Carolina farmers used the grass as forage calling it crop grass. By the 1920's, with the wide emergence in the game of golf, bermudagrass was first planted on golf courses in Florida. The first recognized turf bermudagrass variety in United States was called St. Lucie (Tracy, 1917) which was a fine leaved dwarf variety. This variety had little cold tolerance and could not be used too far north due to an intolerance to low temperature conditions. Arizona grown common bermudagrass emerged from Arizona growers around the 1920s and was increasingly popular in Florida golf courses. In the early 50's the development of the first hybrid bermudagrasses (*Cynodon dactylon* X *C. transvaalensis*) occurred. The first hybrid to be developed was 'Tiffine' in 1953. Since that time, many hybrid and seeded bermudagrasses have been developed by bermudagrass breeders. The cultivars 'Tifway' in 1960 and 'Tifdwarf' in 1965 were developed from the experiment station at Tifton, Georgia. These two

varieties, which remain among the best grasses for golf courses, were developed by Dr. Glenn Burton and colleagues. Dr. Burton was a leader in turfgrass research and development for many years with the USDA-ARS, and as a Professor in Agronomy at University of Georgia. He has to his credit bred and improved 40 grass cultivars, many among the highest quality cultivars today. (Barnes et al., 1995)

Taxonomy of Bermudagrass

The genus *Cynodon* was revised by Harlan et al. (1970). He classified it into nine species and 10 varieties. Those used for turf belong to the taxons *C. dactylon, var. dactylon, C. transvaalensis, C. incompletus,* and *C. magennisii* with the most important being *C.* dactylon and *C.* transvalensis. The few other taxa which are of limited importance as turf are *C. arcuatus, C. barberi and C.dactylon var. Polevansii* (Taliaferro, 1995).

Table 1. Taxonomic classification of the genus Cynodon

Epithet	Chromosome Number	Distribution
C. aethiopicus Clayton Et Harlan	18, 36	East African Rift Valley
C. arcuatus J.S. Presl. Ex C.B. Presl.	36	Malagasy, and Southern India to Northern Australia
C. barberi Rang. et Trad.	18	Southern India
C.dactylon (L) Pers. var dactylon	36	Cosmopolitan
var. afghanicus Harlan et de Wet	18, 36	Afghanistan steppes
var. aridus Harlan et de Wet	18	Southern Africa northward to Palestine; east to South India
var. Coursii (A. Camus) Harlan et de Wet	36	Madagascar
var. elegans Rendle	36	Southern Africa, south of latitude 13° South.
var. polevansii (Stent)	36	Near Barberspan, South Africa
C. incompletes Nees var. incompletes	18	South Africa; Transvaal to Cape
var. hirsutus (Stent) de Wet et Harlan	18, 36	South Africa, Transvaal to Cape
C. nlemfuensis Vanderyst var. nlemfuensis	18, 36	East Africa
Var. robustus Clayton et Harlan	18, 36	East Tropical Africa
C. plectostachyus (K.Schum.) pilger	18	East Tropical Africa
C. transvaalensis Burtt - Davy	18	South Africa
C. X magennisii Hurcombe	27	South Africa

Characteristics of Bermudagrass

Bermudagrass, *C. dactylon* var. *dactylon,* is cosmopolitan in distribution throughout the world from 45 ° north latitude to 45 ° south latitude. It is a perennial sod forming turfgrass and forage grass native to India and Eastern Africa (Beard, 1973; Braun, 1967; Correl and Johnson, 1970, Duble, 1996). In contrast to its utility, bermudagrass has been identified as a weed in disturbed habitat (Harlan, 1969). Bermudagrass cultivars are clonally or seed-propagated and adapted to many temperate areas around the world, including the United States. The replacement cost of bermudagrass in Oklahoma alone was estimated to be around 1.7 billion dollars (Duble 1996) which indicates its wide spread economic importance to the state. Bermudagrass thrives well in diverse soil types and it is highly resistant to wear and tear and recuperates rapidly from injuries. It is a very durable, drought resistant, salt tolerant species which spreads rapidly by above ground stolons and underground rhizomes. Bermudagrass is also reported to possess medicinal value as an astringent, antiseptic, emollient. The leaves and roots of bermudagrass have been used as a folk remedy for treating cancer, cough, cramps, diarrhea, dysentery, epilepsy, head ache, etc.

The development and production of seed propagated bermudagrass cultivars has markedly increased over the past two decades. The increase is evident from the higher number of seeded entries in the National Turf

Evaluation Program (NTEP) from 28 entries in 1986 to 42 entries in the 2002 NTEP test. Interest in seeded cultivars when compared to vegetative cultivars is driven by the economics of reduced seed establishment cost versus clonal production (Evans, 2003).

The increased use of bermudagrass displaced some perennial ryegrass (*Lolium perenne*) use. Perennial ryegrass is susceptible to wide range of fungal diseases like: brown patch, caused by *Rhizoctonia solani*; Pythium disease by *Pythium aphanidermatum*; and gray leaf spot caused by *Pyricularia grisea*. Bermudagrass hybrids have become a better choice in some instances in place of ryegrass because bermudagrasses are resistant to these diseases (Williams and Burrus, 2001). Further, the drastic increase in the number of golf courses in the United States has fueled the rapid increase in demand for improved bermudagrass cultivars.

Development of Improved Bermudagrasses

"The collection and widespread distribution of *Cynodon* germplasm during the first half of the century has provided the raw materials from which superior turf cultivars were derived during the past 50 years"(Taliaferro, 1995). Several hybrids have been developed with improved agronomic and turf characters. Hybrid bermudagrasses are generally sterile producing seed heads with few or no seed. Since most vegetatively propagated cultivars are either completely or highly sterile, further genetic improvement through sexual hybridization of these cultivars is not possible. Hence new techniques like physical or chemical mutagens are used for improving the vegetative bermudagrass cultivars (Powell et al., 1974). Cultivars like Tifsport been developed by these artificial mutation methods with the objective of improving cold tolerance. Natural somatic mutations also occur and are useful for bermudagrass improvement.

Turf breeders have been providing a steady stream of new hybrids as well as improved seeded cultivars with desired characters. The production of seeded bermudagrass cultivars started in Imperial Valley of Arizona around 1920. The commonly grown seeded cultivars here were together referred as Arizona common bermudagrass. This was increasingly popular in Florida golf courses till the 1950's. Since then seeded type bermudagrass usage has been dramatically increasing due to its. Interest in seeded varieties remains

high due to the reduced costs shipping and handling and seed establishment versus clonal production.. To meet the increasing demand of seeded bermudagrass cultivars, the number of seed propagated commercial cultivars has dramatically increased over the past two decades. The NTEP 2002 results indicate that some of the recently developed seeded-type bermudagrass cultivars rival the clonal standard bermudagrass cultivars in quality and other performance characteristics. 'Yukon' exhibits excellent cold tolerance and exceptional level of resistance to SDS caused by *Ophiosphaerella herpotricha* (Fr.) Walker (Martin et al., 2001). The recent release of 'Riviera' is a breakthrough in cold tolerance, quality of turf and seed yield among bermudagrasses.

Evaluation of Improved Cultivars

The National Turf Evaluation Program (NTEP) is the most prestigious program for evaluation of turfgrass species in the world. Turfgrass professionals rely on NTEP data for assessing adaptation and performance characteristics of turfgrass cultivars. The information summarized by NTEP is currently used in thirty countries. (Morris and Shearman, 2004). NTEP data are widely used by turfgrass breeders, researchers and extension specialist to determine adaptation and use of cultivars and experimental lines; Seed companies use NTEP data for sales and advertisement; Government agencies use the NTEP data for placing specifications for bids and purchases; and end users like golf course superintendents, sports turf managers, sod growers, lawn care service operators and ground mangers frequently use the data for evaluating which varieties to use. (Morris and Shearman, 2004).

The most common method of assessing turfgrass quality is by a visual rating system based on the turfgrass evaluator's judgment. Various factors like turfgrass quality, genetic color, turf density, percent living ground cover, turf texture, spring green up, winter color, pest problems, environmental stress, drought stress, winter injury and traffic tolerance are considered in the evaluation process. The performance of a cultivar is measured on a scale using the 1 to 9, where 1 represents poor and 9

represents excellent performance. The results of these evaluations are released in the NTEP official reports (NTEP 2004).

Spring Dead Spot Disease of Bermudagrass

Spring dead spot disease (SDS) is the most common and destructive disease of bermudagrass in the U.S. transition zone and many other similar climatic regions in the world. The disease is characterized by formation of unsightly circular patches of dead turf which ranges from few centimeters to one meter in diameter (Wetzel et al., 1999). Several fungicides are labeled for the control of SDS, but disease control has often been inconsistent (Baird et al., 1998). SDS is undesirable because of the necrotic areas that typically appear in the spring and early summer are visually bothersome to those trying to maintain a beautiful and uniform appearance. Weeds invade these dead areas and persist giving a very bad look to the bermudagrass fields. This is especially true for athletic fields and golf courses where the visual quality of the turf is highly valued. Moreover, the cost and labor for sprigging the spotted areas or killing the invading weeds is high.

SDS was first observed in Stillwater Oklahoma in bermudagrass lawns in during the spring of 1954 by Wadsworth and Young (1960). They were the first to characterize the symptoms of the disease, host range, and possible causal organism, and the first to attempt to control the disease (Kozelnicky,

1974). The following observations were made in their study during early 1970, but few hold true today.

- SDS is observed only on bermudagrass
- SDS is not associated with any particular soil type
- SDS appears more often on intensely maintained bermudagrass.
- Researchers cannot reproduce the symptoms
- The causes of SDS are unknown.

Since 1954, SDS has continued to be found throughout Oklahoma on lawns, golf courses and many other public and private turf areas. Spring dead spot has subsequently been found on buffalograss (*Buchloe dacyloides*) by Tisserat et al., 1999). Additionally, the symptoms of SDS have been reproduced by a number of researchers in the field and greenhouse by using oats infested with *O. herpotricha* and using it as inoculum for SDS field innoculations (Baird et al., 1998 and Martin et al., 2001)

In the fall, when the fungus is actively infecting the tissue, no obvious symptoms of SDS are apparent. It is presumed that most of the damage occurs while the grass is dormant during late fall and winter. Spring dead spot infection during this period are thought to predispose the grass to low temperature stess and destruction.

Partially tolerant varieties have been identified. During early 1960's SDS was known to occur on varieties including: 'U-3', Common, Tiffine

and Tifgreen, with the most extensive damage seen on U-3 (Wadsworth and Young, 1960). Today more tolerant varieties such as: Midlawn, and Yukon are available to greatly reduce but not eliminate the effects of stress. With few exceptions, varieties that are tolerant to SDS are also tolerant to low temperature stress, underscoring the involvement of low temperature stress in disease development.

When SDS was first identified, control of SDS was initiated by the use of chemicals. Wadsworth (1961) indicated that the insecticide Dieldrin was able to control SDS. Two bermudagrass lawns at Stillwater were studied during fall 1965 using eight chemical treatments in which the Dieldrin treatment was found to be more effective (Wadsworth et al., 1967). Studies at Georgia (Wilcoxen, 1976) indicated that the combination of the chemicals, Actidione - Thiram and Daconil 2787 at the rate of three oz per 1000 square feet showed great reduction in number of diseased spots. With a five year study Kozelnicky (1974) proposed the following to reduce the incidence of SDS

1. Nitrogen supply should be minimum
2. Thatch should be controlled
3. Soil compaction avoided.
4. Sensible use of water
5. Timely application of fungicides (early spring)

Various other factors influence the cause and spread of SDS. Higher occurrence of SDS was reported by use of more nitrogen application in summer and fall (Lucas, 1980). Martin et al., (2001) found increased SDS patch area when a higher mowing height was used to manage 'Mirage', 'Jackpot' and 'Yukon' bermudagrasses. Martin (personal communications, 2004) indicated that this increased SDS severity may be linked to increased thatch production at a higher mowing height.

Identification of Causal Agents of Spring Dead Spot Disease

Extensive research was conducted to identify the causal agent of SDS. During the initial studies which focused on the factors leading to disease development, Madison (1970) proposed that close mowing, high fertility, thatch and high traffic are factors that favor SDS development. In Georgia, Kozelnicky et al. (1967) collected the SDS affected roots of bermudagrass and cultured them on water agar and hempseed agar. The most frequently isolated fungal genera were *Rhizoctonia, Pythium, Helminthosporium, Curvularia*, and *Gliocladium*. Dale (1979), in Arkansas, isolated various fungi from seedlings grown in SDS infested soil, and roots of plants growing at the periphery of SDS infected area. He predominantly isolated *Helminthosporium* with occasional occurrence of genera *Polymyxa*, *Olpidium* and *Pythium* in the SDS infected areas. He was not able to

determine the relationship of SDS with any of these fungi but concluded that these fungi could weaken and predispose bermudagrass to winter injury or outright kill bermudagrass plants. The repeatedly isolated fungus from SDS infected soil from Oklahoma was of the species *Helminthosporium spiciferum* (Wadsworth and Young, 1960). Conversely the least isolated fungus from California samples was *H. spiciferu and* most frequently isolated fungi was from the genus *Ophiobolus.* At this point the evidence indicated that causal agent of SDS was not firmly established.

Worf, in (1986) identified a dark ectotrophic fungus in association with rotted crown and roots. He reported formation of necrotic rings and patches and initially named it as necrotic ring spot. He was able to isolate the fungus on potato dextrose agar and identified it as *Leptosphaeria korrae* (Worf, 1986). Similar disease symptoms were identified in California and three dematiaceous fungi similar to *L. korrae* were identified to be the causal agent of SDS (Endo et al. 1985). During 1999, Wetzel sampled two golf courses in Oklahoma and one in Kansas to study the distribution of *Ophiosphaerella* spp that causes SDS. The O. *herpotricha* species was isolated from all three locations and it was the most abundant species found in all the three places.

The disease SDS is currently believed to be caused by three ectotrophic root infecting fungi which include *O. narmari* (Walker and Smith, 1972) *O. herpotricha* (Walker, 1980) and *O. korrae* (Walker and Smith, 1972) *O. herpotricha* is the most common cause of SDS in Oklahoma, and Kansas. *O. korrae* is believed to be the SDS causal fungus in Mississippi, Alabama, North Carolina, Georgia, Tennessee and Virginia (Tisserat, 2000). The three fungi are difficult to distinguish morphologically, however, they can be distinguished by PCR amplification using a specific PCR primers (Tisserat, 2003).

Improving resistance to SDS

To evaluate resistance to SDS of seeded and clonally propagated bermudagrass cultivars, Baird et al. (1998) conducted field and greenhouse studies. In a two year study, the seeded African bermudagrass (*Cynodon transvaalensis*) showed a higher number of live shoots in diseased area than any other bermudagrass tested. Furthermore the seeded cultivars exhibited greater shoot density and higher potential to recover from the disease. Baird further concluded that the African bermudagrass, Guymon, Sundevil, Midlawn, Midfield, Ft. Reno, Mirage and most of the seed propagated entries were the most resistant cultivars to SDS.

Researchers frequently observed a strong correlation between resistance to SDS and tolerance to cold temperatures. To determine the association of

SDS to cold tolerance, Nus (1993) used two SDS causing fungus *Leptosphaeria korrae* (currently *O. korrae*) and *Ophiosphaerella herpotricha*. Differential thermal analysis was used to monitor exotherm temperatures of healthy and infected bermudagrasses. Nus observed that healthy bermudagrass and infected bermudagrass crown supercooled to an average of -4.8 and -4.4 °C respectively. Healthy crown exotherm temperatures were significantly lower than those of fungi infected bermudagrass crowns on all the samples indicating that fungi infected plants are more susceptible to cold damage.

Cultivars of bermudagrass are subjected to winter injury during extreme cold temperatures. Two cold hardy cultivars 'Midiron' and 'MSU' and one susceptible cultivar 'Tifgreen' has been investigated using molecular tools by B. de los Reyes et al (1999). He identified two chitinase genes whose increased expression was associated with cold tolerance. Of the three cultivars he studied, the level of gene expression in the crown tissue was positively correlated with the level of cold hardiness of individual cultivar. (de los Reyes et al., 2001).

To further characterize the temperature response of *O. herpotricha* research was conducted to identify the ideal growth temperature. Crahay et al. (1988) indicated that isolates of *O. korrae* grown on potato dextrose agar media had the greatest growth at 25°C. Tisserat et al. (1989) recorded similar

results and indicated that maximum growth of *O. herp* occurred between 20 C and 25 C. Further, Walker et al., (2003) studied the influence of temperature on the colonization of bermudagrass seedling roots by *O. herpotricha*. Results indicated that colonization was greater at or below 21°C. He found root colonization resulted in dark discoloration of roots and the discoloration varied with the temperature. He also found that after 7-8 days, colonization ranged from 6.47 to 9.56 mm at temperatures 21 C and below. Walker concluded that colonization increased as the temperature decreased and that colonization and mortality of bermudagrass by *O. herpotricha* may occur over a wide range of cool soil temperature.

For better understanding of the SDS infection process with respect to cultural practices in seeded bermudagrasses under different mowing heights, Martin et al. 2001 conducted a three year field experiment using 'Mirage', 'OKS 91-11' (=Yukon) and 'Jackpot' bermudagrasses at Stillwater, OK. The plots were inoculated with *O. herpotricha* isolate KS 188 and mowed to a height of 1.3 and 3.8 cm. Martin et al. reported that the SDS patch area was greater in turf at the higher mowing heights. He further reported that seeded bermudagrass Yukon had a smaller SDS necrotic patch and greater shoot survival density in necrotic patch area than any other cultivar studied.

Using the advanced molecular biology tools including construction of suppressive subtractive libraries and the screening for differential expression

using microarray techniques, researchers have been able to identify changes in gene expression in bermudagrass associated with resistance to *O. herpotricha.* Zhang et al. *2002* (unpublished data). Two seeded cultivars of tetraploid (2n =4X= 36) bermudagrasses Yukon and Jackpot, which are moderately SDS resistant and moderately susceptible, respectively, were used in this study. Zhang concluded that the most common classes of differentially expressed genes between the resistant and susceptible cultivars were associated with the cell signaling pathways and the oxidative burst defense mechanism. She further concluded that there was a marked difference in the global pattern of gene expression in fall and spring. Zhang also identified a number of defense related and signal transduction genes that were induced in the resistant cultivar during the symptom development and recovery in the spring season.

One of the best approaches for reducing SDS is the use of resistant bermudagrass varieties (Anderson et al., 2002). Promising results can be obtained in improving turf quality by developing SDS resistant cultivars for which the knowledge of genetic variations of the existing cultivars is necessary.

Identification of Genetic Variation in Current Bermudagrass Cultivars

In recent years the use of DNA markers to study the genetic variation within and among plant taxa has drastically increased (Clegg; 1990, Paterson et al., 1991, Yang et al., 1996). Molecular markers like RAPD, RFLP, AFLP, SNP, DAF and MHP have been widely used in genetic studies. RAPD was used to study variations among different grasses including switch grass, (*Panicum virgatum)* (Gunter et al., 1996) perennial ryegrass (*Lolium perenne)* (Huff, 1997) creeping bentgrass (*Agrostis palustris*) (Golembiewski et al., 1997) and buffalograss (*Buchloe dactyloides)* (Huff et al., 1993). RAPD was also used to compare the magnitude and structure of genetic variation among tomato accessions (Villand et al., 1998). RFLP was used to determine the phylogenetic relations of tall fescue (*Festuca arundinacea* var. *Genuina Scherb*). AFLP was used by (Wu et al., 2004 - unpublished data) to study the genetic diversity within *C. transvaalensis* accessions and to quantify the genetic relatedness among *C. transvaalensis* and *C. dactylon* accessions. Zhang et al., (1999) used AFLP to study the bermudagrasses genotypes to differentiate a number of released and experimental bermudagrass genotypes.

DNA Amplification and Fingerprinting (DAF)

DAF was developed by Caetano-Anolles et al., (1991) as a high resolution fingerprinting technique that was more powerful than the RAPD technique. DAF utilizes short 5 to 8 base pair oligonucleotide primers verses 10 to 12 base pair primers for RAPD. These shorther primers produce a three-fold increased polymorphism per primer (Williams et al., 1990) and is one of the highest resolution techniques available. Restriction fragment length polymorphism (RFLP) has been widely used as molecular markers in genetic linkage studies and in the DNA fingerprinting studies. However RFLP needs prior knowledge of DNA sequence to be effective, while DAF can be used without such knowledge. Use of amplified fragment length polymorphisms (AFLP) is a newer and very powerful technique that combines restriction digestion and polymerase chain reaction to fingerprint DNA containing organisms. This procedure is especially promising when used with fluorescent probes and automatic DNA sequencing instrumentation. However the costs and labor involved are significantly higher than DAF. DAF is a multiple arbitrary amplicons profiling technique that uses one or more arbitrary primers as short as five

eight to ten base pair long to produce many fragments, or amplicons. The amplicons are resolved by polyacrylamide gel electrophoresis and visualized using highly sensitive DNA silver staining or fluorescent dye techniques. DAF is highly reproducible and provides excellent resolution, high sensitivity and is amendable to automation (Thomas, 1994).

DAF was able to detect genetic differences in a wide variety of organisms including animals, plants and bacteria. Although it is relatively simple to find differences between organisms at the species level DAF can also differentiate those which are closely related like bacterial strains, plant cultivars, near isogenic lines and human individuals (Caetano Anolles, 1991, 1992). Thomas et al., (1994) used the DAF technique for classification of root-knot nematode species and estimation of genotypic diversity and relationships among different species. Twenty single octamer primers of single arbitrary sequence were used in his studies of which, fourteen primers were successfully reported to amplify nematode DNA. Five primers were reported to reveal very conspicuous polymorphisms between species. Phylogenetic analyses of four species showed closer relatedness between *M. arenaria* and *M. javanica* than between *M. arenaria* and *M. incognita*.

Cerny et al., (1996) used DAF to investigate the relationship of five species of Petunia and ten cultivars of the cultivated petunia. From a total of 201 bands scored, 146 loci were polymorphic and distinguished all species and cultivars. Cultivars of the same flower color segregated together within the dendogram. The results demonstrated that the use of use of DAF is very efficient in establishing genetic relationships among closely related species and cultivars of Petunia.

DAF allows the distinction of various turfgrass cultivars in commercial plots (Callahan et al., 1993). DAF has been widely used to characterize bermudagrass species and cultivars of interspecific crosses. In addition, DAF was also used to evaluate genetic diversity, origin of bermudagrass off-types and to certify the authenticity of cultivar stocks. Certain hybrid bermudagrasses have been know for some time to be genetically unstable generating a variety of off-types. DAF was used determine the genetic relationships among off types and the putative cultivar of origin, Tifway (Caetano Anolles, 1997). Results indicated that the offtypes were most likely derived from early contamination of sod rather than genetic instability.

Caetano Anolles (1995) used the DAF technique to study the genetic variations of bermudagrass species and cultivars of interspecific crosses

exhibiting difference in leaf blade textures ranging from coarse to fine. The cluster analysis grouped 13 bermudagrass cultivars studied into several clusters including one containing the African type bermudagrasses and another containing the common type bermudagrasses. The latter group included *C. mageninissii* and the hybrid from an interspecific *C.transvaalensis X C. dactylon*. In addition, the researchers tried to distinguish two cultivars: Tifway and the irradiated induced mutant Tifway II. DAF initially could not distinguished between these closely related cultivars, but by using an extended screen using 81 primers both cultivars were successfully differentiated with a limited number of polymophisms. In the same study, capillary electrophoresis was used to further resolve the DAF amplicons at even higher resolution and sensitivity resulting in detection of polymorphic DNA.

Weaver et al., (1995) used DAF to assess the genetic relationship in centipedegrass (*Eremochloa ophiuroides)*. Cultivars 'Tennessee Hardy', 'Tennessee Tuff', 'Oklawn', 'Centennial' and 'Tifton common' were analyzed using a total of 14 octamer primers. Four out of the fourteen primers were reported to produce highly distinguishable polymorphic patterns. Just two of these primers were able to distinguish all the cultivars studied. The cluster analysis distinctly separated cold tolerant Tennessee

Hardy from the other cultivars. Furthermore, DAF DNA was isolated from the polyacrylamide gels and used as hybridization probes. A 200 base pair band that hybridized to a single amplification product distinguished eight homologous regions in genomic centipedegrass DNA. When it was hybridized to DAF patterns obtained from bermudagrass, bluegrass and zoysia, the 200 base pair fragment and a fragment generated with another primer of weight 175 bp resulted in smearing patterns. The smearing indicated that the putative sequence was present in multiple copies throughout the amplification profile. It was also suggested to be abundant in the heterogeneous grass genomes and represents a group of repetitive sequences. These results by Weaver et al., (1995) indicated that DAF can generate molecular markers that can be used for fingerprinting of centipedegrass cultivars and that DAF amplicons can be used as hybridization probes for further genomic analysis.

DAF was employed to identify the relationships among 18 *Cynodon* Cultivars available in Australia (Ho et al., 1997) by using 20 random primers. All the cultivars showed distinct polymorphism. One of the primers was able to discriminate between all the cultivars except Tifdwarf and it's off-type. The cluster analysis grouped all the *Cynodon* cultivars into two distinct groups. The first group included the hybrids of the common type

bermudagrass, *C. dactylon* and the African bermudagrass, *C. transvaalensis*, while the bermudagrasses from local selection program were clustered into second distinct group.

Caetano Anolles (1998) employed the DAF technique to study the genetic stability of two important *Cynodon* cultivars, 'Tifgreen' and its somatic mutant Tifdwarf. The researchers scanned the DNA of 11 Tifgreen and 8 Tifdwarf cultivars. The analysis revealed that both Tifdwarf and Tifgreen were genetically unstable and that the instability was probably the cause of somatic mutations. However, other Tifgreen and Tifdwarf off-types probably originated from sod contamination.

Assefa et al, (1999) used DAF technique to assess the diversity among a wide range of *Cynodon* species and varieties. Genetic relatedness among 62 cynodon accessions representing eight species was assessed. Ten oligonucleotide primers were used in this study. Each primer revealed polymorphic loci among accessions within species. Of the total 539 bands scored, 496 bands were found to be polymorphic. She identified *Cynodon arcuatus* to be clearly separate from other species by the presence of numerous polymorphic bands. The strongest similarities were found between *C. aethiopicus* and *C. arcuatus, C. transvaalensis and C. plectostachyus* and

C .incompletus and *C.nlemfuensis*. Within taxa, in all instances accessions differing in chromosome number clustered together indicating that 2X and 4X forms to be closely related.

DAF was used to assess authenticity of the U-3 bermudagrass produced in Oklahoma by Anderson et al. (2001). U-3 is a standard turfgrass in the Oklahoma sod production industry. However the identity of the grass presently produced and marketed as U-3 has been questioned. On the request of the sod growers this research was conducted to resolve the concern. The putative original U-3 bermudagrass selected in the early 1930 was compared with bermudagrass currently produced in Oklahoma using DAF. Four samples of putative U-3 and seven samples of Oklahoma U-3 were tested with foundation class Tifway bermudagrass and two commercially labeled Tifway cultivars as reference standards. Results indicated that there exists a wide genetic difference between putative standard U-3 and Oklahoma U-3. Anderson et al (2001) concluded that probable major source of this variability may be attributed to mechanical contamination with vegetative propagules, contamination from viable U-3 seed and possibly genetic mutations.

Objective of the current research

It is always desirable to have cultivars with superior turf quality and increased SDS resistance for which the information concerning the genetic background of the present cultivars is needed. Since DAF has a high potential to identify the genetic variations in bermudagrass cultivars at reasonable cost and effort, DAF was used in this project to study genetic variations among:

- 17 seed propagated cultivars.
- 13 vegetatively propagated cultivars.
- 16 bermudagrass cultivars ranging from highly SDS resistant to SDS susceptible cultivars were studied.

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CHAPTER 2

DNA FINGERPRINTING OF SEEDED CULTIVARS OF BERMUDAGRASS

Accepted for Publication in Crop Science, March 2005

DNA Fingerprinting of Seeded Bermudagrass Cultivars.

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Approved for publication by the Director, Okla. Agric. Exp. Stn. Research supported by the Oklahoma Agric. Exp. Stn. and the United States Golf Association.

Received _____
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ABSTRACT

Bermudagrasses *(Cynodon spp.)* are important for turf and forage in temperate and tropical climates, with cultivars historically propagated clonally. Over the past two decades the number of seed-propagated commercial cultivars has dramatically increased, but information is lacking on the extent of the genetic diversity among these new cultivars. Accordingly, this research was undertaken to assess the genetic relatedness of 17 seed-propagated turf-bermudagrass cultivars using DNA amplification fingerprinting (DAF). Four DAF and four Minihairpin-DAF (MHP-DAF) primers were used in this study. The DAF and MHP-DAF primers amplified 90 and 131 amplicons, respectively. A total of 13 out of the 17 cultivars were practically indistinguishable using the DAF primers with an average similarity (SC) of 0.982, while the MHP-DAF primers distinguished all cultivars readily. Results from the DAF and MHP-DAF analysis indicated that 14 out of the 17 cultivars were related to Arizona common germplasm with average SC of 0.833 in the MHP-DAF analysis. Arizona common germplasm is naturalized to the Colorado River Valley production areas of Arizona and California. The three most distinct cultivars: 'Princess 77', 'Yukon' and 'SWI-11' had an average SC of 0.668. The most distinct

cultivar was 'Yukon' with an average SC of 0.604. Yukon showed 59 DNA signatures not observed in the other varieties studied with DAF and MHP-DAF. These results indicated that a majority of seeded-type bermudagrasses developed over the past two decades depend upon a narrow genetic base, and that several recent cultivars are markedly genetically distinct indicating a recent and significant broadening of the germplasm.

INTRODUCTION

Bermudagrass (*Cynodon dactylon* L. Pers) is a perennial sod-forming turf and forage grass, native to India and eastern Africa. This grass is extensively used in temperate and subtropical regions of the world for agricultural, recreational and residential use Beard (1973 p. 133-142). Historically, the highest quality turf bermudagrass cultivars have been sterile F_1 hybrid plants from crosses between plants of tetraploid (2n=4x=36) *C. dactylon* and diploid (2n=2x=18) *C. transvalensis* Burtt-Davy. These cultivars are commercially propagated by planting either sprigs or sod. Over the past two decades there has been a dramatic increase in the number of seed-propagated cultivars. National Turf Evaluation Program (NTEP) data (NTEP, 2002) indicate some of the recently developed seeded-type bermudagrasses rival the clonal-standard bermudagrass cultivars in turfgrass quality and other performance characteristics.

Several studies have been conducted to examine the genetic relatedness among vegetative propagated bermudagrass cultivars (Caetano-Anolles, 1995 and 1998a; Zhang, 1999), but no information has been published concerning diversity among seeded-type bermudagrasses. Several seeded-type bermudagrass cultivars appear to have originated from the naturalized common form of bermudagrass grown in Yuma County Arizona and the California Imperial Valley and are generally referred to as "Arizona

Common". This bermudagrass is thought to have been introduced to the US southwest desert region at least by the middle of the 19th century (Kneebone, 1966). Baltensperger et al. (1993) indicated that a bermudagrass seed industry started soon after 1900 from bermudagrass naturalized to a region along the Colorado river in Arizona and California. The degree to which current commercial seeded-type bermudagrass cultivars are genetically interrelated is unknown. Accordingly, an estimation of genetic diversity of the seeded-type bermudagrass cultivars would provide important information relative to the need for genetic diversification in breeding programs.

Many techniques have been used to determine genetic relationships, including DNA amplification and fingerprinting (DAF) (Caetano- Anolle's et al., 1997), amplified fragment length polymorphism (AFLP)(Zhang et al., 1999), and randomly amplified polymorphic DNA (RAPD) (Huff, 1997). All these take advantage of the natural variations inherent in plant DNA. While all are capable, there are some advantages to each. AFLP is a very powerful and reproducible technique, and is readily adaptable to automation. However the technique is fairly expensive in terms of reagent cost and equipment, and requires additional steps to perform when compared to DAF. The DAF technique is a reliable, low cost, high-resolution method that is capable of revealing many DNA polymorphisms. The DAF method when

compared to the similar technique known as RAPD produces a many-fold increase in polymorphism per primer (de Vienne et al., 2003).

A variant of DAF that utilizes short minihairpin primers further increases the resolving power of the DAF technique. In one study, the MHP primers detected 5 times as many bermudagrass polymorphisms as conventional DAF primers (Caetano-Anolle's et al., 1995). MHP-DAF primers contain palindromic sequences which hybridizes through intra-primer interactions creating a hairpin and a small looped priming structure (Caetano-Anolle's and Gresshoff, 1994). The MHP-DAF technique uses previously amplified DAF amplicons as template to generate further banding pattern diversity.

DAF has been used successfully to determine the phylogenetic relationships among bermudagrass species (Assefa et al., 1999), provide information on the origin of off-type bermudagrass cultivars (Caetano-Anolles, 1998b), and determine the fidelity of bermudagrass commercially sold as 'U-3'(Anderson et al., 2001), a cultivar originally developed in the early 1930's. Accordingly, this project was undertaken with the objective of determining the genetic relatedness of selected seeded-type bermudagrass cultivars. In this study we analyzed 17 seeded cultivars from different backgrounds using DNA amplification fingerprinting.

MATERIAL AND METHODS

Plant Materials

The seeds of bermudagrass cultivars were obtained from the suppliers listed in Table 1. Approximately 4500 seeds of each cultivar were planted in a 15 cm diameter pot containing Metro mix 250 (Scotts-Sierra, Marysville, OH). The high seeding rate was used to insure that the resulting plant populations would be representative of the cultivars. Plants were fertilized with Peters Professional Peat-Lite (Scotts- Sierra, Marysville, OH) and Iron Chelate (Miller Chemical and Fertilizer Corp., Hanover, PA). The plants were fungicide treated with Chlorothalonil: [2,4,5,6-tetrachloroisophthalonitrile] (trade name: Daconil, Ortho group, Columbus, OH) at a rate of 4.2 ml/L and with Aldecarb: [2-Methyl-2-(methylthio)propionaldehydeO-(methylcarbamoyl oxime] (trade name Temik, Rhone-Poulenc Ag Company, Research Triangle Park, NC).

DNA Isolation

A total of two g of leaf tissue was harvested from a single pot containing each cultivar. The leaf tissue was frozen in liquid nitrogen and ground in a mortar and pestle to a fine powder. Genomic DNA was isolated from 100 mg of powdered leaf tissue using the DNeasy plant mini-extraction kit (Qiagen Inc., Valencia CA) according to directions provided by the supplier.

The DNA concentration was assessed spectrophotometrically at 260 nm and quality was assessed by the 260/280 ratio (Sambrook et al. 1989). If one or more DNA extracts of the batch of 17 cultivars showed a 260/280 ratio less than 1.8 the entire batch was extracted again. The DNA was suspended to a final concentration of 5 ng/L in 0.5X TE and stored at 4° C. DNA quality was further assessed by TBE agarose gel electrophoresis. All samples showed no sign of DNA degradation.

PCR Amplification

Four DAF and four MHP-DAF primers (Table 2) were used to fingerprint the 17 bermudagrass cultivars used in this study. The PCR amplification mixture consisted of 2.5 U of Qiagen *Taq* polymerase (Qiagen Inc., Valencia, CA) 10X PCR buffer which included $MgCl_2$ for a final concentration of 1.5 mM, 250 μM dNTP, 1.5 μM DAF primers (Integrated DNA Technologies Inc, Corelville, IA), and 0.5 ng of template DNA, with the final volume made to 20 μL with sterile distilled water. The DNA template was initially denatured at 94 ° C for 60 seconds. Following denaturation, PCR proceeded at 94° C for 30 seconds, then 30 ° C for 30 seconds and 72° C for 30 seconds, cycling back 39 times. A final extension at 72°C for 60 seconds at the end of the 39 cycles was performed. The PCR

products were visualized on a 1% TBE agarose gel impregnated with ethidium bromide at a final concentration of 0.5 μg/ml.

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 17 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 one μL of DAF PCR product was used instead of the genomic DNA template. We also found that adding 6 mM $MgCl_2$ improved performance of the MHP-DAF.

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 (Cambrex Bio Science Inc., Rockland, ME) 1 X TBE and 7.1 M urea. A total of seven μL of PCR products with three μL of loading buffer containing the tracking dye bromphenol blue were mixed and loaded onto the gel. Molecular markers were loaded on either side of the lanes containing the PCR amplicons. Electrophoresis continued at 80 volts until the bromophenol blue strain reached three-quarters of the length of the gel. The gel was removed and stained with silver using a Bioneer silver staining kit (BioNexus, Oakland, CA) according to manufacturer directions. After staining, the gel was

equilibrated in 10% (v/v) glycerol and 20 % (v/v) ethanol, covered with cellophane and air dried at room temperature for a week prior to analysis. All 17 PCR products were run on the same gel to facilitate accurate band-to-band comparisions.

Data Profiling and Analysis

After silver staining, electrophorectic bands of less than 1.5 kD were scored for their presence (1) or absence (0) for each cultivar. The data were compiled in a Excel spreadsheet and imported into the NTSYS software version 2.0 (Exeter Software, New York, NY) for cluster analysis. Similarity coefficients (SC)(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 replicate experiments.

RESULTS AND DISCUSSION

A total of 90 and 131 bands were scored for DAF and MHP-DAF, respectively (Fig. 1). Over 87% (78 bands) and 79% (103 bands) were found to be polymorphic in the bulked samples using DAF and MHP-DAF, respectively, meaning that the band was present in at least one cultivar but was not observed in others.

The DAF results indicated that 13 out of the 17 bermudagrass cultivars were very closely related to each other (Fig. 2a) with an average SC of 0.982 (data not shown). The other four cultivars, Riveria, Princess, SWI1-1 and Yukon were easily distinguishable using DAF. The technique of DAF alone could not resolve differences between Arizona Common and CD 90160 or differences among 'Mohawk', Savannah, Southern Star, 'Sundevil' and 'Numex Sahara' (Fig. 2a, SC = 1.000). In contrast, the MHP-DAF analysis clearly differentiated among all 17 cultivars (Fig. 2b). The differences between DAF and MHP-DAF were even more dramatic with 14 of the most closely related cultivars in the MHP-DAF analysis showing an average SC of 0.833, while in the DAF analysis these same cultivars showed an average SC of 0.975 (data not shown). The results from the MHP-DAF and DAF analysis indicated that 14 of the cultivars in this study were closely related to Arizona Common. This group included Arizona Common, 'CD90160',

'Jackpot', 'Majestic', Savannah, Southern Star, Sundevil, Mohawk, Riviera, 'Mirage', 'Sydney', 'Pyramid', Numex Sahara, and 'Transcontinental'. According to MHP-DAF analysis, the most closely related cultivars grouped into three clusters, including: Arizona Common and CD90160 (group 1, SC 0.901), Savannah, Southern Star, and Sundevil (group 2, average SC 0.913), and Numex Sahara and Transcontinental (group 3, SC 0.901). The two most similar cultivars were Savannah and Southern Star with a SC of 0.924. The pedigree information available for Savannah (Fraser and Rose-Fricker, 1998) and Southern Star (Samudio and Brede, 2002) indicate that bermudagrass germplasm from Walla Walla, Washington, collected by the respective developers, contributed to the parentage of both cultivars. The use of additional markers may even better differentiate the closely related Arizona Common-type bermudagrasses.

Yukon, Princess 77 and 'SWI-11' were least genetically related to Arizona Common of all the cultivars studied. Futhermore, all three cultivars showed little relationship to each other. Yukon was the most distinct cultivar in this study with an average SC of 0.604 across all cultivars. The least similar cultivar to Yukon was SWI-11 and the most similar was Transcontinental, with SCs of 0.511 and 0.649, respectively. These low SCs indicate thatYukon was the most divergent seeded-type bermudagrass cultivars of those studied. Furthermore, 36 bands from Yukon were not

observed in other cultivars tested, and 23 bands were found in all other bermudagrasses studied except Yukon. Combining those bands not observed with those uniquely observed in Yukon totalled 59 potential DNA signatures representing over 27% of the bands scored. Yukon is a new cultivar recently released by Oklahoma State University. Two other distinct cultivars Princess 77 and SWI-11 had average SCs of 0.689, and 0.712, respectively. Both Princess 77 and SWI-11 showed 7 signatures not observed in other cultivars in the combinded DAF and MHP-DAF studies, or 3% of all bands scored. These DNA signatures may be useful for cultivar maintenance and identification purposes.

The close clustering of the 14 out of 17 cultivars with DAF indicated that most seeded-type bermudagrass cultivars are very closely related. Included in this group is Arizona Common, indicating that many of the cultivars likely originated from breeding populations originally constituted solely, or substantially, from Arizona Common. A second potential reason for some cultivars showing close similarity to Arizona Common relates to mechanical contamination of seed production fields leading to genetic contamination. Seed of many of the cultivars in the study were produced in Yuma Co., Arizona or the Imperial Valley, California where bermudagrass seed production has been concentrated for nearly a century. Preventing the Arizona Common bermudagrass ubiquitous to this region from mechanically

contaminating unique cultivar seed production fields and hybridizing with plants of the unique cultivars is difficult. Seed production fields of cultivars that are less well adapted to the region than Arizona Common can quickly be dominated by the latter. Arizona Common growing as an impurity in seed production fields, or growing in adjacent areas, may hybridize with the cultivars resulting in genetic contamination of the desired cultivar. One of the authors (C. M. Taliaferro) has observed seed production fields of cultivars that were less well adapted to the region than Arizona Common become dominated by the latter within 1 to 3 years contingent on the amount of initial contaminent Arizona Common in the stand. Arizona Common growing as contaminant in cultivar seed-production fields, or growing in adjacent areas, has the potential of hybridizing with the cultivars. Hoff (1967) demonstrated natural crossing between Arizona Common and giant bermudagrass (*C. dactylon* var. *aridus*), the two major forms of bermudagrass traditionally grown in the region. However, the progeny resulting from the hybridization of tetraploid Arizona Common and diploid giant bermudagrass plants were sterile triploids. Such hybridization between tetraploid cultivars could produce fertile progeny leading to genetic contamination. Relative to the usually sterile vegetatively-propagated bermudagrass cultivars the potential for genetic changes in seeded-type

bermudagrass cultivars is greater and warrants additional actions to maintain their genetic fidelity.

It should be noted that significant differences exist among the cultivars grouped with Arizona Common for turf quality, cold tolerance, and other performance traits (National Turfgrass Evaluation Program, 1997, 2002). Notably, Riviera, though loosely grouped with Arizona Common on the basis of SC values, has much higher turf quality and broader adaptation due to greater cold tolerance. None of the seed-propagated cultivars in the 1992 NTEP trial had turfgrass quality ratings as high as the vegetatively-propagated standard cultivars in the test. Results from the 1997 NTEP bermudagrass test indicated that the development of Princess and Riviera represented a major gain in turfgrass quality for seeded-type bermudagrasses relative to industry-standard clonal cultivars. The development of these two cultivars suggests that major gains in performance can be achieved by breeding in relatively diverse germplams pools with the desired result of maintenance of genetic diversity among cultivars.

ACKNOWLEDGEMENTS

We appreciate the financial support provided by the United States Golf Association. (USGA). We appreciate the technical support of Carole Anderson. Special thanks to Gary Williams, Sharon Williams and Rose Edwards for maintenance of the bermudagrass cultivars at the Oklahoma State University greenhouse. We gratefully acknowledge the support of colleagues Janice Hironaka, James Enis and Madhavi Dhulipala.

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Table 1. Seeded type bermudagrass cultivars used in this study and their source

Ref. number	Cultivar	Source
1	Arizona Common	Seeds West, Inc., Roll, AZ
2	CD 90160	Cebeco International Seeds, Inc., Halsey, OR
3	Jackpot	Simplot Turf and Horticulture, Boise, ID
4	Majestic	H & H Seed company Inc, Yuma, AZ
5	Mirage	Cebeco International Seeds, Inc, Halsey, OR
6	Mohawk	Seeds West, Inc, Roll, Arizona.
7	Pyramid	Cebeco International seeds Inc, Halsey, OR
8	Princess 77	Seeds West Inc, Roll, AZ
9	Riviera	Oklahoma State University, Stillwater, OK
10	Savannah	Turf Seed Inc, Hubbard, OR
11	Southern Star	Simplot Turf and Horticulture, Boise, ID
12	Sundevil	Simplot Turf and Horticulture, Boise, ID
13	SWI-11	Seeds West Inc, Roll, AZ
14	Sydney	Seeds West Inc, Roll, AZ
15	Numex Sahara	Seeds West Inc, Roll, AZ
16	Transcontinental	Pure Seed Testing, Inc. Hubbard, Or
17	Yukon	Oklahoma State University, Stillwater, OK

Table 2. Sequence of the DAF and MHP-DAF primers used in this study.

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

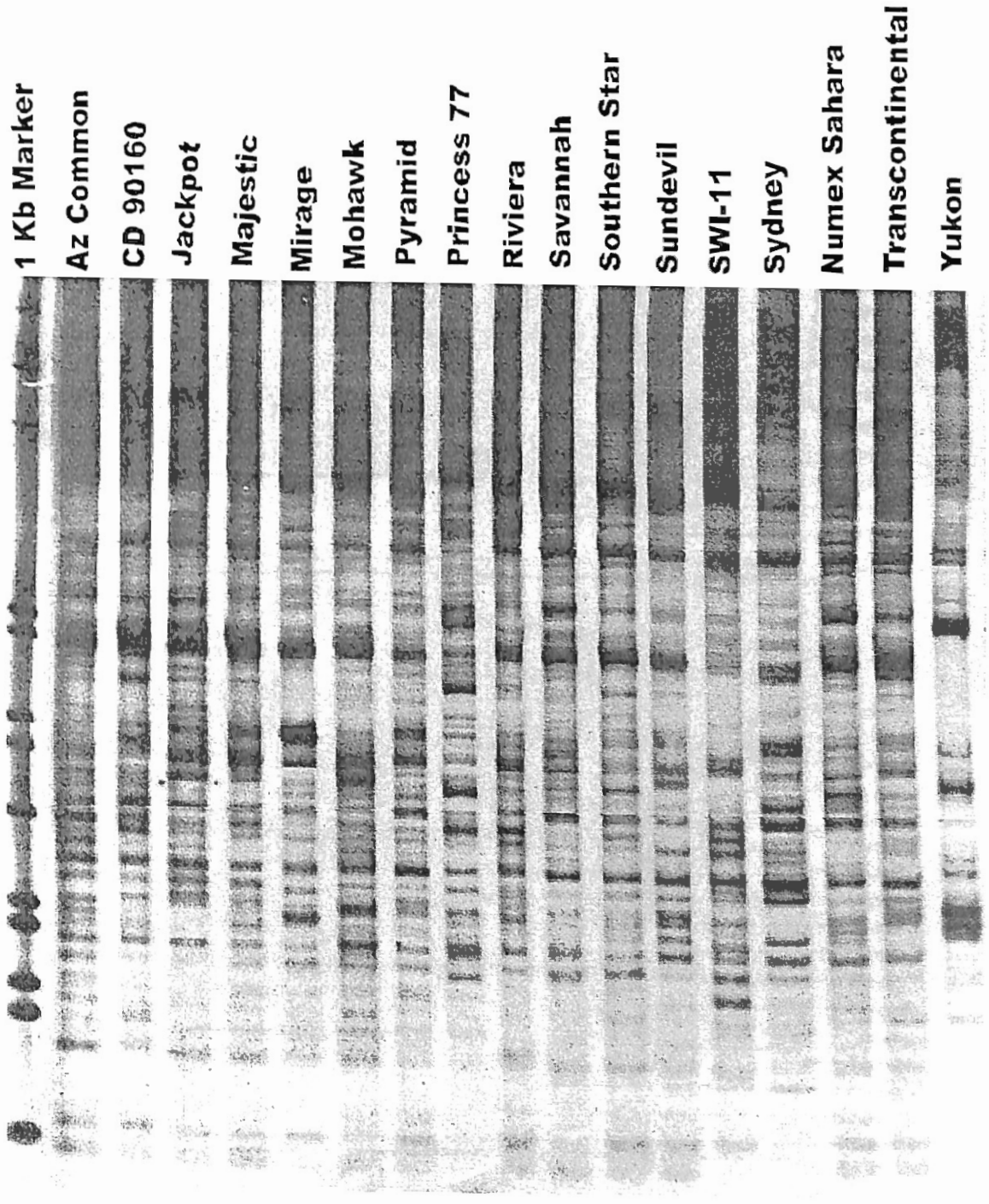

Figure 1. MHP-DAF electrophoresis gel stained with silver.

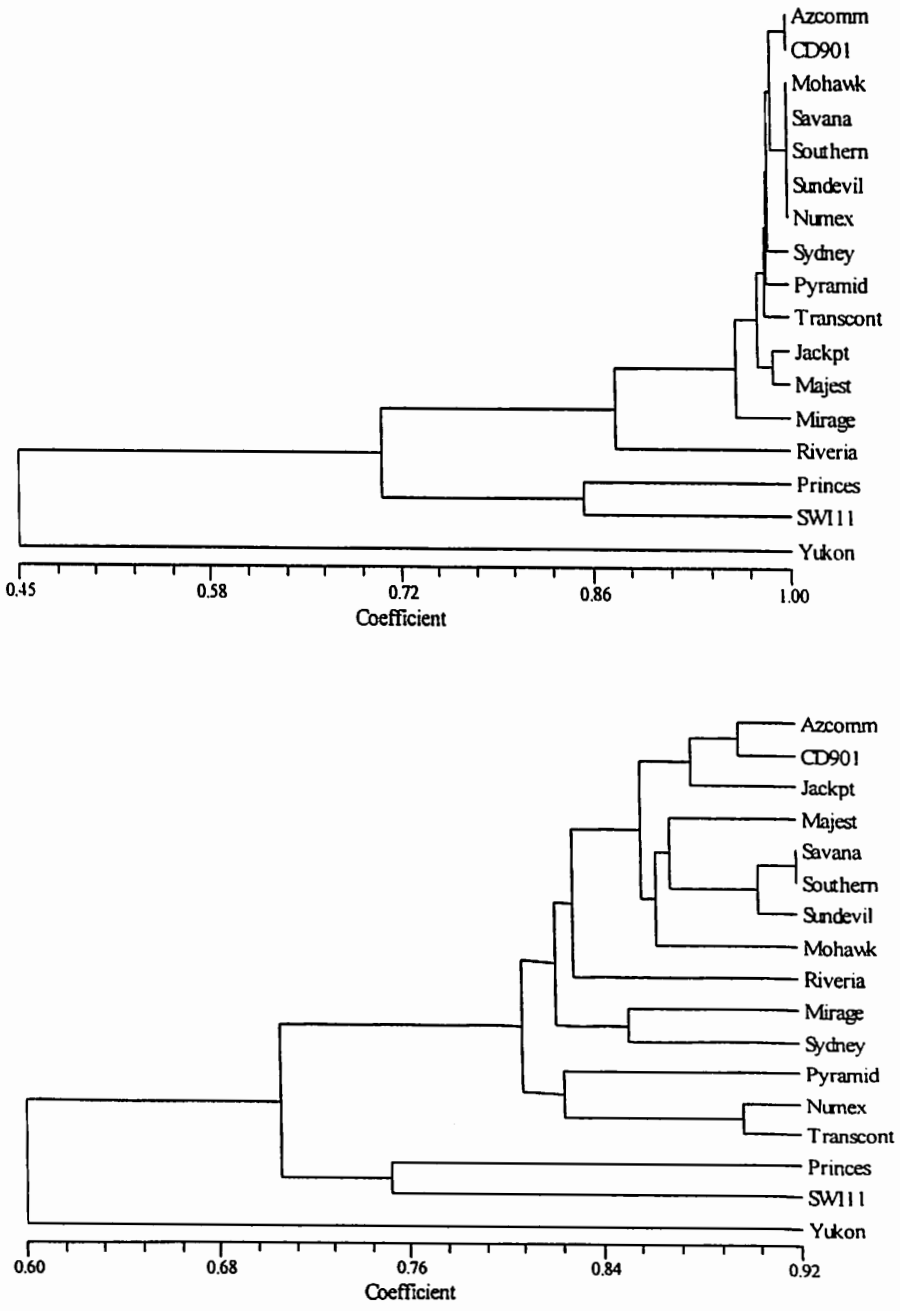

Figure 2. DENDROGRAM from DAF (upper) and MHP-DAF (lower) analysis of 17 seeded type bermudagrass cultivars.

Varieties	Az Comm	CD9010	Jackpot	Majestic	Mirage	Mohawk	Pyramid	Princess 77	Riviera	Savannah	Sout Star	Sundevil	SWI-11	Sydney	Num Sahar	Trans cont	Yukon
Az Comm	1																
CD9010	0.901	1.000															
Jackpot	0.878	0.885	1.000														
Majestic	0.855	0.878	0.870	1.000													
Mirage	0.809	0.817	0.840	0.832	1.000												
Mohawk	0.878	0.855	0.847	0.855	0.855	1.000											
Pyramid	0.786	0.809	0.786	0.824	0.824	0.847	1.000										
Princess 77	0.656	0.664	0.672	0.710	0.710	0.733	0.718	1.000									
Riviera	0.817	0.794	0.878	0.794	0.824	0.863	0.786	0.718	1.000								
Savannah	0.855	0.847	0.855	0.863	0.832	0.870	0.794	0.695	0.824	1.000							
Sout Star	0.870	0.847	0.885	0.893	0.878	0.885	0.840	0.710	0.855	0.924	1.000						
Sundevil	0.855	0.847	0.870	0.863	0.817	0.855	0.809	0.725	0.840	0.908	0.908	1.000					
SWI-11	0.733	0.695	0.733	0.725	0.725	0.733	0.672	0.756	0.733	0.725	0.786	0.740	1.000				
Sydney	0.786	0.809	0.786	0.855	0.855	0.817	0.802	0.687	0.802	0.824	0.870	0.809	0.718	1.000			
Numex Sah	0.802	0.824	0.832	0.840	0.840	0.832	0.832	0.687	0.771	0.794	0.840	0.794	0.702	0.847	1.000		
Trans contin	0.794	0.817	0.855	0.786	0.832	0.824	0.824	0.649	0.779	0.771	0.832	0.756	0.710	0.794	0.901	1.000	
Yukon	0.611	0.603	0.626	0.618	0.603	0.626	0.595	0.542	0.611	0.618	0.603	0.603	0.511	0.626	0.611	0.649	1.000

Table 3. Similarity coefficients (SC) of 18 seeded bermudagrasses using MHP-DAF analysis

CHAPTER 3

GENETIC VARIATIONS IN CLONALLY PROPAGATED BERMUDAGRASS CULTIVARS IDENTIFIED BY DNA FINGERPRINTING.

Genetic Variations in Clonally Propagated Bermudagrass Cultivars Identified by DNA Fingerprinting

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Approved for publication by the Director, Okla. Agric. Exp. Stn. Research supported by the Oklahoma Agric. Exp. Stn. and the United States Golf Association.

Received _____

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ABSTRACT

Vegetatively propagated bermudagrass constitute a major source of turf in the Southern United States and throughout the world. New bermudagrass accessions to be used in breeding were recently introduced from China by researchers at Oklahoma State Agricultural Experimental Station. The objective of this project was to determine the degree of genetic relatedness of these new introduced Chinese accessions with the existing vegetative cultivars commonly grown in the United States. A total of 270 bands were scored using DNA amplification fingerprinting (DAF) and mini-hairpin primer-DAF (MHP-DAF) primers with 97% of the bands being polymorphic. The cluster analysis was able to easily distinguish all cultivars into 4 distinct groups. Group 1 with a similarity coefficient (SC) of 0.77 consisted of the three accessions from China. Group 2 with a SC of 0.81 consisted of 'Patriot' and two promising germplams lines. Group 3 consisted of 'Tifgreen' and 'Baby' with a SC of 0.87. Group 4 consisted of 'Tifsport' and 'Tifway' with a SC of 0.93. Three cultivars, 'Midlawn', 'Quickstand' and 'Tifton 10' were the most unrelated cultivars in the study with SC of 0.62, 0.62 and 0.67, respectively. The study called into question previous understandings about the origin of Tifsport and Baby. Tifsport was indicated to be a radiation induced mutant of Midiron, but in this study Tifsport was most closely related to Tifway, a genetically distant and distinct cultivar to

Midiron. Baby was thought to be a *C. dactylon*, but in this study it is clearly more closely related to the triploid hybrid Tifgreen. The study also indicates the distinctness of the Chinese accessions with the cultivars currently grown in the United States, suggesting that Chinese introductions could be a valuable source of genetic diversity.

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, 1973). There are nine species of *Cynodon* which only two, *C. dactylon* and *C. transvaalensis*, substantially contributes to the gene pools of today's modern-day turf bermudagrasses. 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. These originated 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 parentage for these popular early varieties originated from germplasm from Southern Africa (Taliaferro, 1995), (Juska-FA, 1964). This suggests that many of the most widely used vegetatively propagated bermudagrasses grown today may rely predominantly on a narrow geographically restricted genetic base.

Cynodon dactylon var dactylon is widely adapted and distributed across every continent between 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, 2004). Results indicted that the Chinese accessions constitute a genetically distinct germplasm source when compared to European, African and Australian 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 cultivars.

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, 1995), DNA amplification fingerprinting (DAF) (Caetano-Anolles, 1991), and random amplification of polymorphic DNA [RAPD] Williams, 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, 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, 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, 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, 1995), and hybrid derivatives of two species *C. transvaalensis, C. dactylon* (Caetano Anolles, 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.

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.

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° C for 60 seconds, denatured for 94° C for 30 seconds, annealed at 30° C and extended at 72° C for 60 seconds. The program recycled for 39 times with final extension at

72°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 9μM 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

bromphenol 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 Coefficients (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 replicate experiments

RESULTS AND DISCUSSION

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 four 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 were quite distinct from either of the four groupings, namely: Tifton 10, Midlawn, and Quickstand. Similarity 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 OKC 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 F1 hybrid between *C. dactylon* and *C. transvaalensis*. The *C.dactylon* parent was from the South Carolina golf course and *Cynodon transvaalensis* from East Lakes golf course in Atlanta, developed 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 be more closely related to vegetative cultivar, such as: Tifgreen, rather than a *C. dactylon* cultivar as suggested by the patent originator. Three polymorphic bands were found that distinguishes Baby from Tifgreen and any other cultivar tested. The original 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 (see chapter 4). Furthermore, an AFLP fingerprinting study of many triploid hybrid bermudagrasses, Zhang et al. (1998) 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 induced near sterility or poor seed set (Powell et al., 1974). How this differentiation between Tifsport and Midiron arose is not known, more likely due to misidentification (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 cultivars had 8, 9, and 5 bands present in each cultivar, respectively, but absent in others. Tifton 10 was more closely related to the Chinese accessions (Group 1) and promising breeding lines from Oklahoma State University (Group 2) than to either Midlawn or Quickstand. This may be due to a similar 'Chinese' genetic background among these bermudagrasses.

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 genetically distinct and potentially valuable source of germplasm for variety

development which could be used to increase the genetic diversity of bermudagrasses used in the USA.

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Table 1. Vegetative cultivars of bermudagrass studied in this project.

No	Cultivar or Accessions	Species	Chromosome Number (2n)	Source and Reference
1	A 12205	Chinese Accession	NA	Collected in Guangzhou province of China.
2	A 12250	Chinese Accession	NA	Collected in Nanjing province of China
3	A 12198	Chinese Accession	NA	Collected in Beijing province of China
4	Baby	Accession	NA	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		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

Table 2. Sequence of the DAF and MHP-DAF primers used in this study. MHP-DAF primers amplified templates were 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

Figure 1. Polyacrylamide separation of MHP-DAF amplicons after fluourescent staining with Sybr Gold.

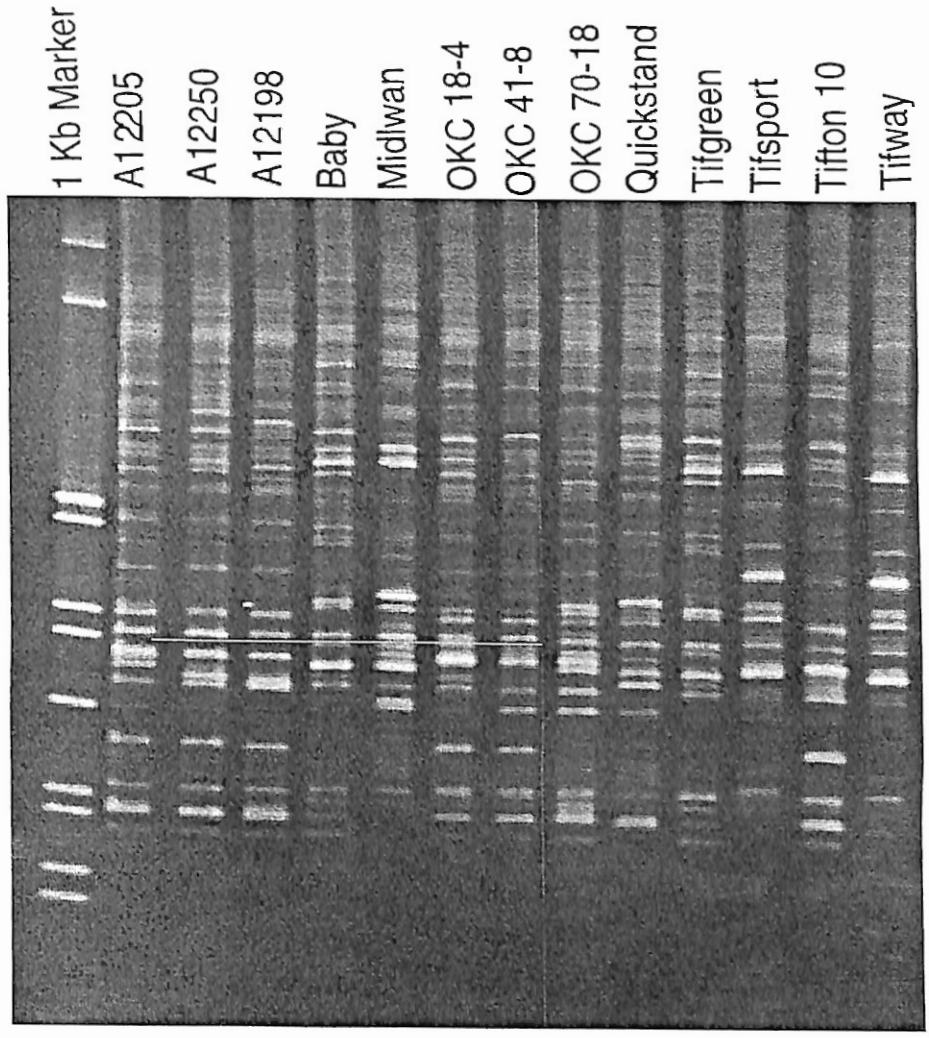

Figure 2. Dendrogram from DAF and MHP-DAF analysis of 13 vegetative cultivars of bermudagrass.

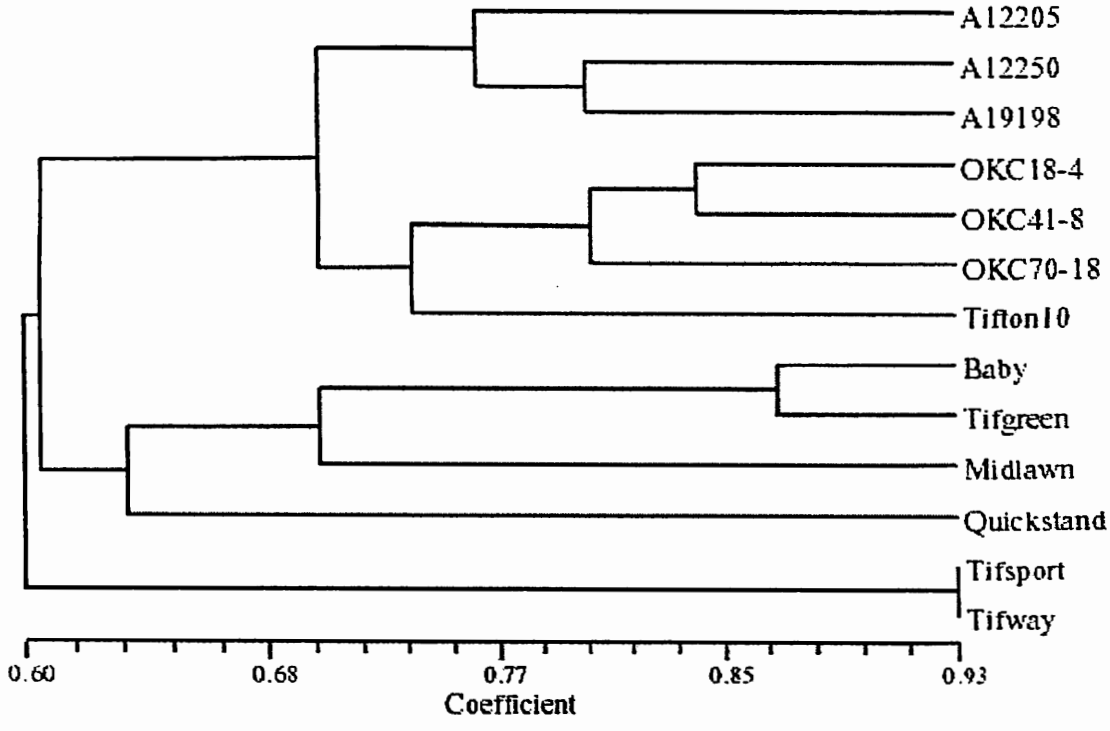

Table 3. Similarity Coefficients for combined DAF and MHP-DAF analysis.

	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

CHAPTER 4

DNA FINGERPRINTING OF RESISTANT AND SUSCEPTIBLE CULTIVARS OF BERMUDAGRASS FOR SRPING DEAD SPOT DISEASE.

DNA Fingerprinting of Resistant and Susceptible Bermudagrass Cultivars to Spring Dead Spot.

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ABSTRACT

Spring Dead Spot (SDS) is among the most destructive diseases of bermudagrass. This project was undertaken to determine the genetic background of selected SDS resistant and susceptible cultivars using DNA amplification fingerprinting (DAF). Four DAF primers were used to produce 82 bands for UPMGA cluster analysis. All cultivars were easily distinguished and separated into four distinct groups. Group one with a similarity coefficient of 0.91 consisted of the seeded varieties most closely related to Arizona Common including: Jackpot, Mirage, Sydney, Numex Sahara, and Pyramid. The second group consisted MidIron, Midlawn and MSU with a SI of 0.87. The third group with an SI of 0.93 consisted of MiniVerde and Tifgreen, and the fourth group the cultivars Tifway and Tifsport with a SI of 0.99. Three cultivars were not closely related to any of the others, or to each other including: Cardinal, Yukon and GN-1. Tifsport reported as a radiation induced mutant of Midiron was unexpectedly found to be practically indistinguishable from Tifway. SDS resistance cultivars, including: MSU, Midiron, Midlawn, Yukon, and Cardinal were was found to be loosely associated indicating a certain level of concentration of SDS resistance within a relatively narrow genetic background. Bands present in SDS resistant cultivars but not detected in susceptibles were excised, and will be evaluated for polymorphic sites useful for breeding purposes.

INTRODUCTION

Bermudagrass (*Cynodon dactylon*) is extensively grown for turf on golf courses, residential lawns, sporting fields, and as forage for livestock. One of the major problems in bermudagrass culture is the occurrence of the fungal disease spring dead spot (SDS) (Tisserat et al., 2004). Spring dead spot is caused by at least three soil-borne fungi including: *Ophiosphaerella herpotricha*, *O. korrae*, and *O. narmari*. All three fungi are ectotrophs meaning that they live on the outside of roots while deriving nutrients from the inside of roots via hyphae that penetrate the root interior (Smiley, 1993). The typical symptoms of the disease includes the appearance of circular patches in the spring ranging in size from several inches to feet depending on the level of resistance of the grass cultivar. The patches remain until rapid spring regrowth results in the recolonization of the dead areas. The patches reoccur with increasing size in the subsequent years only to mysteriously disappear after five to seven years.

Extensive research has been conducted over the past fifty years to develop effective control strategies for SDS (Smith et al., 1989) but as of today there are no successful approaches to satisfactorily control this disease. The use of fungicides has been shown to be expensive inconsistent and ineffective in offering a long standing remedy. Alterations in cultural practices may also reduce, but not control disease occurrence. Higher

incidence of SDS was reported in plots fertilized with nitrogen in the summer and early fall (Lucas, 1980). Similarly, higher mowing heights increased the necrotic patch area when compared to lowing mowing heights (Martin et al., 2001).

One of the most promising approaches for reducing SDS is the use of resistant cultivars (Anderson et al., 2002). Recent NTEP trials of turf bermudagrass cultivars indicate that there is significant genetic variation in a number of seeded and vegetatively propagated bermudagrass cultivars with respect to resistance to SDS (NTEP, 2002). The most resistant vegetative varieties included the *C. transvaalensis* cultivar 'Cardinal' and the *C. dactylon* x *C transvaalensis* triploid hybrid 'Midlawn', while several susceptible included 'Princess' and 'Mini-Verde'. Among the seeded bermudagrass cultivars the most resistant was Riviera, a recent release from Oklahoma State University breeding program. From this data it is clear that significant genetic resistance to SDS exists in current bermudagrass varieties. Unfortunately, information concerning the genetic backgrounds among resistant and susceptible cultivars is fairly limited and must be pieced together from a variety of sources. A better understanding of the genetic background of resistant and susceptible varieties will be helpful to determine if the resistance trait is confined to a narrow or wide range of genetic backgrounds.

Many techniques have been used to determine genetic relationships, including DNA amplification and fingerprinting (DAF) (Caetano- Anolle's et al., 1997) amplified fragment length polymorphism (AFLP) (Zhang et al., 1999), and randomly amplified polymorphic DNA (RAPD) (Huff, 1997). All these take advantage of the natural variations inherent in plant DNA. While all are capable, there are some advantages to each. AFLP is a very powerful and reproducible technique, and is readily adaptable to automation. However the technique is fairly expensive in terms of reagent cost and equipment, and requires additional steps to perform compared to DAF. The DAF technique is a reliable, low cost, high-resolution method that is capable of revealing many DNA polymorphisms. The DAF method when compared to the similar technique known as RAPD produces a many-fold increase in polymorphism per primer (de Vienne et al., 2003).

DAF amplifies various regions of genomic DNA by the polymerase chain reaction (PCR) using single, short 5-8 base pair oligonucleotide primers. The fragments can be easily resolved by polyacrylamide gel electrophoresis (PAGE) and visualized by silver staining, or a suitable DNA intercalating fluorescent dye resulting in a distinct and reproducible gel banding pattern. Different genotypes produce distinct banding patterns that are characteristic of the underlying genetic relationships. DAF has been successful in determining the phylogenetic relationships among bermudagrass species

(Assefa et al., 1999), identifying off type bermudagrass cultivars (Busey et al., 1996), and determining the genetic fidelity of a popular current bermudagrass variety with the original selection produced in the early 1950's (Anderson et al., 2001). In this study we utilize the DAF technique to determine genetic relatedness among a wide range of bermudagrass cultivars differing in resistance to SDS.

MATERIALS AND METHODS

Plant material

Bermudagrass cultivars (Table 1) were grown in a greenhouse in 15 cm pots containing Metro mix 250 (Scotts-Sierra, Marysville, OH). Plants were fertilized with Peters Professional Peat-Lite (Scotts- Sierra, Marysville, OH) and Iron Chelate (Miller Chemical and Fertilizer Corp., Hanover, PA). The plants were fungicide treated with Chlorothalonil: [2,4,5,6-tetrachloroisophthalonitrile] (trade name: Daconil, Ortho group, Columbus, OH) at a rate of 4.2 ml/L and with Aldecarb: [2-Methyl-2-(methylthio)propionaldehydeO-(methylcarbamoyl oxime] (trade name Temik, Rhone-Poulenc Ag Company, Research Triangle Park, NC).

DNA Isolation

Two grams of plant tissue was harvested from the single pot of each cultivar, frozen in liquid nitrogen and ground in a mortar and pestle to a fine powder. The tissue was powdered and mixed so that a 100 mg sample would be representative of the two grams of leaf tissue. Genomic DNA was isolated with the DNeasy plant mini-extraction kit (Qiagen Inc, Valencia CA) according to directions provided by the supplier. The DNA concentration was assessed spectrophotometrically at 260 nm (Beckman Inc, CA) (Sambrook et al., 1989) and quality was assessed by the 260/280 ratio. If one or more extract of the batch 16 cultivars showed 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. Extracted DNA quality was further assessed by TBE agarose gel electrophoresis. All samples showed no sign of DNA degradation.

PCR Amplification

Four DAF primers (Table 2) were used to fingerprint the 16 bermudagrass cultivars used in this study. The PCR amplification mixture consisted of 2.5 U of Qiagen Taq polymerase (Qiagen Inc., Valencia, CA) 10X PCR buffer, 1.5 mM $MgCl_2$, 250 μM dNTP, 1.5 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 distilled water. The PCR mixtures were initially denatured at 94° C for 60 seconds, denatured for 94° C for 30 seconds, annealed at 30° C and extended at 72° C for 30 seconds. The program recycled for 39 times starting at step two. A final extension at 72°C for 60 seconds at the end of the 39 cycles was performed. The PCR products were visualized on a 1% TBE agarose gel stained with Sybr Gold (Molecular Probes) under long wave ultraviolet light. 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 16 reactions then the entire set was re-run until the banding patterns were near equally amplified.

Denaturing Polyacrylamide Gel 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 (Cambrex Bio Science Rockland, Inc, ME) 1 X TBE and 7.1 M urea. Seven □L of PCR products with three □l of loading buffer containing the tracking dye bromphenol blue were mixed and loaded into the gel. Molecular markers were loaded on either side of the PCR reactions. Electrophoresis continued at 80 volts until the bromophenol blue strain reached three quarters of the length of the gel.

The gel was removed and stained with SYBR gold (FMC Bioproducts, Rockland, ME) according to manufacturer directions and visualized under long wave ultraviolet light. All of the 16 PCR products were run on the same gel to facilitate accurate comparision among lanes.

Data Profiling and Analysis

For profiling all 16 bermudagrass strains, a total of 4 DAF primers were used (Table 2). Electrophorectic bands were scored for presence (1) or absence (0). The data were compiled in an Excel spreadsheet and imported into the NTSYS software version 2.0 (Exeter Software New York, NY) for UPMGA cluster analysis. Similarity coefficients were computed by the SIMQUAL module of the NTSYS software. 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, gel staining, data profiling and analysis was replicated for two to three times with near identical results indicating a high degree of reproducibility.

RESULTS AND DISCUSSION

Genetic fingerprinting of 16 bermudagrass varieties differing in SDS resistance was performed to examine the genetic background of cultivars differing in disease resistance. A total of 82 bands were scored using four DAF primers which distinguished almost all cultivars with the single exception being Tifsport and Tifway. The seeded cultivars (*C. dactylon*) and vegetative cultivars (*C. dactylon* X *C. transvaalensis*) as expected grouped separately with the single exception of Yukon. Yukon a recent release from Oklahoma State University was more closely associated with Midiron, Midlawn and MSU vegetative varieties than with the other seeded varieties. The clear separation of vegetative and seeded types is indicative of their different origins, with the seeded cultivars originating from *C. dactylon* germplasm sources and the vegetative varieties from interspecific hybridizations between *C. dactylon* and *C. transvaalensis*.

The DAF analysis separated 13 out of the 16 varieties into four distinct clusters. The first group consisted of seeded types: 'Arizona Common', 'Jackpot', 'Mirage', 'Sydney' 'Numex Sahara' and 'Pyramid'. This cluster had an average similarity coefficient of 0.91 indicating a very close genetic association. The close clustering of these seeded varieties is in support of previous study where 14 out of 17 seeded types were shown to be closely related to Arizona Common (Yerramsetty et al., 2005). The close association

of most seeded varieties with Arizona Common, a bermudagrass that is endemic to the southwestern portion of the USA, suggested the intermixing of bermudagrass genotypes by hybridization or mechanical means over time.

The second group comprised of Midiron, Midlawn and MSU were closely associated with an average similarity coefficient of 0.87. According to the most recent NTEP trials and previous observations, the most resistant bermudagrass varieties to SDS were found within this group (NTEP, 2001). Midlawn and Midiron are triploid hybrid selections developed at Kansas State University by the late Dr. Ray Keen. Midlawn was reported to be developed from material collected from Michigan State University campus and crossed with several unknown African *C. transvaalensis* bermudagrasses. The cultivar MSU was collected directly from Michigan State University campus by Dr. Charles Taliaferro and most probably originated from earlier material collected by Dr. Beal (Dr. Mike Kenna personal communication). All three, Midiron, Midlawn, and MSU are known to possess exceptional cold tolerance, and Midlawn and Midiron are known to be very resistant to SDS. No data exists on the tolerance of MSU to spring dead spot.

The third group includes two vegetative varieties 'Mini-Verde' and 'Tifgreen' with a similarity coefficient of 0.93. Tifgreen is a chance triploid fine textured hybrid derived from selections from a golf course in the North

Carolina, and is susceptible to SDS. Mini-verde is a very dense low growing and very SDS susceptible cultivar (Martin, 2001).

The varieties Tifway and Tifsport comprised Group 4 which were the most closely related varieties in this study with a similarity coefficient of 0.99, practically indistinguishable. Further analysis using the higher resolution mini-hairpin primers failed to differentiate between the two bermudagrass varieties (data not shown). Both Tifway and Tifsport are susceptible to SDS. Tifway is a very popular and high quality but SDS susceptible variety with very dark green color, while Tifsport is an improved triploid developed for increased cold tolerance and with fine leaf texture and high quality of turf characteristics. The limited pedigree information provided by the developer of this variety indicates that Tifway was a chance hybrid between *C transvaalensis* and *C. dactylon.* Tifsport was developed for increased cold tolerance as a gamma radiation induced mutant from Midiron. In this study, however, Tifsport was found to be genetically different from Midiron its putative irradiated parent while being much more closely related to Tifway, a genetically distinct and easily distinguishable cultivar from Midiron. Furthermore, the smaller than expected dissimilarity coefficient of Tifway and Tifsport in differentiation of bermudagrasses by an extensive AFLP studies confirmed these results (Zhang et al., 1999). Producing new varieties by irradiation is an easy and effective method for

improving turfgrasses when parent stock cannot be crossed due to triploid induced sterility or poor seed setting (Powell, 1974). There have been major genetic instabilities observed in vegetatively propagated bermudagrasses in a previous study (Caetano Anolles et al., 1997). Similar results of unusual grouping of two bermudagrasses produced by irradiation were observed in the cultivars Tifeagle and Tifway 2 (Zhang et al., 1999). The reasons for these differences are not clear but may relate to genetic instability in vegetatively propagated bermudas, misidentification of parental stocks, or early contamination of propagation nurseries by the aggressive Tifway.

Three varieties were found to be very distinct from all others including Cardinal, Yukon and 'GN-1'. Cardinal was one of the most resistant cultivar to SDS in the 2001 NTEP trials (NTEP, 2001). Cardinal is a *C. transvaalensis* cultivar that is a very fine textured and very cold tolerant, originating from a chance find on a golf course in Southern Illinois (Diesburg, personal communication). From field observations it appears that most, if not all, *C. transvaalensis* types are very resistant to SDS (personal communications, Charles Taliaferro). Yukon is a new seeded cultivar recently released by Oklahoma State University possessing medium fine textured light green colored leaves and excelling among seeded varieties in SDS resistance and cold tolerance according to the 1996 NTEP trials (NTEP, 1996). In a previous fingerprinting study among seeded varieties

Yukon was found to be the most genetically distinct variety among 17 lines tested (Yerramsetty et al., 2005). GN-1 is a bermudagrass variety originating from Australia. In contrast to Yukon and Cardinal, GN-1 was very susceptible to spring dead spot, and according to our results more genetically related to the Arizona Common-like seeded cultivars than any other type.

SDS resistance was found to be associated with a loosely related cluster including MSU, Midiron, Midlawn, Yukon, and Cardinal. All are among the most SDS and cold tolerant of the bermudagrass varieties. Of the five cultivars, MSU, Midlawn and Midlawn cultivars were the most closely related with an average similarity coefficient of 0.84. Yukon and Cardinal were very distinct from the others and from each other with average similarity coefficients of 0.70 and 0.67, respectively. The cultivars in this cluster are vegetatively propagated being interspecific hybrids, with the exception of Yukon being a seeded type.

A Few bands of interest isolated from DAF gels seem to correlate with SDS resistance. Three bands were identified to be present only in resistant cultivars of Midlawn, Midiron and MSU and were not observed in the other cultivars examined (Figure 2). These are currently being excised and DNA sequenced in order to characterize the DNA basis of the polymorphism(s) associated with these common bands. If the DNA polymorphism is genetically linked with SDS resistance trait it may be t possible to use the

DNA as a marker in a marker assisted selection strategy for enhancing SDS resistance in bermudagrass. Currently, it takes two to three years and extensive labor and expense to evaluate for resistance to SDS among promising breeding lines. If a marker could be found that is highly correlated to SDS resistance then breeding for that trait could commence immediately with the field testing for SDS resistance performed later. Further analysis of these bands may provide a greater understanding of the complex nature of SDS resistance in the three most resistant bermudagrass cultivars in this study. It is also very possible that the bands have no relations to SDS resistance, but are associated with the common genetic background of the Midlawn, Midiron, and MSU cultivars. Further research is necessary to characterize genetic utility of these band in support of breeding for resistance to SDS.

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Table1. Bermudagrass varieties examined for genetic relatedness, their propagation method and infection area with spring dead spot caused by *Ophiosphaerella herpotricha* used in this study

Ref #	Cultivar	Propagation Type	SDS Patch Area in Cm^2
1	Midlawn	Clone	20
2	Cardinal	Clone	25
3	Tifway	Clone	469
4	Tifsport	Clone	472
5	Tifgreen	Clone	486
6	Numex Sahara	Seed	617
7	Arizona Common	Seed	622
8	Jackpot	Seed	681
9	SWI 11	Seed	716
10	Mirage	Seed	746
11	Pyramid	Seed	1105
12	Mini Verde	Clone	1194
13	Michigan	Clone	Data unavailable as not released
14	Midiron	Clone	Resistant cultivar
15	Greg Norman	Clone	1368
16	Yukon	Seed	166

*Dennis Martin 2001

Table 2. Sequence of the DAF primers used in this study.

Primer	Sequence
9110	CAGAAACGCC
9111	GAAACGCC
9112	GTAACGCC
9113	GTAACC

Figure 1. DNA fingerprinting gel with DAF 9111 primer stained with SYBR gold

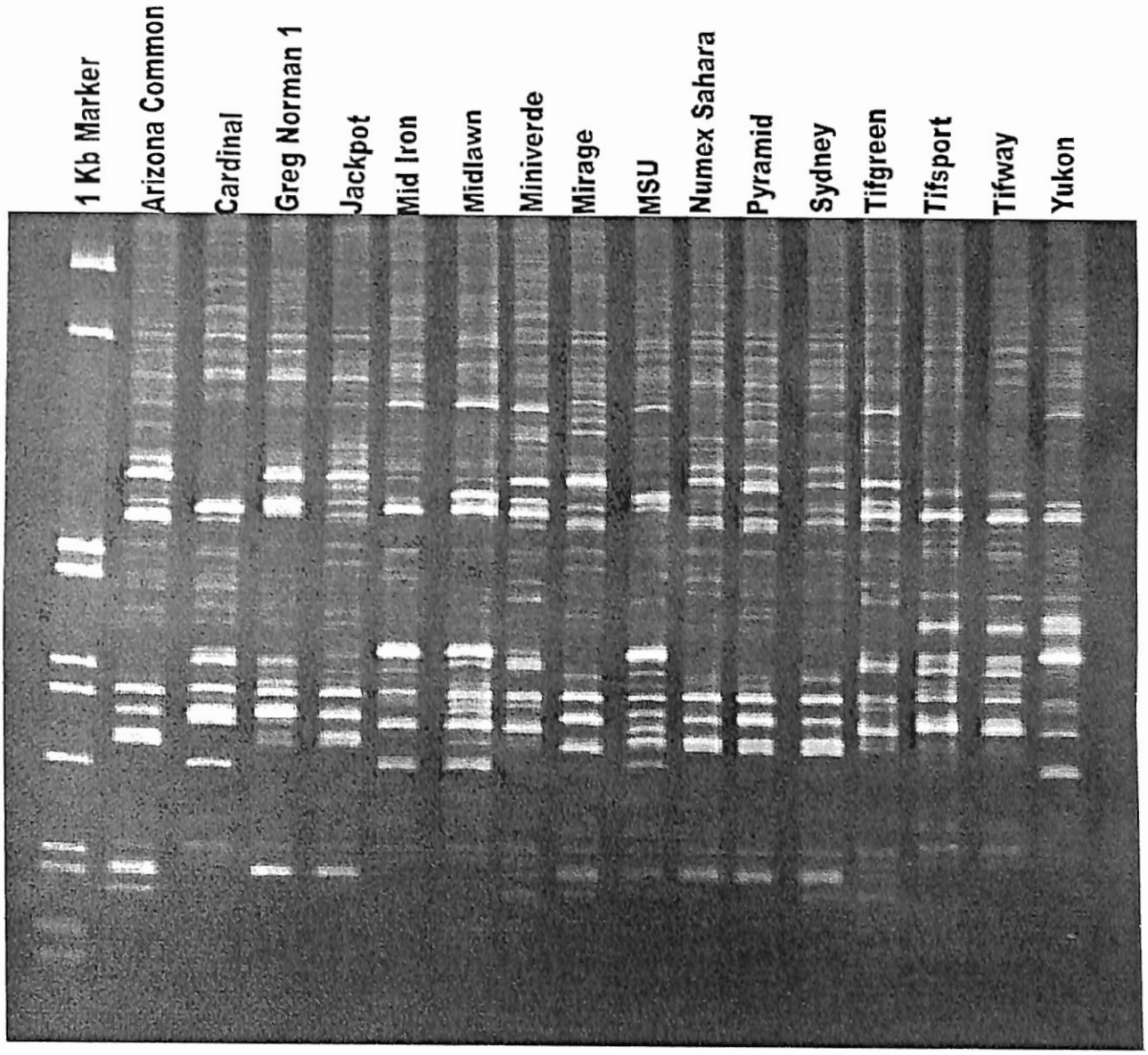
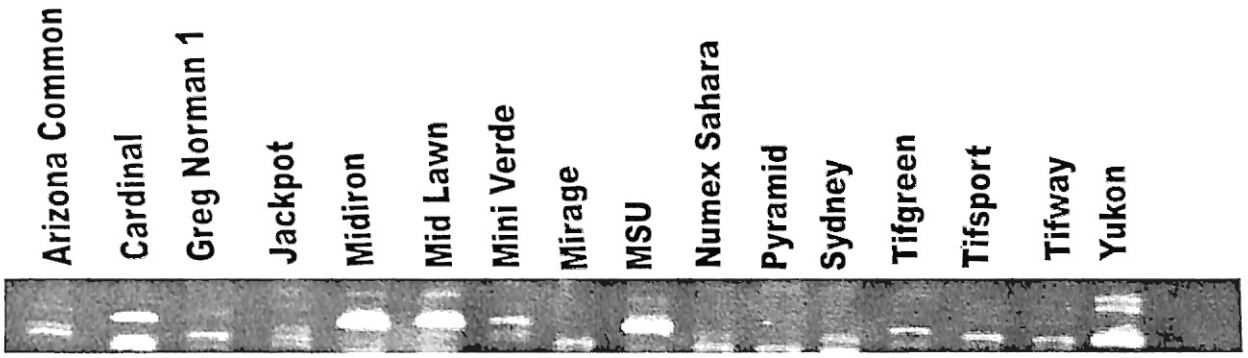


Figure 2: 9111 band 30 Bands found to be present only in the resistant cultivar



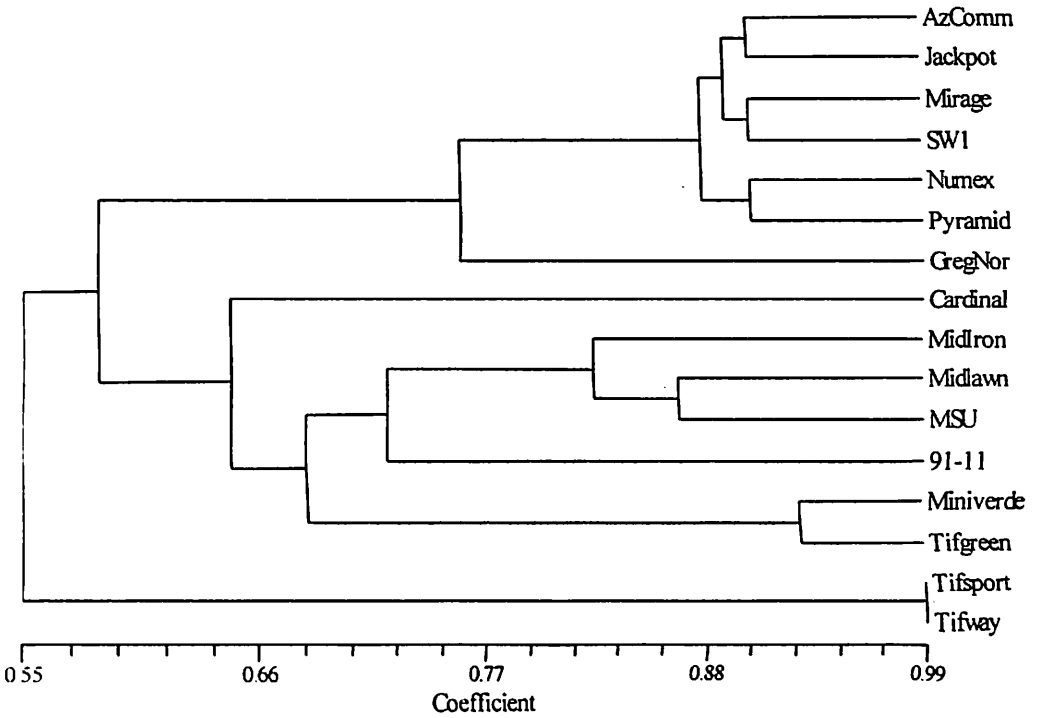


Figure 3. UPMGA based Cluster Analysis with DAF primers

Table 3. Similarity Coefficient with DAAF

	Az Comm	Cardinal	Greg Norman	Jackpot	MidIron	Midlawn	Miniverde	Mirage	MSU	Numex	Pyramid	SW1	Tifgreen	Tifsport	Tifway	Yukon
Az Comm	1															
Cardinal	0.62	1														
Greg Nor	0.77	0.66	1													
Jackpot	0.90	0.65	0.77	1												
MidIron	0.65	0.71	0.66	0.57	1											
Midlawn	0.59	0.67	0.62	0.56	0.84	1										
Miniverde	0.61	0.60	0.57	0.54	0.77	0.71	1									
Mirage	0.88	0.62	0.74	0.90	0.60	0.54	0.59	1								
MSU	0.67	0.66	0.61	0.62	0.80	0.87	0.65	0.62	1							
Num Sah	0.88	0.57	0.74	0.90	0.57	0.56	0.54	0.85	0.62	1						
Pyramid	0.85	0.60	0.72	0.85	0.55	0.49	0.56	0.88	0.57	0.90	1					
SWI 1	0.88	0.65	0.79	0.90	0.60	0.56	0.59	0.90	0.65	0.90	0.90	1				
Tifgreen	0.59	0.60	0.60	0.56	0.77	0.71	0.93	0.56	0.67	0.54	0.54	0.56	1			
Tifsport	0.55	0.56	0.63	0.50	0.63	0.62	0.55	0.52	0.59	0.50	0.48	0.52	0.52	1		
Tifway	0.54	0.55	0.62	0.49	0.62	0.61	0.54	0.51	0.57	0.49	0.46	0.51	0.51	0.99	1	
Yukon	0.57	0.63	0.56	0.52	0.73	0.70	0.60	0.55	0.73	0.52	0.50	0.55	0.57	0.56	0.72	1

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