

Genetic Diversity of Warm-Season Turfgrass: Seashore Paspalum, Bermudagrass, and Zoysiagrass Revealed by AFLPs

Zhenbang Chen^{1*} • Ming Li Wang^{2**} • Clint Waltz¹ • Paul Raymer¹

¹ Crop and Soil Sciences, USDA-ARS, University of Georgia at Griffin-Campus, 1109 Experiment Street, Griffin, GA 30223, USA

² PGRCU, USDA-ARS, University of Georgia at Griffin-Campus, 1109 Experiment Street, Griffin, GA 30223, USA

Corresponding author: * zchen@uga.edu ** mingli.wang@ars.usda.gov

ABSTRACT

Three major types of warm-season turfgrass, including seashore paspalum (*Paspalum vaginatum* Swartz), bermudagrass [*Cynodon dactylon* (L.) Pers.], and zoysiagrass [*Zoysia japonica* Steud., *Zoysia matrella* (L.) Merr., and *Zoysia tenuifolia* auct.] cover many of the golf courts and sport fields in southern regions of the U.S. Improvement of turfgrass cultivars has been mainly based on the selection from natural mutations or genetic variations resulting from recombination of different ecotypes or species (hybrid bermudagrass and zoysiagrass). Genetic diversity among species and among turfgrass cultivars within species (including 10 seashore paspalum cultivars, 14 bermudagrass cultivars, and 24 zoysigrass cultivars and elite lines) was assessed using amplified fragment length polymorphism (AFLP) markers. Among species, the polymorphism level of zoysiagrass is higher than bermudagrass and the polymorphism level of bermudagrass is higher than seashore paspalum. Our results demonstrated that AFLP is one of the useful DNA marker systems for quickly revealing the level of genetic diversity among species and assessing the genetic diversity of different turfgrass cultivars within the species. However, some released turfgrass cultivars could not be differentiated in this report by AFLP markers because they were developed from the parents that are closely related genetically. To enhance turfgrass breeding efficiency, different types of DNA marker systems should be used for evaluating turfgrass germplasm. Based on genetic diversity evaluation, more diverged parents should be selected and used to make crosses for developing new turfgrass cultivars.

Keywords: cultivar improvement, DNA markers, genetic diversification, turfgrass cultivars

Abbreviations: AFLP, amplified fragment length polymorphism; SSR, simple sequence repeat

INTRODUCTION

Turfgrass is a unique group of plant species cultivated for ornamental decoration around us, for recreation in golf courses and sports fields, and for land coverage and protection. Turfgrass covers 50 million acres in the U.S. and turfgrass industry plays an important role in the U.S. economy, with more than \$580 million in annual seed sales, second only to corn hybrid seeds (*Zea mays* L.) (Kidd 1993; Lee 1996). While contributing to soil development, stabilization, and improvement, and erosion control, turfgrass is also used to beautify the earth, enrich our lives, and provide recreation and enjoyment for people. Turfgrass is maintained on lawns, estates, parks, golf courses, playing fields and public grounds. Since the establishment of turfgrass culture in North America and the rest of the world, cultivar improvement has always been a central issue. As the result of the persistent efforts of breeders, significant achievements have been made in turfgrass breeding. Since 1946, more than 245 warm-season and cool-season cultivars have been registered in the USA (Lee 1996). These achievements have contributed to the establishment and growth of the turfgrass industry and the development of turfgrass science (Beard 1973).

Among 40 grass species used as turfgrass, seashore paspalum (*Paspalum vaginatum* O. Swartz), bermudagrass (*Cynodon* spp. Rich.), and zoysiagrass (*Zoysia tenuifolia* auct.) are warm-season species covering most of the non-agriculture land in the southern part of the United States. Seashore paspalum is a sustainable turfgrass because of its tolerance to abiotic stresses, especially to high salt concentration and drought (Carrow and Duncan 1998; Huang *et al.* 1997), and water logging (Colman and Wilson 1960).

Distinguishing seashore paspalum cultivars solely upon morphological traits is difficult and has proven challenging for breeders and inspectors charged with certifying cultivar purity. Genetic diversity among different ecotypes was assessed using Amplified Fragment Length Polymorphism (AFLP) markers (Chen *et al.* 2005) and transferred simple sequence repeat (SSR) markers (Wang *et al.* 2006). Bermudagrass is a highly self-incompatible and long-lived perennial grass (Forbes and Burton 1963; Burton and Hart 1967; Taliaferro 2003). Genetic variation within the variety *dactylon* is extensive. The genetic diversity of *Cynodon dactylon* var. *dactylon* (common bermudagrass) was assessed among 28 accessions by AFLPs (Wu *et al.* 2004). Since common bermudagrass is a widely distributed and extensively used species in the world, more accessions (especially for commercial cultivars) need to be characterized and evaluated genetically to cover its full spectrum of genetic variation. Zoysiagrass is a self-pollinated but favoring in out-crossing species, native to coastal area of China, Japan and other parts of Southeast Asia (Shoji 1983). There are three principal species of zoysiagrass used for turfgrass: *Zoysia japonica*, *Zoysia matrella*, and *Zoysia tenuifolia*. They are different in both morphological traits (such as leaf texture and physiological traits, salt tolerance, cold tolerance) and growth characteristics (Duble 1996; Patton *et al.* 2007). An AFLP-based linkage map of zoysiagrass has been constructed (Cai *et al.* 2004) but the genetic diversity within the species has not been well evaluated.

Turfgrass improvement traditionally has relied on conventional breeding methods, in which the accessible genetic material is restricted by sexual reproduction (Casler and Duncan 2003). Biotechnology is being used to supplement and complement traditional methods of plant im-

Table 1 Selected turfgrass materials.

Cultivar name	Type of turfgrass	Source	Cultivar name	Type of turfgrass	Source
Adlayd	Seashore paspalum	Griffin, GA	Meyer	Zoysia grasses	NTEP, Griffin, GA
Aloha	Seashore paspalum	Griffin, GA	Emerald	Zoysia grasses	NTEP, Griffin, GA
Azul	Seashore paspalum	Griffin, GA	himeno	Zoysia grasses	NTEP, Griffin, GA
Sea Spray	Seashore paspalum	Griffin, GA	J-37	Zoysia grasses	NTEP, Griffin, GA
Salam	Seashore paspalum	Griffin, GA	Zenith	Zoysia grasses	NTEP, Griffin, GA
Supreme	Seashore paspalum	Griffin, GA	PZA-32	Zoysia grasses	NTEP, Griffin, GA
Durban	Seashore paspalum	Griffin, GA	PZB-33	Zoysia grasses	NTEP, Griffin, GA
SeaIsle2000	Seashore paspalum	Griffin, GA	companion	Zoysia grasses	NTEP, Griffin, GA
Sea Dwarf	Seashore paspalum	Griffin, GA	PST-R7ZM	Zoysia grasses	NTEP, Griffin, GA
SeaIsle1	Seashore paspalum	Griffin, GA	PST-R7MA	Zoysia grasses	NTEP, Griffin, GA
GATM-1	Bermudagrass	Griffin, GA	GN-Z	Zoysia grasses	NTEP, Griffin, GA
GACL-1	Bermudagrass	FL	DALZ 0102	Zoysia grasses	NTEP, Griffin, GA
Champion	Bermudagrass	FL	DALZ 0104	Zoysia grasses	NTEP, Griffin, GA
Quality	Bermudagrass	FL	BMZ230	Zoysia grasses	NTEP, Griffin, GA
Tifgreen	Bermudagrass	Tifton, GA	DALZ9604	Zoysia grasses	NTEP, Griffin, GA
Tifwarf	Bermudagrass	Tifton, GA	DALZ0105	Zoysia grasses	NTEP, Griffin, GA
Tifsport	Bermudagrass	Tifton, GA	DALZ0101	Zoysia grasses	NTEP, Griffin, GA
Tifeagle	Bermudagrass	Tifton, GA	Zorro	Zoysia grasses	NTEP, Griffin, GA
ST-5	Bermudagrass	Tifton, GA	6186	Zoysia grasses	NTEP, Griffin, GA
Tifway(B)	Bermudagrass	Tifton, GA	Chinese common	Zoysia grasses	NTEP, Griffin, GA
Tifway(F)	Bermudagrass	Tifton, GA	Zeon	Zoysia grasses	NTEP, Griffin, GA
05-139	Bermudagrass	Tifton, GA	EI Toro	Zoysia grasses	NTEP, Griffin, GA
T-10	Bermudagrass	Tifton, GA	Empress	Zoysia grasses	NTEP, Griffin, GA
T-11	Bermudagrass	Tifton, GA	Empire	Zoysia grasses	NTEP, Griffin, GA

provement by bridging the individual gene pools in nature and thereby creating opportunities for breeders to utilize the genetic material from heterologous sources. Introgression of genetic traits from unrelated plant species or even other kingdoms has become a realistic breeding objective (Asay and Slepner 1989; DeBlock 1993). Plant biotechnology has been applied to turfgrass improvements during the past two decades. The achievements made in turfgrasses can be categorized as applications of molecular markers to assist breeding practices, *in vitro* culture, genetic engineering, and the use of fungal endophytes to improve turfgrass performance. Breeders have been emphasizing the development of better quality, and faster growth with wide adaptability to diverse environments. Elite lines or ecotypes have been developed mainly by selection of natural genetic mutations. However, genetic study on these species has lagged behind other crops because of poor pollination (Sano *et al.* 1979; Sano 1983; Sano 1990), and difficulty to get a normally segregated population (Jenczewski *et al.* 1997; Bert *et al.* 1999; Cai *et al.* 2004). The situation is changing as molecular techniques are employed to select diverse germplasm in turfgrass breeding programs (Yaneshita *et al.* 1999; Cai *et al.* 2004). The objective of this study is to evaluate the genetic diversity of commercial cultivars within and among seashore paspalum, bermudagrass, and zoysiagrass using AFLP technology. Our research indicates that DNA fingerprinting using AFLP provides a quick method for identification for commercial cultivars and also provides useful information for future turfgrass improvement efforts.

MATERIALS AND METHODS

Plant materials

Names and collection sites for 10, 14, and 24 commercial cultivars and elite lines from seashore paspalum, bermudagrass, and zoysiagrass, respectively are listed in **Table 1**. Most of these 48 turfgrass commercial cultivars and elite lines were planted and maintained in the greenhouses of the University of Georgia at either Griffin or Tifton campus.

DNA extraction

Newly emerged fresh leaves were collected from the established perennial plants and kept on ice till DNA extraction. DNA was extracted using the CTAB method (Doyle and Doyle 1990) with

some modifications. About 0.2 g of leaf tissue was used for DNA extraction. Leaf tissue were grounded in 700 µl of 2% CTAB buffer with Cermeric Bids (MP Biomedicals, OH, USA) using a Retsch® MM300 shaker (Haan, German) at a frequency of 30/s for 4 min. After grounding, the samples were incubated in a water bath for 30 min at 65°C. After incubation, the isolation of genomic DNA followed the protocol of the CTAB method. After extraction, the quality of DNA was examined on a 1% agarose gel. The DNA concentration was quantified with a Hoefer DyNA Quant 200 Fluorometer (California, USA) and then diluted to the concentration of about 50 ng/µl for the next step of the experiment.

Fingerprinting by AFLP

Amplified Fragment Length Polymorphism (AFLP) was carried out according to the method of Vos *et al.* (1995) with modifications. Approximately 200 ng of genomic DNA were used for digestion with *EcoRI* and *MseI*. Amplified DNA fragments were separated on a 5% denatured acrylamide gel using ABI PRISM™ 377 DNA sequencer. Gel images were generated using GeneScan 3.0 (Applied Biosystems, California, USA). To make sure the collected fingerprinting results reliable, at least two gel images were produced and collected from two separate experiments for each primer combination.

Data analysis

The clear bands from the gel images were scored manually and POPGENE software (Ver. 1.31, Microsoft Window-based Free-ware for Population Genetic Analysis) was used for calculation of the genetic distance. The results were confirmed with NTSYS-pc (version 2.0, Numerical Taxonomy and Multivariate Analysis System) software. Similarity matrix was calculated by utilizing SIMQUAL. Sequential, agglomerative, hierarchical and nested clustering was carried out with unweighted pair-group method and arithmetic average (UPGMA) method. A simple matching coefficient was used to calculate the similarity matrix in NTSYS-pc.

RESULTS AND DISCUSSION

Polymorphism level among different types of turfgrass

Genomic DNA from different types of turfgrasses were digested with restriction enzymes, *EcoRI* and *MseI*, ligated with different adaptors and then amplified. The amplified

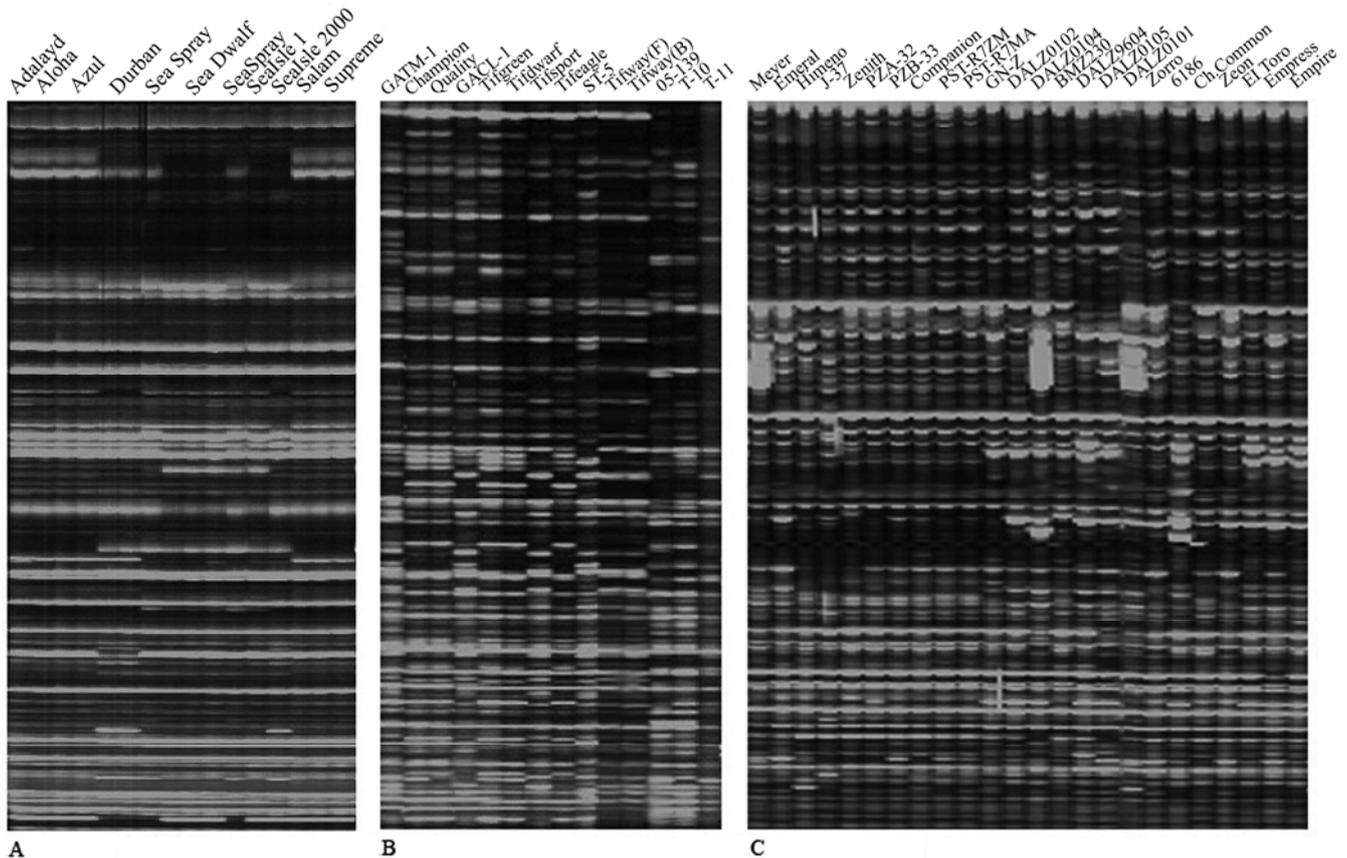


Fig. 1 Fingerprinting profile of turfgrass cultivars from seashore paspalum, bermudagrass, and zoysiagrass. (A) Gel image from seashore paspalum; (B) gel image from bermudagrass; (C) image from zoysiagrass. Primer combination of AAC-CAC was used for all three types of turfgrass.

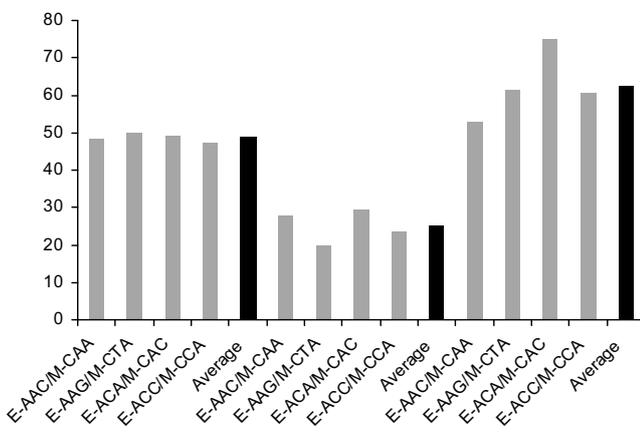


Fig. 2 Percentage of polymorphic bands from four different primer combinations for three types of turfgrass.

DNA fragments were separated on 5% acrylamide gels. As one example, the fingerprinting from each turfgrass species (seashore paspalum, bermudagrass, and zoysiagrass) among different cultivars is shown in **Fig. 1A**, **1B**, and **1C**, respectively. From the observation of fingerprinting profiles, it seemed that the level of polymorphism (ranking from high to low) among different cultivars within each type of turfgrass revealed by AFLP should be zoysiagrass, bermudagrass, and seashore paspalum. Then, recordable polymorphic and non-polymorphic bands were counted from different primer combinations and the percentage of polymorphic bands was calculated and is shown in **Fig. 2**. On average, the highest polymorphism level was revealed within zoysiagrass (62.5%), followed by bermudagrass (48.8%) and seashore paspalum (25.2%). In general, when AFLP technique is employed, polymorphisms can be easily revealed among cultivars within zoysiagrass, whereas poly-

morphisms can not be easily revealed among cultivars within seashore paspalum. These results were consistent with former reports (Chen *et al.* 2005; Wang *et al.* 2006). A comparison of four different primer combinations shows that E-ACA/M-CAC seems to be a better primer combination to reveal polymorphism (75.0%) in zoysiagrass than other primer combinations. In contrast, there is a less significant difference ($P > 0.01$) for revealing polymorphism among different primer combinations in bermudagrass (from left to right 48.5, 50.0, 49.2 and 47.4 in **Fig. 2**). For any species, the level of polymorphisms revealed within a species may depend on how many samples assayed and how well the samples represented. More well-represented (i.e. diverged) samples are to be assayed and a higher level of polymorphism within a species will be revealed. The level of polymorphism revealed within a species may also relate to its means of pollination (Wang *et al.* 2005). Within the grass family, a higher level polymorphism will be revealed within a cross-pollinated species than a self-pollinated species. Taken together, the number of cultivars assayed and the ways of pollination employed of zoysiagrass may explain a higher level of polymorphism revealed from zoysiagrass than seashore paspalum and bermudagrass.

Genetic diversity within each type of turfgrass

Seashore paspalum: With four primer combinations, 73 polymorphic fragments were identified among 10 cultivars within seashore paspalum. The primer combinations (E-AAC/M-CAA and E-ACA/M-CAC) revealed a percentage of polymorphisms (27.8 and 29.4%), higher than other primer combinations (E-AAG/M-CTA and E-ACC/M-CAA; 20.0 and 23.5%). A dendrogram was generated from these polymorphic DNA bands and shown in **Fig. 3A**. Ten cultivars were classified into four small groups. “Sea Spray” as group I was different (with about 50% dissimilarity) from the other nine cultivars. Sea Dwalf, SeaIsle 1 and SeaIsle 2000 as group II clustered together. Durban as group III was

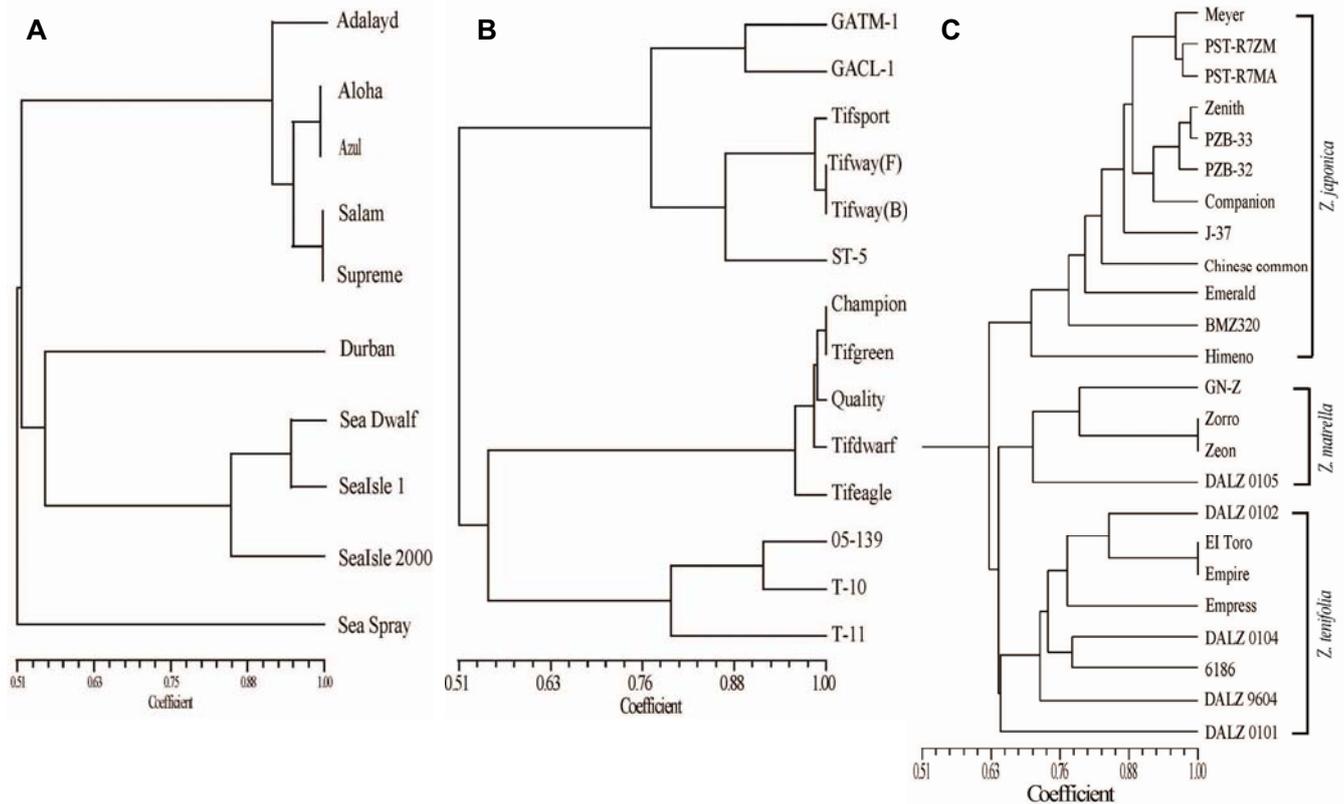


Fig. 3 Dendrograms from different types of turfgrass. (A) Seashore paspalum; (B) Bermudagrass; (C) Zoysiagrass.

related to group II. Adalayd, Aloha, Azul, Salam and Supreme as group IV clustered together. Within this group, there was not much genetic difference between Aloha and Azul, and between Salam and Supreme, respectively. In order to further distinguish these two pairs of cultivars, more DNA markers are required. Since “Sea Spray” is different from other seashore paspalum cultivars, it may be a good parent to use for making crosses for developing new seashore paspalum cultivars.

Bermudagrass: With four primer combinations, 128 polymorphic fragments were identified among 14 cultivars within bermudagrass. The primer combination could affect the polymorphism level revealed from the same set of cultivars but for bermudagrass in this experiment, the primer combination seemed to not very significantly affect the revealed polymorphism level. The revealed polymorphism level for each primer combination was 48.5, 50.0, 49.2, 48.8%, respectively (from left to right in Fig. 2). A dendrogram was generated from these 128 polymorphic DNA bands and shown in Fig. 3B. Fourteen bermudagrass accessions were classified into three major groups. Cultivars 05-139, T-10 and T-11 clustered together as group I but T-11 was at least 15% different from 05-139 and T-10 genetically. Five cultivars (Champion, Tifgreen, Quality, Tifdwarf, and Tifeagle) clustered together as group II and these five accessions were very similar genetically (with over 90% similarity). Champion, Tifgreen, Tifdwarf and Tifeagle have been historically used as grasses for golf course putting greens, because of their tolerance to low mowing heights (e.g. 5 mm or less). Six cultivars (GATM-1, GACL-1, Tifsport, Tifway-F, Tifway-B, and ST5) clustered together as group III and can be classified into three subgroups (a, b, and c). ST-5 as subgroup IIIa was at least 10% different from three cultivars (Tifway-B, Tifway-F, and TIFSPORT) as subgroup IIIb. Within this subgroup, there was no much genetic difference between Tifway-B and Tifway-F. GATM-1 and GACL-1 as subgroup IIIc were very different from other two subgroups. In bermudagrass breeding programs, parents can be selected from different groups to make crosses for developing new cultivars.

Zoysiagrass: With four primer combinations, 226 polymorphic fragments were identified among 24 cultivars within zoysiagrass. Obviously, E-ACA/M-CAC was a good primer combination to reveal polymorphisms (75.0%) in zoysiagrass. A dendrogram was generated from these 226 polymorphic DNA bands and shown in Fig. 3C. Twenty-four zoysiagrass cultivars were classified into three major groups. Eight cultivars (from DALZ 0101 to DALZ 0102) from *Z. tenuifolia* clustered together as group I. Four cultivars (from DALZ 0105 to GN-z) from *Z. matrella* clustered together as group II. Twelve cultivars (from Himeno to Meyer) from *Z. japonica* clustered together as group III. Each group represents a species: group I for *Z. tenuifolia*, group II for *Z. matrella*, and group III for *Z. japonica*. Intriguingly, the genetic difference among three groups was less than 8%, whereas the genetic difference within group (or species) I, II, and III was at least 35, 38, and 38%, respectively (Fig. 3C). One of our explanations for this phenomenon is the means of pollination. All three zoysiagrass species are favoring out-pollination and high rate of out-pollination will keep high level of genetic diversity within the species. Another explanation is that comparison of the species with high levels of polymorphisms will lead to a small difference among species within *Zoysia* genus. Since a high level of genetic diversity exists within a species (or a group), there is a potential to develop new cultivars by selecting diverged parents to make crosses within the species.

Repeatability of AFLPs

AFLPs, to some extent, are fingerprinting of PCR products generated from different pairs of 4-mer primer combinations. The PCR conditions used are critical for repeatability of AFLPs. A highly stringent PCR condition (for example, 60°C annealing temperature) was employed in our experiments. Therefore, our results from AFLPs are highly repeatable. However, there were always some minor bands or nonspecific bands amplified randomly, which were not repeatable. This is also common to SSR markers and other PCR-based markers. To reduce the minor bands, more stringent conditions were used when the second/selective PCR

was conducted. The annealing temperature of 60°C was used instead of a low temperature for touch-down process. The major bands were reproducible between different experiments and there were always some minor bands observed (data not shown). The adjustment of minor and major bands is subjective so only the major and repeated bands should be recorded and used for fingerprinting analysis. To make sure AFLPs are repeatable, it is necessary to repeat the whole process in separate experiments for collecting gel images for fingerprinting analysis.

In the present study, the genetic diversity of 40 warm-season turfgrass cultivars has been assessed by AFLP analysis. Narrow genetic variation has been detected in both seashore paspalum and bermudagrass cultivars. To enlarge the genetic base of seashore paspalum and bermudagrass cultivars, diverged genetic materials need to be used as parents and introduced into turfgrass breeding programs. Before introduction, the diverged genetic materials (accessions) need to be identified first from the germplasm collection. However, in comparison with other major crop species, there are very limited genomic resources (for example, DNA markers and sequences, known-function genes for useful agronomic traits) available in warm-season turfgrass. This is greatly hindering the genetic studies on exploiting germplasm and identifying diverged genetic materials in warm-season turfgrass. Therefore, there is urgent need to develop genomic resources for warm-season turfgrass. Once genomic resources are available, the genetic resources from warm-season turfgrass can be adequately characterized genetically and more diverged genetic materials can be identified. Utilizing more diverged genetic materials as parents in the future breeding programs will eventually help to enlarge the genetic base and develop the new cultivars of warm-season turfgrass.

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