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DNA amplification fingerprinting analysis of bermudagrass (*Cynodon*): genetic relationships between species and interspecific crosses

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Abstract We have used DNA amplification fingerprinting (DAF) to study the genetic variation of bermudagrass (*Cynodon*) species and cultivars of interspecific crosses that exhibit leaf-blade textural characteristics ranging from coarse to fine. Arbitrary octamer primers produced complex and reproducible amplification profiles with high levels of polymorphic DNA. Phylogenetic analysis using parsimony (PAUP) and unweighted pair group cluster analysis using arithmetic means (UPGMA) grouped 13 bermudagrass cultivars into several clusters, including one containing the African-type bermudagrasses (*C. transvaalensis*) and another containing the common-type bermudagrasses (*C. dactylon*). The latter group included *C. magennissii* ('Sunturf') and a interspecific *C. transvaalensis* × *C. dactylon* cross ('Midiron'), 2 cultivars that exhibited leaf textural characteristics closer to the common-types. All other *C. transvaalensis* × *C. dactylon* crosses grouped between the African and common types. An extended screen of 81 octamer primers was needed to separate cultivar 'Tifway' from the irradiation-induced mutant 'Tifway II'. The use of either template endonuclease digestion prior to amplification or arbitrary mini-hairpin primers increased detection of polymorphic DNA and simplified the task of distinguishing these closely related cultivars. Alternatively, the use of capillary electrophoresis (CE) resolved fingerprints adequately and detected products with high sensitivity, thereby promising to increase throughput and the detection of polymorphic DNA. When used to fingerprint samples from commercial sources, DAF identified bermudagrass plant material on the basis of unique reference profiles generated with selected primers. DAF represents an excellent technique for bermudagrass cultivar verification, seed certification, varietal protection, and for the identification of mistakes in plantings, mislabeled plant materials, and contamination or substitutions of sod fields.

Key words Bermudagrass · Arbitrary primers · DAF · Capillary electrophoresis · Genetic relationships

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Introduction

The genus *Cynodon* (bermudagrass) constitutes several species of very important sod-forming warm-season (C_4) grasses used extensively in the Turf industry throughout the world (Anonymous 1972; Beard 1973; Juska and Hanson 1964; Turgeon 1980). Primary areas of use are lawns, golf courses, tennis and bowling greens, athletic fields, industrial and institutional grounds, parks and playgrounds, highway medians and roadsides, and for steep bank erosion control. The principle bermudagrass species used for Turf are *C. dactylon* (L.) Pers. (common types), *C. transvaalensis* Burtt-Davy (African types), and *C. magennissii* Hurcombe (Magennis type). Essentially all intensively maintained Turf situations, such as golf courses, athletic fields, and tennis and bowling greens, utilize the *C. dactylon* and *C. transvaalensis* species, and especially interspecific crosses of these. None of the *Cynodon* species are native to the United States but are believed to have been introduced from Africa in 1751 (Beard 1973). The center of origin of *Cynodon* appears to be located along the Indian Ocean coasts of East Africa and Asia.

Each turfgrass cultivar being marketed results from the evaluation of thousands of selections from interspecific crosses, normally of two species, such as *C. dactylon* × *C. transvaalensis*. However, the thousands of experimental selections from a particular cross commonly exhibit a wide variety of morphological characteristics ranging from

coarse- to fine-textured leaves, and other plant parts (Beard 1973). A cultivar derived from a *C. dactylon* × *C. transvaalensis* cross, for example, can exhibit very fine textured leaves characteristic of *C. transvaalensis* but can also carry the unexpressed genes for coarse leaves typical of *C. dactylon*. Thus, it would be desirable to know the genetic relationship of the primary bermudagrass species and selected cultivars used for Turf. It should also be noted that the breeding of the bermudagrasses has been accomplished without the use of modern molecular techniques and usually without much knowledge of the genetics of selected characters. Therefore, it would be advantageous to use molecular markers in marker-assisted selection strategies to guide the introgression of desirable genes from related species, much as it has been done for many agricultural crops.

The amplification of anonymous genomes with arbitrary oligodeoxyribonucleotides has proved a versatile and universal method for detecting polymorphisms for genetic mapping, phylogenetic analysis, population biology, and general fingerprinting applications (reviewed in Caetano-Anollés 1993, 1994). These short primers produce diagnostic fingerprints by targeting multiple amplicons of an arbitrary nature. DNA amplification fingerprinting (DAF) uses primers as short as 5 nucleotides (nt) and generates relatively complex amplification profiles (Caetano-Anollés et al. 1991). In contrast, other techniques such as random amplified polymorphic DNA (RAPD) analysis produce simple profiles (Williams et al. 1990). DAF has been coupled to restriction endonuclease digestion of template DNA (Caetano-Anollés et al. 1993) or to the use of structured primers containing mini-hairpin sequences at their 5′ terminus (Caetano-Anollés and Gresshoff 1994a) to increase the detection of polymorphic DNA and allow fingerprinting of small fragments of DNA generated by cloning or polymerase chain reaction (PCR) amplification. These tailoring strategies are useful in those cases where polymorphisms are to be detected between organisms that are closely related, such as near-isogenic lines (NILs), or spontaneous or induced mutants. In the study presented here we have used DAF, with and without such tailoring strategies, to determine the genetic and phylogenetic relationships of 13 cultivars and species of bermudagrass that morphologically appear to span species and cultivars of interspecific crosses with leaf-blade textural characteristics ranging from coarse to fine. This study constitutes the first step to help define genetic relationships within this important turfgrass genus and is the first report of capillary electrophoresis being used to analyze DAF profiles.

Materials and methods

Seven cultivars from three species of *Cynodon* (*C. dactylon*, *C. transvaalensis*, and *C. magennissii*) and 6 different *C. dactylon* × *C. transvaalensis* interspecific crosses were used in this study (Table 1). The 2 *C. transvaalensis* selections used were derived from a polycross of *C. transvaalensis* plant introductions 289922, 291591, 290905, and 290812. The selections could be differentiated morphologically but possesed fine-textured leaves typical of *C. transvaalensis* (Anonymous 1972; Beard 1973; Juska and Hanson 1964). The coarse-leaf-textured common bermudagrass and cv 'Sunturf' were

Table 1 Bermudagrass cultivars used in this study

Cultivar	*Cynodon* species	Chromosome number	Leaf texture (mm)[a]	Reference[b]	Source[c]
Ctr2570	*C. transvaalensis*	2n = 18	fine (1.2)	1,2,3	a
Ctr2747	*C. transvaalensis*	2n = 18	fine (1.2)	1,2,3	a
Tifgreen	*C. dactylon* × *C. transvaalensis*	2n = 27[d]	fine (1.8)	1,3,4	b
Tifdwarf[e]	*C. dactylon* × *C. transvaalensis*	2n = 27[d]	fine (1.7)	1,5	b
Tiffine	*C. dactylon* × *C. transvaalensis*	2n = 27[d]	fine (1.8)	1,3,6	c
Tifway	*C. dactylon* × *C. transvaalensis*	2n = 27[d]	medium (2.1)	1,3,7	b
Tifway II[f]	*C. dactylon* × *C. transvaalensis*	2n = 27[d]	medium (2.1)	8,9	b
Midiron	*C. dactylon* × *C. transvaalensis*	2n = 27[d]	coarse (3.0)	10	d
Sunturf	*C. magennissii*	2n = 27[g]	medium (2.0)	1,3,11	e
Tifton 10	*C. dactylon*	2n = 6× = 54	coarse (3.5)	12	f
Texturf 10	*C. dactylon*	2n = 36	coarse (3.0)	1,3	g
Vamont	*C. dactylon*	2n = 36	coarse (3.2)	–	h
Common	*C. dactylon*	2n = 36	coarse (3.5)	1,2,3	e

[a] Classification of leaf texture as defined in Beard (1973). Leaf blade width at the base of the blade (average of 10 measurements in mm) is given in parentheses

[b] References: 1, Anonymous (1972); 2, Beard (1973); 3, Juska and Hanson (1964); 4, Hein (1961); 5, Burton (1966b); 6, Hein (1953); 7, Burton (1966a); 8, Kinney and Flatt (1981); 9, Hanna (1990); 10, Keen (1971); 11, Huffine (1957); 12, Hanna et al. (1990)

[c] Cultivar source: a, C.M. Taliaferro, Oklahoma State Univ, Stillwater, Okla.; b, T. Hollifield, Georgia Crop Improvement Assoc, Athens, Ga.; c, J.B. Beard, Texas A&M Univ, College Station, Tex.; d, K.N. Morris, USDA, Beltsville, Md.; e, L.M. Callahan, Univ, Tennessee, Knoxville, Tenn.; f, W.W. Hanna, Georgia Coastal Plains Exp Stan, Tifton, Ga.; g, M.C. Engelke, Texas A&M Agric Exp Stn, Dallas, Tex; h, D. Whitt, Virginia Crop Improvement Assoc., Richmond, Va.

[d] Natural somatic mutant from 'Tifgreen'

[e] Gamma irradiation-induced mutant from 'Tifway'. Plants were grown from dormant sprigs treated with 9,000 rad[Co^{60}], sectors chosen for further testing, and finally selected

[f] F_1 sterile triploid hybrid

[g] Natural sterile triploid hybrid between *C. transvaalensis* and *C. dactylon*

from turf plots over 30 years of age maintained at the University of Tennessee. 'Sunturf' is a direct increase of 'Magennis' (*C. magennisii*). 'Magennis' is considered to be a *C. transvaalensis*×*C. dactylon* hybrid (Juska and Hanson 1964). The 6 interspecific *C. dactylon*×*C. transvaalensis* crosses were chosen because they exhibited a range of leaf texture and a variety of growth characteristics. Each essentially is an F_1 sterile triploid hybrid. Two of these cultivars are mutations, 'Tifdwarf' being a natural somatic mutation out of 'Tifgreen', and 'Tifway II', an irradiation-induced mutation out of 'Tifway' (also known as 'Tifway 419'). DNA was isolated from the leaves of these cultivars using the Dellaporta et al. (1983) procedure as modified by Sayavedra-Soto et al. (1995).

DAF was conducted using unstructured (Caetano-Anollés and Bassam 1993) or structured mini-hairpin (Caetano-Anollés and Gresshoff 1994a) primers. DAF reactions (20 µl) containing 3 µ*M* primer, 0.3 units/µl Ampli*Taq* Stoffel fragment DNA polymerase (PE-Cetus, Norwalk, Conn.), 0.1 ng/µl of template DNA, deoxynucleoside triphosphates, magnesium and a suitable buffer, were amplified in a recirculating hot-air thermocycler (Bios, New Haven, CT) for 35 cycles of 30 s at 96°C, 30 s at 30°C, and 30 s at 72°C. In template endonuclease cleavage multiple arbitrary amplicon profiling (tecMAAP), DNA was digested with *Hin*fI, *Msp*I, and *Bst*UI (all endonucleases with 4-bp specificities) prior to amplification (Caetano-Anollés et al. 1993).

Amplification products were separated using polyester-backed 5% polyacrylamide-7 *M* urea slab minigels (Caetano-Anollés and Bassam 1993). Concentration, integrity, and the structure of oligomers was confirmed by electrophoresis at room temperature in polyester-backed 20% polyacrylamide-7 *M* urea slab mini-gels. DNA was detected at the picogram level by silver staining (Bassam et al. 1991) according to a slightly modified protocol (Bassam and Caetano-Anollés 1993). In some cases, silver-stained profiles were scanned with an Apple Color One Scanner (Apple Computer, Cupertino, Calif.) and the Ofoto program (version 2.02; Light Source Computer Images Inc., Salinas, Calif.), and were analyzed and evaluated using the Think Pascal program *Image* for the Macintosh computer (Version 1.45; Wayne Rasband, NIH; Internet, wayne@helix.nih.gov) using a gel analysis macro.

DNA fragments were also separated by capillary electrophoresis (CE) using an ABI 270A-HT CE system (Applied Biosystems, Foster City, Calif.) fitted with a fused-silica capillary (75 µm ID, 70 cm, 50 cm effective length) and ABI DNA Fragment Analysis Sieving separation chemistry, modified by the addition of 20% urea and 10 µ*M* ethidium bromide. Prior to analysis, the capillary was flushed with 0.3 *N* NaOH, distilled deionized (DI) water, 5 N HCl, and DI water for 1 min each, and then rinsed with sieving polymer for 8 min. Electrophoresis was run at 210 V/cm and 30°C, using reverse polarity, and products detected at a wavelength of 260 nm. All sample injections were performed electrokinetically at –7 KV for 5 s. Samples were prepared for analysis by membrane dialysis. Amplification reactions were placed as a single drop on top of a VS 0.025 µm membrane (Millipore Inc., Bedford, Mass.) floating on water and allowed to dialyze for 20 min. Size calibration used a 100-bp ladder (Gibco BRL, Gaithersburg, Md.).

The genetic relationships between cultivars were explored by phylogenetic analysis using parsimony (PAUP, version 3.1; Swofford 1993). Bands (≤500 bp in length) in DAF gels loaded with diluted amplification reactions were scored as present (1) or absent (0), and entered as unordered, nondirected, and unweighted characters (Wagner mode for binary characters). The branch-and-bound algorithm was used to identify minimal trees (an exhaustive search was not possible due to time and RAM restraints). The significance of phylogenetic results was assessed by the bootstrap analysis (Felsenstein 1985) of the PAUP program. The dataset was analyzed by 100 bootstrap replications of branch-and-bound searches, with "furthest" stepwise addition sequence, MULPARS option, and 50% consensus calculation. Distance matrices showing absolute and relative number of non-shared bands and homoplasy values were calculated. Minimum trees were rooted by the midpoint rooting mode, since no suitable out-group could be included in the analysis. The dataset was also examined by unweighted pair group cluster analysis using arithmetic means (UPGMA), and a dendrogram was constructed. For this purpose, the NSYS-pc program (Numerical taxonomy and multivariate analysis system; version 1.7, Exeter Software, Setauket, N.Y.) was run using the distance estimator of Dice (Dice 1945).

Results and discussion

DAF of bermudagrass

We used a DAF protocol for turfgrasses that renders highly reproducible DNA fingerprints (Weaver et al. 1994). The protocol resulted from an iterative optimization of amplification parameters and has been used succesfully to fingerprint centipedegrasses [*Eremochloa ophiuroides* (Munro) Hack.], Zoysia (*Zoysia japonica* Steud.), rough bluegrass (*Poa trivialis* L.), and St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze] (Callahan et al. 1993b; Weaver et al. 1995; L.M. Callahan, unpublished). DNA from replicate plants were used to generate DAF profiles in independent experiments performed by different operators, sometimes done over 2 years apart. Figure 1A shows reproducible fingerprints generated in different experiments from different DNA isolations obtained from 'Tifdwarf' bermudagrass leaves. Similarly, repeated amplification of a DNA sample rendered indistinguishable fingerprints (data not shown). DAF consistency has been previously shown for the amplification of DNA from prokaryotic (Bassam et al. 1992; Jayarao et al. 1992) or eukaryotic (Caetano-Anollés 1994; Caetano-Anollés and Gresshoff 1994a; Gresshoff and McKenzie 1994) organisms.

Amplification with selected primers allowed distinction of the majority of bermudagrass cultivars described in Table 1 (Fig. 1B). These results validate the use of DAF as a reliable procedure for DNA typing of important bermudagrass cultivars.

Genetic relationships between bermudagrass cultivars

DNA fingerprints obtained with 11 unstructured arbitrary octamer primers were used to study the 13 bermudagrass cultivars. An average of 28.7±5.4 (mean±standard deviation) amplification products (≤500 bp in length) were obtained per primer. Both the number of polymorphisms detected and the number of unique fingerprint patterns were considerably high. From a total of 316 bands scored, 252 were polymorphic (i.e. the band was missing in at least one of the cultivars). All primers generated polymorphic products, with an average percentage of polymorphic loci per primer of 78±9%. However, the total number of bands shared by more than half of the cultivars (at least 7 out of 13) was 181, with an average percentage of loci per primer of 58±10%. Variation was ascribed mostly to polymorphisms within the *C. dactylon* and *C. magennissii* cultivars. The number of unique fingerprint patterns generated by the different primers ranged from 10 to 12 (77–92% of cultivar fingerprints). Cultivars 'Tifway' and 'Tifway II' could not be distinguished with these primers. However,

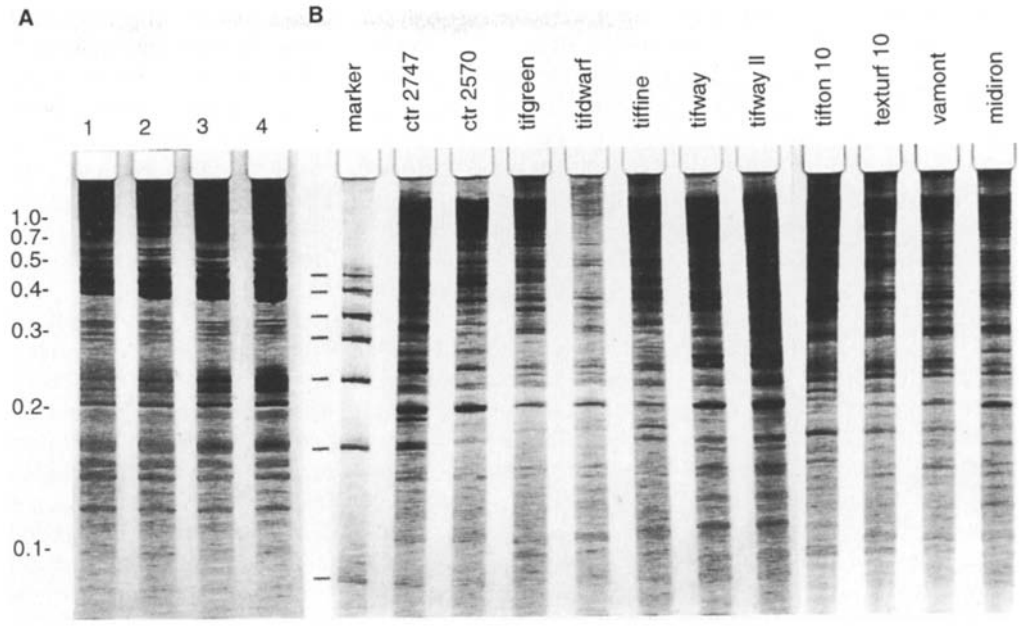

Fig. 1A, B DNA amplification profiles of bermudagrass cultivars. **A** Reproducible DNA amplification of cv 'Tifdwarf' amplified with primer GTTACGCC (8–9). DNA was isolated from different grass samples and amplified independently in May (*lane* 1), June (*lane* 2), July (*lane* 3) and September (*lane* 4) of 1994. **B** Amplification profiles of 13 *Cynodon* cultivars generated using primer GACGTAGG (8–5). Molecular weights are given in kb

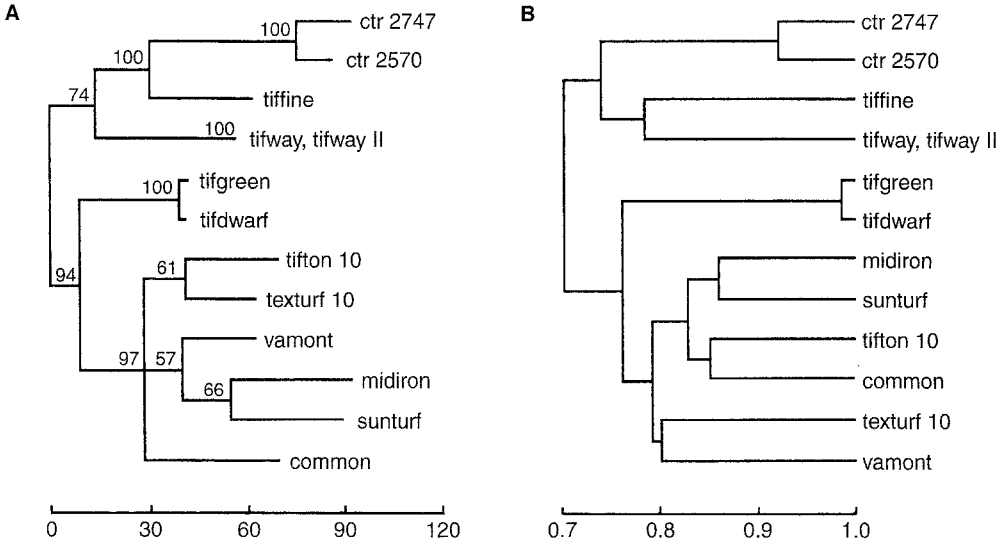

Fig. 2A, B Genetic relationships between bermudagrass cultivars. **A** Phylogram generated using PAUP showing a single minimal tree detected using the branch-and-bound algorithm. The scale indicates branch length between nodes and represents the number of character state changes. Bootstrap confidence values are indicated at each node. **B** Dendrogram generated by cluster analysis using UPGMA. The scale indicates relative genetic similarity of cultivars

cv 'Tifgreen' and its natural somatic mutant 'Tifdwarf' were differentiated by 5 polymorphisms generated with 3 primers. The average level of band-sharing ranged from 0.475 to 0.747 for the 11 primers tested, with an overall value of 0.591±0.076 (mean±standard error). While about 80% of the sampled loci were polymorphic, the estimated divergence of the sampled genomes based on cultivar-to-cultivar band-sharing depicted actual nucleotide divergence based on established criteria (Clark and Lanigan 1993). Compared to centipedegrass (Weaver et al. 1995), bermudagrass cultivars exhibited a much higher level of DNA polymorphism, suggesting higher genetic diversity in bermudagrass. However, DNA polymorphic content was comparable to that obtained in zoysiagrass (G. Caetano-Anollés, unpublished data).

Parsimony analysis was used to determine genetic relationships by analyzing band-sharing of the 316 loci scored. PAUP resulted in a single minimal phylogenetic tree (Fig. 2A). Bootstrap confidence values indicate that most groupings were robustly supported (data not shown). Because phylogenetic analysis implies a natural bifurcating evolutionary tree without hybridizations between its elements (Felsenstein 1988), parsimony analysis that includes hybrid plant material should be interpreted with caution, and if used, contrasted to distance matrix methods. However, cluster analysis using UPGMA rendered dendrograms analogous to those produced with PAUP (Fig. 2B). Cultivars were grouped into several clusters, one containing the African-type bermudagrasses and quite distinct from another containing the common types (Fig. 2). Clearly, all coarse-textured *C. dactylon* cultivars grouped together, including *C. magennissii* (Sunturf) and one interspecific *C. dactylon*×*C. transvaalensis* cross (Midiron), two cultivars that exhibit medium-to-coarse leaf texture

characteristics of *C. dactylon*. *C. magennissii* is a natural triploid hybrid between *C. transvaalensis* and *C. dactylon*. The rest of the interspecific crosses, all exhibiting fine-to-medium leaf texture, either grouped together with the fine-textured *C. transvaalensis* cultivars or were grouped separately. Within the *C. dactylon* group, lower bootstrap confidence values appeared not to support strongly any particular topology (Fig. 2A). The analysis was unable to separate the closely related cultivars 'Tifway' and 'Tifway II' and placed 'Tifgreen' and 'Tifdwarf' as very closely related.

Our results are in agreement with the breeding history of bermudagrass cultivars. African bermudagrasses (*C. transvaalensis*) were crossed with common-type bermudagrasses (*C. dactylon*), initially to introgress the desirable characters of fine leaf texture, wear tolerance, and dark-green leaf color. The interspecific hybridization between these bermudagrass species has resulted in the development of a number of Turf-type cultivars with the improved low temperature hardiness characteristics of common bermudagrass. The interspecific hybrids analyzed in this study were expected to express a range of morphological charactersistics between African and common types that would be reflected in their fingerprint phenotypes. Phenetic and cluster analysis confirmed such a correlation. The observation that medium-to-coarse-textured cv 'Midiron' and 'Sunturf' were unexpectedly placed within the monophyletic *C. dactylon* group and not outside this cluster indicates that they still retain and express much of the genome of common-type bermudagrass despite containing a set of chromosome material from a species clearly distinct at the molecular level. The distribution of cultivars within groups mimicked that inferred by leaf texture. Since arbitrary primers target genomic sequences in an unrestricted manner (Caetano-Anollés 1994), such correlation suggests that leaf texture is a complex trait determined by loci more or less randomly distributed throughout the bermudagrass genome. Finally, the agreement between the DAF-derived tree and the breeding history of cultivars validates the use of arbitrarily amplified DNA for genetic analysis at the species and cultivar level.

Fingerprint tailoring: finding genetic differences between an irradiation mutant and its parent

The cultivar 'Tifway' is a chance hybrid that appeared in a seed lot of *C. transvaalensis* supplied by D. Meredith (African Explosives & Chemical Ind., Johannesburg, South Africa) in 1954, and was assumed to result from a *C. transvaalensis*×*C. dactylon* cross. Since then, it has been widely used, especially for golf course fairways and athletic fields, because of its many desirable characteristics. An irradiation mutant of it, 'Tifway II', was released in 1981 as a more denser and weed-free turf. Though very difficult to differentiate from its parent, it has also been widely used for lawns, fairways, tees, and football fields. One of our goals was the identification of these cultivars by DNA fingerprinting. Because of their close genetic identity, our initial screen was unable to distinguish between these two cultivars. We therefore tested 81 unstructured octamer primers (98.8% rendered complex DAF fingerprints), 7 of which generated 2 polymorphic bands present in 'Tifway' and 8 present in 'Tifway II' (Table 2).

The identification of cultivars such as 'Tifway' and its irradiation mutant obviously requires a major investment of effort and resources. In order to facilitate distinction of closely related cultivars, accessions, or anonymous plant material, we tested two strategies known to increase the generation of polymorphic DNA: template endonuclease digestion (Caetano-Anollés et al. 1993) and the use of mini-hairpin primers (Caetano-Anollés and Gresshoff, 1994). Restriction endonuclease cleavage of the template DNA prior to amplification, a strategy known as tecMAAP, has been used for the identification of near-isogenic soybean lines and closely related plant accessions (Caetano-Anollés et al. 1993). tecMAAP produced 2 polymorphic bands after independent amplification of the restricted 'Tifway' and 'Tifway II' template with only 7 primers (Table 2). Similarly, we amplified DNA from 'Tifway' and its mutant using arbitrary primers having compact hairpin structures at their 5' termini. These terminal mini-hairpins are short DNA segments that form extraordinarily stable hairpin structures consisting of a loop of 3–4 nucleotides (nt) and a 2-nt stem (Hirao et al. 1992). Mini-hairpin primers have the property of doubling detection of polymorphic DNA when fingerprinting genomic DNA from centipedegrass (Caetano-Anollés and Gresshoff 1994a). In this study, DNA amplification with 7 mini-hairpin decamers generated 4 additional polymorphisms (Table 2).

Both strategies increased two-to-four fold the generation of polymorphic DNA. The reasons for such an increase are still speculative, but may stem from an increase in the size of the genome being probed during annealing or restriction. Digestion of the template could result in the differential destruction of amplicons and selective amplification of those products that lack internal restriction sites (Caetano-Anollés 1994). The annealing of mini-hairpin primers may be influenced by secondary DNA structure, as suggested by the fact that annealing interactions established at the amplicon termini condition the success of certain amplicons over others (Caetano-Anollés and Gresshoff 1994a). Regardless of their mode of action, the two tailoring strategies simplified the task of separating closely related bermudagrass cultivars. Therefore, they should be used successfully in those cases when standard primers are unable to provide unique fingerprint phenotypes.

Capillary electrophoretic analysis of DAF products

Polyacrylamide or agarose gel electrophoresis can be used effectively to resolve DAF products (He et al. 1994). However, there is a need to increase throughput while decreasing experimental labor. For example, semi-automatic separation of DAF products was possible using miniaturized electrophoresis and staining devices (Baum et al. 1994). The procedure allowed the processing of over 160 samples

Table 2 Analysis of *C. dactylon* × *C. transvaalensis* cultivars 'Tifway' and 'Tifway II' by DNA amplification fingerprinting

Primers			DAF		DNA polymorphisms			
Characteristic	Number tested	Number active	Loci[a]	Loci · primer^{-1} (range)	Length (category, intensity)[b]	Primer	Total no.	%polymorphism · loci^{-1}
Unstructured octamers[c]	81	80	2563	31.6 ± 7.5 (0–52)	115 (II, 3) 117 (II, 3) 120 (I, 3) 290 (II, 2) 105 (II, 2) 340 (II, 2) 170 (II, 2) 120 (I, 3) 60 (II, 3) 120 (II, 2)	OR1 OR1 OR1 OR1 OR4 OR17 OR30 OR33 OR36 OR58	10	0.39
Unstructured octamers-tecMAAP[d]	7	7	201	28.7 ± 3.8 (23–36)	120 (II, 2) 200 (II, 2)	8–5 8–12	2	1.00
Mini-hairpin decamers[e]	7	7	202	28.9 ± 2.9 (25–33)	205 (I, 2) 150 (I, 2) 340 (II,2) 300 (II, 2)	HP10 HP30 HP30 HP31	4	1.98

[a] Amplification products scored were in the 50- to 700-bp range

[b] Length of polymorphic bands are given in bp. Polymorphic DNA category [presence in 'Tifway' (I) or in 'Tifway II' (II)] and intensity [primary (1), secondary (2), or tertiary (3), as defined in Bassam et al. (1992)] is given in parentheses

[c] Amplified with the set of primers used in Fig. 2 [CCTGTGAG (8–2), GTAACGCC (8–4), GACGTAGG (8–5), GAAACGCC (8–8), GTTACGCC (8–9), GTATCGCC (8–10), GTAACCCC (8–12), ACCCAACC (8–16), AATGCAGC (8–20), CCGAGCTG (8–23), and GCAGGTGG (8–26)] and with GAGCCTGT (8–1), AACGGGTG (8–3), GATCGCAG (8–6), CTAACGCC (8–7), TGGTGAGG (8–17), GCTGGTGG (8–21), GAGGGTGG (8–22), CCTGCTGG (8–28), CCTGGAGG (8–29), CGCGGCCA (8–42), and 64 primers that include all sequence variants of GTCCANNN (OR series). Sequence of polymorphic primers in the series: GTCCATTT (OR1), GTCCAGTT (OR4), GTCCATTA (OR17), GTCCAAGA (OR30), GTCCATTC (OR33), GTCCAATC (OR34), GTCCAGTC (OR36), and GTCCAACG (OR58)

[d] The template was restricted and then amplified with primers 8–4, 8–5, 8–7, 8–8, 8–9, 8–10, and 8–12

[e] Amplified with mini-hairpin primers GCGAAGCCTG (HP10), GCGAAGCCTC (HP29), GCGAAGCCTT (HP30), GCGAAGCCAG (HP31), GCGAAGCCCG (HP32), GCGAAGCCAC (HP34), and GCGAAGCCCC (HP36)

per day per unit, and increased the resolution of low-molecular-weight products. Similarly, fluorophore-labeled amplification products were separated and identified in real time with an ABI362 Gene Scanner (Applied Biosystems, Foster City, Calif.). DAF profiles generated with 5′-end FAM-fluorescently labeled primers from *Zoysia japonica* cultivars allowed the effective identification of diagnostic DNA polymorphisms (Caetano-Anollés et al. 1992). In this study we have explored CE as a direct (real time) separation procedure of amplification products generated from the use of unmodified oligonucleotide primers.

Highly reproducible DNA profiles were generated when DAF products were resolved using the ABI DNA Fragment Analysis Sieving separation chemistry in the presence of urea and ethidium bromide. The generation of DNA profiles required the dialysis of samples prior to electrokinetic loading. CE separated DAF products with high resolution and reproducibility within the size range of the molecular mass standards (100–1500 bp) used for calibration (Fig. 3). Furthermore, electrophoretograms showed reproducible peaks of high molecular weight, indicating that while our DAF protocol allowed reproducible amplification of products over 1500 bp in size, CE resolved these products efficiently. We compared CE electrophoretograms and scanned DAF silver-stained profiles produced from bermudagrass cvs 'Tifgreen' and 'Tifdwarf' (Fig. 3). CE was more efficient in detecting amplification products of less than 100 bp in length. For example, a group of four monomorphic bands (with retention times ranging from 17 to 19 min; Fig. 3) were reproducibly detected in all 11 bermudagrass cultivars examined (data not shown). These amplification products can serve as reliable markers for the genus. However, polyacrylamide gel electrophoresis and silver staining was unable to detect them. Furthermore, silver-stained profiles could not clearly distinguish the 2 bermudagrass cultivars. In contrast, CE detected the existence of a single polymorphic fragment present in 'Tifdwarf', the somatic mutant.

The high resolution and reproducibility of CE gives the technique the potential for its use in routine DNA analysis. Separation is complete in about 30 min, while automatic loading increases throughput with unattended operation. These features make CE superior to traditional slab gel methods. Fingerprinting analysis of bermudagrass cultivars by CE requires a reference sample and molecular mass markers. Parallel calibration with these markers may decrease the precision of molecular weight assigment, unless an internal reference marker is added to the samples. The internal marker can align molecular mass standards and DNA profiles to be analyzed. Monomorphic products within the samples can also serve as alignment standards.

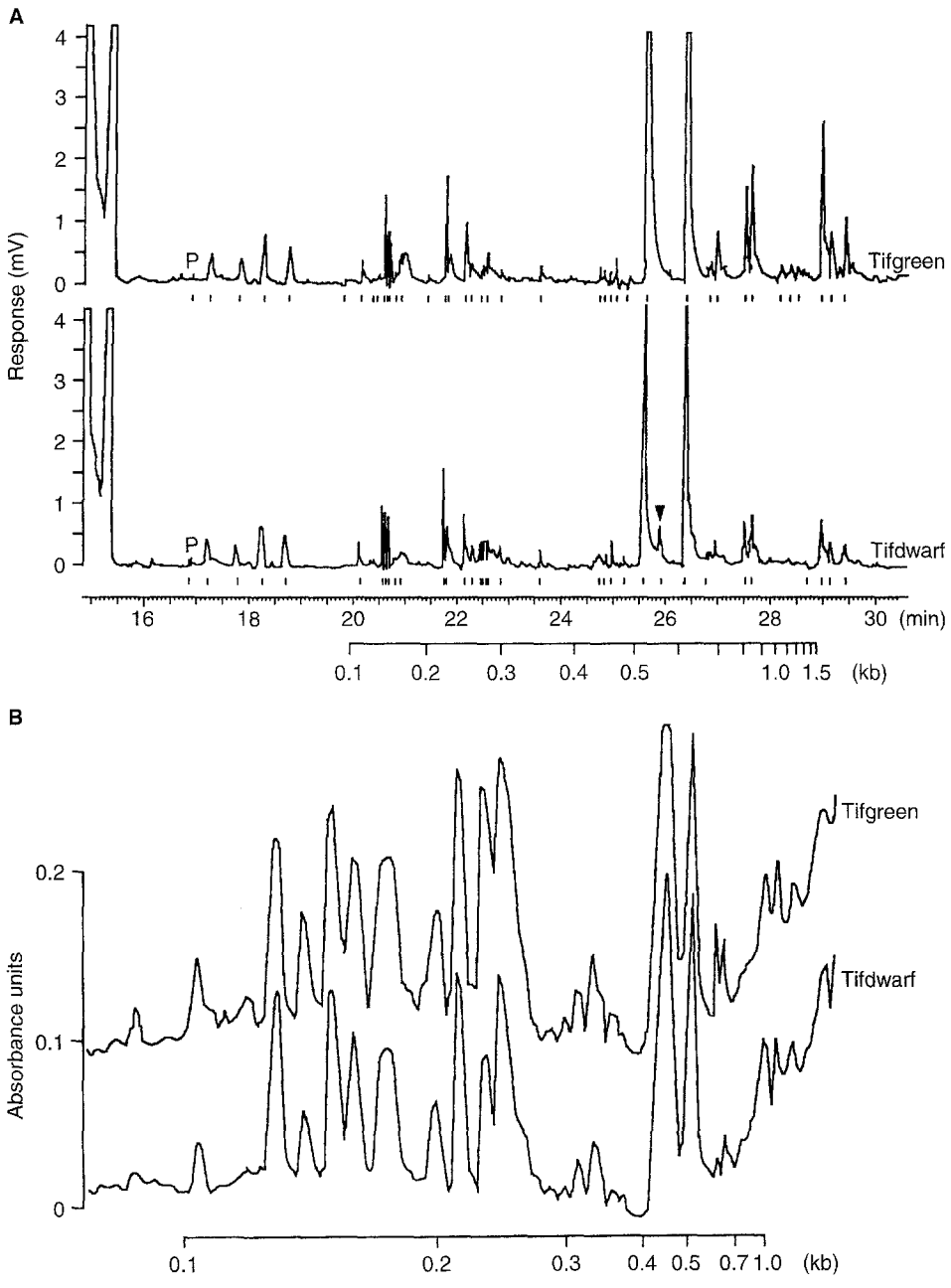

Fig. 3 Comparison of DNA amplification profiles resolved by capillary electrophoresis (**A**) and polyacrylamide gel electrophoresis (**B**). DNA from cvs 'Tifgreen' and the natural mutant 'Tifdwarf' were amplified with primer GTTACGCC (8–9) (see Fig. 1A). Amplification products were dialyzed and analyzed by CE as described in Materials and methods. Alternatively, amplification products were resolved in 7 *M* urea-5% polyacrylamide gels (following a 1/25 dilution), stained with silver, and the scanned profiles analyzed with the *Image* program. A DNA polymorphic peak present in 'Tifdwarf' is indicated by an *arrowhead*

DAF as a tool for cultivar protection and certification

We have used the primers here described to fingerprint samples from commercial sources. DAF allowed us to identify mistakes in plantings, mislabeled plant materials, and contamination of sod fields. Cultivar material has been verified by contrasting the fingerprints with those generated from known stocks of established cultivars (data not shown). In this way, we have detected cultivar substitutions and contaminations (especially in cv 'Tifway') but also confirmed the identity of cultivars supplied by nurseries or those seeking varietal protection. A database of DNA fingerprints from foundation stocks and important cultivars used in the turfgrass industry is now under construction (Callahan et al. 1993a,b) with the purpose of assigning unknowns, verifying seed material for certification, and identifying closely related turfgrass hybrids and cultivars in a mixture. In the comparison with standard fingerprints, exclusions remain categorical while inclusions are probabilistic. It is, however, imperative that results be verified under rigid guidelines to assure the validity of fingerprints for commercial or legal purposes. Verification requires amplification from different DNA isolations, preferably from different sampling locations, using at least 2 different primers in two independent experiments.

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